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EDITORS' NOTE

We take this opportunity to welcome Dr. John Romeo as the third co-editor of the *Journal of Chemical Ecology* and to announce this news to the chemical ecology community, effective with this January 1997 issue (Volume 23, Number 1). Dr. Romeo is currently Professor and Chairman, Department of Biology, University of South Florida, Tampa, Florida. John is well known to most workers in chemical ecology because of his research work in phytochemistry and plant chemical ecology. He has been the editor of the newsletter of the International Society of Chemical Ecology for several years, and has additional editorial experience in his background.

Continue to submit all manuscripts to the Gainesville, Florida mailing address (on the inside back cover), as this office will continue to perform certain administrative functions.

We received 276 manuscripts in 1995 for processing and we anticipate about that many for 1996. It has been clear to us for some time that, if we were to continue our own research programs, teach, advise graduate students, and keep up with the generally growing number of manuscripts in a timely fashion, additional editorial help was needed. Our publisher, Plenum Press, graciously agreed to this appointment.

We welcome you, John, to the editorship team. Sharpen up several pencils!

James L. Nation
David A. Jones
Editors

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EFFECT OF SUBSTITUENT AND RING CHANGES IN NATURALLY OCCURRING NAPHTHOQUINONES ON THE FEEDING RESPONSE OF LARVAE OF THE MEXICAN BEAN BEETLE, *Epilachna varivestis*

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Abstract—Behavioral evaluation of the antifeedant effect of 10 naturally occurring 1,4-naphthoquinones on larvae of the Mexican bean beetle, *Epilachna varivestis* Mulsant, was undertaken concurrently with that of a series of synthetic analogs and model compounds in order to assess structure–activity relationships. Plumbagin, 1,4-naphthoquinone, juglone, menadione, and naphthazarin, which were found to be active at 0.3% concentrations, were also bioassayed at 0.1, 0.05, and 0.01% at which concentration 1,4-naphthoquinone still retained some activity. The model studies suggest that two structural features might be operative independently against *E. varivestis*: one consisting of a properly substituted naphthoquinone moiety and the other requiring a benzo- or naphthohydroquinone. Within the naphthoquinone group, the relative activity is determined by a substituent effect which is the outcome of a complex interplay of electronic, steric, electrochemical, and positional requirements. Among the model compounds, 2-chloro-3-amino-1,4-naphthoquinone and α-naphthylamine displayed appreciable activity even at 0.01%. The results should enable selection of plant sources for naphthoquinones possessing larval inhibition properties.

Key Words—Antifeedant, Mexican bean beetle, *Epilachna varivestis*, secondary metabolites, naphthoquinones, hydroquinones.

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INTRODUCTION

Previous studies of larval growth inhibition of the spiny bollworm, *Earias insulana* (Boisd.), by a series of secondary plant compounds including the naphthoquinone lawsone (Weissenberg et al., 1986), led us to consider the potential activity of other naturally occurring naphthoquinones. Quite a large number of such compounds have been isolated from natural sources comprising bacteria, fungi, animals, and higher plants, and many of them were found to possess biocidal activity (Thomson, 1971). The present report deals with the effect exerted by 10 naphthoquinones (**1a–1j**) (Figure 1) widely distributed in nature on the feeding response of larvae of the Mexican bean beetle, *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae), a severe pest of snap and lima beans in North and Central America (Kogan, 1977). The insecticidal activity of these naphthoquinones has had sporadic investigation, and the early work done *inter*

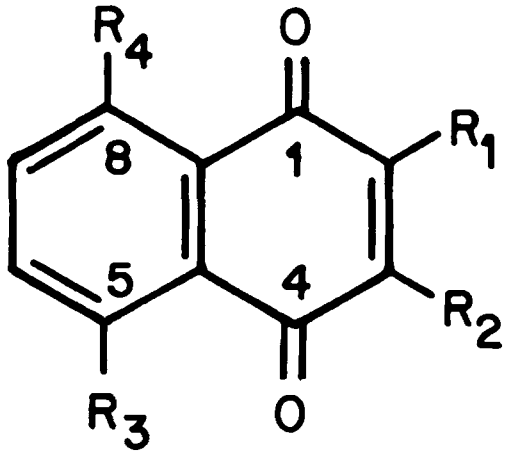

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FIG. 1. General structure of 1,4-naphthoquinones (**1a–1m**): **a** (1,4-naphthoquinone), **b** (juglone), **c** (naphthazarin), **d** (lawsone), **e** (menadione), **f** (plumbagin), **g** (shikonin), **h** (phthiocol), **i** (vitamin K_1), **j** (lapachol), **k** (2,3-dichloronaphthoquinone), **l** (2-chloro-3-aminonaphthoquinone), **m** (2-potassium sulfonate-naphthoquinone). **1a.** $R_1 = R_2 = R_3 = R_4 = H$. **1b.** $R_1 = R_2 = R_4 = H$; R_3, OH. **1c.** $R_1 = R_2 = H$; $R_3 = R_4 = OH$. **1d.** R_1, OH; $R_2 = R_3 = R_4 = H$. **1e.** R_1, CH_3; $R_2 = R_3 = R_4 = H$. **1f.** R_1, CH_3; R_3, OH; $R_2 = R_4 = H$. **1g.** R_1, $CHOHCH_2CH{=}C(CH_3)_2$; R_2, H; $R_3 = R_4 = OH$. **1h.** R_1, CH_3; R_2, OH; $R_3 = R_4 = H$. **1i.** R_1, CH_3; R_2, $CH_2CH{=}C(CH_3)-CH_2CH_2CH_2CH(CH_3)_3-CH_3$; $R_3 = R_4 = H$. **1j.** R_1, OH; R_2, $CH_2CH{=}C(CH_3)_2$; $R_3 = R_4 = OH$. **1k.** $R_1 = R_2 = Cl$; $R_3 = R_4 = H$. **1l.** R_1, Cl; R_2, NH_2; $R_3 = R_4 = H$. **1m.** R_1, SO_3K; $R_2 = R_3 = R_4 = H$.

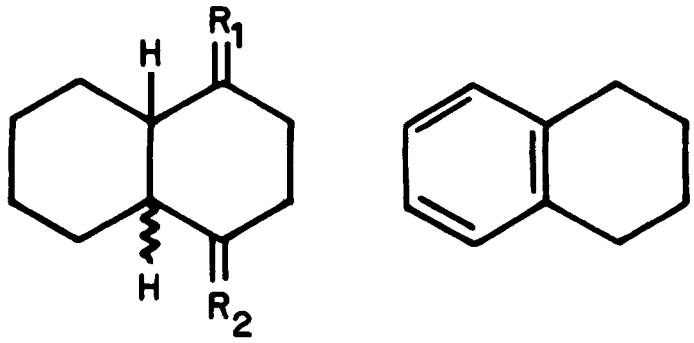

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FIG. 2. General structure of decahydronaphthalene (decalin) derivatives (decalin, **2a**; *trans*-decalin-1,4-dione, **2b**) and molecular structure of tetrahydronaphthalene (tetralin; **3**). **2a.** $R_1 = R_2 = H_2$ (5α- and 5β-H). **2b.** $R_1 = R_2 = O$ (5α-H).

alia on some of the compounds that were active in our study has been summarized (Norris, 1986; Hassanali and Lwande, 1989). The compounds include 1,4-naphthoquinone itself (**1a**), which was demonstrated to occur in plants (Müller and Leistner, 1976), and whose annular frame, devoid of any other substitution pattern, provides a convenient reference structure for comparison to the variously substituted quinonoid and/or benzenoid rings appearing in other naphthoquinones, including the related synthetic derivatives **1k–1m,** and in some analogs and model compounds, **2–8** (Figures 2–4), tested concurrently. We wish to present herein, along with our results, certain considerations on structure–activity relationships concerned with the annular effect on the activity, as well as an extensive appraisal of the substituent effect.

METHODS AND MATERIALS

Test Compounds

Twenty-six pure substances including naphthoquinones and model compounds were bioassayed (Tables 1 and 2). The following were of commercial origin: 2,3-dichloro-1,4-naphthoquinone, juglone, lawsone, menadione, naphthazarin, *p*-naphthohydroquinone, 1,4-naphthoquinone, vitamin K_1 (Fluka AG, Buchs, Switzerland), 1,4-benzoquinone, decalin, hydroquinone, lapachol, quinhydrone (Aldrich Chemical Co., Milwaukee, WI, USA), α-naphthol, α-naphthylamine (B.D.H. Ltd., Poole, UK), 1,3-dihydroxynaphthalene, 2-potassium sulfonate-1,4-naphthoquinone (Eastman Kodak Co., Rochester, NY,

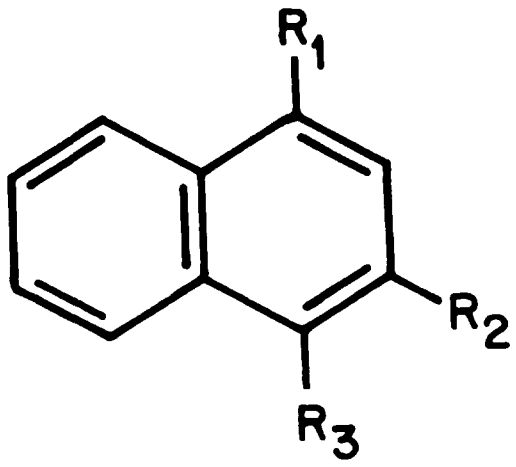

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FIG. 3. General structure of naphthalene derivatives (**4a–4g**): **a** (naphthalene), **b** (α-naphthylamine), **c** (α-naphthol), **d** (1,3-dihydroxynaphthalene), **e** (1,4-dihydroxynaphthalene), **f** (1,4-diacetoxynaphthalene), **g** (carbaryl). **4a.** $R_1 = R_2 = R_3 = H$. **4b.** R_1, NH_2; $R_2 = R_3 = H$. **4c.** R_1, OH; $R_2 = R_3 = H$. **4d.** $R_1 = R_2 = OH$; R_3, H. **4e.** $R_1 = R_3 = OH$; R_2, H. **4f.** $R_1 = R_3 = OAc$; R_2, H. **4g.** R_1, $OCONHCH_3$; $R_2 = R_3 = H$.

USA), 2-chloro-3-amino-1,4-naphthoquinone (Frinton Laboratories, S. Vineland, NJ, USA), 2,3-epoxy-2,3-dihydro-1,4-naphthoquinone (Janssen, Beerse, Belgium), naphthalene (Frutarom, Haifa, Israel), plumbagin (Carl Roth GmbH & Co., Karlsruhe, Germany), and tetralin (Riedel-De Haën AG, Seelze-Hannover, Germany). Three compounds were gifts: 5α-decalin-1,4-dione (Dr. Clive Newton, Stanford University, CA, USA), phthiocol (Prof. Kazuhiro Maruyama, Kyoto University, Japan) and shikonin (Dr. Hiroshi Fukui, Kyoto University,

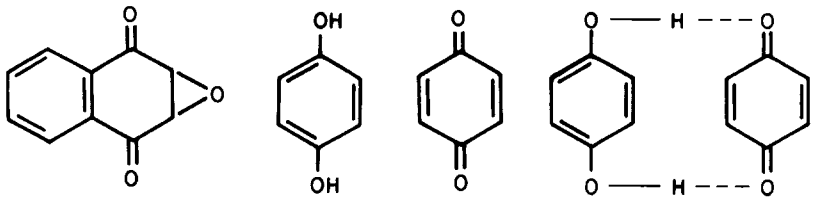

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FIG. 4. Molecular structures of 2,3-epoxy-2,3-dihydro-1,4-naphthoquinone (**5**), hydroquinone (**6**), 1,4-benzoquinone (**7**), and quinhydrone (**8**).

Japan). *p*-Naphthohydroquinone diacetate was prepared according to a known procedure (Fieser, 1948). The test compounds were dissolved in methyl alcohol to give solutions of appropriate concentration for bioassays.

Chemical Modifications of 1,4-Naphthoquinones and Subsequent Application of the Products Formed in Situ

Reduction with Sodium Borohydride. Solutions of naphthoquinones **1a, 1b,** and **1d–1f** (30 mg each) in methyl alcohol (10 ml) were treated separately with $NaBH_4$ (5 mg; Fluka). The color of solutions changed instantly. After 5 min at room temperature, the solutions of the corresponding naphthohydroquinones formed *in situ* (TLC evidence) were applied separately on leaves, as described for the bioassay. Controls with solutions of $NaBH_4$ (5 mg) in methyl alcohol (10 ml) were run in parallel.

Epoxidation with Sodium Hypochlorite. Solutions of naphthoquinones **1a, 1b,** and **1d–1f** (30 mg each) in methyl alcohol (10 ml) were treated separately with NaOCl (0.5 ml of an ~ 1 N solution in 0.1 N NaOH; B.D.H.). Color change occurred instantly. After 5 min at room temperature, the solutions of the corresponding epoxides formed *in situ* (TLC evidence) were applied separately on leaves, as described for the bioassay. Controls with solutions of NaOCl (0.5 ml) in methyl alcohol (10 ml) were run in parallel.

Insect Rearing Technique

Larvae of *E. varivestis* were reared on bean leaves (*Phaseolus vulgaris* L. var. 'Brittle Wax') at 25 $\pm$ 1°C, as described previously (Ascher et al., 1987).

Antifeedant Bioassay

The experiments were conducted with 20- to 30-mg *E. varivestis* larvae on bean leaves, in a previously described test device (Ascher et al., 1987). Solutions of the test compounds in methyl alcohol at various concentrations were pipetted on leaves and then spread over the entire leaf area with a small paint brush. Controls with methyl alcohol-treated leaves and with untreated leaves were run in parallel. The larvae (20 per treatment) were allowed to feed on the treated and control leaves for 48 hr at 21–23°C and then reweighed. Following the 48-hr feeding period the injured, moribund, or dead insects were noted and discarded, and the survivors were weighed. The test compounds were first bioassayed at a primary screening concentration of 0.3% (w/v) and the most active compounds were subsequently tested at 0.1, 0.05, and 0.01%. Percentage larval starvation was calculated from the ratio of the mean weight change of larvae to 100% starvation assessed by considering the mean weight loss of starved larvae (Ascher and Nissim, 1965).

TABLE 1. MEAN WEIGHT CHANGE AND STARVATION OF *Epilachna*

1,4-Naphthoquinone						
		0.3			0.1	
		Weight change (mg)[a]		Starvation (%)[a]	Weight change (mg)	
Structure	Name	Control	Treatment		Control	Treatment
1a	1,4-Naphthoquinone	8.2 ± 0.8	−8.3 ± 1.0	102.4 ± 6.0[b]	13.5 ± 1.6	−4.2 ± 0.8
1b	Juglone	10.7 ± 0.8	−7.4 ± 0.7	97.3 ± 4.5	15.2 ± 0.9	10.9 ± 1.5
1c	Naphthazarin	8.6 ± 1.0	−5.4 ± 0.8	84.8 ± 6.1	8.6 ± 1.0	3.5 ± 1.0
1d	Lawsone	11.4 ± 0.7	−2.3 ± 0.4	71.0 ± 5.6	10.6 ± 1.4	0.5 ± 1.4
1e	Menadione	8.9 ± 1.0	−5.6 ± 0.3	85.9 ± 3.6	13.6 ± 0.8	8.2 ± 0.8
1f	Plumbagin	9.3 ± 0.7	−9.1 ± 0.9	106.9 ± 6.5	10.9 ± 0.9	−2.5 ± 0.8
1g	Shikonin	12.6 ± 1.9	1.4 ± 1.0	54.6 ± 1.0		
1h	Phthiocol	9.7 ± 1.1	−3.8 ± 1.1	76.7 ± 4.3		
1i	Vitamin K_1	12.2 ± 1.3	8.7 ± 1.1	17.4 ± 4.9		
1j	Lapachol	8.6 ± 0.6	−2.8 ± 0.7	69.0 ± 6.2	12.1 ± 0.9	8.4 ± 2.2
1k	2,3-Dichloronaphthoquinone	9.7 ± 0.7	−7.6 ± 0.7	98.3 ± 3.8	9.7 ± 0.7	−4.2 ± 0.7
1l	2-Chloro-3-amino-naphthoquinone	10.0 ± 0.7	−3.4 ± 0.8	74.9 ± 2.6	10.0 ± 0.7	−0.7 ± 0.8
1m	2-Potassium sulfonate-naphthoquinone	7.8 ± 0.8	4.5 ± 0.7	21.0 ± 4.2		

[a] Value ± standard error.
[b] Values of percentage starvation higher than 100% are presumably due to stress-induced weight loss, in addition to that caused by starvation.

RESULTS AND DISCUSSION

The results of the antifeedant bioassays of naturally occurring naphthoquinones against *E. varivestis* are presented in Table 1. Plumbagin, 1,4-naphthoquinone, juglone, menadione, and naphthazarin were found to be highly active antifeedants at 0.3% concentration. Lesser activity was shown by phthiocol, lawsone, lapachol, and shikonin, and practically none by vitamin K_1. The most active compounds were further bioassayed at lower concentrations. At 0.1%, 1,4-naphthoquinone and plumbagin still showed marked activity, followed by lawsone. At 0.05%, lawsone displayed *ca.* 50% activity, whereas 1,4-naphthoquinone and plumbagin retained about 30% activity. At 0.01%, only 1,4-naphthoquinone still possessed *ca.* 30% activity.

The quinonoid structure is associated with a wide range of biological activity, and variously substituted naphthoquinones—both natural and synthetic—

varivestis LARVAE TREATED WITH 1,4-NAPHTHOQUINONES

Concentration (%, w/v)						
	0.05			0.01		
Starvation (%)	Weight change (mg)		Starvation (%)	Weight change (mg)		Starvation (%)
	Control	Treatment		Control	Treatment	
82.7 ± 8.6	13.5 ± 1.6	7.6 ± 2.6	27.6 ± 9.2	13.5 ± 1.6	6.8 ± 1.4	31.3 ± 7.1
18.6 + 9.3	15.2 ± 0.9	14.2 ± 1.4	4.3 ± 5.1	15.2 ± 0.9	10.8 ± 1.5	19.0 ± 5.6
30.9 ± 6.9						
54.6 ± 8.8	10.6 ± 1.4	1.8 ± 0.7	47.5 ± 5.7	10.6 ± 1.4	7.3 ± 1.8	17.8 ± 10.1
25.1 ± 3.8						
71.3 ± 4.3	10.9 ± 0.9	5.2 ± 1.4	30.3 ± 2.0	10.9 ± 0.9	9.5 ± 1.5	7.4 ± 7.7
18.5 ± 4.5						
78.9 ± 3.8	9.7 ± 0.7	6.7 ± 1.3	17.0 ± 6.6	9.7 ± 0.7	8.6 ± 1.1	6.2 ± 6.2
59.8 ± 4.7	7.3 ± 0.5	−4.4 ± 0.3	77.0 ± 1.9	7.3 ± 0.7	−3.1 ± 0.7	68.4 ± 4.4

were shown to display insecticidal, fungicidal, herbicidal, molluscicidal, termiticidal, miticidal, aphicidal, antiviral, antibacterial, antibiotic, coccidiostatic, antitrypanosomal, antineoplastic, anticercarial, antimalarial, tuberculostatic, and tumor-inhibitor effects (Marston et al., 1984; Clark, 1985; Jacobsen and Pedersen, 1986; Spencer et al., 1986). Naphthoquinones are involved in cellular respiration and photosynthesis, participating competitively with the cell components in the cell electron transport (Holmes et al., 1964; Ambrogi et al., 1970). Many of them are believed to act on oxidative phosphorylation (Jacobsen and Pedersen, 1986). Since it was inferred that the presence of certain substituents in the quinone ring, or a vacant quinonoid position, would be essential for activity (Sankaram et al., 1975), a model study was undertaken to investigate the relevance of electronic, steric, electrochemical, and location effects of various substituents in both the quinone and the phenyl nucleus, as well as that of the quinonoid and benzenoid moieties, to antifeedant activity for the larvae of *E. varivestis*; the conclusions arrived at are discussed below.

TABLE 2. MEAN WEIGHT CHANGE AND STARVATION OF *Epilachna varivestis* LARVAE TREATED WITH MODEL COMPOUNDS AND ANALOGS OF NAPHTHOQUINONE DERIVATIVES

Compound		Concentration	Weight change (mg)[a]		Starvation
Structure	Name	(%, w/v)	Control	Treatment	(%)[a]
2a	5α-Decalin-1,4 dione	0.3	5.5 ± 0.4	3.8 ± 0.9	12.7 ± 0.6
2b	Decalin	0.3	20.1 ± 1.3	17.7 ± 1.9	8.6 ± 6.0
3	Tetralin	0.3	9.7 ± 1.9	9.5 ± 1.0	1.1 ± 1.8
4a	Naphthalene	0.3	5.5 ± 0.5	5.0 ± 0.9	3.7 ± 3.7
4b	α-Naphthylamine	0.3	10.0 ± 1.0	−3.4 ± 0.5	62.4 ± 2.8
4c	α-Naphthol	0.3	5.5 ± 0.5	−1.3 ± 0.5	50.7 ± 4.0
4d	1,3-Dihydroxynaphthalene	0.3	6.9 ± 0.7	3.9 ± 0.9	20.3 ± 6.3
4e	1,4-Dihydroxynaphthalene	0.3	6.7 ± 0.4	−8.0 ± 0.6	100.0 ± 4.0
4e	1,4-Dihydroxynaphthalene[b]	0.3	6.7 ± 0.4	−8.3 ± 0.5	100.5 ± 5.5
4f	1,4-Diacetoxynaphthalene	0.3	6.9 ± 0.7	−5.8 ± 0.8	85.8 ± 8.0
4f	1,4-Diacetoxynaphthalene	0.46[c]	5.5 ± 1.1	−7.9 ± 1.0	100.0 ± 4.6
5	2,3-Epoxy-2,3-dihydro-naphthoquinone	0.3	9.9 ± 0.7	−3.5 ± 0.6	75.0 ± 5.3
6	Hydroquinone	0.3	9.6 ± 0.7	−8.0 ± 1.3	100.0 ± 4.3
7	1,4-Benzoquinone	0.3	10.4 ± 0.6	6.2 ± 0.8	22.9 ± 0.8
8	Quinhydrone	0.3	10.4 ± 0.6	2.2 ± 0.8	44.8 ± 0.8

[a] Value ± standard error.
[b] In the presence of vitamin C (0.5%).
[c] Concentration equivalent to 0.3% of the corresponding free diol.

Effect of Substituent Location

The results of our work suggest that substitution at position 2 of the quinone ring of naphthoquinone **1a** (methyl, hydroxyl, and sulfonate groups) decreases activity markedly (**1e, 1d,** and **1m**). However, introduction of the hydroxyl substituent at position 5 in the phenyl ring of **1a** reduces activity only slightly (**1b**), the latter thus becoming much more active than the isomeric **1d.** Substitution at both quinonoid positions 2 and 3 with chlorine tends to diminish activity very slightly (**1k**), but an appreciable decrease occurs when one of the chlorine substituents is replaced in the latter by an amino group (**1*l***). When a hydroxyl group is already present in position 5 of naphthoquinone, as in the case of juglone (**1b**), introduction of a methyl substituent at C-2 enhanced activity (**1f**). Further addition of a *peri* hydroxyl group to **1b** to yield naphthazarin (**1c**) seems to decrease the antifeedant effect, and substitution at C-2 in the last system with a hydroxymethylpentenyl substituent significantly reduces the activity (**1g**).

Examination of the results obtained with the other group of naphthoquinones, which are already substituted at C-2 in the quinone ring, shows that

introduction of the hydroxyl group at C-5 in the phenyl ring of 2-methylnaphthoquinone (**1e**) enhances the activity markedly (**1f**), whereas further substitution at the vicinal C-3 position of the quinonoid system with hydroxyl or phytyl substituents leads to a considerable reduction in effectiveness (**1h** and **1i,** respectively). In the case of 2-hydroxynaphthoquinone (**1d**), further substitution at C-3 with a methyl group slightly increases activity (**1h**), while a 3-methylbutenyl group slightly lowers it (**1j**).

Stereoelectronic Substituent Effect

The quinone ring of naphthoquinones is the structural feature assumed to be involved in much of their antifeedant activity (Norris, 1986), and electronic and steric effects exerted by substituents at C-2 and C-3 could conceivably influence their mechanism of action on the target pathogen by affecting the strong electron-withdrawing character of the quinone carbonyls. Yet, under the conditions of our bioassay, the unsubstituted naphthoquinone **1a** proved to be one of the most active antifeedant substrates against larvae of *E. varivestis* (Table 1), and the presence of either an electron-repelling (methyl) or an electron-attracting (hydroxyl) group at one of the β positions resulted in a decrease in activity (**1a** vs **1e** and **1d,** respectively). The 2-hydroxy group becomes strongly acidic as a result of the increased stability of the anion, due to interaction of the *p*- and *o*-quinone anions induced by the *ortho* quinoid character of the resultant system (Moore and Scheuer, 1966), which should affect the stability of the ring. Steric hindrance by larger substituents at C-2 might also interfere with the participation of the quinonoid system in any biocidal mechanism and, thus, decrease activity (**1g, 1i,** and **1m**).

Activity is retained when the position next to the hydroxyl substituent in the quinone ring is occupied by an electron-repelling alkyl group (**1h** and **1j**). All the same, further substitution of menadione (**1e**) at the vicinal position of the quinonoid system with either an electron-attracting (hydroxyl) or an electron-repelling (phytyl) group, leads to a decrease in activity (**1d** and/or **1i**). Disubstitution of naphthoquinone on the quinoidal ring with electronegative chlorine substituents (**1k**) retains the high activity, presumably due to an increased degree of conjugation, which would facilitate addition–elimination reactions (Clark, 1985). However, the activity level drops appreciably when one of the beneficial chlorine substituents is replaced by the weaker electronegative amino group (**1l**).

Turning now to substitution into the aromatic ring, it appears that addition of a strong electron-attracting hydroxyl group at the *peri* 5 position does not affect activity much (juglone; **1b**), whereas a second *peri*, strongly hydrogen-bonded hydroxyl group at C-8 seems to decrease it (naphthazarin; **1c**), probably owing to rapid tautomerism of the naphthazarin system, which results in simultaneous occurrence of benzenoid and quinonoid properties in both rings (Moore

and Scheuer, 1966). Conversely, although the strong electron-withdrawing character of the quinone carbonyls contributes to isolating the aromatic moiety from the quinoidal double bond and its substituents (Singh et al., 1968), addition of a *peri* 5-hydroxyl group to 2-methyl-naphthoquinone (**1e**) raises the activity appreciably (plumbagin; **1f**). On the other hand, substitution of juglone (**1b**) at one of the β positions of the quinoidal ring with an electron-repelling methyl group brings about a slight increase in activity (plumbagin; **1f**). When naphthazarin is substituted at one of the β positions with an alkyl group (shikonin; **1g**), the principal tautomer shows quinonoid character of the ring bearing the substituents, as substantiated by C-13 NMR evidence (Papageorghiu, 1980); yet the activity drops markedly, and this may well be due to steric hindrance by the bulkier alkyl group present at C-2 in shikonin (**1g**).

Electrochemical Effect of Substituent

The quinoidal moiety of naphthoquinones displays an electrochemical system prone to reversible reduction-oxidation reactions of the quinol–quinone type, characterized by a redox potential (E_0) or a related half-wave potential ($E_{1/2}$). Substituents in both rings of the naphthoquinone frame might conceivably modify the electron density around the quinoid carbonyl groups and thus cause a shift in either E_0 or $E_{1/2}$ values which could, arguably, be correlated with the deterrent effect. Accordingly, a substituent which reduces the E_0 of a deterrent naphthoquinone, would be expected to reduce the latter's deterrent effect (Norris, 1969). For substituents on the aromatic ring (and even in the *peri* position), the mutual interaction with the quinoid carbonyl groups is less pronounced than for the substituents on the quinoid ring (Fieser and Fieser, 1935), and they therefore have a lesser effect on the $E_{1/2}$ shift (Zuman, 1962). Indeed, substituents at C-2 in the quinoidal ring reduce E_0 more than they would at other positions (Fieser and Fieser, 1935). Among them, electron-attracting groups, which increase the electron density at the quinoid grouping as a result of their effect being transmitted through the conjugated system of the aromatic ring, shift $E_{1/2}$ toward negative values, whereas electron-repelling groups, due to their electron unsaturation, cause a reduction in the electron density at the quinoid ring which facilitates reduction and, consequently, cause $E_{1/2}$ to shift toward positive values (Vladimirtsev and Stromberg, 1957). In fact, examination of our results suggests that reduced E_0 might account for the decrease in deterrent effect with both electron-repelling (methyl) and electron-attracting (hydroxyl) substituents at C-2, the former being more of a deterrent than the latter, as would be expected. Likewise, the $E_{1/2}$ value of the most active naphthoquinones in our assays fits quite well the activity range between -160 and -530 mV suggested for competitive participation in electron transport with cell components in bac-

teriostasis (Holmes et al., 1964; Ambrogi et al., 1970). Insofar as antimicrobial (Ambrogi et al., 1970) and antifeeding (Norris, 1986) activity imply reaction between a membrane receptor protein and the electrophilic 1,4-naphthoquinones by a combination of redox interaction and Michael-type (1,4-addition) reaction, $E_{1/2}$ can provide only an initial indication, other factors involved being stereoelectronic features of the compounds, their hydrogen bonding ability, and their increased degree of conjugation. Inasmuch as behavioral effects of plant secondary metabolites elicit different responses from different pathogens, most compounds found to be active in our study also exhibited antifeeding activity—albeit not necessarily in the same order of relative feeding inhibition—in early studies done with other insects such as *Scolytus multistriatus* (European elm bark beetle) (Norris, 1969), *Periplaneta americana* (American cockroach) (Norris et al., 1970), *Culex pipiens quinquefasciatus* (mosquito) (Desmarchelier and Fukuto, 1974), *Acalymma vittata* (F.) (striped cucumber beetle) (Reed et al., 1981), *Aedes aegypti* (mosquito) (Hassanali and Lwande, 1989), and *Actias luna* (luna moth) (Thiboldeaux et al., 1994).

Summing up, the substituent influence on the activity of naphthoquinones is seemingly the outcome of a complex interplay of electronic, steric, electrochemical, and positional effects operating in the reaction between the insect lipoprotein receptors and naphthoquinones, which is involved in energy transduction and in the insect perception of the latter compounds as antifeedants (Norris, 1986, 1988). Other factors include electrophysiological, biophysical, and biochemical aspects (Norris, 1988). Different potential functions have been variously suggested for plumbagin (**1f**), such as inhibition of chitin synthetase (Kubo et al., 1983), of ecdysone 20-monooxygenase (Mitchell and Smith, 1988), and of ecdysteroid production (Joshi and Sehnal, 1989), as well as interference with the neuroendocrine system of the insect and its integration of molting processes (Gujar and Mehrotra, 1988), induction of sterilization and of immunodepression (Saxena and Tikku, 1988), an antilipid peroxidative effect (Sankar et al., 1987), and deactivation of polyphenol oxidase due to formation of six-membered chelate complexes (Sankaram et al., 1975). The antifungal action of lawsone (**1d**) was attributed to inhibition of nitrate reductase (Tripathi et al., 1980). Antimicrobial naphthoquinones were found to inhibit the succinate oxidase system and respiration by acting in the region of cytochrome *c*/cytochrome *b* in the respiratory enzyme chain, to inhibit and/or uncouple oxidative phosphorylation (Howland, 1963), to inhibit electron transfer by acting as vitamin K or ubiquinone antagonists (Holmes et al., 1964), and to inhibit mitochondrial electron transport (Fieldgate and Woodcock, 1973). The synthetic 2,3-dichloro-1,4-naphthoquinone (**1k**) is employed commercially as an agricultural fungicide under the name dichlone, and its activity is presumably linked with the ability to react with vital amines and thiols present in biological systems (Sankaram et

al., 1975) and to form hydrogen bonds (Ambrogi et al., 1970). Furthermore, fungicide activity was shown for juglone (Hedin et al., 1980) and for naphthoquinones found in bark-inhabiting beetle larval defensive secretions and suggested to act under bark as effective fungicides (Dettner, 1993).

Effect of Rings

Bicyclic Frame. Attempts were made to assess the relevance, if any, of the bicyclic backbone to activity. Thus, the saturated bicyclic derivatives decalin (**2a**) and *trans*-decalin-1,4-dione (**2b**) (the latter having the arrangement of the carbonyl groups found in 1,4-naphthoquinones) did not display activity (Table 2). Likewise, aromatization of one ring (tetralin; **3**) or both (naphthalene; **4a**) did not induce activity.

Quinoidal Ring. Another set of experiments was devised in order to evaluate the importance of the quinone ring for activity. The quinoidal moiety is, of course, characterized by the 1,4-carbonyl groups conjugated to the 2,3-double bond. Any modification of these substituents would conceivably alter the quinoidal character of the compounds and, in the process, demonstrate its relevance to activity. The most convenient modifications are reduction of the carbonyl groups and epoxidation of the conjugated double bond. We chose sodium borohydride as the reducing agent and sodium hypochlorite as the epoxidizing agent. The progress of reactions was followed by TLC and color change, and the products were first bioassayed *in situ*, in parallel with the appropriate controls containing the same reagents and solvents, in order to ascertain quickly whether the activity would be affected. It was found that the quinone system was readily reduced to the related hydroquinone using a slight excess of sodium borohydride, enough to prevent both air-oxidation of the product back to the original quinone, and its possible degradative hydrogenolysis (Moore et al., 1966). Likewise, the reaction of naphthoquinones with sodium hypochlorite led to the formation of the corresponding epoxides after only a few minutes (Marmor, 1963). Consequently, both reactions were carried out for periods of 5 min, in order to minimize possible formation of by-products, and submitted immediately to bioassays. Five model naphthoquinones, variously substituted at both rings, were selected for these experiments (**1a, 1b, 1d–1f**). The results showed that naphthoquinones unsubstituted at the quinoidal ring (**1a** and **1b**) experienced a significant drop in activity following both reactions (down to 50–60% starvation), while the 2-substituted naphthoquinones (**1d–1f**) under these conditions displayed practically the same activity as before, for reasons which were not immediately apparent (*vide infra*). At any rate, all five compounds still showed a notable level of activity after the reactions (even **1a** and **1b**), which might indicate that the reaction products would act by yet another mechanism, obviously different from that of the original naphthoquinones.

Since the main products of the reduction and/or epoxidation of naphthoquinone **1a** are *p*-naphthohydroquinone (1,4-dihydroxynaphthalene; **4e**) and 2,3-epoxy-2,3-dihydronaphtoquinone (**5**), respectively, the assays were also conducted with pure samples of these compounds, and the results indicated appreciable activity for both of them, especially the former (Table 2). High activity was also recorded when **4e** was bioassayed in the presence of vitamin C as antioxidant to prevent oxidation back to **1a,** thus demonstrating that the naphthoquinone derivative is not involved in this case. Clearly, besides the activity induced by the properly substituted naphthoquinone system, there is another mechanism of activity at stake here, related to the hydroquinone moiety.

In order to gain more insight into this intriguing assumption, we prepared the 1,4-diacetate of naphthohydroquinone (**4f**) where, of course, both hydroxyl groups are derivatized and thus unable to undergo reoxidation to the quinone system. We bioassayed the diacetate at the same concentration as the other compounds and also at a concentration equivalent to that of the corresponding free diol, and in both cases high activity was observed. Even if not involved in back-oxidation to 1,4-naphthoquinone, the *para* arrangement of the two hydroxyl groups seems to be important for activity, since the isomeric 1,3-dihydroxynaphthalene (**4d**) proved to be largely inactive. Contrariwise, the isomeric 1,5-dihydroxynaphthalene, along with **4e,** were found in another bioassay to display antifungal activity comparable to that of juglone (Hedin et al., 1980). α-Naphthol (**4c**), which has a 1-hydroxyl group only, showed some weak activity. Additional substitution in the hydroquinone ring at the C-2 position (**1d–1f**) would apparently not interfere with activity (*vide supra*), whereas discrepancy between the results of the crude reaction products of **1a** and the related pure compounds (Table 2, **4e** and **5**) might be ascribed to the presence of degradation products in the former.

More evidence on the relevance of the hydroquinone moiety to activity was obtained by bioassaying the single-ring hydroquinone (**6**) which exhibited high activity, comparable to that of naphthohydroquinone (**4e**). Conversely, the related 1,4-benzoquinone (**7**) was found in our experiments to be virtually inactive, whereas quinhydrone (**8**), which is the equimolecular adduct of **6** and **7,** displayed some activity intermediate between that of the components alone (Table 2). Incidentally, α-naphthylamine (**4b**), which has a 1-amino group, displayed moderate activity even at a 0.01% concentration (results not shown), perhaps through a carbaryl (**4g**)-like mechanism. This result recalls similar activity exerted by the synthetic 2-chloro-3-amino-1,4-naphthoquinone **1l** (Table 1) and suggests a possible effect of the amino group.

Our results show that two structural features might independently be operative against *E. varivestis:* one consisting of a properly substituted naphthoquinone moiety and the other requiring a benzo- or naphthohydroquinone. Within the naphthoquinone group, the relative activity is determined by a substituent

effect emerging from a subtle balance of steric, electronic, electrochemical, and positional requirements. The results should enable selection of plant sources for naphthoquinones possessing larval inhibition properties, notwithstanding that the ecological effect of plant–pathogen interaction would be settled by the global biochemical features of plant tissues rather than by individual secondary metabolites (Roddick et al., 1988). Likewise, the high activity showed by hydroquinones warrants further study to assess their potential for practical application. The activity displayed by epoxynaphthoquinone (**5**) is puzzling, especially compared with the inactive decalin-dione (**2a**), and deserves more investigation.

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(*Z*)6,(*E*)8-HENEICOSADIEN-11-ONE: SYNERGISTIC SEX PHEROMONE COMPONENT OF DOUGLAS-FIR TUSSOCK MOTH, *Orgyia pseudotsugata* (McDUNNOUGH) (LEPIDOPTERA: LYMANTRIIDAE)

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Abstract—Three candidate sex pheromone components, (*Z*)6,(*Z*)9-, (*Z*)6,(*E*)8-, and (*Z*)6,(*E*)9-heneicosadien-11-one (Z6Z9, Z6E8, and Z6E9) were identified in pheromone gland extracts of female Douglas-fir tussock moths (DFTM), *Orgyia pseudotsugata* (McDunnough). Their occurrence in subnanogram quantities in extracts and structural conversion during analytical procedures and bioassays complicated chemical identifications. Complete identification required comparative analyses of stereoselectively synthesized and female-produced dienones by coupled gas chromatographic-electroantennographic detection (GC-EAD), high-performance liquid chromatography (HPLC) and coupled GC-mass spectrometry (MS). Determination of the pheromone component was contingent upon an experimental design that minimized structural rearrangement of dienones before and during the field test. In a 40-min field experiment, acetonitrile solutions of each of the above dienones were carried on Dry Ice to traps and were syringed onto cotton release devices below trap lids. In combination with the previously known sex pheromone component of DFTM, (*Z*)6-heneicosen-11-one (Z6), Z6E8 was the only synergistic dienone and the mixture was highly attractive. Because Z6 by itself

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attracts seven species of tussock moths (two sympatric with DFTM), a blend of Z6 and Z6E8 may impart specificity to DFTM pheromone communication. In commercial lures, this binary blend may facilitate species-specific, sensitive monitoring and efficacious control by mating disruption of this important forest defoliator.

Key Words—Lepidoptera, Lymantriidae, *Orgyia pseudotsugata*, tussock moth, (*Z*)6,(*Z*)9-heneicosadien-11-one, (*Z*)6,(*E*)8-heneicosadien-11-one, (*Z*)6,(*Z*)9-heneicosadien-11-one, sex pheromone, synergism.

INTRODUCTION

The Douglas-fir tussock moth (DFTM), *Orgyia pseudotsugata* (McDunnough), is one of the most important defoliators of Douglas-fir and true firs in Western North America (Wickman, 1978; Mason and Wickman, 1988). For example, when 279,000 ha of forest were defoliated in 1974, the Environmental Protection Agency (Federal Register 39, No. 44, pp. 8377–8381) provided an emergency exemption that allowed application of the banned insecticide DDT to 161,000 ha to contain the outbreak. Following the discovery of the sex pheromone component (*Z*)6-heneicosen-11-one (Z6) (Smith et al., 1975), DFTM infestations have been detected and monitored with traps baited with synthetic pheromone (Daterman, 1978, 1980, 1982; Daterman et al., 1979; Shepherd and Otvos, 1986). However, Z6 unspecifically attracts seven species of tussock moths, including whitemarked tussock moth (WMTM), *O. leucostigma* (J. E. Smith), rusty tussock moth, *O. antiqua* (L.), and western tussock moth, *O. cana* Edwards (Daterman et al., 1976; Grant, 1977). Moreover, Z6 is less attractive than unmated DFTM females, supporting the contention that female DFTM produce an additional sex pheromone component (Daterman et al., 1976). This paper describes the identification and field testing of a highly synergistic DFTM sex pheromone component.

METHODS AND MATERIALS

Laboratory Analyses, Instruments, and General Procedures

DFTM male and female pupae were collected around Kamloops, British Columbia. WMTM pupae were reared in Sault Ste. Marie, Ontario, and sent to Simon Fraser University. Male and female pupae were kept separately in filter paper-lined petri dishes at 24°C and under a 14:10 (L:D) photoperiod. At the onset of the scotophase for DFTM or photophase for WMTM (Grant et al., 1975), pheromone glands of 2-day-old virgin females were removed and extracted for 5 min in hexane.

Aliquots of 1 female equivalent (FE) of gland extract were analyzed by

coupled gas chromatographic–electroantennographic detection (GC–EAD) (Arn et al., 1975), using a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25-mm ID) coated with either DB-210 or DB-5 (J&W Scientific, Folsom, CA 95630. GC–mass spectrometry (MS) of synthetic or antennally active compounds in full-scan or selected ion monitoring (SIM) modes with isobutane for chemical ionization (CI) employed an HP 5985B GC–MS equipped with a DB-210 column.

Fractionation of DFTM pheromone extract by high-performanc liquid chromatography (HPLC) employed a Waters LC 625 high-performance liquid chromatograph equipped with a Waters 486 variable-wavelength UV–visible detector set at 220 nm, an HP 3396 series II integrator, and a Nova-Pak C_{18} (3.9 × 300-mm) column with 1 ml/min of acetonitrile flow. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Bruker AMX-400 spectrometer at 400.13 and 100.62 MHz for ^{1}H and ^{13}C NMR spectra, respectively. ^{1}H chemical shifts are reported as parts per million (ppm; δ) relative to TMS (0.00 ppm). ^{13}C chemical shifts are referenced to $CDCl_3$ (77.0 ppm). Two-dimensional correlated spectroscopy (COSY) was used to identify J-coupled protons, while total correlation spectroscopy (TOCSY) was used to identify spin coupled networks. Combined use of both techniques allowed unambiguous assignment of all resonances, even for the mixed rearranged products. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) was then employed to assign the *E* or *Z* stereochemistry about the olefin bonds.

Elemental analyses were performed using a Carlo Erba Model 1106 elemental analyzer. Chemicals obtained from commercial sources were used without further purification unless otherwise indicated. All moisture- and air-sensitive reactions were conducted under argon. Column chromatography refers to flash chromatography using silica gel 60 (230- to 400-mesh, E. Merck, Darmstadt) (Still et al., 1978). Thin-layer chromatography (TLC) was conducted on aluminum-backed plates precoated with Merck silica gel 60F-254 as the adsorbent and visualized by treatment with an acidic solution of 1% $Ce(SO_4)_2$ and 1.5% molybdic acid followed by gentle heating.

Syntheses

1,4-Decadiyne (1) (Scheme 1, Figure 1). 1-Heptyne (7.2 g, 75 mmol) was added to freshly prepared ethylmagnesium bromide (83 mmol) in Et_2O (150 ml). The mixture was refluxed 3 hr, then cooled to 0°C and CuI (1.25 g, 6.7 mol) in THF (200 ml) was added. After stirring 2 hr, propargyl tosylate (15.75 g, 75 mmol) in THF (50 ml) was added. After 1.5 hr, the reaction mixture was allowed to warm to room temperature (rt) and was stirred 1 hr. A solution of NH_4Cl (15 g in 200 ml of H_2O) was added and the organic layer separated. The remaining aqueous layer was extracted (3 × 50 ml) with ether. Extracts were

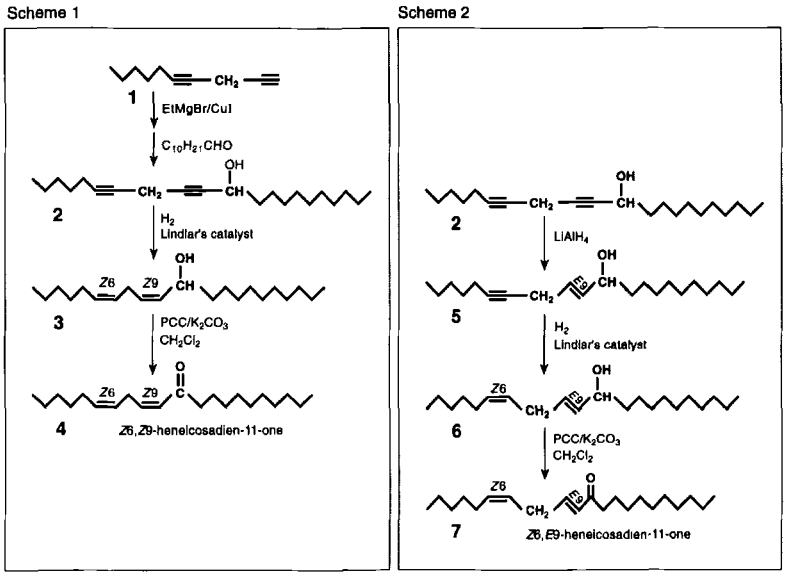

FIG. 1. Routes for the syntheses of (*Z*)6,(*Z*)9-heneicosadien-11-one (**4**; Scheme 1) and (*Z*)6,(*E*)9-heneicosadien-11-one (**7**; Scheme 2).

combined with the organic phase, dried over Na_2SO_4, and distilled *in vacuo* to yield **1** (7 g, 92–96°C/15–18 mm) with a GC purity of 93%. (lit: 75–77°C/12 mm) (Rachlin et al., 1961; Verkruijsse and Hasselaar, 1979). Immediately after distillation the compound began to turn red–yellow and it was used without further purification.

6,9-Heneicosadiyne-11-ol **(2)** *(Scheme 1).* A freshly prepared solution containing 1 equiv of ethylmagnesium bromide was added to **1** (6.13 g, 46 mmol) in ether (150 ml) and the mixture was refluxed 2 hr. After cooling the reaction mixture to −30°C, 0.1 equiv of CuI was added and the mixture stirred 2.5 hr. Following dropwise addition of undecanal (9.41 ml, 46 mmol), the mixture was stirred 1 hr at −20°C, and overnight at rt, and then quenched by the addition of aqueous NH_4Cl. Extraction (3 × 100 ml) with ether:hexane (1:1), separation and washing of organic layers with saturated NaCl, drying, and chromatography (ether/hexane) gave **2** (11.8 g, 85% yield), which was immediately used for the next step. *Anal.* Calcd. for $C_{21}H_{36}O$: C, 82.85; H, 11.92. Found: C, 82.35; H, 11.80.

(Z)6,(Z)9-Heneicosadien-11-ol **(3)** *(Scheme 1).* Alcohol **2** (4.5 g, 14.8 mmol) was hydrogenated using P-2 Ni (or Lindlar catalyst with quinoline), with GC monitoring of the reaction until intermediate monoynes had disappeared.

The resulting dienols were separated by chromatography [SiO_2 (200 g) plus Ag NO_3 (40 g)] using Et_2O/hexanes/toluene (5:45:50) as eluents. Through differential retainment of isomers, pure **3** (2 g, 44% yield) was obtained. *Anal.* Calcd. for $C_{21}H_{40}O$: C, 81.74; H, 13.07. Found: C, 81.90; H, 12.95. ^1H NMR: 5.27–5.49 (m, 4H), 4.46 (dt, 1H), 2.83 (m, 2H), 2.04 (m, 2H), 1.19–1.63 (m, 24H), 0.85 (m, 6H). ^{13}C NMR: 132.94, 130.83, 130.32, 127.15, 67.78, 39.75, 37.54, 31.57, 31.53, 29.61, 29.31, 29.27, 27.26, 26.09, 25.38, 22.66, 22.63, 22.55, 14.04, 13.99.

(Z)6,(Z)9-Heneicosadien-11-one ***(4)*** *(Scheme 1).* Alcohol **3** (100 mg, 3.25×10^{-1} mmol) in CH_2Cl_2 (0.8 ml) at rt was stirred with K_2CO_3 (34 mg, 2.4×10^{-1} mmol) and PCC (105 mg, 4.9×10^{-1} mmol). After 45–50 min of vigorous stirring, hexanes (5 ml) were added and the mixture was flushed through a silica column (20 g) using hexane:ether (99:1) as eluent. The dienone containing fraction, as monitored by GC, was evaporated *in vacuo* at 20–25°C to give **4** (90 mg, 91% yield): *Anal.* Calcd. for $C_{21}H_{38}O$: C, 82.28; H, 12.50. Found: C, 81.92; H, 12.70. ^1H NMR (taken immediately after evaporation): 6.13 (dt, 1H), 5.98 (dt, 1H), 5.44 (m, 1H), 5.36 (m, 1H), 3.38 (dd, 2H), 2.43 (t, 2H), 2.03 (dt, 2H), 1.60 (m, 2H), 1.18–1.38 (m, 20H), 0.85 (m, 6H).

(E)9-Heneicosen-6-yn-11-ol ***(5)*** *(Scheme 2, Figure 1).* Fresh alcohol **2** (3.04 g, 10 mmol) was refluxed 20 hr in ether with lithium aluminum hydride (0.7 g, 20 mmol), and the reaction mixture quenched by slow addition of 2 N NaOH. The precipitate was filtered and washed (3 × 150 ml) with ether. Organic solutions were combined, dried, and evaporated to yield **5** (2.5 g, 82% yield, 97% by GC). ^1H NMR: 5.72–5.79 (M, 1H), 5.60–5.68 (m, 1H), 4.10 (dt, 1H), 2.92 (m, 2H), 2.17 (m, 2H), 1.18–1.55 (m, 24H), 0.87 (m, 6H).

(Z)6,(E)9-Heneicosadien-11-ol ***(6)*** *(Scheme 2).* Alcohol **5** (3.04 g, 10 mmol) was added to a suspension of Lindlar catalyst (0.4 g, 5% Pd on calcium carbonate, poisoned with lead), hexanes (100 ml), and quinoline (1 ml). The presaturated hydrogen mixture was vigorously stirred at rt and hydrogen bubbled through 6 hr, after which the reaction was complete, as monitored by GC over a 10-min period. Ten percent HCl (50 ml) was added with stirring, followed by ether/hexane (1:1) extraction. Combined extracts were washed with water, dried, and evaporated. Purification of crude dienol on a silver column using Et_2O/hexanes/toluene (5:45:50) as eluents yielded **6** (2.4 g, 78%). *Anal.* Calcd. for $C_{21}H_{40}O$: C, 81.74; H, 13.07. Found: C, 81.62; H, 12.93. ^1H NMR: 5.58–5.66 (m, 1H), 5.31–5.51 (m, 3H), 4.04 (dt, 1H), 2.77 (dd, 2H), 2.02 (q, 2H), 1.19–1.57 (m, 24H), 0.81 (m, 6H).

(Z)6,(E)9-Heneicosadien-11-one ***(7)*** *(Scheme 2).* This ketone was obtained (85% yield) from **6** following the same procedure as for conversion of compound **3** to **4** in Scheme 1. ^1H NMR: 6.79 (dt, 1H), 6.10 (td, 1H), 5.54 (m, 1H), 5.37 (m, 1H), 2.95 (t, 2H), 2.51 (dd, 2H), 2.02 (dt, 2H), 1.59 (m, 2H), 1.19–1.48 (m, 20H), 0.88 (m, 6H).

(Z)2-Octenyltriphenylphosphonium Chloride (10) (Scheme 3a, Figure 2). To (Z)2-octenol **(8)** (10 g, 78 mmol) and collidine (10.4 ml, 78 mmol) in DMFA (50 ml) under argon at −30 to −40°C, methanesulfonyl chloride (6 ml, 78 mmol) in DMFA (20 ml) was added dropwise. After stirring 1 hr, the mixture was warmed to 0°C and stirred 1 hr. LiCl (0.1 mol) was added and after 3 hr the mixture was warmed to rt overnight. Water (100 ml) was added and the mixture extracted with hexanes (5 × 50 ml). Combined extracts were washed (2×) with water, dried, and evaporated to yield crude (Z)2-octenyl chloride, which was purified by silica (100 g) column chromatography. Evaporation of the hexane eluent gave the chloride (11.5 g, 95% pure by GC), which was dissolved in acetonitrile (100 ml) to which triphenylphosphine (21 g, 80 mmol) was added. The mixture was refluxed overnight, the solvent evaporated, and the precipitate washed (3 × 100 ml) with hexane/ether (3:1) to yield **10** (26.4 g, 83% yield), m.p. 147–149°C (recrystallized from ethyl acetate). *Anal.* Calcd. for $C_{26}H_{30}ClP$: C, 76.36; H, 7.39. Found: C, 76.44; H, 7.50. ^1H NMR: 7.62–7.88 (m, 15H), 5.70 (m, 1H), 5.37 (m, 1H), 4.69 (m, 2H), 1.63 (m, 2H), 1.10 (m, 2H), 1.03 (m, 4H), 0.74 (t, 3H).

4-Acetoxytetradec-1-ene (4) (Scheme 3b, Figure 2). To freshly prepared vinylmagnesium bromide (30 mmol) in ether (200 ml) was added undecanal (4.8 ml, 22.8 mmol) in ether (100 ml) at 0°C. The mixture was stirred 1 hr and quenched with 2 N aqueous NH_4Cl. The organic layer was separated and the aqueous layer extracted (3 × 50 ml) with ether. Combined extracts and organic fractions were dried over Na_2SO_4 and evaporated to 4-hydroxytetradec-1-ene **(13)** (Matsuda et al., 1989) (4.5 g, 95% by GC). Without further purification, acetic anhydride (3.3 ml, 35 mmol) in pyridine (20 ml) and a catalytical amount of *N,N*-dimethyl-4-aminopyridine (10 mg) were added. The mixture was stirred overnight and evaporated at low pressure. Water (20 ml) was added and the product extracted (4 × 75 ml) with ether. Extracts were combined and dried to yield crude **14** (Chang et al., 1990), which was purified by column chromatography (Et_2O/hexanes, 1:5) (5.22 g, 95% yield from alcohol). *Anal.* Calcd. for $C_{16}H_{30}O_2$: C, 75.54; H, 11.89. Found: C, 75.60; H 12.20.

3-Acetoxytridecenal (15a) (Scheme 3b). 4-Acetoxytetradec-1-ene **(14)** (3 g, 11.8 mmol) was added to $NaIO_4$ (15 g, 71 mmol) and OsO_4 (60 mg) in 100 ml of dioxane/water (9:1). After 8 hr, water (100 ml) was added to the vigorously stirred solution and organic compounds were extracted (4 × 50 ml) with ether. Combined extracts were washed, dried, and evaporated at 35–37°C *in vacuo*. The resulting **15a** became colored when exposed to air, and according to NMR it was contaminated with 5–7% 2-tridecenal **(15b)** (Chang et al., 1990) [^1H NMR −CH=O 9.77 (**t**) and 9.53 (*d*)]. (Attempts to purify **15a** by chromatography led to further elimination of acetic acid and formation of up to 70% of **15b.**)

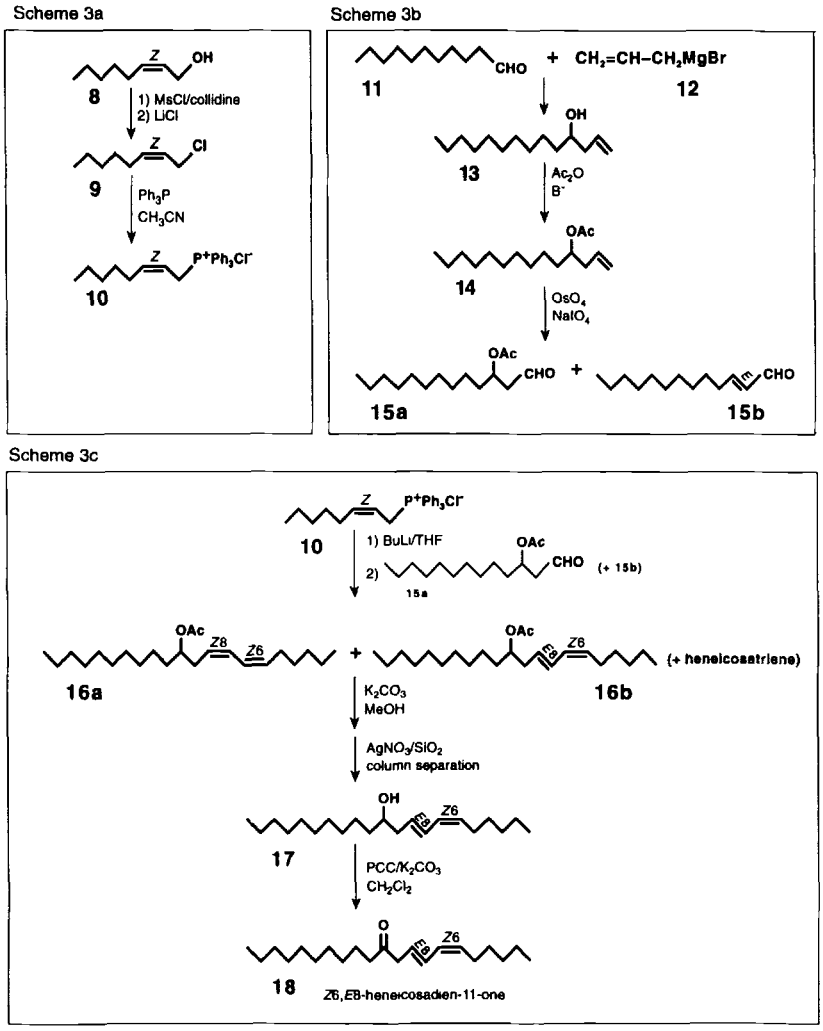

FIG. 2. Routes for the syntheses of (Z)2-octenyl-triphenylphosphonium chloride (**10**; Scheme 3a), 3-acetoxytridecenal (**15a**; Scheme 3b), and (*Z*)6,(*E*)8-heneicosadien-11-one (**18**; Scheme 3c).

(Z)6,(E)8- and (Z)6,(Z)8-Heneicosadien-11-acetates (16a, 16b) (Scheme 3c, Figure 2). All the crude **15a** (Scheme 3b) was dissolved in THF (100 ml) and added slowly to a stirred solution (−78°C) of ylid formed by treating **10** (Scheme 3a) (4.9 g, 12 mmol) in THF (100 ml) with 2.5 M butyllithium in

hexane (5.2 ml) for 2 hr at −70°C. The mixture was warmed to −30 to −20°C and stirred 2 hr, warmed at rt, and quenched with 2 N aqueous NH_4Cl, extracted with ether, dried, and evaporated *in vacuo*. Compounds **16a** and **16b** and a C_{21}-triene formed a 4:6:1 ratio (GC). Diene acetates were purified by chromatography (yield, 2.9 g, 70%). Even with argentation, chromatography separation of *Z,E* and *Z,Z* isomers was difficult. *Anal.* Calcd. for $C_{23}H_{42}O_2$ (mix of isomers): C, 78.79; H, 12.07. Found: C, 78.44; H, 12.12.

(Z)6,(E)8-Heneicosadien-11-ol (17) (Scheme 3c). A mixture of **16a** and **16b** (1 g, 2.85 mmol) was stirred 3 hr with K_2CO_3 (200 mg) in CH_3OH (10 ml). Water (20 ml) was added and alcohols were extracted with ether. Extracts were combined, dried, and evaporated *in vacuo* (0.89 g, 92% yield). The alcohols were separated on an argentation silica column and eluted with a mixture of Et_2O/hexanes/toluene (1.5:48.5:50). Seventy milligrams of earlier-eluting **17** was collected and the remaining mixture, containing both isomers, was used in subsequent column separation. *Anal.* Calcd. for $C_{21}H_{40}O$: C, 81.74; H, 13.07. Found: C, 81.68; H, 13.28. ^{1}H NMR: 6.42 (m, 1H), 5.97 (dd, 1H), 5.64 (m, 1H), 5.37 (dt, 1H), 3.62 (m, 1H), 2.34 (m, 2H), 2.16 (m, 2H), 1.23–1.51 (m, 24H), 0.87 (dt, 6H).

(Z)6,(E)8-Heneicosadien-11-one (18) (Scheme 3c). This ketone (95% yield) was obtained from **17** following the same procedure as for conversion of compound **3** to **4** in Scheme 1. *Anal.* Calcd. for $C_{21}H_{38}$: C, 82.28; H, 12.50. Found: C, 81.82; H, 12.80. ^{1}H NMR: 6.40 (m, 1H), 5.97 (dd, 1H), 5.73 (dt. 1H), 5.41 (dt, 1H), 3.20 (d, 2H), 2.43 (t, 2H), 2.14 (m, 2H), 1.58 (m, 2H), 1.21–1.40 (m, 20H), 0.88 (m, 6H). An improved alternative synthesis for **18** employs $Pd(PPh_3)_4$-catalyzed coupling of (*Z*)-1-iodo-1-heptene with (*E*)-4-(tert-butyldimethylsiloxy)-1-tetradecenylboronic acid (Wimalaratne and Slessor, unpublished).

Field Experiments

Field experiments were conducted in mature Douglas-fir stands west and north of Kamloops, British Columbia. Unitraps (Expts. 1 and 2) (Phero Tech Inc., Delta, British Columba V4G 1E9) or sticky 2-liter Delta milk cartons (Gray et al., 1984) (Expts. 3–6) were suspended from Douglas-fir trees 1.5 m above ground in complete randomized blocks with traps and blocks at >20-m intervals. A small cube of vapona (18.5% dichlorvos; Green Cross, Division of Ciba Geigy Canada Ltd., Mississauga, Ontario L4Z 2Z1) in Unitraps assured retention of captured moths. Traps were baited with cotton dental wicks (Richmond Dental Company, Charlotte, NC 28234) (Expts. 1 and 3–6) cut to 1-cm length or with gray sleeve stoppers (West Company, Lionville, PA 19341) (Expt. 2) baited with candidate pheromone components. Z6 purchased from Phero Tech Inc., was >98% chemically and geometrically pure.

Experiment 1 tested Z6 (1 μg) alone and in combination with Z6E8, Z6Z9, or Z6E9 at 0.01 μg each (Figure 4). During peak flight activity of DFTM males, these HPLC-purified dienones in acetonitrile were carried on Dry Ice to traps and were syringed onto cotton wicks affixed to Unitrap lids. DFTM trap catches were recorded 40 min later. In all subsequent experiments lures were also prepared in the field. The second experiment tested Z6 (100 μg) alone and in combination with Z6E8 (1 μg) (Figure 5). The third experiment tested Z6 (10 μg) alone and in combination with Z6E8 at increasing doses of 0.01, 0.1, and 1 μg (Figure 6). Experiment 4 tested Z6 plus Z6E8 at 10:1 and 10:10 ratios.

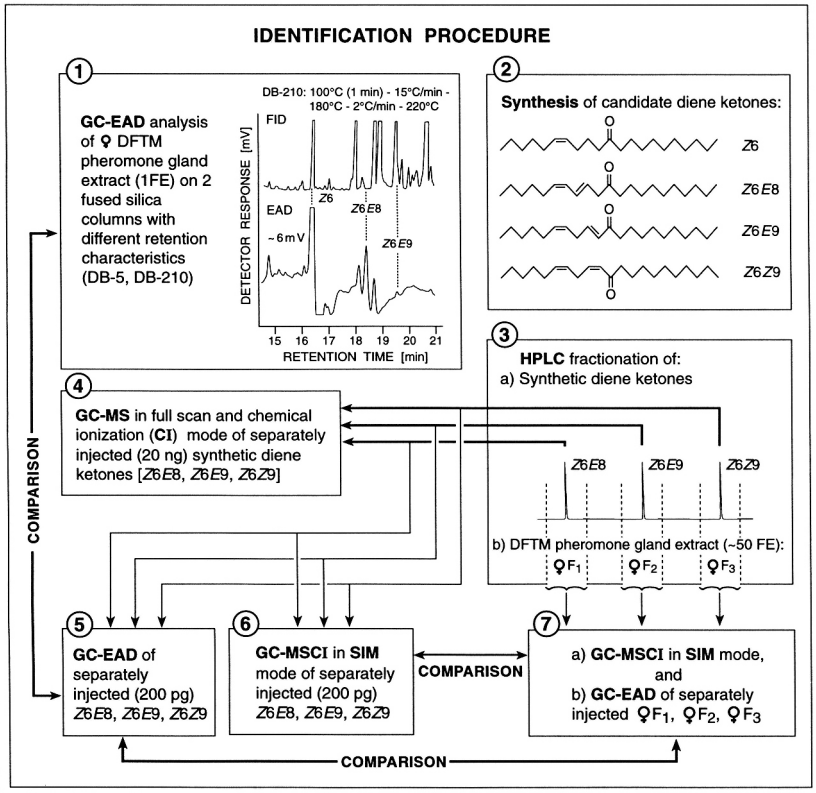

FIG 3. Procedure for the identification of (*Z*)6,(*E*)8-, (*Z*)6,(*E*)9, and (*Z*)6,(*Z*)9-heneicosadien-11-one (Z6E8, Z6E9, Z6Z9) in female Douglas-fir tussock moth (DFTM) pheromone gland extract. FE, female equivalent of pheromone extract; female F1, fraction 1 of female DFTM pheromone gland extract; GC-EAD, gas chromatographic—electroantennographic detection; HPLC, high-performance liquid chromatography; GC-MSCI, gas chromatographic-mass spectrometric analyses in chemical ionization mode; SIM, selected ion monitoring mode.

The fifth experiment tested Z6 (10 μg) in combination with Z6E8 (1 μg) versus either compound alone at 10 μg each (Figure 7). The sixth and final experiment tested Z6 alone at 1, 10, 100, or 500 μg and in binary combination with Z6E8 at 10:1 ratios (Figure 8).

RESULTS AND DISCUSSION

WMTM pheromone analyses by GC-EAD revealed antennal responses to Z6 and several unknown components, one of which gave GC-MS fragmentation ions indicative of a diunsaturated C_{21} ketone. Because a diene ketone, 1,6-heneicosadien-11-one, had previously been identified in DFTM pheromone extracts (Smith et al., 1978), and considering that (*Z*)6,(*Z*)9-diene epoxides are common pheromones in geometrid and arctiid moths (Arn et al., 1992), we synthesized (*Z*)6,(*Z*)9-heneicosadien-11-one (Z6Z9) (Figure 3, box 2). Synthetic Z6Z9 in solution at room temperature and during GC analyses rearranged to (*Z*)6,(*E*)8- and (*Z*)6,(*E*)9-heneicosadien-11-one (Z6E8, Z6E9) and other dienones, as determined by NMR spectroscopy and syntheses of the tentatively

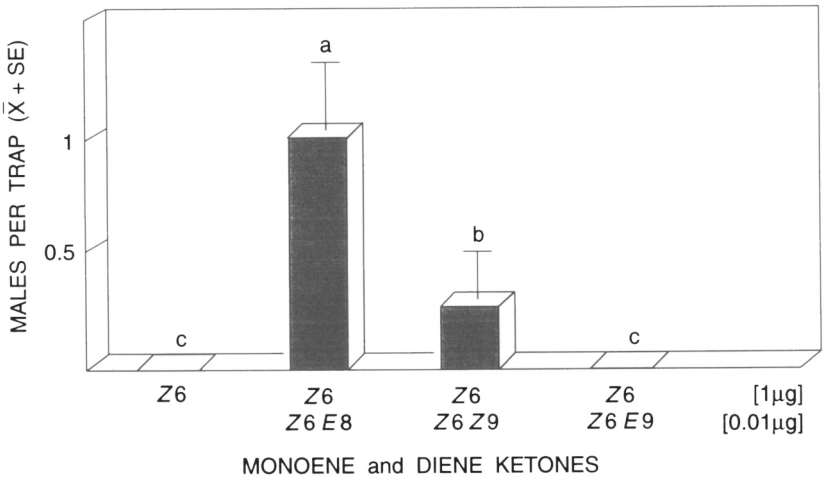

FIG. 4. Capture of male Douglas-fir tussock moths during a 40-min experiment in Unitraps baited with (*Z*)6-heneicosen-11-one (*Z6*) alone and in combination with either (*Z*)6, (*E*)8-, (*Z*)6,(*E*)9-, or (*Z*)6,(*Z*)9-heneicosadien-11-one [Z6E8, Z6E9, Z6Z9]. Kamloops, British Columbia, 7 September 1993, 1830 to 1910; 12 replicates. The experimental time was confined to 40 min to minimize the rearrangement of Z6Z9 to Z6E8 while allowing capture of sufficient numbers of male DFTM to reveal the synergistic pheromone component (most attractive treatment). Bars with the same superscript are not significantly different. Nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) *t* test, $P < 0.05$] (SAS/STAT, 1988).

identified rearrangement products. Identical GC retention times of Z6Z9 and Z6E8 indicated conversion of Z6Z9 to Z6E8, similar but not analogous to deconjugations reported for α,β-unsaturated ketones (Ricard et al., 1986).

GC-EAD analyses of DFTM pheromone extract revealed several compounds consistently eliciting responses by male DFTM antennae, with Z6 being most abundant and (thus) most EAD active (Figure 3, box 1). Comparative GC-EAD on DB-210 and DB-5 columns of pheromone extract and synthetic dienones resulted in retention time matches of two EAD-active, female-produced compounds with synthetic Z6E8 and Z6E9, respectively.

With known HPLC retention times of synthetic Z6E8, Z6E9, and Z6Z9 (which do not rearrange during HPLC), corresponding fractions of DFTM pheromone extract were isolated (Figure 3, box 3). Each synthetic dienone was then analyzed by GC-MS in full scan and CI mode to determine diagnostic ions for SIM (Figure 3, box 4), which provides highly increased sensitivity. GC-MSCI-SIM of synthetic dienones and corresponding DFTM extract fractions (Figure 3, boxes 6 and 7a) resulted in good retention time and ion ratio matches between synthetic and female-produced Z6E8 and Z6E9, respectively [GC-MSCI retention time, *m/z* (relative intensity): synthetic Z6E8—18.6 min, 307 (100), 308

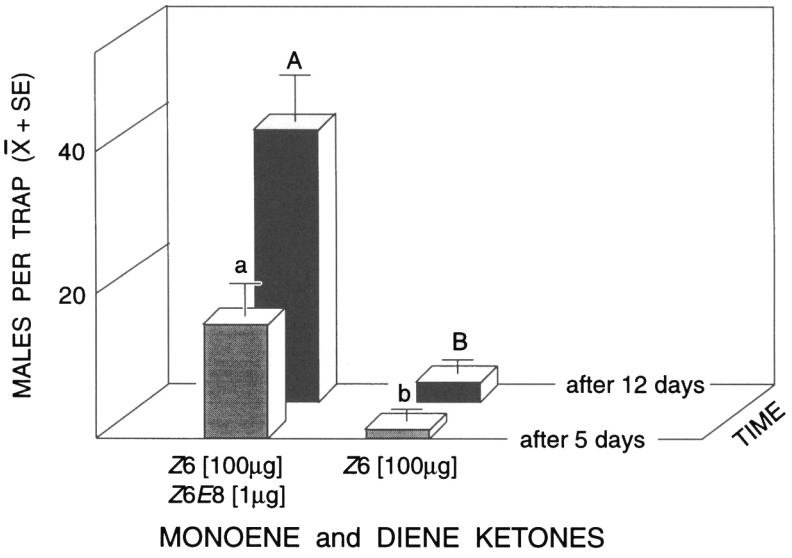

FIG. 5. Capture of Douglas-fir tussock moth males in Unitraps baited with rubber septa impregnated with (*Z*)6-heneicosen-11-one (Z6) alone or in combination with (*Z*)6,(*E*)8-heneicosadien-11-one (Z6E8). Vinsulla, British Columbia, 5–10 September, 1994; 12 replicates. Lures were not changed after 5 days. For each trapping period, treatments were significantly different. Nonparametric Mann-Whitney test, $P < 0.05$.

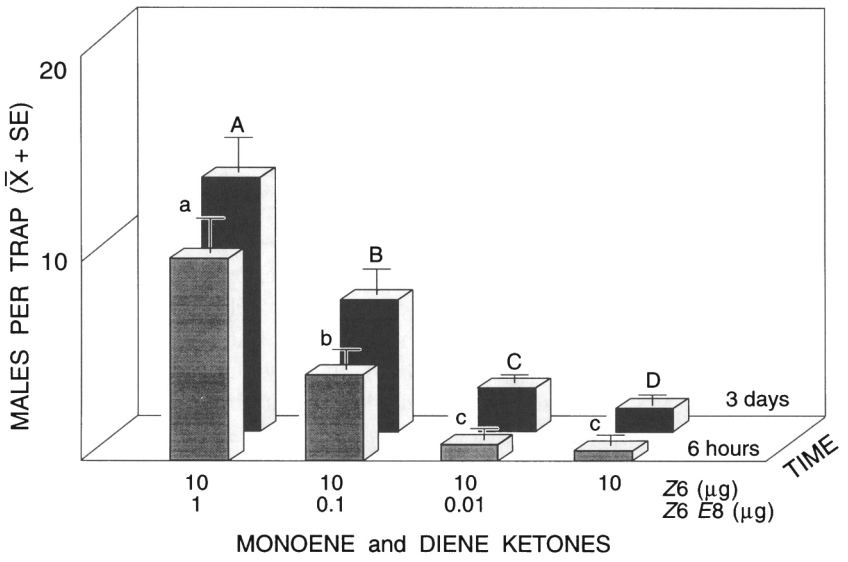

FIG. 6. Captures of Douglas-fir tussock moth males in 2-liter Delta milk carton traps baited with cotton wicks impregnated with (Z)6-heneicosen-11-one (Z6) alone and in combination with (*Z*)6,(*E*)8-heneicosadien-11-one (Z6E8) at increasing proportions. Lures were not changed between trapping periods. Vinsulla, British Columbia, 10–13 September, 1994; 10 replicates. For each trapping period, bars with the same superscript are not significantly different. Nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [(Bonferroni (Dunn) *t* test, $P < 0.05$].

(24); DFTM gland extract—18.6 min, 307 (100), 308 (26); synthetic Z6E9—19.7 min, 307 (100), 308 (23); DFTM gland extract—19.7 min, 307 (100), 308 (33); synthetic Z6Z9[5]—18.6 min, 307 (100), 308 (27); DFTM gland extract—18.6 min, 307 (100), 308 (21)]. During GC–MSCI–SIM, HPLC-fractionated synthetic Z6Z9 and DFTM-dienone in the corresponding HPLC extract fraction (Figure 3, box 3) rearranged to compounds with identical retention and ion ratio characteristics, thereby confirming the presence of Z6Z9 in the DFTM pheromone extract. Moreover, comparative GC–EAD of synthetic dienones (Figure 3, box 5) and corresponding DFTM extract fractions (Figure 3, box 7b) revealed comparable antennal response patterns.

Twenty-four pre-screening field tests, evaluating various trap designs (sticky traps and nonsaturating Unitraps), release devices (rubber septa, cotton, and silica), lure doses (0.01–0.1 μg), and experimental times (40 min to 4 days), led to the critical experiment which determined the synergistic DFTM phero-

[5]Synthetic (*Z*)6,(*Z*)9-heneicosadien-11-one was injected but, due to thermal instability, rearranged on injection, primarily to (*Z*)6,(*E*)8-heneicosadien-11-one.

mone component. Syringing HPLC-purified and Dry Ice-stored dienones onto cotton wicks affixed to lids of presuspended traps, and recording the number of trap-captured DFTM males 40 min later, Z6 alone and in combination with Z6E9 had not attracted any males (Expt. 1, Figure 4). Attraction of males to Z6E8 in combination with Z6 (Expt. 1, Figure 4) indicated that this dienone is a potent sex pheromone component in DFTM. The apparent attractiveness of Z6Z9 in combination with Z6 can be attributed to partial rearrangement of Z6Z9 to Z6E8 during the 40-min test. Attractiveness of Z6 plus Z6E8 dispensed from rubber septa greatly exceeded that of Z6 alone (Expt. 2, Figure 5). Increasing doses of Z6E8 added to Z6 resulted in increasing captures of DFTM males (Expt. 3, Figure 6). At 10:1 or 10:10 ratios, Z6 plus Z6E8 (Expt. 4) attracted on average 16.2 ± 1.7 (SE) and 22.2 ± 2.5 DFTM males (10 replicates; nonparametric Mann–Whitney test, $P > 0.05$). While Z6 or Z6E8 singly were not very attractive, combined they were highly affective in attracting DFTM males (Expt. 5, Figure 7). In the final dose—response experiment, Z6 (10 μg) plus Z6E8 (1 μg) was as attractive as 500 μg of Z6 alone (Expt. 6, Figure 8). Because population densities of sympatric *Orgyia* spp. were very low, congeners of the DFTM were not captured in any of the experiments.

Following the discovery of (Z)6,(Z)9-nonadecadien-3-one in *Peribatodes*

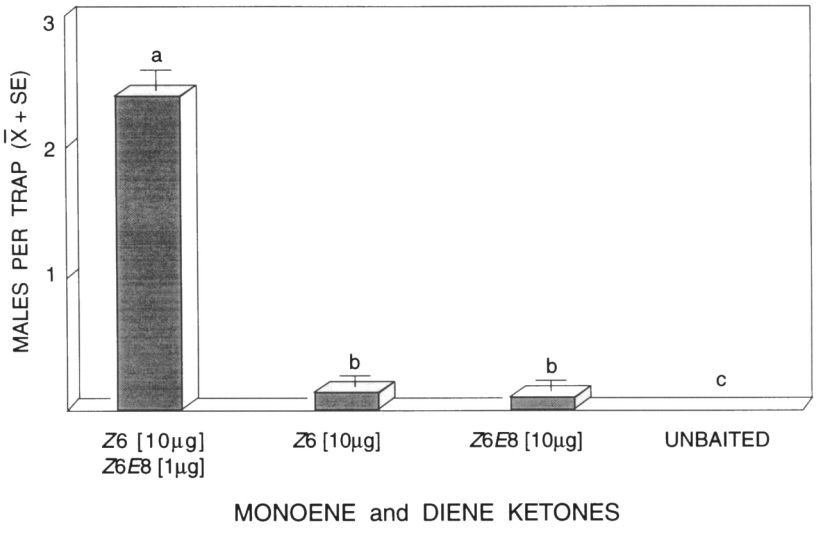

FIG. 7. Captures of Douglas-fir tussock moth males in 2-liter Delta milk carton traps baited with cotton wicks impregnated with (Z)6-heneicosen-11-one (Z6), (Z)6,(E)8-heneicosadien-11-one (Z6E8), or both combined. Vinsulla, British Columbia, 17 September 1994; 1400 to 1845; 10 replicates. Bars with the same superscript are not significantly different. Nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) t test, $P < 0.05$].

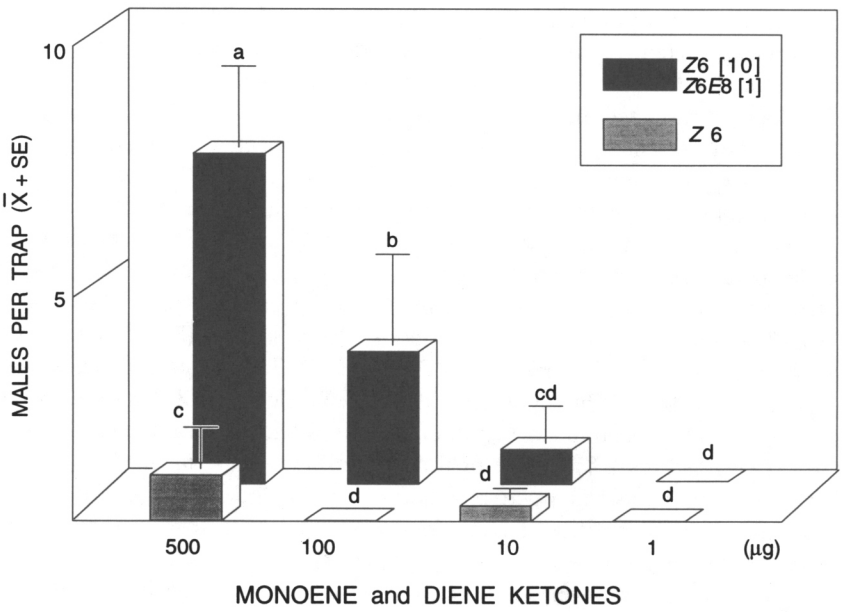

FIG. 8. Captures of Douglas-fir tussock moth males in 2-liter Delta milk carton traps baited with cotton wicks impregnated with increasing doses of (*Z*)6-heneicosen-11-one (Z6) alone or in 10:1 combination with (*Z*)6,(*E*)8-heneicosadien-11-one, Vinsulla, British Columbia, 17–24 September 1994; five replicates. Bars with the same superscript are not significantly different. Nonparamteric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student Newman Keuls test, $P < 0.05$).

rhomboidaria (Buser et al. 1995), Z6E8 is the second dienone sex pheromone component found in the Lepidoptera. Chemical lability and subnanogram quantities of dienones in DFTM pheromone extract required comparative GC–EAD, HPLC, and GC–MSCI–SIM of synthetic and DFTM-produced dienones for identifications. Determination of Z6E8 as the synergistic DFTM pheromone component was contingent upon an experimental design that minimized rearrangement of dienones prior to and during the field test (Expt. 1). Because Z6 is a congeneric sex pheromone component (attractant) in *Orgyia* and *Dasychira* tussock moths (Arn et al., 1992), a blend of Z6 plus Z6E8 (at ratios of 10:0.1, 10:1, or 10:10) may impart specificity to DFTM pheromone communication. It will be of great interest to investigate additional sex pheromone components in other tussock moths. Female WMTM, for example, do not utilize Z6E8 for pheromone communication (Slessor and Grant, unpublished), but their pheromone gland extracts greatly lose attractiveness within 24 hr at room temperature (Slessor and Grant, unpublished), suggesting the presence of a (thermo)labile,

as yet unknown sex pheromone component. Following commercial formulation, the identified two-component DFTM pheromone blend may allow species-specific and highly sensitive monitoring of DFTM populations. It may also greatly enhance the efficacy of pheromone-based control of this important forest defoliator (Sower et al., 1990; Hulme and Gray, 1994).

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OVIPOSITION AND ELECTROANTENNOGRAM RESPONSES OF *Dioryctria abietivorella* (LEPIDOPTERA: PYRALIDAE) ELICITED BY MONOTERPENES AND ENANTIOMERS FROM EASTERN WHITE PINE

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Abstract—Volatile emissions from twigs of eastern white pine, *Pinus strobus* L., stimulated oviposition by *Dioryctria abietivorella* (Grote) in laboratory bioassays. Chemical identification and percentage composition of monoterpenes emitted by twigs were determined by capillary GC and GC/MS analysis of headspace and of volatiles entrained on Porapak Q. Enantiomeric excess of optically active monoterpenes was determined by capillary GC analysis with a β-cyclodextrin chiral column. Four of the six most abundant monoterpenes identified in twig volatiles elicited a significant oviposition response. Myrcene and car-3-ene [predominantly the (+)-enantiomer] were the most active compounds in both electroantennogram (EAG) and oviposition bioassays. In contrast, (−)-limonene elicited a significant oviposition response but was the least stimulating monoterpene in EAG tests. Female EAG responses to monoterpenes generally increased with age and mating and were larger than those from males of the same age.

Key Words—Electroantennogram, enantiomers, headspace, insect-plant interaction, monoterpenes, oviposition, Porapak Q, volatile emissions

INTRODUCTION

Dioryctria abietivorella (Grote) [long confused with the European *D. abietella* (Denis & Schiffermüller) in older literature] is economically important in Canada

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and the United States because it attacks many valuable *Pinus* species, including eastern white pine, *P. strobus* L. (Heinrich, 1956; Hedlin et al., 1980). Larvae are cryptic internal feeders on both terminal shoots and seed cones and are not easily detected before visible damage occurs. Consequently, *D. abietivorella* is a difficult species to control. Use of sex pheromones to monitor males, or oviposition attractants and stimulants to monitor gravid females, would provide useful pest management tools (Grant, 1990; Grant and Turgeon, 1994), but neither of these semiochemical agents is currently available for *D. abietivorella*. Successful use of host attractants as a monitoring tool and oviposition disruptant for another pyralid species, *Amyelois transitella*, has been reported (Walker) (van Steenwyk and Barnett 1985, 1987).

Females of *Dioryctria* species are attracted and their oviposition stimulated by host volatiles emitted from pruning cuts and tree wounds (Wright et al., 1975; Jactel et al., 1994; Valterová et al., 1995), from damage caused by other insects (Heinrich, 1956), or from rust galls (Fatzinger and Merkel, 1985; Hanula et al., 1985). Monoterpenes, major volatile constituents of coniferous trees, are important host finding and oviposition cues for some Lepidoptera (Åhman et al., 1988; Leather, 1987; Städler, 1974; Ross et al., 1995). Individual host monoterpenes and blends of monoterpenes stimulate oviposition of the southern pine coneworm, *Dioryctria amatella* (Hulst) (Fatzinger and Merkel, 1985; Hanula et al., 1985). Many monoterpenes are optically active and hence specific enantiomers may be important as oviposition cues (Valterová et al., 1995). The recent availability of chiral capillary columns allows convenient enantiomeric analysis of monoterpenes at the nanogram level (Ochocka et al., 1991; König et al., 1992). We report the identification and enantiomeric composition of monoterpenes emitted by eastern white pine and describe their effects on the oviposition and electroantennogram (EAG) responses of *D. abietivorella* adults.

METHODS AND MATERIALS

Insects. D. abietivorella were obtained from a >8-year-old laboratory colony (Canadian Forest Service, Sault Ste. Marie, ON) which had been established originally from moths emerging from white pine shoots damaged by the white pine weevil, *Pissodes strobi* (Peck). New field-collected insects were added to the colony periodically to maintain genetic diversity. Oviposition in screened rearing cages was induced by supplying fresh white pine twigs covered with several layers of cotton gauze. Females oviposited on the gauze, which facilitated recovery of eggs. Larvae were reared on an artificial diet developed for the spruce budworm, *Choristoneura fumiferana* (Clemens) (McMorran, 1965). Pupae were sexed and placed in separate containers to collect virgin moths. Pupae and moths were maintained at 50% RH under a 16L:8D photoperiod in

an incubator held at 22°C during the photophase and 20°C during the scotophase.

Bioassays. Oviposition bioassays were conducted in screen cages in a manner similar to other oviposition assays with *Dioryctria* spp. (Fatzinger and Merkle, 1985; Hanula et al., 1985). The cages were 36 cm wide × 24.5 cm deep × 30.5 cm high and were housed in incubators under light and temperature conditions described above. A wire was strung across the width of the cage near the top and about 8 cm from the front to support a test stimulus and a control, which were held 20 cm apart.

Host stimuli were evaluated in several laboratory experiments. The first experiment tested two 5-cm pieces of freshly cut white pine twigs (samples were collected between 29 Oct. 1992 and 3 Jan. 1993), wrapped together in several layers of white cotton gauze (cheesecloth). Twigs were cut from branches taken from the midcrown of mature trees. A different tree was used to supply twigs for each replicate bioassay. Volatiles emitted by the cut ends served as surrogates for volatiles emitted by pruned or insect-damaged trees. The twigs were compared to a control, which consisted of a 6-ml glass vial, approximately the same diameter as the combined twigs, and also covered with gauze. The second experiment evaluated individually the six major monoterpenes identified in the volatile emissions of white pine twigs. In the case of optically active monoterpenes, only the (−)-enantiomers were tested because they predominated in host emissions. An exception was car-3-ene, which was available commercially only as the (+)-enantiomer (Aldrich Chemical Co., Milwaukee, WI). Test monoterpenes were pipetted into separate 6-ml vials fitted with a cap containing a hole. Equivalent release rates for each monoterpene were obtained by adjusting the size of the hole in the cap, by adjusting the volume of monoterpene (50 or 100 μl), and in some cases by also diluting the monoterpene with mineral oil (Table 2). The release rate of each monoterpene tested was estimated by daily weight-loss measurements (mg/day) averaged over a 4-day period. Bioassay cages contained a vial with one of the test monoterpenes and a control consisting of either an empty, gauze-covered vial or one containing mineral oil if the test monoterpene was diluted with it. The third experiment tested a blend of four monoterpenes [(−)-α-pinene, (−)-β-pinene, myrcene, and (-)-limonene] whose volatile composition (as determined by GC analysis of headspace above the mixture) matched 96.5% of the volatile composition (see below) of one batch of twigs that had been collected in November when bioassays of the twigs were performed. The monoterpene blend was placed in a gauze-covered vial and suspended in a bioassay cage along with a gauze-covered control vial.

For each test, five pairs of virgin moths <1 day old were placed in a bioassay cage. Honey solution in a vial with a cotton wick was available at the bottom of the cage. Treatment and control vials were replaced after 4 days and then every 3 days for an additional three consecutive changes. *Dioryctria* are

long-lived species that lay eggs singly. Thus experiments lasted for 16 days to allow time for the newly emerged insects to mature, mate, and lay eggs. Assays of similar duration were used by Fatzinger and Merkle (1985) and Hanula et al. (1985) in their study of *D. amatella* oviposition. The number of eggs on the gauze covering the test and control vials were recorded after each change in vials. At the end of an experiment, the cage was divided into two halves, one containing the treatment and the other the control, and the number of fertile eggs (including hatched eggs) on the screen in both sections was recorded. Experiments were conducted throughout the year and were replicated 6–12 times. Data were analyzed by the Wilcoxon matched-pairs, signed-rank test (Zar, 1984).

Electroantennograms (EAGs). EAG bioassays were conducted to determine the degree of concordance between the behavioral bioassay and the sensory responses to host kairomones. EAGs were obtained as described by Grant (1971). Stimuli consisted of the six major monoterpenes identified in white pine twig emissions. Each monoterpene was serially diluted with hexane. Ten microliters of a test solution containing 10 μg of monoterpene were deposited inside a clean glass tube cartridge (6-mm ID.) which was subsequently connected to an air-stream directed at an antennal preparation as soon as the solvent had evaporated. The order of chemical stimuli presented to each antennal preparation was random. Responses from each preparation were considered a block, and blocks were nested within levels of moth classes (age, sex, mating status), while terpenes and moth classes were crossed in a nested-factorial design (Montgomery, 1984). Moth classes included 1- and 4-day-old virgin females, 4-day-old mated females, and 4-day-old virgin males. Mated females were obtained by pairing virgin males and females 1 day before EAG testing. Mating status of females was confirmed by dissecting them to check for the presence of spermatophores. Data were analyzed by ANOVA with SAS 3.1 for Windows software (SAS/STAT, 1990) and significantly different means were separated by Fisher's LSD test.

Gas Chromatography (GC). Percentage composition and identification of monoterpenes volatilized from white pine twigs were determined by headspace analysis (Corkhill, 1988) and entrainment on Porapak-Q (hereafter PQ) (Byrne et al., 1975). In both cases, 1- to 2-cm-diameter white pine twigs were cut into 6-cm lengths and placed in 0.5-L clean glass Mason jars (5 twigs/jar), which were held under ambient light and temperature conditions. The jars were fitted with modified lids that allowed headspace sampling with a syringe through a rubber septum and/or collection of volatiles on PQ. In the latter case, air passed into the jar through an inlet glass tube containing 0.9 g activated charcoal and exited the jar through a glass tube containing 100 mg PQ. A vacuum pump connected to the outlet provided a regulated airflow ($\approx$ 100 ml/min) through the jar and bed of PQ. The PQ was extracted with 3 $\times$ 1 ml of hexane containing a known quantity of (−)-fenchone (Aldrich Chemical Co.) as an internal stan-

dard. Twigs used for the PQ analysis were collected in July and August, when *D. abietivorella* females normally oviposit, and were refrigerated at 4°C for up to 48 hr, which matched their storage prior to bioassay. Twigs used for headspace analysis (and bioassays) were collected weekly between October and January and held in a freezer until used. The composition of monoterpenes emitted from a vial containing the synthetic blend of four monoterpenes was determined by GC analysis of the headspace after the vial had equilibrated for 1 hr at room temperature.

Capillary GC analyses were performed on a Varian 3500 GC equipped with an SPI injector and an HP-1 column (25 m × 0.20-mm ID × 0.33-μm film thickness; Hewlett Packard, Mississauga ON). Column temperature was held at 40°C for 2 min, increased to 175°C at a rate of 2.5°C/min and held for 10 min, and then increased to 230°C at a rate of 30°C/min and held for 8 min. Injector temperature was held at 70°C for 0.2 min and increased to 250°C at a rate of 180°C/min. Enantiomeric composition of the optically active monoterpenes was determined with a β-cyclodextrin chiral column (β-Dex-120; 30 m × 0.25-mm ID × 0.25-μm film thicknesses; Supelco, Mississauga ON) connected to a 1-m deactivated fused silica retention gap (0.53-mm ID, Supelco) in a Varian 3700 GC with an injector temperature of 250°C and a detector temperature of 300°C. Column temperature was held at 35°C for 1 min, then increased at 1°C/min to 160°C, which was held for 30 min. Identities of compounds were verified by comparing their retention times with authentic standards as follows: (+)-α-pinene, (−)-α-pinene, (+)-β-pinene, (−)-β-pinene, (−)-camphene, (+)-camphene, car-3-ene [predominantly (+)-enantiomer], (+)-limonene, (−)-limonene, myrcene, (−)-borneol, and (−)-bornyl acetate obtained from Aldrich Chemical Co. and (−)-α-phellandrene, tricyclene, and (+)-sabinene from Fluka Chemicals (Ronkonkoma, NY). β-Phellandrene was not available commercially but a small sample of (+)-β-phellandrene was obtained by extraction from lovage, *Levisticum officinale* (König et al., 1992) (prepared in our laboratory by E. Brockerhoff). (−)-β-phellandrene and (−)-car-3-ene were obtained from W. König (Germany), and α-terpinolene from A. Rocques (France). The identification of monoterpenes were confirmed by GC/MS on a Hewlett-Packard 5989A mass spectrometer fitted with a nonpolar DB-5ms column (30 m × 0.25 mm, 0.1-μm film; J&W Scientific, Folsom, CA). Mass spectra were matched against a computerized library of reference spectra.

RESULTS

GC Analysis of Monoterpenes. Capillary GC analysis of PQ extracts showed that the most abundant monoterpenes in the white pine emissions were α-pinene, β-pinene, myrcene, and limonene, with smaller amounts of camphene, β-phel-

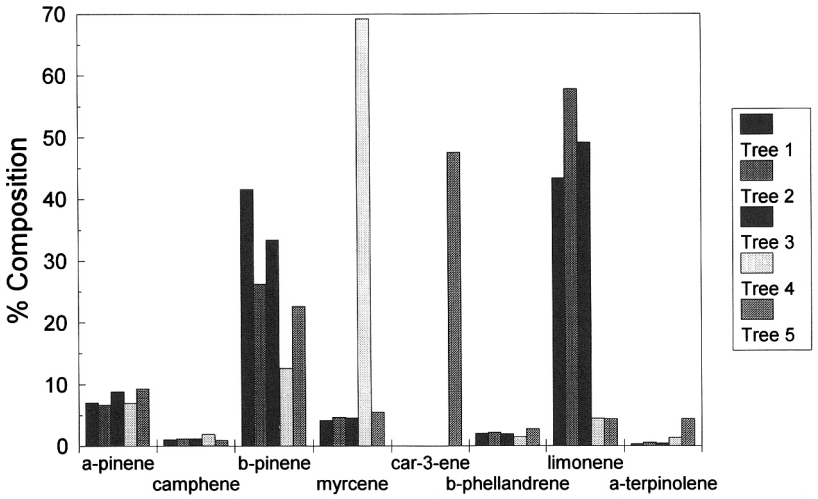

FIG. 1. Composition of major monoterpenes in volatile emissions from white pine twigs obtained from five different trees. Emissions were trapped on Porapak Q and analyzed on a capillary HP-1 column.

landrene, and α-terpinolene (Figure 1) and traces of sabinene, borneol and bornyl acetate. Car-3-ene was absent in four of five trees analyzed by PQ entrainment but represented a high proportion (48%) of the monoterpene composition of the one tree in which it was present (Figure 1). The intertree variation of β-pinene, myrcene, and limonene in the PQ samples was high compared to the other monoterpenes. With the exception of sabinene, the identity of all monoterpenes was confirmed by GC/MS analysis, with a $>90\%$ match between the sample mass spectrum and the library spectrum of an authentic sample. Also present in the PQ extracts were small quantities ($<1\%$ of the monoterpenes) of six additional terpenoid compounds, four of which were tentatively identified by GC/MS as *trans*-caryophyllene (94% match), β-selinene (90% match) or α-humulene (87% match), β-cubebene (93% match), and cadinene (91% match). Authentic standards were not available to confirm retention times of these compounds.

GC analysis of the headspace of white pine twigs from eight trees (data not shown) provided essentially the same composition of the major monoterpenes as found in the PQ extracts except that α-pinene, camphene, and β-pinene tended to represent a greater proportion of these terpenes and the ratio of α-pinene to β-pinene was consistently much greater ($\sim 1:1$) (Figure 2). Car-3-ene was present in only three of the eight trees sampled and represented 28% of the monoterpenes in one tree but $<0.1\%$ of the monoterpenes in the other two trees.

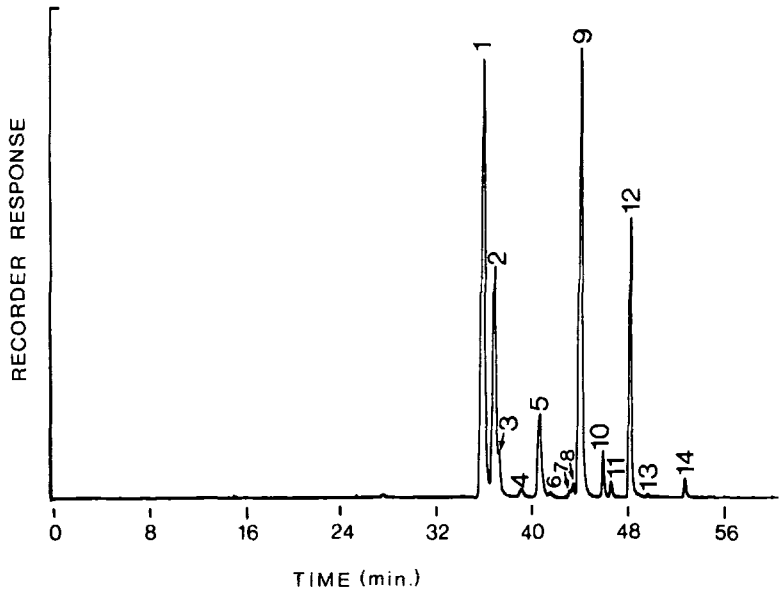

FIG. 2. Chromatogram from β-cyclodextrin chiral column of monoterpenes in headspace of white pine twigs. Car-3-ene was not present in this extract. Peak numbers refer to the following monoterpenes: 1, (+)-α-pinene; 2, (−)-α-pinene; 3, β-myrcene; 4, tricyclene/(+)-sabinene; 5, (−)-camphene/(−)-sabinene; 6, (+)-camphene; 7, (−)-α-phellandrene; 9, (−)-β-pinene; 10, (−)-limonene; 11, (+)-limonene; 12, (−)-β-phellandrene; 13, γ-terpinene; 14, terpinolene.

Not all enantiomers of optically active monoterpenes could be resolved on the chiral β-cyclodextrin column under our operating conditions (Figure 2). For example, tricyclene coeluted with (+)-sabinene, (−)-camphene with (−)-sabinene, and the enantiomers of car-3-ene could not be resolved. However, by comparing results from the chiral and nonchiral columns, it was possible to establish the enantiomeric composition of the most abundant optically active monoterpenes (except car-3-ene), as well as some of the minor terpenes (Table 1). In all resolvable cases, the (−)-enantiomer was predominate. Only in the case of α-pinene did the (+)-enantiomer occur as a substantial proportion (1 : 2) of the enantiomeric ratio in PQ extracts (Table 1).

Effect of White Pine Twigs and Monoterpenes on Oviposition. In the bioassay cages significantly more eggs were deposited on the cotton gauze wrapped around white pine twigs ($\bar{X} = 82.6 \pm 21.6$; $N = 9$) than on the control gauze (1.7 ± 0.8; $N = 9$) (Wilcoxon test, $P < 0.05$). Females also laid significantly more eggs on the screen of the cage nearest the twigs (166.3 ± 43.9) than on the screen in the control section (61.1 ± 15.2). While the number of eggs laid

TABLE 1. ENANTIOMERIC COMPOSITION OF OPTICALLY ACTIVE MONOTERPENES EMITTED BY INDIVIDUAL EASTERN WHITE PINE TREES

	% (−)-Entantiomer					
Monoterpene	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	Mean
α-Pinene	73.0	68.5	61.2	71.7	68.2	68.5
Camphene[a]	100	100	100	100	100	100
β-Pinene	87.1	98.4	98.3	96.0	95.6	95.1
Car-3-ene	—	—	—	NR[b]	—	—
β-phellandrene[a]	100	100	100	100	100	100
Limonene	98.2	65.1	94.8	79.3	67.5	81.0
Borneol[a]	100	100	100	100	100	100

[a]These compounds occur at low levels (see Figure 1) so that detection of (+)-enantiomers is unlikely.

[b]Not resolved. The enantiomers of car-3-ene could not be resolved on the chiral β-cyclodextrin column. Car-3-ene was present in only one of the five trees sampled.

on the cage was greater than on the test substrate, when surface areas of the test substrate (30 cm^2) and the cage (5450 cm^2) are taken into consideration, it is clear that the distribution of eggs was concentrated on the gauze-covered twigs (Figure 3).

In tests with individual monoterpenes, females laid significantly more eggs on the gauze wrapped around vials emitting car-3-ene [predominantly (+)-enantiomer], myrcene, (−)-limonene, or (−)-β-pinene than on the gauze around the controls (Table 2). Of these, car-3-ene and myrcene appeared to be the most effective stimuli. Vials emitting (−)-α-pinene or (−)-camphene did not have a stimulating effect on oviposition. Indeed, overall fewer eggs were produced with these individual monoterpenes than with the others. In tests with the four-component blend of monoterpenes [which, by headspace analysis, consisted of 15% (−)-α-pinene, 55.5% (−)-β-pinene, 20.5% myrcene, and 5.5% (−)-limonene], females laid significantly more eggs on the substrate emitting the blend (69.7 ± 21.6; $N = 9$) than on the control gauze (1.7 ± 0.8; $N = 9$) (Wilcoxon test, $P \leq 0.05$). The level of oviposition (Figure 4) on the substrate emitting the blend was similar to that elicited by white pine twigs (Figure 3).

As in the twig bioassays, more eggs were laid on the screen of the bioassay cage than on the cotton gauze around vials emitting monoterpenes. However, when surface area of the respective substrates was taken into consideration, females clearly concentrated their eggs on the stimulating test substrates, as shown for the monoterpene blend (Figure 4). Test stimuli also affected the

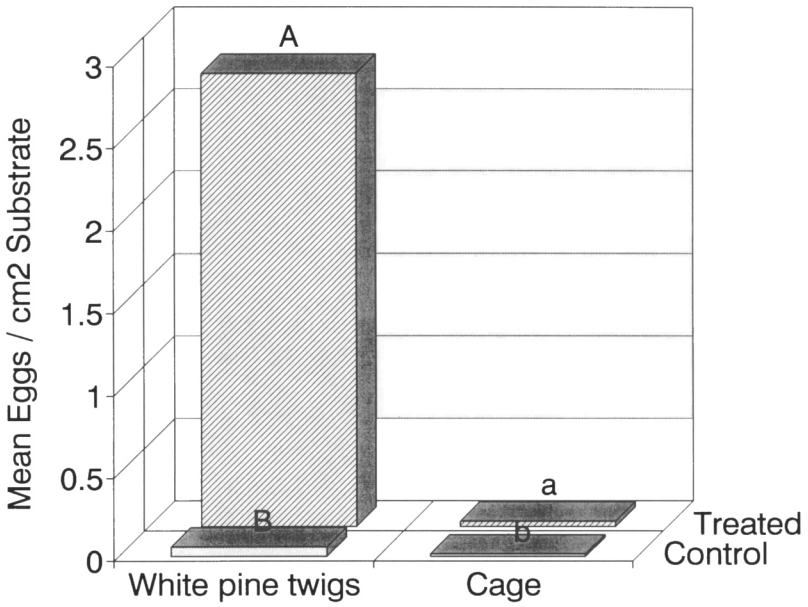

FIG. 3. Effects of white pine twigs on distribution of *Dioryctria abietivorella* eggs in bioassay cage. Oviposition preference is expressed as mean eggs/cm^2 substrate to compensate for difference in surface area of treatment and control substrates versus the cage. Bars in the same row with different letters indicate a significant difference ($P < 0.05$) between white pine twigs and control or between treatment and control sides of the cage (Wilcoxon matched-pairs, signed-rank test).

distribution of eggs laid on the cage walls, as usually more eggs were deposited in the section of the cage containing the test stimulus than in the section with the control. This distribution was statistically significant for bioassays of the twigs (Figure 3) and the monoterpene blend (Figure 4) but not for bioassays of the individual monoterpenes (Table 2).

EAG Responses to Monoterpenes. Of the six most abundant (and commercially available) monoterpenes emitted by white pine twigs, car-3-ene [predominantly (+) enantiomer] and myrcene elicited the largest EAG responses in each moth group (age, sex, mating status) (Table 3). (−)-Camphene followed, eliciting a greater EAG response than (−)-β-pinene and (−)-α-pinene (significantly so in most cases), which in turn elicited significantly greater responses than (−)-limonene. Mated 4-day-old females generally produced larger EAGs in response to monoterpenes than 4-day-old virgin females, 4-day-old virgin males, or 1-day-old virgin females, but differences were significant in only a few cases.

TABLE 2. MEAN ± SE EGGS DEPOSITED BY *Dioryctria abietivorella* FEMALES ON GAUZE-COVERED VIALS OR ON THE SCREEN OF THE BIOASSAY CAGE IN RESPONSE TO INDIVIDUAL MONOTERPENES

Compound	% purity[a]	Release rate (mg/day)	N	Vial[b]		Cage[b]	
				Treatment	Control	Treatment	Control
Myrcene	70[c]	4.7	10	40.5 ± 17.3 a	1.5 ± 0.6 b	127.3 ± 38.0 a	92.8 ± 33.3 a
(+)-Car-3-ene	93[d]	5.7	8	36.1 ± 23.1 a	4.8 ± 3.5 b	142.6 ± 35.5 a	121.6 ± 26.2 a
(−)-Limonene	95	4.5	8	17.0 ± 3.3 a	4.0 ± 3.0 b	182.4 ± 27.0 a	165.8 ± 36.3 a
(−)-β-Pinene	98	5.1[e]	12	8.3 ± 3.1 a	4.3 ± 1.9 b	156.1 ± 19.1 a	164.4 ± 17.0 a
(−)-α-Pinene	97	6.0[e]	8	3.0 ± 1.6 a	0.8 ± 0.4 a	104.1 ± 29.6 a	81.0 ± 17.8 a
(−)-Camphene	97	5.9[e]	6	0.5 ± 0.2 a	0.3 ± 0.2 a	71.2 ± 15.8 a	76.2 ± 14.2 a

[a]Purity and major impurities were determined by headspace analysis on HP-1 and DB-225 capillary GC columns.
[b]Pairs of treatment and control means followed by same letter are not significantly different, $P \leq 0.05$, Wilcoxon matched-pairs, signed-rank test.
[c]Impurities include car-3-ene (1.5%), limonene (11.2%), and β-pinene (5.4%).
[d]Compound is an estimated 70% (+)-enantiomer. Impurities include limonene (1.1%) and β-pinene (2.5%).
[e]These monoterpenes were diluted with mineral oil to obtain the indicated release rate.

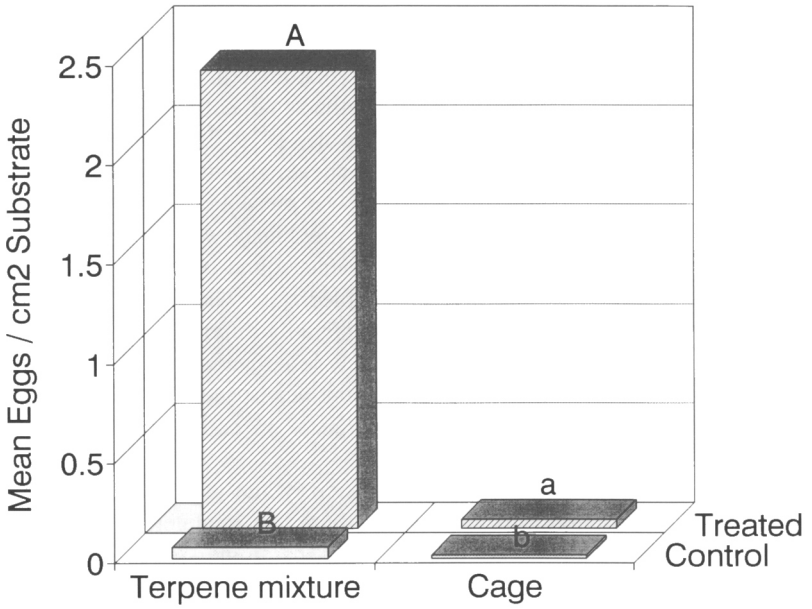

FIG. 4. Effects of four-component monoterpene mixture [15% (−)-α-pinene, 55.5% (−)-β-pinene, 20.5% myrcene, and 5.5% (−)-limonene] on distribution of *Dioryctria abietivorella* eggs in bioassay cage. Oviposition preference is expressed as mean eggs/cm^2 substrate to compensate for difference in surface area of treatment and control substrates versus the cage. Bars in the same row with different letters indicate a significant difference ($P < 0.05$) between monoterpene blend and control or between treatment and control sides of the cage (Wilcoxon matched-pairs, signed-rank test).

TABLE 3. MEAN ± SE (N = 15) EAG RESPONSES (mV) OF *Dioryctria abietivorella* MOTHS OF VARIOUS AGES (DAYS) AND MATING STATUS TO MAJOR MONOTERPENES (10 μg AT SOURCE) EMITTED BY WHITE PINE TWIGS[a]

Monoterpene	Virgin female (1 day)	Virgin male (4 days)	Virgin female (4 days)	Mated female (4 days)
Myrcene	$^{a}1.66 \pm 0.09^{B}$	$^{a}1.79 \pm 0.11^{B}$	$^{a}1.97 \pm 0.16^{AB}$	$^{a}2.40 \pm 0.16^{A}$
(+)-Car-3-ene	$^{a}1.79 \pm 0.11^{B}$	$^{a}1.78 \pm 0.14^{B}$	$^{a}2.07 \pm 0.15^{AB}$	$^{a}2.41 \pm 0.15^{A}$
(−)-Limonene	$^{d}0.59 \pm 0.07^{A}$	$^{d}0.59 \pm 0.07^{A}$	$^{d}0.81 \pm 0.10^{A}$	$^{d}0.81 \pm 0.09^{A}$
(−)-β-Pinene	$^{c}1.18 \pm 0.09^{A}$	$^{c}1.08 \pm 0.11^{A}$	$^{b}1.43 \pm 0.10^{A}$	$^{b}1.54 \pm 0.09^{A}$
(−)-α-Pinene	$^{c}1.14 \pm 0.07^{A}$	$^{c}1.07 \pm 0.11^{A}$	$^{c}1.21 \pm 0.08^{A}$	$^{bc}1.32 \pm 0.08^{A}$
(−)-Camphene	$^{b}1.43 \pm 0.12^{A}$	$^{b}1.51 \pm 0.10^{A}$	$^{b}1.62 \pm 0.11^{A}$	$^{b}1.65 \pm 0.14^{A}$

[a]Means followed by different capital letters in a row indicate a significant difference among moth groups (age, sex, and mating status) at $P \leq 0.05$, Fisher's LSD test. Means preceded by different lowercase letters in a column indicate a significant difference at $P \leq 0.05$ by Fisher's LSD test.

DISCUSSION

The major monoterpenes identified in volatiles emitted by eastern white pine, a major host of *D. abietivorella*, were the same as the major constituents found in leaf oils and oleoresin (Bridgen et al., 1979; Ekundayo, 1988; von Rudloff, 1985). These were α-pinene, camphene, β-pinene, myrcene, car-3-ene, limonene, and α-terpinolene. Car-3-ene was present either in high amounts (28–48%) or essentially absent, as observed previously with oleoresin analysis (Bridgen et al., 1979). The ratio of components found by headspace analysis was similar to that reported for leaf oils and oleoresin, whereas the composition found in the PQ-collected samples were substantially different. In particular, the ratio of α-pinene to β-pinene was considerably less than 1 : 1 in the PQ extracts but close to 1 : 1 in leaf oil extracts and in the headspace samples. These differences in composition could reflect differences in collection techniques or seasonal differences in host material.

So far as we know, the chirality of eastern white pine monoterpenes has not been reported previously. Of the optically active monoterpenes that could be resolved on the chiral GC column, the (−)-enantiomers were predominant. In western white pine, *P. monticola* Douglas, which is also a host for *D. abietivorella* (Haverty et al., 1986), the (−)-enantiomers are also predominant except for car-3-ene and β-phellandrene, which exist primarily as the (+)-enantiomers (Hunt and von Rudloff, 1977). Thus the monoterpene enantiomers, including (+)-car-3-ene, used in the oviposition and EAG bioassays corresponded to the predominant enantiomers emitted by host species.

The bioassay results show that *D. abietivorella* females prefer to oviposit on or near white pine twigs, indicating that host volatiles either attract gravid females or stimulate their oviposition, or both. The static bioassay used in this study did not allow differentiation of these behaviors. Females showed a significant oviposition response to four of the major monoterpenes [myrcene, car-3-ene, (−)-limonene, (−)-β-pinene] that were found in the emissions of white pine twigs. β-Phellanderene was not commercially available in pure form for these tests. A four-component blend of monoterpenes, which by headspace analysis mimicked the composition of major monoterpenes emitted by one batch of white pine twigs, elicited an oviposition response similar to that of twigs and greater than that of any of the individual monoterpenes tested; however, direct (i.e., competitive) comparisons were not performed to confirm this conclusion.

Monoterpenes have been shown to stimulate oviposition in the southern pine coneworm, *Dioryctria amatella* (Hulst). Hanula et al. (1985) found that α-pinene and myrcene individually, and mixtures of α-pinene, myrcene, and limonene in ratios matching those emitted by fusiform rust galls (*Cronartium quercuum*) on loblolly pine, *P. taedea* L., were oviposition stimulants. The addition of β-pinene and β-phellandrene, also constituents of rust gall emissions,

to the stimulating blend did not increase oviposition. Fatzinger and Merkel (1985) showed that a blend of seven terpenes, which matched the composition found in rust galls extracts, stimulated oviposition by *D. amatella*, although not to the same extent as the galls themselves. Together, these three studies support the hypothesis that monoterpene emissions by host conifers contribute to attacks by *Dioryctria* species by stimulating oviposition. An important question not answered by these studies is whether these host compounds are involved in host finding by attracting females over some distance, as observed with another pyralid species, *A. transitella* (Phelan and Baker, 1987).

The EAG results from *D. abietivorella* were not consistent with the behavioral results. While car-3-ene and myrcene were the two best oviposition stimulants and also the two most EAG-active compounds, (−)-limonene, an oviposition stimulant, proved to be considerably less effective in EAG tests than (−)-α-pinene or (−)-camphene, both of which individually failed to stimulate oviposition and may even have suppressed it. These differences could be due to differences in the inherent stimulating effectiveness of the compounds or to differences in their volatility in the EAG bioassay. Further electrophysiological testing, possibly including single cell recordings, will be required to resolve this question. EAG responses might also have been affected by the purity of the test compounds. Car-3-ene and, to a greater extent, myrcene had higher levels of impurities (additional monoterpenes) than the other monoterpenes tested (see Table 2). Thus, they may have acted as monoterpene blends rather than single compounds, which may account for their stronger EAG and behavioral activity. EAG responsiveness was apparently affected by age, sex, and mated status. As expected, mated females were more sensitive to monoterpenes than males, immature females, or virgin females of the same age. These differences in sensitivity related to age, sex, and mating may indicate hormonal influence on antennal sensory receptors. This effect is not well documented in Lepidoptera and merits further study (Ramaswamy, 1994).

Although we have shown that several individual host monoterpenes and at least one blend stimulated oviposition by *D. abietivorella* females, it remains to be shown precisely what characteristics of the host monoterpenes provide the key cues that regulate host finding and host acceptance (oviposition). Several possibilities have been reported for other lepidopteran pests of conifers. For example, Leather (1987) reported that the ratio of α-pinene to β-pinene determined the attractiveness of lodgepole pine, *P. contorta* Douglas, to females of the pine beauty moth, *Panolis flammea* (Denis & Schiffermüller). Städler (1974) found that the specific enantiomers of pinenes, namely, (+)-α-pinene and (−)-β-pinene, stimulated oviposition by the spruce budworm, whereas others [(−)-α-pinene and (±)-α-pinene] did not. Yet another possibility was reported by Wright et al. (1975), who suggested that varieties of Scotch pine, *P. sylvestris* L., that contain significantly lower concentrations of car-3-ene or terpinolene

may be unattractive as potential hosts to females of the Zimmerman pine moth, *Dioryctria zimmermani* (Grote).

The composition of monoterpenes emitted by white pine twigs from different trees was highly variable, particularly with respect to myrcene, car-3-ene, and limonene. As all three compounds were effective oviposition stimulants for *D. abietivorella*, their emission at high rates, either individually or in combination, could signal the suitability of a potential host for ovipositing *D. abietivorella*. The chiral composition of white pine monoterpenes was relatively less variable and, therefore, may be less important as a host signal. An optimum blend of monoterpenes seems unlikely to be a key host cue because *D. abietivorella* is attracted to a wide variety of other pine and nonpine host species (Hedlin et al., 1980), which, although they emit many of the same monoterpenes and enantiomers as found in white pine, they do so in considerably different ratios (von Rudloff, 1975; Ekundayo, 1988; Grant, unpublished data). Valterová et al. (1995) were unable to find any relationship among monoterpene compositions (including enantiomers) that accounted for resistance or susceptibility of pine species in Cuba to *Dioryctria horneana* Dyar.

More extensive testing of individual monoterpenes and monoterpene mixtures is needed to answer the above questions and to understand how monoterpenes serve as host cues for *D. abietivorella*. It is possible that other volatile terpenoids, some of which were detected at very low levels in our extracts, could influence oviposition. Also, an improvement in the bioassay is needed, as females laid more eggs on the screen of the cage than on the oviposition substrates even when white pine twigs were used as a stimulus. Poor air circulation in the cages within the environmental chambers may have been a cause of this problem, but similar egg distributions were obtained when cages were placed in an airflow provided by a wind tunnel to eliminate saturating the cage with test odors (unpublished data). Alternatively, the release rates of monoterpenes may have been too high and repelled most ovipositing moths, but low egg deposition was also observed with vials containing lower quantities of monoterpenes (unpublished data) and with gauze-covered twigs, which presumably provided a suitable release rate of volatiles (as well as releasing additional, potentially important volatile compounds not tested in the bioassays). However, the gauze may have covered essential contact chemical stimuli on the twigs such as cuticular lipids and other surface compounds, which have been suggested as oviposition stimuli for lepidopteran species attacking conifers (Grant and Langevin, 1994; Ross et al., 1995). It remains to be determined how important tactile and visual cues are in selection of oviposition sites by *D. abietivorella* and whether they interact with chemical cues.

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FEEDING DETERRENTS AND SENSITIVITY SUPPRESSORS FOR *Pieris rapae* LARVAE IN WHEAT GERM DIET

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Abstract—Rejection of wheat germ diet by cabbage-reared *Pieris rapae* larvae is explained by the fact that the diet contains strong feeding deterrents to the larvae. These deterrents were present mainly in hexane and butanol (BuOH) fractions of diet extracts, but the post-BuOH water fraction also showed some deterrent activity. Although minor diet components such as sorbic acid had a weak deterrent effect, wheat germ was primarily responsible for the activity. Seven compounds isolated from the BuOH extract of wheat germ were deterrent to the larvae. UV spectra suggested that most of these compounds are apigenin-based flavones. Fractionation of the hexane extracts showed that most of the active compounds were methanol-soluble, and HPLC of the MeOH fractions revealed the presence of active compounds that were absent in the BuOH extract of wheat germ, as well as other compounds that were common to both fractions. Acceptance of nasturtium by *P. rapae* larvae reared on wheat germ diet is explained by a type of "cross habituation" of the larvae to feeding deterrents. When larvae were reared on cabbage leaves treated with chemical fractions from wheat germ diet, they readily accepted nasturtium, which is normally refused by cabbage-reared *P. rapae* larvae, due to the presence of strong feeding deterrents in the plant. A high percentage of diet-reared larvae also fed on *Erysimum cheiranthoides* and *Iberis amara*, which are normally rejected. The chemical constituents encountered in wheat germ can almost completely account for the larval acceptance of nasturtium. The suitability of wheat germ diet for rearing phytophagous insects for behavioral assays must be questioned.

Key Words—*Pieris rapae*, Lepidoptera, Pieridae, wheat germ diet, feeding deterrent, habituation.

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INTRODUCTION

Artificial diets containing wheat germ have been used widely for rearing insects for a variety of purposes. The suitability of wheat germ diets for mass-rearing of insects has been examined for many species (e.g., Message and Zucoloto, 1989; Gunasena et al., 1989; Filho et al., 1991; Tamhankar and Dongre, 1992; Vargas et al., 1994). Similar diets have been used also as basic food sources to monitor the physiological or toxicological effects of insecticides, nutrients, or other nonnutritional chemicals on *Ostrinia nubilalis* (Lynch et al., 1973), *Trogoderma granarium* (Kraszpulski et al., 1987), *Homoeosoma electellum* (Rogers et al., 1987), *Spodoptera eridania* (Johnson and Bentley, 1988), and *Tribolium castaneum* (Oppert et al., 1993). Insects reared on wheat germ diets have been used for investigations of physiological factors (Pannabecker et al., 1992) and larval density effects (Lasota and Kok, 1986) or for field experiments (Wilson, 1990). Jones et al. (1986) found that although wheat germ diets are commonly used for insects with chewing mouthparts, they can be used also to support insects with piercing-sucking mouthparts such as *Oncopeltus fasciatus*.

Variable results have been obtained for different insect species that fed on wheat germ diet. When reared from the neonate larval stage, some species such as *Helicoverpa armigera* (Wu, 1985), *Spodoptera frugiperda* (Mielitz et al., 1986), *Agrotis segetum* (Wu et al., 1988), *Heliothis assulta* (Wu et al., 1990), and *Lymantria dispar* (Sheppard and Friedmand, 1992) perform better on wheat germ diets than on their host plants. Individuals of the tomato pinworm (*Keiferia lycopersicella*) reared on wheat germ diet compare favorably with those reared on tomato foliage in terms of larval growth and development, pupal weight, and adult fecundity (Burton and Schuster, 1986). However, Schroeder et al. (1986) found that larval weights of *O. nubilalis* were 1.7-fold greater on pinto bean diet than on wheat germ diet. Furthermore, although *Melitara prodenialis* larvae feed and grow on a wheat germ diet, they fail to complete development (Carlton and Kring, 1994). Some insects will refuse to feed on wheat germ diet after feeding on a plant (e.g., David and Gardiner, 1966), but others may perform better (Hajek, 1989).

Chemical constituents of wheat germ have been shown to affect insect behavior. For example, Tamaki et al. (1971) reported that wheat germ contained both aggregation and feeding stimulants for adults of the confused flour beetle, *Tribolium confusum*, and three triglycerides were identified as the semiochemicals eliciting aggregation. Nara et al. (1981) found that volatile constituents of wheat germ oil were responsible for initiating aggregating behavior of *Trogoderma glabrum* larvae. Feeding by *Trichoplusia ni* larvae on a wheat germ diet has been shown to be stimulated by proteins, sugars, wheat germ oil, and inorganic salts (Gothilf and Beck, 1967). However, when wheat germ was extracted with ether that contained peroxides, strong repellent activity was found

for adult *T. confusum* (McGinnis and Loschiavo, 1973). A hexane extract of an artificial diet containing wheat germ was deterrent to feeding by *Manduca sexta* larvae, and wheat germ was found to be the source of one of the deterrent components, since omission of wheat germ increased the acceptability of the diet (Städler and Hanson, 1978). However, no further attempts have been made to isolate and test the factors that are feeding deterrents to phytophagous insects in wheat germ diet.

Recent studies conducted in our laboratory have demonstrated remarkable differences in the response of cabbage- and diet-reared *Pieris rapae* larvae to feeding deterrents. While cabbage-reared larvae refuse to feed on nasturtium (*Tropaeolum majus*), which contains strong feeding deterrents, larvae reared on a wheat germ diet readily accept this plant (Renwick and Huang, 1995). Extracts prepared from nasturtium are strongly deterrent to feeding by cabbage-reared larvae but have no deterrent effect on larvae reared on the diet. Similarly, diet-reared larvae are not sensitive to erysimoside, cymarin, 2-*O*-β-D-glucosyl cucurbitacin E, and chlorogenic acid, which act as potent feeding deterrents to cabbage-reared larvae (Huang and Renwick, 1995a). Moreover, larvae refuse to feed on wheat germ diet when transferred from a cabbage plant (Huang and Renwick, unpublished). The mechanisms controlling these behavioral differences are unknown. This study was designed to determine whether wheat germ diet contains factors that are feeding deterrents to cabbage-reared *P. rapae* larvae, and to isolate the active compounds. Experiments were also designed to test whether plants that are unacceptable to cabbage-reared larvae would be acceptable to larvae reared on wheat germ diet, and to explain why such diet-reared larvae are insensitive to some deterrents.

METHODS AND MATERIALS

Plants and Insects. Cabbage (*Brassica oleracea* L. var. Golden Acre, 4 to 6 weeks old), nasturtium (*T. majus* var. Double Gleam; 3 to 4 weeks old), *Erysimum cheiranthoides*, and *Iberis amara* plants for food or bioassays were grown in an air-conditioned greenhouse maintained at ca. 25°C, with supplemental light provided by 400-W multivapor high-intensity discharge lamps. *P. rapae* larvae for feeding deterrent assays were obtained from colonies started from field-collected insects each summer and maintained in the laboratory at ca. 22°C under fluorescent lights providing a photoperiod of 16:8 hr light–dark. Larvae were reared on cabbage plants. Naive neonate larvae were obtained by allowing butterflies to oviposit on a strip of Parafilm (Webb and Shelton, 1988).

Extraction and Fractionation of Diet Materials. A wheat germ diet described by Bell et al. (1979) was used. The combined dry constituents of whole diet or wheat germ were extracted in boiling ethanol for 5 min, cooled, homogenized,

and filtered. The ethanolic extract was evaporated to dryness under reduced pressure and fractionated with *n*-hexane and water. The aqueous extract was then partitioned three times with 1-butanol. The hexane fraction, the butanol fraction, and the postbutanol water fraction were concentrated under reduced pressure at ca. 50°C and stored in the refrigerator until used.

HPLC. HPLC systems were as follows. System 1: Varian MicroPak MCH-10 semipreparative reversed-phase C_{18} column (50 × 0.8 cm); gradient program, 35% methanol (MeOH) in H_2O from 0 to 25 min, increased linearly to 40% at 27 min, 42% at 45 min, and 100% at 50 min, 3.3 ml/min. System 2: Same column as system 1; gradient program, 0% MeOH in H_2O at 0 min, 25% at 10 min, 30% at 25 min, and 100% at 30 min, 3.3 ml/min. System 3: Phenomenex Bondex10 C_{18} column (30 × 0.78 cm); gradient program, 0% CH_3CN in H_2O at 0 min, 10% at 2 min, 15% at 25 min, and 100% at 30 min, 2.5 ml/min. System 4: Same column as system 3; gradient program, 0% CH_3CN in H_2O at 0 min, 15% from 2 min, 17% at 25 min, and 100 at 28 min, 2.5 ml/min. System 5: Same column as system 3; gradient program, 0% CH_3CN in H_2O at 0 min and 20% from 2 to 40 min, 2.5 ml/min. System 6: Same column as system 1; gradient program, 0% CH_3CN in H_2O at 0 min, 30% at 30 min, and 100% at 40 min,.3.0 ml/min. System 7: Same column as system 1; gradient program, 0% MeOH in H_2O from 0 to 5 min and 100% from 12 to 16 min, 3.3 ml/min. System 8: Same column as system 3; gradient program, 20% CH_3CN in H_2O from 0 to 20 min, increased to 50% at 25 min and 100% from 40 min to 60 min, 2.5 ml/min. A diode array detector (Hewlett Packard Model 1040A) was used to monitor the eluate at 254 nm for all the above HPLC systems, and UV absorption spectra of individual compounds were recorded.

Thin-Layer Chromatography. Preparative thin-layer chromatography (TLC) was carried out on 20 × 20-cm, 0.5-mm-thick, Merck silica gel 60 plates. The sample was loaded on each plate in a line, and the plate was developed in a solvent system consisting of tert-butanol–acetic acid–water (7 : 1 : 1). The plates were dried with a hairdrier after development, and bands were visualized using UV light. The bands were individually collected and washed with methanol.

Feeding Deterrent Bioassays. Twenty microliters of solutions of the test extracts, fractions, or compounds was applied in MeOH, except for the hexane extract and its fractions, which were applied in 20% methylene chloride in MeOH, with a Rainin battery-operated motorized pipette onto each side of a cabbage leaf disk (340 mm^2) made with a No. 14 cork borer. Some diet components (such as agar) were not soluble in MeOH, and suspensions were used. For each disk (both sides), 0.0001, 0.001, 0.005, and 0.01 gram diet equivalents (gde) (dry weight) or gram wheat germ equivalents (gwge) of extract, fraction, or component were used. The isolated compounds were used at a dose of 3 μg/disk. Control discs were treated with the solvent alone. The tests were conducted

in ice cream cups (250 ml), each containing a paraffin wax layer at the bottom, which was covered with a moist filter paper. Two treated and two control disks were arranged alternately in each cup and supported on No. 3 insect pins. Four early fourth instars were introduced in the center of the cup and the cup was covered with a perforated plastic lid. The cups were then placed in a translucent polystyrene box with moist paper towels at the bottom. The covered box was kept in an incubator maintained at 28°C. After 8 hr the disk areas were measured using a LiCor-3100 area meter. The area consumed was obtained by subtracting the remaining portion of each eaten disk from the average measurement of all disks that showed no sign of feeding.

Nasturtium Acceptance Assays. Acceptance assays as described by Renwick and Huang (1995) were employed to determine whether diet constituents are responsible for the lack of sensitivity of larvae to the deterrents in nasturtium. Test fractions from extracts of whole diet or wheat germ were applied to cabbage foliage by spraying in MeOH [for butanol (BuOH) extract] or 20% methylene chloride in MeOH (for hexane extract) solution. Control leaves were sprayed with MeOH alone. Neonate larvae hatching on Parafilm were transferred to the cabbage leaves with a small camel hair brush. They were allowed to feed until they became early second instars and were then tested for their acceptance or rejection of nasturtium. The tests were carried out using individual leaves in 250-ml ice cream cups. The leaf petiole was inserted into a beaker of water through a hole punched in the bottom of the cup. One test larva was placed on each leaf, and the cup was covered with a plastic lid. Observations were made after 24 and 48 hr to record whether or not feeding had occurred.

Erysimum *and* Iberis *Acceptance Assays.* Bioassays for testing acceptance of *E. cheiranthoides* and *I. amara* by *P. rapae* larvae were performed as for nasturtium acceptance assays. However, larvae were reared on cabbage or wheat germ diet until they were tested as early third instars, and observations were made at 24 hr after the transfer.

Design and Analysis. For feeding deterrent assays, a replicate consisted of one cup with four larvae, and 5 to 30 replications were performed for each bioassay. Deterrent activities were compared by calculating a feeding deterrent index (FDI) based on the area consumed of treated and control leaf disks, as follows:

$$\text{FDI} = 100(\text{Control} - \text{Treated})/(\text{Control} + \text{Treated})$$

The proportion of disk area consumed, treated:treated + control, was calculated, and the data were subjected to arcsine square root transformation. Differences between treatments and controls were analyzed by a one-sample t test on the transformed data, under the null hypothesis that equal feeding occurred on treated and control disks.

RESULTS

The results clearly show that wheat germ diet contains strong feeding deterrents to cabbage-reared fourth instars of *P. rapae*. In a choice test, larvae were almost completely deterred from feeding on cabbage disks (340 mm^2) treated with the hexane fraction of whole diet extract at a dose of 0.01 gde/disk (Figure 1). When the dose was decreased 10-fold, to 0.001 gde/disk, this material was still significantly deterrent, and a feeding deterrent index (FDI) of 69.9 was obtained. Similar activity was found for the BuOH fraction from whole diet. No feeding occurred on leaf disks treated with 0.01 gde of the BuOH fraction compared with the control disks treated with solvent (MeOH) alone (Figure 1). A FDI of 75.2 was obtained for this fraction when tested at 0.001 gde/disk. The post-BuOH water fraction was much less deterrent to the larvae compared with the hexane and BuOH fractions, although a significant difference was found

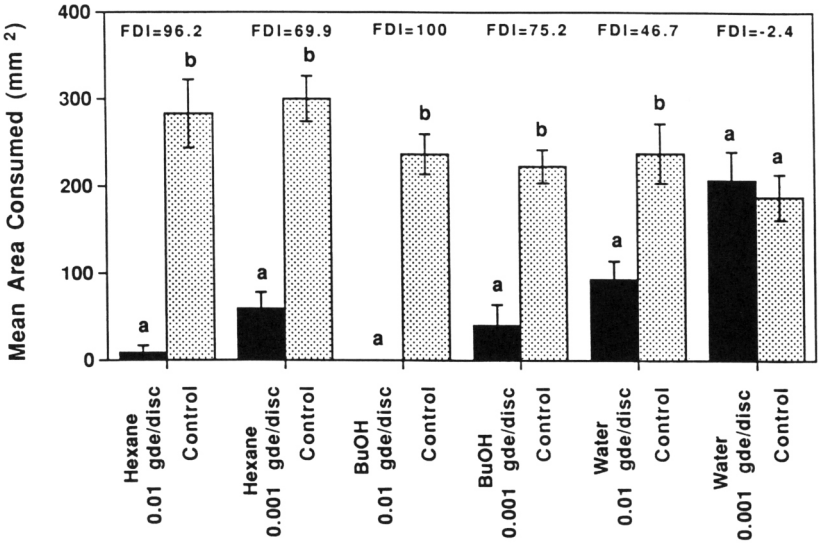

FIG. 1. Feeding by fourth instars of *P. rapae* on cabbage leaf disks treated with hexane, BuOH, or post-BuOH water fractions from extracts of whole wheat germ diet. Both sides of a leaf disk (340 mm^2) were treated with 0.01 or 0.001 gram diet equivalents (gde) of the extracts. Control disks were treated with the solvent (MeOH or 20% methylene chloride in MeOH) alone. Replicated 10 times. A replication consisted of one bioassay cup with two test and two control leaf disks and four larvae. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P > 0.05$), under the null hypothesis that feeding occurred evenly on control and treated disks. Mean feeding deterrent indexes (FDI) are shown. FDI = 100 (Control − Treated)/(Control + Treated).

between the treatment of 0.01 gde/disk and the control ($P < 0.05$, FDI = 46.7; Figure 1). This fraction was not active as a feeding deterrent at 0.001 gde/disk ($P > 0.05$, FDI = −2.4).

When all the minor diet components were tested at 0.01 gde/disk, similar FDIs were found for methylparaben (16.9), salt mix (15.3), and sorbic acid (16.4) (Figure 2). However, only the treatment with sorbic acid was significantly different from its control. Aureomycin, casein, and vitamins were not deterrent to the larvae. Instead, agar acted as a strong stimulant, giving a FDI of −34.9 (Figure 2). Because none of these minor components contributed significantly to the deterrence of the whole diet, further studies were focused on extracts of the major component, wheat germ.

When the hexane fraction from wheat germ at 0.01 gde/disk was tested, a FDI of 100 was obtained (Figure 3). The larvae were still strongly deterred from feeding by this extract at 0.001 gde/disk (FDI = 73.3), but no significant activity was detected at 0.0001 gde/disk (FDI = 7.0, $P > 0.05$). Strong deterrent activity was also found for the BuOH fraction of wheat germ when a dose

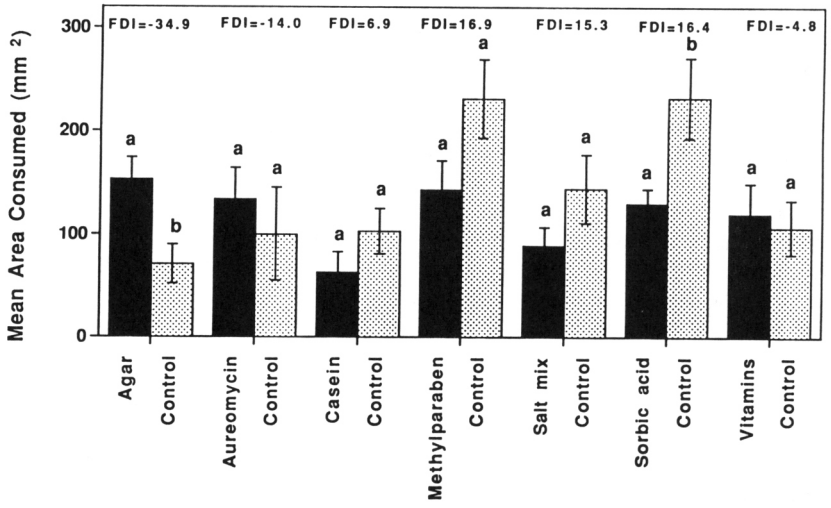

FIG. 2. Feeding by fourth instars of *P. rapae* on cabbage leaf disks treated with minor components of wheat germ diet. Both sides of a leaf disk (340 mm^2) were treated with 0.01 gram diet equivalents of a component. Control disks were treated with the solvent (MeOH) alone. Replicated 10 times. A replication consisted of one bioassay cup with two test and two control leaf disks and four larvae. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P > 0.05$), under the null hypothesis that feeding occurred evenly on control and treated disks. Mean feeding deterrent indexes (FDI) are shown. FDI = 100(Control − Treated)/(Control + Treated).

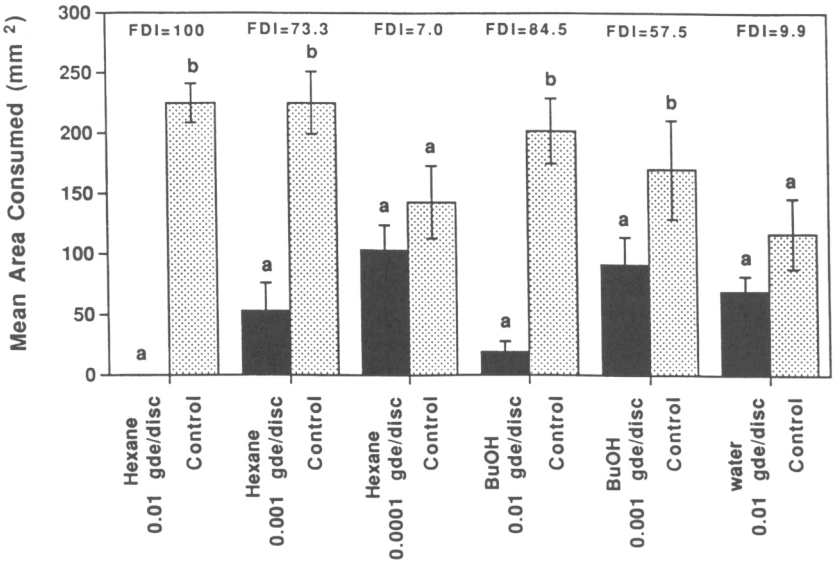

FIG. 3. Feeding by fourth instars of *P. rapae* on cabbage leaf disks treated with hexane, BuOH, or post-BuOH water fractions from extracts of wheat germ. Both sides of a leaf disk (340 mm^2) were treated with 0.01, 0.001, or 0.0001 gram diet equivalents (gde) of the extracts. Control disks were treated with the solvent (MeOH or 20% methylene chloride in MeOH) alone. Replicated 20 times for BuOH extract at 0.01 gde/disk and 10 for all others. A replication consisted of one bioassay cup with two test and two control leaf disks and four larvae. Means (±SE) with the same letters in each series are not significantly different according to a one-sample t test ($P > 0.05$), under the null hypothesis that feeding occurred evenly on control and treated disks. Mean feeding deterrent indexes (FDI) are shown. FDI = 100(Control − Treated)/(Control + Treated).

of 0.01 gde/disk was used (Figure 3). This material was still significantly deterrent to the larvae at 0.001 gde/disk (FDI = 57.5). The post-BuOH water fraction, even at the higher dose (0.01 gde/disk), was not active as a feeding deterrent (FDI = 9.9, $P > 0.05$; Figure 3). Since wheat germ contains the most active feeding deterrents to *P. rapae* larvae compared with other diet components, and these deterrents can be extracted into hexane and BuOH, subsequent work was directed toward the isolation of active constituents of these two fractions.

Many peaks were detected from the BuOH extract of wheat germ by HPLC system 1 (Figure 4). Eight fractions, A through H (Figure 4), were collected and tested for their feeding deterrent activities for *P. rapae* larvae. As shown in Figure 5, most of the fractions had some degree of deterrence to larval feeding. Significant differences ($P < 0.05$) were found when fraction B (FDI

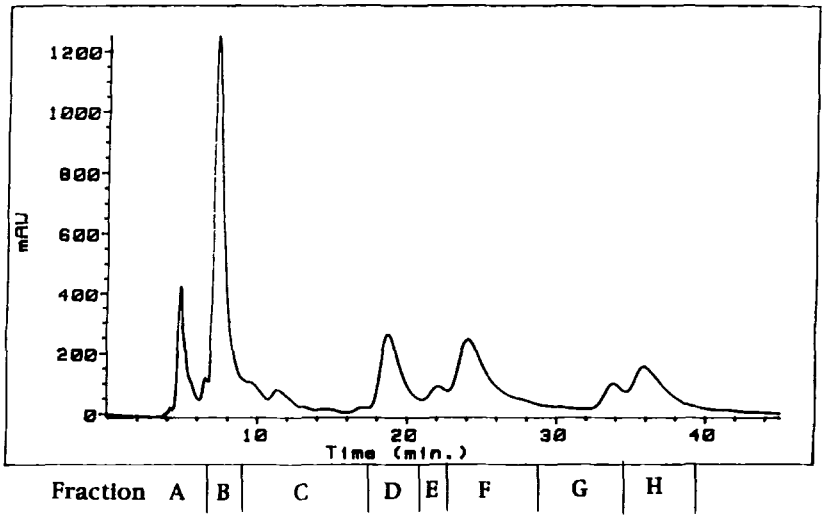

FIG. 4. HPLC separation of BuOH fraction from wheat germ extract. UV monitoring at 254 nm.

= 33.3), D (FDI = 34.7), F (FDI = 31.4), or H (FDI = 36.3) was compared at 0.01 gwge/disk with the solvent alone. Although FDIs of 27.0, 18.3, and 27.4 were obtained for fractions A, E, and G, respectively, the differences between these treatments and the controls were not significant ($P > 0.05$).

The major compounds in fractions, B, E, F, G, and H from the BuOH extract of wheat germ were purified by HPLC and correspondingly named compounds, **B, E, F, G,** and **H.** Compound **B** was purified by running fraction B on HPLC system 2 and then system 3. System 4 was used for compounds **E** and **F,** and system 5 for **G** and **H.** Although fraction D gave a single peak on HPLC system 1 (Figure 4), a shoulder was detected when system 6 was used. None of the HPLC systems used in this study gave complete separation of these two compounds, but a preparative TLC system was effective in separating them. The compound with a lower R_f (0.39) was named **D1,** and another ($R_f = 0.56$) **D2.** Because a small amount of TLC material was found in the samples collected, HPLC system 7 was used for final purification of these two compounds. The UV spectra of compounds **D1, D2, E, F, G,** and **H** were typical of apigenin-based flavones: λ_{max} (MeOH) nm 272, 330–336. Apigenin, apigenin-7-glucoside and apiin gave similar spectra under the same conditions. However, based on their retention times on HPLC system 1, the isolated compounds are more polar than the authentic samples, and further work will be needed to complete their identification.

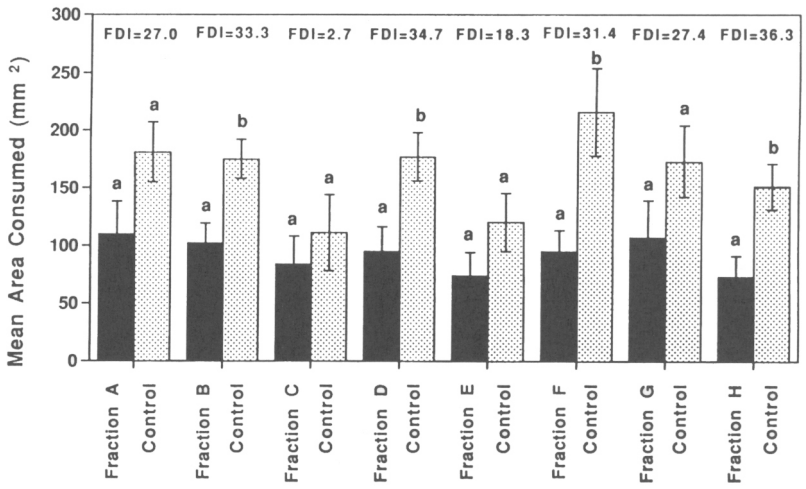

FIG. 5. Feeding by fourth instars of *P. rapae* on cabbage leaf disks treated with HPLC fractions (Figure 4) from BuOH fraction of wheat germ extract. Both sides of a leaf disk (340 mm^2) were treated with 0.01 gram wheat germ equivalents (gwge) of the extracts. Control disks were treated with the solvent (MeOH) alone. Replications: 30 for fraction B, 11 for fractions C and G, 10 for fractions A, D, and E, 9 for fraction F, and 5 for fraction H. A replication consisted of one bioassay cup with two test and two control leaf disks and four larvae. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P > 0.05$), under the null hypothesis that feeding occurred evenly on control and treated disks. Mean feeding deterrent indexes (FDI) are shown. FDI = 100(Control − Treated)/(Control + Treated).

Table 1 shows the relative deterrent activities of all these compounds to feeding by *P. rapae* larvae. Compounds **B, D1, D2, E, F,** and **H** at 3 μg/disk were significantly deterrent ($P < 0.05$), giving FDIs ranging from 28.6 to 42.3. No significant difference was found between the treatment and the control when compound **G** was used at the same level. When combinations of compound **F** and one of the other compounds were tested at 3 μg each/disk, FDIs were slightly higher for **F + B, F + D1, F + D2,** and **F + E** than for **F** alone (Table 1). However, a lower FDI resulted when **F** was combined with **G.** A much higher FDI (76.2) was obtained when **F + H** was tested. The combination of **G** and **H** also gave a slightly higher FDI compared with either one alone. None of the combinations containing three (**D2 + F + H**), four (**B + D2 + F + H** or **D1 + D2 + F + H**) or five (**B + D1 + D2 + F + H**) compounds completely blocked feeding by the larvae, and the highest FDI (82.7) obtained (Table 1) was similar to that for the BuOH extract of wheat germ at 0.01 gde/disk (Figure 3).

TABLE 1. FEEDING DETERRENCY TO *Pieris rapae* LARVAE OF COMPOUNDS ISOLATED FROM THE BuOH EXTRACT OF WHEAT GERM

Compound[a]	Replications[b]	Mean area (mm^2 ± SE) consumed		FDI[c]	$P < 0.05$[d]
		Treatment	MeOH control		
B	10	92 ± 15	177 ± 30	28.6	*
D1	10	70 ± 22	120 ± 19	33.7	*
D2	10	82 ± 27	136 ± 14	42.3	*
E	16	66 ± 17	140 ± 17	39.2	*
F	22	74 ± 13	175 ± 18	40.1	*
G	20	97 ± 14	140 ± 18	17.6	NS
H	20	77 ± 17	156 ± 25	31.5	*
F + B	15	56 ± 18	193 ± 20	44.5	*
F + D1	10	75 ± 25	171 ± 18	44.8	*
F + D2	15	67 ± 19	251 ± 24	58.6	*
F + E	10	87 ± 28	191 ± 36	43.6	*
F + G	10	89 + 21	169 ± 24	31.3	*
F + H	14	27 ± 9	165 ± 21	76.2	*
G + H	8	64 ± 18	145 ± 27	45.6	*
D2 + F + H	10	31 ± 13	160 ± 29	72.9	*
B + D2 + F + H	10	29 ± 15	162 ± 27	78.7	*
D1 + D2 + F + H	10	38 ± 18	250 ± 19	79.0	*
B + D1 + D2 + F + H	10	31 ± 16	250 ± 19	82.7	*

[a] Isolated and purified from corresponding fractions shown in Figure 4. Each cabbage leaf disk (340 mm^2) was treated with 3 μg each of the compounds.

[b] A replication consisted of one bioassay cup with two test and two control leaf disks and four larvae.

[c] Mean feeding deterrent index. FDI = 100(Control − Treated)/(Control + Treated).

[d] According to a one-sample t test, under the null hypothesis that feeding occurred evenly on control and treated disks. Significant difference between control and treatment is indicated with an asterisk. NS, not significant.

After the solvent was removed from the hexane extract of wheat germ, an oily residue was obtained. This residue was successively extracted with water, MeOH and chloroform, and the resulting fractions were used in feeding assays. The MeOH-soluble fraction was highly deterrent to *P. rapae* larvae when tested at a dose of 0.005 gwge/disk (FDI = 92.3; Figure 6). The water- and chloroform-soluble fractions were not active as feeding deterrents at the same dose (FDI = −10.2 and −7.5, respectively). The MeOH-soluble material was therefore further separated by HPLC system 8, and two fractions were collected and tested. Fraction I, which gave an HPLC profile similar to that from the BuOH extract of wheat germ (also run on system 8 for comparison), at 0.005 gwge/disk was more deterrent to the larvae (FDI = 85.1) than was fraction II (FDI

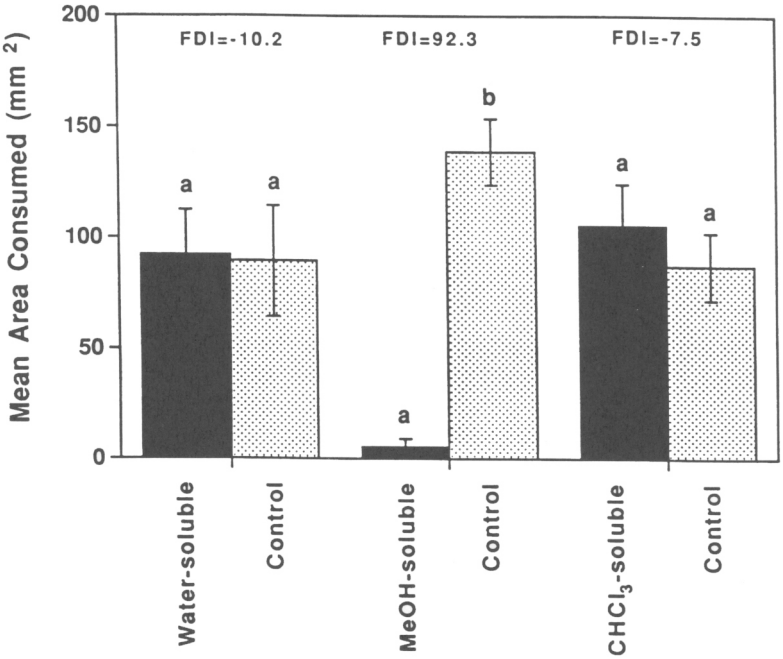

FIG. 6. Feeding by fourth instars of *P. rapae* on cabbage leaf disks treated with fractions from hexane extract of wheat germ. Both sides of a leaf disk (340 mm^2) were treated with 0.005 gram wheat germ equivalents (gwge) of a fraction. Control disks were treated with the solvent (20% methylene chloride in MeOH) alone. Replicated 10 times. A replication consisted of one bioassay cup with two test and two control leaf disks and four larvae. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P > 0.05$), under the null hypothesis that feeding occurred evenly on control and treated disks. Mean feeding deterrent indexes (FDI) are shown. FDI = 100(Control − Treated)/(Control + Treated).

= 35.3) (Figure 7), although a significant difference ($P < 0.05$) was still obtained between fraction II and its control. Fraction II contained some less polar compounds which, based on the HPLC profile, were absent in the BuOH extract from wheat germ.

Cabbage-reared *P. rapae* larvae refused to feed on *E. cheiranthoides* and *I. amara*, but 44 and 48 of 70 third instars reared on wheat germ diet fed on these two plants, respectively, within 24 hr after transfer. However, all these feeding larvae eventually died within 5 days. The results indicate that these plant species contain toxic materials or are nutrient-deficient, and are not suitable for *P. rapae* larval growth and development.

A previous study (Renwick and Huang, 1995) showed that cabbage-reared

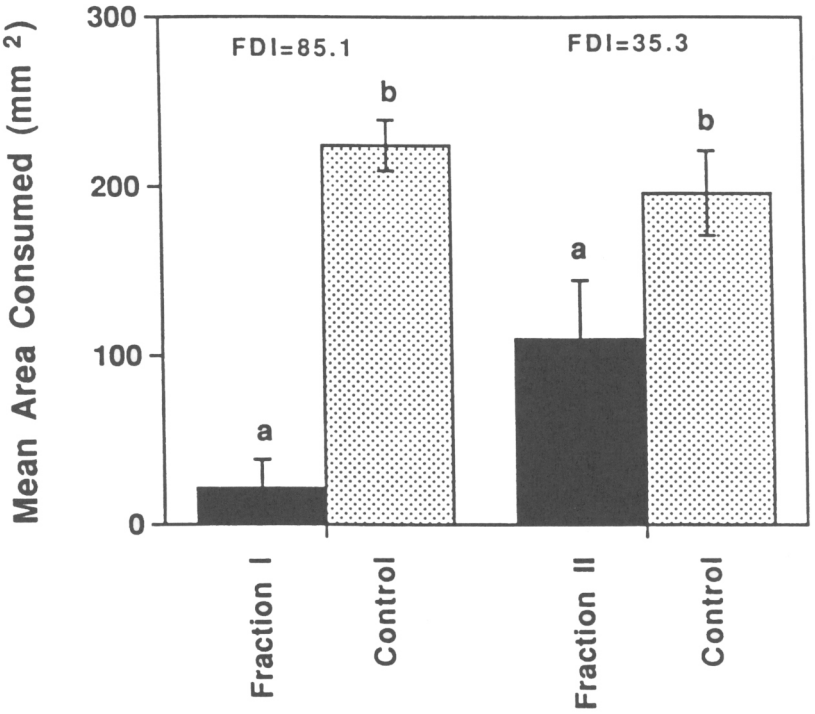

FIG. 7. Feeding by fourth instars of *P. rapae* on cabbage leaf disks treated with HPLC fractions from the MeOH-soluble part (Figure 6) of wheat germ hexane extract. Both sides of a leaf disk (340 mm^2) were treated with 0.005 gram wheat germ equivalents (gwge) of a fraction. Control disks were treated with the solvent (20% methylene chloride in MeOH) alone. Replicated seven times. A replication consisted of one bioassay cup with two test and two control leaf disks and four larvae. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P > 0.05$), under the null hypothesis that feeding occurred evenly on control and treated disks. Mean feeding deterrent indexes (FDI) are shown. FDI = 100(Control − Treated)/(Control + Treated).

P. rapae larvae refused to feed on nasturtium, due to the presence of strong feeding deterrents. But larvae reared on the wheat germ diet accepted this plant without incident. To test if chemical constituents in the diet are responsible for the acceptance of nasturtium, extracts from the whole diet or wheat germ were sprayed onto cabbage leaves which were then used as the larval food source. Larvae were fed, starting as naive neonates on the cabbage leaves, and as early second instars they were individually tested for their acceptance of nasturtium (Figure 8). The results from observations made at 24 and 48 hr after transfer to

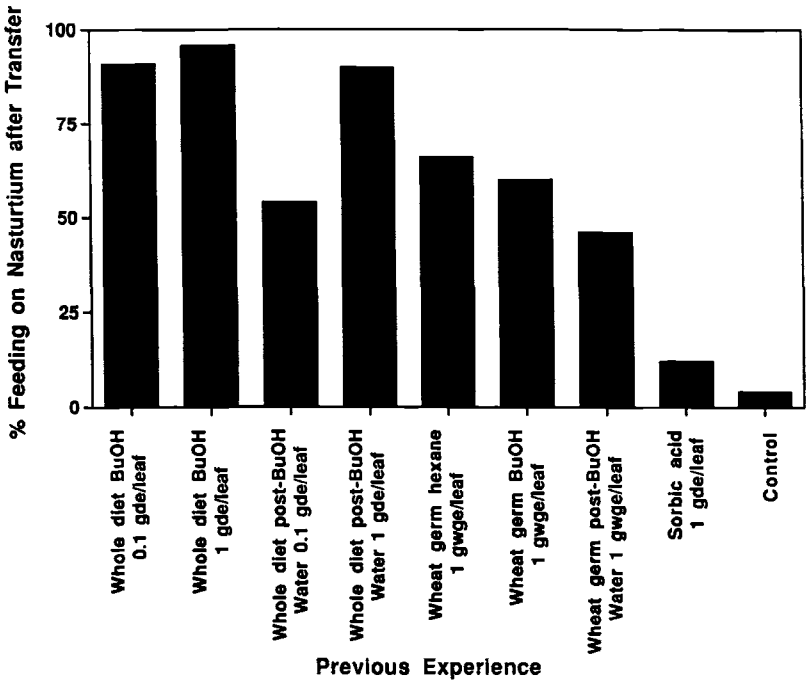

FIG. 8. Induced feeding by second instars of *P. rapae* on nasturtium. Neonate larvae were reared on cabbage leaves (ca. 120 cm^2) treated with 0.1 or 1 gram diet equivalents (gde) or gram wheat germ equivalents (gwge) of hexane, BuOH, or post-BuOH water fractions from extracts of whole diet or wheat germ or of sorbic acid. Control larvae were reared on cabbage leaves treated with MeOH alone. Individual early second instars were transferred to nasturtium leaves. The percentage of larvae feeding on nasturtium is shown based on observations made at 48 hr after transfer. Fifty larvae were tested for each treatment.

nasturtium were similar, and only those from the second observation are shown. Only 2 of 50 (4%) control larvae reared on cabbage leaves treated with the solvent alone fed on nasturtium, but 96% of larvae reared on leaves treated with 1 gde/leaf of BuOH fraction of whole diet accepted the plant. When a dose of 0.1 gde/leaf of this extract was used, the percentage acceptance of nasturtium was still very high (90%). The post-BuOH water extract of whole diet at 0.1 and 1 gde/leaf also caused larvae to feed on nasturtium, giving 54 and 90% acceptance, respectively. A majority of the larvae reared on cabbage leaves treated with the hexane and BuOH extracts of wheat germ accepted nasturtium (66 and 60%, respectively). The post-BuOH water extract of wheat germ also resulted in 46% acceptance. Sorbic acid, which is a weakly deterrent component

of the diet (Figure 2), caused only 12% of the larvae to feed on nasturtium (Figure 8).

DISCUSSION

Rejection of wheat germ diet by cabbage-reared *P. rapae* larvae is explained by the fact that the diet contains strong feeding deterrents to the larvae. These deterrents were found primarily in the hexane and the BuOH extracts, but the post-BuOH water extract also showed some activity (Figure 1). Although minor components such as sorbic acid had a weak deterrent effect, wheat germ was shown to contain the most active compounds (Figures 2 and 3). Seven compounds isolated from the BuOH extract of wheat germ had some deterrent activity on the larvae (Table 1). When tested in combinations of two, the highest feeding deterrent index (76.2) was obtained from compounds **F** + **H.** However, none of the combinations of three or more compounds could completely block feeding by larvae in choice assays. The most active deterrents in the hexane extract were found to be MeOH-soluble (Figure 6), and based on HPLC this fraction contained some other active compounds which did not appear in the BuOH extract. Although a hexane extract of wheat germ diet had previously been shown to be deterrent to tomato-reared *M. sexta* larvae (Städler and Hanson, 1978), the deterrence was much weaker than for our cabbage-reared *P. rapae* larvae. Furthermore, wheat germ was not the most active deterrent to *M. sexta* compared with some other diet components. Städler and Hanson (1978) showed that wheat germ was less deterrent than salt mix, yeast, or formaldehyde.

P. rapae larvae reared on wheat germ diet readily accepted *E. cheiranthoides* and *I. amara*, which were refused by cabbage-reared larvae. A similar phenomenon occurred when larvae with different dietary experiences were transferred to nasturtium (Renwick and Huang, 1995). A previous study by Schmidt et al. (1987) showed that bud-fed *Helicoverpa zea* larvae generally preferred feeding on cotton flower buds, whereas larvae reared on wheat germ diet preferred feeding on blooms. When *M. sexta* is reared on artificial diet, the larvae accept a wider range of potential hostplants (Schoonhoven, 1967). These diet-reared larvae are not behaviorally "naive" but are induced to feed preferentially by the chemical constituents of the diet (Städler and Hanson, 1978). These results suggest that hostplant selection behavior can be distinctly different for insects reared on artificial diets or on natural host plants.

Effects of dietary experience on the susceptibility of insects to insecticides has been known for some time. Plant allelochemicals can act as cytochrome P-450 inhibitors, thus increasing the effectiveness of toxins (Ahmad et al., 1986). Differences in cytochrome P-450 activity in gypsy moth larvae that fed on wheat germ diet or oak leaves could be attributed in part to inhibitors encountered in

the normal food plants (Ahmad and Forgash, 1978). In studies with *Plutella xylostella*, Shelton et al. (1991) found that larvae reared on artificial diet were consistently more susceptible to the pesticides methomyl and permethrin. Also, enzyme induction by dietary constituents can result in tolerance of other plant compounds that are normally toxic (Brattsten et al., 1977). Similarly, dietary experience may affect the susceptibility of insects to ingested pathogens. For example, *Lymantria dispar* larvae inoculated on the foliage of different host plant species showed significantly different susceptibility to the gypsy moth nuclear polyhedrosis virus, and these differences in mortality rates were strongly correlated with differences in foliage chemistry (Keating et al., 1988). A subsequent study (Keating et al., 1989) demonstrated that larvae consuming the virus on diets with additional casein or salts, on diets made more acidic with HCl, or on diets containing tannins may be as much as four times less susceptible than larvae on normal diets. Other diet nutrients and plant allelochemicals such as ascorbic acid (Pristavko and Dovzhenok, 1974), phenolics and quinones (Felton et al., 1987), and terpenes (Morris, 1972) have also been shown to affect the responses of insects to pathogens. Based on these findings, diet neutrality, often suggested as a working assumption in earlier studies (Städler and Hanson, 1978), can no longer be assumed for *P. rapae*, and might also be questioned for other insects. Therefore, although it is easier and cheaper to rear insects on artificial diets, the choice of rearing method must depend on the ultimate use of the colony (Shelton et al., 1991).

Feeding deterrence of potential host plants is an important mediator of plant–insect interactions (Usher et al., 1988). Studies of habituation have been prompted by consideration of the practical use as well as theoretical aspects of feeding deterrents, since repeated contact of a feeding insect with a deterrent-containing food source will cause increased acceptance of that food over time (reviewed by Bernays, 1983; Szentesi and Jermy, 1989). In the present study, acceptance of nasturtium by *P. rapae* larvae reared on wheat germ diet is explained by the effect of "cross habituation" (Huang and Renwick, 1995a) of the larvae to feeding deterrents in nasturtium. Nasturtium is refused by cabbage-reared *P. rapae* larvae due to strong feeding deterrents in the plant (Renwick and Huang, 1995; Huang and Renwick, 1995b). However, when larvae were reared on cabbage leaves treated with chemical factors from wheat germ diet, they readily accepted nasturtium as a food resource (Figure 8). The BuOH extract of the whole diet was more active than the post-BuOH water extract in causing larvae to feed on nasturtium. This difference was consistent with the potency of the feeding deterrent activities of these extracts to cabbage-reared larvae (Figure 1). Tests of the individual diet components indicate that chemical constituents of the wheat germ can account for most of the "induction" of larval feeding on nasturtium. The only minor component tested in nasturtium accept-

ance bioassays was sorbic acid (a weak feeding deterrent), and correspondingly weak activity was detected. These results clearly show that *P. rapae* larvae exposed to constituents of wheat germ diet become cross-habituated to the feeding deterrents in nasturtium. Cross-habituation was previously found in a related study (Huang and Renwick, 1995a) where responses of *P. rapae* to feeding deterrents were compared for larvae reared on cabbage, wheat germ diet, or nasturtium. Cabbage-reared larvae were strongly deterred by erysimoside, cymarin, 2-*O*-β-D-glucosyl cucurbitacin E, and chlorogenic acid. However, nasturtium-reared larvae showed limited sensitivity to these compounds, and larvae reared on wheat germ diet were not affected. Jermy et al. (1987) suggested that larvae of a polyphagous strain of *Mamestra brassicae* could not become habituated to the majority of nonhost plants, which contained a mixture of deterrents. We also found that cabbage-reared *P. rapae* larvae could not cope with the defense formed by the multiple feeding deterrents in nasturtium (Renwick and Huang, 1995; Huang and Renwick, 1995b). However, although cabbage-reared *P. rapae* larvae cannot become habituated to the deterrents in wheat germ diet, most of the naive neonate larvae feed and grow well on the diet, and the resulting larvae readily accept nasturtium as a food plant. We can, therefore, conclude that freshly emerged first instars play a critical role in the rejection or acceptance of a food source (Chapman and Bernays, 1989) and that larval sensitivity to feeding deterrents depends largely on dietary experience. We also suggest that wheat germ diet is a strong suppressor of sensitivity for *P. rapae* larvae and is not suitable for rearing this insect species to assay for feeding deterrent activity. The compounds responsible for this suppression of sensitivity have been tentatively characterized as apigenin derivatives, and future studies will focus on their identification.

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ANTIFEEDANT COMPARISONS OF GABA/ GLYCINERGIC ANTAGONISTS FOR DIABROTICITE LEAF BEETLES (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—The phagostimulatory sensitivity of diabroticite (Coleoptera, Chrysomelidae, Galerucinae) species to cucurbitacins is not correlated with Cucurbitaceae specialization, indicating that other factors, including the absence of feeding deterrents, may influence host-plant affinities among these beetles. Quinoline, indole, and isoquinoline alkaloids and sesquiterpene lactones believed to antagonize γ-aminobutyric acid/glycine Cl^- ionophores mediating chemoreception were tested on squash blossom disks for antifeedant activity to four diabroticite species with different host plant specializations. Most alkaloids were antifeedant below 30 nmol/disk. Antifeedant concentrations of sesquiterpene lactones were higher than alkaloids for all species. Oligophagous *Diabrotica virgifera virgifera* was more sensitive to quinoline alkaloids than polyphagous *D. undecimpuntata howardi*. *Diabrotica virgifera virgifera* was also more sensitive to the indole alkaloids strychnine, brucine, eburnamonine, and vincamine than *D. u. howardi*. The closely related *D. barberi* had sensitivities similar to those of *D. v. virgifera* but the more distantly related *Acalymma vittatum* was less sensitive to the antifeedants than *D. v. virgifera*. The isoquinoline alkaloid hydrastine was uniformly antifeedant to all diabroticites. All the GABA/glycine neurotoxicants tested against diabroticites were feeding deterrents and suggest that beetles share a common antifeedant mechanism.

Key Words—Feeding deterrents, antifeedants, *Diabrotica*, Chrysomelidae, alkaloids, sesquiterpenoids, chemoreception, GABA receptor, glycine receptor.

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INTRODUCTION

Few taxon-specific phagostimulants have been characterized for phytophagous insects, which suggests that host plant recognition is mediated by a relative lack of feeding deterrents compared to phagostimulants. Studying the ecological significance of a plant secondary metabolite often begins with determining its antifeedant activity against an herbivore (Bernays and Chapman, 1994; Dethier, 1980; Jermy, 1966; Mitchell, 1988; Schoonhoven et al., 1992). For diabroticite beetles the opposite occurred; early attention was devoted to characterizing phagostimulants, demonstrated by the compulsive and extreme phagostimulation of nortriterpenoid cucurbitacins that act as feeding deterrents to other insects (Metcalf et al., 1980; Tallamy and Krischik, 1989). Only recently has attention returned to characterization of diabroticite feeding deterrents (Chou and Mullin, 1993; Landis and Gould, 1989; Mullin et al., 1991, 1992, 1994; Xie et al., 1991, 1992).

Among the most potent insect feeding deterrents are quinoline and indole alkaloids, sesquiterpene lactones, diterpenoids, and triterpenoids including azadirachtin (Schoonhoven, 1982; Jermy, 1990; Jain and Tripathi, 1993; Frazier and Chyb, 1995). Our investigations into chemoreception of western corn rootworm, *Diabrotica virgifera virgifera* LeConte, suggest that a receptor protein pharmacologically similar to a GABA (γ-aminobutyric acid)/glycine ionophore complex mediates gustation in the insect peripheral nervous system (Mullin et al., 1992; 1994; Chyb et al., 1995). Using quantitative structure–activity relationships (QSAR) we can predict which alkaloidal compounds are antifeedant by analogy to minimal structural requirements for known GABA or glycine antagonists. Strychnine and hydrastine, glycine, and $GABA_a$ antagonists, respectively, are among the more potent antifeedants discovered for *D. v. virgifera* (Mullin et al., 1992, 1994).

Here we determine if closely related diabroticite beetles with different host plant affinities share similar behavioral sensitivities to antifeedants evaluated against *D. v. virgifera*. Species include northern corn rootworm, *Diabrotica barberi* Smith & Lawrence, which share the same univoltine life cycle and larval oligophagous habit on roots of corn and a few other grasses as *D. v. virgifera*. Adults of both species feed on pollen and floral tissue of many plants but use what is readily available, usually corn. Based on their winter egg diapause and larval host plant affinity, both *D. v. virgifera* and *D. barberi* are placed within the *D. virgifera* group (Krysan, 1986). Another *Diabrotica* sp. tested was the multivoltine southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber. Larvae and adults are polyphagous and do not possess an egg diapause. This species is placed within the *D. fucata* group. The last diabroticite species tested was the multivoltine striped cucumber beetle, *Acalymma vittatum* (F.). Adults overwinter, and although known to feed on many plants in early spring,

they are most abundant on cucurbits where available, and cucurbits are the only known larval hosts (Houser and Balduf, 1925).

All diabroticites tested here share an affinity for nortriterpenoid cucurbitacins, which are potent phagostimulants, but they have different sensitivity thresholds (Metcalf et al., 1980). We wanted to determine if behavioral differences in antifeedant sensitivities exist among diabroticite species by testing stereoisomers and structural modifications of some of the more potent antifeedants for *D. v. virgifera*.

METHODS AND MATERIALS

Beetles

Diabrotica spp. and *A. vittatum* were collected from squash blossoms in a 0.2-ha polyculture of 'Bodacious' sweet corn (*Zea mays* L.), 'Giant gray stripe' sunflower (*Helianthus annuus* L.), and 'Blue Hubbard' winter squash (*Cucurbita maxima* Duchesne) at the Russell E. Larson Research Farm, Rock Springs, PA, during July–September, 1993–1995. Field-collected beetles were maintained in plastic boxes on store-bought acorn squash fruit and iceberg lettuce for 2–4 weeks at 25°C under a 16:8 light–dark photoperiod. Northern corn rootworms were collected from sunflowers where they were more abundant.

Squash Blossom Bioassay

Cucurbita maxima 'Blue Hubbard' vines were grown in plastic pots under halide lamps and a 16:8 light–dark photoperiod in a greenhouse maintained at 27 ± 4°C. Staminate flowers were collected daily and peduncles placed in water and kept at 4°C for up to 5 days to ensure enough fresh flowers for bioassay. Disks (1.5-cm diameter) were cut from petals on either side of the midvein with a cork borer (mean weight ≈ 37.9 mg). Chemicals were applied to the adaxial surface of disks in 8 × 1-μl aliquots of methanol carrier solvent with a Hamilton (Reno, NV) syringe repeating dispenser. Two solvent-control and two treated disks on top of moist filter paper were placed in a 2 × 10-cm polystyrene petri dish along with four beetles. Consumption of each disk was visually rated on a 0 (no feeding) to 10 (complete feeding) scale at 5, 24, and 48 hr. A minimum of three concentrations for each chemical was tested and replicated at least four times.

Data Analysis

Consumption ratios of treated to solvent control were calculated for each replicate at 5 and 24 hr. At least three concentrations were tested within the linear phase of the log dose-response. Concentrations where consumption ratios

remained at maximum or minimum values were discarded, except for the lowest concentration producing the highest consumption ratio and the highest concentration producing the lowest consumption ratio.

For each replicate within the linear phase of the dose–response, consumption ratios at 24 hr were regressed against the log nmol applied to disk to obtain a linear response. Four estimates of the ED_{50} (nanomoles antifeedant required to reduce the consumption ratio 50%) were obtained and the mean and standard error calculated. Mean separations were carried out with the Mann–Whitney two-sample rank test (quinoline alkaloids) or Mood's median test on MINITAB Version 8.2 (State College, PA).

Chemicals

The structures of chemicals used here are presented in Figure 1. Parthenolide was purchased from Aldrich (Milwaukee, WI). Argophyllin A was extracted and isolated by Chou and Mullin (1993). All other chemicals were purchased from Sigma (Saint Louis, MO). The quinoline alkaloids (quinine, quinidine, cinchonine, cinchonidine) were chloride salts as were strychnine and hydrastine ([1R,9S]-β-Hydrastine). All other chemicals were free bases.

RESULTS

Diabrotica v. virgifera was more sensitive to three of the quinoline alkaloids than *D. u. howardi*. Both species were equally sensitive to quinidine (Table 1). *Diabrotica u. howardi* was least sensitive to strychnine of the diabroticite species and significant differences in strychnine sensitivity existed among three of the species (Table 2). Species sensitivity to the dimethoxylated analog of strychnine, brucine, was different from that of strychnine. *Acalymma vittatum* and *D. u. howardi* were significantly less sensitive to brucine than *D. v. virgifera* or *D. barberi* (Table 2). Estimated ED_{50}'s for both eburnamonine and vincamine were higher for *D. u. howardi* than *D. v. virgifera* or *D. barberi*. No sensitivity differences were detected between the latter two species (Table 2). *Acalymma* sp. was also less sensitive to vincamine and eburnamonine than the two species in the *D. virgifera* subgenus; *Acalymma*'s sensitivity to these two alkaloids was similar to that of *D. u. howardi* (Table 2). Compared to the other alkaloids in Table 2 the spectrum of species sensitivity to hydrastine was relatively uniform. *Diabrotica barberi* was the most sensitive beetle to hydrastine but *D. v. virgifera* had an ED_{50} similar to those of the two least sensitive species, *D. u. howardi* and *A. vittatum*. Sensitivities to indole, quinoline, and isoquinoline alkaloids were within the same order of magnitude for *D. v. virgifera*. In contrast, we observed much more variation in the sensitivity to alkaloids with *D. u. howardi* and *A. vittatum* (Tables 1 and 2).

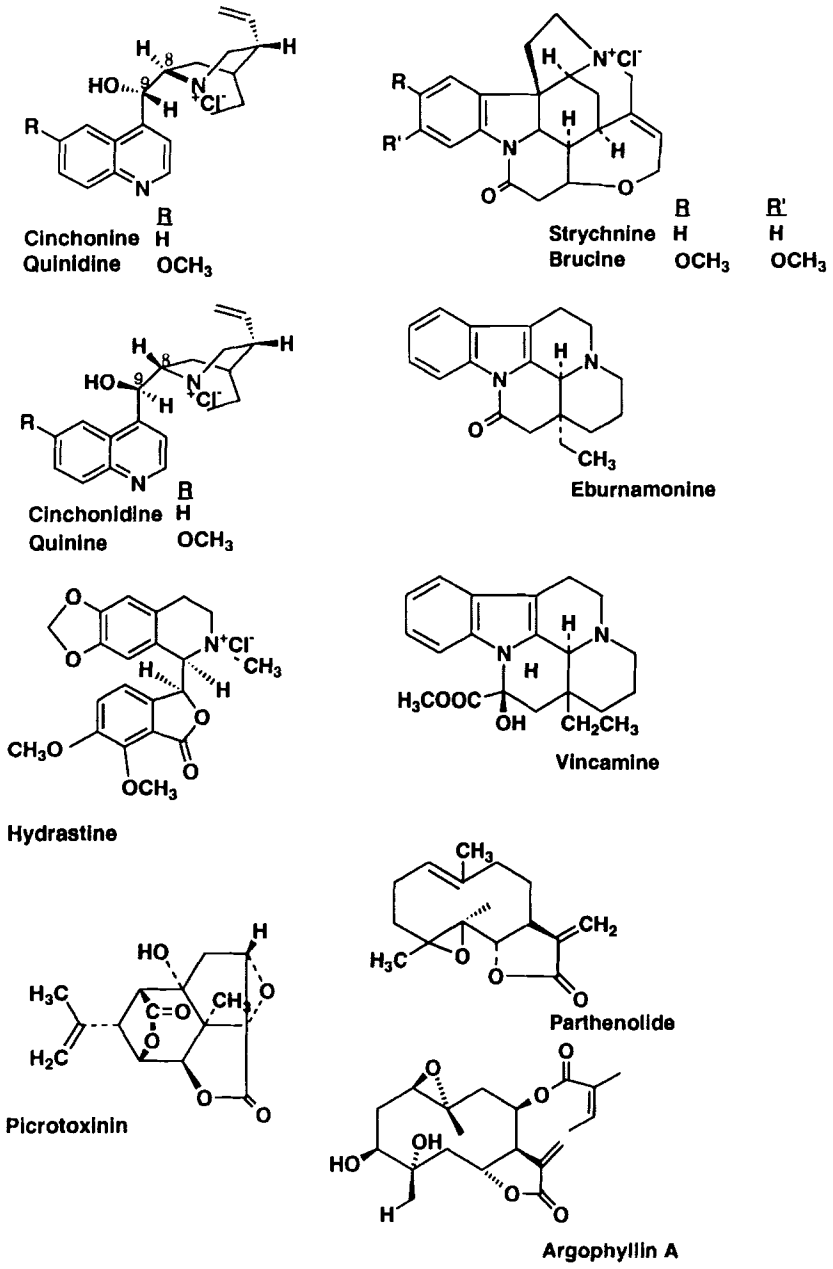

FIG. 1. Chemical structures of antifeedants used in this study.

TABLE 1. ED_{50} (NMOL/DISK ± SE) CALCULATED FOR QUINOLINE ALKALOIDS

Chemical	*D. virgifera*	*D. howardi*	
Quinine	1.2 ± 0.2	141.9 ± 17.8	*[a]
Quinidine	4.7 ± 0.4	5.6 ± 0.3	NS
Cinchonine	2.4 ± 0.1	40.1 ± 4.9	*
Cinchonidine	2.9 ± 0.4	43.9 ± 1.5	*

[a] *, significant ($P < 0.1$) pairwise differences; NS, not significantly different (Mann Whitney, two-sample rank test).

TABLE 2. ED_{50} (NMOL/DISK ± SE) CALCULATED FOR INDOLE AND ISOQUINOLINE ALKALOIDS[a]

Chemical	*D. virgifera*	*D. barberi*	*D. howardi*	*A. vittatum*
Strychnine	1.1 ± 0.2c	2.9 ± 0.6b	15.1 ± 2.2a	5.6 ± 1.8b
Brucine	1.9 ± 0.3b	0.3 ± 0.1b	23.5 ± 2.9a	32.0 ± 11.3a
Eburnamonine	4.3 ± 1.7ab	1.2 ± 0.5b	9.4 ± 1.3a	9.6 ± 2.9a
Vincamine	5.4 ± 1.9b	5.4 ± 2.7b	15.2 ± 0.9a	21.6 ± 4.8a
Hydrastine	4.6 ± 2.0ab	1.9 ± 0.3b	3.0 ± 0.6b	4.2 ± 0.4a

[a] Means followed by different letters within each row are significantly different ($P < 0.1$).

In general, all beetle species were less sensitive to sesquiterpenoids than alkaloids; however, there were exceptions (Table 3). *Diabrotica u. howardi* had similar sensitivities to the quinoline alkaloids, cinchonine and cinchonidine, but lower sensitivities to quinine and the three sesquiterpenoids. *Diabrotica v. virgifera* was less sensitive to picrotoxinin than the other diabroticite species. Parthenolide sensitivity was similar among the three *Diabrotica* spp. but lower for *Acalymma* (Table 3). The ED_{50} response of three *Diabrotica* spp. to argophyllin

TABLE 3. ED_{50} (NMOL/DISK ± SE) CALCULATED FOR SESQUITERPENE LACTONES[a]

Chemical	*D. virgifera*	*D. barberi*	*D. howardi*	*A. vittatum*
Picrotoxinin	143.0 ± 22.3a	39.1 ± 14.3b	55.3 ± 22.1b	69.1 ± 25.3ab
Parthenolide	25.6 ± 4.5b	19.7 ± 4.0b	40.7 ± 15.3ab	82.1 ± 16.5a
Argophyllin A	98.1 ± 17.1a	29.7 ± 5.3b	50.5 ± 19.8ab	

[a] Means followed by different letters within each row are significantly different ($P < 0.1$).

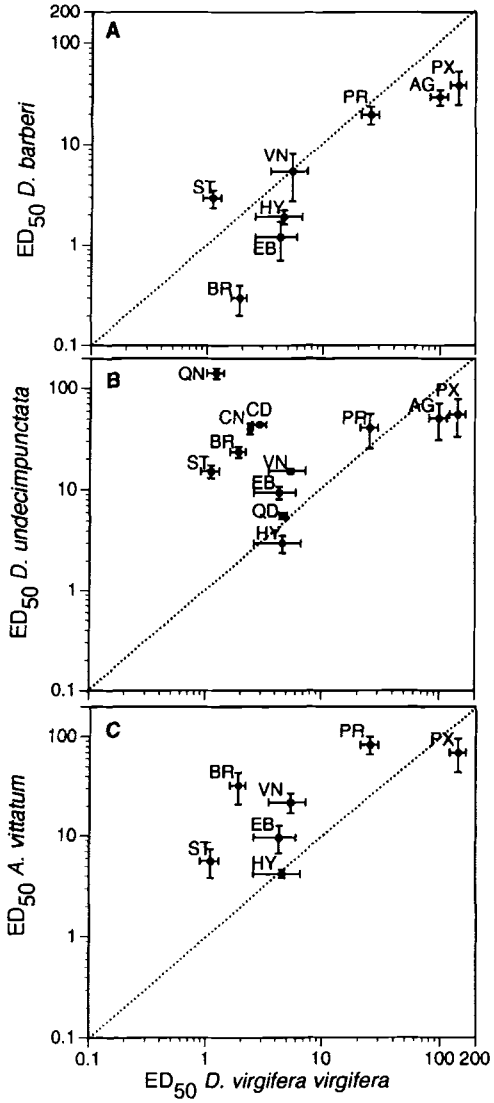

FIG. 2. Comparisons of sensitivities ($ED_{50} \pm SE$) to antifeedants between *D. v. virgifera* and (A) *D. barberi*, (B) *D. u. howardi*, and (C) *A. vittatum*. The dotted diagonal line in each panel indicates equal sensitivity between species to a given compound. AG, argophyllin A; BR, brucine; CD, cinchonidine; CN, cinchonine; EB, eburnamonine; HY, hydrastine; PR, parthenolide; PX, picrotoxinin; QD, quinidine; QN, quinine; ST, strychnine; VN, vincamine.

A was similar to their response to picrotoxinin. *D. barberi* was more sensitive to argophyllin A than the two other *Diabrotica* spp. (Table 3).

Figure 2 summarizes ED_{50} comparisons between *D. v. virgifera* and the three other diabroticite species. Points above or below the diagonal line of equal sensitivity indicate that the species being compared to *D. v. virgifera* was less or more sensitive to each compound, respectively. The ED_{50} for every compound, except strychnine and vincamine, was lower for *D. barberi* than for *D. v. virgifera* (Figure 2A). The opposite occurred for comparisons between *D. v. virgifera* and *D. u. howardi* and *A. vittatum*. With the exception of hydrastine and two sesquiterpenoids, *D. u. howardi* was less sensitive to every compound than *D. v. virgifera* (Figure 2B). Except for picrotoxinin, *A. vittatum* was less sensitive to antifeedants than *D. v. virgifera* (Figure 2C).

Diabrotica barberi was more sensitive to alkaloids and sesquiterpenoids than *D. v. virgifera* (Figure 2). Polyphagous *Diabrotica u. howardi* was less sensitive to most alkaloids than *D. v. virgifera* but was more sensitive to two of the three sesquiterpenoids than *D. v. virgifera*. *Acalymma vittatum* was less sensitive to most compounds than *D. v. virgifera* (Figure 2). All beetle species were uniformly sensitive to hydrastine (Table 2, Figure 2).

DISCUSSION

All sesquiterpene lactones and quinoline, isoquinoline, and indole alkaloids tested here were diabroticite antifeedants. Overall, beetles were more sensitive to alkaloids than sesquiterpenoids. Consistent differences in antifeedant sensitivity between polyphagous beetles and oligophagous beetles were not found. Although wide variations in individual behavior within a species make relationships between antifeedant sensitivity and host plant breadth less clear, polyphagous insects tend to be less sensitive to antifeedants than oligophagous or monophagous insects (Bernays and Chapman, 1977; 1994; Cottee et al., 1988; Schoonhoven, 1982; Simmonds et al., 1990), a trend that we found here. Interestingly, the closely related *D. v. virgifera* and *D. barberi* shared more similar antifeedant sensitivities than the more distantly related *D. u. howardi* and *A. vittatum*.

We chose compounds that were potent antifeedants with a putative common mode of action. Antifeedants not targeting GABA/glycine-gated ionophores may be useful for future studies. Dethier and Bowdan (1989) concluded that rejection behavior of blowflies, *Phormia regina* Meigen, evoked by eight different alkaloid classes was a result of interaction at different target sites rather than a common one. This should not be surprising, considering the many different pharmacological activities of alkaloids (Wink, 1993).

Adult diabroticites may have similar gustatory mechanisms because they

share an affinity for cucurbits, which may also result in similar antifeedant sensitivities. However, the cucurbit feeding habit does not explain interspecific differences in threshold concentrations of cucurbitacins that evoke diabroticite feeding on silica substrates. Among the diabroticite beetles, *Diabrotica u. howardi* is the most sensitive species to six cucurbitacins; *Diabrotica* spp. within the *virgifera* subgenus have intermediate sensitivity and *Acalymma* spp. are least sensitive to cucurbitacins (Metcalf et al., 1980). The relative insensitivity of *Acalymma* to both cucurbitacins and antifeedants is an interesting similarity between the earlier study and ours.

A common mechanism may underlie the antifeedant activity of compounds tested. We proposed the involvement of a GABA/glycine-gated chloride (Cl^-) ionophore in insect gustation (Mullin et al., 1992, 1994). Agonists, including short-chain neutral amino acids (i.e., GABA, glycine, alanine) bind to a receptor protein that allows efflux of Cl^- and generation of a receptor potential. Antagonists, such as the ones studied here, prevent channel opening and do not evoke receptor potentials or generate electrical impulses that stimulate feeding.

Variability of individual responses was inherent in behavioral assays and made comparisons between species difficult. Greatest variation in estimated ED_{50}'s were observed among sesquiterpenoids and the species least sensitive to antifeedants, i.e., *D. u. howardi* and *A. vittatum*. This may, in part, be caused by the shallow dose–consumption curves (not shown). Another source of variability was the different consumption rates of beetle species which could influence the ratio of control to test disks consumed. However, antifeedant sensitivity was greater at 5-hr observations and reflected the same trend 19 hr later, even though the earlier data were more variable. Thus, our experimental goal to compare ED_{50}'s between species was best met by comparisons at 24 hr.

Phagostimulatory cucurbitacins, amino acids, or other undefined compounds in squash blossom disks may have confounded our ability to estimate ED_{50}'s accurately for each compound. Concentrations of these phagostimulants in squash blossom petals were of unknown variability. Nevertheless, most compounds tested remained strongly antifeedant even after 48 hr, and thus sensory input from these compounds overrode sensory input from blossom phagostimulants or through habituation. Most antifeedant bioassays use sucrose for phagostimulation, but this sugar does not sufficiently stimulate diabroticites to feed on cellulose disks (unpublished). That the compounds tested here show antifeedant activity even in the presence of highly phagostimulatory cucurbitacin demonstrates the potency of these antifeedants.

Altering the structure of model antagonists, for example, through stereochemistry (e.g., quinine vs quinidine), addition of methoxy groups (e.g., quinidine vs cinchonine, strychnine vs brucine), and modification of indole lactam linkages (e.g., eburnamonine vs vincamine), did not markedly affect antifeedant activity for any species. The most significant structural alteration in antifeedant

activity occurred in *D. u. howardi* between the diastereomers quinine and quinidine, in which both chiral centers at carbons 8 and 9 are reversed (Figure 1, Table 1).

Wink (1993) recently summarized the antifeedant effects of many alkaloids tested here. Notable differences occur, even if there are difficulties comparing results from other studies because of various experimental designs, substrates, media, phagostimulants, and units reported. Except for cinchonine, the quinoline alkaloids we tested reduced *Spodoptera littoralis* Boisd. feeding (Krug and Proksch, 1993). However, of all the alkaloids tested in our study, honeybees, *Apis mellifera* L., were most sensitive to cinchonine (Detzel and Wink, 1993). Such large differences in the efficacy of alkaloidal antifeedants were not seen in adult *D. v. virgifera*. Except at the highest concentration tested (1%), berberine (benzylisoquinoline alkaloid), brucine, strychnine, cinchonidine, and chinchonine were not strong antifeedants to the polyphagous ctenuchid, *Syntomis mogadorensis* Blachier (Schneider and Wink, 1990). When tested against the oligophagous and polyphagous locusts, *Locusta migratoria* (L.) and *Schistocerca gregaria* Forskål, respectively (Cottee et al., 1988), the estimated ED_{50}'s for quinine on glass-fiber disks were higher than the ED_{50} estimated for *D. v. virgifera* on squash blossom disks. *Diabrotica u. howardi* was less sensitive to quinine than the above three species. Beetles in this study were less sensitive to picrotoxinin and agrophyllin A than reported by Mullin et al. (1992).

Electrophysiology of chemosensillar responses to hydrastine and strychnine on *D. v. virgifera* galeae determined that taste cells are sensitive to these alkaloids at concentrations below 10^{-7} M (<0.1 nmol/disk). Picrotoxinin evokes responses at 10^{-6} M. Behavioral responses to these compounds have higher thresholds than electrophysiological responses (Chyb et al., 1995, personal communication). The importance of galeal chemoreceptors and their sensitivity to antifeedants is also demonstrated by *D. v. virgifera*'s inability to discriminate between cucurbitacin and strychnine when both galeae are ablated (Chyb et al., 1995; Eichenseer and Mullin, 1996). When strychnine and hydrastine were tested at the same concentration on galeal chemosensilla, there were similar rates of response, and small differences in the firing rate of galeal chemosensory cells evoked large changes in behavioral rejection to antifeedants (Chyb et al., 1995). Electrophysiological comparison with other diabroticites would define the relative role of peripheral vs central neural mechanisms that evoke an antifeedant response. Similar qualitative sensitivities among closely related beetles suggest a common peripheral mechanism involved in antifeedant chemoreception.

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DISRUPTION MECHANISMS OF PHEROMONE COMMUNICATION IN THE EUROPEAN GRAPE MOTH *Lobesia botrana* DEN & SCHIFF. III. SENSORY ADAPTATION AND HABITUATION

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Abstract—Disruption experiments were carried out under vineyard conditions and in the laboratory. Males from laboratory cultures were conditioned in an atmosphere permeated with pheromone (E7 Z9-12Ac), marked externally with fluorescent powder, and released in the middle of vine plots. They were then trapped in a series of traps baited with virgin females or dispensers loaded with various amounts of pheromone. Over 10,000 males were released in these experiments between 19 May and 22 July. Electroantennography tests were used for studying olfactory sensitivity in pheromone-permeated air by measuring the EAG responses of male antennae in constant pheromone airflows. The conclusion is that both sensory adaptation and central nervous system habituation mechanisms acted simultaneously in air laden with pheromone, but they are probably not relevant when concentrations are of the same order of magnitude as those obtained under field conditions in which mating disruption methods are used for controlling the European grape moth.

Key Words—*Lobesia botrana*, European grape moth, sex pheromone, mating disruption, insect marking technique.

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INTRODUCTION

Several mechanisms may be involved in mating disruption with pheromone according to Cardé (1990), including loss of sensitivity of the antennal receptors (sensory adaptation), loss of sensitivity in the central nervous system (habituation), camouflaging of the female's odor trail, competition between dispensers and females, and unbalanced components in the synthetic pheromone. We demonstrated that two of the hypotheses, namely, trail camouflaging and competition, are sufficient to account for the overall disruption process and that unbalanced components were of no relevance in the European grape moth (Schmitz et al., 1995a,b). In the present paper, we tested whether loss of sensitivity (sensory adaptation and habituation) also plays a role under conditions used for field testing mating disruption (Stockel et al., 1994). Laboratory tests in chambers known as disruption meters showed that fewer females mated in pheromone-laden airflow (24%, vs 89% in the control) despite the proximity of males and females. However, this occurred only when pheromone concentrations in the air were far higher than those that can be obtained in vineyards (Carles et al., 1979) (37 ng of pheromone per liter of air in the example mentioned). The insects in the above trial remained in the pheromone-enriched air, and as a consequence, all of the disruption mechanisms could have occurred. We wanted to determine whether previous exposure affected the behavior of insects once they returned to normal conditions without the pheromone or with pheromone concentrations that are realistic for a vineyard treated for mating disruption. Field experiments using previously conditioned males and electroantennography were the two experimental methods that we used.

METHODS AND MATERIALS

Vineyard Trials

A joint experiment set up to study the mechanisms of mating disruption was used (Schmitz et al., 1995a,b). The experiment was carried out to assess the competition between pheromone sources and females according to variation of doses, distances, and pheromone blends. In order to determine the influence of a previous exposure of males to the pheromone, conditioned moths were marked, released, and retrapped.

Preparation of Released Males. All moths were reared on a semisynthetic diet under controlled conditions (Stockel et al., 1989). Males were conditioned by pheromone either in the laboratory or in a vineyard plot before trials. In the laboratory, they were exposed to a constant pheromone flow in a "confusiomètre," a device described previously (Carles et al., 1979) that is a glass tube (15-cm diameter × 40 cm) in which constant air flows over a pheromone dis-

penser (BASF type). The pheromone loss from the dispenser, calculated by weighting the dispenser before and after the trial, was 0.3 mg/hr. The males were thus conditioned during a 3-hr period in 1990 and an 8-hr period in 1991.

In the vineyard, moths were held for 8 hr in muslin enclosures placed in the center of a 200-m^2 vine plot equipped with BASF pheromone dispensers (1 dispenser/5 m^2). Each dispenser contained 500 mg pheromone. In both cases, reference insects were conditioned under similar conditions without pheromone exposure. After conditioning, moths were marked with a fluorescent powder (Daygloo Color Corp., Cleveland, OH, USA) by putting the insects in contact with felt on which dye had been sprinkled. Insects were then transported to the experimental vineyard, where they were released 1 hr before dusk. The total length of time between the end of conditioning and the start of the activity period was 1.5 hr.

Traps. Only an INRA type trap was used (Stockel, 1977). This trap is suitable to use with live females or a pheromone dispenser as bait. Before the experiment, the females coming from the laboratory culture were conditioned for 48 hr to the natural photoperiod. They were then isolated in small wire cages provided with water. Each cage was placed in the middle of a trap and kept for 3 days (time of one replicate).

Two types of dispensers were used. Polyisoprene septa (Ets Leune, Orsay, France) were used for doses between 1 and 1000 μg, and polyethylene dispensers supplied by BASF for the 500-mg dose in the mating disruption tests. The major pheromone compound, (*E*)-7,(*Z*)-9-dodecadienyl acetate (E-7,Z-9-12Ac) was used as one bait, and the second bait consisted of a blend of E-7,Z-9-12Ac (80%), (E)-7, (Z)-9-dodecadienol (E-7,Z-9-12ol) (16%), and (Z)-9-dodecanyl acetate (Z-9,12Ac) (4%). This blend is similar to the female secretion.

Experimental Protocol. In 1990 experiments were carried out in a 2500-m^2 vine plot at Château Baret (Graves region). In 1991 another plot 500 m away from this vineyard, and separated by buildings, was added for the pheromone blend experiment. In each plot two trap nets were set up (Figure 1). Four traps containing a virgin female were located in each quadrant (A, B, C, and D) of the plot, 5, 15, 25, and 35 m away from the center, where the males were released. Four other traps baited with a dispenser were also arranged according to a rectangular pattern around each female trap located 25 m away from the center. The size of these rectangles differed between quadrants as shown in Figure 1.

Timetable. Each trial lasted 3 days, with the same dosage in all traps. The dispensers were then changed to test another dose. The experimentation took place during two summers. For each dose, five replicates were made in 1990 and four in each plot in 1991. For each trial, 60 to 110 males were released depending on the capacity of the laboratory culture.

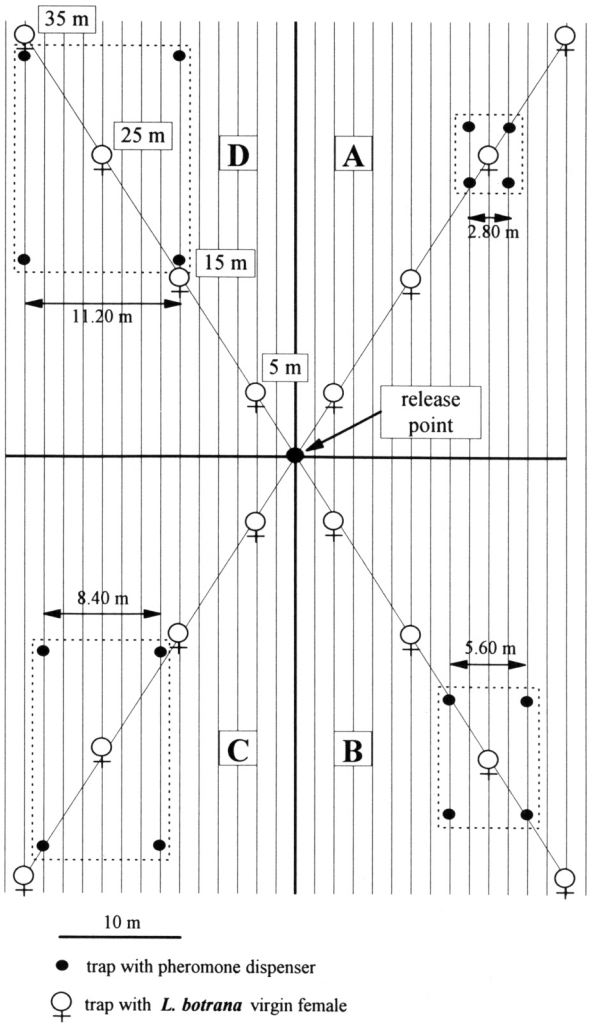

FIG. 1. Experimental design for trap placement for trapping of *L. botrana* males released from the center of a vineyard.

Evaluation. Trapped males were counted in the traps after the first and the third evenings. They were calculated as a percentage of the total released males at the first evening and of remaining moths after the first captures at the third evening. Differences between results were assessed with a chi-square test.

Electroantennography (EAG) Tests

Insect Preparations. Male insects aged 24 to 72 hr were slightly anesthetized with CO_2 and immobilized on polyurethane foam. EAGs were recorded with the antennae still attached in order to improve longevity. Three or four terminal antennal segments were removed with fine scissors and the recording electrode was placed over the distal end of the antenna. The reference electrode was inserted into the neck.

Stimulation Apparatus. The stimulation setup (Figure 2) included a continuous airflow humidified by bubbling through water. The airflow was split into three branches which were later converged into a glass tube with three perpendicular inlets (length = 12 cm, int. Æ = 8 mm) where mixing occurred. The first flow or vector flow (V.F.) was set to 5.2 ml/sec with a flowmeter (FM_1). The second flow or constant flow (C.F.) was further split into two flows by a solenoid valve (SV_1). Part of the C.F. flow was directed toward a vial (F_1; 14-ml vol) containing a rubber septum (Ref. 4010200, Ets Leune, Orsay, France) impregnated with 1 mg of synthetic pheromone E-7,Z-9-12Ac (isomeric purity, 95–98%; synthesized by the Laboratoire des Médiateurs Chimiques, INRA,

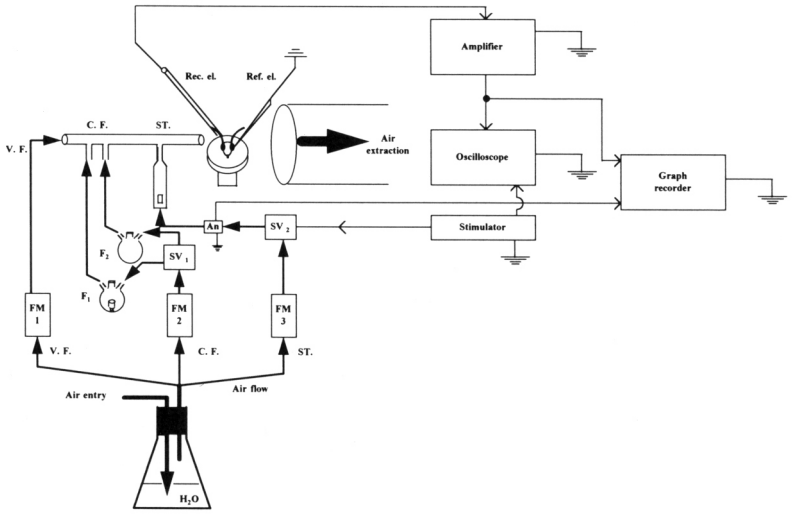

FIG. 2. Electroantennography design: FM, flow meter; SV_1 and SV_2, solenoid valves; An, anemometer; F_1, vial containing the rubber septum for dispensing pheromone E-7,Z-9-12Ac used for generating the continuous flow; V.F., vector airflow; C.F., constant pheromone flow; ST., stimulation test; Rec. el., recording electrode; Ref. el., reference electrode.

Versailles, France). The remainder of the C.F. flow was directed toward a similar vial (F_2) without a septum. A flowmeter (FM_2) was located upstream from the solenoid valve in order to modify the rate of flow. The third flow or stimulation flow (ST) was directed through a Pasteur pipette (5-mm ID) containing a piece of filter paper (144 mm^2) impregnated with 2, 20, or 200 ng of E-7,Z-9-12Ac. The latter served as a stimulation cartridge. A solenoid valve (SV_2) located upstream of the cartridge was controlled by a stimulator so that air could be pulsed through the cartridge for 1 sec at 2.8 ml/sec (FM_3).

Recording Device. The electrodes were glass capillary tubes filled with Roeder's electrolyte (NaCl, 9 g; KCl, 0.2 g; glucose, 4.36 g; H_2O, 1000 ml). Electrical contact was provided by a chloridized silver wire inserted in the capillary. The electrode signals were amplified before input into an oscilloscope. Stimulation was visualized with an anemometer (AN; thermistor probe) located upstream from the stimulation cartridge and connected to the second channel of the oscilloscope. The output from both channels was also sent to a graphical recorder.

Experimental Protocol. The insects were stimulated (ST) with known amounts of pheromone for 1 sec every 2 min, before, during, and after a 6-min exposure to a constant flow of pheromone (C.F). About 15 to 25 repetitions were done.

EAGs were measured at three stimulation dosages (2, 20, and 200 ng of E-7,Z-9-12Ac) and each dose was tested at three constant flow concentrations. The first four stimulations of the sequence were also carried out at doses of 0.1 and 1 ng, each at the three constant flows of 1.14, 2.6, and 7.4 ml/sec so as to expose the antennae to three pheromone concentrations after mixing with the vector flow (V.F.: 5.2 ml/sec). Although pheromone concentrations were not measured, if a value of 1 is attributed to the lowest flow, then the relative concentrations in the other flows would be 2.5 and 6. A set of control insects was exposed in constant airflow, without pheromone, and then stimulated with doses of 0.1, 1, 2, 20, 200, and 2000 ng. As a rough indication, the 2.5 concentration triggered an EAG response of over 8 mV, which is equivalent to the response obtained with a cartridge containing 2000 ng of E-7,Z-9-12Ac.

Statistical Tests. Differences in responses over time and between treatments were compared with nonparametric Kruskal–Wallis tests followed by a multiple-comparison test based on the experimentwise error rate of Noether (1976) cited by Scherrer (1984).

RESULTS

Vineyard Trials

Figure 3 shows the inhibition of attractiveness on the evening following release after the males had been conditioned for 3 or 8 hr in the disruption meter.

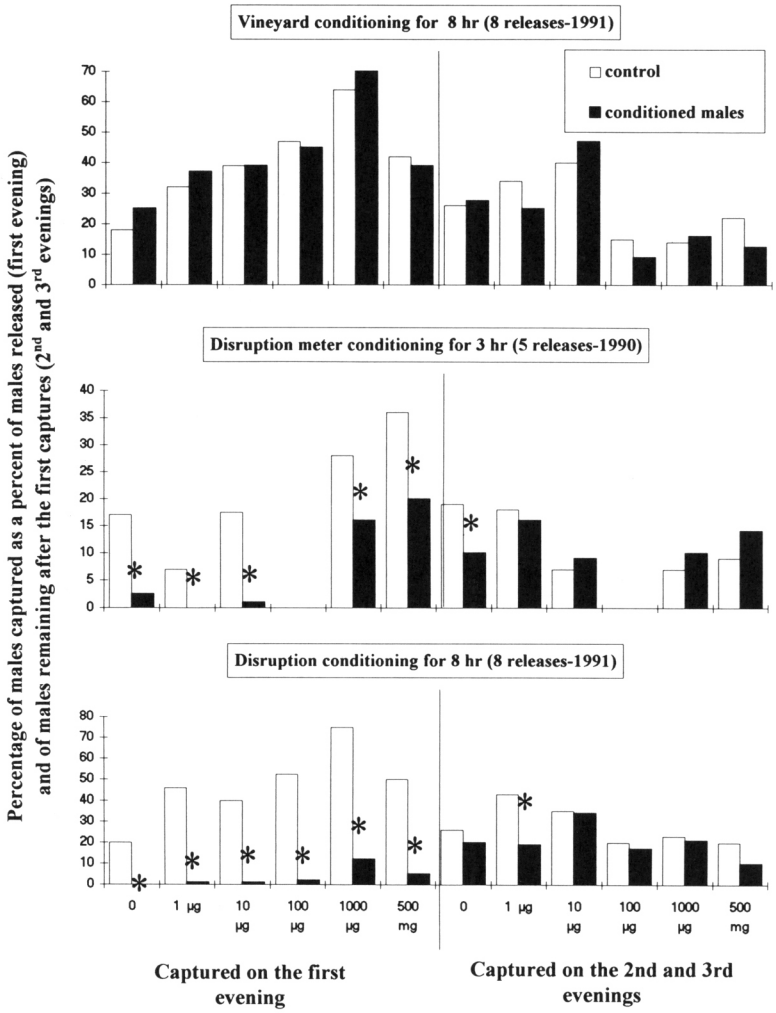

FIG. 3. Male capture rates by the 32 traps (16 baited with one female and 16 with various amounts of pheromone bait as a function of conditioning before release). Significant differences at $P = 0.05$ (χ^2 test) are noted with an asterisk.

Inhibition was almost-total when the baits were females alone or females and dispensers loaded with less than 100 μg of pheromone and remained significant for all doses in dispensers. Inhibition disappeared almost completely on the two following evenings, with significant differences only for low doses of bait. The results did not differ from controls when insects were conditioned in a vineyard plot with dispensers.

Electroantennography Tests

Relationship Between Dose and Response. The dose-response curves in Figure 4 show that the amplitude of the EAGs decreased as the load of pheromone in the constant flow increased for all doses tested. The stimulation threshold increased from 0.1 to 2 ng. For the constant flow levels 1 and 2.5, the stimulation threshold was 1 ng. From this value, the curve increased only for stimulations over 20 ng. The experiment was conducted with the control stim-

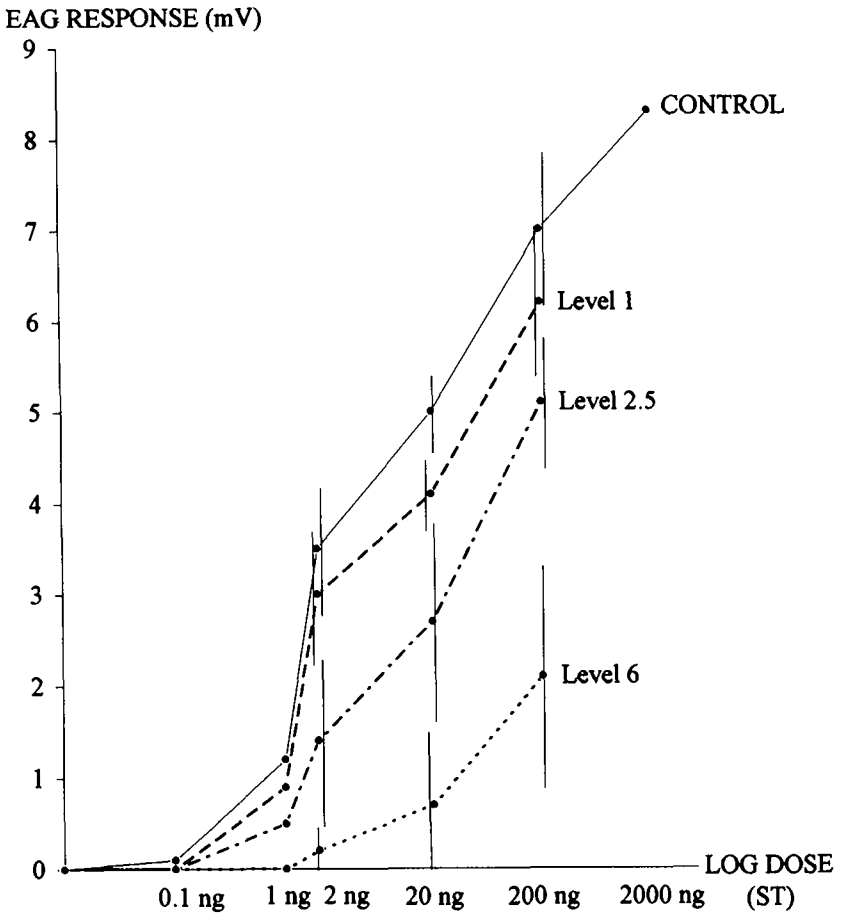

FIG. 4. EAG response of male *L. botrana* antennae to E-7,Z-9-12Ac under pure air (control) or air containing various amounts of pheromone at ratios of 1, 2.5, or 6 (15 to 25 repetitions).

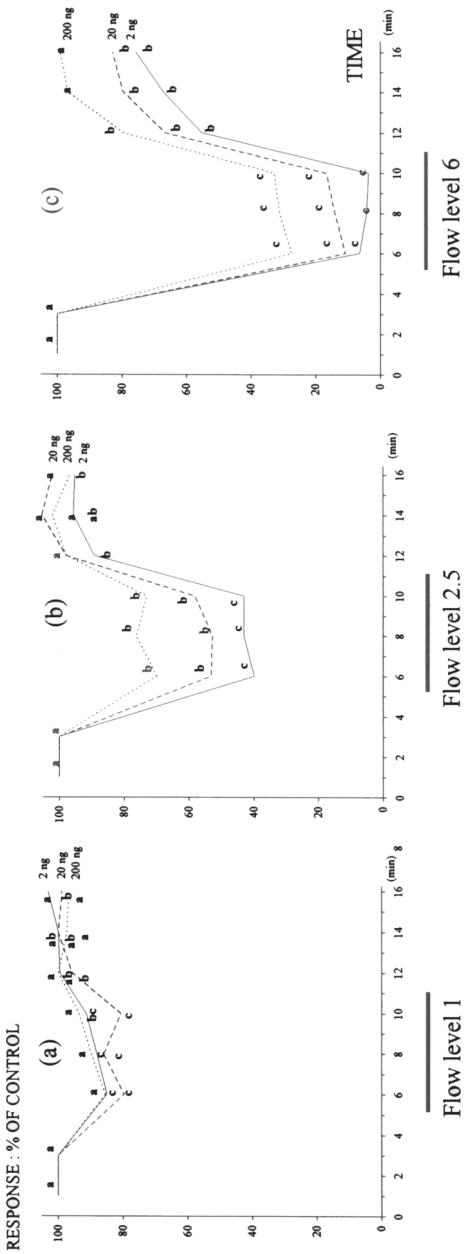

FIG. 5. Changes in antennal responses in male *L. botrana* to periodic stimulations before, during and after exposure to a constant flow (C.F.) containing one of the three concentration levels: 1(a), 2.5 (b), and 6(c) of E7,Z9-12Ac. Responses are expressed as a percentage of the response without the pheromone. On every curve, the values bearing the same letter do not differ at $P = 0.05$ (Kruskal–Wallis test and multiple comparisons based on the experimentwise error rate). Significance differences to level of sensory inhibition according to constant flow level or pheromone stimulation are noted in Tables 1 and 2.

TABLE 1. DIFFERENCES BETWEEN INTENSITIES OF STIMULATION ACCORDING TO THE CONSTANT FLOW LEVEL

	Stimulation intensity (ng)[a]		
Constant flow level	2	20	200
1	88.0a	82.0a	89.4a
2.5	41.6a	52.0ab	72.1b
6	4.5a	13.6ab	30.2b

[a]Values in a row followed by the same letter are not significantly different at $P = 0.05$.

ulation in order to detect a possible plateau of response, but even at a dose of 2000 ng, a plateau of response was not reached.

Inhibition of Responses During and After Exposure to a Constant Pheromone Flow. The data in Figure 5 show the EAGs to the various stimulation pulses at three levels of stimulation (2, 20, and 200 ng) as a percentage of the signal before exposure to the constant flow (mean of three pulses), before, during, and after a 6-min exposure to a constant flow containing each pheromone concentration. For constant flow level 1, responses to stimulations were similar to the controls, and not significantly different (Table 1). On the contrary, responses at levels 6 and 2.5, to high and low stimulation extremes (200 and 2 ng, respectively), were significant.

For low stimulations at 2 and 20 ng, the differences between the three levels of constant flows were significant (Table 2) and the highest inhibition was related to the highest constant flow. Finally, for highest stimulation (200 ng), only the highest constant flow was different.

Inhibition of the EAGs was reversible. The amplitude of the EAGs increased

TABLE 2. DIFFERENCES BETWEEN CONSTANT FLOW LEVELS ACCORDING TO THE INTENSITY OF STIMULATION

	Constant flow level[b]		
Stimulation intensity (ng)	1	2.5	6
200	89.4a	72.1a	30.2b
20	82.0a	52.0b	13.6c
2	88.0a	41.6b	4.5c

[a]Values in a row followed by the same letter are not significantly different at $P = 0.05$.

rapidly after the exposure to constant flow was over. Responses reached 70% of the pretreatment amplitude after a recovery period of 5 min following exposure to the highest constant flow.

DISCUSSION

The amplitude of EAGs increased as the dose of E-7,Z-9-12Ac increased in the range 0.1 to 2000 ng. The olfactory organs of *L. botrana* therefore seem capable of encoding a wide range of concentrations spanning a 20,000-fold range. Previous observations demonstrated that very high doses are attractive to males around the dispensers in experimental disruption areas (Schmitz et al., 1995a; Roehrich and Carles, 1987). In many moths flight is often arrested by large amounts of pheromone compounds (Baker et al., 1981, 1988).

Decreases in responses of the olfactory receptors of males exposed to a constant pheromone flow, as revealed by EAGs, indicate that adaptation occcurred in *L. botrana*. The adaptation was reversible, and disappeared within a few minutes after the constant flow stopped. Similar results have been described previously in other species (Kuenen and Baker, 1981; Sanders, 1985). In most cases, recovery of EAGs generally occurred after a few seconds (Sauer et al., 1992). In the present work at the high doses of pheromone (level 6), the delay observed for recovery (5 min) may have been due to contamination of glassware.

A contradiction seems to appear between these results and those of vineyard trials using insects conditioned by pheromone. In the latter case, the inhibition period of males lasted up to 3 hr after the end of pheromone application (1.5 hr before release and 1.5 hr during the dusk activity). It is clear that under these conditions we did not test the same physiological mechanism in the two experiments. The EAG experiments suggest "adaptation" of sensory organs, while the vineyard tests suggest "habituation" of the central nervous system.

However, habituation was observed only when the amounts of pheromone used were far greater than can be obtained with mating disruption methods in vineyards. Concentrations in the olfactometer disruption tests were of the order of 4 μg/l. For instance, Flint et al. (1990) estimated that air contained a few nanograms of pink bollworm pheromone per cubic meter when 60 mg/ha/hr was diffused under field conditions. Moreover, moths exposed during 8 hr in a vineyard treated with pheromone dispensers were not inhibited after their release in a reference plot. With the pheromone doses used in vineyard tests, habituation does not play any role in mating disruption.

Although it was not possible to evaluate concentrations of pheromone that induced adaptation in olfactory receptors and thus caused lower EAGs, they were probably above those present in natural conditions. Inhibition induced by the level 1 pheromone concentration is poor and not significantly different from

the reference. Adaptation probably plays no role in mating disruption, except possibly very close to the pheromone dispensers.

The experiments suggest that direct action of pheromones on the sensory organs and nervous system of grape berry moth has no influence on efficiency of mating disruption in vineyard trials, and supports the explanation of Schmitz et al. (1995a), who characterized disruption as competition between females and dispensers and camouflage of their pheromone trail.

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ANTINUTRITIVE AND OXIDATIVE COMPONENTS AS MECHANISMS OF INDUCED RESISTANCE IN COTTON TO *Helicoverpa zea*

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Abstract—Induced resistance in cotton (*Gossypium hirsutum*) foliage and squares to herbivory by *Helicoverpa zea* (Lepidoptera: Noctuidae) is reported in this study. Induced resistance was indicated by decreased larval growth when larvae fed on previously damaged foliage or squares compared to the controls. Herbivory caused a significant decline in host nutritional quality as shown by a reduction in protein and most amino acids in both foliage and squares. Peroxidase, ascorbate oxidase, and diamine oxidase activities increased in both damaged foliage and squares, whereas levels of the nutritional antioxidant, ascorbate, were depressed after larval feeding. Larval feeding also markedly enhanced lipoxygenase activity and lipid peroxides in square tissues. Moreover, feeding damage altered the quantitative levels of phenolic compounds in foliage and squares. These results indicate a significant shift in the oxidative status of cotton plants following herbivory as indicated by increased oxidative enzyme activity, decreased levels of the nutritional antioxidant ascorbate, and increased levels of phenolic prooxidants (i.e., chlorogenic acid) and lipid peroxides.

Key Words—Oxidative stress, nutritional stress, induced resistance, chlorogenic acid, insect-plant interactions, herbivory, tannins, polyphenol oxidase, lignin, phenolics, *Gossypium hirsutum*, *Helicoverpa zea*.

INTRODUCTION

Research on the phytochemical basis of arthropod resistance in cotton has been focused primarily on constitutive factors. These factors include condensed tan-

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nins (Chan et al., 1978b; Reese et al., 1982; Zummo et al., 1983, 1984), flavonoids (Hedin et al., 1992), anthocyanins (Hedin et al., 1983b), phenolic acids (Lege et al., 1995), and/or terpenoid aldehydes such as gossypol (Chan et al., 1978a). However, in the case of tannins (Hedin et al., 1983b; Smith et al., 1992) and gossypol (Chan et al., 1978a), there has been uncertainty as to their efficacy in resistance against *Heliothine* pest species.

Induced resistance to arthropods is well documented in cotton using spider mites (Karban and Carey, 1983; Karban, 1986a,b; Brody and Karban, 1989; Karban and Niiho, 1995) and lepidopterans (Karban, 1988), although a phytochemical basis for induced resistance has not been reported. Our laboratory is investigating the phytochemical basis of induced resistance to *Helicoverpa zea* in several regionally important argonomic (e.g., soybean, cotton) and feral (e.g., geranium, clover) hosts. Our initial studies in soybean revealed that induced responses include primary compounds (e.g., proteins, protease inhibitors, vitamins), secondary metabolites (e.g., phenolics), and reactive oxygen species (e.g., hydroxyl radical, hydrogen peroxide) (Bi et al., 1994; Felton et al., 1994a,b; Bi and Felton, 1995). Foliar herbivory by *H. zea* on soybean increased lipoxygenase (LOX), peroxidase (POD), ascorbate oxidase (AOX), and diamine oxidase (DAO) activities (Bi et al., 1994; Felton et al., 1994b; Bi and Felton, 1995). A severe decline in the nutritional quality of foliar protein also occurred in wounded plants (Bi et al., 1994).

The current study was initiated to determine if prior feeding by *H. zea* (1) induces *H. zea* resistance in cotton foliage and squares and (2) induces the formation of phytochemicals (e.g., gossypol, tannins, etc.) implicated in *H. zea* resistance in cotton foliage and squares. The results reported here indicate a novel mechanism for *H. zea* resistance in cotton.

METHODS AND MATERIALS

Plants and Insects

Eggs of *H. zea* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on artificial diet until used in the experiment (Chippendale, 1970).

Cotton (*Gossypium hirsutum*) seeds (cv. Deltapine 50) were soaked in water for 6 hr and then incubated at 28°C for 24 hr. Germinated seeds were sown in 2-L plastic pots filled with soil mixture (Redi Earth Peat-Lite Mix) in a greenhouse. Plants were watered every 2 days, and fertilizer (N:P:K = 20:20:20) was used weekly. Greenhouse conditions were (1) a 14-hr photophase, using high-pressure sodium light (1000 W), and (2) a day temperature of 33 ± 2°C and a night temperature of 20 ± 2°C.

In a field experiment, cotton plants at the four-node stage were transplanted with the above-mentioned soil mixture to three rows at the Agricultural Experiment Station of the University of Arkansas, Fayetteville. Row spacing was 0.96 m; row length was 10 m. Plants were spaced at 0.30-m intervals. Five grams of Osmocote fertilizer was placed in each planting hole at the time of transplanting. The plants were watered regularly.

Induction of Resistance

To determine if *H. zea* feeding induces resistance, a single fourth instar was placed on each of 40 four-node-stage plants. Plants were placed individually in screen cages to prevent larval escape. Forty control plants were identically treated except that larvae were excluded. Larvae were starved for 24 hr prior to infesting the plants. The levels of defoliation in the wounded plants were visually estimated at less than 30%. After 72 hr, two terminal fully expanded leaflets from each damaged plant and the same-positioned two leaflets from each control plant were excised with a single-edge razor blade. The 72-hr time period was chosen because preliminary experiments indicated that resistance to *H. zea* was not induced at 24 to 48 hr postdamage.

The excised leaves from each plant were placed in a 500-ml clear plastic container (Fabri-Kal Co., Kalamazoo, MI) with two layers of moist filter paper (Whatman No. 1) in the bottom. A newly molted fourth instar or 10 neonates were placed in each container. Leaves from 20 damaged and 20 control plants were used for the single fourth-instar treatment, and the leaves from the other plants were used for the 10-neonate treatment. Containers were randomly placed in an incubator at 28°C. Each fourth instar was weighed at the beginning of the test and again after 48 hr. There were no significant differences between the initial weights of the larvae used in the treatment and control containers. The neonates were weighed 96 hr after being placed in the containers.

To evaluate the induced physiological and biochemical responses in cotton foliage to feeding by *H. zea*, 20 damaged and 20 control plants as described above were used. Fully expanded terminal foliage from each of 10 damaged and 10 control plants was individually assayed for LOX, POD, AOX, DAO, reduced ascorbate (ASC), and the oxidized form of ASC, dehydroascorbic acid (DHA). The terminal foliage of the remaining plants was excised and immediately placed in plastic bags, which were held on dry ice. After being fully frozen, the foliage was freeze-dried and then ground to powder for assays of amino acids, protein, lipid peroxides, and phenolic compounds as described below.

In a second test, conducted in the field, 16 15-node-stage plants were used: 8 control and 8 treated plants. Each plant was enclosed in an organdy-covered cage (1 × 1 × 1 m). Plants were damaged by placing eight fourth-instar *H.*

zea on each treatment plant. After 5 days, the original larvae had pupated or died. Five newly molted fourth instars were weighed individually and placed for 3 days on each of the control or treatment plants. The larvae were then collected (the collection rate was ca. 85%) and weighed individually. Because of the movement of larvae on plants in their respective cages and the inability to identify original individuals, the larval relative growth rate (RGR) was calculated using the mean initial weight rather than individual weights (Felton et al., 1994a). The preparation of larval midgut epithelium for chemical assay was as described by Summers and Felton (1994). Midgut tissue of each larva was rendered, and the epithelium was separated from the lumen contents and peritrophic envelope. Each replicate consisted of three midgut epithelia, which were pooled and homogenized in 1.4 ml of ice-cold 0.01 M potassium phosphate, pH 7.0. A total of 10 replicates was respectively prepared for treatment or control group. Following centrifugation for 10 min at 5000*g*, the supernatants were used to assay for antioxidants and hydroperoxides as described below. Aliquots for ascorbic acid assay were immediately removed after centrifugation and combined with an equal volume of 10% metaphosphoric acid.

Squares were also used for bioassay because they represent the preferred food source for later instars of *H. zea*. Three damaged squares from each of eight treated plants and three same-positioned squares from each of eight control plants were excised. Each square was placed individually in a 500-ml container as described above with a newly molted fourth-instar *H. zea* for 48 hr in an incubator at 28°C. The RGR was then computed.

Two fresh squares from each of the eight damaged or eight control plants were assayed for LOX, POD, AOX, and DAO. Another two squares from each of the eight damaged or eight control plants were freeze-dried, ground to powder, and pooled together for treatment or control group. The powder was assayed for phenolics, lipid peroxides, protein, amino acids, ASC, and DHA concentrations. Preliminary data indicated that lypophilization did not significantly affect the ASC:DHA ratio in square tissue compared to using freshly prepared tissue.

Temporal Changes in Damage-Induced Foliar Oxidative Enzymes

To evaluate the temporal effect of mechanical damage on foliar oxidative enzymes, cotton plants at the four-node stage were artificially damaged with a cork borer (diameter, 9 mm). The terminal, fully expanded leaflet was punched with three holes on each side of the main vein, and the terminal semiexpanded leaflet was punched with two holes on each side of the main vein. Twenty plants were damaged and 20 plants were used as controls. The leaf with six holes from treatment plants and the same-positioned leaf from control plants were assayed for AOX, POD, and DAO at 24, 48, 72, and 96 hr after treatment. Five treatment plants and five control plants were individually assayed at each time period.

Plant Enzymes, Protein, and Amino Acids

To assay for plant enzymes, 1 g of foliage or squares was homogenized in 10 ml of 0.1 M ice-cold potassium phosphate buffer, pH 7.0, containing 1% PVP and 0.5 mM EDTA. The homogenate was centrifuged at −2°C for 20 min at 10,000*g*. The supernatant was used immediately as the enzyme source for the assays described below. A SLM-AMINCO 3000-diode array spectrophotometer with rate analysis software was used for all assays.

To assay for LOX, 0.25 mM linoleic acid was used as a substrate, and the rate of change in absorbance at 234 nm was measured (Grayburn et al., 1991) as modified by Bi and Felton (1995). LOX was assayed at the optimal pH of 7.0 (0.1 *M* potassium phosphate buffer), which was determined in a preliminary experiment.

Polyphenol oxidase (PPO) activity was determined following the procedure of Ryan et al. (1982). A 50-μl aliquot of foliar homogenate was assayed with 1 ml of 3.0 mM chlorogenic acid or caffeic acid in 0.1 M potassium phosphate buffer, pH 7.0. The increase in absorbance was monitored at 470 nm.

POD activity was assayed with guaiacol as the hydrogen donor according to the procedure of Ridge and Osborne (1970). Fifty microliters of enzyme solution were mixed with 1 ml of substrate containing 1 mM H_2O_2 and 2 mM guaiacol in 0.1 M potassium phosphate buffer, pH 7.0. POD acivity was estimated at 470 nm.

DAO activity was measured with 10 mM putrescine (diaminobutane) in 0.1 M potassium phosphate buffer, pH 7.0, following Bi and Felton (1995). Absorbance of the Δ^1-pyrroline complex was measured at 430 nm with an extinction coefficient of 1.86 mM^{-1} cm^{-1} (Federico et al., 1985).

AOX activity was measured according to the procedure of Felton and Summers (1993). An extinction coefficient of 14.7 mM^{-1} cm^{-1} was used for ASC.

The protein content of plant tissue was determined by a modified Bradford (1976) assay. Ten milligrams of tissue powder were dissolved in 4 ml of 0.1 N NaOH. One percent soluble PVP was added to Coomassie brilliant blue G-250 reagent to prevent the interference of phenolics. Absorbance of the reaction mixture was then read at 595 nm using bovine serum albumin (Sigma Chemical Co.) as a standard.

Amino acid analyses of the square and foliar tissues were performed by Commonwealth Biotech, Richmond, VA. Duplicate samples were subjected to acid hydrolysis following standard protocols and analyzed on a fully automated Hewlett Packard Amino Quant pre-column amino acid analysis system. Values reported are the means of two analyses.

Chemical Antioxidants

The concentrations of ASC and DHA were determined by a spectrophotometric assay (Law et al., 1983) as fully described by Bi and Felton (1995).

To assay for nonprotein thiols, a method from Hu (1994) was adopted. A 0.2-ml sample was mixed in a 10-ml test tube with 0.6 ml of Tris base (0.25 M)–EDTA (20 mM) buffer (pH 8.2), followed by the addition of 40 μl of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 3.16 ml of absolute methanol. The color was developed for 20 min at 25°C, followed by centrifugation of 3000*g* for 10 min. Absorbance of the supernatant was measured at 412 nm and subtracted from a DTNB blank and a blank containing the sample without DTNB. An extinction coefficient of 13.6 mM^{-1} cm^{-1} was used.

Lipid Peroxidation

Lipid peroxidation of the midgut epithelium was assayed by the FOX2 method (Wolff, 1994; Summers and Felton, 1994). Lipid peroxidation for cotton squares and foliage was specifically determined following Auerbach et al. (1992) using a kit (Kamiya Biomedical Co., Thousand Oaks, CA). The assay takes advantage of the ability of lipid peroxides to oxidize *N*-benzoyl leucomethylene blue to methylene blue in the presence of hemoglobin. The lipid peroxides were quantified spectrophotometrically at 660 nm with cumene hydroperoxide as a standard.

Plant Phenolics

To measure the concentration of condensed tannins, the vanillin–HCl method (Makkar and Becker, 1993) was followed. Twenty milligrams of tissue powder were vigorously vortexed in 4 ml of 50% methanol for 3 min. After centrifugation at 5000*g* for 20 min, 0.25 ml of supernatant was added to 1.5 ml of vanillin reagent (4%, w/v, in methanol), followed by the addition of 0.75 ml concentrated HCl. The reaction was carried out in a 30°C water bath for 20 min and the absorbance was then read at 500 nm. Catechin (Sigma Chemical Co.) was used as a standard. The assay was replicated 15 times from the pooled sample of tissue powder.

Total flavonoids were quantified after extraction of the tissue powder with 70% aqueous acetone following Hedin et al. (1992). Rutin (Sigma Chemical Co.) was used as a standard. The assay was replicated 15 times from the pooled sample of tissue powder.

Estimation of lignin content was performed by the thioglycolic acid procedure as described by Barber and Ride (1988) with some modifications. Ten milligrams of tissue powder were extracted four times in absolute methanol in a 48-hr period. Air-dried residue was suspended in 2 ml of 0.5 M NaOH and incubated at 25°C for 24 hr to hydrolyze cell wall-bound phenolic acids. The resulting mixture was neutralized with 0.5 ml of 2.0 N HCl followed by centrifugation for 10 min at 3000*g*. The pellet was then washed in 10 ml of water,

collected by centrifugation, and resuspended in 10 ml of absolute methanol. After centrifugation at 3000*g* for 10 min, the residue was air-dried and then treated with 5 ml of 2.0 N HCl and 0.5 ml of thioglycolic acid in a screw-top test tube at 95°C for 4 hr. The solid material was collected by centrifugation and then washed in 5 ml of water and recollected by centrifugation. The resulting solid was incubated with 5 ml of 0.5 M NaOH for 16 hr. After centrifugation, the solid was washed twice in 2 ml of water, followed by centrifugation. The NaOH extract and water washes were combined, and 1 ml of 37% HCl was added. To precipitate the ligninthioglycolic acid (LTGA), the mixture was held at 4°C for 48 hr. The precipitated LTGA was collected by centrifugation and resuspended twice in 2 ml of 0.1 N HCl and each held for 48 hr at 4°C. After centrifugation, the pellet was dissolved in 1 ml of 0.5 M NaOH at 25°C for 24 hr. The insoluble material was then removed by centrifugation at 10,000*g* for 3 min and the absorbance was read at 280 nm. The lignin assay was replicated five times per treatment from the pooled sample of tissue powder.

To assay for gossypol and related terpenoid aldehydes, 20 mg of tissue powder were extracted in a 4-ml mixture of cyclohexane, ethyl acetate, and acetic acid (500:500:1) following Hedin et al. (1992). Gossypol (Sigma Chemical Co.) was used as a standard. The assay was replicated 15 times from the pooled sample of tissue powder.

To specifically analyze for other phenolic compounds (i.e., chlorogenic acid, *p*-coumaric acid, syringic acid, gallic acid, ferulic acid, *p*-hydroxybenzoic acid, and rutin), the extraction technique employed was that described by Murphy and Stutte (1978) and Guerra (1981). Two 100-mg samples of foliar or square powder from treatment or control were vigorously extracted for 1 min in 1 ml of 50% (v/v) methanol three times, and each extraction was followed by a centrifugation for 5 min at 5000*g*. The resulting supernatants were filtered through a 0.45-μm nylon filter. The filtrate from the first sample was added to the same volume of 2.0 N HCl and hydrolyzed for 1hr in a boiling water bath. The nonhydrolyzed sample was directly injected for HPLC analysis. After hydrolysis, the sample was extracted with ca. 1 ml of diethyl ether three times, and the combined extracts were dried under nitrogen gas. The resulting sample was resuspended in 0.2 ml of 80% (v/v) methanol and filtered with a 0.45-μm nylon filter. For the hydrolyzed sample, 10 μl were injected for the test separation. For the nonhydrolyzed foliage, 100 μl were injected, and for the nonhydrolyzed square, 50 μl were injected for separation.

Separation of phenolic compounds was accomplished by reverse-phase high-pressure liquid chromatography (modified from Murphy and Stutte, 1978) using a Nova-Pak C_{18}, 3.8 × 150-mm stainless-steel column. The separation solvents were as follows: initial, butanol:methanol:acetic acid:double-distilled (dd) water (0.25:1.25:2:96.5); final, butanol:methanol:acetic acid:dd H_2O

(5:25:2:68). Both solvents contained 18 mM ammonium acetate. The separation was programmed with a linear gradient for 30 min from the initial solvent to 50% of the final solvent, followed by a 20-min linear gradient from 50% to 100% of the final solvent. Mobile-phase flow was 1 ml min^{-1}. Absorbance at 254 and 280 nm was monitored by a multiwavelength detector. Peak areas, peak heights, and retention times were determined by a computing integrator. Sample peaks were identified by the comparison of their retention times and their 254:280-nm absorbance ratios with those of sample standards (Sigma Chemical Co.). Contents were determined by standard curves of each compound and were expressed on a per dry weight basis. The analysis was replicated three times from the pooled sample of tissue powder.

Statistics

The least significant differences (LSD) test in a one-way completely randomized ANOVA (SAS Institute, 1988) was used to analyze the data.

RESULTS

Induced Resistance in Foliage and Squares due to Herbivory

Resistance was significantly induced in foliage and squares by *H. zea* larval feeding (Tables 1 and 2). The RGR of fourth instars was reduced by 10% when they fed on excised foliage from previously damaged plants, 38% on squares excised from damaged plants, and 24% on damaged but intact plants compared to larvae on the respective controls. The larval weight of neonates was decreased 61% when larvae fed on damaged foliage compared to control foliage. The larval survivorship was not significantly affected by treatment.

Temporal Changes in Damage-Induced Foliar Oxidative Enzymes

Twenty-four hours following mechanical damage to the terminal leaflets, the activities of AOX and DAO were unchanged relative to the undamaged control ($P > 0.05$), while POD was unaffected by mechanical damage throughout the test period of 96 hr (Figure 1; $P > 0.05$). At 48 hr, AOX activity was 47% higher ($P = 0.009$) than the control, but DAO was still unaffected by treatment ($P > 0.05$). At 72 hr following wounding, AOX was 48% higher ($P = 0.003$), and DAO was more than twofold higher than the controls ($P = 0.001$). At 96 hr posttreatment, AOX was 49% ($P = 0.026$) higher than the control, while DAO was not significantly different from the control ($P > 0.05$). The significant increases in DAO and AOX at 72 hr correspond with data indicating that resistance is induced after at least a 48 hr lag following initial damage.

TABLE 1. EFFECT OF PREVIOUS HERBIVORY BY *H. zea* ON COTTON PLANTS ON FOURTH-INSTAR *H. zea* RELATIVE GROWTH RATE[a]

Larval diet	Control	Damaged	$LSD_{.05}$	% reduction
Excised foliage (mg day^{-1} mg^{-1})	0.50 (0.01)	0.45 (0.01)	0.03	10
Excised squares (mg day^{-1} mg^{-1})	0.50 (0.01)	0.31 (0.03)	0.02	38
Intact plants (mg day^{-1} mg^{-1})	0.25 (0.02)	0.19 (0.02)	0.03	24

[a]Fourth instars fed on excised foliage or squares for 48 hr in laboratory and fed on intact plants for 72 hr in field. Numbers in parentheses are standard errors.

TABLE 2. EFFECT OF PREVIOUS HERBIVORY BY *H. zea* ON COTTON FOLIAGE ON NEONATE *H. zea* GROWTH[a]

Treatment	Larval weight (mg)[b]	Survivorship (%)
Control	9.2 (0.6) a	80 a
Damaged	3.6 (0.2) b	83 a

[a]Means in columns followed by different letters significantly different at $LSD_{.05}$. Numbers in parentheses are standard errors.
[b]Larval weight was measured at 96 hr after neonate feeding on excised foliage in the laboratory.

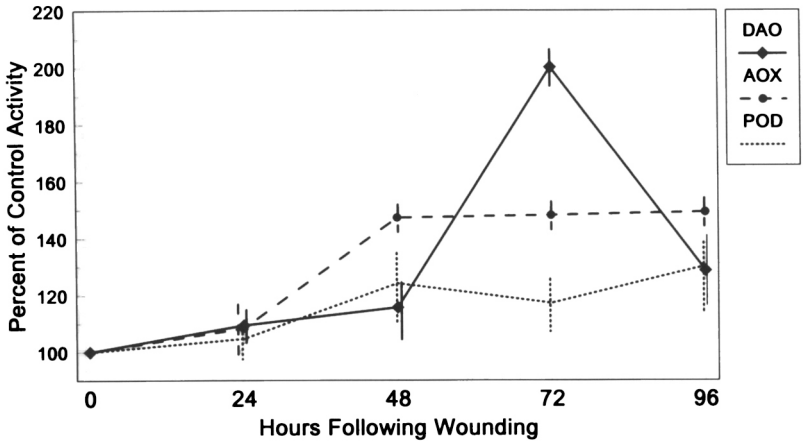

FIG. 1. Temporal changes in foliar oxidative enzymes following mechanical damage. DAO, diamine oxidase; AOX, ascorbate oxidase; POD, peroxidase. Vertical bars represent standard errors.

Oxidative Responses in Foliage and Squares to Herbivory

Larval *H. zea* feeding increased the activities of several oxidative enzymes in cotton foliage and squares (Tables 3 and 4). Foliar POD activities increased by two-fold, AOX by two-fold, and DAO by one-fold compared with the control foliage. However, the feeding did not significantly increase the activity of foliar LOX. Activity of PPO assayed with chlorogenic acid or caffeic acid as substrates was not detectable in either damaged or control foliage (Table 3). The feeding also significantly increased activities of the assayed oxidases in squares including POD by four-fold, AOX by two-fold, LOX by two-fold, and DAO by one-fold (Table 4).

Feeding by *H. zea* also resulted in significant losses in foliar antioxidant levels as indicated by a 19% loss of reduced ASC and 82% higher DHA compared to controls (Table 3). Foliar reduced ASC:DHA decreased from 13.6 to 6.0, reflecting the significant change in redox status. However, foliar lipid peroxides were not significantly increased by the herbivory (Table 3). The redox status in the squares was also modified by *H. zea* feeding as shown by a 7% decrease in reduced ASC concentration and 260% enhancement of DHA level in comparison with those in control squares (Table 4). Reduced ASC:DHA in squares declined following herbivory from 19.1 to 4.9 (Table 4). In damaged squares, a 45% increase in lipid peroxides occurred compared to the control treatment (Table 4).

TABLE 3. EFFECT OF HERBIVORY BY *H. zea* ON OXIDATIVE STATUS IN COTTON TERMINAL FOLIAGE[a]

Phytochemical	Control	Damaged	LSD_{05}	% change
AOX (nmol min^{-1} g^{-1} fresh weight)	101.0 (11.6)	205.0 (15.7)	44.1	+103.0
DAO (μmol min^{-1} fresh weight)	1.89 (0.13)	2.30 (0.09)	0.35	+21.7
LOX (nmol min^{-1} g^{-1} fresh weight)	11.2 (0.5)	13.8 (1.8)	5.5	+23.2
POD (ΔOD min^{-1} g^{-1} fresh weight)	82.0 (7.0)	180.0 (12.0)	31.1	+119.5
PPO (ΔOD min^{-1} g^{-1} fresh weight)	ND	ND	—	
Lipid peroxides (nmol g^{-1} dry weight)	3450.5 (54.0)	3543.4 (41.1)	156.4	+2.7
Total ASC (mg g^{-1} fresh weight)	1.60 (0.05)	1.40 (0.07)	0.18	−12.5
Reduced ASC (mg g^{-1} fresh weight)	1.49 (0.07)	1.20 (0.05)	0.18	−19.5
DHA (mg g^{-1} fresh weight)	0.11 (0.03)	0.20 (0.02)	0.08	+81.8
Reduced ASC:DHA	13.6	6.0	—	−55.8

[a]ND, not detectable. Numbers in parentheses are standard errors. AOX, ascorbate oxidase; DAO, diamine oxidase; LOX, lipoxygenase; POD, peroxidase; PPO, polyphenol oxidase; ASC, ascorbate; DHA, dehydroascorbate.

TABLE 4. EFFECT OF HERBIVORY BY *H. zea* ON OXIDATIVE STATUS IN COTTON SQUARES[a]

Phytochemical	Control	Damaged	$LSD_{.05}$	% change
AOX (nmol min^{-1} g^{-1} fresh weight)	49.0 (8.9)	82.0 (8.1)	29.3	+67.3
DAO (μmol min^{-1} g^{-1} fresh weight)	1.50 (0.11)	2.18 (0.24)	0.65	+45.3
LOX (nmol min^{-1} g^{-1} fresh weight)	112.2 (18.0)	187.0 (17.1)	60.0	+66.7
POD (ΔOD min^{-1} g^{-1} fresh weight)	0.26 (0.05)	0.92 (0.14)	0.37	+253.8
Lipid peroxides (nmol g^{-1} dry weight)	1548.3 (91.0)	2244.3 (97.0)	325.4	+45.0
Total ASC (mg g^{-1} dry weight)	7.05 (0.05)	7.47 (0.04)	0.15	+6.0
Reduced ASC (mg g^{-1} dry weight)	6.70 (0.05)	6.21 (0.05)	0.15	−7.3
DHA (mg g^{-1} dry weight)	0.35 (0.05)	1.26 (0.07)	0.19	+260.0
Reduced ASC:DHA	19.1	4.9	—	−74.3

[a]Numbers in parentheses are standard errors. AOX, ascorbate oxidase; DAO, diamine oxidase; LOX, lipoxygenase; POD, peroxidase; ASC, ascorbate; DHA, dehydro-ascorbate.

Changes in Protein and Amino Acids in Foliage and Squares Following Herbivory

Herbivory reduced protein content as indicated by a 10.5% loss of foliar protein and a 14.0% decrease in protein level in squares (Table 5). The herbivory also caused changes in amino acid levels in foliage and squares as shown by decreased contents of most amino acids and an altered relative molar ratio of several amino acids (Table 6). Total amino acids declined by 16.3% in foliage and 12.2% in squares (Table 6). The herbivory enhanced molar ratios of foliar amino acids Ser, Gly, Pro, and Hyp and square amino acids Thr, Ser, Glx, Gly, Ala, Met, Leu, Phe, His, and Hyp. Larval feeding caused quantitative losses in all amino acids in foliage and squares with the exception of an increase in Ser and Hyp in squares.

TABLE 5. EFFECT OF HERBIVORY BY *H. zea* ON PROTEIN CONTENT OF COTTON FOLIAGE AND SQUARES[a]

Tissue	Control	Damaged	$LSD_{.05}$	% reduction
Foliage (mg g^{-1} dry weight)	233.2 (4.4)	208.8 (2.1)	4.4	10.5
Square (mg g^{-1} dry weight)	68.6 (0.5)	59.0 (0.8)	2.2	14.0

[a]Numbers in parentheses are standard errors.

TABLE 6. EFFECT OF HERBIVORY BY *H. zea* ON AMINO ACIDS IN COTTON FOLIAGE AND SQUARES

	Molar ratio (%)				nmol amino acids/mg sample			
	Foliage		Square		Foliage		Square	
	Damaged	Control	Damaged	Control	Damaged	Control	Damaged	Control
As(x)	12.00	12.15	18.93	20.17	18.69	226.1	116.3	141.5
Thr	4.69	4.77	4.39	4.08	73.1	88.7	27.0	28.6
Ser	5.20	5.17	5.77	5.04	81.0	96.2	35.4	35.4
Glx	10.68	10.97	11.11	10.78	166.4	204.2	68.3	75.6
Gly	11.29	9.96	8.72	8.32	175.9	185.4	53.6	58.4
Ala	8.79	8.95	7.87	7.36	136.9	166.6	48.4	51.6
Val	6.14	6.27	5.66	5.67	95.6	116.7	34.8	39.8
Met	1.28	1.74	1.75	1.73	20.0	32.4	10.8	12.1
Ile	4.63	4.83	4.35	4.44	72.1	89.9	26.7	31.1
Leu	8.72	8.81	7.43	7.31	135.9	163.8	45.7	51.3
Tyr	2.54	2.72	1.95	1.96	39.5	50.6	12.0	13.8
Phe	4.30	4.39	3.47	3.37	66.9	81.7	21.3	23.6
His	1.74	1.91	1.65	1.46	27.2	35.5	10.1	10.3
Lys	6.02	6.09	5.48	5.88	93.9	113.2	33.7	41.3
Arg	4.12	4.32	3.79	4.06	64.1	80.4	23.3	28.5
Pro	7.56	6.66	5.31	5.92	117.7	123.8	32.6	41.5
Hyp	0.30	0.28	0.57	0.46	4.6	5.2	3.5	3.3
				Totals	1557.7	1860.4	603.3	687.5

Changes in Phenolics in Foliage and Squares due to Herbivory

Larval *H. zea* feeding on cotton altered the levels of several foliar phenolic compounds, including condensed tannins, which decreased 31% compared with the control foliage. Total flavonoids and terpene aldehydes were decreased very slightly (<5%) by herbivory (Table 7). Herbivory also significantly decreased levels of *p*-coumaric acid by 13.8%. Conversely, herbivory raised levels of syringic acid by 103.6%, chlorogenic acid by 59.6%, ferulic acid by 11%, and rutin by 10.7%.

Changes in phenolic compounds also occurred in cotton squares following the herbivory (Table 8). Larger decreases in terpene aldehydes (35.4%), *p*-hydroxybenzoic acid (34.3%), *p*-coumaric acid (10.6%), rutin (15.4%), and ferulic acid (46.0%), and a small loss (5.9%) of total flavonoids, were observed in wounded squares compared to control squares. In contrast, an increase in gallic acid (25.2%) and chlorogenic acid (28.9%) were found in damaged squares compared to the control treatment. Condensed tannins in squares were relatively unaffected by treatment.

TABLE 7. EFFECT OF HERBIVORY BY *H. zea* ON PHENOLIC COMPOUNDS IN COTTON TERMINAL FOLIAGE[a]

Phenolic	Control	Damaged	$LSD_{.05}$	% change
Condensed tannins (mg g^{-1} dry weight)	10.2 (0.1)	7.0 (0.1)	0.3	−31.3
Total flavonoids (mg g^{-1} dry weight)	43.2 (0.2)	42.1 (0.1)	0.5	−2.5
Lignin (ABS g^{-1} dry weight)	83.39 (5.93)	80.21 (4.59)	16.08	−3.8
Syringic acid[b] ($\mu g\ g^{-1}$ dry weight)	2.8 (0.9)	5.7 (0.5)	2.8	+103.6
p-Coumaric acid[b] ($\mu g\ g^{-1}$ dry weight)	140.5 (1.0)	121.1 (3.6)	9.6	−13.8
Ferulic acid[b] ($\mu g\ g^{-1}$ dry weight)	22.3 (0.4)	24.8 (0.8)	2.7	+11.2
Chlorogenic acid ($\mu g\ g^{-1}$ dry weight)	996.0 (43.8)	1590.1 (56.2)	224.1	+59.6
Rutin ($\mu g\ g^{-1}$ dry weight)	1094.3 (19.7)	1211.4 (37.3)	117.1	+10.7
Terpene aldehydes (mg g^{-1} dry weight)	2.7 (0.02)	2.6 (0.02)	0.06	−3.7

[a] Numbers in parentheses are standard errors.
[b] Phenolic compound assayed from hydrolyzed sample.

Effect of Induced Responses on Oxidative Status of Insect Midgut

The ingestion of previously damaged plant tissue by larvae affected the oxidative status of the midgut (Table 9). Evidence that oxidative damage occurred in larvae ingesting damaged tissue was indicated by the fact that total ASC was decreased by 15%, reduced ASC was decreased by 19%, and the DHA was increased by 100%. Accordingly, midgut reduced ASC:DHA decreased from 29.00 in larvae feeding on control plants to 12.4 in larvae on wounded plants. The levels of nonprotein thiols were significantly decreased in the damaged treatment by 21.7%. Total hydroperoxides, an indicator of oxidative stress, were more than 39% higher in the midgut of larvae feeding on the damaged plants compared to larvae on control plants.

DISCUSSION

Previous feeding damage on cotton foliage and squares induced resistance to neonate and fourth-instar *H. zea* measured in excised leaves and squares or intact plants (Table 1 and 2). These results provide further evidence of induced resistance in cotton to several species of herbivores (Karban, 1986a, 1988; Karban and Carey, 1983). We previously reported that induced responses in soybean by *H. zea* feeding included changes in primary compounds, secondary

TABLE 8. EFFECT OF HERBIVORY BY *H. zea* ON PHENOLIC COMPOUNDS IN COTTON SQUARES[a]

Phenolic	Control	Damaged	$LSD_{.05}$	% change
Condensed tannins (mg g^{-1} dry weight)	132.8 (0.3)	132.2 (0.5)	1.3	−0.5
Total flavonoids (mg g^{-1} dry weight)	18.5 (0.2)	17.4 (0.2)	0.6	−5.9
Lignins (ABS g^{-1} dry weight)	110.23 (9.67)	135.93 (4.52)	21.86	+23.3
p-Hydroxybenzoic acid[b] ($\mu g\ g^{-1}$ dry weight)	49.2 (2.6)	32.3 (1.6)	8.6	−34.3
p-Coumaric acid[b] ($\mu g\ g^{-1}$ dry weight)	36.9 (0.5)	33.0 (0.0)	1.3	−10.6
Gallic acid ($\mu g\ g^{-1}$ dry weight)	603.7 (14.5)	756.0 (11.1)	50.6	+25.2
Ferulic acid ($\mu g\ g^{-1}$ dry weight)	36.3 (0.0)	19.6 (3.6)	14.7	−46.0
Chlorogenic acid ($\mu g\ g^{-1}$ dry weight)	2144.5 (27.7)	2763.2 (106.4)	305.4	+28.9
Rutin ($\mu g\ g^{-1}$ dry weight)	3458.9 (12.5)	2926.3 (18.7)	62.4	−15.4
Terpene aldehydes (mg g^{-1} dry weight)	8.2 (0.3)	5.3 (0.1)	0.6	−35.4

[a]Numbers in parentheses are standard errors.
[b]Phenolic compound assayed from hydrolyzed sample.

TABLE 9. EFFECT OF FEEDING BY *H. zea* ON PREVIOUSLY DAMAGED COTTON PLANTS ON THE OXIDATIVE STATUS OF THE LARVAL MIDGUT EPITHELIUM[a]

Chemicals in larval midgut epithelium	Control	Damaged	$LSD_{.05}$	% change
Total ASC (mg g^{-1} fresh weight)	1.50 (0.06)	1.28 (0.06)	0.18	−14.7
Reduced ASC (mg g^{-1} fresh weight)	1.45 (0.06)	1.18 (0.05)	0.17	−18.6
DHA (mg g^{-1} fresh weight)	0.05 (0.01)	0.10 (0.02)	0.04	+100.0
Reduced ASC:DHA	29.0	12.4		−57.3
Free thiols (μmol g^{-1} fresh weight)	4.15 (0.20)	3.25 (0.37)	0.88	−21.7
Total hydroperoxides (nmol g^{-1} fresh weight)	594.7 (60.4)	827.2 (59.0)	182.9	+39.1

[a]Numbers in parentheses are standard errors. ASC, ascorbate; DHA, dehydroascorbate.

metabolites, and reactive oxygen species (Bi et al., 1994; Bi and Felton, 1995). Similar coordinated responses were observed in cotton.

One component of induced resistance may involve a significant shift in the oxidative status of the host plant. Activities of several oxidative enzymes were strikingly induced in tissues damaged by *H. zea* (Table 3 and 4). POD catalyzes the oxidation of mono- and/or dihydroxyphenolics to orthoquinones in the presence of H_2O_2 (Gaspar et al., 1981; Pierpoint, 1983; Mayer, 1987; Felton et al., 1989; Duffey and Felton, 1989; 1991). Superoxide radicals and H_2O_2 may be formed in the reactions (Elstner, 1987). DAO catalyzes the oxidation of di- and/or polyamines, such as putrescine and spermidine, with the formation of corresponding amino aldehydes, ammonia and H_2O_2 (Smith and Baker, 1988). AOX oxidizes ASC to form DHA and reactive oxygen such as hydroxyl radicals (Chiou, 1984; Halliwell et al., 1987; Uchida et al., 1989; Baysal et al., 1989; Felton and Summers, 1993). LOX catalyzes the hydroperoxidation of polyunsaturated lipid such as linoleic and linolenic acids (Schewe et al., 1986; Hatanaka et al., 1987; Mack et al., 1987; Vick and Zimmerman, 1987). At the same time, reactive oxygen species (e.g., singlet oxygen, hydroxyl radicals, superoxide anion) are produced (Kanofsky and Axelrod, 1986; Chamulitrat et al., 1991). The reactive oxygen species, particularly hydroxyl radicals, are highly reactive and can cause lipid, DNA, and protein oxidation (Fridovich, 1978; Halliwell and Gutteridge, 1985; Imlay et al., 1988).

Phenolic metabolism in cotton tissues was altered by larval feeding. Herbivory resulted in increased levels of syringic acid, chlorogenic acid, ferulic acid, and rutin in foliage, whereas gallic acid and chlorogenic acid levels increased in squares (Tables 7 and 8). Chlorogenic acid, in particular, is susceptible to oxidation and may produce reactive oxygen species causing oxidative stress to the herbivore (Ahmad, 1992; Summers and Felton, 1994). Also, the enhanced levels of chlorogenic acid provide more substrate for POD to produce quinones (Felton and Duffey, 1991a). The quinones are potent alkylators and irreversibly combine with nucleophilic side chains of amino acids (Felton and Duffy, 1991a,b). The greatest increase was in chlorogenic acid, which is the most toxic of the phenolic compounds identified by HPLC in this study (Summers and Felton, 1994; unpublished data). Ingestion of chlorogenic acid by *H. zea* causes increased levels of lipid peroxidation and oxidized protein and release of free iron in the larval midgut (Summers and Felton, 1994).

Consistent with the oxidative shift in redox status of damaged plant tissues, larvae feeding on damaged cotton plants showed substantial evidence of oxidative damage to their midgut epithelium (Table 9). Lipid hydroperoxides were elevated by 39%, free thiols declined by 22%, and DHA levels increased by 90% in larvae feeding on wounded plants compared to their control counterparts. The primary cause of oxidative damage to the midgut may be products of

oxidative enzymes (e.g., quinones, lipid hydroperoxides, reactive oxygen species), phenolic prooxidants (e.g., chlorogenic acid, gallic acid, rutin), and/or depletion of dietary antioxidants (e.g., ascorbate) (Summers and Felton, 1994; Bi and Felton, 1995).

A second component of induced resistance may involve a significant decline in host nutritional quality. The decline in quality following larval feeding is illustrated by a significant decline in protein in both foliar and square tissues (Table 5). Most amino acids were also decreased in wounded tissues (Table 6). Moreover, the amino acid composition, as reflected by molar ratios, is altered by herbivory (Table 6). In damaged foliage, the greatest decline in the molar ratios of amino acids was with Met, followed by His and Tyr. These amino acids contain reactive nucleophilic side chains and are susceptible to attack by oxidized phenolic products formed by POD and/or PPO (Felton et al., 1992). The molar ratios of these three amino acids in square tissues were unaffected by the herbivory, which was consistent with the low levels of POD in square tissues. Additionally, reactive oxygen species produced by phenolic oxidation and the above mentioned oxidative enzyme-catalyzed reactions may damage nutrients such as lipids, amino acids, proteins, and ascorbate (Bi et al., 1994; Bi and Felton, 1995). Additionally, the essential nutrient ASC declined in the wounded tissues (Tables 3 and 4).

A third component of induced resistance may involve lignification and cell wall strengthening. The changes in ferulic acid and *p*-coumaric acid levels in damaged squares may be related to increased lignin formation (Table 8) (Pascal, 1972; Hagerman and Butler, 1991). These phenolic acids are dehydrogenated and then serve as substrates for a POD-catalyzed oxidation. The H_2O_2 that is required for the oxidation may be supplied by a second POD activity and/or DAO activity (Hagerman and Butler, 1991; Federico and Angelini, 1986, 1988; Angelini and Federico, 1989; Angelini et al., 1990). The phenolic radicals generated from POD activity condense to form lignin (Hagerman and Butler, 1991). The enhanced ratios of Hyp and Ser in both the damaged foliage and the square may be indicative of increased synthesis of insoluble hydroxyproline-rich proteins since these proteins contain a highly repetitive peptide sequence with Ser–Hyp (Table 6); (Varner and Lin, 1989). The decrease in ASC may result from hydroxylation of proline residues by prolyl hydroxylase because this enzyme utilizes ASC as an electron donor (Table 4); (Gara et al., 1991). Hydroxyproline-rich proteins are important cell wall proteins that comprise 0.2 to 10% of the primary wall of higher plants (Varer and Lin, 1989; Varner and Burton, 1980). The increased ratio of Gly in the wounded tissue may relate to the formation of another major class of cell wall proteins, glycine-rich proteins (Table 6); (Varner and Lin, 1989). The strengthened cell wall caused by lignification and insoluble cell wall proteins may serve as a defensive barrier by

reducing the digestibility of cell wall carbohydrates and proteins for larval *H. zea* (Brisson et al., 1994).

Increases in constitutive resistance factors such as condensed tannins, flavonoids, or terpene aldehydes were not observed in wounded tissues. There are discrepancies in the literature associated with tannin toxicity to *Heliothine* species. For instance, condensed tannins in cotton terminal leaves were not correlated with resistance to *Heliothis virescens*, although artificial diets containing tannin are quite toxic (Hedin et al., 1983a). Also, the elevated levels of condensed tannins in cotton breeding lines did not act as a feeding deterrent or reduce the performance of neonate larvae of the bollworm/budworm complex (Smith et al., 1992). Our preliminary data suggest a possible explanation for these discrepancies because the observed decline in condensed tannins may be due to enzymatic oxidation. We have found a polyphenol oxidase-type activity in cotton that preferentially oxidizes condensed tannins.

The oxidative responses and changes in phenolic compounds induced in cotton tissues by *H. zea* feeding may also be associated with signal transduction for eliciting plant defenses. Acetosyringone, a derivative of syringic acid, is a known plant signal molecule controlling gene expression in tissue culture (Jacq et al., 1993), whereas ferulic acid and its glycoside are potent inhibitors of ethylene synthesis (Shih et al., 1989). The increase in ferulic acid in damaged foliage (Table 7) may inhibit damage-induced ethylene synthesis and prevent foliage abscission. Ethylene can be synthesized from LOX-catalyzed oxidation of linolenic acid (Osborne, 1978). A decrease in polyamines such as putrescine in damaged plant tissue is a step subsequent to ethylene enhancement and several defensive responses (Belles et al., 1993). LOX is also involved in the initial phase of jasmonic acid biosynthesis (Ueda and Kato, 1980; Vick and Zimmerman, 1987; Enyedi et al., 1992). Jasmonic acid is an important signal in many plants, inducing the synthesis of several plant defenses such as proteinase inhibitors, chalcone synthase, proline-rich cell wall proteins, phenylalanine ammonia lyase, and alkaloids (Greelman et al., 1992; Enyedi et al., 1992; Hamberg and Gardner, 1992).

In summary, induced resistance in cotton includes changes in the oxidative status, nutritive value, cell wall lignification, and secondary metabolism of leaf and reproductive tissues. Although not identical to the induced responses observed in soybean (Bi and Felton, 1995), we suggest that this coordinated response may represent a multicomponent mechanism for plant defense against herbivores.

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ALKALOIDAL GLYCOSIDASE INHIBITORS AND DIGESTIVE GLYCOSIDASE INHIBITION IN SPECIALIST AND GENERALIST HERBIVORES OF *Omphalea diandra*

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Abstract—Generalist herbivores of the neotropical liana *Omphalea diandra* (Euphorbiaceae) were compared to the specialist herbivore, larvae of the uraniid moth *Urania fulgens*, with respect to their ability to accumulate the alkaloidal glycosidase inhibitors (AGIs) produced by the plant and the resistance of their digestive glycosidases to inhibition by these AGIs. The generalist herbivores did not accumulate the AGI aglycones 2*R*,5*R*-dihydroxymethyl-3*R*,4*R*-dihydroxypyrrolidine (DMDP) and 2,6-dideoxy-2,6-imino-D-glycero-L-gulo-heptitol (HNJ) to the levels detected in larvae of *U. fulgens*, which contained 0.05–0.11% dry weight DMDP and 0.17–0.35% HNJ. Glucosides of DMDP and HNJ that were synthesized by *O. diandra* were either absent from both the generalist and the specialist herbivores or present at low levels (less than 0.01%), even though HNJ-glucoside was often the most abundant AGI in the foliage. Analyses of the herbivores' feces indicated that failure to accumulate AGIs was due to the compounds being metabolized rather than excreted. The digestive glycosidases of *U. fulgens* larvae were more resistant

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to inhibition by AGI aglycones than those of the generalist herbivores. Similarly, sucrose and maltose hydrolysis in two of the generalist lepidopteran herbivores, larvae of *Panthiades ballus* and *Theope virgilius*, was more resistant to inhibition by DMDP than in larvae of *Spodoptera littoralis*, a lepidopteran which does not encounter *O. diandra* in nature. There was little difference in the susceptibility to AGIs of glycosidases from the generalist coleopteran *Rhabdopterus fulvipes*, which naturally feeds on *O. diandra*, compared with the coleopteran *Dermestes maculatus*, which does not. The glucoside of HNJ was found to be a very potent inhibitor of trehalase activity in all the insects examined. AGIs are considered to reduce the nutritional value of *O. diandra* to nonadapted herbivores rather than be acutely toxic. Nevertheless, *U. fulgens* does appear to be unique among *Omphalea*-feeding insects in its ability to accumulate AGIs, suggesting that it gains some advantage from storing these compounds.

Key Words—*Urania fulgens*, Lepidoptera, Uraniidae, *Omphalea diandra*, Euphorbiaceae, alkaloidal glycosidase inhibitors, glycosidases, sequestration, generalist/specialist herbivores.

INTRODUCTION

Omphalea diandra L., a neotropical liana belonging to the family Euphorbiaceae, synthesizes alkaloidal glycosidase inhibitors (AGIs). These are nitrogen-containing compounds that mimic the structures of simple sugars and inhibit the action of glycosidase enzymes (Fellows et al., 1989). Examples of AGI-producing plants have been found in several plant families, and to date, four AGIs have been isolated from foliage of *O. diandra*: 2*R*,5*R*-dihydroxymethyl-3*R*,4*R*-dihydroxypyrrolidine (DMDP), 2,6-dideoxy-2,6-imino-D-glycero-L-gulo-heptitol (HNJ), 1,5-dideoxy-1,5-imino-D-mannitol (DMJ), and 1,5-dideoxy-1,5-imino-D-glucitol (DNJ) (Kite et al., 1988, 1990).

Some AGIs, such as DMDP, elicit antifeedant behavior in herbivorous insects, and it has been suggested that they have a function in protecting plants against insect attack (Fellows et al., 1986). For example, levels of DMDP in leaves of *O. diandra* generally exceed 0.01% dry weight, and this concentration of DMDP, when incorporated into a suitable diet, was sufficient to deter the feeding of the locusts *Locusta migratoria* and *Schistocera gregaria* (Blaney et al., 1984), and larvae of various lepidopterans such as *Spodoptera littoralis*, *Heliothis virescens*, and *Helicoverpa armigera* (Simmonds et al., 1990). It also caused mortality in larvae of the bruchid beetle *Callosobruchus maculatus* (Evans et al., 1985).

Nevertheless, Smith (1982) noted severe herbivore damage on wild plants of *O. diandra* and identified the herbivore responsible as larvae of the uraniid moth *Urania fulgens* (Walker); the only other herbivores which he observed on *O. diandra* at that time were leaf-cutting ants (*Atta* sp.) which did not consume

the leaves directly. Larvae of *U. fulgens* are specialist (monophagous) herbivores since they will feed only on foliage of certain *Omphalea* species (Lees and Smith, 1991). Not only do they appear to tolerate the effects of the AGIs in their diet, but they have been found to accumulate two of them: DMDP and HNJ (Kite et al., 1990). These AGIs are retained through to the adult stage, where they may serve as the moth's own defense compounds. Larvae of a uraniid moth from Australia, *Alcides metaurus*, also accumulate DMDP and HNJ from their foodplant, *Omphalea queenslandia* (Kite et al., 1991), and it is probable that all uraniids which utilize *Omphalea* species as their larval foodplants also exploit the plant's defensive chemicals in this way.

In this paper we report on a more detailed field survey which found other herbivores feeding on the foliage of *O. diandra*. We examined whether these herbivores contain AGIs to discover if the ability to accumulate these compounds is unique to uraniid moths. We also studied the sensitivity to AGIs of the digestive glycosidases of *Omphalea*-feeding herbivores to detect any adaptation to these biologically active compounds.

METHODS AND MATERIALS

Experimental Material. Insects observed to be feeding on *O. diandra* growing wild in Panama were collected together with samples of the foliage they had been eating and, when possible, their feces. Larvae were left to void their gut contents before being killed and freeze-dried. Some larvae were allowed to pupate after feeding on a diet of *O. diandra* so that either pupae or adults could be analysed.

Analysis for AGIs. Insect specimens were crushed in 70% (v/v) aqueous methanol (1 ml per 25 mg dry weight tissue) and extracted for 15 hr at 4°C. Samples of leaves and feces were powdered in bulk and 25-mg samples were extracted as above. AGIs were purified from the extracts by ion-exchange chromatography as described previously (Kite et al., 1990) and analyzed by gas chromatography/mass spectrometry (GC/MS) of the trimethylsilyl derivatives. These were prepared by reacting the dry AGI residue from 20 mg dry weight of tissue with 30 μl of Sigma-Sil A (Sigma Chemical Co. Ltd.) at 70°C for 30 min and then at room temperature (22°C) for 15 hr to ensure complete derivatization of the AGI-glycosides. Split 1-μl injections were made onto a 25 m $\times$ 0.2-mm-ID $\times$ 0.25-μm (film thickness) BPX5 capillary column (SGE Ltd.) and chromatographed using an oven temperature program of 160–200°C at 4°C/min and then 200–300°C at 8°C/min and a helium carrier gas pressure of 20 psi. Electron impact mass spectra (70 eV) of the column eluate were recorded by an ion trap detector (Finnigan-MAT) and AGIs were quantified against standards.

Partial Characterization of AGI-glycosides. Two late-eluting compounds noted in GC/MS analyses of AGIs in foliage of *O. diandra* were purified by column chromatography of the AGI residue on neutral aluminium oxide against 0–50% acetone in water. Quantities isolated pure were insufficient for structural determination but hydrolysis with 2 M hydrochloric acid (1 hr at 100°C) yielded DMDP and glucose for one compound and HNJ and glucose for the other. The aglycones were identified by GC/MS analysis as above and the sugar by standard paper chromatographic techniques (Harborne, 1984). Hence the plant compounds were considered to be O-glucosides of DMDP and HNJ.

Gut Glycosidase Inhibition. Freeze-dried larval guts of *U. fulgens* and entire specimens of the other herbivores were homogenized in 7.5 mM maleate buffer (pH 6.0) and dialyzed against 250x the volume of the buffer at 4°C. The dialysis fluid for *U. fulgens* was changed six times over 3 days in order to reduce the concentrations of AGIs to levels sufficiently low that they gave less than 10% inhibition of mouse sucrase activity, an enzyme particularly sensitive to AGIs (Scofield et al., 1986). For the other insects only three or four changes of dialysis fluid were used, as the concentrations of AGIs after this number of changes were insufficient to inhibit mouse sucrase activity. For comparative purposes, the entire adult leather beetle *Dermestes maculatus* (Degeer) and the guts of the fifth and sixth instars of the lepidopteran *Spodoptera littoralis* (Boisd.) were homogenized and dialyzed (three changes) as above. Also, homogenates were made from freeze-dried larvae of another uraniid moth, *Alcides metaurus* (Hopffer), followed by six changes of dialysis buffer. The dialyzed homogenates were stored at −70°C. The ability of the AGIs to inhibit glucosidase activity in the homogenates was tested using the methods described by Scofield et al. (1986) and Nash et al. (1988); assays were performed at 37°C in a 50 mM maleate buffer (pH 6.0).

RESULTS

In addition to larvae of *U. fulgens*, we found seven other insect herbivores feeding on *O. diandra*, including larvae of the lycaenid butterfly *Panthiades ballus* (Reakirt); larvae of two riodinid butterflies, *Theope virgilius* (Fab.) and *Nymula mycone* (Hewitson); larvae of two hag moths (Limacodidae) belonging to the genera *Sibine* H.-S. and *Phobetron* Hb.; adults of the stingless trigonid bee *Trigona fusipennis* (Friese); and adults of the leaf beetle *Rhabdopterus fulvipes* (Jac.) (Chrysomelidae: Eumolpinae). These herbivores were more frequently observed feeding on other plant species, and so can be considered generalist herbivores.

Observation on the feeding behavior of *U. fulgens* larvae on *O. diandra* indicated that first and second instars were gregarious and ate the epidermis of

usually the underside of young (mid- to fully-expanded) leaves, not usually the very youngest leaves. Third to fifth instars tended to be solitary and ate into the lamina of leaves of various ages. Late instars often "trenched" the leaf petiole, so that the leaf hung down, prior to eating the lamina. The feeding activity of *U. fulgens* could cause almost-complete defoliation of some plants. The lycaenid and riodinid larvae and the larva of *Sibine* sp. that were noted on *O. diandra* were solitary and were generally feeding on the younger leaves. They ate only small portions of the leaf, never the entire lamina as did *U. fulgens*. Larvae of *T. virgilius* were also observed to be feeding from the extrafloral nectaries and were often attended by ants. Adults of *R. fulvipes* ate into the central portions of younger leaves producing chevron-shaped holes, whereas adults of *T. fusipennis* chewed the abaxial veins of leaves of various ages and fed on the exudate. Larvae of *Phobetron* sp. were the only generalist herbivores that were observed to eat *O. diandra* leaves with apparent indifference, but they produced large quantities of feces compared to the other herbivores and were not abundant on *O. diandra*, so they inflicted only minor damage.

All the generalist lepidopteran larvae gathered from *O. diandra* completed their larval development successfully on a diet that consisted exclusively of *O. diandra* leaves. Those that were not harvested at the pupal stage emerged as adults. When examined for AGIs, 8 of 15 adults or pupae reared from these larvae either lacked detectable amounts of AGIs (detection limit, 0.0001% dry weight) or contained levels below the limit of accurate quantification (0.001% dry weight) (Table 1). The remainder contained one or two AGIs at levels between 0.01 and 0.001% dry weight and these occurrences of higher levels were not confined to any one species. In contrast, adults of *U. fulgens* consistently contained DMDP and HNJ at levels significantly greater (Mann–Whitney *U* test, $P < 0.01$ for both DMDP and HNJ when *P. ballus* was compared to *U. fulgens*); mean levels were 0.03% DMDP and 0.1% HNJ.

Levels of AGIs in the generalist lepidopteran larvae and adults of *R. fulvipes* and *T. fusipennis* were again mostly below the limit of either detection or quantification (Table 2). The exceptions were two larvae of *P. ballus* in which some AGIs occurred at levels between 0.001 and 0.01% dry weight (one of these also contained 0.03% DMJ), two larvae of *T. virgilius* analyzed together that contained 0.007% HNJ, and one analysis of *R. fulvipes* adults that contained 0.002% DMDP. In contrast, larvae of *U. fulgens* contained levels of 0.05–0.11% DMDP and 0.17–0.35% HNJ. Levels of AGIs in the foliage that the herbivores had been eating varied among samples, but the levels of individual AGIs in the foliage on which *U. fulgens* larvae had been feeding were within the range of the samples analyzed for the generalist herbivores (Table 2).

The ratio of AGI concentration in the feces of the herbivores relative to the foliage that was being eaten are given in Table 3. The feces of the riodinid larvae (*N. mycone* and *T. virgilius*) and *R. fulvipes* were much depleted in AGI

TABLE 1. LEVELS (% DRY WT) OF ALKALOIDAL GLYCOSIDASE INHIBITORS (AGIs) IN ADULTS OR PUPAE OF LEPIDOPTERA THAT HAD BEEN REARED FROM LARVAE FEEDING ON *Omphalea diandra* (ANALYTICAL ERRORS LESS THAN 10%)

	Level (% dry wt) of AGI[a]					
Species	DMDP	DMJ	DNJ	HNJ	DMDP gluc	HNJ gluc
Urania fulgens						
Adults[b]	0.03	0.001	Trace	0.1	nd	nd
(SE)	(0.004)	(0.0006)	(−)	(0.02)	(−)	(−)
Panthiades ballus						
Adult	0.009	nd	nd	nd	nd	nd
Adult	nd	nd	nd	0.005	nd	nd
Adult	0.007	0.002	nd	nd	nd	nd
Pupa	nd	nd	nd	nd	—	—
Pupa	nd	nd	nd	nd	—	—
Pupa	nd	nd	nd	nd	—	—
Pupa	0.008	0.005	Trace	Trace	nd	nd
Theope virgilius						
Adult	nd	nd	nd	nd	nd	nd
Adult	nd	nd	nd	nd	nd	nd
Adult	0.004	nd	0.004	0.005	0.009	0.002
Pupa	nd	nd	nd	nd	—	—
Nymula mycone						
Adult	0.004	Trace	nd	nd	nd	nd
Adult	nd	nd	nd	Trace	Trace	nd
Adult	Trace	Trace	nd	Trace	Trace	Trace
Sibine sp.						
Pupa	0.007	nd	nd	0.004	nd	nd

[a] Trace, less than 0.001% dry wt; nd, not detectable; −, no data.
[b] Mean values for eight individuals.

TABLE 2. LEVELS (% DRY WT) OF ALKALOIDAL GLYCOSIDASE INHIBITORS (AGIs) IN INDIVIDUAL SPECIALIST (LARVAE OF *Urania fulgens*) AND GENERALIST HERBIVORES OF *Omphalea diandra* COMPARED TO THE FOLIAGE THEY HAD BEEN EATING (ANALYTICAL AND/OR SAMPLING ERRORS WERE LESS THAN 10%)

		Levels (% dry wt) of AGI[a]					
Herbivore	n[b]	DMDP	DMJ	DNJ	HNJ	DMDP gluc	HNJ gluc
Urania fulgens							
Larvae	2	0.11	0.09	0.05	0.35	nd	0.001
Foliage		0.02	0.01	0.03	0.08	0.001	0.001
Larva	1	0.05	0.008	0.03	0.17	Trace	0.001
Foliage		0.04	0.02	0.02	0.03	0.06	0.16
Panthiades ballus							
Larva	1	0.008	0.03	Trace	0.002	nd	0.007
Foliage		0.07	0.04	0.006	0.10	0.09	0.18
Larva	1	nd	nd	nd	nd	nd	nd
Foliage		0.06	0.03	0.007	0.07	0.16	0.18
Larva	1	0.005	Trace	0.008	0.004	nd	nd
Foliage		0.08	0.04	0.006	0.05	0.09	0.15
Theope virgilius							
Larva	1	nd	–	–	nd	–	–
Foliage		0.02	–	–	0.05	–	–
Larva	2	nd	nd	nd	0.007	nd	nd
Foliage		0.05	0.06	0.007	0.13	0.03	0.10

TABLE 2. CONTINUED

Herbivore	n[b]	Levels (% dry wt) of AGI[a]					
		DMDP	DMJ	DNJ	HNJ	DMDP gluc	HNJ gluc
Nymula mycone							
Larva	1	Trace	nd	nd	Trace	nd	nd
Foliage		0.01	0.001	0.02	0.08	0.04	0.14
Sibine sp.							
Larva	1	nd	nd	nd	nd	nd	nd
Foliage		0.02	0.03	0.009	0.05	0.27	0.30
Phobetron sp.							
Larva	1	nd	–	–	nd	–	–
Foliage		0.27	–	–	0.23	–	–
Trigona fusipennis							
Adults	10	Trace	nd	nd	Trace	nd	nd
Foliage		0.12	0.005	0.05	0.06	0.04	0.16
Rhabdopterus fulvipes							
Adults	10	0.002	Trace	Trace	Trace	nd	nd
Foliage		0.08	0.02	0.006	0.12	0.06	0.13
Adults	10	Trace	Trace	Trace	Trace	nd	Trace
Foliage		0.08	0.008	0.05	0.05	0.07	0.22

[a]Trace, less than 0.001% dry wt; nd, not detectable; –, no data.
[b]Number of individuals analyzed together. *U. fulgens* larvae were first and second instars.

TABLE 3. RATIO OF ALKALOIDAL GLYCOSIDASE INHIBITOR (AGI) CONCENTRATION IN THE FECES OF HERBIVORES OF *Omphalea diandra* TO THE CONCENTRATION IN THE FOLIAGE BEING EATEN (ALL INSECTS WERE LARVAL STAGES EXCEPT *Rhabdopterus fulvipes*)

	Feces : foliage [AGI] ratio[a]					
Herbivore	DMDP	DMJ	DNJ	HNJ	DMDP gluc	HNJ gluc
Urania fulgens	0.25	0.50	0.30	0.16	0	0
	0.40	1.00	0.45	0.33	0	0
Panthiades ballus	0.66	0.50	0.33	0.35	0	0
	0.63	0.75	3.33	1.00	0.44	1.06
Theope virgilius	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Nymula mycone	0.06	0.02	<0.01	0.02	0	0
	<0.01	0	0	<0.01	<0.01	<0.01
Rhabdopterus fulvipes	<0.01	<0.01	<0.01	<0.01	0	0

[a] A ratio of less than 0.01 indicates that AGI levels in the feces were too low for accurate calculation.

aglycones giving low feces : foliage ratios. The ratio for the AGI aglycones was higher for *U. fulgens* and *P. ballus*, and for some AGI aglycones the feces contained a concentration that was equal to or greater than the concentration in the foliage. With the exception of one of the *P. ballus* larvae, the AGI-glycosides were always greatly depleted in the feces of all the herbivores.

The effects of the AGIs found in *O. diandra* on the hydrolysis of disaccharides by gut glycosidases of the *Omphalea* herbivores are shown in Tables 4–7. Data for two other AGIs which do not occur in *O. diandra*, 1,4-dideoxy-1,4-imino-D-arabinitol (AB-1) and (1*S*,6*S*,7*R*,8*R*,8a*R*)-1,6,7,8-tetrahydroxyoctahydroindolizidine (castanospermine), and two herbivores which would not encounter *O. diandra* in nature, *Spodoptera littoralis* larvae (Lepidoptera) and *Dermestes maculatus* adults (Coleoptera), are included for comparison. Also included are the results obtained for the Australian uraniid moth *Alcides metaurus*.

None of the AGIs had any effect (i.e., less than 50% inhibition of substrate hydrolysis at a concentration of 3.3×10^{-4} M) on the hydrolysis of sucrose by *U. fulgens* and *A. metaurus* preparations (Table 4). In contrast, hydrolysis of sucrose by the gut glycosidases of *T. virgilius* and *P. ballus* was inhibited by DMDP. The sucrase of these species did, however, appear to be more resistant than the enzyme from *S. littoralis*. Sucrose hydrolysis in *R. fulvipes* was inhibited by the majority of the AGIs tested, the exceptions being castanospermine and DMJ. Sucrase activity in *D. maculatus* showed the same spectrum of inhi-

TABLE 4. CONCENTRATIONS (M) OF ALKALOIDAL GLYCOSIDASE INHIBITORS (AGIs) GIVING 50% INHIBITION OF SUCROSE HYROLYSIS BY PREPARATIONS OF SPECIALIST AND GENERALIST HERBIVORES OF *Omphalea diandra* (*Spodoptera littoralis* LARVAE AND *Dermestes maculatus* ADULTS INCLUDED FOR COMPARISON)

		Concentration (M) of AGI required to inhibit sucrase activity by 50%						
		AGIs from *Omphalea diandra*					Other AGIs	
Herbivore	Type[a]	DMDP	DMJ	DNJ	HNJ	HNJ gluc	AB-1	Cast
Urania fulgens	SLO	NI[b]	NI	NI	NI	NI	NI	NI
Alcides metaurus	SLO	NI	NI	NI	NI	NI	NI	NI
Panthiades ballus	GLO	4.1×10^{-5}	NI	NI	NI	NI	NI	NI
Theope virgilius	GLO	3.5×10^{-5}	NI	NI	NI	NI	2.4×10^{-4}	NI
Phobetron sp.	GLO	8.0×10^{-5}	NI	NI	NI	NI	3.0×10^{-4}	NI
Spodoptera littoralis	GL	2.2×10^{-6}	NI	NI	2.2×10^{-4}	NI	1.0×10^{-4}	NI
Rhabdopterus fulvipes	GCO	1.4×10^{-6}	NI	1.0×10^{-5}	1.0×10^{-5}	5.3×10^{-5}	1.5×10^{-6}	NI
Dermestes maculatus	GC	6.4×10^{-7}	NI	5.3×10^{-6}	5.6×10^{-6}	2.5×10^{-5}	1.4×10^{-6}	NI

[a]Herbivore type codes: S, specialist; G, generalist; L, lepidopteran larva; C, coleopteran adult; O, *Omphalea*-feeding.
[b]Less than 50% inhibition at 3.3×10^{-4} M.

bition but was more sensitive to AGIs than sucrase activity in *R. fulvipes*. In all the insects, sucrase activity was always higher than either maltase or lactase and higher than trehalase in all but *A. metaurus*.

Maltose hydrolysis in *U. fulgens* was resistant to all AGIs tested, except for poor inhibition by AB-1 (Table 5); maltase activity in *A. metaurus* was too low to be useful in these assays. Four of the AGIs inhibited maltase activity in *P. ballus*, *T. virgilius*, and *S. littoralis*, with DMDP and DNJ being less effective inhibitors in the generalist Lepidoptera from *O. diandra* than in *S. littoralis*, while inhibition by HNJ and AB-1 was similar in all three species. Maltose hydrolysis in *R. fulvipes* was susceptible to the above four AGIs and also HNJ-glucoside and castanospermine and, with the exception of DMDP, was more susceptible to the AGIs than maltase activity from *D. maculatus*.

Trehalase activity in *U. fulgens* and *A. metaurus* again showed consistently higher resistance to inhibition by the various AGIs than the trehalase activity in the other insects (Table 6). Nevertheless, HNJ-glucoside proved to be such a potent inhibitor of trehalase activity that, even in *U. fulgens*, it was still an effective inhibitor at submicromolar concentrations; only in *A. metaurus* was

TABLE 5. CONCENTRATIONS (M) OF ALKALOIDAL GLYCOSIDASE INHIBITORS (AGIs) GIVING 50% INHIBITION OF MALTOSE HYROLYSIS BY PREPARATIONS OF SPECIALIST AND GENERALIST HERBIVORES OF *Omphalea diandra* (*Spodoptera littoralis* LARVAE AND *Dermestes maculatus* ADULTS INCLUDED FOR COMPARISON)

		Concentration (M) of AGI required to inhibit maltase activity by 50%						
		AGIs from *Omphalea diandra*					Other AGIs	
Herbivore	Type[a]	DMDP	DMJ	DNJ	HNJ	HNJ gluc	AB-1	Cast
Urania fulgens	SLO	NI[b]	NI	NI	NI	NI	2.7×10^{-4}	NI
Panthiades ballus	GLO	5.6×10^{-5}	NI	2.1×10^{-4}	1.4×10^{-4}	NI	4.9×10^{-5}	NI
Theope virgilius	GLO	1.9×10^{-5}	NI	2.1×10^{-4}	1.7×10^{-4}	NI	2.6×10^{-5}	NI
Phobetron sp.	GLO	1.6×10^{-5}	NI	1.3×10^{-4}	—	NI	—	NI
Spodoptera littoralis	GL	7.9×10^{-6}	NI	8.6×10^{-5}	1.3×10^{-4}	NI	2.9×10^{-5}	NI
Rhabdopterus fulvipes	GCO	9.9×10^{-6}	NI	3.9×10^{-6}	3.7×10^{-6}	1.6×10^{-5}	1.5×10^{-5}	3.1×10^{-4}
Dermestes maculatus	GC	7.6×10^{-6}	NI	2.5×10^{-5}	2.6×10^{-5}	1.1×10^{-4}	—	NI

[a] See Table 4, footnote *a*.
[b] Less than 50% inhibition at 3.3×10^{-4} M.

there a marked increase in resistance to this AGI. Lactase activity in *U. fulgens* and *A. metaurus* again showed resistance to the AGIs which are found in *O. diandra* (Table 7).

Overall, the gut glycosidases of the two specialist insects were more resistant to the AGIs occurring in the plant than the glycosidases of the generalist insects feeding on *O. diandra*. Sucrose and maltose hydrolysis in the generalist lepidopteran larvae was more resistant to some of the *O. diandra* AGIs than in *S. littoralis*; however, there was little difference in the susceptibility to AGIs of gut glycosidases from the *Omphalea*-feeding coleopteran *R. fulvipes* compared to *D. maculatus*. The glycosidases from both coleopterans were generally more susceptible to AGIs than those from the lepidopterans.

DISCUSSION

The captive-bred adults of *U. fulgens* examined in this study contained lower levels of AGIs than reported previously for wild-caught, migrating *U. fulgens*, in which mean levels of 0.09% DMDP and 0.27% HNJ were determined (Kite et al., 1990). Nevertheless, there were still significantly higher

TABLE 6. CONCENTRATIONS (M) OF ALKALOIDAL GLYCOSIDASE INHIBITORS (AGIs) GIVING 50% INHIBITION OF TREHALOSE HYROLYSIS BY PREPARATIONS OF SPECIALIST AND GENERALIST HERBIVORES OF *Omphalea diandra* (*Spodoptera littoralis* LARVAE AND *Dermestes maculatus* ADULTS INCLUDED FOR COMPARISON)

		Concentration (M) of AGI required to inhibit trehalase activity by 50%						
		AGIs from *Omphalea diandra*					Other AGIs	
Herbivore	Type[a]	DMDP	DMJ	DNJ	HNJ	HNJ gluc	AB-1	Cast
Urania fulgens	SLO	NI[b]	NI	NI	1.5×10^{-4}	2.4×10^{-7}	3.6×10^{-5}	NI
Alcides metaurus	SLO	NI	NI	NI	NI	1.1×10^{-5}	3.8×10^{-4}	NI
Panthiades ballus	GLO	1.8×10^{-4}	NI	2.4×10^{-5}	1.1×10^{-5}	1.4×10^{-8}	9.4×10^{-6}	4.7×10^{-5}
Theope virgilius	GLO	6.6×10^{-5}	NI	3.3×10^{-5}	8.6×10^{-6}	1.1×10^{-8}	—	1.6×10^{-4}
Spodoptera littoralis	GLO	1.3×10^{-4}	NI	5.4×10^{-5}	3.4×10^{-5}	4.2×10^{-8}	4.1×10^{-5}	2.3×10^{-5}
Rhabdopterus fulvipes	GCO	1.8×10^{-4}	NI	1.8×10^{-4}	3.5×10^{-5}	7.4×10^{-8}	—	—
Dermestes maculatus	GC	2.4×10^{-4}	—	1.3×10^{-4}	3.3×10^{-5}	1.6×10^{-8}	6.3×10^{6}	1.6×10^{-4}

See Table 4, footnote *a*.
[b]Less than 50% inhibition at 3.3×10^{-4} M.

TABLE 7. CONCENTRATIONS (M) OF ALKALOIDAL GLYCOSIDASE INHIBITORS (AGIs) GIVING 50% INHIBITION OF LACTOSE HYROLYSIS BY PREPARATIONS OF SPECIALIST AND GENERALIST HERBIVORES OF *Omphalea diandra* (*Spodoptera littoralis* LARVAE INCLUDED FOR COMPARISON)

		Concentration (M) of AGI required to inhibit lactase activity by 50%						
		AGIs from *Omphalea diandra*					Other AGIs	
Herbivore	Type[a]	DMDP	DMJ	DNJ	HNJ	HNJ gluc	AB-1	Cast
Urania fulgens	SLO	4.6×10^{-5}	NI[b]	NI	NI	NI	NI	4.6×10^{-5}
Alcides metaurus	SLO	3.5×10^{-5}	NI	2.0×10^{-4}	NI	NI	3.0×10^{-4}	1.2×10^{-5}
Spodoptera littoralis	GL	2.7×10^{-6}	—	—	1.9×10^{-4}	—	1.4×10^{-4}	NI

[a] See Table 4, footnote *a*.
[b] Less than 50% inhibition at 3.3×10^{-4} M.

levels of DMDP and HNJ in the *U. fulgens* adults compared to adults of generalist lepidoptera that had been reared from *O. diandra*; in these, AGIs often could not be detected. Some lepidopterans which contain plant-derived toxins do so only in the larval stage and eliminate them before or during pupal development (Rothschild et al., 1975). This did not occur in the generalist lepidopterans that fed on *O. diandra*, as the larvae also contained only low levels of AGIs. It is possible that these small amounts of AGIs resided in residual undigested *O. diandra* in the gut; *U. fulgens* larvae contained DMDP and HNJ at concentrations higher than in the foliage. These observations conform with studies on the herbivores of milkweeds, *Asclepias* spp., where generalist herbivores also either lack toxic cardenolides or contain much lower levels than the specialist monarch butterfly *Danaus plexippus* or the milkwood bug *Oncopeltus fasciatus* (Isman et al., 1977). Nevertheless, sequestration of plant toxins is not always confined to specialist herbivores; for example, the polyphagous lubber grasshopper *Romalea guttata* accumulates numerous plant-derived compounds (Jones et al., 1987).

Neither larvae nor adults of *U. fulgens* contained the glucosides of DMDP and HNJ that were detected in *O. diandra* during this study, even though HNJ-glucoside was often the most abundant alkaloid in the plant. This discrimination against glycosides is in contrast to other sequestration systems, in which herbivores preferentially accumulate glycosides from the host plant, for example, the sequestration of iridoid glycosides from Plantaginaceae by checkerspot but-

terflies, cyanogenic glycosides from *Passiflora* by heliconiines, and azoxyglycosides from cycads by *Eumaeus atala* (Bowers, 1988, and references therein). This strategy reflects the generally less toxic nature of glycosides compared to the corresponding aglycones, and herbivores adapted to the host plant restrict the activity of the glycosidases which would otherwise release the toxic aglycone, as occurs in nonadapted herbivores or predators (Blum, 1983). With AGIs, the glycosides may show greater biological activity than the aglycones. For example, in this study, HNJ-glucoside was about 1000 times more active against insect trehalases than HNJ, a property confirmed for an HNJ-glucoside (MDL) in a number of other insect species (Scofield et al., 1995a,b).

A herbivore which is adapted to feed on a plant containing chemicals that would be deleterious to nonadapted herbivores, and which does not sequester the plant toxins, must either excrete the toxins completely, metabolize them to nontoxic products, or avoid ingesting high levels. Herbivores feeding on *Nicotiana tabacum* either excrete nicotine or metabolize it to nontoxic continine (Self et al., 1964), and the squash beetle *Epilachna borealis* exhibits leaf-trenching behavior on cucumber to isolate cucurbitacins (Tallamy 1985). Underlying these strategies are various levels of physiological and biochemical resistance, depending on the degree of adaptation to the toxin-producing plant and the degree of activity of the toxin. None of the generalist herbivores of *O. diandra* appeared to excrete completely all the AGIs they ingested (this strategy must result in a feces:diet ratio of AGI concentrations greater than 1 on a dry weight basis), and there was no evidence that the generalist lepidopteran larvae or *R. fulvipes* avoided ingesting AGIs, for example, by selecting leaves that were depleted in AGIs. Thus an ability to metabolize these alkaloids is inferred from the results. The *Trigona* bees may have shown behavioral avoidance of AGIs since they fed on the exudate from the abaxial veins, probably from the phloem. Larvae of *U. fulgens* also showed behavioral adaptations since late instars often trenched the petiole of a leaf before eating the lamina. This action possibly stemmed the flow of latex to the lamina.

Since *U. fulgens* larvae were found to accumulate DMDP and HNJ, one might have expected their feces to be very depleted of these AGIs, but this appeared not to be the case. Whether *U. fulgens* larvae simply excreted any AGI aglycones which they did not accumulate, or whether some were metabolized, cannot be determined from the data. The AGI-glucosides were clearly metabolized efficiently by *U. fulgens* larvae since they were not detectable in the feces. This also seemed to be the case for the generalist herbivores, with the exception of the data obtained for one of the *P. ballus* larvae. Thus, an inverse correlation between the accumulation of AGIs and the efficiency of AGI metabolism is suggested from a comparison of the fate of AGI aglycones in specialist versus generalist herbivores and free AGIs versus AGI-glucosides in *U. fulgens*. In this respect, it is interesting to note that, of the generalist her-

bivores, AGIs were most frequently observed in *P. ballus*, which excreted more AGIs than the other generalist herbivores. Selective restriction of AGI metabolism may, therefore, be one adaptation to the accumulation of AGIs by specialist *Omphalea* herbivores.

A further adaptation of *U. fulgens* to *O. diandra* is shown by the gut glycosidase inhibition data. The glycosidases of the generalist herbivores had a lower tolerance to AGIs than the specialists. In particular, sucrose and maltose hydrolysis in *U. fulgens* (and *A. metaurus*) was highly resistant to inhibition by the AGIs occurring in *O. diandra*, while sucrose hydrolysis in the generalist herbivores was susceptible to inhibition by DMDP and maltose hydrolysis was inhibited by DMDP, DNJ, and HNJ. Such target-site resistance to plant toxins has also been reported for the specialist herbivores of *Asclepias* spp. For example, it has been shown that a Na,K-ATPase of the large milkweed bug, *Oncopeltus fasciatus*, is insensitive to cardenolides (Moore and Scudder, 1985). Maltase and sucrase activities in the generalist Lepidoptera found on *O. diandra* were, however, still more resistant to several of the AGIs than the corresponding enzyme activities in the lepidopteran *S. littoralis*, which suggests there had been some adaptation to AGIs. Larvae of *S. littoralis* do not encounter *O. diandra* in nature, and when larvae are presented with leaves of this species in the laboratory, they show rejection behavior and do not eat them (M. Simmonds, personal communication). For the generalist beetle *R. fulvipes* there was less evidence of adaptation of its enzymes to the inhibitory effects of the AGIs present in *O. diandra*.

The only AGI from *O. diandra* showing potent inhibition of a gut glycosidase from *U. fulgens* was HNJ-glucoside, which inhibited trehalase activity, although again, the inhibition was less than for the generalist herbivores. In most insects trehalose is the immediate carbohydrate reserve from which glucose can readily be liberated by trehalase to provide energy. Thus an inhibitor of trehalase would be expected to disrupt the metabolism of an insect, and some trehalase inhibitors have been shown to be insecticidal when injected into *S. littoralis* (Asano et al., 1990). As *U. fulgens* does not exhibit target-site resistance to HNJ-glucoside, it appears to overcome the potentially lethal effects of HNJ-glucoside in its diet by metabolizing the compound in the gut. It is interesting to note that trehalase activity in *A. metaurus* was much more resistant to HNJ-glucoside inhibition than in the other species tested; trehalase was also the most active of the disaccharidases tested in this species.

In the spectrum of activities displayed by the defensive compounds produced by plants, AGIs probably fall between specific acute toxins, such as the cardiac glycosides of *Asclepias*, and nonspecific general digestion inhibitors, such as tannins. Acute toxicity of AGIs has yet to be demonstrated, but undoubtedly the presence of AGIs reduces the digestibility of *O. diandra* and other plants which synthesize them, and this, coupled with their antifeedant effects,

is probably sufficient to deter many potential generalist herbivores or reduce the nutritional value of the foliage. Although it has yet to be demonstrated that *U. fulgens* gains protection from accumulating AGIs, the evidence found in this study of nonaccumulation by generalist herbivores and the evolution of target-site resistance mechanisms does tend to indicate that they gain some advantage from doing so.

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LEARNED RECOGNITION OF PREDATION RISK BY *Enallagma* DAMSELFLY LARVAE (ODONATA, ZYGOPTERA) ON THE BASIS OF CHEMICAL CUES

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Abstract—We studied two populations of damselfly larvae (*Enallagma boreale*): one population cooccurred with a predatory fish (northern pike, *Esox lucius*); the other did not. Damselflies that cooccurred with pike adopted antipredator behavior (reduced activity) in response to chemical stimuli from injured conspecifics, and to chemical stimuli from pike, relative to a distilled water control. Damselflies from an area where pike do not occur responded only to chemical stimuli from injured conspecifics. In a second set of experiments, we conditioned pike-naive damselflies to recognize and respond to chemical stimuli from pike with antipredator behavior. Damselfly larvae that were previously unresponsive to pike stimuli learned to recognize pike stimuli after a single exposure to stimuli from pike and injured damselflies or pike and injured fathead minnows (*Pimephales promelas*). The response to injured fathead minnows was not a general response to injured fish because damselfly larvae did not respond to chemical stimuli from injured swordtails (*Xiphophorus helleri*), an allopatric fish. Taken together, these data suggest a flexible learning program that allows damselfly larvae to rapidly acquire the ability to recognize local predation risk based on chemical stimuli from predators, conspecifics, and heterospecific members of their prey guild.

Key Words—Injury-released chemical alarm pheromone, alarm signal, antipredator behavior, predator-prey, learned predator recognition, cross-species reactions, prey guild, damselfly, *Enallagma boreale*, fathead minnow, *Pimephales promelas*, northern pike, *Esox lucius*.

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INTRODUCTION

Chemical stimuli are an important source of information for assessing predation risk for animals living in aquatic habitats, especially at night, in turbid water, or in highly structured habitat when visual information is unavailable (Hara, 1992; Smith, 1992; Dodson et al., 1994).

The chemical ecology of predator–prey interactions involving odonates remains relatively unexplored. Odonate larvae (dragonflies and damselflies) live in structured aquatic habitats and are subject to predation by fish (Morin, 1984; Pierce et al., 1985; Dixon and Baker, 1988; Henrikson, 1988; McPeek, 1990a, b; Johansson and Samuelsson, 1994) including northern pike, *Esox lucius* (Allen, 1939; Lawler, 1965). Juvenile pike readily eat damselflies in the laboratory (personal observation).

Individuals from populations that frequently encounter predators typically exhibit stronger antipredator responses than individuals from populations that rarely experience predators (Giles and Huntingford, 1984; Magurran, 1986; Mathis et al., 1993; Gelowitz et al., 1993; Matity et al., 1994; Chivers et al., 1995a). Population differences in antipredator behavior could result from evolved, genetic differences between populations in response to differing selection pressures, such as the presence or absence of a predator species (e.g., Seghers, 1974; Seghers and Magurran, 1995), or from learned recognition of a predator species by its prey (Chivers and Smith, 1994a). European minnows (*Phoxinus phoxinus*), fathead minnows (*Pimephales promelas*), and brook stickleback (*Culaea inconstans*) learn to associate chemical cues from injured conspecifics with those from a novel predator (Göz, 1941; Magurran, 1989; Mathis and Smith, 1993a; Chivers and Smith, 1994a, b; Chivers et al., 1995b) or from risky habitats (Chivers and Smith, 1995). It is not known whether learned predator recognition occurs in invertebrates.

Members of a prey guild are species that share habitat and predators. Injury-released chemical cues produced by one species indicate predation risk to all species in the guild (Snyder, 1967). Examples of cross-species responses to injury-released chemical cues among prey guild members are known for gastropods (Stenzler and Atema, 1977) and some fishes (Smith et al., 1991; Mathis and Smith, 1993b; Chivers and Smith, 1994c; Chivers et al., 1995a; Wisenden et al., 1994, 1995a). It has not yet been tested whether animals can learn to recognize novel predator cues by associating them with injury-released chemical cues from heterospecifics.

In this study we quantify foraging activity of damselfly larvae (Odonata, Zygoptera) before and after exposure to test stimuli in order to test four hypotheses. First, we test if damselflies decrease their activity in response to injured conspecifics, a reliable indicator of predation risk. Reduced movement and foraging are common responses of prey animals, including damselflies, to

the presence of predators (Ware, 1973; Heads, 1985; Pierce et al., 1985; Convey, 1988; Dixon and Baker, 1988; McPeek 1990a, b; see reviews by Lima and Dill, 1990; Johnson, 1991). To verify that the response of damselflies to stimuli from injured conspecifics represents antipredator behavior, we exposed damselflies to chemical stimuli from a known predator, northern pike. We used two populations of damselfly larvae, one that cooccurs with pike and one that does not.

The second hypothesis tested in this study is that damselflies that cooccur with pike should decrease foraging activity in response to pike stimuli, whereas damselflies that do not cooccur with pike should not respond to pike stimuli.

Third, we test to see if damselflies respond with antipredator behavior to alarm pheromones from injured heterospecific prey species (fathead minnows) from the same prey guild.

Fourth, we test to see if an antipredator response to a chemical cue can be acquired by learning. We present naive damselfly larvae with a novel predator cue (northern pike) in combination with chemical stimuli either from injured damselflies or from injured fathead minnows.

METHODS AND MATERIALS

In December 1994 we collected damselfly larvae from two locations in south-central Saskatchewan, Canada. Feedlot Pond is located on the University of Saskatchewan campus in Saskatoon and does not contain northern pike. Pike Lake is an oxbow lake of the South Saskatchewan River and contains pike and other species. Damselflies were maintained in 20-liter aquaria at approximately 18°C on a 12L : 12D-hr photoperiod and were fed daily with brine shrimp nauplii (*Artemia franciscans*). Damselflies were maintained in the laboratory for a minimum of 2 weeks prior to testing.

All damselfly larvae were keyed to *Enallagma boreale* using Merritt and Cummins (1984) and Walker (1953). Species identification of *Enallagma* based on larval characteristics can be unreliable. Adult male damselflies emerging from the two study sites were collected in June 1995 and identified on the basis of the morphology of male genitalia using Walker (1953). Of 22 male *Enallagma* collected from Feedlot Pond, 15 were *E. boreale* and 7 were *E. cyathigerum*. Of 45 male *Enallagma* collected from Pike Lake, 44 were *E. boreale* and 1 was *E. cyathigerum*. Because the larvae of *E. boreale* and *E. cyathigerum* are morphologically identical (McPeek, 1990b), animals tested in this study could have included some individuals of *E. cyathigerum*. Because cross-species reactions are evident (see results of experiment 2), we believe the possibility that more than one species of *Enallagma* were used in these experiments is unlikely to have affected the conclusions.

Experiment 1: Population Differences. Thirty damselflies from each population were placed individually into 5-liter round opaque plastic containers (diameter, 18 cm) that contained 2 liters of dechlorinated tap water. No substrate or structure was provided. Mean ± SD length of the 60 damselflies (measured from tip of the head to tip of the abdomen, excluding caudal lamellae) was 17.0 ± 7.2 mm. Experiments began after the damselflies had acclimated to the test chambers for a minimum of 36 hr. Prior to the experiment, we did not feed the damselflies after placing them into the testing chambers. We began by injecting 5 ml of a standard concentration of brine shrimp culture (approximately 1200 shrimp nauplii) into each container. We waited 5 min before conducting observations to allow the damselflies to adjust to the presence of the observer and establish a regular pattern of feeding behavior. We recorded the frequency of three conspicuous behaviors associated with foraging (feeding strikes, head turns, and walking movements) for 5 min before and 5 min after the injection of one of three test stimuli: (1) 10 ml of distilled water (control stimulus), (2) 10 ml of pike stimulus, or (3) 10 ml of water containing stimuli of injured conspecifics.

A feeding strike was scored every time a damselfly extended and retracted its prementum (feeding apparatus that bears mandibles). A head turn was recorded whenever a damselfly shifted its head position by an arc of more than 22.5° (1/4 of a 90° arc) in any direction. A walking movement was a short walk that entailed the lifting of a tarsus (foot) and planting it in a new location. Bouts of walking not separated by more than 1 sec were counted as a single "move." Typically, damselflies used short walks of one to several steps while feeding on brine shrimp. Moves greater than one body length (N = 1 in 900 min) and undulatory swimming (N = 6 in 900 min) were rare.

We tested 30 individuals from each population of damselflies, 10 in each of the three treatment conditions (control, pike, and conspecific). For each trial we calculated the change in the number of feeding strikes, head turns, and walking movements by subtracting the number performed before exposure to the stimulus from the number performed after exposure to the stimulus. For each damselfly population (and in subsequent experiments) we used separate Kruskal–Wallis one-way analysis of variance tests to compare the significance in the change in each foraging behavior (Siegel and Castellan, 1988). These analyses were followed by post hoc nonparametric multiple comparisons with alpha level adjusted for nonindependent pairwise comparisons (Siegel and Castellan, 1988). One-tailed statistical tests were used because we predicted that damselflies would reduce the frequency of conspicuous behavior in response to water containing stimuli from pike or injured conspecifics.

We prepared the conspecific stimuli by crushing two damselflies (one from each population), with a mortar and pestle and then adding 20 ml of distilled water. We used 10 ml from each 20-ml solution of conspecific stimulus to test one damselfly from each population. In all cases the conspecific stimulus was used within 15 min of preparation.

To ensure that damselflies in our study did not respond to stimuli in the pike's diet (Mathis and Smith, 1993a; Brown et al., 1995), we fed the pike a diet of swordtails for three feedings (once every 5 days) prior to collecting the stimulus. Approximately 12 hr after the final feeding, the pike (fork length, 18 cm) was placed into a clear plastic collection chamber (26 × 8 × 8 cm) that contained 1200 ml of dechlorinated tap water. The collection chamber was aerated but not filtered. After 3 days the pike was removed from the chamber and the pike stimulus water was frozen at approximately −20°C until needed. Previous studies have shown that the use of multiple pike to generate pike stimulus is no better than using a single pike (Gelowitz et al., 1993).

Experiment 2: Learned Recognition of a Novel Predator Cue. Damselfly larvae were collected from Feedlot Pond in December 1994, transferred to laboratory aquaria, and maintained at 18°C on newly hatched brine shrimp for at least a week before being used in experiments. Three days before the experiment began, one damselfly larva was placed into each of 30 5-liter plastic containers filled with 2 liters of dechlorinated tap water. On Day 1 of the experiment, each larva was tested for its response to one of three sets of paired stimuli: (1) 10 ml of pike stimulus combined with 5 ml of water containing injured conspecific damselflies, (2) 10 ml of pike stimulus combined with 5 ml of skin extract from fathead minnows, or (3) 10 ml of pike stimulus combined with 5 ml of distilled water (Control). Damselfly stimulus was made by crushing two damselflies in 10 ml of distilled water to produce two 5-ml aliquots for two trials. Pike stimulus was prepared as described for experiment 1. Fathead minnow skin extract was prepared by killing two minnows by a blow to the head and making 25 vertical superficial cuts on each side of the body using a razor blade. Each fish was rinsed with 5 ml of distilled water. We prepared stimulus from pairs of fish and used the resulting 10 ml of rinse water as stimulus for two trials. Thus one minnow was used per trial. The stimulus was used immediately. The mean ± SD standard length of fish used as stimulus was 3.4 ± 0.2 cm.

We began each trial by injecting 5 ml of live brine shrimp into the center of the container. After 5 min, we recorded the frequency of foraging behavior for 5 min using the same criteria as described for experiment 1. After 5 min, we gently introduced one of the test solutions of paired stimuli into the container and monitored foraging behavior for another 5 min. At the end of Day 1, we emptied each container and replaced the water with fresh dechlorinated tap water.

We repeated the same procedure the next day using the same damselflies. The protocol was similar to the first day except that no additional stimuli accompanied the 10 ml of pike stimulus.

Mean ± SD length of the 10 damselfly larvae used for each treatment in this experiment was as follows: damselfly, 14.3 ± 0.9 mm; fathead minnow, 15.2 ± 0.9 mm; and distilled water (control), 15.2 ± 1.3 mm.

Experiment 3: Specificity of Response to Fish Alarm Cues. In this experiment, we exposed damselflies to skin extract of swordtails (*Xiphophorus helleri*) to test if the response to fathead minnow skin was specific to fathead minnows or a general response to chemical stimuli from injured fish. Swordtails are tropical fish, allopatric to the damselflies used in this experiment, and thus unfamiliar to the test animals. Swordtails are phylogenetically unrelated to fathead minnows and lack the specialized epidermal alarm substance cells present in fathead minnows (Smith, 1992).

The experimental protocol was similar to the first two experiments. We used damselflies from Feedlot Pond (where pike are absent). One damselfly was placed into each of 30 5-liter containers 3 days before the experiment began. At the start of a trial, 5 ml of live brine shrimp were added to the center of the container. After waiting 5 min, we recorded the number of feeding strikes, head turns, and walking movements for 5 min. Then we added one of three stimuli: (1) 15 ml of chemical stimuli from an injured conspecific damselfly, (2) 15 ml rinse of chemical stimuli from an injured swordtail, or (3) 15 ml of distilled water. The frequency of foraging behaviors was recorded for another 5 min. Mean $\pm$ SD length of the 10 damselfly larvae used for each treatment in this experiment was as follows: damselfly, 16.3 $\pm$ 0.9 mm; swordtail, 15.6 $\pm$ 1.6 mm; and distilled water, 16.2 $\pm$ 1.9 mm.

Damselfly stimulus was prepared as described for the first experiment except 30 ml were used instead of 20 ml to prepare stimulus for each set of two trials. Swordtail skin extract was prepared by killing each fish by a blow to the head, making 25 vertical cuts on each side of its body, and rinsing with 15 ml of distilled water. One swordtail was used per trial. Mean $\pm$ SD standard length of the 10 swordtails used in the experiment was 4.0 $\pm$ 0.28 cm.

RESULTS

Experiment 1: Population Differences. For damselflies that do not cooccur with pike, there was a significant effect of the treatments on feeding strikes [Kruskal–Wallis (KW) = 8.68, $P < 0.02$], head turns (KW = 13.50, $P < 0.01$), and walking movements (KW = 14.26, $P < 0.001$). Damselflies reduced their frequency of feeding strikes, head turns and walking movements in response to conspecific stimuli relative to pike stimuli or distilled water (Figure 1; $P < 0.05$ for all comparisons). There was no significant difference in the change in feeding behavior between the distilled water and the pike stimulus treatments ($P > 0.15$ for all comparisons). Slight increases occurred in control trials because some brine shrimp that settled during the first 10 min were resuspended upon introduction of the test stimulus thus stimulating foraging activity.

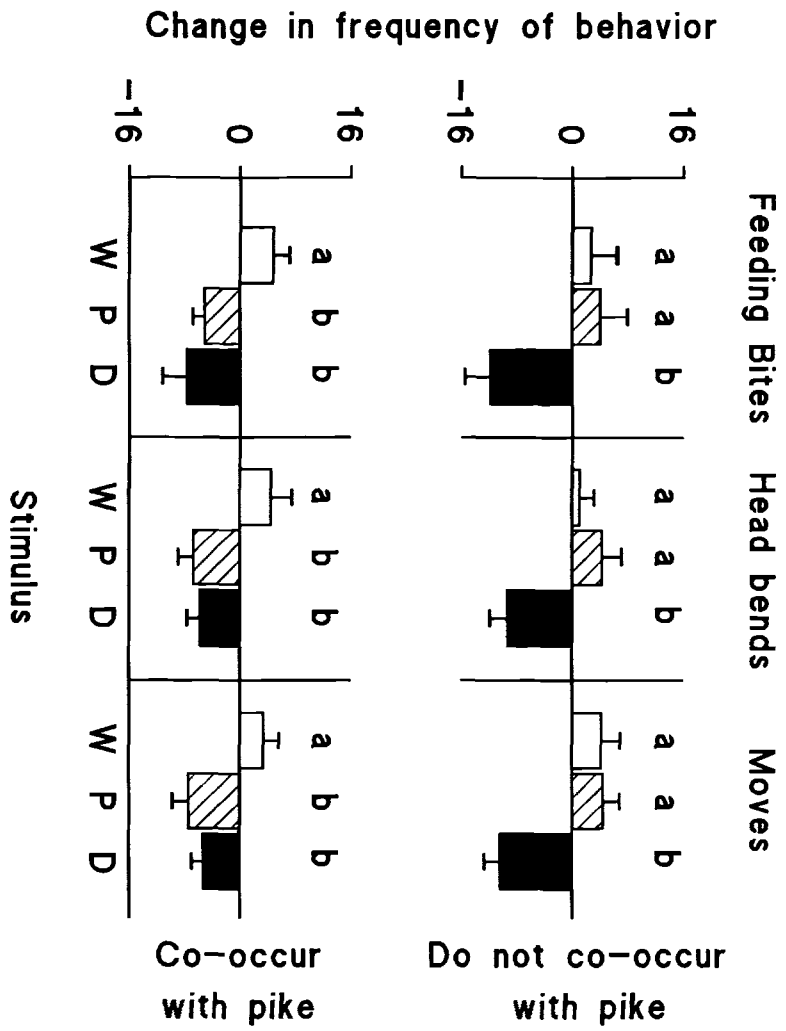

FIG. 1. Mean (±SE) change in frequency of feeding strikes, head turns, and walking movements by damselflies that cooccur with pike and those that do not, following exposure to a control of distilled water (W; open bars), pike stimulus (P; hatched bars), or injured conspecific damselflies (D; solid bars). For each behavior, bars with different letters are significantly different from others in that population (KW post hoc multiple-comparison test, $P < 0.05$).

For the population that cooccurs with pike, there was a significant overall effect of the treatments on feeding strikes (KW = 9.58, $P < 0.01$), head turns (KW = 7.98, $P < 0.02$), and walking movements (KW = 12.51, $P < 0.01$). Damselflies reduced their frequency of feeding strikes, head turns, and walking movements in response to conspecific or pike stimuli relative to distilled water (Figure 1; $P < 0.05$ for all comparisons). There was no significant difference between the response to conspecific and pike stimuli ($P > 0.15$ for all comparisons).

Experiment 2: Learned Recognition of a Novel Predator Cue. Chemical stimuli presented to damselflies along with pike stimuli had a significant overall effect on feeding strikes on Day 1 (conditioning trials) of the experiment (KW = 12.43, $P < 0.01$), head turns (KW = 9.23, $P < 0.01$), and walking movements (KW = 9.79, $P < 0.01$). Damselflies performed significantly fewer feeding strikes ($P < 0.05$), head turns ($P < 0.05$), and waking movements ($P < 0.01$) after exposure to stimuli from pike plus injured damselfly than after exposure to stimuli from pike plus distilled water (Figure 2). Exposure to fathead minnow skin extract along with pike stimuli resulted in a significant decrease in the frequency of feeding strikes, head turns, and walking movements compared to control trials (Figure 2; $P < 0.05$ for all comparisons). There were no significant differences between the damselfly and the minnow treatments ($P > 0.30$).

When the same damselflies were subsequently presented with pike stimuli alone on Day 2 (test trials), there was a significant difference among groups in the frequency of feeding strikes (KW = 9.25, $P < 0.01$), head turns (KW = 9.05, $P < 0.02$), and walking movements (KW = 17.43, $P < 0.001$). Pairwise comparisons between groups showed that for each behavior, activity was significantly lower for damselflies previously exposed to stimuli from pike plus injured damselfly or pike plus injured minnow compared to control trials ($P < 0.05$). There was no significant difference between damselflies previously conditioned with stimuli from injured damselflies and damselflies previously conditioned with fathead minnow skin (Figure 2; $P > 0.30$). Therefore, damselfly larvae that were previously unresponsive to pike stimuli learned to recognize pike stimuli after a single exposure to stimuli from pike and injured damselflies or pike and injured fathead minnows.

Experiment 3: Specificity of Response to Fish Alarm Cues. There was a significant overall effect of the test stimuli on the frequency of feeding strikes (KW = 13.8, $P < 0.01$), head turns (KW = 19.3, $P < 0.001$), and walking movements (KW = 15.0, $P < 0.001$). After exposure to injured damselflies, the frequency of all three behaviors was significantly reduced ($P < 0.05$ for all comparisons) in comparison with either distilled water or swordtail skin extract (Figure 3). There was no significant difference in damselfly behavior between the distilled water and the swordtail treatments ($P > 0.30$ for all comparisons).

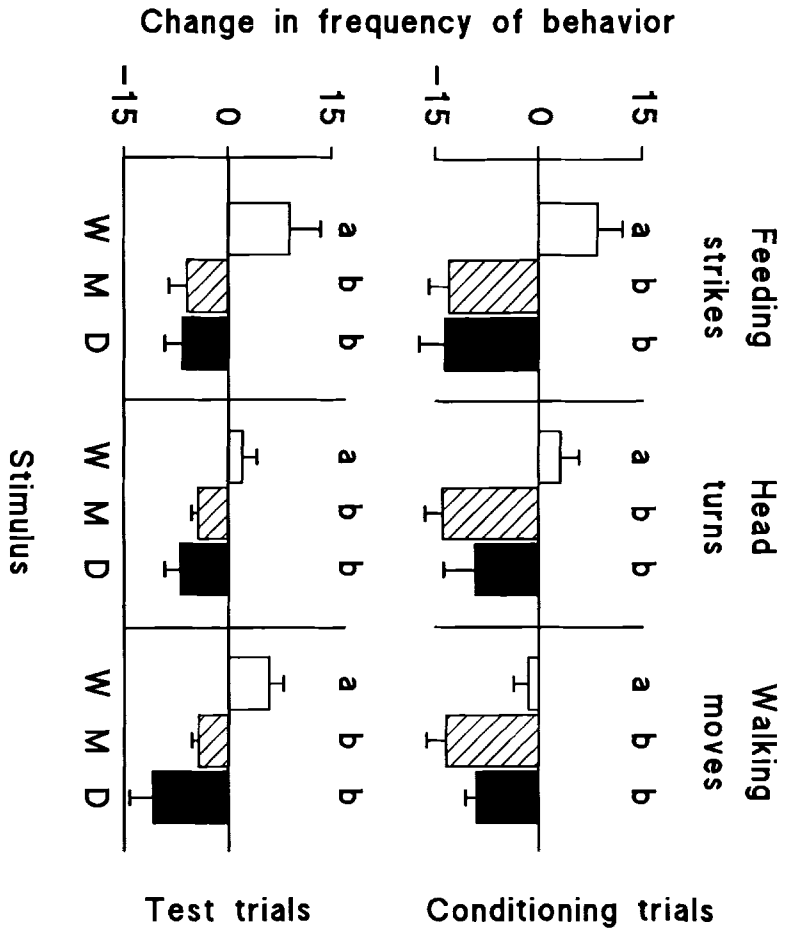

FIG. 2. Right: Mean (±SE) change in frequency of feeding strikes, head turns, and walking movements for damselflies presented with chemical stimuli from pike and distilled water (W; open bars), pike and injured fathead minnows (M; hatched bars), or pike and injured conspecific damselflies (D; solid bars). Left: Mean (±SE) change in frequency of feeding behaviors for the same damselflies when presented later with chemical stimuli from pike alone. Bars denote conditioning stimuli described above. For each behavior, bars marked with different lowercase letters are significantly different from others in that set of trials (KW post hoc multiple-comparison test, $P < 0.05$).

Damselflies did not appear to recognize chemical stimuli from swordtail skin as an indicator of danger, and thus it is not likely that the response to fathead minnow skin extract in experiment 2 represented a general response to stimuli released from injured fish.

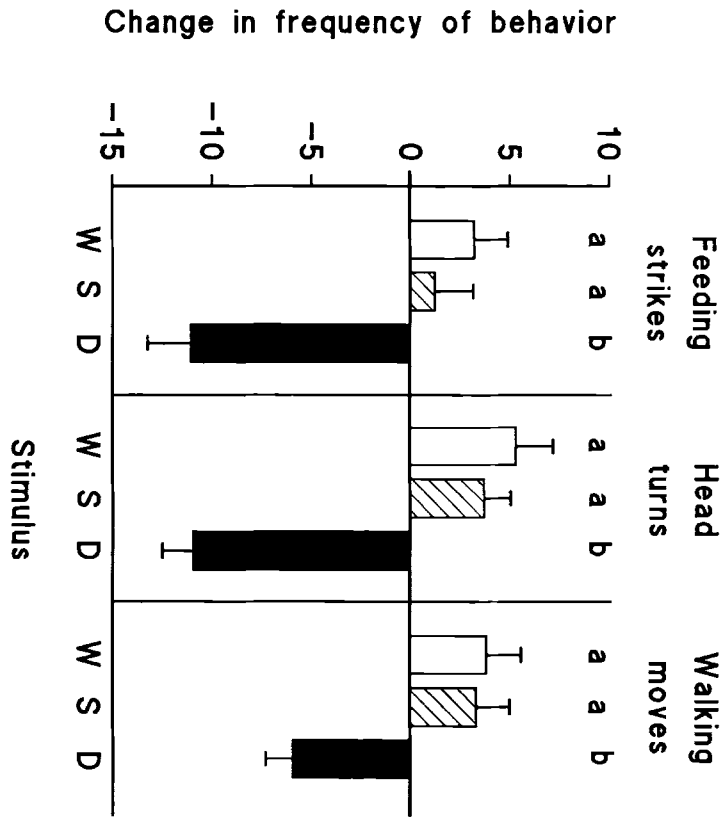

FIG. 3. Mean (±SE) change in frequency of feeding strikes, head turns, and walking movements for damselflies presented with distilled water (W; open bars), chemical stimuli from injured swordtails (S; hatched bars), or chemical stimuli from injured conspecific damselflies (D; solid bars). For each behavior, bars marked with different lowercase letters are significantly different (KW post hoc multiple-comparison test, $P < 0.05$).

DISCUSSION

To our knowledge this is the first study to demonstrate that a member of the order Odonata responds to injured conspecifics with an antipredator response. Damselflies from both populations decreased activity in response to stimuli from injured conspecifics alone. We do not know if the antipredator response to stimuli from injured conspecifics is species-specific or if it is a general response to damaged odonate or insect tissue. Because we prepared the stimulus from whole damselflies, we do not know whether or not conspecifics are sensitive only to stimuli from a particular part of the damselfly body. In ostariophysan

fishes such as fathead minnows, specialized epidermal club cells contain alarm pheromone (Smith, 1992). In mud snails (*Nassarius obsoletus*), the foot, viscera, and blood all elicit an alarm reaction from conspecifics (Atema and Stenzler, 1977).

Predator recognition is of obvious survival value. Early recognition of predation risk allows animals to adopt cryptic behavior and decrease the chances of their being detected and attacked by a predator (Snyder, 1967; Hews, 1988; Mathis and Smith, 1993c; Lima and Dill, 1990; Wisenden et al., 1995b). Upon recognition of the pike cue, damselflies reduced movements associated with feeding; as a consequence, there was an increase in crypsis.

Motion attracts predators. Movement by *Chaoborus* larvae more than doubles the maximum distance at which they can be detected by white crappie (*Poxomis annularis*) (Wright and O'Brien, 1982). Three-spine (*Gasterosteus aculeatus*) and 10-spine (*Pungitius pungitius*) stickleback preferentially attack larval damselflies in motion, especially in the absence of structured refuge (Convey, 1988). Once detected by predacious fish, *Enallagma* damselflies rarely escape (McPeek, 1990b).

In the first experiment, pike stimuli caused damselflies that cooccur with pike to decrease conspicuous behaviors associated with feeding, whereas damselflies that do not occur with pike did not respond. Although *E. boreale* are vulnerable to predation by centrarchids (McPeek, 1990b), naive *E. boreale* do not respond to their presence (McPeek, 1990a). *Enallagma boreale* are excluded from lakes that contain sunfish (*Lepomis gibbosus, L. Macrochirus*), probably due to predation pressure from these fishes (McPeek, 1989; 1990a, b). Our study sites do not contain centrarchids, suggesting that *E. boreale* are able to coexist with pike and the other fish species that occur at our study sites.

Data from experiments 1 and 2 show that damselfly larvae learned to recognize pike as predators on the basis of chemosensory cues when pike odor was presented simultaneously with chemical alarm cues. To our knowledge, this is the first demonstration of learned predator recognition in an insect. These data suggest a robust learning mechanism by which damselflies can acquire knowledge of predation risk across their geographical distribution, regardless of the types and abundance of predators present in any one locale. An important aspect of this learning is that it occurred after a single presentation and therefore minimized the cost of acquiring this information (Johnston, 1982; Menzel et al., 1984). Repeated naive encounters with a predator would greatly increase the risk of predation.

Experiment 3 established that the response to fathead minnow skin was not a general response to injured fish, suggesting that damselflies use injury-released chemicals from heterospecifics to assess predation risk. To our knowledge, this is the first demonstration of learned predator recognition based on cues from an injured heterospecific from a different phylum and reinforces the concept of

shared information relating to predation risk among members of a prey guild (Snyder, 1967). Presumably, damselfly larvae used in this experiment had at some point in their lives in Feedlot Pond transferred the association between conspecific alarm cues and predation risk to cues from an injured minnow. In this study, we demonstrated that damselflies were able to transfer an association between injured minnow skin and predation risk to an association between chemical stimuli from pike and predation risk.

Our data were collected from two populations, one with and one without pike. We cannot extrapolate from a single population in each condition to make general statements about all damselfly populations that may or may not cooccur with pike. However, these data serve to illustrate that population differences in predator recognition can result from experience, and not strictly as the result of an evolved, genetic trait. Because we did not train damselflies from Pike Lake, a study of laboratory reared damselflies from the Pike Lake population would be needed to determine if the antipredator response to pike stimuli in this population is genetically based or a learned response.

We cannot say from these data how long damselfly larvae may retain the learned recognition of pike stimuli. The response observed in this study resulted from a single exposure of a predatory cue paired with known indicators of predation risk. Presumably, the pairing of predator cues with stimuli from injured conspecifics occurs frequently in nature, which would serve to reinforce recognition of predator cues. Recognition of predator cues could in turn be used by damselfly larvae to extend the range of recognized chemical cues that indicate risk. This could be achieved by pairing predator cues with injury-released chemicals from heterospecific members of its prey guild, for example, the *Enallagma boreale/cyathigerum* species complex, or phylogenetically distant species such as fathead minnows.

Future research should be directed toward tests of the generality of this phenomenon among aquatic taxa and evolutionary constraints on the classes of stimuli that can be associated with predation risk (Chivers and Smith, 1994b).

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TWO REGIONAL STRAINS OF A PHORETIC EGG PARASITOID, *Telenomus euproctidis* (HYMENOPTERA: SCELIONIDAE), THAT USE DIFFERENT SEX PHEROMONES OF TWO ALLOPATRIC TUSSOCK MOTH SPECIES AS KAIROMONES

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Abstract—The egg parasitoid, *Telenomus euproctidis* Wilcox (Hymenoptera: Scelionidae), is phoretic on females of two allopatrically distributed tussock moths, *Euproctis pseudoconspersa* (Strand) and *Euproctis taiwana* (Shiraki) (Lepidoptera: Lymantriidae). Crossing experiments between the two regional parasitoid strains indicated no evidence for their reproductive isolation. More wasps were found on the locally occurring host, *E. pseudoconspersa*, than on *E. taiwana*, when virgin females of the two moth species were exposed concurrently in the field for 24 hr in Ibaraki Japan. In Ibaraki, many wasps were caught in traps baited with the synthetic sex pheromone of *E. pseudoconspersa*, 10,14-dimethylpentadecyl isobutyrate (10Me14Me-15:iBu), but none with that of *E. taiwana*, (Z)-16-methyl-9-heptadecenyl isobutyrate (16Me-Z9-17:iBu) or blank traps. In Okinawa, Japan, more wasps were found on *E. taiwana* than on *E. pseudoconspersa*, and many wasps were caught in traps baited with 16Me-Z9-17:iBu, but only a few with 10Me14Me-15:iBu, and none with blank traps. These results suggest that local wasp strains discriminate between the two sex pheromones, and they strongly prefer the sex pheromone of the moth occurring at their location.

Key Words—*Telenomus euproctidis*, phoresy, kairomone, sex pheromone, *Euproctis taiwana*, *Euproctis pseudoconspersa*, egg parasitoid, tussock moth.

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INTRODUCTION

Phoresy by the egg parasitoid *Telenomus euproctidis* has been reported in females of two allopatric tussock moth species; one is *Euproctis taiwana* (Arakaki, 1990), which occurs on the Ryukyu Islands of Japan and Taiwan (Inoue et al., 1982), and the other, *Euproctis pseudoconspersa* (Arakaki et al., 1995), occurs in Honshu, Shikoku, Kyushu Islands of Japan, Korea, Taiwan, and China (Inoue et al., 1982). In Japan, there is no overlap in geographical distribution between these two tussock moth species.

Two compounds, (*Z*)-16-methyl-9-heptadecenyl isobutyrate (16Me-Z9-17:iBu) and 16-methylheptadecyl isobutyrate (Yasuda et al., 1995), have been identified from the abdominal tip of female *E. taiwana*. The synthetic pheromone component of *E. taiwana*, 16Me-Z9-17:iBu, attracted female *T. euproctidis* and male *E. taiwana* (Arakaki et al., 1996). This finding strongly suggests that the wasp uses host sex pheromone as a host-location kairomone.

Three compounds, 10,14-dimethylpentadecyl isobutyrate (10Me14Me-15:iBu), 14-methylpentadecyl isobutyrate, and 10,14-dimethylpentadecyl *n*-butyrate (Wakamura et al., 1994), were identified from the abdominal tip of female *E. pseudoconspersa*. The major component, 10Me14Me-15:iBu, attracted wild male moths. It is noteworthy that the sex pheromones of the two tussock moth species have no compound in common. Whether the wasp (the PC strain) on *E. pseudoconspersa* uses the moth's sex pheromone as a kairomone to locate its host moth, as do wasps (the TW strain) phoretic on *E. taiwana* (Arakaki et al., 1996), is still unknown. Furthermore, it is unknown if these two parasitoid strains are attracted to the sex pheromone of the host species with which they cooccur or that of both tussock moth species.

In this paper, we show that the PC strain of the parasitoid does use the sex pheromone of *E. pseudoconspersa* as a primary cue for host location. In addition, we examine the response of the local parasitoid strains to the sex pheromones of the two allopatric tussock moth species in the field at two locations.

METHODS AND MATERIALS

Insects

Larvae of *E. pseudoconspersa* were collected from a wild population on trees of the Japanese camellia, *Camellia japonica* L., at Tsukuba, Ibaraki (Honshu Island), Japan, in 1995. They were reared on camellia leaves until pupation at 25 ± 0.5°C under a 16:8-hr L:D photocycle. The *E. taiwana* culture was derived from a wild population in a cabbage field at Naha, Okinawa (Ryukyu Islands), Japan, in 1994. They were maintained on an artificial diet (Insecta LF,

Nihon Nosan Kogyo Co. Ltd., Kanagawa) for successive generations in the laboratory under same conditions as above. To avoid allergic reactions while handling this moth, we removed the urticating bristles by soaking the cocoons in 1% sodium hypochlorite solution for ca. 10 min and then washed the pupae in tap water. The pupae were sexed and kept in separate containers until emergence.

Crossing Experiments between Two Parasitoid Strains

The two *T. euproctidis* strains were established from females of *E. pseudoconspersa* collected in Ibaraki and females of *E. taiwana* collected in Okinawa in 1994. They were maintained in the laboratory on eggs of the corresponding host species. Adult wasps were held in test tubes (1.7-cm diameter × 13 cm) with a ball of cotton moistened with diluted honey at 25°C. Crosses were made between the ninth generation of the TW strain and the third generation of the PC strain at 25 ± 1.5°C under a 16:8-hr L:D photocycle. One virgin male and one virgin female of the same or a different strain were put together in a test tube with diluted honey. Egg masses consisting of 20 eggs of *E. taiwana* were each exposed to a single wasp for oviposition. Each crossing was replicated 20 times. From F_1 adults that emerged from each egg mass, mated females were randomly selected and placed individually in test tubes with an unparasitized egg mass of 20 eggs (20 replicates). The copulation rate was evaluated indirectly by counting the number of pairs producing female offspring instead of direct observation of copulation behavior. Unmated females produce only male offspring.

Comparison of Responses of Parasitoids to Two Tussock Moth Species

Field experiments were conducted at the National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki, in October 1995 and at Okinawa Prefectural Agricultural Experiment Station, Naha, Okinawa, in April 1995. Treatments consisted of plastic cups containing a single 1-day-old virgin female of either *E. pseudoconspersa* (N = 10 replicates) or *E. taiwana* (N = 10) and were repeated twice, which resulted in 20 replications for each species. Each 120-ml plastic cup with a moth had two windows, 2.5 × 1.5 cm, on opposite sides and each window was covered with 3-mm-mesh plastic net. These windows allowed the moth's pheromone to diffuse into the air surrounding the cup. They also allowed attracted parasitoids to enter the cup. These 10 cups with moths were hung on camellia trees in Tsukuba and on hibiscus trees in Naha, at 5-m intervals, 1.2 m above the ground, at around 1100 hr, and replicated twice (20 traps per moth species). After 24 hr of exposure in the field, all cups were collected, individually held in plastic bags, and stored in a freezer for

1 hr to kill the moths and parasitoids. The anal tufts of each moth were then inspected for parasitoids under a dissecting microscope, and the number of parasitoids per moth was counted.

Comparison of Responses of Parasitoids to Two Synthetic Sex Pheromones

Traps baited with synthetic sex pheromone for *E. pseudoconspersa* or *E. taiwana* or unbaited controls were placed in the field at Ibaraki in October 1995 and at Okinawa in June 1995.

The treatments consisted of 80 μg of 10Me14Me-15:iBu for *E. pseudoconspersa* and 300 μg of 16Me-Z9-17:iBu for *E. taiwana*. These doses were determined to be optimal for male moth attraction in previous experiments (Wakamura et al., 1994; Yasuda et al., 1995). Chemicals were dissolved in hexane and applied to a 2 × 5-cm piece of filter paper (Toyo No. 2). The filter paper was then placed 3 cm above a sticky plate in a sticky trap (Sankei Chemical Co., Kagoshima, Japan). As a control, a piece of filter paper treated with solvent only was placed in the sticky trap. The traps were deployed at 5-m intervals, 1 m above the ground. The baits and sticky plates were replaced with fresh lures at approximately 1800 hr daily. Trap locations were exchanged systematically to cancel out the possible effect of trap positions on trap catches. The number of male moths and the number and sex of *T. euproctidis* caught on the sticky plates were counted with the aid of a dissecting microscope. The treatments (= two pheromone-baited traps and one control trap) were replicated at three sites and repeated three times in Okinawa and Ibaraki.

RESULTS

Crossing Experiments Between the Two Wasp Strains

All females crossed with males of the other strain produced female offspring, indicating that they had mated and that the strains are reproductively compatible (Table 1). The percentage of eggs yielding parasitoids, the sex ratio, and the developmental period in F_1 and F_2 offspring were comparable to the results from the intrastrain crosses.

Comparison of Responses of Parasitoids to Two Tussock Moth Species

In Ibaraki, a mean of 0.65 wasps of *T. euproctidis* was found on virgin females of *E. pseudoconspersa*, and 0.10 on those of *E. taiwana* (Table 2). On the contrary, in Okinawa, a mean of 1.25 wasps was found on virgin females of *E. taiwana*, and 0.15 on those of *E. pseudoconspersa*. These differences in the number of parasitoids on two tussock moth species were statistically significant ($P < 0.05$, t test). All parasitoids found on the moths were female.

TABLE 1. CROSSING EXPERIMENT BETWEEN TWO LOCAL PARASITOIDS PHORETIC ON THE TUSSOCK MOTHS *E. taiwana* AND *E. pseudoconspersa*[a]

Crossing combination	No. of pairs tested	No. of copulated pairs	Percentage of eggs yielding parasitoids	Mean sex ratio[b]	Mean developmental period (*d*)[c]
TW × TW	20	20	98.2	0.13	15.1
TW × PC	20	20	98.5	0.13	15.1
PC × TW	20	19	98.2	0.14	15.1
PC × PC	20	20	98.3	0.14	15.1
F_1(TW × PC)	20	19	99.2	0.13	15.0
F_1(PC × TW)	20	20	97.8	0.14	15.1

[a]TW, parasitoids originated from eggs of *E. taiwana;* PC, parasitoids originated from eggs of *E. pseudoconspersa*. For calculating sex ratio and developmental period, data from uncopulated pairs were excluded.
[b]No. of males/(No. of females + No. of males).
[c]Time from deposition of eggs to adult emergence.

Comparison of Responses of Parasitoids to Two Synthetic Sex Pheromones

In Ibaraki, 1.2 wasps of *T. euproctidis* per trap per day were caught on average with the synthetic sex pheromone of *E. pseudoconspersa*, 10Me14Me-15:iBu, but no wasp was captured in traps baited with the sex pheromone of *E. taiwana*, 16Me-Z9-17:iBu, or blank traps (Table 3). These differences were statistically significant ($P < 0.01$, Tukey's multiple comparison). A similar trend was observed for male moths captured. Many male moths of *E. pseudoconspersa* were caught with 10Me14Me-15:iBu, but no moth was caught in traps baited with 16Me-Z9-17:iBu or blank traps.

TABLE 2. COMPARISON OF RESPONSES OF THE EGG PARASITOID *T. euproctidis* TO CAGED VIRGIN FEMALE TUSSOCK MOTHS, *E. pseudoconspersa* AND *E. taiwana*, IN THE FIELD (IBARAKI, OCTOBER 13–14; OKINAWA, JUNE 15–16, 1995)[a]

Locality	No. of egg parasitoids (mean ± SE) found on	
	E. pseudoconspersa	*E. taiwana*
Ibaraki	0.65 ± 0.22 (20)	0.10 ± 0.07 (20)
Okinawa	0.15 ± 0.11 (20)	1.25 ± 0.41 (20)

[a]Moths were exposed from 1100 for 24 hr. Differences were significant at the 5% level by *t* test. Values in parentheses are the total numbers of moths examined.

TABLE 3. COMPARISON OF ATTRACTIVENESS BETWEEN SYNTHETIC SEX PHEROMONAL COMPONENTS FOR *E. pseudoconspersa* (10Me14Me-15:iBu) AND *E. taiwana* (16Me-Z9-17:iBu) TO THE EGG PARASITOID *T. euproctidis* AND MALE *E. pseudoconspersa* IN THE FIELD (IBARAKI, OCTOBER 17–19, 1995)[a]

Bait	Mean ± SE[b] No. of wasps caught/trap/day	No. of male moths caught/trap/day
10Me14Me-15:iBu[c]	1.2 ± 0.4 a	23 ± 2.3 a
16Me-Z9-17:iBu[d]	0 b	0 b
Blank	0 b	0 b

[a]Data are means of three traps for three days.
[b]Means in a column followed by the same letter are not significantly different at the 1% level (Tukey's multiple comparison).
[c]10,14-Dimethylpentadecyl isobutyrate.
[d](Z)-16-Methyl-9-heptadecenyl isobutyrate.

In Okinawa, a mean of 2.0 wasps per trap per day was caught with 16Me-Z9-17:iBu, but only 0.2 with 10Me14Me-15:iBu, and no wasp with blank traps (Table 4). The differences among the treatments were statistically significant ($P < 0.05$, Tukey's multiple comparison). Many *E. taiwana* male moths were caught in the traps baited with 16Me-Z9-17:iBu, but no moths were captured in traps baited with 10Me14Me-15:iBu or in blank traps.

TABLE 4. COMPARISON OF ATTRACTIVENESS BETWEEN SYNTHETIC SEX PHEROMONAL COMPONENTS FOR *E. pseudoconspersa* (10Me14Me-15:iBu) AND *E. taiwana* (16Me-Z9-17:iBu) TO THE EGG PARASITOID *T. euproctidis* AND MALE *E. taiwana* IN THE FIELD (OKINAWA, JUNE 15–17, 1995)[a]

Bait	Mean ± SE[b] No. of wasps caught/trap/day	No. of male moths caught/trap/day
10Me14Me-15:iBu[c]	0.2 ± 0.2 b	0 b
16Me-Z9-17:iBu[d]	2.0 ± 0.8 a	3.6 ± 1.0 a
Blank	0 b	0 b

[a]Data are means of three traps for three days.
[b]Means in a column followed by the same letter are not significantly different at the 5% level for wasps and at the 1% level for male moths (Tukey's multiple comparison).
[c]10,14-Dimethylpentadecyl isobutyrate.
[d](Z)-16-Methyl-9-heptadecenyl isobutyrate.

DISCUSSION

Phoresy by the parasitoid *T. euproctidis* has been reported on females of two tussock moth species which occupy different geographical ranges in Japan: the one on *E. taiwana* (Arakaki, 1990) is distributed in the Ryukyu Islands, and the one on *E. pseudoconspersa* (Arakaki et al., 1995) is found in Honshu, Shikoku, Kyushu Islands. No overlap occurs in distribution between the two wasp strains or between the two moth species.

According to morphological examination by K. Yamagishi, no differences were found between the two local parasitoids. To clarify the taxonomic status of these wasps, we conducted a crossing experiment between them. All female wasps crossed with males of a different local strain produced female offspring, indicating that mating occurred. Percentages of eggs yielding parasitoids, sex ratios, and developmental rates of the F_1 and F_2 offspring were comparable to those of the intrastrain combinations. Thus, we found no evidence to suggest reproductive isolation between the two local parasitoid strains.

The phoretic egg parasitoid in Okinawa is attracted to females of *E. taiwana* and the synthetic sex pheromone of that moth, 16Me-Z9-17 : iBu (Arakaki et al., 1996). This evidence strongly suggests that *T. euproctidis* uses the sex pheromone of *E. taiwana* to locate host female moths. Likewise, *T. euproctidis* in Ibaraki was attracted to females of *E. pseudoconspersa* and the synthetic sex pheromone of that moth, 10Me14Me-15 : iBu. This also suggests that *T. euproctidis* in Ibaraki uses the sex pheromone of *E. pseudoconspersa* to locate a host female moth.

More egg parasitoids were found on *E. pseudoconspersa* than on *E. taiwana* in Ibaraki, while in Okinawa, more wasps were found on *E. taiwana* than on *E. pseudoconspersa*. Similar responses of the parasitoid to synthetic sex pheromonal components of the two tussock moth species occurred, which indicates that the two populations of wasps can distinguish the sex pheromones of the two allopatric tussock moth species, but they show strong preference for the sex pheromone of the locally occurring host. A small number of *T. euproctidis* wasps also were attracted to virgin females of the other host species or the synthetic sex pheromone of that host. Over a short distance, wasps might use common substances for host location, such as hydrocarbons from the scales of moths which are well-known contact kairomones (e.g., Lewis et al., 1971, 1975; Nordlund et al., 1977).

T. euproctidis is recorded as a natural enemy of four *Euproctis* moths: *E. pseudoconspersa* (Wilcox, 1920; Minamikawa, 1956; Yasumatsu and Watanabe, 1965; Mizuta, 1981), *E. taiwana* (Arakaki, 1990), *E. similis* (Yasumatsu and Watanabe, 1965), and *E. subflava* (Yasumatsu and Watanabe, 1965). *E. similis* and *E. subflava* are distributed in Hokkaido, Honshu, Shikoku, and Kyushu Islands in Japan, and their distribution overlaps with that of *E. pseu-*

doconspersa, except for Hokkaido. Therefore, it is possible that *T. euproctidis* is also phoretic on two additional tussock moth species and uses their sex pheromones as kairomones. The sex pheromone compounds of three tussock moth species, *E. similis* (Yasuda et al., 1994), *E. pseudoconspersa* (Wakamura et al., 1994), and *E. taiwana* (Yasuda et al., 1995), were identified in Japan, with no compound common to their sex pheromones (Yasuda et al., 1995).

If *T. euproctidis* females locate all four tussock moth species using each moth's sex pheromone, how might this situation occur in nature? Two possibilities come to mind: (1) preferences may be genetically fixed, with wasps specialized as a strain in locating each tussock moth, or (2) the parasitoids may switch their preferences in response to seasonal changes in the environment or local abundance of the host species. Many adult parasitoids have the capability of learning, and exhibit great flexibility in their responses to learned environmental cues (Vet and Groenewold, 1990; Turlings et al., 1993; Godfray, 1994). Moreover, some adult parasitoids can learn to associate novel chemicals with the presence of hosts (Vinson et al., 1977; Lewis and Tumlinson, 1988; Lewis and Takasu, 1990).

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THE EFFECT OF VOLATILE METABOLITES OF LIPID PEROXIDATION ON THE AGGREGATION OF REDLEGGED EARTH MITES *Halotydeus destructor* (ACARINA: PENTHALEIDAE) ON DAMAGED COTYLEDONS OF SUBTERRANEAN CLOVER

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Abstract—Redlegged earth mites (*Halotydeus destructor*) aggregated in larger numbers on cotyledons of subterranean clover (*Trifolium subterraneum* L.) previously damaged either by mite feeding or by mechanical injury than on undamaged cotyledons. This effect lasted for up to 7 days. The total volatile fractions derived from crushed cotyledons and its three major components, 2-(*E*)-hexenal, 1-octen-3-ol, and 1-octen-3-one, were tested for their effect on the aggregation of mites. Significantly more mites gathered on detached cotyledons treated with the metabolites at low concentrations than on controls, with 2-(*E*)-hexenal being the most effective. Mites were repelled by higher concentrations of the metabolites and 1-octen-3-one, the most active, killed mites at high concentrations. Fewer mites aggregated on DGI007 (resistant) than on Dalkeith (susceptible) cotyledons treated with droplets of the metabolites. The three volatile metabolites were recovered from the headspace of undamaged and of damaged cotyledons. Crushed cotyledons of Dalkeith produced higher levels of 2-(*E*)-hexenal and lower levels of 1-octen-3-one than undamaged cotyledons. The results suggest that damage-induced metabolites enhance the aggregation of redlegged earth mites at low concentrations and reduce aggregation at high concentrations.

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Key Words—Subterranean clover, *Trifolium subterraneum*, redlegged earth mite, *Halotydeus destructor*, cotyledons, attraction, repellance, lipid peroxidation products, volatiles.

INTRODUCTION

Lipid peroxidation is an immediate response of plant tissues to damage and it has been suggested that it has a role in defense against invading bacteria (Croft et al., 1993), fungi (Zeringue and McCormick, 1989; Ohta et al., 1990; Andersen et al., 1994) and mites (Hildebrand et al., 1986; Kasu et al., 1994; Jiang et al., 1996a). A number of volatile metabolites arising from peroxidation of fatty acids have been identified from different plants (de Lumen et al., 1978; Vick and Zimmerman, 1987; Gardner, 1991; Slusarenko et al., 1991; Hatanaka, 1993). It seems likely that these compounds (damage-induced volatiles) can affect herbivores not only through immediate contact at the feeding site but also at a distance.

Our investigations on the resistance of cotyledons of subterranean clover (subclover, *Trifolium subterraneum* L.) to the redlegged earth mite (*Halotydeus destructor*) indicated that the amount of 1-octen-3-one, one of the damage-induced compounds, is positively correlated with resistance to the mite (Jiang et al., 1996a). Moreover, subclover cotyledons of either susceptible or resistant varieties, after mechanical damage, were found to be preferred by mites to undamaged ones (Jiang and Ridsdill-Smith, 1996).

The aim of the present work was to examine if damage-induced volatile compounds affect mites that did not initiate the damage. The release of volatile compounds from damaged sites of a cotyledon was simulated by presenting the volatile compounds as fine droplets on the upper surfaces of the cotyledons. Both resistant and susceptible varieties were examined in this regard using the total volatile metabolites collected from crushed cotyledons of a susceptible variety and with the three individual compounds placed on undamaged cotyledons.

METHODS AND MATERIALS

Plant Material. Seeds of subclover (variety Dalkeith, susceptible; DGI007, resistant) were sown in pots with standard soil mix (Ridsdill-Smith and Gillespie, 1993). The pots were moved to a glasshouse after seed germination. Seedlings 10–13 days after seeding were used for experiments.

Redlegged Earth Mites. Mites collected from pastures near Perth in winter were used in experiments involving feeding damage to cotyledons and to test the attraction to the damaged cotyledons. For the tests in which volatile com-

pounds were applied to the cotyledons, mites cultured on vetch (cv. Blanchefleur) were used during summer. Mites were staged and young adults, starved for 1 hr in a humid vial (15°C) before experiments, were used.

Damage to the Cotyledons of Dalkeith (Susceptible) and DGI007 (Resistant). Damage was induced either by allowing mites to feed on the cotyledons or by mechanical injury. For testing cotyledons damaged by feeding after 1.5 hr, single cotyledons were detached from a seedling and placed on a moistened sand–loam mixture in a petri dish (diameter, 5.5 cm), adaxial face upward, with 20 mites for 1 hr. Mites were then removed with a compressed air sucker. For testing feeding damaged cotyledons at intervals after damage, 200 mites were released into a pot containing 15 seedlings and allowed to feed on the cotyledons for 18 hr. Mites were then removed with a compressed air sucker. For mechanical damage, a spiral metal screw (with increasing diameter up to 2 mm) was pressed onto the upper part of the cotyledons and each cotyledon was thus damaged in 10 places. Damaged and undamaged seedlings were grown in separate pots in a glasshouse and detached when ready to test at 1.5 hr (feeding damage), 3 hr (mechanical damage), and 1, 4, and 7 days after damage.

Pairwise Choice Tests with Damaged Cotyledons. Cotyledons were cut from the seedlings on the day of each 3 hr experiment. A damaged cotyledon was paired, nearly touching, with an undamaged one on moistened sand–loam in a petri dish (diameter, 5.5 cm). Twenty mites were tapped gently from a small vial into the petri dish, at an equal distance from both cotyledons, and mite numbers on either cotyledon were counted every 20 min in the first hour and then every 30 min afterward for 2 hr (seven observations). Feeding damage to cotyledons was scored at the end of the experiment with a 1–10 rating system (10 to 100% damage to the upper surfaces of a cotyledon). There were 10 replicates of each treatment.

Pairwise Choice Tests with Volatile Compounds. Cotyledons of two varieties (Dalkeith, susceptible; DGI007, resistant) were treated with the volatile fraction collected from the headspace of crushed cotyledons of Dalkeith (susceptible) or with the three individual volatile compounds, 2-(*E*)-hexenal, 1-octen-3-ol, and 1-octen-3-one (Jiang et al., 1996a). To simulate the *in vivo* situation, where there is a mixture of volatile compounds with cell sap, volatile compounds were dissolved in 1% glucose and 5% Tween 80 (a detergent) (Jiang et al., 1996b). Application of a glucose and Tween solution alone to a cotyledon had no effect on the number of mites gathering and/or feeding in choice tests (unpublished). The volatile fraction from Dalkeith cotyledons was prepared at different concentrations, one of which was equal to that estimated to be present *in vivo* (calculated on 94% water content in cotyledons). The lowest concentration tested was 1% and the highest 400% of that in the crushed cotyledons. 2-(*E*)-Hexenal and 1-octen-3-ol, purchased from Sigma Chemical Company, and 1-octen-3-

one, prepared by oxidation of 1-octen-3-ol (Jiang et al., 1996a), were diluted to concentrations of 1, 10, 50, 100, 1000, and 10,000 ppm. The control solution contained no test compound(s). Cotyledons were cut from the petioles of seedlings and the upper surface was treated with the compounds in one of two ways. One single droplet (5 μl) was placed at the basal part of the cotyledon with a syringe. Mites do not feed on this part during the 3-hr test period (Jiang and Ridsdill-Smith, 1996). Alternatively, 5 droplets (0.05 μl each, delivered with a micropipet) were placed on the nonbasal part of a cotyledon, 2 droplets on each side of the cotyledon and 1 droplet in the middle, to simulate the release of volatiles from cotyledons damaged by mites or by mechanical injury. For the choice test, two cotyledons, one treated with a solution of the volatile compound(s) and the other with the control solution, were paired as described above. Twenty mites were released into the petri dish and numbers on each cotyledon counted every 20 min for the first hour and every 30 min after that for 2 hr. There were 10 replicates of each treatment.

Headspace Collection of Volatile Compounds from Cotyledons. The volatile compounds were collected from the headspaces of detached cotyledons (5 g) of the variety Dalkeith (susceptible) after the following treatments: (1) intact cotyledons as a control; (2) cotyledons cut in half (damaged) with a pair of scissors; and (3) cotyledons crushed in liquid nitrogen, as described previously (Jiang et al. 1996a). From cut or intact cotyledons, volatiles were collected for a total of 8 hr since 6 hr gave insufficient material for analysis. Airflow was maintained at a rate of about 500 ml/min except for the intact cotyledons, where the flow rate was doubled.

Gas Chromatography (GC) Analysis. A Hewlett-Packard 5790A GC instrument (BP 1 column; 0.25 μm; 0.22 mm $\times$ 25 m) equipped with FID (H_2, 1.5 ml/min; 40°C for 5 min and then programmed to 250°C at 20°C/min) was used (Jiang et al., 1996a). The quantity of the volatiles collected from different treatments was estimated after correction for airflow rate, on the assumption that there was a linear relationship between production and collection of volatiles.

Data Analyses. Mite preference in the choice test for each treatment was calculated from the average of seven observations as follows: [(mite number on treatment $-$ mite number on control)/total number of mites] $\times$ 100. Mite preference at 20 min and the average of seven observations during 3 hr is presented. Differences between means were examined with paired t tests. In the experiments in which cotyledons were damaged by mite feeding, a considerable amount of the upper surface had been consumed and new mites had a restricted feeding area. Mite numbers on these cotyledons peaked at 1.5 hr after commencing the choice test and then declined. Average mite preference for these tests was calculated with the data obtained within 1.5 hr (four observations).

RESULTS

Enhanced Attraction of Mites to Damaged Cotyledons. Significantly more mites gathered on cotyledons of both susceptible Dalkeith and resistant DGI007, damaged either by feeding mites or mechanical injury, than on undamaged counterparts (Table 1). Mites readily distinguished between the damaged and the undamaged cotyledons within 20 min. The preference for damaged cotyledons was maintained for up to 4 days for Dalkeith and 7 days for DGI007. Scores of mite feeding were higher, especially for the mechanically damaged cotyledons, than for the undamaged counterparts (not shown).

Enhanced Attraction of Cotyledons Treated with Volatile Compounds. A greater number of mites gathered on cotyledons treated with 5 droplets of the volatile fraction from Dalkeith (susceptible) at concentrations equal to (on DGI007, resistant) or four times higher (on Dalkeith, susceptible) than that occurring in a crushed cotyledon, but not at lower concentrations of the compounds (Table 2). The effect was not apparent after 20 min.

After treatment with 5 scattered droplets of the three individual volatile compounds, significantly more mites gathered on the cotyledons of Dalkeith (susceptible) treated with between 10 and 1000 ppm of 2-(*E*)-hexenal or 1 ppm of either 1-octen-3-ol or 1-octen-3-one over 3 hr (Table 3). The average mite preference was significant for 2-(*E*)-hexenal at 50 and 1000 ppm and for 1-octen-3-one at 1 ppm. At 10,000 ppm, 2-(*E*)-hexenal was deterrent at 20 min and

TABLE 1. MITE PREFERENCE (%) FOR COTYLEDONS IN CHOICE TESTS OF DALKEITH (SUSCEPTIBLE; sus) AND DGI007 (RESISTANT; res), DAMAGED EITHER BY MITE FEEDING OR MECHANICAL INJURY, OVER TIME (1.5 HR–7 DAYS) COMPARED TO CONTROLS[a]

	Mite preference				
Treatments	1.5 hr	3 hr	1 day	4 days	7 days
Dalkeith (sus)					
Feeding damage	46.8**		37.5***	30.4	−0.7
Mechanical injury		28.2*	20.4*	23.8**	4.0
DGI007 (res)					
Feeding damage	35.1*		52.7**	40.7*	37.3*
Mechanical injury		16.5	21.2	17.8	17.2

[a]Negative values indicate mite preference for the nondamaged cotyledons. Mite numbers were counted seven times over 3 hr in all treatments except those using cotyledons damaged by mites feeding where mite numbers were counted over 1.5 hr. Significance is indicated at levels of 0.05 (*), 0.01 (**), and 0.001 (***).

TABLE 2. MITE PREFERENCE FOR THE COTYLEDONS OF DALKEITH (SUSCEPTIBLE) AND DGI007 (RESISTANT) TREATED WITH 5 DROPLETS (EACH 0.05 μl; MICROPIPET) OF VOLATILE COMPOUNDS COLLECTED FROM CRUSHED DALKEITH COTYLEDONS[a]

Treatment and conc. (%)	Mite preference (%) at 20 min	AMN ± SE during 3 hr, treatment/ control	Average mite preference (%) over 3 hr
Dalkeith			
1	21.9	6.6 ± 0.9/5.4 ± 0.3	10.0
10	7.7	5.9 ± 0.5/4.8 ± 0.2	10.3
100	5.6	7.4 ± 0.8/5.9 ± 1.0	11.3
400	−4.5	7.9 ± 0.6/5.5 ± 0.3	17.9*
DGI007			
1	−33.3	4.8 ± 0.3/5.3 ± 0.1	−4.9
10	−12.8	4.6 ± 0.2/5.1 ± 0.5	−0.5
100	18.3	4.6 ± 0.6/2.0 ± 0.4	39.4*
400	15.1	3.7 ± 0.8/2.5 ± 0.6	19.4

[a]Concentration applied was a percentage of that *in vivo* produced by crushed cotyledons of Dalkeith. AMN, average mite numbers from seven observations over 3 hr. Significance is indicated at a level of 0.05 (*).

became ineffective at longer times, 1-octen-3-ol showed no effect, and 1-octen-3-one deterred mites from gathering throughout the 3 hr.

With similar treatment, cotyledons of DGI007 (resistant) showed a distinctly different effect on mites from those of Dalkeith (susceptible). From 1 to 1000 ppm, 2-(*E*)-hexenal conferred no significant attraction to cotyledons, although relatively higher mite numbers occurred on treated cotyledons for 3 hr at 100 ppm (Table 4). A similar effect occurred with 1-octen-3-ol at 1 and 50 ppm and with 1-octen-3-one at 1 and 10 ppm. At 1000 ppm, 1-octen-3-ol rendered cotyledons significantly deterrent at 20 min and 1-octen-3-one throughout 3 hr.

When each compound was applied as a single droplet (5 μl) on the base of a cotyledon of Dalkeith (susceptible), 2-(*E*)-hexenal showed a clear concentration-dependent effect (Table 5), with reduced attraction at 1000 and 10,000 ppm. The effect was significant at 20 min. 1-Octen-3-ol had no effect at and below 1,000 ppm but repelled mites at 10,000 ppm. In both treatments with 2-(*E*)-hexenal and 1-octen-3-ol at 10,000 ppm, no mites gathered on either treated or untreated (neighboring) cotyledons at 20 min. 1-Octen-3-one showed attractive effects at 10 ppm at 20 min, deterred mites at 1000 ppm, and killed all mites in the petri dishes at 10,000 ppm within 20 min.

Comparison of Volatile Compounds from Cotyledons. The volatile fraction collected from the headspace of crushed cotyledons consisted mainly of five

TABLE 3. MITE PREFERENCE FOR THE COTYLEDONS OF DALKEITH (SUSCEPTIBLE) TREATED WITH 5 DROPLETS (EACH 0.05 μl; MICROPIPET) OF 2-(*E*)-HEXENAL, 1-OCTEN-3-OL, AND 1-OCTEN-3-ONE AT SIX CONCENTRATIONS[a]

Treatment and conc. (ppm)	Mite preference (%) at 20 min	AMN ± SE over 3 hr, treatment/control	Average mite preference (%) over 3 hr
2-(*E*)-Hexenal			
1	19.6	7.5 ± 1.3/5.4 ± 1.1	16.3
10	39.3*	7.2 ± 1.1/3.8 ± 1.0	32.1
50	3.8	5.2 ± 0.8/2.7 ± 0.9	25.3*
100	35.1	4.9 ± 1.2/3.0 ± 0.8	24.1
1000	32.1*	6.4 ± 0.9/3.4 ± 0.5	30.6*
10,000	−36.4**	4.4 ± 0.9/3.9 ± 0.8	6.0
1-Octen-3-ol			
1	29.8*	5.0 ± 1.1/3.2 ± 1.0	22.0
10	27.3	6.0 ± 0.8/4.9 ± 0.8	10.1
50	−15.0	6.0 ± 1.1/5.4 ± 0.9	4.6
100	−1.7	5.6 ± 0.7/4.4 ± 0.5	12.0
1000	2.0	5.3 ± 0.6/3.6 ± 0.7	19.1
10,000	14.3	5.3 ± 0.6/6.6 ± 0.9	−10.9
1-Octen-3-one			
1	40.6**	8.8 ± 0.8/2.8 ± 0.5	51.7***
10	−20.0	5.8 ± 0.9/4.0 ± 0.6	18.4
50	26.3	4.9 ± 0.7/5.5 ± 0.8	−5.8
100	21.3	5.1 ± 0.3/4.5 ± 0.4	0.63
1000	3.7	5.0 ± 0.5/4.6 ± 0.7	4.2
10,000	−24.4	2.0 ± 0.5/5.6 ± 1.2	−47.3*

[a]Negative values indicate mite preference for the control. AMN, average mite numbers from seven observations over 3 hr. Significance is indicated at levels of 0.05 (*), 0.01 (**), and 0.001 (***).

compounds, of which 2-(*E*)-hexenal, 1-octen-3-ol, and 1-octen-3-one accounted for more than 77%. These three compounds were also detected in the headspace of intact cotyledons and cotyledons cut in half, but two more compounds were detected from the latter. The proportions of different compounds in different treatments are presented in Table 6, showing that 2-(*E*)-hexenal was the major compound produced by crushed cotyledons and 1-octen-3-ol by intact cotyledons and cotyledons cut in half.

DISCUSSION

Damage caused by either mite feeding or mechanical injury of subclover cotyledons enhanced the preference of these to new mites for up to seven days. GC analysis indicated that volatile metabolites, including 2-(*E*)-hexenal in par-

TABLE 4. MITE PREFERENCE FOR THE COTYLEDONS OF DGI007 (RESISTANT) TREATED WITH 5 DROPLETS (EACH 0.05 μl; MICROPIPET) OF 2-(*E*)-HEXENAL, 1-OCTEN-3-OL, AND 1-OCTEN-3-ONE AT FIVE CONCENTRATIONS[a]

Treatment and conc. (ppm)	Mite preference (%) at 20 min	AMN ± SE during 3 hr, treatment/control	Average mite preference (%) over 3 hr
2-(*E*)-Hexenal			
1	−7.5	5.2 ± 1.0/4.8 ± 1.1	4.0
10	2.9	3.0 ± 0.2/2.9 ± 0.7	1.7
50	−11.4	3.3 ± 0.6/3.6 ± 0.6	−4.3
100	18.3	5.0 ± 0.7/3.6 ± 0.7	16.3
1000	−18.8	3.8 ± 0.4/3.9 ± 0.5	−1.3
1-Octen-3-ol			
1	21.8	4.9 ± 0.6/3.6 ± 0.6	15.3
10	−4.2	3.8 ± 0.7/4.1 ± 0.6	−3.8
50	15.5	4.5 ± 0.9/2.8 ± 0.4	23.3
100	−35.7	3.9 ± 0.6/3.6 ± 0.3	4.0
1000	−34.6*	4.0 ± 0.4/4.0 ± 0.3	0.0
1-Octen-3-one			
1	5.7	3.8 ± 0.6/2.9 ± 0.3	13.4
10	20.0	4.7 ± 0.8/3.2 ± 0.7	19.0
50	0.0	3.0 ± 0.6/4.3 ± 0.4	−17.8
100	12.5	3.7 ± 0.3/3.6 ± 0.5	1.4
1000	−26.1	2.4 ± 0.4/4.4 ± 0.5	−29.4**

[a]Negative values indicate mite preference for the control cotyledons. AMN, average mite numbers from seven observations over 3 hr. Significance is indicated at levels of 0.05 (*) and 0.01 (**).

ticular, were produced from damaged cotyledons. When these volatile metabolites were applied to susceptible and resistant cotyledons at concentrations equal to, or higher than, the *in vivo* level found in crushed Dalkeith (susceptible), more mites gathered on the treated cotyledons. The three dominant metabolites, 2-(*E*)-hexenal, 1-octen-3-ol, and 1-octen-3-one, placed at low concentrations as small droplets on Dalkeith (susceptible) cotyledons favored the gathering of mites over 3 hr. This suggests that the susceptibility of a subclover variety can be increased by increasing the concentration of these compounds. However, too high a concentration of 1-octen-3-ol and 1-octen-3-one deterred the mites from aggregating. On the other hand, increasing the amounts of these compounds available in a resistant species (DGI007) had no significant effect until high levels of 1-octen-3-one were achieved.

In tests in which a single large droplet of each of the three metabolites was added to the basal part of cotyledons of the Dalkeith variety, low concentrations (< 100 ppm) had little or no effect, but higher concentrations deterred aggre-

TABLE 5. MITE PREFERENCE FOR COTYLEDONS OF DALKEITH (SUSCEPTIBLE) TREATED ON THE BASE WITH 1 DROPLET (5 μl; MICROPIPET) OF 2-(*E*)-HEXENAL, 1-OCTEN-3-OL, AND 1-OCTEN-3-ONE AT FOUR CONCENTRATIONS[a]

Treatment and conc. (ppm)	Mite preference (%) at 20 min	AMN ± SE over 3 hr, treatment/control	Average mite preference (%) over 3 hr
2-(*E*)-Hexenal			
10	24.1*	8.3 ± 0.7/4.8 ± 0.8	26.0*
100	40.0*	8.4 ± 0.5/5.4 ± 0.5	21.7**
1000	−40.7*	3.7 ± 0.6/5.9 ± 0.8	−22.9
10,000	No mites	1.2 ± 0.2/3.1 ± 0.9	−44.2*
1-Octen-3-ol			
10	7.0	6.2 ± 0.5/6.3 ± 0.6	−0.8
100	−10.6	6.5 ± 0.6/5.7 ± 0.5	6.5
1000	−12.7	5.6 ± 0.6/5.7 ± 0.7	−0.9
10,000	No mites	1.0 ± 0.5/3.1 ± 1.0	−51.2*
1-Octen-3-one			
10	42.9**	4.7 ± 0.2/4.6 ± 0.6	1.1
100	−6.0	4.5 ± 0.3/5.3 ± 0.6	−8.1
1000	−31.7*	2.2 ± 0.7/6.7 ± 1.2	−50.6*
10,000		All mites dead at 20 min	

[a]Negative values indicate mite preference for the control. AMN, average mite numbers from seven observations over 3 hr. Significance is indicated at levels of 0.05 (*) and 0.01 (**).

gation of the mites on the treated cotyledons. The most dramatic effect was observed with 1-octen-3-one, which strongly deterred mites at 1000 ppm and killed mites at 10,000 ppm. The treatment was such that high local concentrations of the metabolites were achieved, and increasing the level of these metabolites resulted in a susceptible variety becoming a resistant one.

TABLE 6. PROPORTION (%) OF 2-(*E*)-HEXENAL, 1-OCTEN-3-OL, AND 1-OCTEN-3-ONE IN THE TOTAL VOLATILE METABOLITES COLLECTED FROM THE HEADSPACE OF DETACHED DALKEITH COTYLEDONS WITH OR WITHOUT DAMAGE (CUTTING AND CRUSHING)[a]

Treatment	2-(*E*)-Hexenal	1-Octen-3-ol	1-Octen-3-one	Others
Intact	4.3	39.5	11.0	45.2
Cut in half	4.2	10.3	3.4	82.1
Crushed	>60.0	14.0	3.0	<23.0

[a]Volatiles from intact and cut-in-half cotyledons were collected over 8 hr, and those from crushed cotyledons over 6 hr.

These results are in agreement with previous observations. The crushed cotyledons of the DGI007 variety (resistant) produce higher amounts of 2-(*E*)-hexenal, 1-octen-3-ol, and 1-octen-3-one than those of Dalkeith (susceptible) (Jiang et al., 1996a). Of the three volatile metabolites, 1-octen-3-one induced the highest repellence, consistent with previous studies showing that cotyledon resistance to these mites is correlated with production of 1-octen-3-one (Jiang et al., 1996a). When this metabolite was presented in a membrane sachet, mites were deterred by high concentrations but attracted by low concentrations.

The picture that is emerging is as follows. Mites engaged in feeding probably trigger the production of damage-induced volatile compounds. In susceptible varieties, the level of these compounds is such that they enhance aggregation of mites including those in the vicinity not initially involved in feeding. In resistant varieties, the increased production of 1-octen-3-one has a deterrent effect, particularly to mites directly involved in feeding who face the highest concentration of metabolites. On the other hand, these compounds emanating from damaged cells produce a concentration gradient and may attract mites from some distance away. This would explain the observation that more mites aggregate on damaged cotyledons of the resistant variety DGI007 than on undamaged ones. In drawing these conclusions, it is important to realize that host factors, such as cotyledon toughness of DGI007, could also play a role in reducing the number of mites aggregating (Jiang and Ridsdill-Smith, 1996).

The production of C_6 and C_8 volatile metabolites as a result of lipid peroxidation induced by damage to plant tissues is well-known (de Lumen et al., 1978; Hatanaka, 1993). Since the three volatile metabolites identified here were also detected in the headspace of intact cotyledons, this suggests that the detached cotyledons were slightly damaged by biotic or abiotic factors, or that the cotyledons emitted some volatiles through the stomata. 1-Octen-3-ol and 1-octen-3-one were dominant in the headspace of intact cotyledons. It is possible that both metabolites are produced more readily than 2-(*E*)-hexenal when the cotyledons are slightly damaged, and it is tempting to suggest that C_8 compounds are produced mainly in epidermal cells and C_6 compounds in subepidermal cells. Enhanced production of lipid peroxidation products including hexenal, hexenol, octenol, and hexenyl acetate has been reported for a number of plants after damage by spider mites (Hildebrand et al., 1986; Dicke et al., 1990; Dicke and Sabelis, 1992; Kasu et al., 1994) and insects (Blaakmeer et al., 1994). For the redlegged earth mite, preference for cotyledons after mechanical damage is comparable to preference for those with mite feeding damage, suggesting that both treatments initiated similar plant responses in terms of production of volatile compounds.

A selection pressure for evolution of damage-induced volatile compounds by plants may have been invasion by either herbivores or pathogens (Croft et al., 1993; Kasu et al., 1994; Jiang et al., 1996a) whose attacks induced the

release of volatiles by peroxidation of fatty acids. If this is so, the volatile-based effect of damaged plant tissues attracting the redlegged earth mite can be regarded as an adaptation of the mite to the defensive responses of the host plant, except for cases of varieties which can produce high amounts of the compounds, thus effectively showing resistance by repelling the mites.

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CHEMOCOMMUNICATION IN *Phyllonorycter ulmifoliella* (HBN.) (LEPIDOPTERA: GRACILLARIIDAE): PERIODICITY, SEX PHEROMONE, AND INHIBITORS

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Abstract—(*Z*)-10-Tetradecenyl acetate (Z10-14:OAc) from abdominal tip extracts of virgin females of the tentiform leafminer moth *Phyllonorycter ulmifoliella* (Lepidoptera: Gracillariidae) was identified by gas chromatography and mass spectrometry. The biological activity of the component was confirmed by field tests with synthetic compounds. As a sex pheromone component this ester is novel both in the family Gracillariidae and in the superfamily Gracillarioidea. Field trapping of *P. ulmifoliella* with synthetic Z10-14:OAc at dosages of 1 and 0.2 mg/dispenser led to catches of approximately 9000 and 3000 male moths, respectively. The attractivity of the Z10-14:OAc was strongly inhibited by a 10% admixture of either (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), (*E*)-9-tetradecenyl acetate (E9-14:OAc), or (*E*)-11-tetradecenyl acetate (E11-14:OAc). Addition of 10% (*E*)-10-tetradecenyl acetate (E10-14:OAc) to the sex pheromone reduced attractivity, but significantly less than the inhibitors previously mentioned. The pheromone releasing (or "calling") behavior of virgin *P. ulmifoliella* females was recorded under laboratory conditions. Calling activity started about half an hour before lights-on and the maximum number of calling females was registered half an hour after the start of photophase. A high level of pheromone releasing activity lasted for about 2 hr and ceased about 5 hr after the start of photophase. Chemocommunication activity in the light period of day is assumed to be an adaptation which allows this phyllonoryctid to avoid inhibitors emitted as pheromones by many other species. A scheme of probable interactions by means of semiochemicals between *P. ulmifoliella* and other lepidopterans is

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presented and the appearance of Z10-14:OAc as a sex pheromone component in Lepidoptera during evolution of the order is discussed.

Key Words—*Phyllonorycter ulmifoliella*, Lithocolletinae, (*Z*)-9-tetradecenyl acetate, (*E*)-9-tetradecenyl acetate, (*Z*)-10-tetradecenyl acetate, (*E*)-10-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, field trapping, pheromone identification, calling behavior.

INTRODUCTION

Most of the tentiform leafminer moths of the genus *Phyllonorycter* are monophagous or oligophagous insects. Individuals of a few species of the genus *Phyllonorycter* quite often cooccur on the same host plant, and competition between them is highly probable. Differences in form, color, and location of a mine on a host-plant leaf make it easy to identify the moths before adult emergence and to collect a single species for pheromone identification by convenient methods of chemical analysis. These peculiarities make the moths convenient objects for investigation of interspecific interactions including those mediated by semiochemicals in adults.

About 150 species of the genus *Phyllonorycter* are known to occur in the European part of the Palaearctic region (Medvedev, 1981) and over 1000 species have been found in the world (Kuznetzov, 1979). Only a few of these species are important pests in orchards and recreation parks. Despite the abundance of species within the genus, the sex pheromone has hitherto been identified for only one of them. A binary mixture of (*E*4,*E*10)-4,10-dodecadienyl acetate (E4,E10-12:OAc) and (*E*)-10-dodecenyl acetate (E10-12:OAc) has been proved to be the sex pheromone of *P. mespilella* (Hubner) (Gries et al., 1993). It is obvious that data in this field are very scarce. This induced us to start an investigation of the chemical communication systems in phyllonoryctids, beginning with *P. ulmifoliella*. This species is not a pest of economic importance. However, research on pest species alone will hardly allow a closer approach to the solution of chemoecological, evolutionary, or chemotaxonomic problems.

P. ulmifoliella is one of very few polyphagous gracillariids. It feeds on plants of a few families which belong to two different orders (Kuznetzov, 1975). In Lithuania *P. ulmifoliella* is narrowly oligophagous, feeding on plants of the genus *Betula* only (Ivinskis et al., 1985). As this leafminer and its host plants are widely spread in different habitats throughout the Palaearctic region (Medvedev, 1981), the moths may interact with many other species by means of semiochemicals.

In this paper we present data on the periodicity of pheromone emission in *P. ulmifoliella* females, on the identification of their main pheromone component, and on field tests of the activity of this compound as well as some closely related synthetic substances, including binary mixtures.

METHODS AND MATERIALS

Insects. Leaves containing *P. ulmifoliella* mines were collected from birch trees, *Betula pendula* Roth., near Vilnius (Eastern Lithuania) in November 1992, just before the shedding of leaves. The mined leaves collected were placed in wooden boxes on a 6- to 8-cm layer of a moistened peat moss. The upper parts of the boxes were covered with plastic film. Small ventilation gaps were made in the walls on two sides of each box. The peat moss was wetted regularly. The boxes with the leaves were kept outdoors during the winter. In the spring the mined leaves were placed in vials of 32-cm^3 volume (three mines per vial) and were held at a temperature of 17 $\pm$ 2°C during scotophases and 22 $\pm$ 2°C during photophases. The light : dark regime was 16 : 8 hr/day. Following emergence, adults were collected daily immediately after the onset of photophase and were then sexed and placed in individual holding vials provided with a solution of 5% sugar in water.

Periodicity of Pheromone-Releasing or "Calling" Behavior of Females. To determine the daily periodicity as well as the time and duration of maximum calling activity, 18 females were transferred to individual vials with one end covered with a net of mill-silk and the other with a sponge. Observation and registration of the behavior were started at the beginning of the first photophase after adult emergence. The registration lasted for a period of 4 days under the same light/dark cycle and temperature conditions as mentioned above. The behavior was registered half-hourly during the last scotophase hour and in the photophase until the females stopped calling. During the resting period, observations were made hourly. In the scotophase the observations were made under red light with an electric incandescent bulb of 40 W at a height of about 2.5 m above the experimental vials.

Extraction of a Pheromone. Within 2 hr after the onset of calling, the females (2–4 days old) were placed in a refrigerator for 10 min to become inactivated. The abdominal tip containing the sex pheromone gland was everted under mechanical pressure, excised, and washed twice with 10 μl of pentane (Merck p.a.) for 15 min. The solution was removed with a syringe, combined, concentrated to approximately 10 μl, and stored at -14°C. In total 128 calling females were used for extraction.

Chemical Analysis. GC analyses of the pheromone gland extract were conducted on a GCV PYE Unicam instrument modified for capillary gas chromatography with a split injector (180°C), a flame ionization detector (FID; 220°C), and a DB Wax J&W fused silica capillary column (30 m $\times$ 0.25-mm ID, 0.25-μm film thickness). Helium was used as the carrier gas, with an inlet pressure of 110 kPa. The temperature program was 60°C for 4 min, followed by 4°C/min to 180°C, and maintained at 180°C thereafter. A Finnigan 4500 MS instrument connected to a Varian 3400 gas chromatograph was used for the

GC–MS analyses. It was operated with a split/splitless injector (180°C), splitless mode for 30 sec, and with a DB-1 capillary column (30 m × 0.25-mm ID, 0.25-μm film thickness). The temperature program was 40°C for 4 min, followed by 4°C/min to 200°C, and maintained at 200°C thereafter. Helium was used as the carrier gas, with an inlet pressure of 110 kPa. Electron impact (EI) mass spectra were determined at 70 eV with the ion source at 150°C.

The structure of the compound, extracted from the female sex pheromone glands, was identified by comparison of its mass spectral data and GC retention times with those of authentic standards.

Field Tests. All compounds used in the field tests were obtained from Tartu University (Estonia). Their isomeric and chemical purities were determined by GC and are presented in Table 1. The compounds were tested alone and/or in binary mixtures at the ratios 10:1, 1:1, and 1:10 (see Table 2) under field conditions. Each compound and each mixture were dissolved in hexane (Merck p.a.) and impregnated into a red rubber tube dispenser (18 × 15 mm) at a dose

TABLE 1. PURITIES OF SYNTHETIC COMPOUNDS USED IN THE FIELD TESTS

Compound	Isomeric purity (%)	Total purity (%)
	Summer 1993	
*E*10-12:OH	>99	91
*E*10-12:OH	>99	90
*Z*10-12:OAc	>99	93
*Z*10-12:OH	>99	94
*E*9-14:OAc	>99	>99
*Z*10-14:OAc	>99	91
*E*10-14:OH	>99	80
	Summer 1994	
*Z*5-14:OAc	>99	98
*Z*5-14:OH	>99	99
*Z*7-14:OAc	>99	99
*Z*9-14:OAc	>99	>99
*Z*9-14:OH	>99	>99
*E*9-14:OAc	>99	>99
*Z*10-14:OAc	>99	98
*E*10-14:OAc	>99	97
*E*10-14:OH	>99	>99
*Z*11-14:OAc	>99	>99
*Z*11-14:OH	>99	96
*E*11-14:OAc	>99	97
*E*11-14:OH	>99	>99

TABLE 2. ATTRACTION OF *Phyllonorycter ulmifoliella* MALES TO SYNTHETIC COMPOUNDS UNDER FIELD CONDITIONS AT FOUR LOCALITIES[a]

		Number of males trapped			
		1993			1994
Compound(s)	Ratio	Locality I	Locality II	Locality III	Locality IV
Z10-12:OAc		1 a	—	—	—
*E*10-12:OAc		0 a	0 a	—	—
*E*10-12:OH		10 a	0 a	—	—
Z5-14:OAc		—	—	—	0 a
Z5-14:OH		—	—	—	0 a
Z7-14:OAc		—	—	—	0 a
Z9-14:OAc		—	—	—	0 a
Z9-14:OH		—	—	—	0 a
*E*9-14:OAc		—	0 a	0 a	0 a
Z10-14:0Ac		3956 b	4009 c	1079 c	441 f
*E*10-14:OAc		—	—	—	0 a
*E*10-14:OH		—	0 a	—	0 a
Z11-14:OAc		—	—	—	0 a
Z11-14:OH		—	—	—	0 a
*E*11-14:OAc		—	—	—	0 a
*E*11-14:OH		—	—	—	0 a
*E*10-12:OAc/*E*10-12:OH	10:1	2 a	—	—	—
*E*10-12:OAc/Z10-12:OH	1:1	28 a	—	—	—
*E*10-12:OAc/*E*10-14:OH	1:10	29 a	—	—	—
Z9-14:OAc/Z9-14:OH	10:1	—	—	—	0 a
Z9-14:OAc/Z9-14:OH	1:1	—	—	—	1 a
Z9-14OAc/Z9-14:OH	1:10	—	—	—	0 a
Z9-14:OAc/*E*9-14:Ac	10:1	—	—	—	29 abc
Z9-14:OAc/*E*9-14:Ac	1:1	—	—	—	5 ab
Z9-14:OAc/*E*9-14:Ac	1:10	—	—	—	3 a
Z10-14:OAc/Z9-14:OAc	10:1	—	—	—	64 c
Z10-14:OAc/Z9-14:OAc	1:1	—	—	—	28 abc
Z10-14:OAc/Z9-14:OAc	1:10	—	—	—	3 a
Z10-14:OAc/Z9-14:OH	10:1	—	—	—	372 ef
Z10-14:OAc/Z9-14:OH	1:1	—	—	—	401 ef
Z10-14:OAc/Z9-14:OH	1:10	—	—	—	27 abc
Z10-14:OAc/*E*9-14:OAc	10:1	—	36 b	52 b	69 cd
Z10-14:OAc/*E*9-14:OAc	1:1	—	7 a	8 a	6 ab
Z10-14:OAc/*E*9-14:OAc	1:10	—	1 a	0 a	3 a
Z10-14:OAc/*E*10-14:OAc	10:1	—	—	—	349 e
Z10-14:OAc/*E*10-14:OAc	1:1	—	—	—	15 ab
Z10-14:OAc/*E*10-14:OAc	1:10	—	—	—	0 a
Z10-14:OAc/*E*10-14:OH	10:1	—	29 ab	—	465 f
Z10-14:OAc/*E*10-14:OH	1:1	—	—	—	156 d
Z10-14:OAc/*E*10-14:OH	1:10	—	—	—	33 bc
Z10-14:OAc/Z11-14:OAc	10:1	—	—	—	354 ef

TABLE 2. CONTINUED

Compound(s)	Ratio	Number of males trapped			
		1993			1994
		Locality I	Locality II	Locality III	Locality IV
*Z*10-14:OAc/*Z*11-14:OAc	1:1	—	—	—	45 bc
*Z*10-14:OAc/*Z*11-14:OAc	1:10	—	—	—	3 a
*Z*10-14:OAc/*E*11-14:OAc	10:1	—	—	—	41 bc
*Z*10-14:OAc/*E*11-14:OAc	1:1	—	—	—	30 bc
*Z*10-14:OAc/*E*11-14:OAc	1:10	—	—	—	2 a
*E*10-14:OAc/*Z*9-14:OAc	10:1	—	—	—	1 a
*E*10-14:OAc/*Z*9-14:OAc	1:1	—	—	—	0 a
*E*10-14:OAc/*Z*9-14:OAc	1:10	—	—	—	0 a
*E*10-14:OAc/*Z*9-14:OH	10:1	—	—	—	0 a
*E*10-14:OAc/*Z*9-14:OH	1:1	—	—	—	0 a
*E*10-14:OAc/*Z*9-14:OH	1:10	—	—	—	0 a
*E*10-14:OAc/*E*9-14:OAc	10:1	—	—	—	0 a
*E*10-14:OAc/*E*9-14:OAc	1:1	—	—	—	0 a
*E*10-14:OAc/*E*9-14:OAc	1:10	—	—	—	2 a
*E*10-14:OAc/*E*10-14:OH	10:1	—	—	—	0 a
*E*10-14:OAc/*E*10-14:OH	1:1	—	—	—	0 a
*E*10-14:OAc/*E*10-14:OH	1:10	—	—	—	0 a
*E*10-14:OAc/*Z*11-14:OAc	10:1	—	—	—	0 a
*E*10-14:OAc/*Z*11-14:OAc	1:1	—	—	—	0 a
*E*10-14:OAc/*Z*11-14:OAc	1:10	—	—	—	0 a
*E*10-14:OAc/*E*11-14:OAc	10:1	—	—	—	0 a
*E*10-14:OAc/*E*11-14:OAc	1:1	—	—	—	0 a
*E*10-14:OAc/*E*11-14:OAc	1:10	—	—	—	0 a
Control		3 a	0 a	0 a	2 a
Total		4029	4082	1139	2950

[a]Each figure denotes the total number of moths trapped by the lure indicated. If numbers in the same column are followed by different lowercase letters, the difference between them is statistically significant at $P < 0.05$. (—) Not tested.

of 1 and 0.2 mg/dispenser for the 1993 and 1994 tests, respectively. Each lure was fixed in an opaque white delta trap, measuring 10 × 11 × 10 cm, which contained an exchangeable bottom (11 × 18 cm) that was coated with sticky material (Atracon A trap and Pestifix glue, both from Flora Co., Tartu, Estonia). The traps were fixed on shrub or tree branches 1.5 to 2.5 m above the ground and were inspected and rotated within each replication every 2 days. The distance between the traps was at least 25 m.

The field tests were carried out in the vicinity of Vilnius (Eastern Lithuania)

at the following four localities. (I) July 2 to August 9, 1993. Four replications of each compound and mixture listed in Table 2 were tested. The traps were placed on mountain ashes (*Sorbus aucuparia* L.) in a mixed forest. (II) July 20 to August 23, 1993. Three replications of each compound and mixture mentioned in Table 2 were tested. The traps were fixed to branches of goat willows (*Salix caprea* L.) at the edge of a mixed forest, bordering on a rye field. (III) August 9 to August 23, 1993. This test included three replications of each compound and mixture listed in Table 2. The traps were fixed on branches of alder trees (*Alnus incana* Mnch.) at the edge of a mixed forest, next to a meadow. (IV) July 20 to August 17, 1994. Three replications of each compound and mixture listed in Table 2 were deployed with traps fixed 2–2.5 m above the ground on the branches of different shrubs and trees close to birch trees in a mixed forest.

Identification of the Species. The host-plant species was identified by the key from the Lithuanian flora (Natkevičaitė-Ivanauskienė, 1976).

Moths captured were identified by analysis of their wing pattern. When both wings were covered with sticky material, the moths were rinsed in hexane before identification. In case a wing pattern was erased, the moths were identified by analysis of their genitalia (Medvedev, 1981). Representative specimens are kept in the insect collection at the Institute of Ecology, Vilnius, Lithuania.

Statistical Analysis. Data from the field tests were transformed by the formula $(x + 1)^{0.5}$, where x is the number of moths captured per trap. The values thus obtained were analyzed by ANOVA, and Duncan's multiple-range test was used to identify significantly different mean values.

RESULTS

Pheromone-Releasing or "Calling" Behavior of Females. The following features of the calling posture of virgin *P. ulmifoliella* females are characteristic: the abdominal tip is curved dorsally, with the distal segments telescopically protruded and the pheromonal gland exposed. The wings are slightly spread and the antennae are mostly cuddled to the wings.

Periodicity of Chemocommunication. P. ulmifoliella females start their pheromone-releasing activity on the second day after emergence, at the very beginning of a photophase (Figure 1). Less than 10% of the females started calling before the light was on. The peak of the pheromone-releasing behavior was registered half an hour after the beginning of photophase and the high level of the activity was maintained for about 2 hr. The daily calling period of the females lasted about 5 hr altogether (Figure 1).

Chemical Analysis of the Extract from the Sex Pheromone Glands. After a systematic search by GC–MS, using the key ion fragments for acetates *m/z* 61,

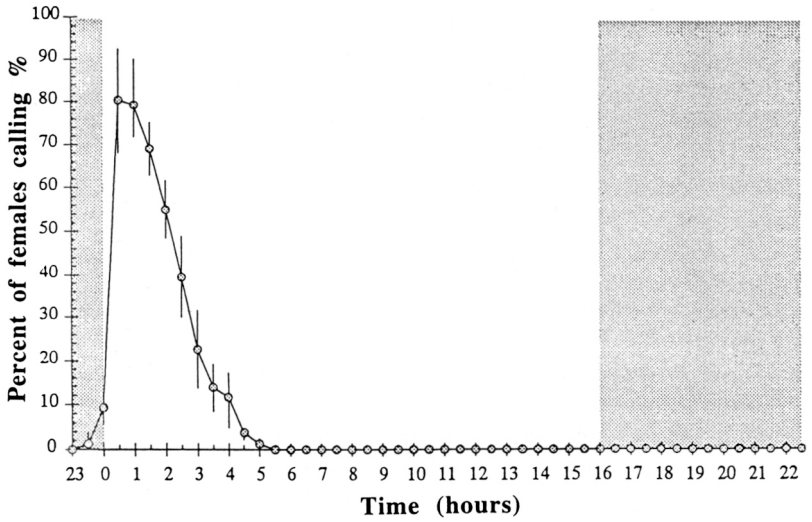

FIG. 1. Daily periodicity of the calling of *Phyllonorycter ulmifoliella* females under laboratory conditions. The behavior of 18 virgin females was recorded during a period of 4 days. The females were not found "calling" during the first day after emergence. One-day results were counted as single tests starting from the second day after emergence. Vertical bars represent standard deviations (SD). Shaded areas indicate scotophases.

alcohols *m/z* 31, and unsaturations *m/z* 82 and *m/z* 96, only one compound was detected in the extract from the sex pheromone glands of the females. To discriminate fatty acids from acetates, the key ion fragment *m/z* 60 has been used (Figure 2). The fragments *m/z* 61 and *m/z* 194 (M-60) in the mass spectrum of the compound detected were typical of an aliphatic tetradecenyl acetate and the abundant fragments *m/z* 82 and *m/z* 96 indicated a monounsaturated carbon chain (Figure 3). The position of the double bond was determined by comparing the retention time on GC of the pheromone component from the extract with those of a number of synthetic isomers of the monounsaturated tetradecenyl acetates. The retention time of the extract constituent coincided with that of synthetic Z10-14:OAc on two columns—on the polar column as retention times 39.82 and 39.81, and on the nonpolar column as 39.15 and 39.15—but differed significantly from the retention times of all the closely related reference compounds (Table 3). The retention times of all the other double-bond isomers, not listed in Table 3, differed more. The estimated amount of pheromone component analyzed was about 1 ng per female.

Field Trapping. In all, 9250 and 2950 *P. ulmifoliella* males were trapped during the field tests in 1993 and 1994, respectively. Z10-14:OAc was the only

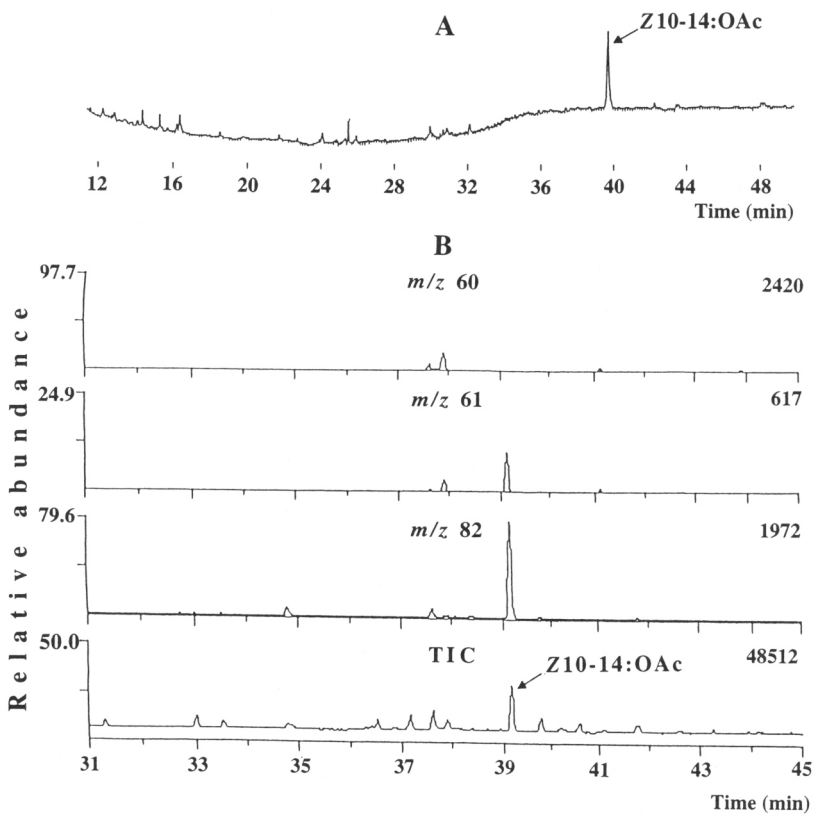

FIG. 2. Chromatogram records of pheromone gland extract from *Phyllonorycter ulmifoliella* females. (A) Gas chromatogram (FID detector, DB Wax J&W fused silica capillary column; 30 m × 0.25-mm ID, 0.25-μm film thickness). (B) Mass chromatogram, where *m/z* 61 and *m/z* 82 are key ion fragments indicating acetates and unsaturations, respectively; *m/z* 60 is an ion fragment characteristic of fatty acids that we used to discriminate them; TIC represents the total ion (*m/z* 30–400) chromatogram DB-5 capillary column; 30 m × 0.25-mm ID, 0.25-μm film thickness); and the number at the upper-right corner of the chromatogram represents the total abundance of the ion indicated.

compound tested alone that was attractive for *P. ulmifoliella* males (Table 2). None of the binary mixture was significantly more attractive than Z10-14:OAc alone. Thus, Z10-14:OAc should be considered the main sex pheromone component of *P. ulmifoliella*. Admixture of at least 10% of either Z9-14:OAc, E9-14:OAc, E10-14:OAc, or E11-14:OAc to the main sex pheromone component inhibited its attractivity for *P. ulmifoliella* males (Table 2). It is interesting to

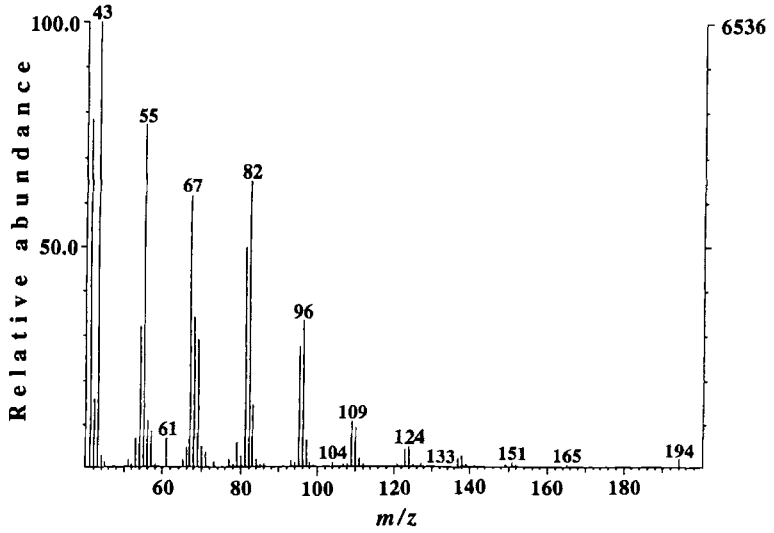

FIG. 3. Mass spectrum of (Z)-10-tetradecenyl acetate from pheromone gland extract of 64 *Phyllonorycter ulmifoliella* females.

TABLE 3. GAS CHROMATOGRAPHIC RETENTION TIMES OF Z10-14:OAc, ITS DIASTEREOMER, THE CLOSEST DOUBLE-BOND POSITION ISOMERS, AND THE COMPOUND EXTRACTED FROM THE PHEROMONE GLANDS OF *Phyllonorycter ulmifoliella* FEMALES (TWO CAPILLARY COLUMNS WERE USED)

Compound	Retention time (min)	
	Polar column (DB wax)	Nonpolar column (DB-5)
From extract	39.82	39.15
Z9-14:OAc	39.50	39.08
Z10-14:OAc	39.81	39.15
*E*10-14:OAc	39.41	39.10
Z11-14:OAc	40.21	39.23
*E*11-14:OAc	39.65	39.18
*E*12-14:OAc	40.13	39.33

note that all of the inhibitors thus revealed are alkenyl acetates and contain the same functional groups as the pheromone component. Among the inhibitory acetates, E10-14:OAc is significantly less potent than the rest (Table 2).

Six alcohols (E- and Z10-12:OH, Z9-14:OH, E10-14:OH, E- and Z11-14:OH), tested either as single compounds or in binary mixtures with acetates, were inactive. The only exception was the binary mixture of the pheromone component with E10-14:OH when applied at a dosage of 1 mg/dispenser at the ratio 10:1 during tests in 1993 at locality II (Table 2). The mixture was significantly less attractive than Z10-14:OAc alone. However, the inhibitory effect was not confirmed in the 1994 tests, when dispensers were loaded with lower dosages of the chemicals.

DISCUSSION

The "calling" posture of *P. ulmifoliella* females is the same as known in two other species within the same genus, *P. emberizaepennella* (Bouche) (Būda et al., 1991) and *P. junoniella* (Z.) (Būda and Mozūratis, unpublished data). *P. ulmifoliella* should be grouped among the species that actively call early in the morning.

Tentiform leafminer moths are very small, thus it is not surprising that females contain very small amounts of pheromones. In *P. mespilella* about 1–2 ng of its main pheromone component was found, while the minor component detected by electroantennographic registration (GC–EAD) (Gries et al., 1993) was far below the detection limits of gas chromatography with a flame ionization detector and of mass spectroscopy.

In the family Gracillariidae, Z10-14:OAc had not been identified previously as a female sex pheromone in any other species. However, the compound effectively attracts males of *P. orientalis* (Kumata) (Ando et al., 1977), *P. klemanella* (F.) (Booij and Voerman, 1984), and *P. ringoniella* (Matsumura) (Sugie et al., 1986). With the last two species, the compound is most effective in binary mixtures with E11-14:OAc and (*E*4,Z10)-4,10-tetradecadienyl acetate (E4,Z10-14:OAc), respectively.

Z10-14:OAc is a sex pheromone component in *Grapholita funebrana* Tr. (Guerin et al., 1986), *Planotortrix excessana* (Walker) (Foster and Dugdale, 1988), and *Notocelia uddmanniana* L (Witzgall et al., 1991). All belong to the family Tortricidae.

Ecological Aspects. Our field trapping experiments not only showed the activity of the compound identified from *P. ulmifoliella* females glands, but also revealed four sex pheromone inhibitors. All of these were known earlier as sex pheromone components in other lepidopteran species belonging to different families. As the host plant of *P. ulmifoliella* is widely spread in the temperate

climatic zone and common in extremely varied habitats, the possibility of interspecific interactions by semiochemicals between *P. ulmifoliella* and other lepidopteran species is highly probable if their activity periods overlap. A scheme of possible antagonistic interactions between *P. ulmifoliella* and moths of other species that might be mediated by five semiochemicals is summarized in Figure 4. The data presented suggest that the inhibitors we have found not only serve as a premating barrier among closely related species (at least in the genus *Phyllonorycter*), but also can be effective antagonists in competition for the most suitable calling period between *P. ulmifoliella* and many other lepidopterans.

The presence of a large number of species which release pheromones disturbing the "normal" communication in a particular species can cause significant ecological pressure and force this species to shift its pheromone communication period to one free of inhibitors, perhaps, as in *P. ulmifoliella*, to the beginning of the light period of the day, which seldom is used by moths communicating by chemical signals. The shift toward a period free of inhibitors should be adaptive.

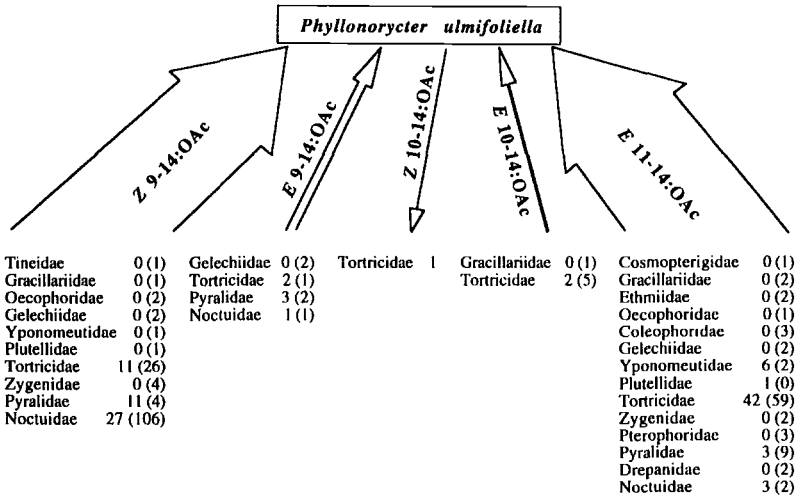

FIG. 4. Possible antagonistic interactions by means of semiochemicals between *Phyllonorycter ulmifoliella* and other moth species from different families. Each arrow indicates that a compound functions as a sex pheromone in one species and as an inhibitor in another species; the arrow points toward the species perceiving the compound as an inhibitor. The number following the name of the family indicates the number of species within that family, for which the compound is known to be a sex pheromone component; the number of species for which the compound is known as a sex attractant (i.e., potentially can be a pheromone constituent) is indicated in parentheses. The width of an arrow corresponds to the total number of species which are known to use the compound in their sex pheromone. Based on data from Arn et al., (1992, 1993).

Evolutionary Aspects. Although there is probably no animal group that has a better-studied pheromone communication system than Lepidoptera, the evolution of pheromone communication still remains little understood even in that order (for recent publications see Phelan, 1992; Löfstedt, 1993), and there is no general agreement on the selection forces operating. Likewise, there are very few reports correlating the first appearance of pheromone compounds with evolution of lepidopterans.

The families Gracillariidae and Tortricidae, representatives of which use Z10-14:OAc as a sex pheromone component, belong to different superfamilies, both included in the taxonomic group Ditrysia. Among the ditrysian lepidopterans, the superfamilies Tineoidea and Gracillarioidea (after Robinson, 1988; Scoble, 1992) are considered to belong to the first or most primitive lineage of superfamilies (Scoble, 1992). Moreover, the family Gracillariidae was formerly included in the superfamily Tineoidea, which was regarded as one of the most primitive families within Ditrysia. In the case of the pheromone component Z10-14:OAc, the ancestor may be common to the entire Ditrysia group (Figure 5).

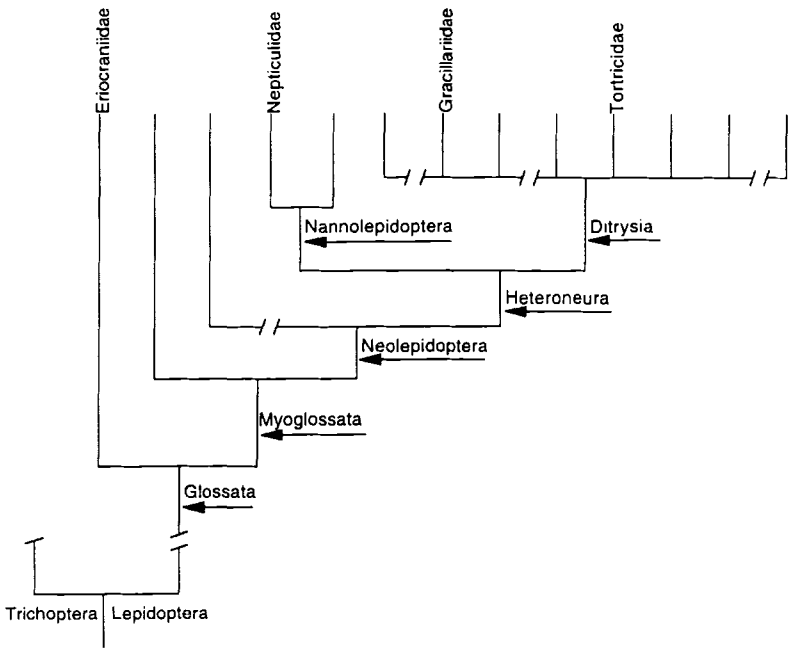

FIG. 5. Part of the phylogenetic tree of Lepidoptera, indicating the positions of different taxa including the families related to the evolution of Z10-14:OAc as a sex pheromone (Gracillariidae and Tortricidae) and those using much simpler structures (Eriocraniidae and Nepticulidae). Adapted from Nielsen (1989).

A recent publication on the first identification of sex pheromone structures in the family Eriocraniidae (Zhu et al., 1995) indicates that those structures have much shorter (i.e., C_7) carbon chains and that Eriocraniidae are more closely related to caddisflies (Trichoptera) than to any other taxa of Lepidoptera (Myoglossata) (Figure 5). So it is evident that Z10-14:OAc did not evolve earlier than Myoglossata. Another pheromone identification in a representative of the family Nepticulidae (Tóth et al., 1995) indicates that nepticulid moths use longer (nine carbons in chain) communication compounds than eriocraniids, but still much shorter-chain ones than used by ditrysians. If only shorter-chain compounds occur in species from families belonging to the same taxonomic group as Nepticulidae (Nannolepidoptera), it would indicate that Z10-14:OAc evolved in Ditrysia only. If Z10-14:OAc should be found in any family belonging to Nannolepidoptera, the evolutionary appearance of Z10-14:OAc as a sex pheromone component must have taken place during an earlier period, when Neolepidoptera or even Myoglossata was formed.

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DEVELOPMENT AND PHEROMONE COMMUNICATION SYSTEMS IN HYBRIDS OF *Agrotis ipsilon* AND *Agrotis segetum* (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Hybrids were obtained by crossing males of the turnip moth *Agrotis segetum*, which has a period of arrested development in the larval stage, with females of the migrant and nondiapausing black cutworm, *Agrotis ipsilon*. No progeny were obtained by crossing females of *A. segetum* with males of *A. ipsilon*. Backcrosses were successful only by crossing hybrids with both sexes of *A. ipsilon*. No larval oligopause occurred when hybrid larvae were reared under conditions inducing arrest in *A. segetum* (12L:12D, 21°C). Wind tunnel tests showed interattractiveness of F_1 hybrids with *A. ipsilon* but not with *A. segetum*. Single sensillum recordings of pheromone-sensitive hairs on the antennae of males of the parent species revealed differences in the distribution of sensilla. F_1 and F_2 hybrids exhibited the same receptor cell types and distribution as *A. ipsilon*. Gas chromatographic analysis of female pheromone gland extracts revealed that F_1 hybrids were also very close to *A. ipsilon*.

Key Words—Lepidoptera, Noctuidae, *Agrotis ipsilon*, *Agrotis segetum*, hybridization, pheromone, diapause, migration, sexual isolation.

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INTRODUCTION

The black cutworm, *Agrotis ipsilon*, is an important pest on seedling corn and numerous crops throughout the world (Rings et al., 1975). It is a migrant noctuid. The European populations of the insects fly from north Africa to reproduce in southern Europe (Onyango Odiyo, 1975). Adults arising from this spring generation move towards the northern parts of Europe. In autumn, a second generation flies southward (Causse et al., 1989). (Z)-7-Dodecen-1-yl acetate (Z7-12:Ac) and (Z)-9-tetradecen-1-yl acetate (Z9-14:Ac) have been identified as sex pheromone components in American populations (Hill et al., 1979). Further investigations with Asian and European populations revealed that the addition of (Z)-11-hexadecen-1-yl acetate (Z11-16:Ac) to binary mixtures of Z7-12:Ac and Z9-14:Ac increased the catches of males in the field and stimulated the behavioral responses of males in a wind tunnel (Wakamura et al., 1986; Causse et al., 1988).

The turnip moth, *A. segetum*, is a very important pest of corn and other crops throughout Europe (Cayrol, 1966). Last instars undergo a winter arrestment of development classified as an oligopause (Mansingh, 1971). This species is autochtonous. Twenty-two compounds have been identified in extracts of female pheromone glands (see the exhaustive review by Arn et al., 1992). However, a ternary mixture of (Z)-5-decen-1-yl acetate (Z5-10:Ac), Z7-12:Ac, and Z9-14:Ac is sufficient to mimic the pheromone blend (Bestmann et al., 1978; Arn et al., 1980; Toth et al., 1980; Löfstedt et al., 1982). (Z)-5-Dodecen-1-yl acetate (Z5-12:Ac) was recently found to be a fourth sex pheromone component (Wu et al., 1995).

Development rhythms of both these noctuid species differ from the typical developmental periodism, as seems to be the case for most Agrotinae that have been studied (Danilevskii, 1961). *A. ipsilon* does not show any regulated larval or pupal diapause (Beck, 1986). Larvae may survive many months at mildly cold conditions in a state of "nondiapause dormancy" (Beck, 1988). *A. segetum* is a nonmigrant noctuid which does not have a classical state of diapause. Rather larvae exhibit an oligopause characterized by a longer larval period and an increase in the thermal threshold for pupation (Buès et al., 1989) that is linked with short-photoperiod and low-temperature conditions. Northern populations thus show a stronger delay in development than southern populations (Buès et al., 1989). The ecological importance of migration and its selective value are similar to that of diapause. Both strategies are methods of escape or resistance to unfavorable environmental conditions. According to the classification by Novak and Spitzer (1972), *A. segetum* would fit the type 2a characterization, in which a species splits into two partial populations, migrants and autochtons. The larger part of the population of an area is formed by a partially indigenous population, and only a small proportion of the population is nondiapausing and

of migratory origin. *A. ipsilon* would be classified as type 3, a nondiapausing species that is not autochtonous in central and northern Europe. Larvae and adults of both species can be differentiated easily by their morphology, but several traits related to reproduction (calling, mating and oviposition behavior) are very similar (Cayrol, 1966).

The two species may be sympatric in distribution; males of both species can be trapped concomitantly in the same localities during the same periods of the year (Gabarra, 1990; Hmimina, 1990; Popescu, 1990; Tsitsipis, 1990; Vasilev, 1990). Moreover, capture in the field of males of *A. segetum* to traps baited with lures aimed at *A. ipsilon* were recorded in 1992 and 1993 in the south of France at a 1:10 ratio (Barthès, personal communication).

Can these two species hybridize in the field as a result of "errors" in species recognition? We tested the capacity of the two species to hybridize in the laboratory, and crosses between the two species allowed us to initiate a study of interspecific isolation mechanisms, in particular, of their pheromone communication systems. We investigated and report here on pheromone perception and production in hybrids and parental species.

METHODS AND MATERIALS

Insects. Larvae of *A. ipsilon* and of *A. segetum* were reared on an artificial diet and maintained using procedures described by Poitout and Buès (1974), with a photoperiod of 16L:8D, at 21°C and 60% RH. A photoperiod of 12L:12D and 21°C were used to induce arrestment of development in *A. segetum* last instars. Laboratory strains were regenerated each year by adding adults caught near Avignon (France) by light and sex pheromone traps during the northward migration. Male and female pupae of each species were held in different chambers and checked each day for emergence. Sexed adults were kept separately in plastic boxes and had access to 20% sucrose solution.

Crossing Experiments. Pairs were formed before the end of the photophase. Crossing was performed by placing one female of a given species into a plastic box containing one male of the other species. Backcross experiments were conducted using hybrid males and females and both sexes of the parent species, and the incidence of mating among species, F_1, and backcross progeny was determined. Mating behavior was observed as previously described (Gadenne, 1993). The occurrence of mating was checked by location of the spermatophore in the female. If eggs were fertile, they became reddish the day following oviposition and the developmental germ band could be observed through the eggshell. Fertility of eggs was confirmed by checking for hatching after maturation at 28°C. Fertility was considered as the percentage of mated females laying viable eggs.

Anatomy of the Male Genitalia. The anatomy of the male genitalia of the F_1 and F_2 hybrids was examined and compared to that of their parent species. Emphasis was given to quantitative diagnostics cues to determine the status of individuals. Male genitalia were dissected, cleared in boiling KOH, and mounted on a microscope slide. Digitized pictures were obtained from a Cohu 4912 videocamera mounted on a stereomicroscope and were viewed to obtain measurements with a MacIntosh microcomputer equipped with a Image Grabber/24 board and Optilab software (Graftek Company).

The most relevant observations could be made on the valvae and the juxta. A total of nine measurements was taken on each individual (Figure 1). The measurements included *h1*, the distance between the costa and the basal part of

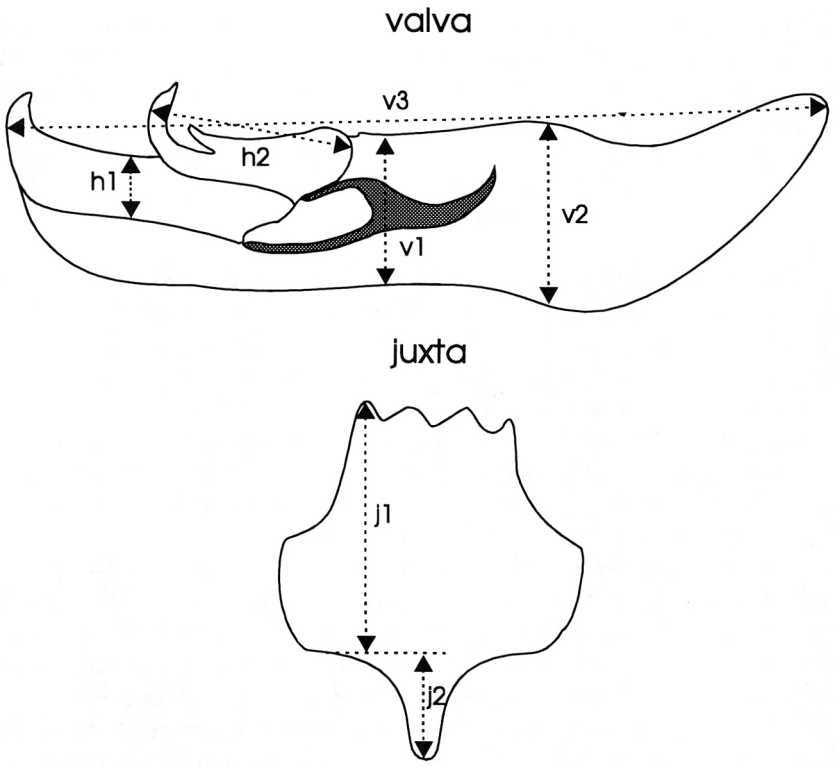

FIG. 1. Schematic representation of male *Agrotis* valva and juxta showing eight of the nine measures used for a quantitative comparison of the genitalia morphology. h1, width of the harp; h2, ampulla length; j1, juxta height; j2, height of the inferior process of the juxta; v1 and v2, valva width at two levels; v3, valva length; shaded area, surface of the clasper.

the harp; *h2*, the length of the dorsal process of the harp (ampulla); *v1*, the width of the valva at the level of the clasper; *v2*, the width of the valva before its constriction at the base of the cucculus; *v3*, the total width of the valva; *sc*, the surface of the clasper (shaded area in Fig. 1); *j1*, the height of the juxta; *j2*, the size of the inferior process of the juxta; and *t*, the size of the teeth on the basal crested process of the penis vesica. The square root of *sc* was calculated to equalize the variances of the data.

Behavioral Experiments. We used a wind tunel to test the responsiveness of day 4 virgin F_1 hybrid males to gland extracts of day 4 virgin females of *A. ipsilon* and *A. segetum* or of F_1 hybrids. Similarly we tested the responsiveness of day 4 virgin males of *A. ipsilon* and *A. segetum* to gland extracts of day 4 virgin F_1 hybrid females. Procedures were derived from Gadenne et al. (1993). Pheromone glands were dissected at midscotophase, which is the time of maximum calling for both species (Löfstedt et al., 1982; Gadenne, 1993). The glands were extracted in 50 μl hexane for 1 hr. Virgin day 4, sexually mature, individual males were exposed once to gland extracts [1 female equivalent (FE)]. Gland extracts were dispensed on a filter paper and placed in the airflow upwind to a cage containing one single male in the wind tunnel. Responsiveness of males to the pheromone gland extract was estimated using the following scale: (0) no response, (1) clasper eversion, (2) interrupted flight, (3) complete flight toward the source, and (4) landing on the source. Responsiveness of tested males was compared by calculation of the mean score of each group. Statistical differences between treatments was assessed by the *G* test, $P \leq 0.05$.

Chemical Analyses. The number of female hybrids obtained by hybridization was very low and we could not perform gas chromatography coupled with mass spectrometry (GC/MS) analysis of pheromonal gland extract of female hybrids. Only GC analyses of hybrid females were therefore performed and compared with those of female *A. ipsilon* for which a more detailed GC/MS analysis was performed (Picimbon, 1995).

For pheromone analysis, the pheromone glands of day 4 *A. segetum* and of F_1 hybrid females were excised at midscotophase and were then transferred individually into 15 μl of hexane containing 10 ng of internal standard (tridecanyl acetate; 13:Ac). After 1 hr in hexane, a gland was removed, and the extract was concentrated under nitrogen and subsequently injected (1 FE) in a Hewlett-Packard HP 5890 gas chromatograph equipped with a 30-m × 0.25-mm-ID Supelcowax 10 capillary column (Supelco France). The temperature program started at 80°C and increased to 220°C at 5°C/min, with a final hold time of 20 min.

Standards consisting of 13:Ac, Z5-10:Ac, Z7-12:Ac, (Z)-8-dodecen-1-yl acetate (Z8-12:Ac), Z9-14:Ac, (Z)-11-hexadecen-1-yl acetate (Z11-16:Ac), and (Z)-11-hexadecenol (Z11-16:OH) were purchased from Interchim (Montluçon, France).

Single Sensillum Recordings. Electrophysiological responses of pheromone-specialist olfactory neurons were recorded from sensilla on the male antennae. Single sensillum recordings were carried out using the tip recording technique with standard procedures (Kaissling, 1974; Van Der Pers and Den Otter, 1978). Live male adults or isolated antennae were used. Screenings were performed to characterize receptor cell types by their responses to pheromone compounds or pheromone analogs. Compounds were presented at 0.5 μg on a filter paper inserted in a glass cartridge. The stimulus was delivered by a 1-sec puff of air blown through the cartridge and over the sensilla. The antenna was continuously flushed by a stream of humidified pure air (1.5 liters/min, 100% RH). Acquisition and analysis of data were performed on a PC-AT microcomputer with programs developed in the laboratory.

RESULTS

Crossing Experiments. Results of crossings are summarized in Table 1. Crossings of female *A. segetum* with male *A. ipsilon* were unsuccessful. Although some matings did occur, no larvae were obtained. On the contrary, pairing of

TABLE 1. CROSSING EXPERIMENTS BETWEEN *Agrotis ipsilon*, *Agrotis segetum*, AND THEIR HYBRIDS

	Males			
Females	*segetum*	*ipsilon*	F_1 hybrid	F_2 hybrid
segetum				
Mating (%)	>70	3	0	
Fertility (%)	>60	0	0	
	(N = hundreds)	(N = 227)	(N = 86)	
ipsilon				
Mating (%)	4	>75	40	75
Fertility (%)	1	>65	30	27
	(N = 367)	(N = hundreds)	(N = 57)	(N = 40)
F_1 hybrid				
Mating (%)	0	69	73	
Fertility (%)	0	29	20	
	(N = 75)	(N = 51)	(N = 49)	
F_2 hybrid				
Mating (%)		57		56
Fertility (%)		40		29
		(N = 35)		(N = 79)

female *A. ipsilon* with male *A. segetum* produced viable F_1 hybrids. Nevertheless, of 367 pairs, only 15 matings were recorded and 4 females laid fertile eggs. Wing design of F_1 hybrids generally resembled that of *A. ipsilon*. However, the wing pattern in some individuals was close to that of *A. segetum*.

The fertility of F_1 hybrids was low, and only 25 of 83 mated F_1 hybrid females laid fertile eggs. However, an F_2 generation of hybrids was obtained. Backcross experiments were performed as indicated in Table 1. Only pairs formed with F_1 hybrids and *A. ipsilon* produced offspring. No progeny could be obtained when F_1 hybrids were crossed with *A. segetum*. In F_1 hybrids × *A. ipsilon* pairings, fertility was low. Only 15 of 51 F_1 hybrid females laid fertile eggs when paired with *A. ipsilon* males, and only 17 of 57 *A. ipsilon* females laid fertile eggs when paired with F_1 hybrid males. Crossing of F_2 hybrids was successful, leading to F_3 progeny, although the fertility was low (23 of 79 F_2 hybrid females laid fertile eggs). Backcross of F_2 hybrids with adults of *A. ipsilon* produced viable progeny (Table 1).

Anatomy of Male Genitalia. The male genitalia of *A. segetum* and *A. ipsilon* are different from each other and several traits allow species diagnosis (Pierce, 1909). The uncus is shorter, wider, and swollen in *A. segetum*, its apex is blunted, and the more apical bristles are shorter than in *A. ipsilon*. The vesica of *A. segetum* is swollen and bears a strong crested process, and the vinculum is bulky with a narrower saccus.The juxta of *A. segetum* is rectangular and shows a more developed ventral process, in contrast to the juxta of *A. ipsilon*, which is roughly hexagonal with a short ventral process. The valvae of *A. segetum* are relatively shorter with parallel edges, the corona bears shorter bristles, and the harp is wider and covers entirely the sacculus. The ampulla is shorter than in *A. ipsilon*. In *A. segetum*, the clasper is wide and its tip is blunted and turned ventrally, while in *A. ipsilon* the clasper is tapered and its tip turned dorsally, and the harp is narrower and does not cover entirely the sacculus.

Preliminary observations revealed that F_1 male genitalia morphology was intermediate between *A. ipsilon* and *A. segetum* phenotypes. This was further confirmed by quantitative analysis (Table 2) that indicated intermediate values for the nine measures. The width of the harp (h1; mean = 440.5 ± 35.3 μm) and the length of the ampulla (h2; mean = 861.4 ± 40.4 μm) were intermediate between the narrower harp (h1; mean = 328.2 ± 29.9 μm) but longer ampulla (h2; mean = 1029.7 ± 41.5 μm) in *A. ipsilon* and the corresponding dimensions in *A. segetum* (h1 = 611.5 ± 79.6 μm; h2 = 750.3 ± 44.8 μm). The square root of the clasper surface well reflected the shape differences of this piece between the parent species. In F_1, this parameter was intermediate between *A. ipsilon* and *A. segetum*. Distribution of F_1 individuals (Figure 2) in a scheme constituted by the h1:h2 ratio and the square root of the clasper surface confirmed the intermediate morphology of the harp. Values for F_2 genitalia were generally closer to that of *A. ipsilon*.

TABLE 2. MEAN VALUES OF THE NINE PARAMETERS SELECTED FOR A QUANTITATIVE ANALYSIS OF THE GENITALIA MORPHOLOGY OF MALE *Agrotis ipsilon*, *A. segetum*, AND OF THEIR F_1 AND F_2 HYBRIDS (MEAN μm AND SD)

	A. segetum (N = 25)		F_1 (N = 24)		F_2 (N = 21)		*A. ipsilon* (N = 30)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Harp								
h1	611.5	79.6	440.5	35.3	407.8	77.2	328.2	29.9
h2	750.3	44.8	861.4	40.4	921.8	72.2	1029.7	41.5
h1/h2	0.8	0.10	0.51	0.03	0.45	0.10	0.32	0.03
Valva								
v1	732.2	73.9	672.7	70.0	629.1	47.5	608.0	44.5
v2	818.0	84.8	817.7	74.0	856.8	87.2	891.1	69.6
v1/v2	0.90	0.07	0.82	0.08	0.74	0.09	0.68	0.04
v3	3314.2	143.4	3387.1	125.0	3423.4	136.0	3696.4	134.5
Clasper surface	415.9	31.4	317.4	17.5	309.8	25.3	269.6	12.4
Juxta								
j1	281.1	25.2	329.8	20.5	374.1	48.1	437.0	33.8
j2	234.6	21.2	194.7	23.8	157.6	30.7	164.0	24.0
j1/j2	0.84	0.11	0.59	0.09	0.44	0.13	0.38	0.07
Vesica teeth	40.5	5.1	27.6	5.8	25.7	6.1	17.0	3.2

Although they were intermediate between the parent species phenotypes, the three measures of the valvae, v1, v2, and v3, of F_1 hybrids (Table 2) were closer to those of *A. segetum*. The v1/v2 ratio close to 1 reflected the relatively straight shape of valvae in *A. segetum*, in contrast to the swelled valvae of *A. ipsilon* (v1/v2 = 0.68). Valvae were shorter in *A. segetum* and the hybrids than in *A. ipsilon* (Table 2, v3).

The two measures of the juxta, j1 and j2, reflected the intermediate position of hybrids between the relatively rectangular juxta with a long process in *A. segetum* and the more hexagonal juxta with a shorter process in *A. ipsilon* (Table 2). Likewise, the teeth on the vesica in hybrids were intermediate to those in the parents (Table 2). Distribution of individuals about the ratio j1/j2 and penis toothlength confirmed this intermediate position, with a tendency for some F_2 to cluster very close to *A. ipsilon* (Figure 2).

Behavior. Flight tunnel experiments were performed with males (M) of the parental species, *A. ipsilon* (i) and *A. segetum* (s), and of hybrids (h) exposed to female (F) gland extract (1 FE) of parental species or of hybrids (Table 3). The mean response of each experimental group was obtained by scoring individual male responses. This procedure revealed two groups (Figure 3). One

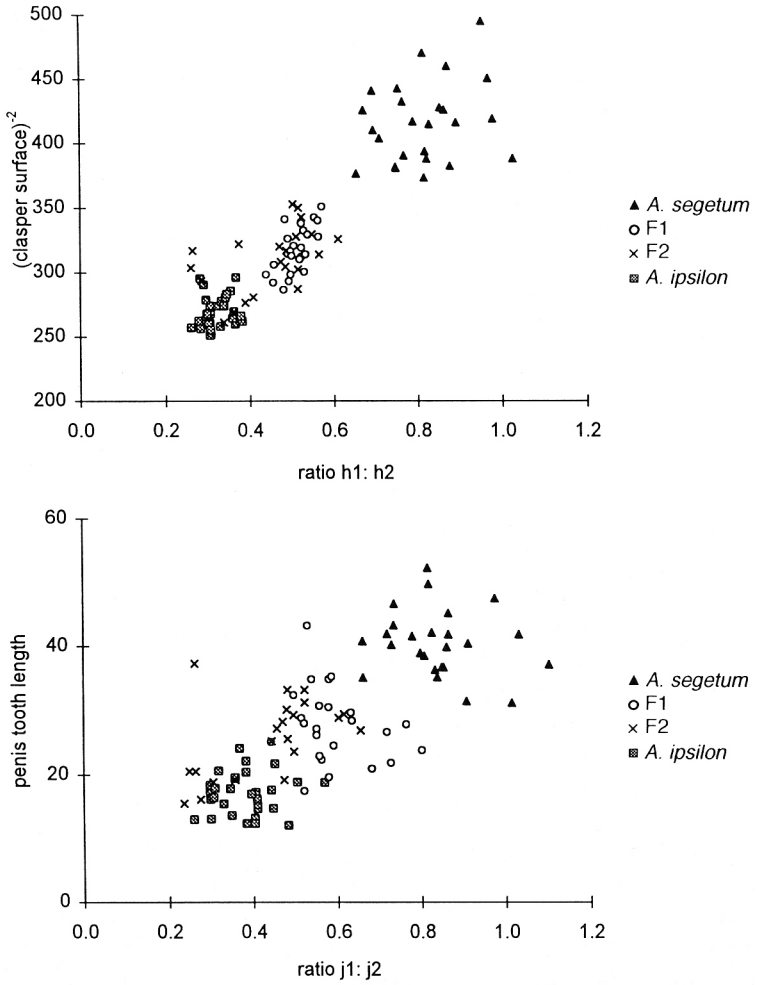

FIG. 2. Distribution of individual male *A. ipsilon*, *A. segetum*, and F_1 and F_2 hybrids according to the shape of the harp and the square root of the clasper surface (top) or the shape of the juxta and the size of the penis vesica teeth (bottom). Scales are in micrometers.

group of tested pairs (Fs/Mi, Fi/Ms, Fh/Ms, Fs/Mh, and Fh/Mh) showed a low response. Males were not attracted to gland extracts of heterospecific females. Hybrid males showed a very low response when tested with gland extracts of female *A. segetum*, and gland extracts of hybrid females did not attract male *A. segetum*. With gland extracts of hybrid females, hybrid males exhibited a very

TABLE 3. FLIGHT TUNNEL EXPERIMENTS WITH *Agrotis ipsilon*, *Agrotis segetum*, AND THEIR HYBRIDS[a]

Female gland extracts	Males	Number	Score 1, No response (%)	2, Clasper eversion (%)	3, Interrupted flight (%)	4, Complete flight (%)	5, Landing on source (%)
A. ipsilon	*A. segetum*	21 (a)	100	0	0	0	0
A. ipsilon	*A. ipsilon*	29 (b)	24	27	17	11	21
A. ipsilon	Hybrids	20 (b)	50	10	10	10	20
A. segetum	*A. segetum*	24 (b)	16	25	9	5	45
A. segetum	*A. ipsilon*	30 (a)	100	0	0	0	0
A. segetum	Hybrids	20 (a)	80	20	0	0	0
Hybrids	*A. segetum*	19 (a)	95	5	0	0	0
Hybrids	*A. ipsilon*	26 (b)	50	11	8	11	20
Hybrids	Hybrids	20 (a)	85	0	5	10	0

[a]Numbers followed by the same letter (in parentheses) are not statistically different (G test, $P \leq 0.05$).

weak response, and 85% of the males did not react. In the second group, hybrid tested pairs (Fh/Mi, Fi/Mh) showed interattractiveness not statistically different from that of homospecific pairs (Fi/Mi, Fs/Ms) (Figure 3).

Larval Development. When reared under a 16L:8D photoperiod at 21°C, larvae of *A. ipsilon* pupated in 32.8 ± 2.6 days (N = 72). Larvae of *A. ipsilon* did not enter diapause when reared under a 12L:12D photoperiod at 21°C and pupation was obtained in 29 ± 1.5 days (N = 415). Under these conditions, last instars of *A. segetum* displayed arrestment of development, and pupation occurred only after 64 ± 9.7 days (N = 385). When reared under a 16L:8D photoperiod at 21°C, larvae of *A. segetum* pupated in 37.4 ± 3 days (N = 136). When hybrids were reared under 12L:12D at 21°C, there was no diapause and pupation occurred in 31 ± 1.8 days (N = 281).

Single Sensillum Recordings. The antennal flagella of both *A. ipsilon* and of *A. segetum* have segments with branches. Single sensillum recordings revealed different specificities of receptor neurons present in the sensilla located either on the branches or on the stem. Four types of sensilla were found in *A. ipsilon* (Figures 4 and 5). A majority of the sensilla housed one neuron responding only to Z7-12:Ac. Some sensilla that contained two neurons responding to Z5-10:Ac and to Z8-12:Ac, respectively, were found on the antenna stem. A third type of sensillum, located on the branches, contained a neuron responding to Z9-

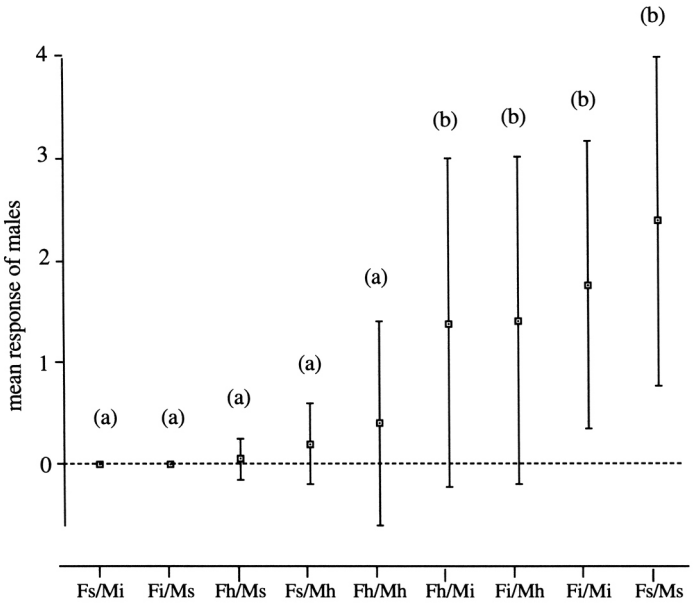

FIG. 3. Mean response of males of *A. ipsilon* (Mi) and of *A. segetum* (Ms), and of F_1 hybrids (Mh) submitted to gland extracts of females of *A. ipsilon* (Fi), and of *A. segetum* (Fs) or of F_1 hybrids (Fh) in a wind tunnel. For each group, the mean response is the mean score of attractiveness of the tested males. See Methods and Materials for details. Bars with the same letter are not statistically different (*G* test, $P \leq 0.05$).

14:Ac. In addition, one neuron responding to (Z)-9-dodecen-1-yl acetate (Z9-12:Ac) was found in a single olfactory hair (Renou et al., 1996).

Three types of sensilla were found on the antennae of *A. segetum* (Figures 4 and 5). Sensilla housing a neuron tuned to Z7-12:Ac were found on branches and stem. A majority of the sensilla housed two neurons, responding to Z5-10:Ac and to Z8-12:Ac, respectively. Most of them were on the branches. A third type of sensillum located on the stem contained a cell tuned to (Z)-5-dodecen-1-yl acetate (Z5-12:Ac).

In the F_1 hybrids, a large majority of sensilla containing a Z7-12:Ac-responding neuron was found on the branches. A few sensilla were found to house a neuron responding to Z9-14:Ac. Sensilla with neurons responding to Z5-10:Ac and Z8-12Ac were found only on the stem. This pattern was very similar to that of *A. ipsilon*. In addition, a few stem sensilla contained a receptor

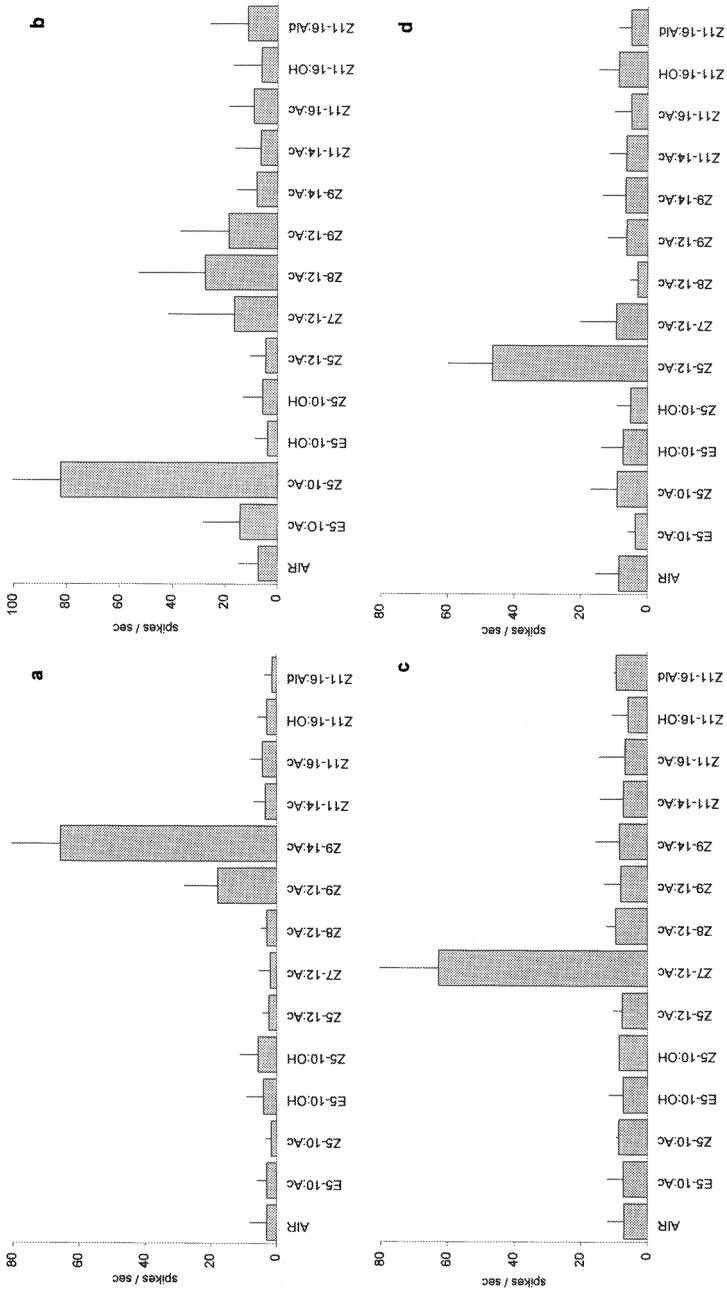

FIG. 4. Response profiles of the four main types of pheromone-sensitive trichoid sensilla found on the antennae of *A. ipsilon*, *A. segetum*, and their hybrids. (a) Z9-14:Ac sensillum, mean responses of three sensilla recorded in male *A. ipsilon*. (b) Z5-10:Ac sensillum, mean of 15 sensilla in *A. ipsilon*. (c) Z7-12:Ac sensillum, mean of 30 sensilla in *A. ipsilon*. (d) Z5-12:Ac sensillum, mean of eight sensilla in *A. segetum*. Responses were measured as the number of spikes fired in 1 sec. Error bars: standard deviation.

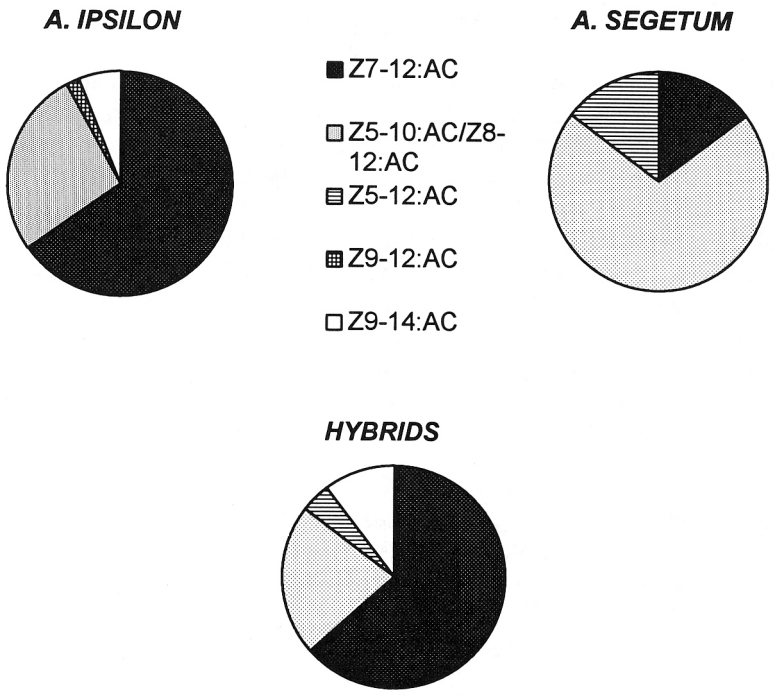

FIG. 5. Populations of each sensillum type in *A. ipsilon*, in *A. segetum*, and in their hybrids. Population proportions were estimated from a sample of 50 trichoid sensilla recorded in *A. ipsilon*, 54 sensilla in *A. segetum*, and 68 sensilla in the F_1 hybrids.

cell tuned to Z5-12:Ac (Figures 4 and 5). The main difference in the pheromone detection system of *A. ipsilon* and *A. segetum* was found in the proportion of sensilla containing neurons tuned to Z7-12Ac or Z5-10:Ac, and their distribution between antennae branches and stem. This character was used to determine the phenotype of individuals of the first and second generation of hybrids. For each male moth, 10 sensilla located in different positions on the branches were sampled and the cell responses to Z5-10:Ac, Z5-12:Ac, Z8-12:Ac, or Z9-14:Ac were recorded. The great majority of the sensilla in 15 F_1 males contained neurons responding to Z7-12:Ac. In addition, from 0 to 2 of the 10 sampled sensilla responded to Z9-14:Ac. A total of 25 males belonging to the second generation of hybrids was tested. All of them had sensilla containing neurons responding either to Z7-12:Ac (the great majority) or to Z9-14:Ac.

Pheromone Gland GC Analysis. Pheromone levels produced in hybrid females were very low (less than 1 ng) compared to that found in parental species (Hill et al., 1979; Löfstedt et al., 1982: Zhu et al., 1995; Picimbon, 1995).

Furthermore, the number of females available was not sufficient for mass extracts. Thus, a complete identification of the components of the pheromone blend was not feasible. Instead, chemical structures were tentatively attributed to peaks from the hybrids on the basis of their coelution time with standards and by comparison with the pheromone blend of their parent species. We analyzed the content of the pheromone gland of hybrids and compared it with the gland content of their parents (Table 4). GC analysis of gland extracts of *A. ipsilon* revealed the presence of peaks coeluting with the following components: Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, Z11-16:OH, and traces of Z5-10:Ac and Z8-12:Ac (Picimbon et al., 1994). GC/MS analysis confirmed the presence of the first five components (Picimbon, 1995). GC analysis of gland extract from female *A. segetum* confirmed the presence of Z5-10:Ac, the major pheromonal compound in the French strain, in agreement with previous chemical

TABLE 4. GC ANALYSIS OF SEX PHEROMONE GLAND OF FEMALES OF *Agrotis ipsilon*, *Agrotis segetum*, AND THEIR HYBRIDS

Pheromone gland analysis		
French *Agrotis ipsilon* (Picimbon, 1995)	F_1 hybrids	French *Agrotis segetum* (Löfstedt et al., 1986)
		10:Ac
Z5-10:Ac		Z5-10:Ac[a]
		10:OH
		Z5-10:OH
		12:Ac
		Z5-12:Ac
Z7-12:Ac[a,b]	Z7-12:Ac	Z7-12:Ac[a]
Z8-12:Ac		
		Δ11-12:Ac
		12:OH
		Z7-12:OH
		14:Ac
		Z5-14:Ac
		Z7-14:Ac
Z9-14:Ac[a,b]	Z9-14:Ac	Z9-14:Ac[a]
Z11-14:Ac[b]		
		16:Ac
Z11-16:Ac[a,b]	Z11-16:Ac	Z11-16Ac
Z11-16:OH[b]	Z11-16:OH	18:Ac

[a]Component present in the pheromone blend used in field experiments.
[b]Components identified by GC/MS analysis.

analyses (Löfstedt et al., 1986) and field data (Arn et al., 1980). GC analyses of a hexane extract of the glands of six hybrid females showed peaks coeluting with synthetic Z7-12:Ac, Z9-14:Ac, Z11-16:Ac, and Z11-16:OH. No peak with a retention time similar to that of Z5-10:Ac was detected.

DISCUSSION

Interspecific hybridization studies have been performed in various Lepidoptera, including *Papilio* species (Ae, 1960), some Arctiidae (Bacheler and Habeck, 1974), *Heliothis* species (Hardwick, 1965; Laster, 1972: Teal and Oostendorp, 1995), *Euxoa* species (Byers and Hinks, 1978; Teal et al., 1978; Byers et al., 1981), *Spodoptera* species (Monti et al., 1995), and tussock moths (Lymantridae) (Grant et al., 1975). Grant et al. (1975) also compared the pheromone system of hybrid tussock moths with that of their parents. Sex-controlled inheritance was suggested from studies of sex pheromone responses of aldehyde- and acetate-producing *Choristoneura* species (Tortricidae) and of their hybrids (Sanders et al., 1977). Genetic inheritance of male olfactory response to sex pheromone was studied in two tortricid species of *Ctenopseustis* and their hybrids (Hansson et al., 1989).

Hybridization between *Heliothis subflexa* and *H. virescens* was attempted as a means to produce sterile males for biological control of the latter species (Laster et al., 1988; Teal and Oostendorp, 1993). Female hybrids produced low amounts of pheromone possibly due to an alteration in the hormonal system regulating pheromone production (Teal and Oostendorp, 1995). Although the rate of hybrid production in our experiments was low, we were able to induce matings and to obtain viable offspring by pairing males of *A. segetum* with females of *A. ipsilon*. We have not been able to produce hybrids by crossing males of *A. ipsilon* with females of *A. segetum*. Crossings were more difficult when *H. virescens* females were paired with *H. subflexa* males, partly because of the poor performance of the *H. subflexa* males (Proshold and LaChance, 1974). Hybrids were successfully produced only when males of *H. zea* were paired with females of *H. armigera* (Hardwick, 1965).

Quantitative analysis of the morphology of genitalia provided reliable clues for the identification of potential hybrids in field populations of *Agrotis* in our work. The mean position occupied by the F_1 hybrids for all the measured parameters confirmed the intermediate morphology of male genitalia. The results indicated codominance of the parent phenotypes. However, in the F_2 generation, measurements were closer to *A. ipsilon* than to *A. segetum*, and we did not find any individual with the *A. segetum* phenotype.

Flight tunnel experiments showed that female gland extracts of a species attracted only conspecific males. In the field, however, some *A. segetum* males

were trapped with synthetic lures intended to catch *A. ipsilon* males. Therefore, the specificity of pheromone attractiveness in the two species observed in the laboratory contrasts with the cross-attraction observed in the field. Field cross-attraction could be due to lower specificity of synthetic sex attractants compared to that of the natural gland extracts used in the wind tunnel experiments. Behavioral cross-attraction was observed between F_1 hybrid males tested with gland extracts of *A. ipsilon* females, and *A. ipsilon* males were attracted to gland extracts of F_1 hybrid females. Preliminary analysis of the pheromone blend of the hybrid females showed that the hybrid blend seemed closer to that of *A. ipsilon* rather than to the *A. segetum* pheromone blend. In particular, the absence of Z5-10:Ac in the pheromone blend of the hybrids may explain the lack of attraction of the male *A. segetum* to the gland extracts of hybrid females. F_1 hybrid males responded very weakly to gland extracts from F_1 hybrid females, showing differences in pheromone synthesis and pheromone detection of F_1 hybrids. This might indicate that there are some discrepancies between pheromone production by females and response specificity to pheromone blends in males. Relatively little is known about the genetic control of pheromone blend composition in insects, but three major genetic factors control the traits involved in the sexual communication system of the European corn borer, *Ostrinia nubilalis* (Roelofs et al., 1987).

The pheromonal system in the French population of *A. segetum* is different from that in other European populations (Arn et al., 1983; Löfstedt et al., 1986). Löfstedt et al. (1986) hypothesized that this could be due to an "adaptation to varying interspecific interactions within the geographic range of this species." The European populations of *A. ipsilon* seem to be homogeneous, based upon electrophoretic analyses performed by Buès et al. (1994).

A. ipsilon encounters *A. segetum* from populations with varying pheromone blends during its migration, and hybridization could occur with the *A. segetum* having the closest pheromonal system. The species *A. ipsilon* and *A. segetum* in France constitute an excellent model system for a genetic study of the role of pheromone as reproductively isolating mechanisms. Z8-12:Ac, which is a strong inhibitor of pheromone attractiveness in *A. segetum* (Arn et al., 1980), could play a role in reproductive isolation between the two species. It would be interesting to test the capacity of *A. segetum* males from other locations in Europe (those more likely to contain various ratios of Z5-10:Ac, Z7-12:Ac, and Z9-14:Ac) to mate with *A. ipsilon* from France. *A. ipsilon* and hybrids do not have larval arrestment of development. *A. segetum* larvae exhibited a nontypical diapause phenomenon. Contrary to southern populations, which show a short delay of development when exposed to short photoperiods and low temperatures, larvae of the northern populations of *A. segetum* exhibited a much longer larval period (Buès et al., 1989). It would be interesting to compare the development

of the progeny of crosses of *A. ipsilon* with that of populations of *A. segetum* showing different levels of arrestment of development.

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SEX PHEROMONE OF THE FRENCH BLACK CUTWORM MOTH, *Agrotis ipsilon* (LEPIDOPTERA: NOCTUIDAE): IDENTIFICATION AND REGULATION OF A MULTICOMPONENT BLEND

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Abstract—The sex pheromone blend of a European strain of the black cutworm moth, *Agrotis ipsilon* (Hufnagel), was investigated. Chemical analyses of pheromone gland extracts from 4- to 8-day-old females showed that individual isolated glands contained only very small amounts of pheromone. High-resolution gas chromatography combined with mass spectrometry (GC–MS) analyses showed the presence of *cis*-7-dodecenyl acetate (Z7-12:Ac), *cis*-9-tetradecenyl acetate (Z9-14:Ac), *cis*-11-tetradecenyl acetate (Z11-14:Ac), *cis*-11-hexadecenyl acetate (Z11-16:Ac), and *cis*-11-hexadecenol (Z11-16:OH) in biologically active pheromone gland extracts. Removing 27-12:Ac, Z9-14:Ac, or Z11-16:Ac from the complete gland extract by GC trapping techniques strongly reduced the attractiveness of the pheromone blend tested in a wind tunnel. Lack of *cis*-5-decenyl acetate (Z5-10:Ac) or Z11-16:OH did not affect the blend attractiveness. Chemical and behavioral analyses showed that pheromone components are produced during photophase, at least 2 hr before lights off. Quantitative data showed that decapitation inhibited the production of Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, and Z11-16:OH, but production in decapitated females was stimulated in response to injection of synthetic *Heliothis zea* PBAN (pheromone biosynthesis activating

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neuropeptide) or *A. ipsilon* brain-subesophageal (Br-SEG) homogenates. Moreover, upon injecting BR-SEG homogenates, other minor components were detected, which were tentatively identified as *cis*-8-dodecenyl actetate (Z8-12:Ac) and Z5-10:Ac. Our study demonstrated that Z11-16:Ac is one of the main active components produced by the pheromone gland of this European population of *A. ipsilon*, in addition to Z7-12:Ac/Z9-14:Ac, which were investigated in a previous behavioral analysis. All these data strongly suggest that some polymorphism is present in pheromone communication in different strains of *A. ipsilon*.

Key Words—*Agrotis ipsilon*, sex pheromone, gas chromatography/high-resolution mass spectrometry, pheromone biosynthesis activating neuropeptide, *cis*-7-dodecenyl acetate, *cis*-9-tetradecenyl acetate, *cis*-11-hexadecenyl acetate, polymorphism.

INTRODUCTION

The black cutworm moth, *Agrotis ipsilon*, is a serious pest and threat to many agricultural crops throughout the world (Rings et al., 1975). On the American continent, *A. ipsilon* migrates from southern Louisiana or south-central Texas northward into Iowa, Kansas, and Missouri each year (Showers et al., 1989a, b). Environmental changes in the late summer and early autumn cause the moths to move southward again (Showers et al., 1993). Laboratory and field tests on the North American strain showed that the attractive blend was a mixture of two compounds, *cis*-7-dodecenyl acetate (Z7-12:Ac) and *cis*-9-tetradecenyl acetate (Z9-14:Ac), at a ratio of approximately 5 to 1 (Hill et al., 1979).

The European population of the black cutworm migrates northwards in the spring from northern Africa and southern Europe (Onyango Odiyo, 1975) into northern Europe, where the first generation reproduces. The second generation moves southward in the fall (Causse et al., 1989). Z7-12:Ac and Z9-14:Ac, the most effective lures used on the American *A. ipsilon*, were ineffective on the strain of moths in field studies in France and Japan. Adding *cis*-11-hexadecenyl acetate (Z11-16:Ac) to the two-component blend increased the catch (Causse et al., 1988) and increased the attractiveness of the two-component blend in a wind tunnel when the blend ratio was 20/5/20 (Z7-12:Ac/Z9-14:Ac/Z11-16:Ac) (Wakamura et al., 1986). These results suggested that American, Asian, and European populations of *A. ipsilon* may use different pheromone blends, as has been reported from different populations of the turnip moth, *A. segetum* (Arn et al., 1983; Löfstedt et al., 1982, 1986).

We have investigated the sex pheromone composition of the French strain to check whether Z11-16:Ac is one of the pheromone components produced by European females of *A. ipsilon*. To our knowledge, no previous chemical analyses have been carried out on the pheromone composition of the European strain of *A. ipsilon*. We report here chemical determination by high-resolution GC–

MS, wind tunnel tests, and brain control of the pheromone gland secretion of a French population of *A. ipsilon* females shown to produce the active mixture Z7-12:Ac/Z9-14:Ac/Z11-16:Ac associated with Z11-14:Ac and Z11-16:OH. The discussion deals with the existence of polymorphism in the sex pheromone system used by various populations of *A. ipsilon*, in particular, between those inhabiting American and European regions.

METHODS AND MATERIALS

Insects

The colony of *A. ipsilon* originated from wild moths caught with light traps and female-baited traps in southern France (Camargue area). Black cutworm moth larvae were reared on an artificial diet and kept as described by Poitout and Buès (1974) under a 16L:8D photoperiod at 21°C. Late instars were reared individually to prevent cannibalism. Boxes of pupae were observed every day, and the day on which an adult emerged was taken to be day 0 of adult life. Adults of both sexes were isolated at emergence, placed in plastic boxes with an aqueous 20% sucrose solution, and given a reversed 16L:8D photoperiod. Adults from field catches were introduced into the stock colony each spring.

Decapitation and Hormone Injection Procedures

Pheromone biosynthesis activating neuropeptide (PBAN) is known to induce pheromone biosynthesis in females of Lepidoptera studied so far and it shows biological interspecificity (Sreng et al., 1990; Raina, 1993; Masler and Raina, 1993). Brain–subesophageal ganglion (Br-SEG) extracts from *Lymantria dispar* and *Heliothis zea* showed strong pheromonotropic activity when injected into ligated *H. zea* females (Raina et al., 1987). Based on these results, we decided to use PBAN to isolate the main components of the pheromone secretion of *A. ipsilon*, as has been done with *Cydia splendana* (Frérot et al., 1995).

Day 3 females of *A. ipsilon* were anesthesized with CO_2 and decapitated with microscissors 24 hr before injection of saline, synthetic Hez-PBAN, or Br-SEG extract. The decapitated adults were kept together in a large humidified box until injection treatment or gland excision at day 4. It is known that decapitated females of this species do not produce any sex pheromone (Picimbon et al., 1995).

Day 7 control females and 24-hr decapitated females were injected between the fourth and the fifth abdominal sternites using a 10-μl Hamilton syringe with 2 μl of a solution containing 1 Br-SEG equivalent or with 2 μl of a solution of 5 pmol Hez-PBAN 2 hr before lights-off. Br-SEG extracts were prepared as

described by Picimbon et al. (1995). PBAN was synthesized by Biosystem (Strasbourg, France) based on the purified *H. zea* PBAN sequence (Raina et al., 1989), and the lyophilized PBAN was diluted in saline just before injection.

The pheromone gland was excised from injected experimental and control females 6 hr after Br-SEG, Hez-PBAN, or saline injection, and the glands were extracted with hexane (Sreng et al., 1990; Picimbon et al., 1995). The pheromone titer was analyzed by gas chromatography (GC).

Chromatography and Fraction Collections

Fractions of female gland extracts were obtained in the following manner. The extract from 10 female glands (day 4/8 females) was fractionated by gas chromatography on a 2-m × 3-mm-ID glass column packed with a 3% CP-Sil5 Chromosorb phase (100–120 mesh) in a 300 Girdel gas chromatograph. A temperature program beginning at 80°C was programmed to 180°C at 5°C/min (final time, 30 min). Nitrogen was used as carrier gas at a linear flow velocity of 2 bar. Synthetic standards consisting of Z5-10:Ac, Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, and Z11-16:OH were chromatographed to determine retention times. The column effluent was split to send 2/10 of the extract to the FID detector and 8/10 to a capillary glass tube embedded in Dry Ice for collection of volatiles. Pheromone volatiles from gland extracts were trapped on the cold capillary tube wall, and subsequently eluted with 200 μl of hexane and concentrated to 50 μl. In order to bioassay a blend minus a specific component, we eliminated a specific component from the total gland blend during chromatography by disconnecting the cold capillary trap connection for a 2-min interval beginning 1 min before elution (based on retention time) of a particular compound and ending 1 min after its retention time. The trapped volatiles thus contained a blend minus one of the components detected by chemical analysis: Z5-10:Ac, Z7-12:Ac, Z9-14:Ac/Z11-14:Ac, Z11-16:Ac, or Z11-16:OH. A total collection (0 to 50-min trapped collection from the GC) of pheromone gland extract was used as a control. Collected samples were kept at −20°C until bioassayed.

Bioassay

Behavioral tests were performed in a wind tunnel (2 × 0.63 × 0.63 m). Responsiveness of day 4 males exposed to various experimental chemical sources was assessed during 2-min periods at 21°C under a red filtered light source of less than 2 lux (to which moths showed no reaction) (Picimbon et al., 1995). Male behavior on the downwind release platform was scored using the following sequence: clasper display (CD), incomplete flight (IF) (flight less than 100 cm) or complete flight (CF) (flight greater than 100 but less than 150 cm), and landing on the source (L). Day 4 control males were placed individually in wire-

net cages 150 cm downstream from the pheromone source in the wind tunnel. Wind speeds were maintained at 1 m/sec. Sexually mature males were subjected once to pheromone gland extract from conspecific females or to a fractionated sample. Glandular extracts (1 FE) were obtained from females of various ages (0, 4, 7, and 12 days old) at midscotophase and from day 4 females in the photophase (2 hr before lights off). Samples or gland extracts were dispensed on filter paper (50 μl) that was placed upstream in the wind tunnel. Responsiveness of tested males was compared by calculation of the mean score of each group. Statistical significance of treatments assessed by the G test was accepted at $P \leq 0.05$.

Chemical Analysis

Extraction and Gas Chromatography Analysis. Adult female abdomens (day 4 females) were squeezed until the ovipositor extruded, and individual female sex pheromone glands were then excised in the middle of the scotophase, at the peak time of calling (Swier et al., 1977; Gadenne, 1993). Individual glands were placed in 10 μl of hexane, and the pheromone content was quantified by gas chromatography with 10 ng added tridecanyl acetate (13:Ac) as an internal standard (IS). Glands from day 4, day 7, and day 8 females in photophase were excised 2 hr before lights-off and analyzed by the same procedure. Pooled samples were prepared by immersing 3, 5, or 10 glands in 30, 50, or 100 μl of hexane containing 10, 50, or 100 ng of IS, respectively. A gland was left to soak in the solvent for at least 1 hr before the extract was evaporated under nitrogen, and 2 μl was injected into the chromatograph. The gas chromatograph was a Hewlett–Packard HP 5890 GC equipped with a 30-m × 0.25-mm-ID Supelcowax 10 or a 25-m × 0.25-mm-ID CP-Sil5 column with a split/splitless sample injector and a flame ionization detector. An HP 3396 Series II integrator recorded the data. The temperature program ranged from 80°C for 1 min, then programmed to 180°C at 5°C/min (final time, 20 min), for the Supelcowax 10 column and from 80°C for 1 min, then programmed to 240°C at 5°C/min (final time, 15 min), for the CP-Sil5 column.

Statistical analyses of the GC values were performed with the Statgraphics V5-01 software program and any differences between the means were tested to determine their significance at $P \leq 0.05$ in a Mann–Whitney U test.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. Gas chromatography–mass spectrometry (GC-MS) was carried out using a Hewlett Packard 5989 high-resolution spectrometer. Extracts were prepared by bathing 10 pheromone glands from day 4 to day 7 females excised at midscotophase for 1 hr in 200 μl of hexane containing 100 ng of IS. Samples were concentrated under nitrogen flow prior to the injection of the whole extract (2 μl) into the GC-MS instrument. Based on behavioral tests, the active blend was expected to be the mixture Z7-12:Ac/Z9-14:Ac/Z11-16:Ac, and extracts were analyzed

to verify the presence of the active compounds and their analogues in the gland secretion by selected ion monitoring of *m/z* values 61 ($CH_3COOH_2^+$, for acetates), 31 (CH_2OH^+, for alcohols), 166, 194, and 222 (M^+-CH_3COOH and M^+-H_2O for both monounsaturated acetates and alcohols of 12, 14, and 16 carbons, respectively).

The GC-MS data were obtained after separating the products on a 50-m × 0.2-mm column of CP-Sil5. The column temperature was increased from 80 to 240°C at 5°C/min and held at 240°C for 15 min. The purge valve was opened 1 min after the injection and the carrier gas (helium) pressure was 20 psi. Electron impact (EI) mass spectra were collected at 80 eV with the separator and source at 240°C. Extracts of 10 female pheromone glands (10 FE) were needed for chemical detection using an electron multiplier programmed at 2800 eV to enable detection in the picogram range beginning 15 min into the analysis. The chemical components were identified by comparing their mass spectra with those of authentic standards, and identification was checked subsequently by comparing the GC retention time of the natural product with that of an authentic standard under the same GC conditions as those described above.

The standards used were *cis*-5-decenyl acetate (Z5-10:Ac), *cis*-7-dodecenyl acetate (Z7-12:Ac), *cis*-8-dodecenyl acetate (Z8-12:Ac), *cis*-9-tetradecenyl acetate (Z9-14:Ac), *cis*-11-tetradecenyl acetate (Z11-14:Ac), *cis*-11-hexadecenyl acetate (Z11-16:Ac), and *cis*-11-hexadecenol (Z11-16:OH), and tridecanyl acetate (13:Ac) (Sigma, France).

RESULTS

Effects of Age on Pheromone Production

Data on effect of age upon pheromone production are presented in Figure 1. Pheromone gland extracts (1 FE) from newly emerged (day 0) females of *A. ipsilon* did not attract sexually mature males when tested in a wind tunnel. However, glandular extracts from day 4 females triggered a male response in the behavioral assay, and similar results were obtained with glandular extracts from day 7 females. No sexual attraction was observed in 80% of the males tested with glandular extracts from day 12 females. These results showed that pheromone was found principally in the glands from day 4–day 7 females.

Chemical Identification

The pheromone was expected to contain Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac according to behavioral data from the capture of males in the field (Causse et al., 1988). High-resolution GC-MS analysis on pooled samples with monitoring of diagnostic ions showed that gland-derived peaks coeluted with synthetic

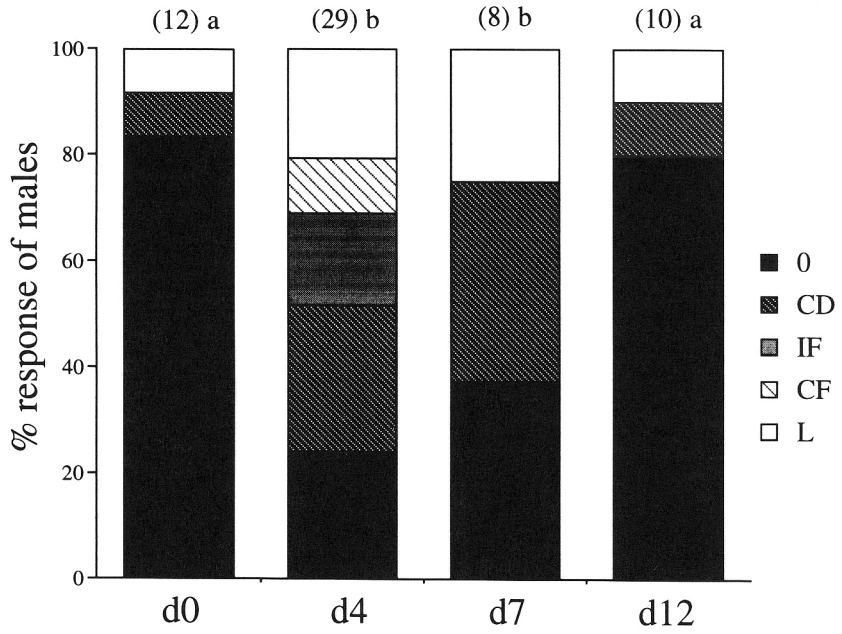

FIG. 1. Pheromonal responsiveness of day 4 males of *A. ipsilon* exposed to hexane gland extracts from day 0 (d0), day 4 (d4), day 7 (d7), and day 12 (d12) females of *A. ipsilon* in scotophase. 0, no response; CD, clasper display; IF, interrupted flight; CF, complete flight; L, landing on the source. Number in parentheses is the number of tested males. Bars followed with the same lowercase letter are statistically similar (statistical *G* test, $p \leq 0.05$).

Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, and Z11-16:OH (Figure 2). Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac were identified from the GC-MS spectra by looking for diagnostic ions corresponding to the alkyl chain and the acetate groups (ion of *m/z* 61) and then comparing the pheromone mass spectra with those of specific synthetic standards (Figures 3B–G). A fourth acetate was identified as Z11-14:Ac. Monitoring the *m/z* 31 ion and the *m/z* 222 ion showed the presence of Z11-16:OH in the pheromone gland extracts. No additional pheromonal components could be detected by monitoring the *m/z* 61 ion, characteristic of acetates.

Quantitative Analysis

Table 1 shows the amount of pheromone components collected from females in midscotophase (mean ± SE). An extract of 1 gland (1 FE) from a day 4 female gave the following values for the gland components: 0.2 ± 0.1 ng of Z7-12:Ac, 0.5 ± 0.3 ng of Z9-14:Ac, 0.3 ± 0.1 ng of Z11-14:Ac, 1.4 ±

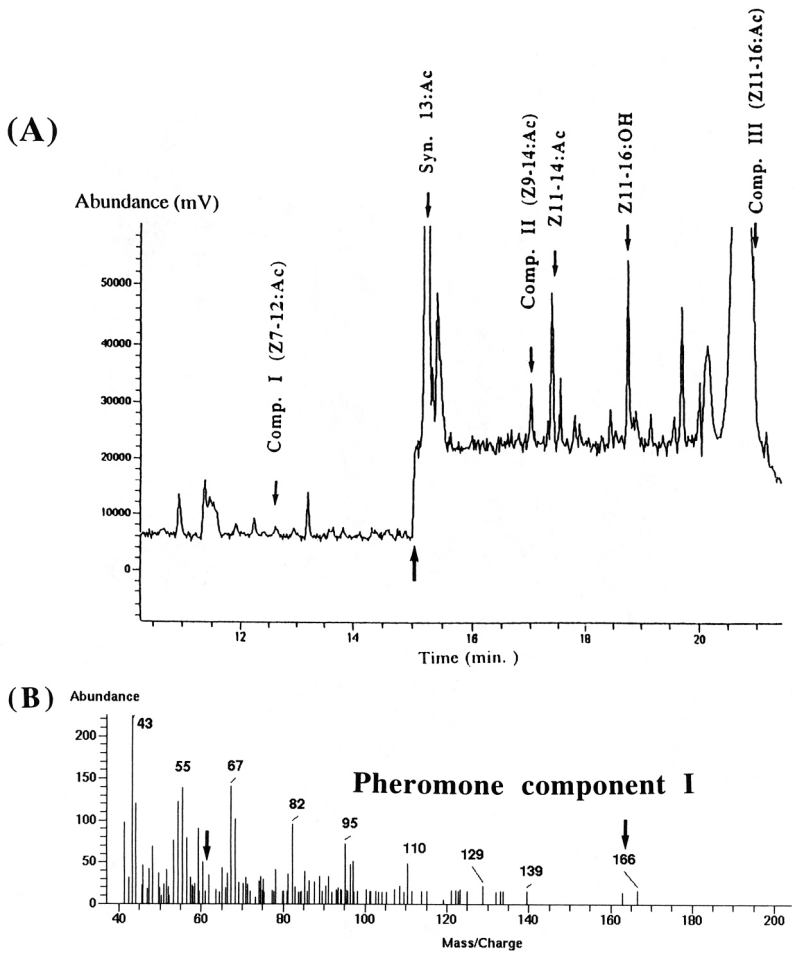

FIG. 2. GC/MS analysis of pheromone gland secretion of females from the French population of *A. ipsilon* and synthetic standards. (A) Total ion current (TIC) by electron impact (EI) of pheromone gland extract (10 FE) from calling day 4 *A. ipsilon* females at midscotophase. (B) Mass spectrum of pheromone component I. (C) Mass spectrum of synthetic *cis*-7-dodecenyl acetate (Z7-12:Ac). (D) Mass spectrum of pheromone component II. (E) Mass spectrum of synthetic *cis*-9-tetradecenyl acetate (Z9-14:Ac). (F) Mass spectrum of pheromone component III. (G) Mass spectrum of synthetic *cis*-11-hexadecenyl acetate (Z11-16:Ac). Arrows in B, D, and F indicate monitored diagnostic ions allowing identification of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac. Conditions: CP-Sil5 capillary column (25 m × 0.25-mm ID); temperature, 80–240°C (5°C/min); carrier gas, helium at a flow rate of 2 ml/min. After a 15-min run, the electron multiplier was increased from 800 to 2800 eV as indicated by the arrow in A. Synthetic 13:Ac was the internal standard.

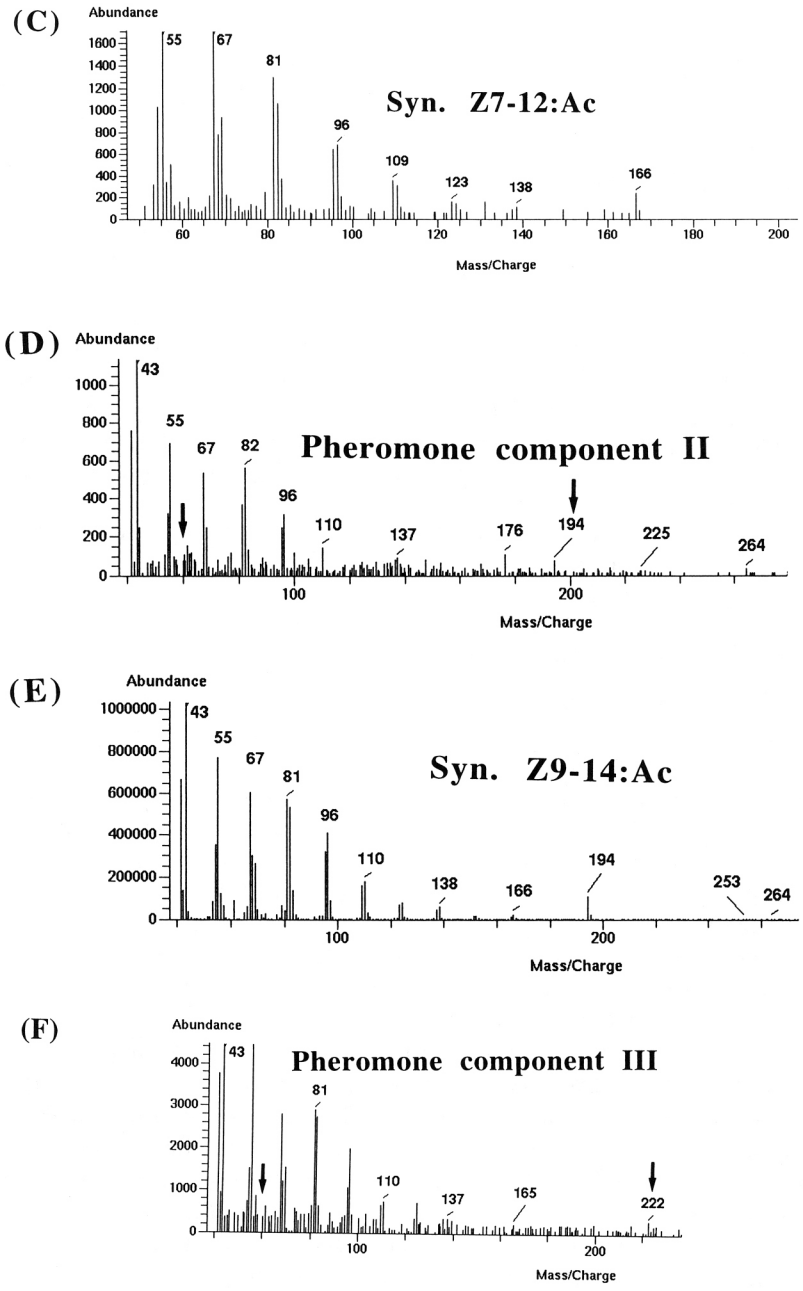

FIG. 2. Continued.

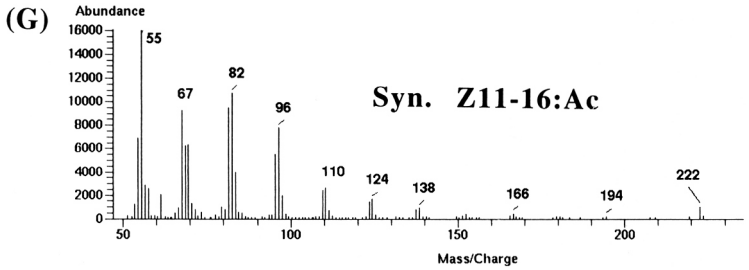

FIG. 2. Continued.

0.3 ng of Z11-16:Ac, and 0.8 ± 0.4 ng of Z11-16:OH per female. Additional data are shown in Table 1 for combined gland extracts from multiple day 4 females, day 7 females, and females sampled during the photophase. Extracts of 1 and 10 glands taken from females in the photophase 2 hr before lights off showed that traces or very small amounts of the five components were produced in the photophase.

Injection of 5 pmol of Hez-PBAN or 1–2 Br-SEG equivalents into the abdomen of control females during photophase did not enhance the production of pheromone (Table 1).

Effects of Fractions of Pheromone Blend on Behavioral Responses of Males

The retention times of Z5-10:Ac, Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:OH, and Z11-16:Ac were 11.5, 16.5, 20.1, 20.1, 24.6, and 29.6 min, respectively, based upon synthetic standards injected into the CP-Sil5 column. Fractions that represented the pheromone blend minus one of the five components were trapped from a GC column and bioassayed in a wind tunnel. The total glandular blend was collected from GC in a 0- to 50-min fraction and used for the control experiments. The total blend (F in Figure 3, 0- to 50-min collection) attracted day 4 males in a wind tunnel but was slightly less attractive than a crude glandular extract from one female in midscotophase. The total blend was much more attractive than a blend lacking Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac (Figure 3).

The 0- to 15- plus 17- to 50-min fractions (the blend lacking Z7-12:Ac) did not induce any pheromone response at all. Similarly, the 0- to 20- plus 22- to 50-min fractions (blend lacking Z9-14:Ac/Z11-14:Ac) and the 0- to 28- plus 30- to 50-min fractions (lacking Z11-16:Ac) failed to attract the males or elicited poor responses. The 0- to 10- plus 12- to 50-min fractions (the blend lacking Z5-10:Ac) and the 0- to 23- plus 25- to 50-min fractions (lacking Z11-16:OH) elicited pheromone activity in a way similar to that of the control fraction (Figure 3), suggesting that the latter two components were not necessary for attraction. These fractionation procedures confirmed that the active components were Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac.

TABLE 1. EVALUATION OF THE RATIO BETWEEN PHEROMONE COMPONENTS IN FEMALE *A. ipsilon*[a]

	n	Pheromone titer (ng/female ± SE)				
		Z7-12:Ac	Z9-14:Ac	Z11-14:Ac	Z11-16:Ac	Z11-16:OH
10-gland extract, day 4 female, control scotophase	3	0.2 ± 0.1	1.5 ± 0.3	0.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
5-gland extract, day 4 female, control scotophase	2	0.1 ± 0.1	0.6 ± 0.5	0.4 ± 0.3	0.8 ± 0.5	0.7 ± 0.6
1-gland extract, day 4 female, control scotophase	9	0.2 ± 0.1	0.5 ± 0.3	0.3 ± 0.1	1.4 ± 0.3	0.8 ± 0.4
1-gland extract, day 7 female, control + Hez-PBAN	5	Trace	0.5 ± 0.2	0.1 ± 0.1	0.5 ± 0.2	0.2 ± 0.1
1-gland extract, day 7 females, control + 2 Eq Br-SEG	5	0.2 ± 0.1	0.6 ± 0.3	Trace	2.3 ± 1.1	1.5 ± 1.3
1-gland extract, day 7 females, control + 1 Eq Br-SEG	5	Trace	0.7 ± 0.1	Trace	0.5 ± 0.2	0.2 ± 0.1
1-gland extract, day 8 females, photophase	5	Trace	1.7 ± 0.5	Trace	Trace	1.1 ± 1.1
10-gland extract, day 4 females, photophase	3	0.1 ± 0.05	0.9 ± 0.4	0.4 ± 0.2	0.7 ± 0.4	0.05 ± 0.03

[a]Levels of GC/MS-identified components in the pheromone gland secretion of day 4/7/8 females of *A. ipsilon* were measured by GC analysis of hexane extracts of 10, 5, or 1 pheromone glands using the internal standard method. Quantitative data were obtained from control females in scotophase or in photophase and from control females treated with Hez-PBAN or a Br-SEG homogenate. Data were then reported in 1 female equivalent (FE). N designates the number of GC-analyzed hexane extracts.

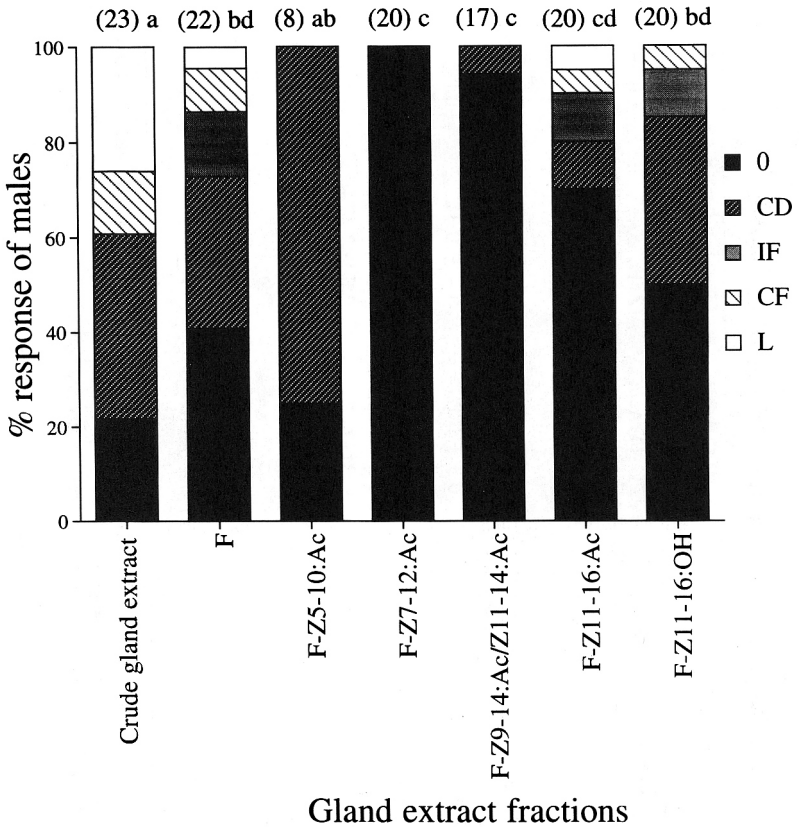

FIG. 3. Pheromonal responsiveness of day 4 *A. ipsilon* males exposed in a wind tunnel to 1 FE hexane pheromone gland extract obtained from day 4 control females at midscotophase to the GC-trapped complete pheromone mixture (F) and to selectively partitioned pheromone mixtures. Lack of Z5-10:Ac, Z7-12:Ac, Z9-14:Ac/Z11-14:Ac, Z11-16:Ac, and Z11-16:OH in the mixture indicated by F-Z5-10:Ac, F-Z7-12:Ac, F-Z9-14:Ac/Z11-14:Ac, F-Z11-16:Ac, and F-Z11-16:OH, respectively. Removal of each component from the complete blend was considered according to results from chemcial identification. Bars labeled with the same lowercase letter are statistically similar (statistical G test, $p \leq 0.05$). 0, no response, CD, clasper display; IF, interrupted flight; CF, complete flight; L, landing on the source. Number in parentheses is the number of tested males.

Influence of Diel Periodicity on Pheromone Production

Sexually mature males exhibited about equal responses to 1 FE of pheromone gland extract from day 4 females during photophase, 2 hr before lights-off, and to 1 FE gland extract prepared in midscotophase from day 4 females (Figure 4).

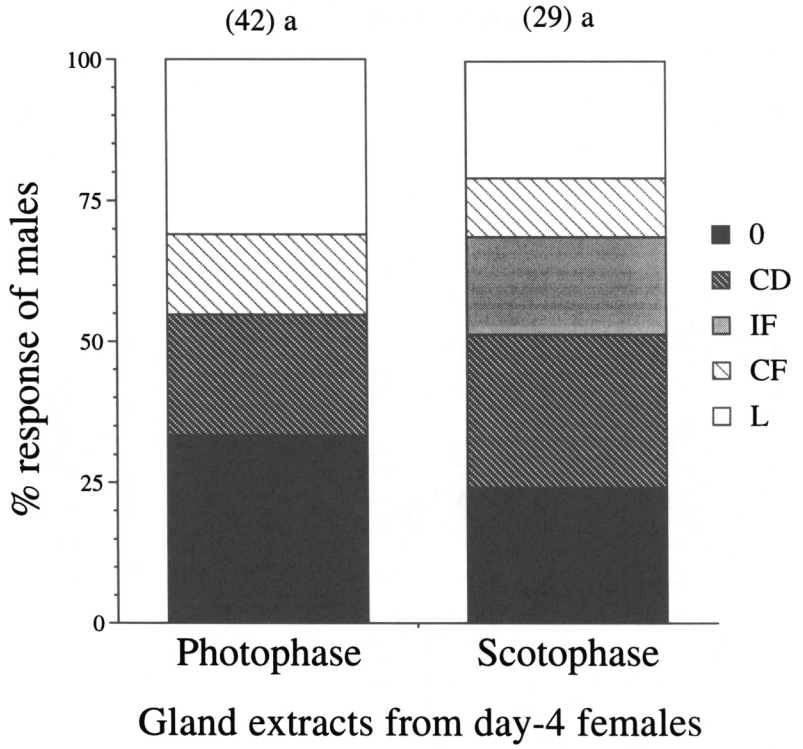

FIG. 4. Pheromonal responsiveness of day 4 males of *A. ipsilon* exposed to hexane gland extracts from day 4 females in photophase, 2 hr before the onset of scotophase, and in scotophase, 4 hr after lights-off. 0, no response; CD, clasper display; IF, interrupted flight; CF, complete flight; L, landing on the source. Number parentheses is the number of tested males. Bars labeled with the same lowercase letter are statistically similar (statistical G test, $p \leq 0.05$).

Effects of Brain Factor

The data are presented in Figure 5. We showed earlier that pheromone production was inhibited in *A. ipsilon* after decapitation but restored with synthetic PBAN or Br-SEG treatment (Picimbon et al., 1995). We therefore tested the effects of decapitation followed by PBAN or Br-SEG treatment on the female pheromone gland secretion. Capillary GC analysis of gland extracts (1–3 FE) showed that decapitation abolished production of Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, and Z11-16:OH, but injection of Hez-PBAN restored the control levels (Figure 5). Production of Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, and Z11-16:OH was inhibited by decapitation, but the level of Z7-12:Ac in decapitated females did not differ significantly from that measured in control females, probably because of strong interspecific variations (two decapitated

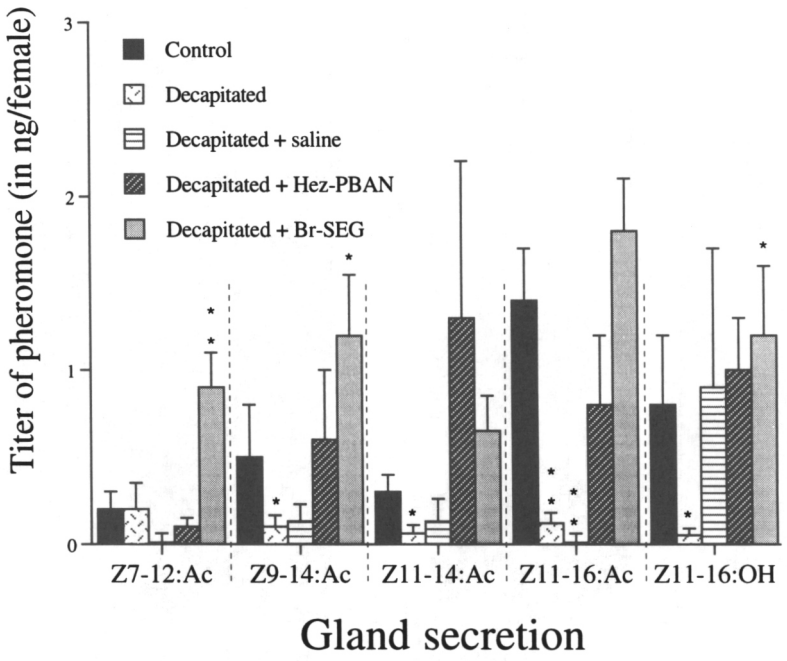

FIG. 5. Pheromone concentration expressed as ng/female of (left to right) Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, and Z11-16:OH of untreated virgin day 4 *A. ipsilon* female, 1 FE gland extract (control) (N = 9); 24-hr decapitated females (N = 19); 24-hr decapitated females injected with saline (N = 6); 24-hr decapitated females injected with 5 pmol of Hez-PBAN (N = 6); and 24-hr decapitated females injected with 1 Br-SEG equiv extract of control females (N = 20). Error bars represent SE. For each analyzed compound, levels from different treatments were compared with respective controls. Statistical analysis used the Mann-Whitney test ($*P \leq 0.05$;$**P \leq 0.01$) comparing each series of values with controls. N designates number of measures.

females produced pheromone). Injecting Br-SEG into decapitated females strongly increased (mean ± SE/female) the production of Z7-12:Ac (0.9 ± 0.2 ng), Z9-14:Ac (1.2 ± 0.4 ng), Z11-14:Ac (0.7 ± 0.2 ng), Z11-16:Ac (1.8 ± 0.3 ng), and Z11-16:OH (1.2 ± 0.3 ng) (Figure 5). Injecting Br-SEG into decapitated females stimulated production of components tentatively identified as Z5-10:Ac and Z8-12:Ac by comparing retention times with synthetic components, but no structural evidence could be obtained from GC-MS analyses.

DISCUSSION

Our behavioral and GC-MS analyses showed that the pheromone gland of the French strain of *A. ipsilon* contains a multicomponent blend consisting of

at least five components: Z7-12:Ac, Z9-14:Ac, Z11-14:Ac, Z11-16:Ac, and Z11-16:OH. A ternary mixture of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac is active, which differs from results found for the North American population (Hill et al., 1979). Our data support the use of the ternary mixture which has already been used for field trapping (Causse et al., 1988). Z11-14:Ac and Z11-16:OH are present in gland extracts but may not be involved in attraction of males. No olfactory neurons tuned to these two components have been detected on the male antennae (Renou et al., 1996).

Alcohols either played no role or reduced the number of *H. subflexa* (Teal et al., 1981), *Cnaphalocrocis medinalis* (Ganeswara Rao et al., 1993), and *Prionoxystus robiniae* (Doolittle and Solomon, 1986) males captured in pheromone traps. They also influenced pheromone-mediated flight when released upwind of the pheromone source in wind tunnel experiments (Liu and Haynes, 1993; Quartey and Coaker, 1993). Z11-16:OH is known to act as a strong inhibitor of *Mamestra brassicae* males (Struble et al., 1980; Subchev et al., 1985, 1987). Z11-16:OH may increase the trap catch yield in *Pseudaletia unipuncta* (Turgeon et al., 1983), possibly by guiding male flight over a short distance to the pheromone-emitting female.

Z11-16:OH might be involved in inhibition of sympatric species with similar pheromonal components, and it may help in sexual isolation of *A. ipsilon*. Although we have no evidence that Z11-16:OH release occurs during calling, the absence of sensory cells tuned to it (Renou et al., 1996) suggests that this component might not be perceived by *A. ipsilon* males, but it may be detected by other males. Lack of Z5-10:Ac in the blend does not reduce attraction of the pheromone mixture. The presence of sensory cells tuned to Z5-10:Ac in both *A. ipsilon* and *A. segetum* antennae suggests that it could act as an inhibitor of male *A. ipsilon*. Z5-10:Ac is a powerful antagonist of the peach twig borer pheromone (Millar and Rice, 1996) and of *Coleophora laricella* (Priesner and Witzgall, 1984). The possible presence and the role of this component need to be elucidated in further studies of the pheromone communication system between *A. ipsilon* and *A. segetum*. In *A. ipsilon* the primary long-distance stimulation seems to be due only to the combined action of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac. It is noteworthy that Z7-12:Ac is a pheromonal component for more than 126 species of insects (Arn et al., 1992) and has also been reported from the volatiles of an elephant (Rasmussen et al., 1996).

Previous attempts to collect airborne volatiles from *A. ipsilon* females during calling were not successful, probably due to the minute amounts of pheromone released, as in *Autographa gamma* (Mazor and Dunkelblum, 1992). Consequently, it cannot be stated which of the components were emitted in the middle of the scotophase.

Further evidence that Z7-12:Ac and Z9-14:Ac play a key role in sexual attraction in *A. ipsilon* is that a large population of specialist cells responsive to Z7-12:Ac or Z9-14:Ac occurs on the antennae of *A. ipsilon* males. Z7-

12 : Ac triggered the largest EAG response when tested on male antennae of the French *A. ipsilon* (Renou et al., 1996) and with the North American strain (Hill et al., 1979). In the Australian strain the largest EAG response was triggered by Z9-14 : Ac (Common, 1958), which suggests that polymorphism exists among the *A. ipsilon* strains as in the European corn borer, *Ostrinia nubilalis* (Klun et al., 1973; Klun and Maini, 1979; Roelofs et al., 1987).

Sex pheromone production is usually dependent on exogenous factors, mainly the photoperiod (Delisle and Royer, 1994; Babilis and Mazomenos, 1992; Tang et al., 1992; Raina et al., 1986; Pope et al., 1984; Haynes et al., 1983). In *A. ipsilon*, glandular extracts from females at the end of the photophase attracted males in a wind tunnel and contained small amounts of Z7-12 : Ac, Z9-14 : Ac, and Z11-16 : Ac. The presence of pheromone in the gland during photophase was described previously in other moth species, for example, in *Lymantria dispar* (Tang et al., 1992; Giebultowicz et al., 1992), in *Argyrotaenia velutinana* (Bjostad et al., 1981), and in *Trichoplusia ni* (Sower et al., 1972). In these species, the action of the pheromonotropic (i.e., PBAN-like) factor could be independent of the photoperiod, whereas PBAN release is affected by the photoperiod in *H. zea* (Rafaeli et al., 1993; Kingan et al., 1992).

Capillary GC analysis showed that individual pheromone glands of *A. ipsilon* contained only a very small amount of material, as occurs in some Israeli Plusiinae (Mazor et al., 1991; Dunkelblum and Mazor, 1993) and in *A. segetum* (Zhu et al., 1995), thus making routine chemical analysis and detection of minor components difficult.

We used PBAN and BR-SEG homogenates to stimulate production of pheromone components. Br-SEG homogenates are known to be a more efficient means of pheromone enhancement than Hez-PBAN (Picimbon et al., 1995). This strong pheromonotropic potency might be attributable to the fact that the PBAN-like factor in *A. ipsilon* may differ from that purified from *H. zea* brain (Raina et al., 1989), or multiple forms of PBAN may exist in *A. ipsilon*, as occurs in *Bombyx mori* (Kitamura et al., 1989, 1990). Injecting Br-SEG into decapitated females increased the peaks coeluting with standards Z7-12 : Ac, Z9-14 : Ac, Z11-14 : Ac, Z11-16 : Ac, and Z11-16 : OH at the ratio of 9 : 12 : 7 : 18 : 12, as found to occur in sexually active day 4 females. Chemical analysis of the female pheromone from the French strain of *A. ipsilon* has confirmed results from the use of sexual traps in France and Japan by showing the presence of Z11-16 : Ac in the pheromone secretion. Males from the American population were caught largely with lures containing only Z7-12 : Ac and Z9-14 : Ac (Hill et al., 1979). These results suggest the existence of some polymorphism among the different strains of *A. ipsilon* throughout the world, in particular, among the European, Asian, and North American populations. Our preliminary chemical analysis of the pheromone gland secretion of females from the North American strain *A. ipsilon* showed the presence of Z7-12 : Ac and Z9-

14:Ac, confirming previous data from Hill et al. (1979), and Z11-16:OH, but no Z11-16:Ac or Z11-14:Ac, has been detected (Picimbon, 1995). The function of Z11-16:OH in *A. ipsilon* species must be investigated. No specialist cells responsive specifically to Z11-16:Ac have been detected in the male receptor system of the European *A. ipsilon*, although we clearly demonstrated here that this component plays a crucial part in the female pheromone, in agreement with behavioral data from Causse et al. (1988). The mode of perception of Z11-16:Ac by the male olfactory system needs to be investigated. Electrophysiological and biochemical studies of male antennae are now under way, in the hope of throwing further light on the *A. ipsilon* pheromone system.

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ESSENTIAL AMINO ACID METHYL ESTERS: MAJOR SEX PHEROMONE COMPONENTS OF THE CRANBERRY WHITE GRUB, *Phyllophaga anxia* (COLEOPTERA: SCARABAEIDAE)

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Abstract—Chiral capillary gas chromatographic–electroantennographic detection (GC-EAD) analysis indicates that L-valine and L-isoleucine methyl esters are the major sex pheromone components released by females of the cranberry white grub, *Phyllophaga anxia* (LeConte). The GC retention times and GC–mass spectrometry of the two natural compounds were identical to those of authentic standards. Of five reproducible GC-EAD active components revealed with female volatiles, the L-valine and L-isoleucine methyl esters elicited the strongest male antennal responses. The ratio of L-valine and L-isoleucine methyl esters was determined to be 3 : 1 by analysis of pheromone gland extracts. Chirality was shown to be critical by GC-EAD, since only the L-form of these amino acid methyl esters elicited an EAD response. In field experiments conducted in Massachusetts, a synthetic 3 : 1 blend of L-valine and L-isoleucine methyl esters on a rubber septum was attractive to *P. anxia* males.

Key Words—*Phyllophaga anxia*, Coleoptera, Scarabaeidae, cranberry white grub, sex pheromone, chirality, L-valine methyl ester, L-isoleucine methyl ester, electroantennographic detection, field trapping.

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INTRODUCTION

The cranberry white grub, *Phyllophaga anxia*, belongs to the family Scarabaeidae, subfamily Melolonthinae, tribe Melonothini. There is a total of 200 species of *Phyllophaga* widely distributed over the world (Westcott, 1964). About 152 species are native to North America (Tashiro, 1987). Larvae of the genus *Phyllophaga* are commonly known as white grubs due to their body color (Ritcher, 1966), whereas adults are referred to as May or June beetles due to their flight period. Several species of *Phyllophaga* are identified as major agricultural pests, and in the northeastern United States and Canada *P. anxia* has been recognized as the most widespread and injurious species (Tashiro, 1987). Damage is caused by larvae feeding on the roots and underground plant parts. They may eventually kill the host plants, which include forest trees, tree nursery seedbeds, strawberry, cranberry, roses, corn, and turfgrass (Metcalf et al., 1951; Westcott, 1964). Because the larvae are aggressive feeders and reach a relatively large size as third instars (25–38 mm), only a few grubs per unit area can be very damaging (Tashiro, 1987). Although insecticides have been used for larval control of *Phyllophaga* (Franklin, 1950; Metcalf et al., 1951), larval injury is still a serious problem.

Although about 950 insect pheromones and sex attractants have been listed for more than 1300 insect species by the early years of this decade (Mayer and McLaughlin, 1991), chemical communication in the *Phyllophaga* has not been reported until now. To aid in the detection and management of *P. anxia* in cranberry, we initiated a study to determine if a sex pheromone is used for mate location. In a preliminary test, we found that female *P. anxia* volatiles elicited male activity. Males were active in the flight tunnel, but none took flight to either the airborne collection or a female source. In an additional study, we discovered that the female volatiles elicited significant male EAG responses and that the active materials could be separated into several fractions by capillary gas chromatography. Here we report the identification of two pheromone components, methyl esters of the essential amino acids, L-valine and L-isoleucine.

METHODS AND MATERIALS

Insects. Cranberry white grub adults and larvae used in these studies were collected from Cranebrook cranberry bog near Wareham, Massachusetts, in the spring and fall of 1994. The individuals used in the preliminary investigations of summer 1994 were collected in early May of that year, while those used in the spring of 1995 were collected in October and overwintered at 10°C until early March, at which time the females were ready to emerge from the soil and

produce pheromone in scotophase. Insects were stored in soil-filled ~30-ml disposable plastic cups.

Pheromone Collections. Two female beetles were introduced each time into an airborne collection apparatus, consisting of one three-neck glass bottle (500 ml) connected to a Super Q (200 mg; Alltech Associates, Inc., Deerfield, IL) trap (15 cm × 0.6-cm OD). The air was filtered with a charcoal (activated carbon, 6–14 mesh; Fisher Scientific) trap (7 cm × 1-cm OD) and then passed through a humidifying bubbler tube before being pulled through the apparatus with a vacuum pump at ~1 liter/min. Female beetles were aerated continuously for 8 hr each day, from 0830 to 1630 in scotophase. The beetles were kept on a diel cycle of 16 hr in photophase at 27°C and 8 hr in scotophase at 18.5°C. The Super Q traps were changed every day. The airborne volatiles were eluted from the Super Q trap with four portions of glass-distilled hexane (0.5 ml/each). The resultant hexane solutions were combined and concentrated under a stream of nitrogen to a volume of about 100 μl. The concentrated extracts were used directly for GC-EAD or fractionation on the capillary GC.

Female Gland Extracts. Gland extracts were obtained by compressing the abdomen of the females until the pheromone gland everted from the abdominal tip. Two excised glands were soaked in 200 μl methylene chloride for 20 min. Extracts were combined and concentrated to a small volume (about 100 μl) in a nitrogen stream.

Instrumentation. Fractionation and purification of the active components were performed on a Hewlett Packard 5880A gas chromatograph equipped with a nonpolar SE-30 Econo-Cap capillary column (30 m × 0.25-mm ID, 0.25-μm film thickness; Alltech Associates, Inc., Deerfield, IL) in the splitless mode. The oven temperature was programmed at 40°C for 5 min, then increased at 10°C/min to 200°C and held for 20 min. Injector and detector temperatures were set at 220°C. Nitrogen was the carrier gas, with the flow rate at 2 ml/min. Septum purge flow rate was set at 3 ml/min and split vent flow rate at 2 ml/min, respectively. All of the gas source pressures were 276 kPa (40 psi) and initial column head pressures were 138 kPa (20 psi). Fractions eluting from the column were collected each minute in Dry Ice-chilled 75-μl micropipets (VWR Scientific Inc., Philadelphia, PA), which were cleaned by heating to 550°C in a muffle oven for 12 hr. Each micropipet was rinsed with a total of 100 μl of glass-distilled hexane to remove trapped fractions.

GC–mass spectrometry (GC-MS) was carried out with a Hewlett Packard 5890 gas chromatograph coupled to a HP 5970B Mass Selective Detector or a Finnigan ion-trap detector (ITD 800) using the same SE-30 capillary column and conditions as above, but with helium as the carrier gas.

Electrophysiological Recordings. Pheromone samples were also subjected to GC-EAD analysis using a HP 5880A gas chromatograph equipped with a

HP-5 capillary column (30 m × 0.25-mm ID, 0.25-μm film thickness; Hewlett Packard, Wilmington, DE). The oven temperature was programmed at 50°C for 1 min, then increased at 15°C/min to 250°C and held for 25 min. A β-DEX 120 fused silica chiral capillary column (30 m × 0.25-mm ID, 0.25-μm film thickness; Supelco, Inc., Bellefonte, PA) was used for chirality analysis. The oven temperature was programmed at 80°C for 2 min, then raised at 1°C/min to 220°C and held for 25 min. The capillary column effluent and nitrogen makeup gas (30 ml/min) were split (ca. 1 : 1) by a Y GlasSeal capillary column connector (Supelco, Inc.) to a flame ionization detector (FID) and an electroantennographic detector (EAD). After one antenna was removed from the beetle, one lamella tip and scape were mounted on an acrylic holding station (Leal et al., 1992) with the club open. Two pure gold wires and isotonic saline solution (0.9% NaCl) were used in all experiments. The output recording electrodes were connected by a short coaxial cable to a high-impedance 1 : 100 amplifier (Bjostad and Roelofs, 1980) with automatic baseline drift compensation. The airstream flowing over the antenna (about 500 ml/min) was humidified by bubbling through distilled water before entering the EAD interface. The temperature of the antenna on the acrylic station was maintained at about 16°C by pumping 10°C ice-cooled water into the insulated layer of the modified condenser containing the acrylic station mounted on the top of GC apparatus. An HP 3390 A integrator was used for EAD recording.

The EAG responses of male antennae were obtained using Ag–AgCl glass electrodes (Roelofs, 1984) filled with isotonic saline solution (0.9% NaCl), and they were displayed on a Tektronix 2212 digital storage and analog oscilloscope and printed by a Tektronix HC 200 nine-pin dot-matrix tekprinter. The same high-input impedance DC amplifier (Bjostad and Roelofs, 1980) used in GC-EAD was employed in EAG.

Chemicals. The conversion of individual amino acids into their methyl esters was achieved by the modified method of Brenner (Brenner and Huber, 1953). The esterification reaction proceeded with a 1.2 mol ratio of thionyl chloride, with cooling at ~4°C provided by an ice bath. Thionyl chloride (8.8 ml, 120 mmol) was slowly added to 30.0 ml of anhydrous methyl alcohol cooled with an ice bath. After the addition was completed, isoleucine (13.1 g, 100 mmol) or valine (11.7 g, 100 mmol) was slowly added in portions with vigorous stirring at 0°C. The resulting mixture was stirred at 0°C for 0.5 hr, then warmed to 40°C and maintained at this temperature while being stirred for another 2 hr. The methyl alcohol was removed *in vacuo*, and the residue diluted with 10 ml of water. The reaction mixture was treated with concentrated ammonium hydroxide until the water layer was basic to litmus, then extracted with three portions of ether (100 ml/each). The organic layer was combined, washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The residue was purified by distillation to give pure products in a 70–90% yield. L-Valine (Grand

Island Biological Company, Grand Island, NY) methyl ester was obtained at 80°C under 15 mm Hg and L-isoleucine (Grand Island Biological Company) methyl ester was obtained at 98°C under 15 mm Hg. The unnatural amino acid (D-valine and D-isoleucine from Aldrich Chemical Company, Inc., Milwaukee, WI; D-allo- and L-allo-isoleucine from Sigma Chemical Co., St. Louis, MO) methyl esters were prepared in small amounts in vials stored at 40°C for 4 hr without stirring, and no distillation was performed. The isomeric purity of all isomers was >90% based on results with the capillary GC (FID). The mixtures of DL-isoleucine purchased from either Aldrich or Sigma actually were a mixture of 1:1 D-allo- and L-isoleucine, which was determined and confirmed by chromatography of their methyl esters on the chiral capillary column.

Field Tests. Field tests were conducted in two cranberry white grub infested bogs in Massachusetts during the 1995 field season. Four Fuji Flavor wing-type traps (Fuji Flavor Co., Ltd., 3-5-8 Midorigaoka, Hamura-Shi, Tokyo 205, Japan) baited with septa containing 0, 1, 3, or 10 mg synthetic pheromone were placed 20 m apart in a line on the edge of each of the two bogs. Traps were hung 1 m high on metal stakes. Trapped beetles were removed twice each week from May 15 until July 14. Traps were rotated once each week when the septa were changed.

RESULTS

Identification of the Major Sex Pheromone Component. Although male *P. anxia* did not fly to the pheromone source in the flight tunnel, the airborne extract elicited the same behavioral responses as the presence of females in a screened cage at the upwind end of the tunnel. The excited males stood on their hindlegs with their prothoracic legs waving while keeping the lamellae of their antennae open. They also walked upwind with their elytra partly open. The males did not exhibit these behavioral responses to a blank control (hexane) under the same conditions. The activity of airborne extract was further demonstrated in EAG experiments. One female equivalent of collected pheromone elicited about a 4-mV EAG response with a male antenna, whereas the control (hexane alone) produced a response of less than 1 mV. The airborne extract did not elicit any significant response from female antennae under the same conditions. This suggests that the electrophysiologically active materials are sex specific and probably involved in the mating communication system.

Purification of the pheromone was carried out on a capillary GC and activity monitored by EAG. After the crude hexane extract had been concentrated by a nitrogen stream, it was applied to a nonpolar SE-30 capillary column and fractions were collected each minute. The EAG consistently revealed significant activity in five fractions (9–10, 11–12, 19–20, 21–22, and 23–24 min). These retention times correspond to the following hydrocarbon standards under the

same conditions: nonane (8.50 min), decane (10.95 min), undecane (12.98 min), pentadecane (19.12 min), heptadecane (21.65 min), and octadecane (23.09 min). Further purification of each active fraction on a polar Carbowax capillary column failed, however, possibly due to the small amount of material.

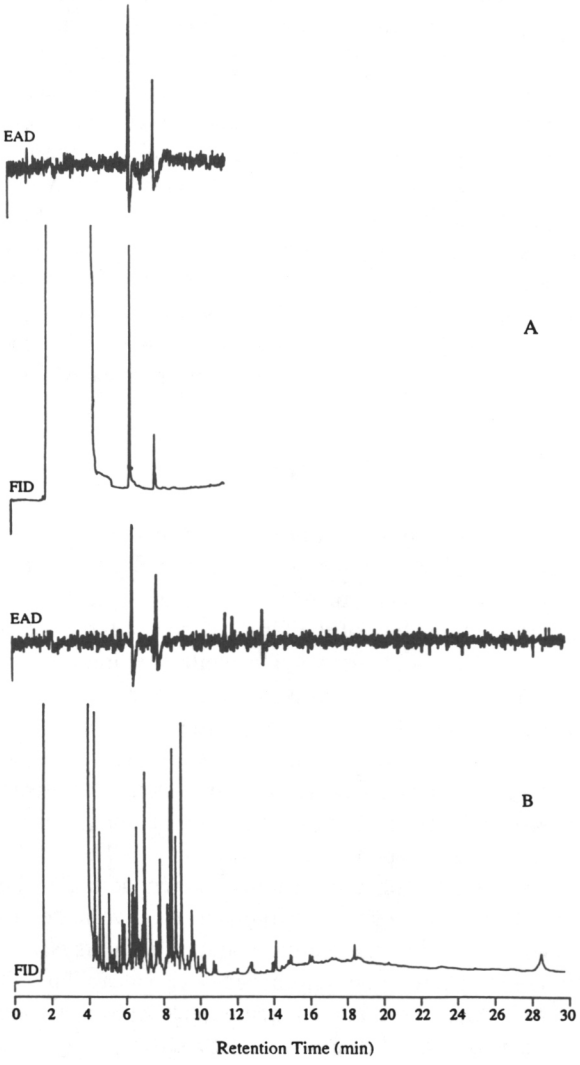
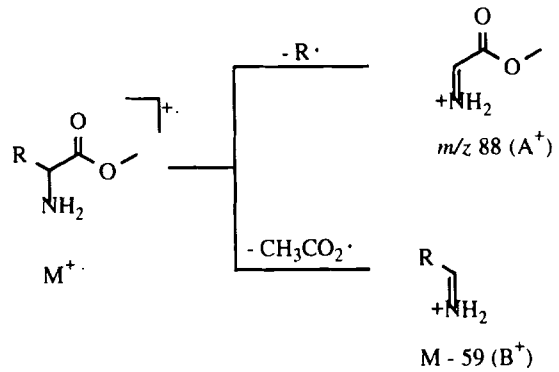


FIG. 1. Reconstructed simultaneous responses of flame ionization detection (FID) and electroanntennographic detection (EAD; antenna of male *P. anxia*) to a mixture of synthetic 3:1 L-valine and L-isoleucine methyl esters (A) and volatiles of female *P. anxia* (B) on a HP-5 capillary column.

Crude airborne extracts were subjected to GC-EAD analysis. Five EAD-active compounds (t_R = 6.27, 7.66, 11.54, 11.96, and 13.48 min) were revealed on a nonpolar HP-5 capillary column (Figure 1B), which were consistent with the GC fractionation studies. However, the ratio of these active peaks could not be determined due to the complexity of the gas chromatographic record and the trace amount of materials. EAD responses to the female-gland methylene chloride extract were obtained only for the two components at the same retention times as the airborne collection at 6.27 and 7.66 min, respectively, and the ratio of the GC peaks was clearly demonstrated as 3:1.

The MS spectrum of the active component corresponding to the first EAD response (t_R = 6.27 min) exhibited a small ion at *m/z* 131 (0.2) along with the following fragments: 41 (8), 55 (46), 72 (100), and 88 (30). The fragment at *m/z* 131 appeared to represent the molecular ion because all fragments at lower mass were chemically reasonable mass differences relative to this signal. This assumption was confirmed by a Finnigan ion-trap detector (ITD 800), in which self-ionization gave rise to the M+1 peak (*m/z* 132) as the highest mass unit. The odd mass value indicated the presence of an odd number of nitrogen atoms in the molecular formula (nitrogen rule). Since there was no evidence for the presence of other heteroatoms and no reason for assuming the presence of three nitrogen atoms in such a small molecule, the most likely molecular formulae were $C_4H_5NO_4$, $C_5H_9NO_3$, and $C_6H_{13}NO_2$. The fragment at *m/z* 88 (M^+-43) probably represented loss of C_3H_7 or CH_3CO. With a base peak at *m/z* 72 (M^+-59), the cleavage of CO_2CH_3 from the molecule probably occurred. Based on the above information, the compound was likely to be an amino acid ester. At this point, we tentatively refined our molecular formula to $C_6H_{13}NO_2$, and proposed the structure to be valine methyl ester.

The cleavage of one of the carbon–carbon bonds next to the amino group was a very prominent fragmentation reaction in all amino acid esters (Scheme 1). The formation of immonium ions is the driving force in this fragmentation.



SCHEME 1. Degradation pathway of the amino acid methyl ester.

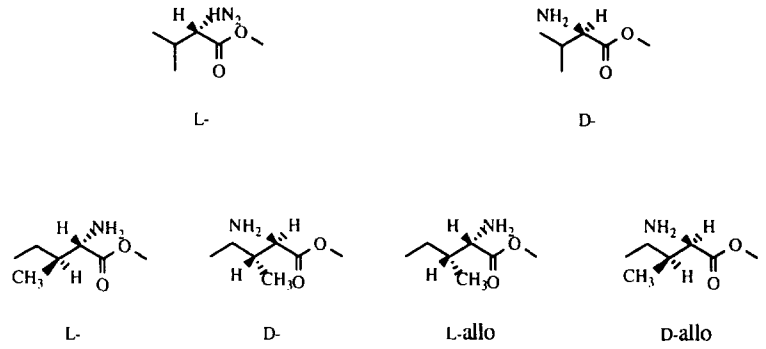
The stability of such ions is so high that the abundance of the molecular ions usually is quite low. Of the two possible pathways, the one in which the carboxyl group is lost is more predominant than the cleavage of group R leading to ion A^+ (*m/z* 88) and gives rise to the M-59 base peak ion B^+ (*m/z* 72). The reason is that the immonium ion A^+ formed during fragmentation is slightly destabilized by the negative inductive effect of a conjunctive carboxyl group and thus gives rise to a lower abundance than ion B^+.

This characteristic fragmentation was clearly shown in the spectrum of the first EAD active peak (t_R = 6.27 min). The ion B^+ (*m/z* 72) corresponding to loss of 59 mass units from molecular *m/z* 131 was the base peak. The ion A^+ (*m/z* 88) attributed to another primary cleavage was a prominent peak with abundance at about 30%. These data supported our proposal of valine methyl ester as the active component at 6.27 min.

An identical fragmentation pattern was observed in the MS spectrum of the second EAG-active component (t_R = 7.66 min). The following fragments were detected: 41 (23), 44 (22), 56 (15), 57 (17), 69 (15), 74 (14), 86 (100), 88 (40), 116 (0.3), 130 (0.1), and 145 (0.1). It was observed that both the base peak B^+ (*m/z* 86) and the highest mass unit (*m/z* 145) were 14 units higher than those for the first EAD-active peak (t_R = 6.27), and the A^+ ion (*m/z* 88) was also found as a prominent peak. The M+1 peak (*m/z* 146) was the highest mass unit detected by a Finnigan ion-trap detector (ITD 800), indicating that *m/z* 145 was the molecular ion. All of these data suggested that this compound is an amino acid methyl ester that possesses one more methylene unit in the R group than the valine methyl ester. Three structural possibilities existed; the R group could be butyl, isobutyl, or *sec*-butyl. Thus, norleucine, leucine, and isoleucine methyl esters were candidates.

To distinguish among these possibilities and verify that the first EAD-active component was valine methyl ester, authentic standards were synthesized. Isomers such as norleucine, leucine, and isoleucine methyl esters were readily distinguished by comparing their GC retention times and MS spectra. Only synthetic L-valine and L-isoleucine methyl esters had retention times (t_R = 6.27 and 7.66 min) and MS spectra that were identical to those of the first and second EAD-active components produced by female *P. anxia*. A synthetic 3:1 blend of L-valine and L-isoleucine methyl esters also elicited the same male antenna EAD responses as the airborne collection (Figure 1A). Thus, valine and isoleucine methyl esters at a 3:1 ratio were identified as the major electrophysiologically active components of *P. anxia*. This synthetic blend was used for the remaining experiments.

Chiral Specificity Characterization. Valine methyl ester has one chiral center, and two enantiomeric forms are possible, L-valine and D-valine methyl esters. The isoleucine methyl ester, however, has two chiral centers, and two pairs of enantiomeric forms are possible, L- and D-isoleucine methyl esters and



SCHEME 2. Stereoisomers used to characterize the specificity of male *P. anxia* antennae. Top: Valine methyl esters. Bottom: Isoleucine methyl esters.

L-allo- and D-allo-isoleucine methyl esters (Scheme 2). The L-forms are the naturally occurring amino acid methyl esters, and the D-forms have physical properties identical to those of L-forms except for their optical activity. To examine the chiral specificity of receptors in male *P. anxia* antennae and identify the chirality of the natural components, all of the possible enantiomers were synthesized individually. Chromatography on chiral capillary columns and electroantennographic detection analyses were employed.

Significantly different GC-EAD responses from D- and L-stereoisomers were observed with 20-ng dosages on the male antenna. Synthetic L-valine methyl ester (t_R = 9.78 min) elicited strong antennal responses, whereas the D-enantiomer (t_R = 9.64 min) was EAD inactive (Figure 2). Interestingly, D-isoleucine methyl ester (t_R = 15.12 min) and D-allo-isoleucine methyl ester (t_R = 14.92 min) diastereomers were EAD inactive, but both L-isoleucine methyl ester (t_R = 15.40 min) and L-allo-isoleucine methyl ester (t_R = 14.74 min) diastereomers elicited equally high EAD responses (Figure 3). Additionally, even though the D-isomers in valine and isoleucine methyl esters elicited no EAD activity, they appeared to affect the EAD responses of the L-isomers negatively when eluting just prior to those isomers (Figures 2 and 3).

Results of the chiral capillary resolution experiments with female-gland extracts were consistent with chiral specificity for synthetic valine and isoleucine methyl esters by GC-EAD. The natural components exhibited retention times identical to those of authentic L-valine and L-isoleucine methyl esters on a β-DEX 120 fused silica chiral capillary column (Figure 4). The possible trace presence of other stereoisomers was beyond the limits of FID detection.

In field experiments conducted in Massachusetts, traps baited with a 3:1 blend of L-valine and L-isoleucine methyl esters at three dosages (1, 3, and 10 mg) on rubber septa proved to be attractive (Table 1). The composition of our

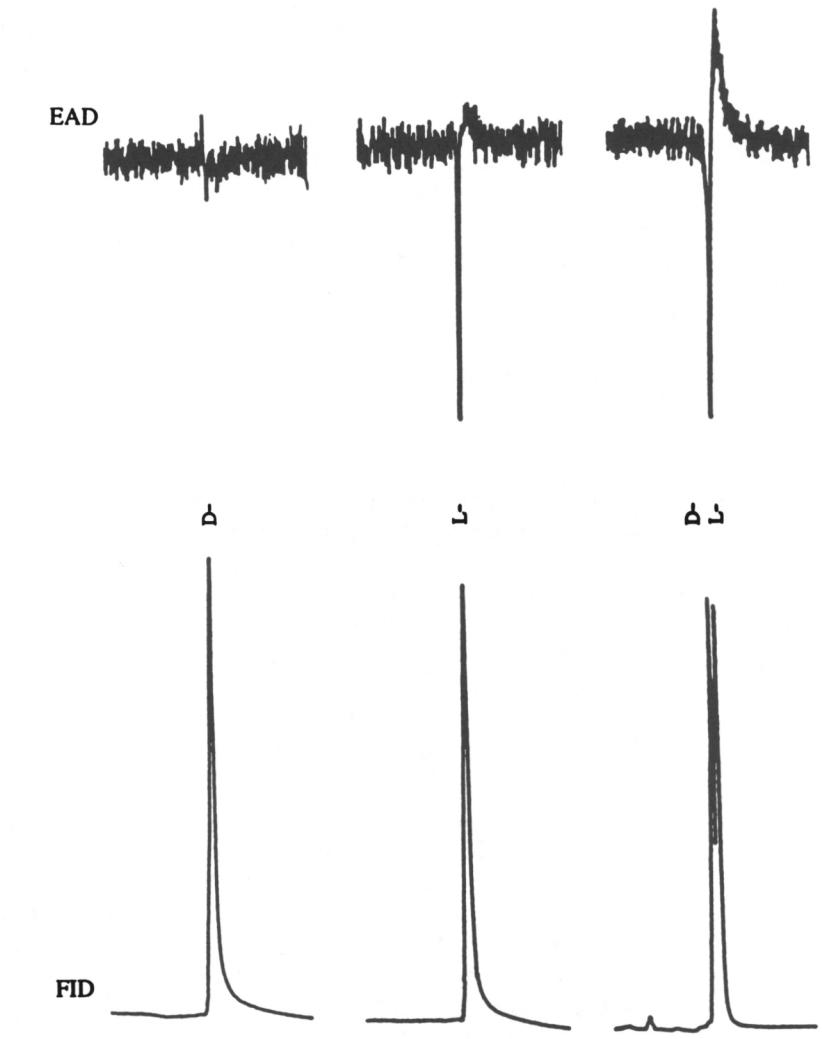


FIG. 2. Partial, reconstructed simultaneous responses from FID and EAG detection using an antenna of male *P. anxia* to synthetic D, L, and DL-valine methyl esters, respectively, on a β-DEX 120 chiral capillary column.

synthetic valine methyl ester was estimated to be 10% D- and 90% L-isomer. Synthetic L-isoleucine methyl ester contained about 3% D-isomer. A total of 1114 male *P. anxia* was caught from the two locations during the 1995 season (May 8 to July 14) (Table 1). It was found that there was a significant difference

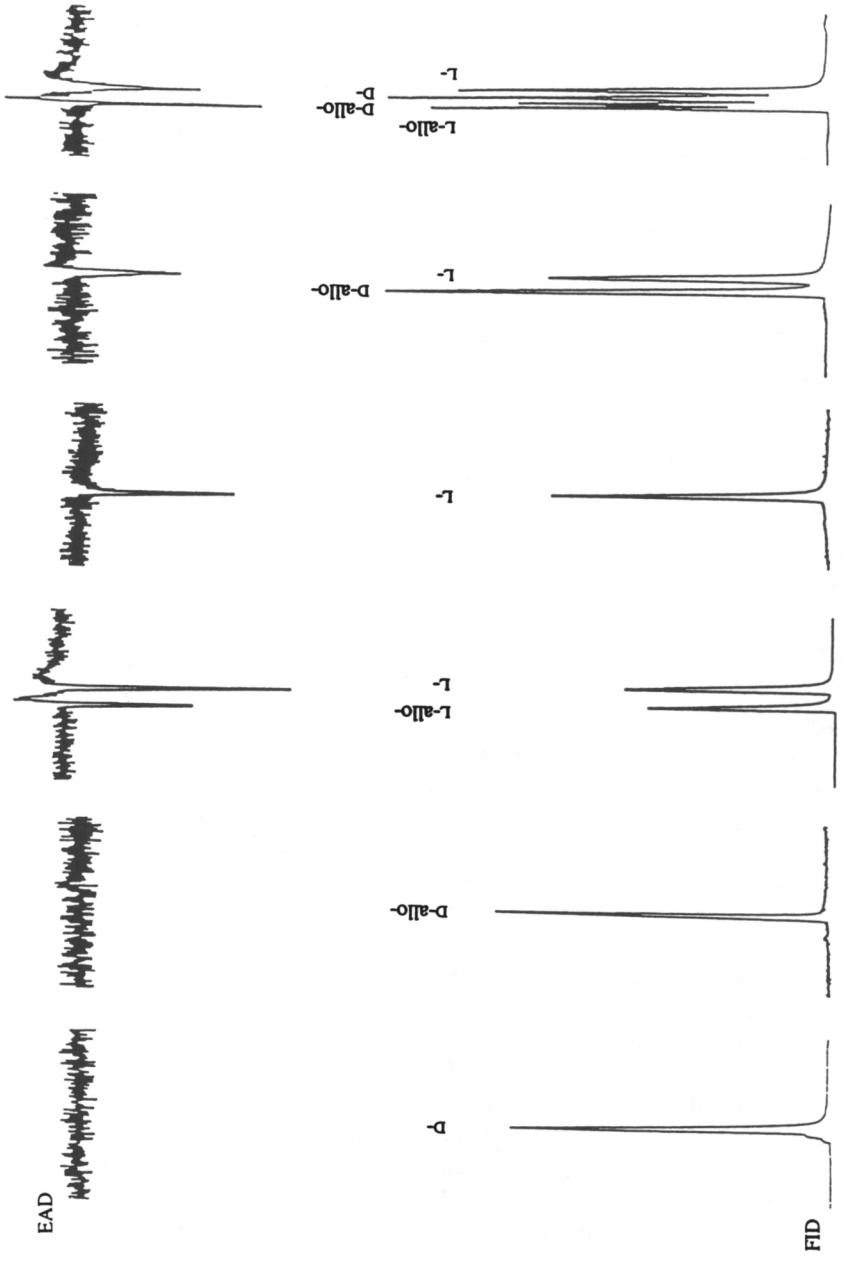


FIG 3. Responses from flame ionization detection (FID) and electroantennographic detection (EAD; antenna of male *P. anxia*) to single or different mixtures of synthetic D, D-allo-, L, and L-allo-isoleucine methyl esters, respectively, on a β-DEX 120 chiral capillary column.

TABLE 1. CAPTURES OF *Phyllophaga* MALES IN TRAPS BAITED WITH A 3:1 BLEND OF L-VALINE AND L-ISOLEUCINE METHYL ESTERS ON A RUBBER SEPTUM[a]

Location	Number of male beetles caught			
	0 mg	1 mg	3 mg	10 mg
Massachusetts				
Terrill	0	34	44	18
Cranebrook	1	279	315	423
Total	1	313	359	441

[a]Test conducted May 15–July 14, 1995.

in trap catch between control (hexane alone, 0 mg pheromone) and pheromone traps at all dosages, whereas there were no significant differences in trap catch between the three dosages at each of the two locations (one-way ANOVA, $P = 0.05$).

DISCUSSION

Our results indicate that the major sex pheromone components of *P. anxia* are L-valine and L-isoleucine methyl esters at a 3:1 ratio. Authentic L-valine and L-isoleucine methyl esters synthesized in our laboratory were active in both electrophysiological experiments and field trapping tests. This apparently represents the first report of chemical communication in the *Phyllophaga.*

In flight tunnel tests the activation responses exhibited by males to the pheromone (females, female airborne extract, or synthetic blend) were similar to that of another beetle, the oriental beetle (Zhang et al., 1994), but none flew to the pheromone source. Insufficient numbers of males restricted further investigation in the wind tunnel. The age of males, light intensity in scotophase, temperature, and humidity could be important factors influencing oriented flight.

In field-trapping experiments, most of the male beetles were caught in the early part of June. Although dosages of 1, 3, and 10 mg were tested, the 1-mg lure was sufficient to trap male *P anxia.*

Our analysis of the *P. anxia* pheromone system also showed evidence of seasonal production of the pheromone. Females that emerged from pupae in August and September could not be induced to produce pheromone before undergoing a winter chilling period, as determined by GC-EAD analysis of airborne samples. In March females began producing up to 60 ng of L-valine methyl ester/gland. In contrast, during this entire period electrophysiological activity in male antennae remained the same.

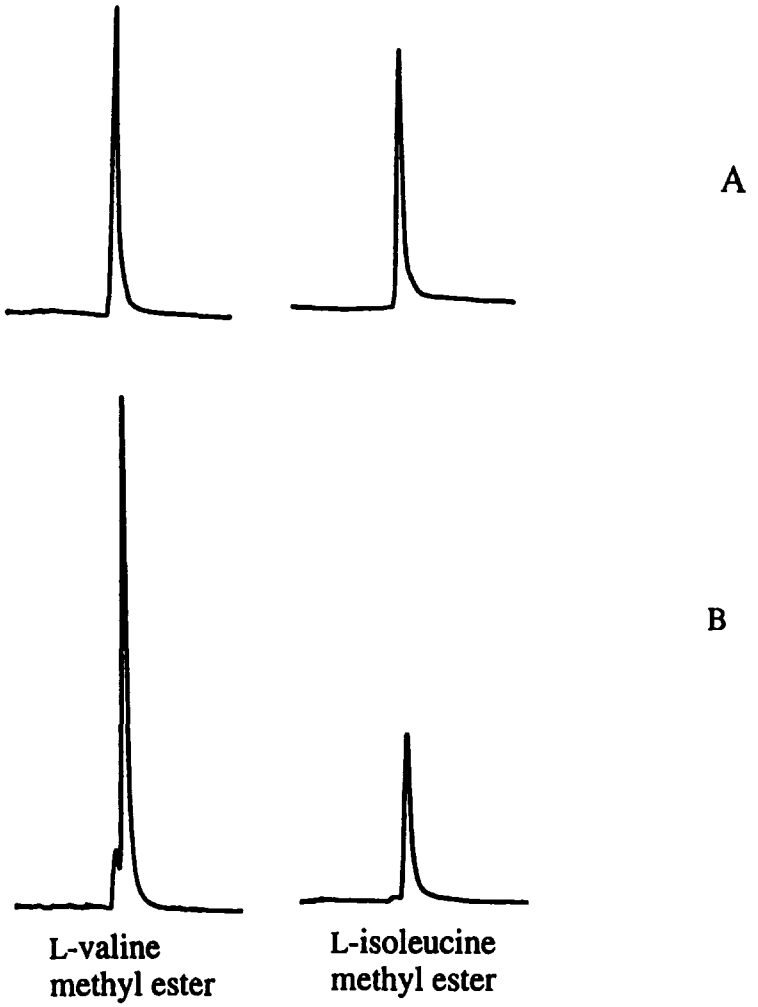


FIG. 4. A partial, reconstructed gas chromatogram (FID) of natural pheromone (A) and synthetic L-valine and L-isoleucine methyl esters on a capillary chiral β-DEX 120 column.

Amino acids are the building blocks from which all proteins are made. With the exception of glycine, the α-carbon of all 20 common amino acids is chiral, and at least two enantiomeric forms are possible. However, only the *S*-enantiomers, usually referred to as L-amino acids, are naturally used to construct proteins. Valine and isoleucine are listed as essential amino acids for insects (Hagen et al., 1984), and females obtain them from their food as the

precursors to synthesize the sex pheromone. The biosynthetic pathway is unknown but may be similar to that found in humans, in which amino acids are methylated by a methyl transferase that uses *S*-adenosylmethionine as the active methyl group donor and reversed by a specific esterase that uses water as the methyl group acceptor. If true, methylation of amino acids would represent one of the simplest sex pheromone biosynthetic pathways for insects.

The stereoselectivity of olfactory cells in *P. anxia* was clearly demonstrated in our chiral-capillary GC-EAD experiments. Only L-isomers elicited EAD responses and all of the D-isomers were electrophysiologically inactive, suggesting that these insect receptors are chiral selective. Female *P. anxia* produces only single enantiomers, L-valine and L-isoleucine methyl esters, as the major sex pheromone components. Therefore, less active or inactive EAG and behavioral responses from their antipodes would be expected (Silverstein, 1988). The D-form amino acid methyl esters may not be synthesized by these insects because their precursors are absent from nature. Electrophyiologically inactive results were obtained from all of the D-forms, D-valine, D-isoleucine, and D-allo-isoleucine methyl esters, in the GC-EAD experiments (Figures 2 and 3). Why the D-forms affect the EAG responses of the L-isomers is unknown. L- and L-allo-isoleucine methyl esters elicited the same EAD responses. This indicates that in this species the receptors specify only the amine-attached chiral center and ignore the chirality of second chiral center. Neither attractancy nor antagonism with the L-allo-isomer was formed.

Although no study of EAD responses to the chirality of valine and isoleucine methyl esters has been reported previously, L-isoleucine methyl ester was found in the large black chafer *Holotrichia parallela* (Mots.) as a sex pheromone component (Leal, 1992). Chirality was not essential for activity, as males ignored antipodes and diastereomers and responded equally well to pure L-isoleucine methyl ester and DL mixtures (Matsuyama et al., 1993), but no data for behavioral responses to L-allo-isoleucine methyl ester with that species were obtained.

In additional preliminary studies with other species we found that *P. hirticula* (Knoch), *P. futilis* (LeConte), and *P. crassissima* (Blanchard) also exhibited high GC-EAD responses to L-valine and L-isoleucine methyl esters. Three other species, *P. fusca* (Froelich), *P. fraterna* (Harris), and *P. forsteri* (Burmeister), were field-trapped in New Jersey with *P. anxia* synthetic pheromone lures during the 1995 season, suggesting that the mating communication system in the *Phyllophaga* genus involves some components in common, but probably additional components in a blend are needed for specificity. Identification of the three additional minor components in *P. anxia* volatiles could provide a species-specific lure for *P. anxia.* A lure compound of the L-valine and L-isoleucine methyl esters could be useful for monitoring a number of *Phyllophaga* spp. populations.

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PERSISTENCE OF ISOFLAVONES FORMONONETIN AND BIOCHANIN A IN SOIL AND THEIR EFFECTS ON SOIL MICROBE POPULATIONS

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Abstract—Persistence of the isoflavones formononetin and biochanin A in soil was investigated by HPLC analysis. Biochanin A disappeared more rapidly than formononetin in nonsterile soil. In soil planted with corn seedlings, the disappearance was dramamtic for both isoflavones. The results suggested that soil microbial populations were able to metabolize these isoflavones. The response of several microbial populations to isoflavone amendments was measured in soil samples obtained from Michigan potato fields. Results suggested that the isoflavones formononetin and biochanin A were able to stimulate the growth of soil microorganisms.

Key Words—Formononetin, biochanin A, microbial population, mycorrhiza.

INTRODUCTION

The degradation of biologically active, natural plant-derived phenolics by soil has been reported earlier (Dalton, 1989). Such studies have almost exclusively explored phenolic acids, because of their association with allelopathic interac-

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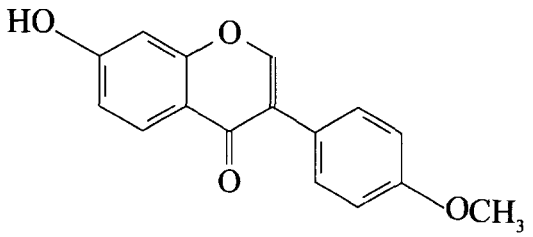
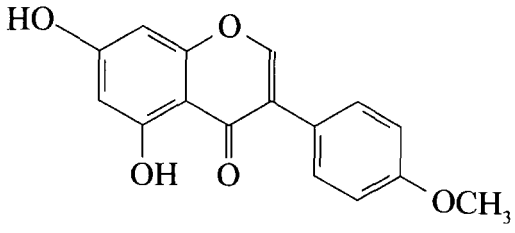
tions in some ecosystems and in cultivated soils (Wang et al., 1967; Patrick, 1971; Sparling et al., 1981; Blum and Shafer, 1988). Phenolic acids enter the soil solution and are metabolized by soil microorganisms (Sparling et al., 1981; Vaughan et al., 1983). Alkaline solutions or water are the preferred extractants for the recovery of added phenolic acids (Wang et al., 1967; Sparling et al., 1981) to determine the active (bioavailable) concentrations remaining in soils (Dalton, 1989). The rate of phenolic acid decomposition, the effects on soil biomass, and the response of soil microbial populations have been reported (Sparling et al., 1981; Blum and Shafer, 1988). Studies on the immediate response of soil biomass to added phenolic acids have indicated that some crops, such as potato, stimulate the growth of phenolic-acid-degrading microorganisms (Blum and Shafer, 1988). Isoflavones, another group of biologically active plant phenolics, have received less attention in this context.

The isoflavones formononetin and biochanin A (Figure 1) occur in shoots and roots of leguminous plants (Gildersleeve et al., 1991; Volpin et al., 1994) and in root exudates of some *Trifolium* and *Medicago* species (Nair et al., 1991; Tsai and Phillips, 1991). These isoflavones are also found as sugar conjugates (Jaques et al., 1985). Formononetin 7-*O*-glucoside has been detected in soils associated with a weed species, *Pluchea lanceolata* (Inderjit and Dakshini, 1992).



Formononetin



Biochanin A

FIG. 1. Structures of formononetin and biochanin A.

Formononetin-7-*O*-glycoside has also been identified in methanolic extracts of alfalfa rhizosphere soil (Leon-Barrios et al., 1993).

Exogenous application of formononetin and biochanin A increases the rate of root colonization by VAM fungi during the early stages of symbiosis as well as plant growth (Nair et al., 1991; Ozan et al., 1996; Siqueira et al., 1991a,b). Since the decomposition rates of these isoflavones and their effects on soil microbial communities are not known, potential application strategies for these isoflavones in agroecosystems are imprecise.

The objectives of the present study are: (1) to determine the general effects of microorganisms on the persistence of formononetin and biochanin A in soil and (2) to determine the response of soil microbial communities to the presence of formononetin and biochanin A.

METHODS AND MATERIALS

Soil Types. The soil types used were soil A (from Alba, Michigan) and soil B (from Crystal Falls, Michigan) and were collected in the spring of 1993. The soils were passed through a 3-mm mesh screen and stored at 24°C until used. The isoflavone persistence experiments were conducted in soil A, and the enumeration of the soil microorganisms in soil B, for reasons that are explained later. The soil test results were as follows: soil A; sandy soil; flow depth, 22.5 cm; soil pH, 7.5; phosphorus, 20 kg/ha; potassium, 31.5 kg/ha; calcium, 643.5 kg/ha; magnesium, 78.2 kg/ha; zinc, 4.48 kg/ha; copper, 2.24 kg/ha; iron, 11.2 kg/ha; and cation exchange capacity, 4.4 me/100 g. For soil B results were: sandy loam; flow depth, 22.5 cm; soil pH, 5.7; phosphorus, 62.2 kg/ha; potassium, 82.2 kg/ha; calcium, 391.1 kg/ha; magnesium, 81.8 kg/ha; zinc, 8.06 kg/ha; copper, 2.24 kg/ha; iron, 11.42 kg/ha; and cation exchange capacity, 9.2 me/100 g.

Extraction of Added Isoflavones from Sterile and Non-sterile Soil. Soil material was prepared by mixing one part soil A with one part silica sand. Soil A (10 g) was transferred into 2.5-cm × 15-cm test tubes. Half of the test tubes containing soil A were autoclaved for 1 hr at 120°C for three consecutive days. Formononetin (American Cyanamid Co.) or biochanin A (Sigma Chemical Co.) was incorporated in powdered form into the soil in the test tubes (0.5 mg/g/tube). After thorough mixing, each tube received 1 ml sterile distilled water to moisten the soil. The test tubes were capped with aluminum foil and kept in the dark at room temperature. At 0, 5, 10, and 15 days, the compounds were extracted from the soil with boiling methanol (25 ml × 3) by stirring the contents of the test tubes over a heat gun (Master Appliances Corp., HG 201). The combined extracts (75 ml) were filtered through Whatman No. 1 filter paper and evaporated to a final volume of 5 ml using a Buchi rotary evaporator. These

concentrated solutions were filtered through 0.2-μm filters (Acrodisc, Milex FGS, Millipore) prior to HPLC analysis.

Extraction of Added Isoflavones from Soil Planted with Corn. Corn seeds (Great Lakes hybrid 582) commercially treated with the fungicides apron and captan, were germinated on moist filter paper in Petri plates. At the end of all experiments, root systems were examined for disease and none was found. Corn seeds were chosen for the experiment because they are not known to produce the isoflavones being studied and mycorrhizal root colonization and growth of corn is increased by formononetin and biochanin A (Siqueira et al., 1991b). Styrofoam cups (0.6-liter capacity) were filled with 0.5 liters (800 g) of soil A. The isoflavones were dissolved first in a small volume of methanol (0.2%) and then added to water. Each cup received 100 ml of formononetin or biochanin A solutions (5 mg/liter) and 50 ml of one-half strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). The corn seedlings were planted into the soil and grown in a greenhouse for three weeks. The plants received 100 ml water on alternate days. At 7, 14, and 21 days after planting, the corn plants were removed from the soil, and the soil particles on the roots were loosened and recovered by shaking. Three 20-g samples of soil were taken from each treatment and were extracted with boiling methanol (75 ml $\times$ 3) as described above. The extracts (225 ml) were evaporated to 5-ml volumes and filtered through 0.2-μm filters before HPLC analysis.

HPLC Analysis. Aliquots of filtered concentrates (5–10 μl) were injected into a HPLC (Millipore Corp.) for recovery studies. Methanol–water (5:1, v/v, 2 ml/min flow rate), under isocratic conditions, was used to elute the isoflavones from the HPLC column (C18, 10 $\times$ 250 mm, 10 μm). The isoflavones were detected at 254 nm with a Waters 490 programmable multiwavelength detector. Peak integrations were made with a Waters 740 Data Module integrator. The isoflavones were identified by comparing retention times with those of pure formononetin and biochanin A dissolved in methanol, respectively. The retention times for formononetin and biochanin A were 12.2 and 15.5 min, respectively. For isoflavone quantitation, the peak areas of the recovered isoflavones were compared to the peak areas of the standard solutions. All percent recovery calculations were based on duplicate HPLC chromatogram readings for three repetitions. The results were analyzed using a one-way analysis of variance for all treatments and times.

Enumeration Media for Microorganisms. Medium ACT, modified from AGM and FMS media (Atlas, 1993) (1 g KH_2PO_4, 400 mg $MgSO_4 \cdot 7H_2O$, 130 mg $CaCl_2 \cdot 2H_2O$, 100 mg NaCl, 100 mg $FeCl_3$, 500 mg KNO_3, 20 μl pimaricin fungicide, and 15 g agar per liter of distilled water) was used for enumeration of actinomycetes. Medium BMS (ACT plus 1 g glucose) was used for enumeration of bacteria. For enumeration of fungi, the medium FGI (ACT

without pimaricin plus 50 mg rose Bengal and 100 mg chloramphenicol per liter) was used. Medium BNA (23 g nutrient agar; Difco laboratories, 20 μl pimaricin, and 50 mg tetrasodium phenolphthalein diphosphate) was also used for enumeration of bacteria including phosphatase-positive colonies. Medium GNB for enumeration of gram-negative bacteria was similar to medium BNA but tetrasodium phenolphthalein diphosphate was substituted with 4 mg of crystal violet. The media were adjusted to pH 7.0 using NaOH before autoclaving.

Enumeration of Soil Microorganisms on Agar Media. The effects of added formononetin and biochanin A on soil microbial communities were determined according to Blum and Shafer (1988) in soil B. This soil was from a relatively low-input Michigan potato field and was used because of the expected increased microbial diversity compared to soil A (see Results). The soil B was placed into 250-ml sterilized Erlenmeyer flasks (10 g soil per flask). The soil was amended with glucose, formononetin, or biochanin A in solid form (0.5 mg/g soil). The flasks were capped with aluminum foil, and the amendments were mixed into the soil by shaking the flasks. Sterile distilled water (1 ml) was added to the soil in each flask. The control soil received only water. Flasks were held at room temperature in the dark. After 3, 5, 10, and 15 days, 20 ml of sterile distilled water were added to each flask. The flasks were placed on an orbital shaker at 150 rpm for 15 min and the extract diluted to 10^{-6} with 10-fold serial dilutions. Petri plates with agar media were spread with diluted soil and kept in an incubator for six days at 30°C in the dark. For enumeration of initial microbial populations in soil A and soil B, 20 ml of water was added to 10 g samples of air-dried soil. Following the 10-fold serial dilutions, the soil suspensions were spread onto selective agar media as described above. The least significant difference (LSD) was calculated between treatments at $\alpha = 0.05$ for each time point by using Michigan State University Statistical Package (MSTAT).

RESULTS

Disappearance of Isoflavones in Soil. The isoflavones were isolated from autoclaved and nonsterile soil A over a 15-day exposure in soil (Figure 2). In sterile soil the amount of recovered isoflavones did not change, but in nonsterile soil a time-dependent decline was observed. The rate of disappearance was faster for biochanin A than for formononetin.

The microbial counts were relatively low in the soil A used for the persistence studies. The air-dried soil contained 30×10^4 colony forming units (CFU)/g dry soil for actinomycetes and 23×10^4 cfu/g dry soil for bacteria. The counts were < 10 cfu g in the lowest dilution (10^{-1}) for gram-negative, phosphatase-positive, bacteria (BNA) and fungi.

In the presence of corn plants, smaller quantities of isoflavones were

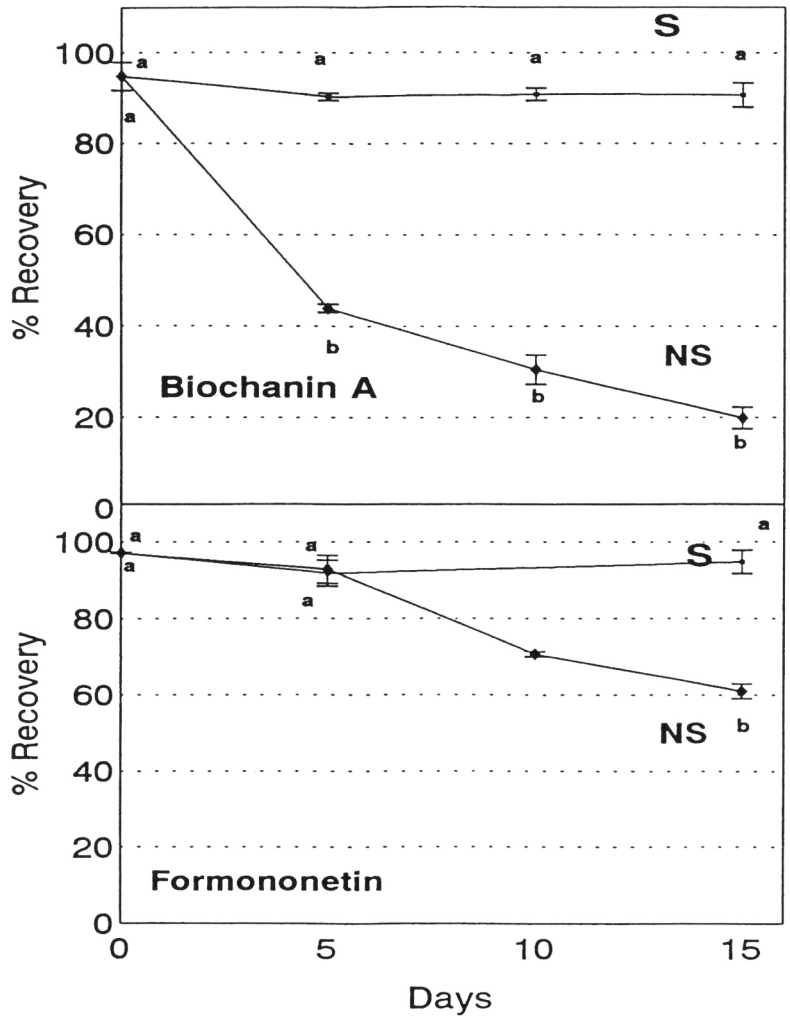


FIG. 2. Recovery of formononetin and biochanin A amendments (0.5 mg/g of soil) from soil A. S: autoclaved soil A; NS: nonsterile soil A. Percent recovery values represent the average of duplicate measurements of three replications. Points followed by different letters are significantly different at the $P = 0.05$ level.

recovered from soil A than from the nonsterile soil A (Figure 3). One week after addition, more than 60% of formononetin was recovered. About 95% of biochanin A was not recovered. Although about 5% of the added formononetin was still detectable after three weeks, biochanin A was not detectable. HPLC of the methanolic extracts of the soil planted with corn seedlings gave several

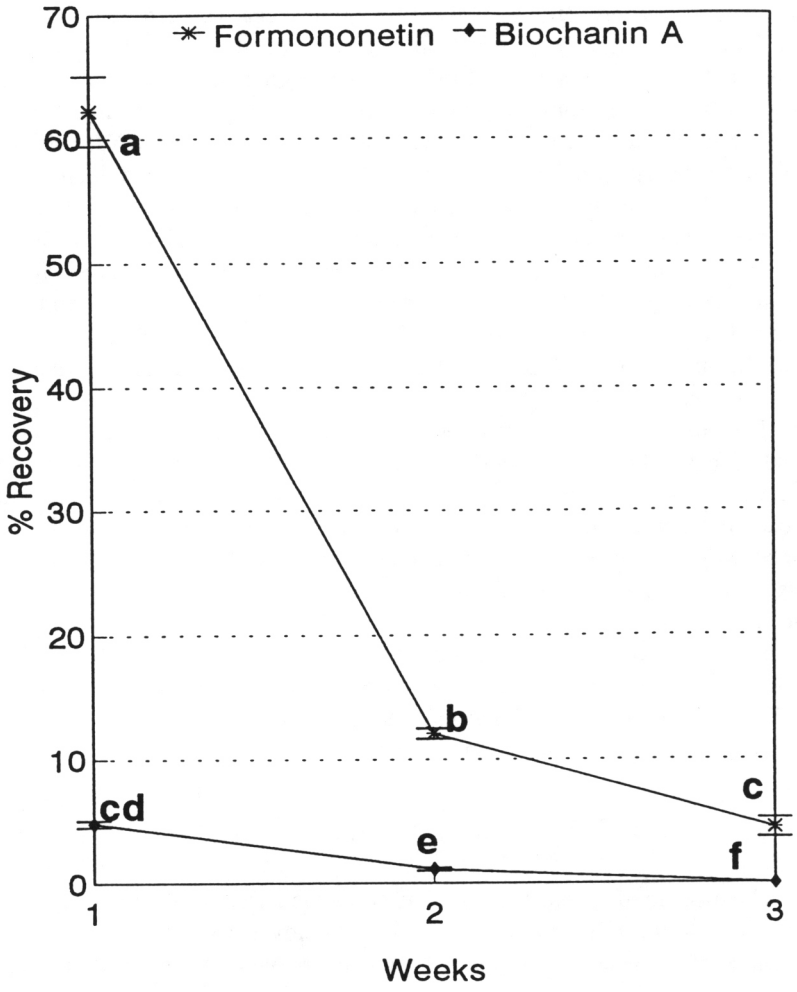


FIG. 3. Recovery of formononetin and biochanin A amendments (625 mg/kg of soil A) from soil A planted with corn seedlings. Percent recovery values represent averages of single measurements of three replicate samples. Points followed by different letters are significantly different at the $P = 0.01$ level.

peaks in addition to formononetin and biochanin A. In these chromatograms, formononetin and biochanin A peaks were confirmed by mixing and coinjecting the methanolic soil extracts with the standard solutions of the isoflavones.

Response of Soil Microbial Communities to Added Isoflavones. The initial microbial populations in soil B (10^4 cfu/g dry soil) were: actinomycetes (ACT), 553.3 ± 42.4; bacteria (BMS), 693.3 ± 100.0; bacteria (BNA), 11.8 ± 1.2;

phosphatase$^+$ bacteria (BNA), 2.53 ± .1; gram-negative bacteria (GNB), 6.26 ± 1.7 fungi (FGI), 3.73 ± 1.3. The response of soil microbial populations to added isoflavones was measured in reference to glucose stimulation (Table 1). For this study, soil B from a low-input potato field was used. The largest increase in actinomycete and bacterial numbers cultured on BMS and BNA media was observed with glucose after three days. Formononetin addition increased the actinomycete population as much as did glucose (Table 1). Formononetin addition increased the number of bacteria cultured on BMS medium as much as did glucose and biochanin A (Table 1). The number of bacteria cultured on BNA medium was the highest in glucose-amended soil (Table 1). On BNA medium, the number of bacteria increased with biochanin A addition only after 10 days, while the number of bacteria in biochanin-A-amended soil was similar to glucose amended soil at 15 days.

The number of phosphatase-positive bacteria increased gradually between 5 and 15 days. The largest increase in numbers of gram-negative bacteria was observed at 10 days with formononetin and glucose (Table 1). At three days, biochanin A and formononetin stimulation was 69% of glucose stimulation. The number of gram-negative bacteria decreased and reached the initial counts at 15 days in biochanin-A-amended soil, whereas the counts were still high with glucose and formononetin.

The fungal community responded to the amendments rather slowly (Table 1). The highest stimulation was observed with glucose at 10 days. Although the number of fungi decreased in glucose and in formononetin amended soil at day 15, it remained high in biochanin A added soil.

DISCUSSION

The mixture of steamed top soil and silica sand used for the recovery studies was similar to the soil material in which white clover plants were grown to test the effect of exogenously applied formononetin on root colonization by VA mycorrhizal fungi and growth (Siqueira et al., 1991a).

In our study, methanol-extractable concentrations of added isoflavones did not vary over 15 days in sterile soil (Figure 2). However, decreasing amounts of isoflavones were recovered in methanol extracts of nonsterile soil. The isoflavones were added on day 0 to nonsterile soil and extracted immediately. The amounts recovered at 5, 10, and 15 days from sterile soil did not differ from the amounts obtained on day 0 (Figure 2). This indicated that the properties of the soil–sand mixture used in the recovery studies were not affected by autoclave sterilization.

Since sterilization can change physical and chemical properties of soils (Lopes and Wollum, 1976; Williams-Linera and Ewel, 1984), Dalton (1989)

TABLE 1. TOTAL NUMBERS OF SOIL MICROORGANISMS (10^4 × CFU/g DRY SOIL) IN SOIL B AMENDED WITH GLUCOSE, FORMONONETIN, OR BIOCHANIN A

Microorganisms	Days			
	3	5	10	15
Bacteria (BMS)				
Control	426.0	630.6	431.3	533.3
Glucose	5080.0	4473.3	380.0	514.0
Formononetin	4953.3	3460.0	533.3	782.0
Biochanin A	4166.6	2080.0	851.3	517.3
$LSD_{0.05}$	587.4	878.0	175.7	121.3
Gram-negative bacteria (GNB)				
Control	3.2	12.6	31.0	3.7
Glucose	48.9	39.8	70.0	30.6
Formononetin	33.8	43.6	82.6	49.3
Biochanin A	34.0	11.4	16.8	5.6
$LSD_{0.05}$	6.8	5.7	18.2	9.5
Phosphatase$^+$ bacteria (BNA)				
Control	10.0	10.9	9.2	6.0
Glucose	9.6	11.0	32.0	53.3
Formononetin	5.4	16.6	24.6	36.6
Biochanin A	8.5	4.2	12.6	90.0
$LSD_{0.15}$	2.6	10.1	8.5	66.0
Bacteria (BNA)				
Control	24.3	32.3	20.7	21.6
Glucose	586.7	420.0	182.0	168.0
Formononetin	233.3	240.0	94.0	80.6
Biochanin A	50.0	21.4	86.0	205.3
$LSD_{0.05}$	187.1	168.4	54.6	104.2
Actinomycetes (ACT)				
Control	1066.6	610.6	502.0	700.6
Glucose	5973.3	4260.0	515.3	721.4
Formononetin	5386.6	3566.6	849.3	1188.6
Biochanin A	4566.6	1820.0	497.3	696.2
$LSD_{0.05}$	737.5	575.0	139.8	78.2
Fungi (FGI)				
Control	10.1	4.5	20.6	13.6
Glucose	15.4	9.9	58.6	19.8
Formononetin	10.3	5.7	27.3	14.2
Biochanin A	8.5	3.0	30.6	28.3
$LSD_{0.05}$	3.0	2.5	12.3	3.6

suggested comparison of immediate recovery of added compounds from sterilized and nonsterilized soils.

Water or sodium acetate solution has been used for the extraction of phenolic acids. Leon-Barrios et al. (1993) have extracted formononetin glycoside from soils using methanol. Refluxing the soil with methanol is highly effective in recovering water-insoluble phenolics.

The decrease in the isoflavones extracted from nonsterile soil suggested that the soil microbial population was able to metabolize these isoflavones. The decrease of isoflavone in nonsterile soil was slow when compared to the disappearance rates of added phenolic acids to soils (Sparling et al., 1981; Blum and Shafer, 1988; Dalton, 1989). This variation in the rate of decline may be a result of the differences in quantity and composition of soil microbial communities. Phenolic acids in the solution phase of soil serve as a carbon source for many soil microorganisms. In fact, some bacterial species preferentially metabolize phenolic acids.

Unlike many phenolic acids, formononetin or biochanin A are not water-soluble compounds. They may dissolve in the micropockets of soil with high pH and then be utilized by soil microbes associated with these microenvironments (Patrick, 1971). In liquid cultures, formononetin is metabolized completely by rhizosphere bacteria (Barz et al., 1970). However, the rate of utilization in soil environments has not been measured.

When soil microbial populations were eliminated by sterilization, there was no decomposition. However, the decomposition was faster in the soil planted with corn seedlings. This is probably due to the mineral nutrient solution applied as fertilizer, and organic metabolites from the root exudates enriched the soil and favored the growth of microorganisms. The observed difference between the rates of decline in the amounts of isoflavones recovered with or without plants was probably due to the stimulation of soil microorganisms in the enriched soil. It is possible, however, that isoflavone uptake by the corn seedlings may have contributed to our result. We believe this potential contribution to be minimal since these two isoflavones are highly immobile in soil (Ozan, 1994).

The disappearance of biochanin A was faster than that of formononetin in both the presence (Figure 3) and absence (Figure 2) of plants. This suggested that biochanin A may be more ready utilized by microorganisms than formononetin.

The response of soil microorganisms to added isoflavones was measured in reference to glucose stimulation. The changes in the numbers of soil microorganisms were monitored at 3, 5, 10, and 15 days (Table 1). The results indicated that the added isoflavones were able to stimulate the growth of some of the soil microorganisms. Formononetin caused a higher increase in numbers of actinomycetes and bacteria than biochanin A. Formononetin stimulation was equal to glucose stimulation as observed in ACT and BMS media.

Bacterial communities had higher growth responses to formononetin, whereas biochanin A amendment caused a prolonged growth response in fungi between 10 and 15 days. The fungistatic effect of biochanin A has been demonstrated by Willeke et al. (1983). Perhaps after the added biochanin A has been decomposed or converted by other soil microorganisms, some fungal species were able to grow in the soil, causing the late prolonged growth response observed in our experiments. However, in recovery studies biochanin A was depleted more quickly than formononetin. This difference in the amount of recovery and response may be due to the initial composition of the microbial communities in the soils used in the experiments or to the effects of microorganisms that cannot be cultured on the selective media used.

Stimulation of indigenous VAM fungi for low-input agriculture may be advantageous since VA mycorrhizal symbiosis is known to benefit many crops for uptake of mineral nutrients such as phosphorus and nitrogen (Sanders and Tinker, 1973; Bowen and Smith, 1981). In natural environments, migration, breakdown, and adsorption of compounds onto soil particles occur simultaneously (Patrick, 1971). These factors would decrease the effective concentrations of the applied isoflavones in the soil. Migration studies of formononetin or other exogenously applied bioactive natural compounds in fields may thus become very complicated due to these multiple variables. Nevertheless, the results of such additional studies would provide valuable information to determine the optimum parameters for soil application of isoflavones for stimulation of indigenous microbial populations.

Acknowledgments—This is a contribution from the Michigan State University Agricultural Experiment Station.

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A NATURAL ALGACIDE FROM SOFT CORAL *Sinularia flexibilis* (COELENTERATA, OCTOCORALLIA, ALCYONACEA)

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Abstract—A crude lipophilic extract and specific pure metabolites of the soft coral *Sinularia flexibilis* have been examined for algacidal properties both in laboratory and field experiments. Laboratory algal bioassays, in which cultures of the common fouling alga *Ceramium codii* were incubated with six different diterpenes isolated from *S. flexibilis*, revealed that 11-episinulariolide exhibits strong algacidal properties. Field experiments carried out with treated settlement tiles confirmed the laboratory findings and provided evidence for the algacidal properties of 11-episinulariolide against several other common reef algae. Sinulariolide, which had previously been reported to inhibit the growth of unicellular algae, was approximately one third as effective as its stereoisomer 11-episinulariolide in the laboratory growth inhibition bioassay and showed no significant algacidal properties at the concentrations used in the field experiments.

Key Words—*Sinularia flexibilis*, *Ceramium codii*, algal bioassay, natural algacide, sinulariolide, 11-episinulariolide, chemical defense, biofouling, marine antifoulants.

INTRODUCTION

In the marine environment, any living or nonliving substrate is constantly in danger of being fouled, with the consequences of reduced fitness or even death

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of the fouled organism or severe damage and loss of efficiency for man-made substrates.

Biofouling, especially by algae, is currently one of the most important problems facing marine technology (Gerhart et al., 1988; Callow, 1986; Anonymous, 1992). Since the 1970s, the traditional way of preventing marine fouling has been by use of tributyltin-based copolymer antifouling paints (Batley et al., 1989). Tributyltin (TBT), the active ingredient in antifouling paints, has high potency against fouling, but also high toxicity towards nontarget marine organisms (Lenihan et al., 1990; Lau, 1991). It bioaccumulates in the food chain and research has demonstrated its severe damaging effects on the reproduction and growth of many marine life forms. This has prompted various countries to regulate or ban its use in antifouling products (Lau, 1991; Batley et al., 1989).

Competition for space on tropical coral reefs is enormous, and overgrowth of one organism by another is a very common phenomenon. The most important mechanism by which surfaces may be colonized and later overgrown by macrophytes and epizooites is by settlement of their spores or free swimming larvae, respectively (Davis et al., 1989). Algae, which cause the biggest problems as fouling organisms on man-made substrates (Callow, 1986), are able to develop on most reef surfaces, but octocorals (soft corals) are rarely fouled (Coll et al., 1987; Gerhart et al., 1988). The ability of octocorals and other sessile invertebrates, such as sponges, to stay markedly free of fouling has been attributed to the occurrence of toxic secondary metabolites, especially terpenes, in their tissues (Sammarco and Coll, 1988). The energy expenditure for the production of significant amounts of these secondary metabolites is well compensated by the numerous functions they fullfil within the organism. Besides possibly functioning as antifouling agents, they may enhance a coral's fitness by serving as a defense against predation (Wylie and Paul, 1989; LaBarre, et al., 1986; Dai, 1990), for communication (Kittredge et al., 1974), and for reproduction purposes (Coll et al., 1989, 1990). The use of secondary metabolites derived from marine invertebrates could possibly provide an alternative to the commercial metal-based antifouling coatings that are in use today.

In this study we have shown that extracts of *Sinularia flexibilis* possess algacidal properties, identified the algacidal agents, and determined their critical concentration against the red alga *Ceramium codii*. Since it has previously been demonstrated that *Sinularia flexibilis* releases its metabolites into the surrounding water in concentrations comparable to the critical concentrations determined in this work (Coll et al., 1982), the results described are of ecological significance. Furthermore, we have demonstrated the possible physiological effect of the terpene on the alga *Ceramium codii* from examination of growth forms of treated and untreated algal samples.

METHODS AND MATERIALS

Collection. Samples of *Sinularia flexibilis* were collected in August and November 1992 at Iris Point, Orpheus Island, Australia (18°34′S; 146°29′E) from about 3 m of water. After collection, the coral samples were frozen (−18°C) as soon as possible and later freeze-dried and weighed.

Extraction and Analysis. The freeze-dried corals were extracted three times with methylene chloride for periods of 1 hr, 8 hr, and 24 hr, respectively. The extracts were then combined and the solvent removed in vacuo to afford the crude extract. Vacuum liquid chromatography (after Coll and Bowden, 1986) was carried out for fractionation of the crude extract, for purification of individual compounds, and prior to separation by HPLC. For elution, step gradients of solvents with increasing polarity (from petroleum ether via methylene chloride and ethyl acetate to methanol) were used. The eluted fractions were subsequently examined by TLC on plastic sheets with 0.2-mm-thick silica gel 60 F_{254} (Merck). Further separation of the fractions was achieved through HPLC with Waters Si-100 7μ 240 × 8-mm and Technisil Silica 5μ 240 × 8-mm columns in tandem, with HPLC grade solvents. Best separation of diterpenes was achieved with diethyl ether–petroleum ether (32 : 68). For the identification of diterpenes, proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained. Optical rotation ($[\alpha]_D$) and melting point determinations were used to confirm the purity of isolated known compounds.

Algal–Terpene Bioassays. The bioassays were done according to Coll et al. (1987). The red alga *Ceramium codii* (Richards) Mazoyer, held in unialgal culture in the Botany Department of James Cook University, was chosen because it is a common alga on the Great Barrier Reef and because its filamentous nature facilitated the determination of the relative growth rate, which was examined in the bioassays. Sterile cultures of the alga were kept in a constant temperature room (28°C) under a light regime of 24 μE/m^2/sec and a 12-hr day–night cycle. The algal samples were lodged in 50-mm glass Petri dishes containing 20 ml nutrient-enriched seawater. The nutrient medium used was a simple inorganic liquid culture medium (Grundmedium; von Stosch, 1964). Branched inocula (6 mm long) with two active growing points, were excised from actively growing cultures. After excision, the inocula were allowed to regenerate for 24 hr.

When regeneration of each inoculum was confirmed, the initial length of each inoculum was measured through microscopy, and the treatment was started. Dimethylsulfoxide (DMSO) was used as solvent for the diterpenes. The volume of DMSO used did not exceed 0.5% (= 100 μl) of the total culture volume, since the presence of higher concentrations of DMSO significantly inhibited algal growth. All bioassays were carried out with controls that had been treated with the same volume of DMSO. For comparison purposes only, controls to

which no DMSO had been added were used to assess any growth inhibition due solely to the use of DMSO as a solvent. Initial concentrations used were chosen to be comparable to those concentrations that might be encountered in close proximity of the surface of the coral. The concentrations of the three main metabolites normally encountered in *Sinularia flexibilis* have been observed to vary with the size of the colony (Maida et al., 1993), however, that study did not monitor the concentrations of other minor metabolites that might have been present. *Sinularia flexibilis* typically yields a dichloromethane extract that contains approximately 8 mg of flexibilide, 6 mg of dihydroflexibilide and 2 mg of sinulariolide per gram dry weight of coral (Maida et al., 1993). In the colony extracted for this work, the yields per gram of dry coral were as follows: flexibilide (6 mg/g), dihydroflexibilide (4 mg/g), sinulariolide (2 mg/g), 11-epi-sinulariolide (0.6 mg/g), 11-episinulariolide acetate (0.6 mg/g), and flexibilene (0.6 mg/g). The lowest concentration (0.6 mg/g) approximates to 0.2 mg/ml volume wet weight of the coral or 200 mg/liter. The concentration of terpenes in soft corals is higher in the polyp tissues than in the connecting coenchyme tissues (Bowden et al., 1992) and both these concentrations are significantly higher than in the basal tissues (i.e., the level of chemical protection is highest in the actively growing polyp region). Coll et al., (1982) demonstrated that *Sinularia flexibilis* releases its metabolites into the surrounding water in concentrations of the order of 1–5 ppm (1–5 mg/liter). Whether this terpene is truly dissolved in seawater surrounding the coral or is associated with mucous strands in the water has not been demonstrated. The initial concentration of pure compounds chosen for testing (10 mg/liter) in laboratory experiments was a compromise concentration, slightly higher than that expected in the water surrounding the coral but significantly lower than the concentration present in the surface tissues of the coral.

Subfractions of the crude extract of *Sinularia flexibilis* were used in preliminary bioassays to determine which fractions inhibited algal growth. Subfractions exhibiting no growth inhibition were discarded and active fractions only were carried over into a further bioassay. The six pure diterpenes previously discussed were also used in this bioassay. Five of these were isolated from the fractions that showed the highest inhibition of algal growth; flexibilene was isolated from a fraction that did not inhibit algal growth. Only those bioassays that involved the pure compounds and the active fractions from which they had been isolated are reported here. The first bioassay used the purified diterpenes at a concentration of 10 mg/liter, and the two subfractions from which the diterpenes had been isolated at a concentration of 100 mg/liter. All treatments and controls were replicated seven times. In a second bioassay the diterpene concentrations used were 0 mg/liter (= control), 1.25 mg/liter, 2.5 mg/liter, 5.0 mg/liter, and 10.0 mg/liter. An "absolute" control with neither DMSO nor terpenes was used to show that any growth inhibition that resulted from the use of DMSO as solvent

was minimal. All treatments and controls were replicated seven times. At the conclusion of the bioassay (four days after initial treatment), the final length of each inoculum was again measured through microscopy.

The growth of algal inocula was expressed as the relative growth rate (RGR) according to the following formula:

$$RGR = \frac{I}{M \cdot d_n},$$

where I is the increase in length of branch system during the assay; M is the mean of initial and final length of branch system; and d_n is the duration of the bioassay in days (n). This formula is a linear modification of the accepted formula for relative growth rate, which is based on dry weight (Hunt, 1982) and has been used previously (Coll et al., 1987). The formula takes into account the fact that larger pieces grow faster than smaller pieces. The mean of initial and final lengths of branch systems has been used so the relative growth rates are not significantly distorted by the fact that during the assay period (four days) the lengths of the systems are increasing, and hence their growth rates will also be increasing.

Field Experiment. A settlement experiment was set up at Iris Point, Orpheus Island, in December 1992 and terminated four weeks later. Commercial glazed ceramic tiles (15 × 15 cm) were used as artificial settlement substrata, as studies by others had revealed that they allow a variety of settled organisms representative of the natural species composition and there are low mortality rates of settling organisms (Lamberti and Resh, 1985; Harriot and Fisk, 1985). Tiles were impregnated with the compound under test by pouring 50 ml of a methylene chloride solution onto the porous surface of the tile at a rate such that most of the solution was absorbed. Concentrations used were 10 g/liter for the crude extract and 200 mg/liter for each of the six pure diterpenes. The amount of DCM extract obtained from the colony of *Sinularia flexibilis* used in our experiments was 7% of its dry weight, or about 2% of its wet weight. Although the surface area of a coral and a porous ceramic tile are difficult to determine for comparison purposes, it was felt that the surface area of a 25-g wet weight coral might be comparable to that of the tile. Such a coral would yield about 0.5 g of extract, and 50 ml of a 10 g/liter extract contains 0.5 g. Assessment of algacidal properties was by comparison with control tiles that had been treated with a 50-ml aliquot of methylene chloride. Control tiles that had not undergone treatment with methylene chloride were also prepared, simply to see whether the methylene chloride had any effect. In addition, to determine whether or not algal growth was influenced by the surface characteristics of the tile and, in particular, whether an extract that was lipophilic in nature would affect growth rates merely by making the tile surface lipophilic, tiles treated with a methylene

chloride solution of sunflower oil (200 mg/liter), and a methylene chloride solution of the lipids (mainly triglycerides) isolated from *S. flexibilis* (200 mg/liter) were also prepared. The solvent (methylene chloride) was allowed to evaporate, and all tiles were dried before the settling experiment commenced. The number of replicates for both controls with and without methylene chloride (DCM) was 16, while eight replicates were used for the crude extract, sinulariolide, flexibilide and the lipids from *S. flexibilis*. For all other treatments (except 11-episinulariolide, where only five replicates were used due to the small quantity of pure terpene available), nine replicates were used. The impregnated tiles then were mounted on stainless-steel bolts, arranged into stacks of three replicate tiles per bolt with the tiles kept 2.4 cm from each other by plastic spacers. Where the number of replicates was not a multiple of three, the additional tile(s) needed were untreated tiles that were not used in the data analyzed. The stack arrangement reduced grazing effects, while still allowing free water flow and sufficient illumination for the settling organisms (after Maida, 1993). After preparation, the tile stacks were attached to plastic racks that were fixed on galvanized steel grids in 5 m depth of water.

Data Collection. Algal coverage on the settlement plates was determined by two techniques. A plastic grid dividing the tile into 400 squares (0.56 cm^2) was made from a solid plastic frame (15 × 15 cm) and nylon line, and the frequency of algal coverage (i.e., each square was listed as with or without the presence of algae) was determined using a dissecting microscope. After the frequency data had been collected, a scalpel was used to quantitatively remove all algae from the tiles. A chlorophyll extraction of the algal samples was then carried out, using techniques described elsewhere (Harborne, 1988). The chlorophyll concentrations were calculated from the following formula: total chlorophyll (mg/liter) = $17.3\ A_{646} + 7.18\ A_{663}$

All results were subjected to a one-way analysis of variance (ANOVA) on a 95% level.

Algal Taxonomy. Identification of algal species was based on microscope studies of fresh specimen. Identifications were carried out by Dr. Ian Price, Department of Botany, James Cook University of North Queensland. The taxonomy was based on criteria described in Price and Scott (1992).

RESULTS

Identification of Diterpenes. Six known diterpenes were isolated and purified by HPLC (Figure 1). The identity of each was confirmed by comparison of proton NMR spectral data with published values and, where possible, the purity was checked by comparison of melting point and $[\alpha]_D$ with literature values.

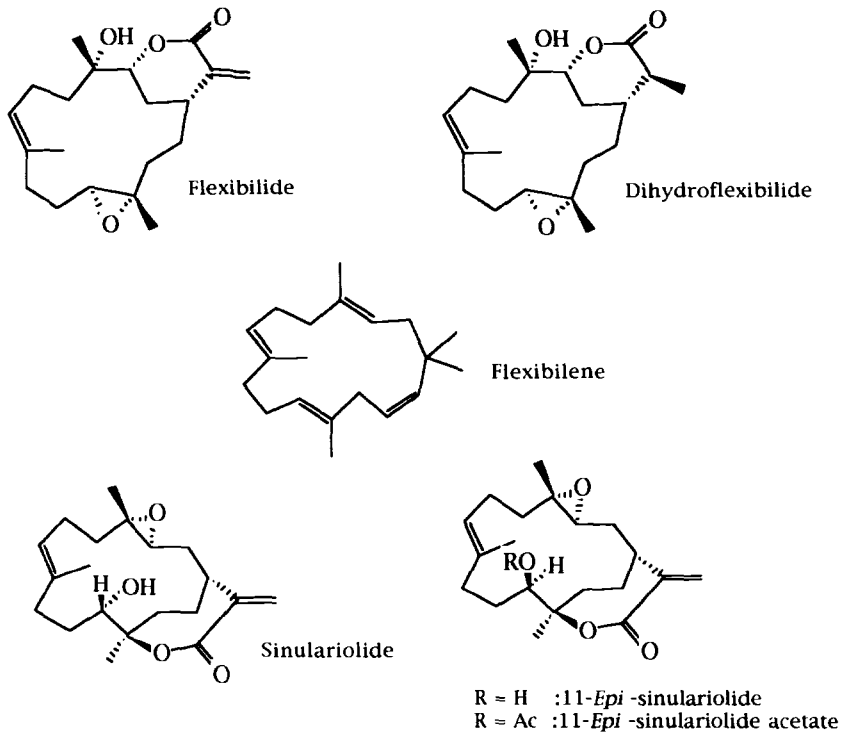

FIG. 1. Structures of diterpenes isolated from *Sinularia flexibilis* and used in this study.

Bioassays. All growth rates discussed are relative to that observed for controls that had DMSO added to them; the growth rate for controls that did not have added DMSO are included only to indicate the magnitude of growth inhibition due to the use of DMSO as solvent. The results of the first bioassay (Figure 2) revealed that treatment with flexibilene and 11-episinulariolide acetate did not result in any significant changes in the growth of the test organism. The diterpenes flexibilide and dihydroflexibilide both promoted the growth of *Ceramium codii* significantly. Sinulariolide, 11-episinulariolide, and the two fractions (4d and 4e, Figure 2) from which the diterpenes had been isolated, exhibited significant growth inhibition by comparison with the controls to which only DMSO had been added. 11-Episinulariolide was more effective than sinulariolide at the concentration used. The growth inhibition by sinulariolide is, however, statistically significant.

To determine the critical toxic concentrations of 11-episinulariolide, sinulariolide, and 11-episinulariolide acetate, the second bioassay was carried out.

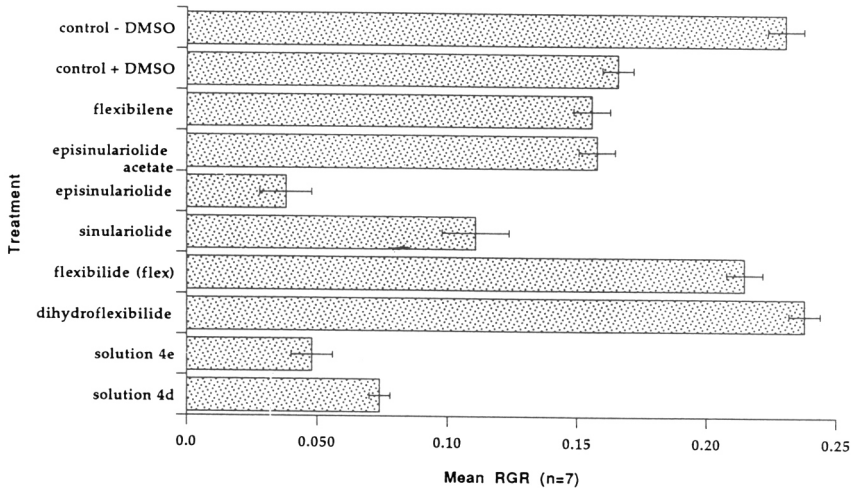

FIG. 2. Influence of specific diterpenes and certain fractions from separation of the coral extract on the relative growth rate of *Ceramium codii*. Concentrations used were 10 mg/liter for pure compounds and 100 mg/liter for fractions from the crude extract.

11-Episinulariolide, sinulariolide, and 11-episinulariolide acetate were tested at concentrations of 0.625, 1.25, 2.5, and 5.0 mg/liter, to elucidate the critical toxic concentration. The results are summarized in Figure 3.

This bioassay provides evidence for growth enhancement by the three tested diterpenes at low concentrations (0.625 and 1.25 mg/liter). All data points except that for 11-episinulariolide acetate at concentration 5.0 mg/liter differed from controls by a statistically significant amount. The critical toxic concentration (the point where the mean RGR curve crosses that of the control) for 11-episinulariolide is at 1.8 mg/liter, approximately 2.5 times lower than for sinulariolide (4.4 mg/liter). 11-Episinulariolide acetate did not show growth inhibition within the concentration range used, but growth enhancement that occurred for the lower concentrations was not apparent at the highest concentration tested.

Field Experiments. For the determination of area covered by algae, the area of each tile was divided into 400 equivalent squares, and each square was assessed for the presence of algae. The results were subjected to a one-way ANOVA test at the 95% confidence level, which showed significant differences in the data set ($P = 0.0002$). The data for each individual treatment were then each separately compared with data for the controls using unpaired t tests with application of the Bonferoni correction for multiple comparisons. All treatments gave coverage that differed significantly from controls (two-tail significance <0.001) except the treatment with 11-episinulariolide acetate, which was not

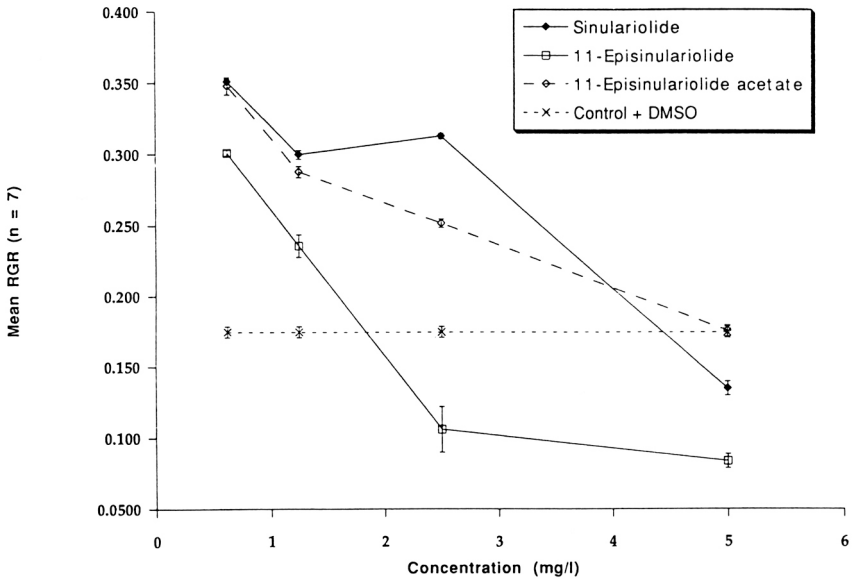

FIG. 3. Sensitivity test for the determination of the critical toxic concentrations.

significantly different from controls (two-tail significance 0.373). The area coverage data are summarized in Figure 4.

Treatment with flexibilide, dihydroflexibilide, the lipids from *Sinularia flexibilis*, and with sunflower oil resulted in a statistically significant enhancement of algal growth on the tiles relative to control tiles that had only been treated with dichloromethane. Tiles impregnated with crude extract of *Sinularia flexibilis*, sinulariolide, and 11-episinulariolide, however, showed statistically significant inhibition of macroalgal growth by comparison with the dichloromethane-treated controls. 11-Episinulariolide exhibited the highest algacidal properties of all compounds tested, followed by sinulariolide and the crude extract, but it should be noted that it is not valid to directly compare the amount of growth inhibition observed for the pure compounds with that for the crude extract, since the concentrations used differed. Tiles not treated with dichloromethane were noted to significantly inhibit the amount of algal coverage observed by comparison with tiles that had been treated with dichloromethane. (Data obtained were similar to those from treatment with the crude *S. flexibilis* extract, but those data are not presented in Figure 4 since all treatments presented in Figure 4 included dichloromethane). It is possible the dicloromethane removed some chemical treatment the tiles had undergone during production.

The results of the determination of the total chlorophyll concentrations from

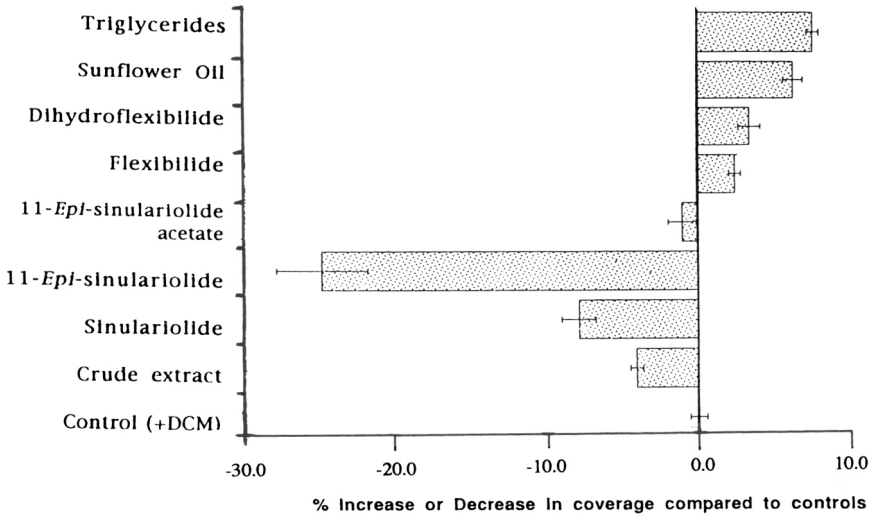

FIG. 4. Influence of different terpenes and lipids on the coverage of settlement plates by macroalgae.

algae growing on the settlement tiles were similar to the previous findings. The chlorophyll levels from tiles treated with crude extract, sinulariolide, and 11-episinulariolide were all lower than control tiles that had been treated only with DCM, but these findings were only significant at the 95% confidence level (one-way ANOVA, $P < 0.0001$) for the tiles treated with 11-episinulariolide. Flexibilide displayed no significant variation from controls (although the area coverage data had indicated increased growth). Settlement tiles treated with 11-episinulariolide acetate, dihydroflexibilide, sunflower oil, and triglycerides all yielded higher chlorophyll concentrations than the controls, despite the fact that area coverage for 11-episinulariolide acetate was not significantly different from controls. Figure 5 summarizes these results.

Taxonomy of Fouling Algae. Taxonomic identification of the fouling algae revealed that the green alga *Enteromorpha* sp. made up approximately 80% of the surface area covered, followed by *Derbesia* sp. a siphonous, creeping green alga, and another creeping green algae, *Cladophora* sp. These three species accounted for approximately 90% of the total surface coverage. The following red algae occurred mainly on the margins of the tiles in approximately similar amounts: *Ceramium sympodiale* Dawson, *Heterosiphonia crispella* var. laxa (Boergesen) Wynne, *Champia* sp. (*Champia? viellardii* Kuetzing), *Dasya* sp., and *Hypoglossum* sp.

Physiological Effects of Treatment with 11-Episinulariolide. The treatment

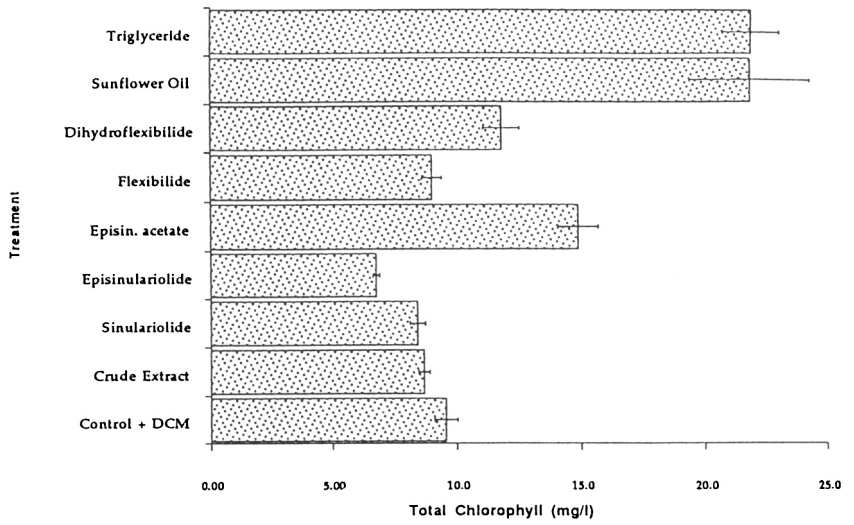

FIG. 5. Total chlorophyll concentrations obtained by extraction of algae scraped from treated settlement tiles (with standard errors indicated).

of cultured *Ceramium codii* with 11-episinulariolide resulted in a loss and/or suppression of growth of hyaline hairs. This is demonstrated on Figure 6.

DISCUSSION

11-Episinulariolide (More et al., 1983) differs from sinulariolide (Tursch et al., 1975) only in the stereochemistry of a single hydroxyl group; however, 11-episinulariolide has been proven to be two to three times more inhibitory towards algal growth than sinulariolide in bioassays. The determination of total chlorophyll concentrations of algae growing on sinulariolide-treated tiles did not result in significant inhibition at the concentration used. This might indicate that the diffusion rate into the surrounding water is such that the concentration fell below the concentration required for growth inhibition during the time coure of the experiment. The growth inhibition observed for sinulariolide against *Ceramium codii* in the bioassays was in a situation where a constant concentration was present throughout the time course of the experiment, while the time period for the field experiment was four weeks. Algae of the family Ceramiales are common fouling algae on the Great Barrier Reef (Morrissey, 1980; Coll et al., 1987; Maida, 1993). The algacidal properties of both 11-episinulariolide and sinulariolide demonstrated against a member of this algal family in laboratory

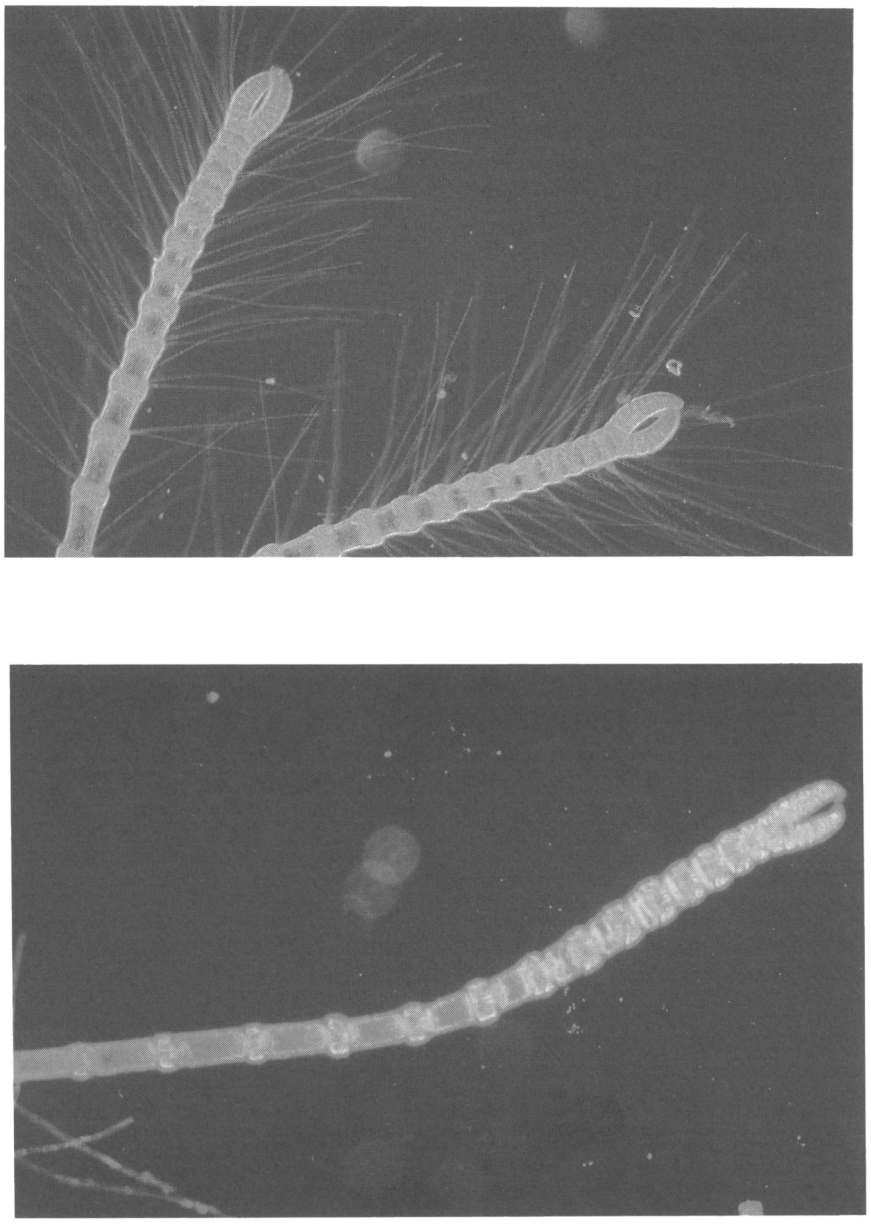

FIG. 6. Hyaline hair loss and/or suppression in *Ceramium codii* as a consequence of treatment with 11-episinulariolide. (top: control alga, bottom: treated alga).

experiments indicates field protection against fouling by these particular kinds of algae. In experiments carried out by Tursch (1976), sinulariolide was proposed to act as an algacide against some unicellular green microalgae (however, no details of concentrations used or experimental set-up were provided). Our laboratory experiments provided some evidence on how the growth inhibition of the algae may occur; the key to the growth inhibition of *Ceriamium codii* seems to be the nearly total suppression of the production of hyaline hairs in the alga (Figure 6). Hyaline hairs are used for the uptake of nutrients from the medium, and result in a two to three times larger surface area for the alga (DeBoer and Whorskey, 1983). The treated algae were in an optimal nutrient environment, but may be unable to fully utilize it due to limited uptake facilities. One can not conclude, however, that this is the only mechanism by which algal growth in Ceramiales is suppressed. A study investigating the effects of flexibilide and other soft coral secondary metabolites on scleractinian corals (*Acroporoa formosa* and *Porites andrewsi*), revealed that concentrations below 5 ppm lead to polyp mortality. This mortality was attributed to the fact that the terpenes caused an initial increase in the respiration of the symbiotic zooxanthellae, followed by an extremely decreased photosynthetic output. This decreased output was attributable to an uncoupling of respiration from oxidative phosphorylation (Webb and Coll, 1983).

The absence of algacidal properties observed for 11-episinulariolide acetate is indeed surprising. One might expect that the acetyl group would be enzymatically hydrolyzed in cells, so that it would be rapidly converted into 11-episinulariolide after absorption. The growth enhancement found, however, indicates that the acetyl moiety is either not rapidly lost or that the terpene is not rapidly absorbed.

The experiments carried out with lipids (sunflower oil and the lipids from *Sinularia flexibilis*) gave the expected results, namely high growth rates as a consequence of the high nutritional value of the compounds. The presence of triglycerides in the soft coral may also account for the insignificant lowering of chlorophyll levels for plates treated with crude extract compared to controls. The nutritional value of the triglycerides may compensate for the growth inhibition by 11-episinulariolide and sinulariolide.

The use of flexibilide and dihydroflexibilide, two cembranoid lactones (Kazlaukas et al., 1975), resulted in enhanced algal growth in both the field and laboratory; dihydroflexibilide generally led to a higher growth promotion.

Enteromorpha sp., which covered about 80% of the tile surface, is not only one of the most common algae in the Great Barrier Reef (Bak and Borsboom, 1984), but together with the brown alga *Ectocarpus* is the main fouling alga on ships (Callow, 1986). 11-Episinulariolide has been shown to suppress the growth of *Enteromorpha* sp, thus making it potentially useful as a substitute for organotin-based antifouling agents used in the shipping industry.

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DYNAMICS OF PREFERENCE BY SHEEP OFFERED FOODS VARYING IN FLAVORS, NUTRIENTS, AND A TOXIN

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Abstract—We conducted two experiments to determine how toxicosis affected preference of sheep for foods varying in flavors, nutrients, and a toxin. The first experiment determined how toxicosis affected the preference of lambs (fed a basal ration of alfalfa pellets) for foods that varied in energy and a toxin. Thirty lambs (10/treatment) were given LiCl by gavage (0, 50, or 100 mg/kg body wt/day), and 1 hr later were offered for 15 min/day foods containing different amounts (low, medium, high) of energy (barley) and a toxin (LiCl) added to alfalfa. The proportions of barley and LiCl changed every three to six days during the 30-day study. The results showed: (1) lambs' food preferences were high > medium > low for barley in the absence of LiCl; (2) lambs quickly regulated intake of foods in response to changes in barley and LiCl concentrations, even with short exposures (15 min/day); (3) lambs maintained intake of LiCl at about 57 mg/kg body wt by adjusting intake of food containing LiCl in accord with the amount of LiCl they received by gavage; and (4) as barley levels increased, intake of foods containing LiCl increased. The second experiment determined the relative influence of flavors, nutrients, and toxins on food preferences of lambs. We did this by treatments in which different flavors (onion and oregano at 1%) were paired with different levels of energy (depending on the addition of wheat to rabbit pellets) or a toxin (LiCl). At six-day intervals, we varied the types of food offered, either changing the nutrient or toxin content and the flavors. The resulting analyses of preference showed lambs markedly preferred foods high in nutrients and low in toxins, regardless of flavor, when changes in food flavor were not correlated with changes in nutrient and toxin concentrations. Thus, in both experiments lambs quickly regulated intake of foods varying in nutrients and a toxin according to the lambs' toxicological and nutritional state. Even with

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brief eating bouts lambs discriminated accurately and exhibited little permanent preference or aversion in postconditioning preference tests. The lambs remained in an unbiased testing mode, sampling anew the food. This is adaptive because the toxin and nutrient contents of plants vary with season and location. Most taste aversion studies emphasize the permanence of aversions and miss the dynamic sampling power of animals.

Key Words—Food preference, flavor, nutrients, toxins, learning, sheep, *Ovis aries*.

INTRODUCTION

Wild and domestic ruminants confront temporal and spatial variation in nutrients and toxins in food (Provenza and Balph, 1990). Animals generally select foods higher in nutrients and lower in toxins than average levels in available foods, indicating that they possess mechanisms to meet these challenges. Taste and odor, and their association with postingestive feedback, appear to be vitally important in directing food selection and intake by ruminants (Provenza, 1995). Ruminants sample small quantities of a novel food or a familiar food whose flavor has changed (Provenza et al., 1993a, 1995) and subsequently adjust intake depending upon the postingestive consequences, thus enabling them to determine changes in concentrations of toxins and nutrients. If toxicity of a food decreases, the taste of the food is no longer paired with aversive postingestive consequences, and intake increases to reflect the nutritional value of the food. Conversely, intake decreases as the toxicity of the food increases.

Animals evidently limit intake of nutritious foods that contain toxins in accord with the amount of a particular toxin they can detoxify (Freeland and Janzen, 1974; Brattsten, 1979; Lindroth, 1988; McArthur et al., 1991; Provenza et al., 1992). For instance, sheep regulate intake of food containing LiCl to 40–60 mg LiCl/kg body wt/day (duToit et al., 1991; Launchbaugh et al., 1993). In previous studies, toxin concentrations were varied, and nutrient concentrations were constant. However, the ratios of nutrients and toxins often change simultaneously with changes in soil moisture and nutrients, plant phenology, and shade (McKey, 1979; Harborne, 1991; Bryant et al., 1983, 1992). In such situations, there is a trade-off in food selection between nutrient requirements and poisoning; which foods are eaten probably depends on an animal's toxicological and nutritional state.

An animal's nutritional needs change depending on the nutrient content of the foods it ingests, and macronutrients (energy and protein) play a primary role in food preference. Lambs strongly prefer foods high in readily fermentable energy, and they acquire strong preferences even for poorly nutritious foods like straw when straw is eaten during intraruminal infusions of energy (Villalba and Provenza, 1996, 1997a) or nitrogen (Villalba and Provenza, 1997b). Nonethe-

less, food preferences depend on what was just eaten. Lambs fed a ration high in energy (grain) subsequently prefer food lower in energy and higher in protein (alfalfa), whereas those fed a ration high in protein (alfalfa) subsequently prefer food high in energy (grain) (Wang and Provenza, 1996a). Lambs maintain a constant ratio of energy to protein in their diet when allowed to select from foods varying in energy and protein (Provenza et al., 1996).

In the following experiments, we addressed two questions related to the interplay between nutritional and toxicological state and the chemical characteristics of the food offered. First, we determined how different degrees of toxicosis (caused by gavage of the toxin LiCl prior to eating) affected selection by sheep of foods varying in energy (barley) and LiCl when sheep were fed a basal ration of alfalfa pellets (high in protein but marginal in energy for growth), and then offered a choice of foods that varied in energy and LiCl. We hypothesized that food preference would be directly related to degree of toxicosis, and we predicted that as the dose of LiCl administered prior to a meal increased, sheep would avoid further intoxication by consuming foods low in LiCl, even though those foods were lower in energy. Second, we determined how sheep selected diets when a food's flavor was not consistently correlated with its nutrient or toxin content. We examined this issue by treatments in which flavors (onion and oregano at 1%) were paired with various levels of energy and LiCl in foods.

METHODS AND MATERIALS

Degree of Toxicosis. This experiment was conducted at the Green Canyon Ecology Center in Logan, Utah, and involved 30 lambs (average body weight 38 kg) that were crosses of Columbia, Rambouillet, Suffolk, Targhee, and Finnish Landrace breeds. The lambs were purchased at 4 to 6 weeks of age, and exposed for two months to the foods used in this study. At 4 months of age, the lambs were placed in individual pens, adjacent to one another. For one week prior to the study, lambs were fed alfalfa pellets ad libitum from 07:00 to 17:00 hr each day, and the intake of each lamb was measured. All lambs had access to water and salt ad libitum.

We varied toxicological status by assigning lambs at random to three treatments (10/treatment): 0, 50, or 100 mg LiCl/kg body wt. LiCl was administered by gavage in gelatin capsules 1 hr before each daily test (15 min/day) during four separate periods (days 4–6, 13–15, 22–24, and 28–30; Table 1). LiCl is absorbed rapidly into the bloodstream and reaches peak levels in the rumen within 60 min of its administration (Provenza et al., 1993b).

The foods offered to lambs during the 30-day trial are shown in Table 1. The foods differed in concentrations of energy and a toxin, depending on the

TABLE 1. FOODS OFFERED AND TIMING OF LiCl ADMINISTRATION TO LAMBS DURING EXPERIMENT 1 (DEGREE OF TOXICOSIS)

Days	Treatment	Foods offered (15 min/day)[a]	Time of LiCl administration
1-3	1	high barley—50A + 50B	
	2	medium barley—75A + 25B	
	3	alfalfa—100A	
4-9	1	high barley—50A + 50B + 1Li	1 hr before testing on days 4-6
	2	medium barley—75A + 25B + 0.5Li + 0.5Na	
	3	alfalfa—100A + 1Na	
10-12	1	high barley—25A + 75B	
	2	medium barley—50A + 50B	
	3	alfalfa—100A	
13-18	1	high barley—25A + 75B + 1Li	1 hr before testing on days 13-15
	2	medium barley—50A + 50B + 0.5Li + 0.5Na	
	3	alfalfa—100A + 1Na	
19-21	1	high barley—25A + 75B + 2Li	
	2	medium barley—50A + 50B + 1Li + 1Na	
	3	alfalfa—100A + 2Na	
22-27	1	high barley—25A + 75B + 2Li	1 hr before testing on days 22-24
	2	medium barley—50A + 50B + 1Li + 1Na	
	3	alfalfa—100A + 2Na	
28-30	1	high barley—25A + 75B + 2Na	1 hr before testing on days 28-30
	2	medium barley—50A + 50B + 2Na	
	3	alfalfa—100A + 2Na	

[a] A is alfalfa pellets, B is rolled barley, Li is lithium chloride, and Na is sodium chloride; numbers represent the proportion of each component. Barley and alfalfa were ground and mixed. Calculated values for digestible energy (DE) and digestible protein (DP) were as follows: 25A:75B—3.1 Mcal/kg DE and 10.1% DP; 50A:50B—2.8 Mcal/kg DE and 10.4% DP; 75A:25B—2.5 Mcal/kg DE and 10.6% DP; 100A—2.3 Mcal/kg DE and 10.9% DP. Calculated values for DE and DP were based on the following values obtained from nutrient requirements of sheep (NRC, 1985): barley 3.35 Mcal/kg DE, 9.8% DP; alfalfa 2.27 Mcal/kg DE, 10.9% DP.

addition of barley (energy) and LiCl (toxin) to alfalfa (Table 1). On days 1-3, three foods [(1) 50% barley and 50% alfalfa, (2) 25% barley and 75% alfalfa, (3) 100% alfalfa] were offered to each lamb. On days 4-9, 1% LiCl was added to food 1, 0.5% LiCl and 0.5% NaCl to food 2, and 1% NaCl to food 3. On days 10-12, the barley concentration was increased in food 1 (i.e., 75% barley and 25% alfalfa) and food 2 (i.e., 50% barley and 50% alfalfa), while food 3 remained the same, i.e., 100% alfalfa. From days 13 to 18, 1% LiCl was again added to food 1, 0.5% LiCl and 0.5% NaCl to food 2, and 1% NaCl to food

3. On days 19–27, LiCl was increased from 1% to 2% in food 1 and from 0.5% to 1% in food 2. On the last three days, 2% NaCl was added to all three foods.

Each lamb was offered 400 g of each food for only 15 min/day to determine how quickly lambs adjusted to changes in nutrient and toxin concentrations in foods. The positions of boxes containing the three foods were changed daily. Intake of each food was measured daily. After each daily trial, lambs were offered alfalfa pellets ad libitum from 09:00 hr to 17:30 hr. The amount of alfalfa pellets consumed by each lamb was measured daily.

The analysis of variance was a factorial with repeated measures (Hicks, 1993). There were three treatments (0, 50, 100 mg LiCl/kg body wt), and three foods (low, medium, high in energy and a toxin). Animals (N = 10) were nested within treatments. We separated the 30-day trial into seven periods (days 1–3, 4–9, 10–12, 13–18, 19–21, 22–27, and 28–30) based on the different foods offered and LiCl treatments, and we analyzed each period separately using the SAS statistical program (Cody and Smith, 1991; Hatcher and Stepanski, 1994).

Variation in Flavors, Nutrients, and a Toxin. This experiment involved 30 lambs raised at the Green Canyon Ecology Center near Utah State University in Logan, Utah. The lambs (average body weight 46 kg) were crosses of Columbia, Rambouillet, Suffolk, Targhee, and Finnish Landrace breeds. At 6 months of age, the lambs were put in individual pens with free access to water and mineral blocks and fed alfalfa pellets ad libitum from 09:00 to 18:00 hr daily. Conditioning and preference testing were conducted for 15 min/day (08:00 to 08:15 hr). The lambs had eaten wheat and rabbit pellets in a previous experiment. Onion and oregano were novel to the lambs; these flavors were added to their alfalfa pellets two days before conditioning began.

The first phase of the experiment evaluated the response of lambs to foods varying in energy and flavors. During nutritional conditioning (days 1–6), lambs were assigned to three treatments (10 lambs/treatment) and were allowed to select between two foods varying in flavors (onion and oregano) and containing different amounts of energy, depending on the addition of wheat (energy) to rabbit pellets: treatment 1—high energy + onion vs. low energy + oregano; treatment 2—intermediate energy + onion vs. intermediate energy + oregano; or treatment 3—low energy + onion vs. high energy + oregano (Table 2). During postnutritional preference tests (days 7–12), all lambs were allowed to choose between two foods with the same levels of energy (intermediate), but flavored with either onion or oregano (Table 2). Onion and oregano were added at 1% concentration.

The second phase of the experiment determined the response of lambs to a toxin. During toxin conditioning (days 13–18), lambs in the three treatments were offered food intermediate in energy, but with the following additional ingredients: treatment 1—onion + NaCl vs. oregano + LiCl; treatment 2—

TABLE 2. INTAKE OF FOODS DURING EXPERIMENT 2 (FLAVORS, NUTRIENTS, AND TOXINS)

Treatment	Foods offered[a]	Intake (g/15 min)	
		Onion-flavored food	Oregano-flavored food
Nutrient conditioning (days 1–6)			
1	75W ON and 25W OR	241a[b]	111b
2	50W ON and 50W OR	169a	156a
3	25W ON and 75W OR	118a	235b
Postnutrient conditioning preference test (days 7–12)			
1	50W ON and 50W OR	187a	178a
2	50W ON and 50W OR	196a	154b
3	50W ON and 50W OR	194a	184a
Toxin conditioning (days 13–18)			
1	50W ON NaCl and 50W OR LiCl	235a	55b
2	50W ON NaCl and 50W OR NaCl	180a	151b
3	50W ON LiCl and 50W OR NaCl	54a	207b
Posttoxin conditioning preference test (days 19–24)			
1	50W ON NaCl and 50W OR NaCl	168a	151a
2	50W ON NaCl and 50W OR NaCl	182a	146b
3	50W ON NaCl and 50W OR NaCl	168a	159a

[a]W is wheat. ON and OR represent onion and oregano at 1% concentration, respectively. NaCl and LiCl were added at 2% concentration. The remainder of each food was rabbit pellets. Wheat and rabbit pellets were ground and mixed. Foods were high (75% wheat: 3.66 Mcal/kg DE and 10.8% CP), intermediate (50% wheat: 3.44 Mcal/kg DE and 10.1% CP), or low (25% wheat: 3.22 Mcal/kg DE and 9.5% CP) in digestible energy (DE) and crude protein (CP). Values for digestible energy and crude protein were based on food values listed in NRC (1985).

[b]a,b: Means with different letters differ ($P < 0.05$). SEM is 12 for nutrient conditioning, 10 for postnutrient conditioning, 12 for toxin conditioning, and 8 for posttoxin conditioning.

onion + NaCl vs. oregano + NaCl; or treatment 3—onion + LiCl vs. oregano + NaCl (Table 2). During posttoxin preference tests (days 19–24), all lambs were again allowed to choose between two foods with the same levels of energy (intermediate) and NaCl, but flavored with either onion or oregano (Table 2). NaCl and LiCl were added at 2% concentration, and onion and oregano were added at 1% concentration.

The analysis of variance was a factorial with repeated measures (Hicks, 1993). There were three treatments, and lambs ($N = 10$) were nested within treatments. Food ($N = 2$) was a split plot in the analysis. We separated the 24-day trial into four periods (conditioning, days 1–6; testing, days 7–12; conditioning, days 13–18; testing, days 19–24) and analyzed each period separately using the SAS statistical program (Cody et al., 1991; Hatcher et al., 1994).

RESULTS

Degree of Toxicosis. Lambs in the control group (no LiCl administered by gavage) changed intake of the three foods in response to changes in concentrations of barley and LiCl in the food (Figure 1). When there was no LiCl in the foods (days 1–3, 10–12, 28–30), consumption was: food 1 (high barley) > 2 (medium barley) > 3 (alfalfa) ($P < 0.01$). When LiCl was added to foods 1 and 2 (days 4–9) and when LiCl concentration increased (days 19–27), intake of foods 1 and 2 decreased, whereas intake of food 3 increased ($P < 0.05$ for food × day interactions). When LiCl increased to 1% in food 2 (days 19–27), consumption was food 1 > 3 > 2 ($P < 0.05$).

Lambs dosed with 50 mg LiCl/kg body wt responded intermediately compared to lambs in the other treatments (Figure 2). In the absence of LiCl in the foods (days 1–3, 10–12, 28–30), food preference was 1 > 2 > 3 ($P < 0.01$). When LiCl was added, lambs decreased intake of foods 1 and 2 and increased intake of food 3 ($P < 0.05$ for food × day interaction), especially after they received LiCl in capsules (days 4–6, 13–15, 22–24). Lambs preference was food 1 > 2 and 3 during days 4–9 and 13–27 ($P < 0.05$).

Lambs dosed with 100 mg LiCl/kg body wt showed the largest fluctuations in intake of the three foods (Figure 3). In the absence of LiCl in foods (days

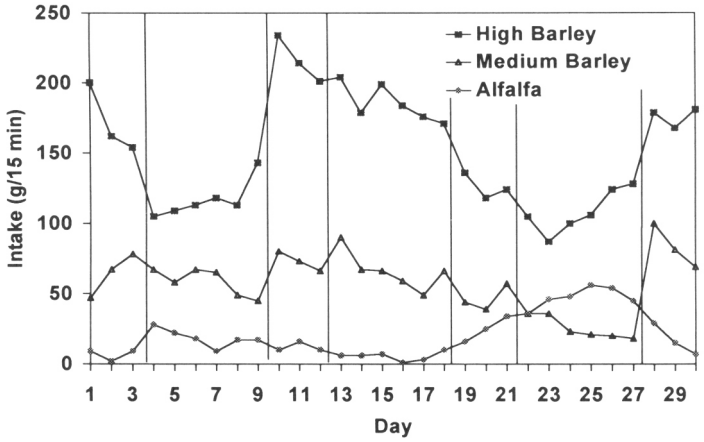

FIG. 1. Intake of three foods by lambs that did not receive LiCl by gavage. Vertical lines represent changes in nutrient and toxin content of the three foods (see Table 1 and text for details). Standard errors were calculated for each feeding period: days 1–3, SEM = 9; days 4–9, SEM = 8; days 10–12, SEM = 9; days 13–18, SEM = 8; days 19–21, SEM = 9; days 22–27, SEM = 7; days 28–30, SEM = 12.

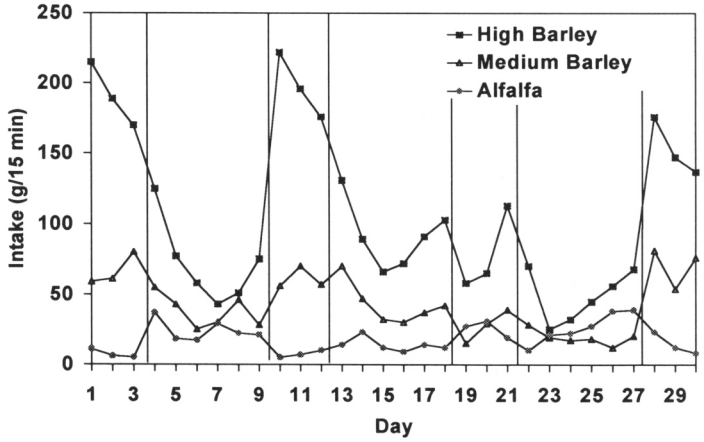

FIG. 2. Intake of three foods by lambs that received 50 mg LiCl/kg body wt by gavage during days 4–6, 13–15, 22–24, and 28–30. Vertical lines represent changes in nutrient and toxin content of the three foods (see Table 1 and text for details). Standard errors were calculated for each feeding period: days 1–3, SEM = 9; days 4–9, SEM = 8; days 10–12, SEM = 9; days 13–18, SEM = 8; days 19–21, SEM = 9; days 22–27, SEM = 7; days 28–30, SEM = 12.

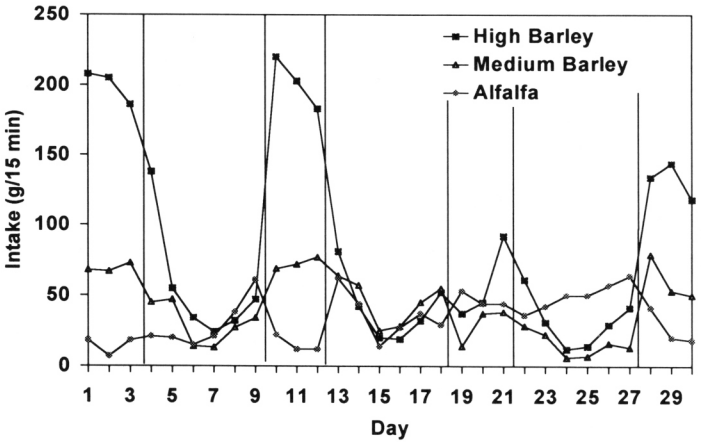

FIG. 3. Intake of three foods by lambs that received 100 mg LiCl/kg body wt by gavage during days 4–6, 13–15, 22–24, and 28–30. Vertical lines represent changes in nutrient and toxin content of the three foods (see Table 1 and text for details). Standard errors were calculated for each feeding period: days 1–3, SEM = 9; days 4–9, SEM = 8; days 10–12, SEM = 9; days 13–18, SEM = 8; days 19–21, SEM = 9; days 22–27, SEM = 7; days 28–30, SEM = 12.

1–3, 10–12, 28–30), their food preference was 1 > 2 > 3 ($P < 0.01$). Consumption of foods 1 and 2 decreased following the addition of LiCl and consumption of food 3 increased ($P < 0.05$ for food × day interaction), especially after they received LiCl in capsules (days 4–6, 13–15, 22–24). During days 5–9 and 13–23, lambs did not differ in intake of the three foods, but during days 24–27, they ate more of food 3 than foods 1 and 2 ($P < 0.05$).

Food selection of lambs experiencing different levels of toxicosis can be summarized as follows. Consumption did not differ when none of the foods contained LiCl (days 1–3, 10–12, 28–30). When LiCl was added to foods 1 (high barley) and 2 (medium barley) (days 4–9 and 13–27), lambs dosed with 50 or 100 mg/kg body wt LiCl ate less than controls of foods 1 and 2 ($P < 0.05$). Lambs dosed with 100 mg/kg body wt LiCl ate less of food 1 than lambs dosed with 50 mg/kg body wt LiCl on days 13–18 and 22–27 ($P < 0.05$), but not on days 4–9 and 19–21 ($P > 0.05$). There was no difference in intake of food 2 by lambs dosed with 50 or 100 mg/kg body wt LiCl ($P > 0.05$). Lambs dosed with 100 mg/kg body wt LiCl consistently ate more of food 3 (alfalfa) than lambs in the control group ($P < 0.05$).

Intake of LiCl by lambs differed when LiCl was added to foods 1 and 2 ($P < 0.05$). Daily intake of LiCl averaged 57, 32, and 21 mg/kg body wt for lambs dosed with 0, 50, and 100 mg/kg body wt LiCl. However, the average daily accumulation of LiCl throughout the trial (from food intake and capsules) was similar (57 mg/kg body wt) for all lambs ($P > 0.05$), and averaged 57, 52, and 61 mg/kg body wt for lambs dosed with 0, 50, and 100 mg/kg body wt LiCl.

LiCl concentrations were the same in foods 1 and 2 during days 4–9 and 13–18, but barley concentrations were increased during days 13–18. Increasing barley concentrations enhanced ($P < 0.05$) intake of LiCl by controls (from 38 to 58 mg/kg body wt), but not ($P > 0.05$) by lambs dosed with 50 (from 24 to 30 mg/kg body wt) or 100 mg/kg body wt LiCl (from 18 to 17 mg/kg body wt) (Figure 4). Intake of LiCl did not increase ($P > 0.05$) when the level of barley was constant, and LiCl increased in foods 1 and 2 (from days 13–18 to days 22–27).

Intake of the basal ration (alfalfa pellets) differed only during days 4–6 when lambs were initially dosed with LiCl. For the 30-day experiment, the average daily intake of alfalfa pellets (from 09:00 hr to 17:30 hr) was 2171 g (SEM = 26), 2047 g (SEM = 27), and 2078 g (SEM = 25) for lambs dosed with 0, 50, and 100 mg/kg body wt LiCl, respectively. Thus, a dose of 50 or 100 mg LiCl/kg body wt did not adversely affect intake of the basal ration by lambs.

Variation in Flavors, Nutrients, and a Toxin. During nutritional conditioning (days 1–6), lambs in treatments 1 and 3 preferred the food highest in energy ($P < 0.05$), regardless of flavor, whereas lambs in treatment 2 ate similar

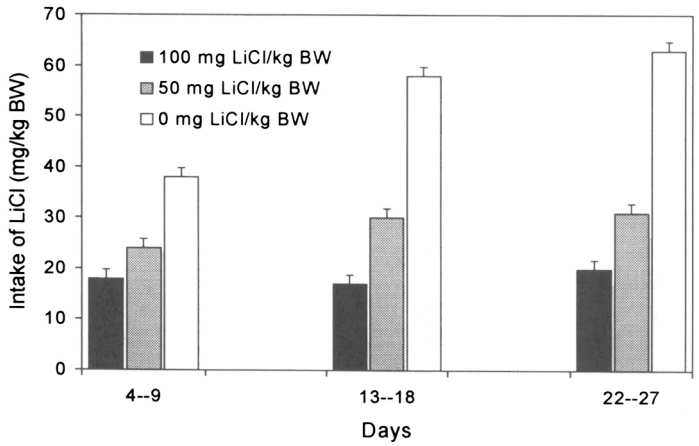

FIG. 4. Average daily intake of LiCl (SEM = 1.8) by lambs during three periods. When the level of LiCl was the same, but barley concentrations increased from days 4–9 to days 13–18, lambs in the control group (0 mg LiCl/kg body wt by gavage) increased intake of LiCl ($P < 0.05$), whereas lambs dosed with 50 mg LiCl/kg body wt or 100 mg LiCl/kg body wt by gavage did not change their intake of LiCl ($P > 0.05$). When the level of barley remained the same, but LiCl increased from days 13–18 to days 22–27, intake of LiCl did not increase by lambs in any of the treatments ($P > 0.05$).

amounts of both foods, both of which provided comparable amounts of energy ($P < 0.01$ for treatment × food interaction; Table 2). During postnutritional testing (days 7–12), when lambs could choose between two foods that provided the same levels of energy but differed in flavors, lambs in treatments 1 and 3 ate similar amounts of both foods ($P > 0.05$), and lambs in treatment 2 showed a slight preference for onion- over oregano-flavored food ($P < 0.05$; Table 2); oregano contains various oils, phenols, and glucopyranosides that likely caused the decrease in preference (Duke, 1992). There was no treatment × food interaction ($P = 0.19$).

During toxin conditioning (days 13–18), when foods contained similar amounts of energy but differed in flavor and toxin (or salt) content, lambs in treatments 1 and 3 preferred the food containing NaCl ($P < 0.05$), regardless of flavor (onion or oregano), and lambs in treatment 2 preferred onion-flavored food ($P < 0.05$; for treatment × food interaction $P < 0.01$; Table 2). During posttoxin preference tests (days 19–24), when all lambs could choose between two foods containing the same levels of energy, but differing in flavors, lambs in treatments 1 and 3 ate similar amounts of both foods ($P > 0.05$), and lambs in treatment 2 again showed a slight preference for onion- over oregano-flavored food ($P < 0.05$; Table 2). There was no treatment × food interaction ($P = 0.27$).

DISCUSSION

Degree of Toxicosis. Freeland and Janzen (1974) hypothesized that generalist herbivores should consume only small amounts of new foods, learning quickly to eat or avoid a specific food after ingesting small quantities. Our results agree with these speculations. Lambs regulated intake of foods according to concentrations of energy (barley) and a toxin (LiCl) after only brief (15-min) daily exposures, but their responses were often delayed when concentrations of LiCl changed (e.g., days 4, 13, 19), during which time they initially tended to ingest more LiCl. When LiCl remained constant, lambs increased intake as barley concentrations increased. When barley level remained constant, they decreased intake as LiCl concentrations increased.

The degree of toxicosis affected food preference. Lambs dosed with 50 or 100 mg LiCl/kg body wt ate less foods containing LiCl than controls, even though these foods contained more energy (barley). Lambs dosed with 100 mg LiCl/kg body wt avoided energy-rich and previously preferred foods that also contained LiCl. The response of lambs dosed with 50 mg LiCl/kg body wt was intermediate to that of lambs dosed with 100 mg LiCl/kg body wt and controls. All lambs maintained a constant level of LiCl in their bodies (about 57 mg/kg body wt), which is consistent with results of Launchbaugh et al. (1993) (62 mg/kg body wt) and Wang and Provenza (1996b) (58 mg LiCl/kg body wt). Collectively, these results indicate that the mass of plant material eaten is determined by the mass of toxin in that material. Most studies report only the concentration of toxin (or nutrient) in a plant tissue and attempt to relate concentration to intake, often with little success. Our present and earlier (Provenza et al., 1996) results indicate that what should be determined is the mass ingested of toxin or nutrient.

Nutritional state also affected food preference. Feeding alfalfa pellets ad libitum as the basal ration (from 09:00 hr to 17:30 hr daily) exceeded lambs' requirements for digestible protein, but scarcely met their requirements for digestible energy. The average daily intake of alfalfa pellets (2099 g) provided 229 g of digestible protein (DP) and 4.8 Mcal of digestible energy (DE), whereas lambs (40 kg body weight) require 107 g DP and 4.9 Mcal DE to gain 0.25 kg/day (NRC, 1985). Their preference for food high in energy is consistent with the hypothesis that preference for food high in energy or protein depends on whether or not a lamb just ate a meal high in energy or protein and with the notion that lambs maintain a constant ratio of energy to protein in their diet when allowed to select from a variety of foods (Provenza et al., 1996). Thus, within the limits of toxin satiation, lambs adjusted food intake on the basis of their nutritional state, a result that supports the notion of nutritional wisdom within the context of plant chemical defense (Wang and Provenza, 1996a,b).

Most studies of food selection treat nutrient selection and toxin avoidance

as separate processes. Lambs dosed with LiCl rejected nutritious foods that aggravated toxicosis, whereas control lambs selected foods high in energy that also contained toxins. Moreover, increased energy content enhanced intake of foods containing toxins. Lambs in the control group ingested 38 mg LiCl/kg body wt during days 4–9 when food 1 contained 1% LiCl and 50% barley, and food 2 contained 0.5% LiCl and 25% barley. When LiCl levels remained constant in these two foods, but barley increased to 75% in food 1 and 50% in food 2 (days 13–18), the daily intake of LiCl increased to 58 mg/kg body wt. LiCl produced prolonged and acute hypoglycemia in rats and cattle (Johnson et al., 1980), which may be alleviated by higher energy concentrations. There is also evidence that nutrients provide substrate to enhance detoxification (McArthur, et al., 1991; Foley et al., 1995; Illius and Jessop, 1995).

Humans cannot reliably distinguish between 0.12 M solutions of LiCl and NaCl, nor can rats distinguish between sodium water and lithium water (Garcia and Rusiniak, 1977), but lambs discriminated between LiCl and NaCl, even though they evidently taste similar to sheep (Hill and Mistretta, 1990). During days 22–27, we added 2% LiCl to food 1, 1% LiCl and 1% NaCl to food 2, and 2% NaCl to food 3. Lambs in the control group preferred food 1 (108 g) > 3 (47 g) > 2 (26 g); lambs dosed with 50 mg LiCl/kg body wt preferred food 1 (49 g) > 3 (26 g) > 2 (19 g); and lambs dosed with 100 mg LiCl/kg body wt preferred food 3 (50 g) > 1 (31 g) > 2 (15 g). In the last three days (28–30), we removed LiCl and added the same concentration of NaCl (2%) to all three foods. Although lambs received LiCl in capsules during the three days, all lambs regained their preference for food 1 > 2 > 3 ($P < 0.001$).

Variation in Flavors, Nutrients, and a Toxin. During conditioning, when foods provided different levels of nutrients or toxins, lambs preferred foods high in nutrients and low in toxins, regardless of flavor (onion or oregano). Even with brief eating bouts, lambs discriminated accurately and exhibited little permanent preference or aversion in postconditioning preference tests. The lambs remained in an unbiased testing mode, sampling anew the nutrient and toxin status of the food. Most taste aversion studies emphasize the relative permanence of aversions and miss the dynamics of foraging in environments where toxins and nutrients vary temporally and spatially.

Food preference results from the interplay between taste and postingestive feedback, determined by an animal's physiological condition relative to a food's chemical characteristics (Garcia, 1989; Provenza, 1995, 1996a,b). Taste (as well as odor and sight) enables animals to discriminate among foods and provides hedonic sensations associated with eating. Feedback calibrates taste in accord with a food's homeostatic utility. Animals prefer foods that contain needed nutrients and they become averse when foods are deficient in nutrients or contain excesses of toxins. The preference for flavor according to postingestive feedback is adaptive because salient plant flavors may not correlate well

with nutrient or toxin content of a plant (Manners et al., 1992). When flavor and nutrients or toxins are highly correlated, herbivores first learn to regulate food intake based on flavor-feedback interactions, and then simply adjust intake on the basis of changes in flavor concentration (Launchbaugh et al., 1993, Wang and Provenza, 1996a,b).

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ARE TROPICAL HERBIVORES MORE RESISTANT THAN TEMPERATE HERBIVORES TO SEAWEED CHEMICAL DEFENSES? DITERPENOID METABOLITES FROM *Dictyota acutiloba* AS FEEDING DETERRENTS FOR TROPICAL VERSUS TEMPERATE FISHES AND URCHINS

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Abstract—Because herbivory is more intense in the tropics, tropical seaweeds may be better defended against herbivory than are temperate seaweeds. A "diffuse" coevolutionary corollary to this hypothesis is that tropical herbivores should be more resistant to seaweed defenses than temperate herbivores because tropical herbivores more commonly encounter heavily defended seaweeds. We begin to test the latter prediction using three newly discovered diterpenoid secondary metabolites from the tropical brown alga *Dictyota acutiloba*. We tested the feeding deterrent properties of these compounds against common herbivorous fishes and sea urchins from warm-temperate North Carolina versus tropical Guam using standardized laboratory feeding assays. The temperate herbivores were deterred by lower concentrations of secondary metabolites than the tropical herbivores. In no case was a tropical herbivore more deterred by a compound than a temperate herbivore, suggesting that

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temperate herbivores may be more strongly affected by seaweed chemical defenses. Feeding by the temperate pinfish *Lagodon rhomboides* was significantly reduced by two of the three diterpenes at a concentration that was only 13–18% of the natural concentration found in the alga. Feeding by four species of tropical fishes (two parrotfishes and two surgeonfishes) was unaffected by metabolite concentrations that deterred the temperate fish. At 100% of natural concentrations, only one of the three compounds deterred the two parrotfishes, and none of the three compounds deterred the surgeonfishes. Contrasts between the temperate sea urchin *Arbacia punctulata* and the tropical sea urchin *Diadema savignyi* showed a similar pattern; low concentrations of acutilol A acetate strongly deterred the temperate, but not the tropical, urchin. Tropical herbivores appear more resistant than temperate herbivores to seaweed chemical defenses.

Key Words—Acutilol diterpenes, biogeography, chemical defenses, *Dictyota*, diffuse coevolution, fish, plant-herbivore interactions, sea urchin, secondary metabolites.

INTRODUCTION

Because marine herbivores can have dramatic effects on populations and communities of benthic macroalgae, seaweeds must avoid, deter, or tolerate herbivory (Lubchenco and Gaines, 1981; Hay, 1985, 1991; Duffy and Hay, 1990; John et al., 1992). An important herbivore deterrent used by many seaweeds is the production of chemical defenses (Hay and Fenical, 1988; Van Alstyne and Paul, 1988; Hay and Steinberg, 1992; Paul, 1992).

There is geographic variation in the numbers and types of seaweed secondary metabolites found in seaweeds. More than 1000 small lipophilic secondary metabolites have been isolated and characterized from tropical algae (Faulkner, 1994 and references therein), but the diversity and concentration of these compounds appear to decrease at higher latitudes (Hay and Fenical, 1988). Recent direct tests of the palatability and chemical deterrencies of temperate versus tropical seaweeds demonstrated that tropical seaweeds were less palatable to both temperate and tropical sea urchins and that the reduced palatability of tropical seaweeds was due to greater deterrent effects of lipophilic, rather than water-soluble, chemical defenses (Bolser and Hay, 1996). Thus, tropical herbivores will be more likely than temperate herbivores to encounter potent lipophilic chemical defenses.

In contrast to lipophilic secondary metabolites, water-soluble phlorotannins (i.e., polyphenolics produced by brown seaweeds) were initially reported to be more abundant in temperate than in tropical seaweeds (Steinberg, 1989, 1992; Van Alstyne and Paul, 1990), but this apparent latitudinal pattern has not held when tested in additional locations (Targett et al., 1992, 1995). The paucity of phlorotannins in some tropical seaweeds was attributed to their ineffectiveness

against tropical herbivores (Steinberg et al., 1991; Steinberg and Van Altena, 1992; Targett et al., 1995). However, phlorotannins from temperate seaweeds do deter some tropical herbivores (Van Alstyne and Paul, 1990), so the ecological functions of algal phenolics are uncertain.

Numerous investigations indicate that tropical herbivores can be food limited in the field (Tsuda and Bryan, 1973; Robertson, 1982; Hay and Taylor, 1985; Carpenter, 1986, 1990; Morrison, 1988; Hay 1991), while food limitation of temperate herbivores appears to be less common (John et al., 1992). Given that tropical herbivores are faced with more limited (Hatcher and Larkum, 1983; Hay, 1985, 1991, Carpenter, 1986) and better defended (Hay and Fenical, 1988; Bolser and Hay, 1996) algal resources than temperate herbivores, we might expect large differences between temperate and tropical herbivores in their exposure to, and resistance to, the lipophilic chemical defenses that occur in seaweeds.

Although the effects of some lipophilic algal metabolites on feeding behavior are known for both tropical and temperate herbivores (Hay and Steinberg, 1992), confounding factors associated with using differing methodologies make it difficult to directly compare the responsiveness of tropical versus temperate herbivores to specific algal defenses. In this investigation, we compare how three newly discovered lipophilic secondary metabolites from the tropical brown seaweed *Dictyota acutiloba* affect feeding by common tropical versus temperate herbivores using standardized feeding assays.

METHODS AND MATERIALS

Dictyota acutiloba was collected from the seaward side of a shallow reef platform at Tunnels Beach on the north side of Kauai, Hawaii, in December 1990, stored in cold ethanol, returned to the Institute of Marine Sciences in North Carolina, and stored at $-30°C$ until processed. A few thalli were kept as vouchers, and the rest of the collection was extracted six times with a 2 : 1 mixture of dichloromethane (DCM)–methanol (MeOH) (Cronin et al., 1995). Individual metabolites were purified using silica-gel vacuum-flash chromatography followed by silica-gel high-performance liquid chromatography (HPLC). Structure assignments of the purified secondary metabolites acutilol A, acutilol A acetate, and acutilol B were achieved by standard spectroscopic methods, which emphasized proton and carbon nuclear magnetic resonance (NMR) spectrometry (Hardt et al., 1996).

The effects of the acutilols from *Dictyota acutiloba* on the feeding rates of warm-temperate Atlantic (North Carolina) and tropical Pacific (Guam) plant-eating fishes and sea urchins were determined by adding known amounts of purified compounds to an artificial food made from freeze-dried algae imbedded

in agar (Hay et al., 1994; Cronin and Hay, 1996). Tests in North Carolina used the pinfish *Lagodon rhomboides* and the sea urchin *Arbacia punctulata* because these represent the most common plant-eating fish and sea urchin in the South Atlantic Bight (Hay and Sutherland, 1988). The parrotfishes *Scarus schlegeli* and *Scarus sordidus*, surgeonfishes *Naso lituratus* and *Naso unicornis*, and the sea urchin *Diadema savignyi* were the tropical herbivores used in assays performed at the Marine Laboratory on Guam. Although the species' geographic ranges of some of our herbivores overlap with *Dictyota acutiloba* (Table 1), it is unlikely that any of the herbivores used in assays had previous exposure to the specific algal metabolites produced by *D. acutiloba* because the alga has not been reported from North Carolina or Guam (Tsuda, 1972; Schnieder and Searles, 1991) and these compounds have not been found in other species. However, all the herbivores could have experienced related compounds because other, chemically rich, species of the widespread genus *Dictyota* occur in both North Carolina and Guam (Meyer et al., 1994; Cronin et al., 1995).

Artificial algal food was made by reconstituting freeze-dried and finely powdered green seaweeds (a 5:4:1) mixture of *Ulva* sp., *Enteromorpha* sp., and *Cladophora* sp.) into an agar base and forming this onto fiberglass window screening material by pouring the agar-algal mixture into two openings in a mold placed over the window screen material [see Figure 1 in Hay et al. (1994) for an illustration of the artificial seaweeds and the procedures used to produce them]. The artificial food approximated the dry mass-wet mass ratio of *Dictyota* spp. For every gram of freeze-dried algae used in a recipe, 9.0 ml of distilled water and 0.18 g of agar were used. For the treatment food strip, compounds were adsorbed onto the dried algal particles before they were embedded in the

TABLE 1. SPECIES USED IN THIS STUDY WITH GEOGRAPHIC RANGE AND SITE OF COLLECTION

Species	Range	Collection site
Dictyota acutiloba	tropical Indo-Pacific, excluding Guam	Kauai, Hawaii
Fishes		
Lagodon rhomboides	Cape Cod to Yucatan	North Carolina
Scarus schlegeli	tropical Western Pacific, excluding Hawaii	Guam
Scarus sordidus	tropical Indo-Pacific, including Hawaii	Guam
Naso lituratus	tropical Indo-Pacific, including Hawaii	Guam
Naso unicornis	tropical Indo-Pacific, including Hawaii	Guam
Urchins		
Abracia punctulata	Cape Cod to Florida	North Carolina
Diadema savignyi	East Africa to Polynesia, excluding Hawaii	Guam

agar (Hay et al., 1994). Artificial food without added compounds was used as a control. In a few assays, we also tested the two minor metabolites at a much higher concentration ($\sim 5.5\times$ natural) to see if the nondeterrent effects of the minor metabolites at natural concentrations were due to them being less potent feeding deterrents than the major metabolite or due to their lower concentration.

The agar and water were mixed and heated to a boil in a microwave oven prior to adding them to the freeze-dried algae. To minimize heating the secondary metabolites, we either: (1) added 30% of the total volume of water to the powdered algae at room temperature and mixed this with the remaining 70% of water and agar that was boiled, resulting in food that was initially 65 $\pm$ 0.5°C (mean $\pm$ 1 SE, $N = 5$, North Carolina assays), or (2) allowed the total volume of heated water and agar to cool prior to mixing in the powdered algae, resulting in food that was initially 64 $\pm$ 0.3°C ($N = 3$) or 67 $\pm$ 1.2°C ($N = 3$), depending on the amount of artificial food being prepared (Guam assays). These small differences in initial temperatures were short-lived, because the food cooled quickly as it was spread into thin strips in the mold. Equal amounts of treated and control food were offered simultaneously to individual herbivores housed in separate aquaria, except for the scarids, which had two fish per replicate aquarium.

To standardize the methodologies, we attempted to use the same batch of freeze-dried green algae collected in North Carolina for all the assays. However, because *Naso* spp. refused to eat adequate amounts of the artificial green algal food, two parts of freeze-dried *Sargassum cristaefolium* (from Guam) were combined with one part of the mixture of green seaweeds to make the artificial algal food for assays with *Naso* spp. To see if artificial diet alone altered the effects of *Dictyota* metabolites on feeding (thus potentially confounding differences between *Naso* spp. and other herbivores with differences generated by the altered composition of the assay food), we also used both diets to assay two compounds against the parrotfish *Scarus schlegeli*.

The amount of food consumed from the test strips was determined by counting the number of window screen squares uncovered by the fishes or sea urchins. Because agar squares were not lost from the screen grid in the absence of grazers, problems associated with autogenic changes (Peterson and Renaud, 1989) were avoided. Data were analyzed with paired-sample t tests. Because other diterpenes from species of *Dictyota* commonly deter herbivores (Hay and Steinberg, 1992), we predicted that the acutilols from *D. acutiloba* would deter feeding by the herbivores. We therefore used directed P values (P_{dir}, *sensu* Rice and Gaines, 1994) to test the statistical significance of our results.

At the end of the assay, uneaten control and treatment foods were extracted with organic solvent, and each extract was analyzed by thin-layer chromatography against standard solutions of the acutilols to assure that each test compound was still present in the treated food.

Our herbivores were captured from natural habitats by hand or with nets and housed in flow-through aquaria during assays. They were fed nonassay foods between feeding experiments because food deprivation can influence the effect of secondary metabolites on herbivore feeding behavior (Cronin and Hay, 1996). Herbivores were used in more than one assay but never more than once per assay. Thus, it is possible that herbivores became sensitized from repeated exposures to compounds, but the space and time involved in housing and acclimating herbivores made it impractical to use new animals for each assay.

RESULTS

Three diterpenoid secondary metabolites were isolated from *Dictyota acutiloba*; acutilol A acetate [~1.1% dry mass (DM)]; acutilol A (~0.2% DM), and acutilol B (~0.2% DM) (Hardt et al., 1996). These previously undescribed compounds are structurally similar to secondary metabolites produced by other *Dictyota* species (compare structures in Figures 1–4 with *Dictyota* metabolites in Faulkner, 1994).

TLC analysis of extracts from uneaten assay food indicated that each secondary metabolite remained in the treated food, that no obvious breakdown products were present, and that the secondary metabolites were absent from the control food. Because treatment and control food differed only in secondary chemistry, differential feeding by herbivores can only be attributed to the presence/absence of the test compound.

Acutilol A acetate deterred feeding of the temperate pinfish *Lagodon rhomboides* at 0.2% DM, or only 18% of natural concentration (Figure 1A). The tropical parrotfishes *Scarus schlegeli* and *S. sordidus* were not affected by acutilol A acetate at 0.2% DM, but were deterred at 1.1% DM, or 100% of natural concentration (Figure 1B and C). In contrast to the previous three fish species, the surgeonfishes *Naso lituratus* and *N. unicornis* were not deterred by natural concentration of acutilol A acetate (Figure 1D and E).

Acutilol A deterred *L. rhomboides* at 0.025% DM or 13% of natural concentration (Figure 2A). In contrast, acutilol A had no effect on feeding by the four tropical fishes at 0.2% DM or 100% of natural concentration. Parrotfishes, however, were deterred by acutilol A at 1.1% DM (Figure 2B–E). This is 5.5× the natural concentration of acutilol A but equals the natural concentration of the major metabolite acutilol A acetate. Thus, levels of acutilol A acetate and acutilol A that strongly deterred feeding by the temperate fish had no effect on feeding by any of the four tropical fishes.

Natural concentration of acutilol B did not influence feeding by any of the fishes (Figure 3). However, as with acutilol A, *Scarus schlegeli* was deterred by acutilol B at 1.1% DM or 5.5× natural concentration (Figure 3B).

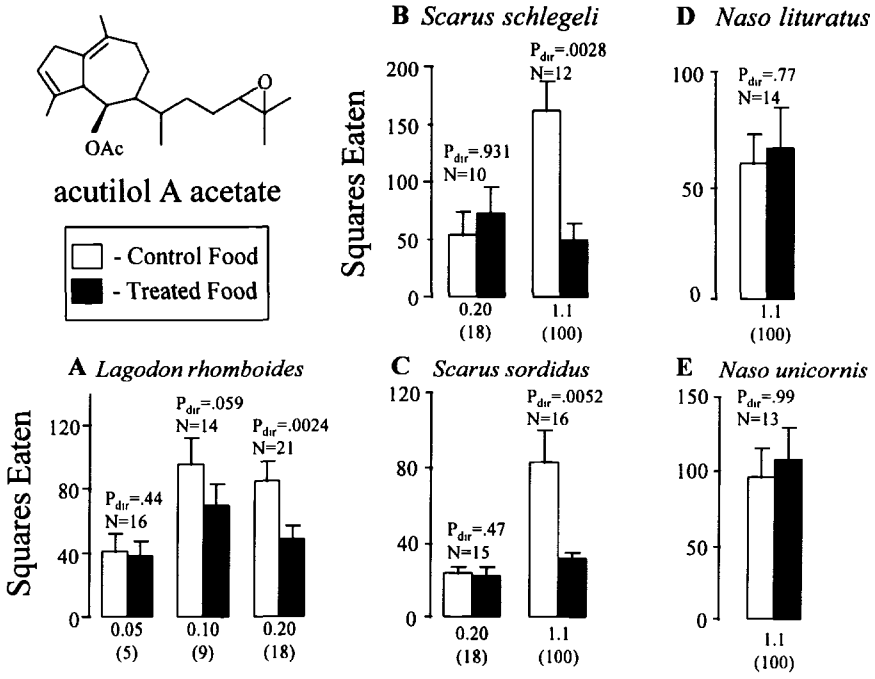

FIG. 1. The effect of acutilol A acetate on feeding by pinfish from North Carolina (A), parrotfishes from Guam (B and C), and surgeonfishes from Guam (D and E). The concentrations of acutilol A acetate tested are given below each pair of bars as both percentage of food dry mass and approximate percentage of the natural concentration (in parentheses) found in *Dictyota acutiloba*. Bars are the means + 1 SE amount of food consumed and each pair of bars represents one feeding assay. P_{dir} values are from paired-sample *t* tests.

The ineffectiveness of the *Dictyota* compounds against *Naso* spp. was not a result of the altered test food (i.e., 2:1 *Sargassum*-green algae). When we used altered food in assays with the parrotfish *Scarus schlegeli*, results were similar to those obtained using the standard food. Acutilol A at 0.2% DM did not affect feeding by the scarid (26.5 ± 7.9 vs. 27.0 ± 7.4 squares, mean ± 1 SE, $P_{dir} = 0.62$; compare to Figure 2B), but 1.1% DM of acutilol B reduced feeding by 52% (64.8 ± 19.2 vs. 31.0 ± 12.8, $P_{dir} = 0.043$; compare to Figure 3B).

The temperate sea urchin *Arbacia punctulata* was deterred by acutilol A acetate at 0.2% DM or 18% of natural concentration, by acutilol A at 0.2% DM or 100% of natural concentration, but not by acutilol B at 100% of natural concentration (Figure 4). The tropical sea urchin *Diadema savignyi* was deterred

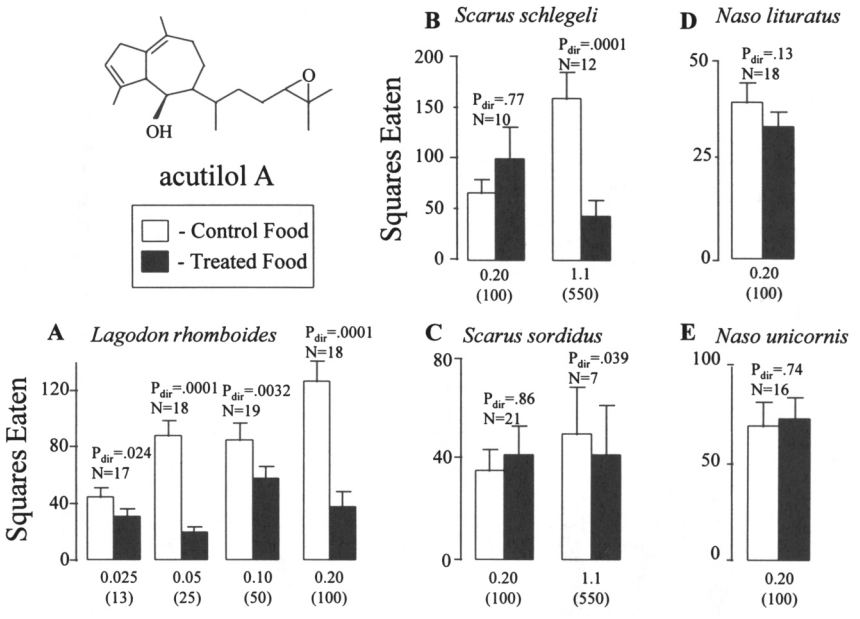

FIG. 2. The effect of acutilol A on the feeding behavior of pinfish (A), parrotfishes (B and C), and surgeonfishes (D and E). Symbols are as in Figure 1.

by acutilol A acetate at 100% of natural concentration but not by the 18% of natural concentration that deterred *Arbacia* (Figure 4D). As with the fishes and the temperate urchin, acutilol B also did not deter *D. savignyi* at 100% of natural concentration. We lacked enough acutilol A to assess the effect of this compound on feeding by *D. savignyi*.

DISCUSSION

Tropical herbivores encounter seaweeds that are richer in lipophilic secondary metabolites than those encountered by their temperate counterparts (Hay and Fenical, 1988). A recent direct experimental evaluation of palatability and chemical defenses of tropical vs. temperate seaweeds found that tropical seaweeds were generally less palatable and better defended chemically than temperate seaweeds and that differences in palatability were due to lipid-soluble, rather than water-soluble, metabolites (Bolser and Hay, 1996). Just as seaweed defenses are believed to evolve in response to intense herbivory (Hay, 1991; Steinberg et al., 1995), diffuse (Janzen, 1980) or guild (Thompson, 1989) coevolutionary theory would indicate that herbivores consistently confronted with

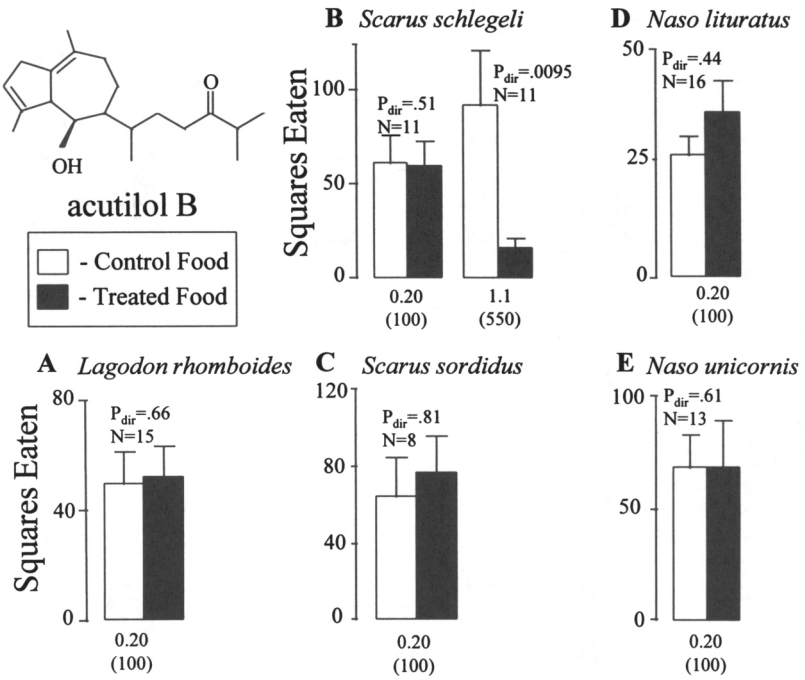

FIG. 3. The effect of acutilol B on the feeding behavior of pinfish (A), parrotfishes (B and C), and surgeonfishes (D and E). Symbols are as in Figure 1.

highly defended seaweeds should evolve increased resistance to algal defenses. Thus, tropical herbivores should be more resistant to seaweed defenses than temperate herbivores.

Consistent with this hypothesis, the compounds we tested were less effective against tropical than temperate herbivores. *Lagodon* from North Carolina was deterred by 13–18% of the natural concentration of acutilol A acetate or acutilol A, while the four tropical fishes were not deterred by these concentrations of these compounds. The two tropical parrotfishes were deterred by acutilol A acetate at 100% of natural concentration but the two species of surgeonfishes were not affected by any of the acutilol diterpenes. The temperate sea urchin *Arbacia* was deterred by 18% of the natural concentration of acutilol A acetate, while the tropical sea urchin *Diadema* was not deterred by this level, but was deterred by 100% of natural concentration. In no case was a tropical herbivore more deterred by a compound than a temperate herbivore. That the five tropical herbivores were less affected by the acutilols than the two temperate herbivores supports the hypothesis that herbivores consistently faced with well-defended

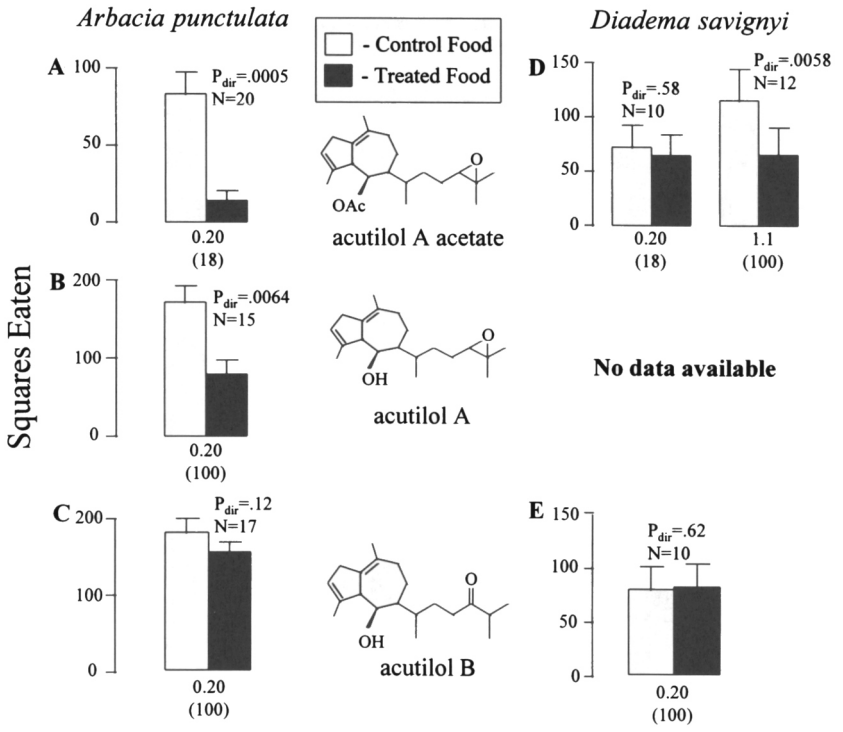

FIG. 4. The effect of acutilol A acetate (A), acutilol A (B), and acutilol B (C) on the temperate sea urchin *Arbacia punctulata* and the effect of acutilol A acetate (D) and acutilol B (E) on the tropical sea urchin *Diadema savignyi*. Symbols are as in Figure 1.

seaweeds may evolve increased resistance to chemical defenses (Estes and Steinberg, 1988; Steinberg and van Altena, 1992; Steinberg et al., 1995). Similarly, temperate herbivores in the North American Pacific region coexist with weakly defended seaweeds and are susceptible to seaweed chemical defenses, while Australasian herbivores coexist with chemically rich seaweeds but are generally resistant to the effects of these secondary metabolites (Steinberg et al., 1995).

Natural concentrations of acutilol A acetate deterred both temperate herbivores and three of the five tropical herbivores, demonstrating that *Dictyota acutiloba* is chemically defended from grazing by some herbivores. However, none of the acutilols deterred feeding by either species of *Naso*. Other investigators also have shown that species of *Naso* and some related rabbitfishes (Siganidae) are tolerant of a wide range of seaweed chemical defenses that deter feeding by other common reef herbivores (Meyer et al., 1994; Schupp and Paul, 1994;

Meyer and Paul, 1995). The tolerance of *Naso* species for our *Dictyota* metabolites is consistent with their known feeding patterns. Species of *Naso* often selectively consume brown seaweeds, including *Dictyota* (Hiatt and Strasburg, 1960; Robertson and Gaines, 1986; Meyer et al., 1994).

Natural concentrations of acutilol A acetate deterred feeding by both species of parrotfishes, but natural concentrations of the very similar compounds acutilol A and acutilol B did not. This difference occurred because the latter two compounds were present in much lower concentrations, not because they were less deterrent at an equivalent dosage. When acutilol A and B were tested separately at 1.1% DM (i.e., equivalent to the natural concentration of acutilol A acetate, but $\sim 5.5\times$ the natural concentration of these metabolites), each significantly decreased feeding by parrotfishes at this higher concentration. This may allow the mixture of these compounds to have additive, or even synergistic (Berenbaum and Neal, 1985; Hay et al., 1994), effects against herbivores. We were unable to test this possibility due to our limited supply of compounds.

The structurally similar diterpenes produced by *Dictyota acutiloba* differed markedly in their effects on feeding by different herbivores. For example, both *Lagodon* and *Arbacia* were significantly deterred by either acutilol A acetate or acutilol A at 0.2% DM; however, acutilol B (a ketone derivative of acutilol A) did not deter *Lagodon* or *Arbacia* at 0.2% DM. Just as deterrent effects varied among related secondary metabolites, the effectiveness of a compound also varied among herbivores. For example, at or below natural concentration, acutilol A acetate deterred the pinfish, both parrotfishes, and both sea urchins, yet the compound was ineffective at reducing feeding by the two surgeonfishes. That seaweeds cooccur with multiple herbivores, any one of which may or may not be deterred by a particular compound, may account for why seaweeds commonly produce multiple secondary metabolites or commonly combine chemical and structural defenses in tropical habitats (Hay et al., 1994; Schupp and Paul, 1994).

Persistent, intense herbivory experienced on tropical reefs (Carpenter, 1986; Hay, 1991) can result in low algal abundance, food limitation for reef herbivores (Tsuda and Bryan, 1973; Robertson, 1982; Hay and Taylor, 1985; Robertson and Gaines, 1986; Morrison, 1988; Carpenter, 1990), and possibly select for herbivores that are tolerant of algal chemical defenses. Similar patterns may occur in certain temperate systems if herbivores escape control by consumers and impose unusually strong selection on seaweeds (Steinberg et al., 1995). Previous studies demonstrated that the responsiveness of marine herbivores to seaweed chemical defenses was related to the herbivore's mobility and size (Hay et al., 1987, 1988, 1990; Duffy and Hay, 1994), taxonomic affiliation (Hay, 1992), or hunger level (Cronin and Hay, 1996). We suggest that consistent exposure to defended prey over evolutionary time may also contribute to an herbivore's response to chemical defenses.

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INFLUENCE OF *Prokelisia* PLANTHOPPERS ON AMINO ACID COMPOSITION AND GROWTH OF *Spartina alterniflora*

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Abstract—The effects of feeding by the planthopper *Prokelisia dolus* on its host, *Spartina alterniflora*, were examined under conditions of both high and low plant-nitrogen subsidy. Phloem feeding by *P. dolus* reduced the concentrations of γ-aminobutyric acid, isoleucine, leucine, lysine, threonine, and valine in *S. alterniflora* leaves. In contrast, glutamic acid was the only amino acid that increased in plants fed upon by planthoppers, and this increase was only observed in plants in the high N-fertilizer treatment. Planthopper feeding reduced the total concentration of amino/imino acids tested, and the concentration of essential amino acids, although this difference was not quite statistically significant. Generally, concentrations of individual amino acids in *Spartina* were higher in the high N-fertilizer treatment. Planthopper feeding and nitrogen fertilization also significantly impacted *Spartina* growth and production. Culm elongation, new leaf production, and tiller elongation were reduced and leaf mortality was increased on plants fed upon by planthoppers. Furthermore, planthoppers showed enhanced survival on the high-N plants.

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Nitrogen fertilization also moderated the effects of sap feeding on plant growth because the reduction of culm elongation associated with planthopper feeding was more pronounced in the low N-fertilizer treatment. Thus, feeding by *P. dolus* adversely affected both the nutritional quality and growth of *Spartina*, effects that were tempered, in part, in plants receiving high nitrogen subsidy. Our results are discussed in the context of feeding-induced changes in plant quality and quantity as possible mechanisms mediating competitive interactions in *Prokelisia* planthoppers.

Key Words—Amino acids, Delphacidae, plant nitrogen, nutritional ecology, planthoppers, plant growth, sap-feeding insects.

INTRODUCTION

Modification of host plant quality by feeding is a possible mechanism underlying intra- and interspecific competition among herbivorous insects (Schultz, 1988; Karban and Myers, 1989; Denno and Roderick, 1992; Damman, 1993; Denno et al., 1995; but see Faeth, 1992). Induced changes in attacked plants that reduce the suitability of hosts for later feeders include diminished nitrogen and carbohydrate availability (Cagampang et al., 1974; McClure, 1980; Inbar et al., 1995), increased levels of deterrents or toxic phytochemicals (Haukioja and Niemelä, 1979; Toumi et al., 1984; Tallamy and Raupp, 1991), diminished plant growth or altered plant structure (Berube, 1980; Fritz et al., 1986; Wellings and Dixon, 1987), and the release of volatiles that attract natural enemies (Price et al., 1980; Vet and Dicke, 1992). Although feeding-induced changes in host plant quality have been studied for mandibulate insects (e.g., Haukioja and Niemelä, 1979; Rhoades, 1983; Niemelä and Toumi, 1987; Tallamy, 1985), this phenomenon has been less well investigated for sap-feeding herbivores (Meyer, 1993; Dixon et al., 1993; Denno et al., 1995).

Poor plant nutrition can have very adverse effects on the performance and fitness of sap feeders such as planthoppers and aphids (McNeill and Southwood, 1978; Dixon, 1985; Denno et al., 1986; Cook and Denno, 1994). A probable mechanism by which sap feeders alter host plant quality for subsequent herbivores is by an induced change in plant nutrition, specifically amino nitrogen (McClure, 1980; Sōgawa, 1982; Denno and Roderick, 1992; Dixon et al., 1993). Sap-feeding insects are known to alter the levels of amino nitrogen in their host plants, and levels of free amino acids in attacked plants may increase, decrease, or remain unchanged depending upon the plant species (Weibull, 1988; Douglas, 1993; Sandström and Pettersson, 1994), plant phenology (Parry, 1977; Prestidge and McNeill, 1982; Dixon et al., 1993; Douglas, 1993), or the density of sap feeders (Cagampang et al., 1974; Sōgawa, 1982; Bacheller and Romeo, 1992). One potentially important consideration is that amino acid levels often increase immediately when plants are attacked, but then decrease with time

(Sōgawa, 1971; Bacheller and Romeo, 1992; Cook and Denno, 1994). Thus, the benefits of such pulses in available nitrogen for herbivores may be rather short-lived and may not be realized by subsequent-feeding herbivores that either attack plants later or have relatively extended developmental periods (Cook and Denno, 1994).

Nitrogen availability in host plant tissues is considered a limiting factor for many herbivores including sap feeders (McNeill and Southwood, 1978; Mattson, 1980; Raven, 1983). Nitrogen subsidy can dramatically increase the amount of total nitrogen and free amino acids available in phloem sap (Prestidge and McNeill, 1982; Pfeiffer and Burts, 1984; Cook and Denno, 1994), and densities of phloem feeders often increase following nitrogen fertilization of hosts (Cheng, 1971; Prestidge, 1982; Denno et al., 1985; Lightfoot and Whitford, 1987; Denno and Roderick, 1990), occasionally reaching outbreak proportions (Kenmore, 1980). What is not well understood is how the amount of nitrogen available to plants interacts with herbivore feeding to influence the concentration of free amino acids for subsequent herbivores.

The monophagous planthopper *Prokelisia dolus* Wilson (Hemiptera: Delphacidae) feeds on the perennial cordgrass *Spartina alterniflora* Loisel. throughout the intertidal salt marshes of North America (Denno and Roderick, 1992; Denno et al., 1995). The soluble protein content of *Spartina* declines as *Prokelisia* densities increase over the summer in the field (Denno et al., 1985). Nitrogen availability to plants is most limited in the high marsh (Cavalieri and Huang, 1981) where *Prokelisia* species occur sympatrically for much of the growing season (Denno and Roderick, 1992). This study explores one possible mechanism underlying the direct and delayed effects of competition between *Prokelisia* planthoppers, namely feeding-induced changes in the amino acid chemistry and growth of their host plant.

To test the hypothesis that sap feeding alters plant physiology, we used a factorial experiment to examine the effect of *P. dolus* on the growth and amino acid composition of *S. alterniflora*. Our specific objectives were to determine: (1) if the amino acid composition and growth of *Spartina* plants differ when they are grown with and without planthoppers, and (2) if nitrogen fertilization modifies any planthopper-induced differences in plant growth or amino acid chemistry.

METHODS AND MATERIALS

Experimental Design. To evaluate the potentially interactive effects of feeding by *Prokelisia dolus*, feeding duration, and nitrogen subsidy on the growth and nutrition of *Spartina*, we compared the growth and amino acid composition of plants grown with or without planthoppers under high or low N-fertilizer regimes. Specifically, we used a 3 × 2 × 2 factorial design with planthoppers

(present, absent, and a cage control), N-fertilizer subsidy (low or high), and feeding duration (nine and 15 days after planthoppers were established on plants) as main factors. Uncaged plants served as cage controls and were used to evaluate any effect of caging on plant growth and chemistry independent of planthopper effects.

Spartina alterniflora plants were grown from seed in sand-filled pots (6.3 cm) placed in plastic-lined flats (1.0 × 0.7 m^2) filled to 3 cm with water. The two nitrogen subsidy treatments (low and high) were established by fertilizing plants biweekly at two rates (1 g/flat or 7 g/flat) with a 3 : 1 mixture of ammonium nitrate (N/P/K, 34 : 0 : 0) and phosphoric acid (0 : 46 : 0) for three months prior to their use in the experiment. Two flats of plants were fertilized for each nitrogen treatment and were maintained in the greenhouse (see Denno et al., 1985, for details). The two fertilizer regimes result in plants with soluble nitrogen contents that span those that occur naturally in the field (Denno et al., 1985, 1986).

Because plants were fertilized prior to the application of planthopper treatments, we measured the dry weight of five randomly selected plants from each N-fertilizer treatment on the same day that the planthopper/cage treatments were applied (day 0). The average biomass value for each N-fertilizer treatment was determined and subtracted from the final values to adjust for initial differences in plant mass between the two N-fertilizer treatments. Prior to the random application of planthopper/cage treatments, all *Spartina* seedlings were thinned to three stems per pot.

Planthoppers were established on plants by placing 200 field-collected adults of *P. dolus* within a cage constructed of clear plastic cellulose butyrate tubing (see Denno and Roderick, 1992, for details). Planthoppers were collected in *Spartina* meadows near Tuckerton, Ocean County, New Jersey, three days prior to their use in the experiment. Each of the six planthopper/cage × fertilizer treatment combinations was replicated at least 10 times. The effect of feeding duration on plant growth and chemistry was evaluated by assessing half of the replicates after nine days of exposure to planthoppers and measuring the other half after 15 days of exposure.

Amino Acid Extraction and Analysis. Plants for amino acid analysis were harvested nine or 15 days after planthoppers were established on treatment plants. Whole-plant samples (leaves from all three stems) were collected in the greenhouse, placed immediately on Dry Ice, and taken to the laboratory where they were lyophilized and then ground in a Wiley mill. Whole-leaf samples, rather than phloem exudate, were used as indicators of the chemical composition of phloem sap.

Powdered leaf material (50 mg) was extracted 3× with 2.0 ml MCW (MeOH–$CHCl_3$–H_2O, 12 : 5 : 1). Combined supernatant was separated into an upper aqueous and a lower $CHCl_3$ phase by the addition of 1.5 ml H_2O and 1.0

ml $CHCl_3$. The aqueous layer was removed, dried under a stream of air, and redissolved in 0.5 ml of 25% EtOH. Samples for the amino acid analyses were prepared by drying 0.1 ml of the above solution under air and redissolving in 0.5 ml of pH 2.0 analyzer buffer (Singh et al., 1973), which is described below.

Amino acids were separated using a Dionex model MBF/SS amino acid/peptide analyzer. The organic nitrogen compounds analyzed included all essential amino acids, nonessential amino acids, and the imino acids proline (Pro) and pipecolic acid (PIP). Extracts were loaded onto a 0.4 × 12.0-cm column packed with DC-5A cation exchange resin (Dionex Corp.). OPA (*o*-phthaldialdehyde) was used as the postcolumn fluorescence detection reagent. Fluorescence was measured with a Gilson Fluorometer with wavelength of excitation 360 nm and emission 455 nm. Integration of peak areas was performed using a Shimadzu C-R3A Chromatopac data processor. Peaks were identified by absolute retention time and quantified by relation to standards of known concentration.

The analyzer was equipped with a modified Dionex secondary amine kit (model SAA 26401) for detecting imino acids. Chl-T was used as the oxidant for opening the imino rings of Pro and PIP prior to reaction with OPA (Bleecker and Romeo, 1982). The oxidation reaction temperature was maintained in a delay coil for 60 sec at a constant 111.5°C by a Haake circulating hot oil bath (model E2).

Two amino acid analyzer runs were performed on each sample, one for the primary amino acids and a second for imino acids. Amino acids were sequentially eluted from the column using six buffers (A–F) of increasing pH in a constant 0.2 M solution of Na^+ cations. Seventeen of 20 protein amino acids and γ-aminobutyric acid were resolved in the system. The amides asparagine and glutamine coeluted with serine (Asx). An abbreviated second run, utilizing buffers A, B, and F and a Chl-T delay coil prior to mixing with OPA detected Pro and PIP (Bleecker and Romeo, 1982).

Amino acid analyzer buffers contained 2.0 g NaOH, 8.76 g NaCl, 0.25 g Na_2EDTA, and 1.0 ml phenol per liter solution. The sample buffer and buffers A, B, and C were brought to the desired pH of 2.0, 3.1, 3.5, and 4.2, respectively, by titrating with formic acid (99%). Eluent D was titrated to pH 7.1 with H_3PO_4 (85%), and eluent E was brought to pH 10.0 with 3.5 g H_3BO_3 and two pellets of NaOH. An additional regenerant buffer, eluent F, contained 4.0 g NaOH, 5.84 g NaCl, 0.25 g Na_2EDTA, and 1.0 ml phenol per liter.

A three-way factorial of analysis of variance was used to evaluate the effects of planthoppers, N-fertilizer levels, time, and their interactions on *Spartina* amino/imino acid composition. Sidak's *t* tests were used to compare interaction means. Prior to final analyses, residuals were examined for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test, Milliken and Johnson, 1984). Throughout the text and tables, means are expressed with one standard error

and amino acid concentrations are expressed as nanomoles per gram *Spartina* dry mass.

Although the experimental treatments were randomized in 3 × 2 × 2 factorial design, data from caged plants and uncaged plants were analyzed separately for ease of presentation of results. The results from the partial analyses did not differ from those of the more complex factorial ANOVA.

Plant Growth. Just prior to their harvest for amino acid analysis, all plants were measured to determine the effects of planthoppers, feeding duration, and fertilization on growth. The following plant parameters were measured on days 9 and 15: culm elongation per stem (average elongation in centimeters for the three stems per pot); leaf gain per stem; number of dead leaves per stem; number of tillers produced per pot; tiller growth (total elongation in centimeters for tillers in the pot); and dry biomass (pooled biomass of all tillers and stems in the pot). Dry biomass measurements (grams) were made on plants harvested for the amino acid analysis (total of the three-stem harvest). After nine and 15 days, we determined planthopper densities and calculated planthopper loads (load = number of planthoppers per gram dry mass *Spartina*) for each replicate. Three-way analyses of variance, as described in the previous section, were used to evaluate differences in plant growth parameters among treatments and treatment combinations.

RESULTS

Planthopper and N-Fertilization Effects on Amino Acid Composition of Spartina. Planthoppers significantly reduced the concentrations of Ile, Leu, Lys, Thr, and Val in plants on which they fed compared to planthopper-free controls (Figure 1; Table 1). Concentrations of Trp were initially increased by planthopper-feeding in the high N-fertilizer treatment but this increase was not observed in the low N-treatment or in either fertilizer treatment after 15 days (significant three-way interaction in Table 1). Planthoppers also reduced, but not quite statistically, the total concentration of essential amino acids ($\bar{X}$ = 5581 ± 376 nmol/g) compared to control plants ($\bar{X}$ = 6320 ± 458 nmol/g; $F_{1,42}$ = 3.69, $P < 0.07$).

Planthoppers significantly reduced the concentrations of GABA and Ala, but they did not significantly reduce the concentration of any other nonessential amino acid (Table 1, Figure 1). In fact, Ala concentrations were only reduced by planthopper feeding after nine days in the low-N treatment, and no significant differences existed between the planthopper and control plants after 15 days (three-way interaction in Table 1). Glutamic acid was the only amino acid that increased significantly in plants fed upon by planthoppers. Concentrations of Glu increased in response to planthopper feeding in plants in the high N-fertilizer treatment; no significant planthopper effect was detected in the low N-fertiliza-

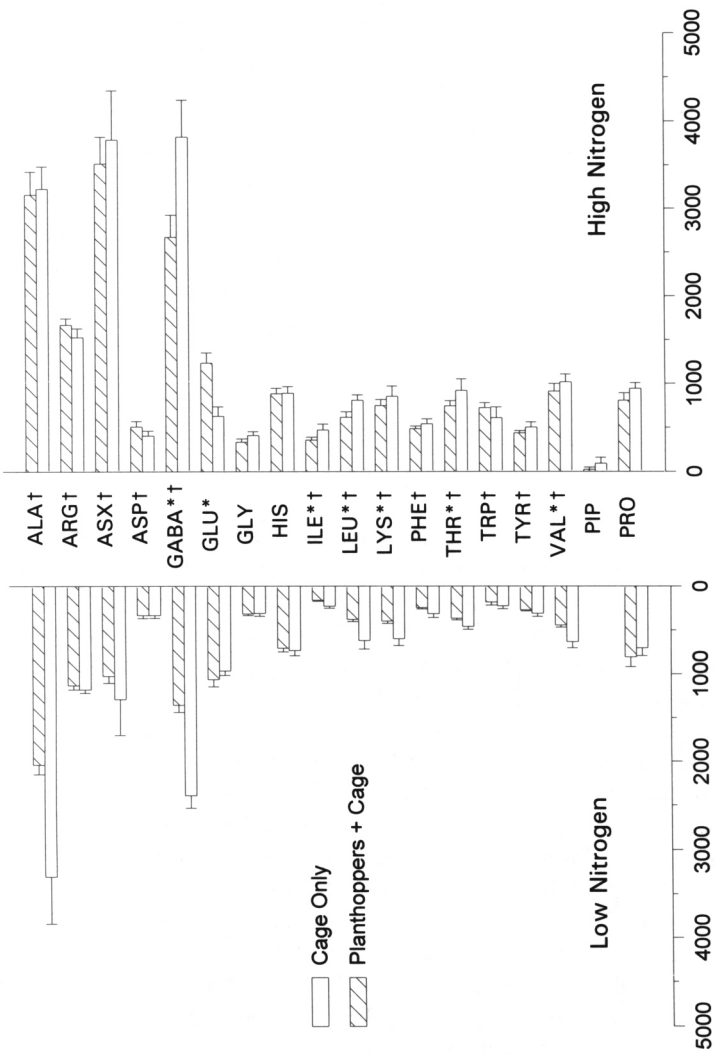

FIG. 1. Amino/imino acid concentrations of *Spartina alterniflora* caged with or without *Prokelisia dolus* planthoppers. Plants were randomly assigned to low or high N-fertilizer treatments. Asterisks (*) indicate significant differences between the planthopper treatment and controls (Table 1). Daggers (†) indicate significant differences between low and high N-fertilizer treatments (Table 1).

TABLE 1. ANALYSES OF VARIANCE RESULTS FOR EFFECTS OF PLANTHOPPERS, N-FERTILIZER TREATMENTS, AND TIME ON CONCENTRATIONS OF ESSENTIAL AND NONESSENTIAL AMINO ACIDS IN *Spartina alterniflora*[a]

		Essential amino acids						
Source of variation	*df*	Arg	His	Ile	Leu	Lys	Phe	Thr
Planthoppers	1	26,993	4,440	98,618***	536,513**	305,047*	44,525	208,736*
Fertilizer	1	2,144,528***	288,800**	554,846***	551,859**	1,030,724***	623,001***	2,079,481***
Planthopper × fertilizer	1	71,443	0.92	7,261	9,845	209,776	128	17,071
Time	1	142,599	7,113	57,273*	128,017	89,582	8,987	57,577
Planthopper × time	1	125,363	815	40,453	26,498	12,216	7,311	88,307
Fertilizer × time	1	335,504*	270,693*	72,758*	6,807	227,487	82,308*	78,733
Planthopper × fertilizer × time	1	28.2	33,245	39,182	50,823	60,457	26,413	126,561
Error	42	49,202	40,193	13,017	47,967	62,874	13,528	52,076

[a] $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

tion treatment (Figure 1; significant planthopper × fertilizer interaction in Table 1).

As expected, N fertilization increased the concentrations of most amino acids assayed (Figure 1). Specifically concentrations of Asp, Leu, Lys, Thr, and Val were significantly greater in plants receiving more nitrogen (Figure 1; Table 1). Concentrations of Arg, Asx, GABA, Ile, Phe, and Tyr were greater in plants in the high N-fertilizer treatment on both sampling days, but the difference was much greater on day 15 (significant fertilizer × time interaction term in Table 1).

There were no statistically significant effects of planthoppers, time, N fertilizer, or interactions on Gly, the two imino acids, or the two sulfur-containing compounds (Met and Cys). Concentrations of total amino/imino compounds in plants on which planthoppers fed were significantly lower ($\bar{X}$ = 15,700 ± 1120 nmol/g) than controls ($\bar{X}$ = 18,100 ± 1390 nmol/g; Table 1). As expected, the total concentration of amino/imino acids was significantly influenced by N-fertilization (significant fertilizer × time interaction term in Table 1). Total amino/imino acids were higher in plants in the high-N treatment on day 9 (high-N $\bar{X}$ = 17,840 ± 1291 nmol/g; low-N $\bar{X}$ = 13,544 ± 1227 nmol/g; $P < 0.05$; Sidak's *t* test), but the effect of fertilization was much greater by day 15 (high-N $\bar{X}$ = 23,077 ± 1551 nmol/g; low-N $\bar{X}$ = 11,838 ± 656 nmol/g; $P < 0.05$; Sidak's *t* test).

Caging Effects on Amino Acid Composition of Spartina. After 15 days, concentrations of Ala, GABA, Glu, and Trp were higher in caged plants than uncaged controls (Table 2). Caged plants in the high N-fertilizer treatment also

Mean square values								
		Nonessential amino acids						
Trp	Val	Ala	Asx	Asp	Gaba	Glu	Tyr	Total amino/imino acids
9,442	271,240*	6,077,317*	1,180,235	17,717	14,577,603***	1,178,319***	32,695	80,188,369*
2,519,911***	2,109,323***	2,982,128	70,662,106***	162,061*	21,906,005***	122,319	372,334***	725,519,220***
69,517	16,835	3,914,761*	30,543	25,827	52,019	670,323**	2,773	8,012,140
161,406	85,509	27,029	2,658,514	232,405**	3,476,736*	1,225,860***	10,162	48,283,362
19,506	19,446	3,945,842*	406	419	1,466,768	36,999	8,927	3,161
284,353*	181,082	7,054,914**	13,629,960***	54,965	2,883,158*	299,115	60,003*	173,195,751**
206,442*	109,831	3,657,559*	4,125,985	262	1,807,394	3,266	31,633	64,405,778
42,282	61,255	856,080	1,088,675	27,755	576,900	91,760	11,820	17,030,000

had higher levels of Arg, His, and total amino/imino acids than did caged plants in the low-N treatment, but no difference in the concentrations of these amino acids existed between control (uncaged) plants in the two N-fertilizer treatments (Table 2). Concentrations of Asx were greater in the high N-fertilizer treatment in both control and caged plants, but within the high-N treatment, caged plants had more than twice the Asx than controls (Table 2). The other amino/imino acids assayed did not show a response to plant caging.

To summarize the cage effects, amino acids levels in caged plants were generally high relative to controls, and this difference was often marked when nitrogen availability to the plant was high (cage × fertilizer interaction in Table 2). This may represent a stress response of plants to the cages because increased concentrations of free amino acids have been associated with plant stress (Cagampang et al., 1974; Stewart and Larher, 1980). In contrast, in the low N-fertilizer treatment, amino acid levels in caged and control plants generally did not differ significantly (Table 2). Thus, in the absence of sufficient nitrogen, *Spartina* plants may not exhibit the same stress response.

Planthopper and N-Fertilizer Effects on Spartina *Growth.* Planthopper feeding negatively impacted *Spartina* growth and development. New leaf production and tiller elongation were reduced and the number of dead leaves were increased on *Spartina* fed on by planthoppers (Table 3, Figure 2). Culm elongation was also significantly reduced by planthopper feeding, but the difference in culm elongation was greater in plants in the low N-fertilizer treatment (control plant $\bar{X} = 8.1 \pm 1.16$ cm; planthopper treatment $\bar{X} = 3.48 \pm 0.332$ cm; $P < 0.01$; Sidak's t test) than in the high-N treatment (control plant $\bar{X} = 4.1 \pm 0.89$ cm; planthopper treatment $\bar{X} = 1.50 \pm 0.262$ nmol/g; $P < 0.001$, Sidak's t test;

TABLE 2. AVERAGE ($\bar{X} \pm 1$ SE) AMINO ACID CONCENTRATIONS (nmol/g) IN CONTROL AND CAGED *Spartina alterniflora* PLANTS AFTER 15 DAYS[a]

		Caged plants	Control plants	$F_{1,16}$
Means comparisons for significant caging effects				
Ala		2950 ± 253	2285 ± 234	5.52*
GABA		3560 ± 436	2800 ± 304	4.89*
Glu		930 ± 94	603 ± 83	8.57**
Trp		496 ± 148	224 ± 62	5.41*
Means comparisons for significant Cage* Fertilizer interactions				
N-Fertilizer				
Arg	High	1720 ± 128*	1370 ± 146†	5.38*
	Low	1205 ± 61	1334 ± 35	
His	High	100 ± 98*	776 ± 56†	7.57*
	Low	640 ± 59	799 ± 57	
ASX	High	4860 ± 490*	2065 ± 523*†	13.82**
	Low	696 ± 121	628 ± 92	
Total	High	25740 ± 1810*	17130 ± 2150†	8.65**
	Low	12550 ± 970	13120 ± 933	

[a] Plants were randomly assigned to low or high N-fertilizer treatments ($n = 10$) and were grown in a greenhouse. Interaction means were compared using Sidak's t tests ($P < 0.05$). For comparisons of interaction means, daggers (†) indicate significant differences between caged and control plants within fertilizer treatments and asterisks (*) indicate significant differences between high and low N-fertilizer treatments within columns.

TABLE 3. ANALYSES OF VARIANCE RESULTS FOR EFFECTS OF PLANTHOPPERS, N-FERTILIZER, AND TIME ON GROWTH (CULM ELONGATION, LEAF GAIN, AND TILLER ELONGATION) AND LEAF LOSS OF *Spartina alterniflora*

		Mean square values[a]			
Source of variation	*df*	Culm elongation	Leaf gain	Tiller elongation	Dead leaves (*N*)
Planthoppers	1	153.5***	3.40***	83.6*	14.50***
Fertilizer	1	106.4***	0.07	36.04	0.02
Planthopper × fertilizer	1	12.3*	0.006	58.3	3.46
Time	1	81.5***	2.07***	66.7*	0.43
Planthopper × time	1	67.3***	0.55	9.28	0.14
Fertilizer × time	1	6.18	0.05	108.9*	5.92*
Planthopper × fertilizer × time	1	6.23	0.12	0.97	0.51
Error	42	2.26	0.16	15.78	0.99

[a] *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

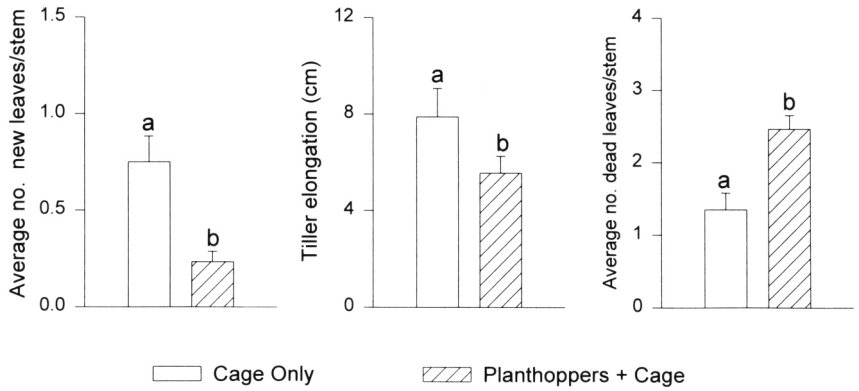

FIG. 2. Number of new leaves per stem, tiller elongation, and dead leaves ($\bar{X} \pm 1$ SE) on *Spartina alterniflora* plants grown with or without *Prokelisia dolus* planthoppers. Significant differences between control and planthopper treatments are indicated by different letters (Table 3).

significant planthopper × fertilization interaction in Table 3). A significant planthopper × time interaction also existed for culm elongation (Table 3) because this parameter did not differ between controls ($\bar{X} = 3.6 \pm 0.62$ cm) and the planthopper treatment on day 9 ($\bar{X} = 2.34 \pm 0.373$ cm; $P > 0.05$; Sidak's t test), but was reduced in the planthopper treatment by almost 75% by day 15 (planthopper treatment $\bar{X} = 2.58 \pm 0.397$ cm; control plant $\bar{X} = 8.6 \pm 1.13$ cm; $P < 0.01$; Sidak's t test).

Because N fertilizer had been applied to *Spartina* plants three months prior to the application of the planthopper/cage treatments, it was not surprising that the initial dry biomass readings (on day 0) from plants in the high-N treatment were almost three times ($\bar{X} = 0.276 \pm 0.069$ mg/plant) that observed in the plants in the low treatment ($\bar{X} = 0.094 \pm 0.0216$ mg/plant; $F_{1,8} = 6.33$, $P < 0.036$). After subtracting this initial difference, the final dry biomass values did not differ between plants in the two fertilizer treatments ($F_{1,42} = 0.43$, $P > 0.50$). Planthoppers slightly reduced the dry biomass of plants on which they fed ($\bar{X} = 0.72 \pm 0.053$ mg) compared to controls ($\bar{X} = 0.77 \pm 0.057$ mg) but this difference was not statistically significant ($F_{1,42} = 0.64$, $P > 0.40$).

Nitrogen fertilization of *Spartina* plants appeared to enhance the survival of *P. dolus*. Planthopper densities were greater on plants in the high-N treatment ($\bar{X} = 45.8 \pm 3.83$ individuals/stem) than in the low-N treatment ($\bar{X} = 35.5 \pm 2.72$ individuals/stem; $F_{1,25} = 12.65$, $P < 0.01$), and planthopper densities in both fertilizer treatments were higher on day 9 ($\bar{X} = 69.1 \pm 1.25$ individuals/

stem) than on day 15 ($\bar{X}$ = 38.8 ± 0.84 individuals/stem; $F_{1,25}$ = 14.93, $P < 0.001$).

Planthopper loads were also influenced by N fertilization. On day 9, planthopper loads were higher on plants in the high N-fertilizer treatment ($\bar{X}$ = 265 ± 37.1 individuals/g *Spartina* dry mass) than in the low-N treatment ($\bar{X}$ = 175 ± 17.4 individuals/g *Spartina* dry mass; $P < 0.05$, Sidak's t test; $F_{1,25}$ = 10.36, $P < 0.01$), but by day 15, loads were similar between the two fertilizer treatments (high-N $\bar{X}$ = 116 ± 11.2; low-N $\bar{X}$ = 161 ± 18.5 individuals/g *Spartina* dry mass; $P > 0.05$; Sidak's t test). The lack of difference in planthopper loads between the two fertilizer treatments on day 15 was due to the reduced biomass of plants in the low nitrogen treatment rather than to any increase in planthopper survival.

Caging Effects on Spartina *Growth.* A significant interaction between caging and fertilization effects existed for both culm elongation ($F_{1,32}$ = 4.26, $P < 0.05$) and new leaf production ($F_{1,32}$ = 3.99, $P < 0.05$). Culm elongation in the high-N treatment did not differ between caged plants ($\bar{X}$ = 4.1 ± 0.89 cm) and the uncaged controls ($\bar{X}$ = 3.6 ± 0.78 cm; $P > 0.05$, Sidak's t test). In contrast, in the low N-fertilizer treatment, culm elongation was greater for the caged plants ($\bar{X}$ = 8.1 ± 1.16 cm) than for uncaged plants ($\bar{X}$ = 5.4 ± 0.96 cm; $P < 0.05$, Sidak's t test). This difference may have been the result of etiolation by plants stressed in the low N-fertilizer treatment.

The number of new leaves produced by caged plants in the high N-fertilizer treatment ($\bar{X}$ = 0.80 ± 0.223) was significantly less than that produced by control plants ($\bar{X}$ = 1.30 ± 0.144; $P < 0.05$, Sidak's t test), but caging did not affect new leaf production in the low fertilizer treatment ($\bar{X}$ = 0.70 ± 0.161) compared to controls ($\bar{X}$ = 0.63 ± 0.135; $P > 0.05$, Sidak's t test). Dry plant biomass ($F_{1,32}$ = 2.40, $P > 0.05$) and the number of dead leaves ($F_{1,32}$ = 0.26, $P > 0.05$) did not differ between caged and control plants.

Although tiller production did not differ between caged and control plants ($F_{1,32}$ = 3.23, $P > 0.05$), tiller elongation was negatively affected by caging. Tiller elongation of caged plants ($\bar{X}$ = 7.9 ± 1.18 cm) was dramatically less than that of controls ($\bar{X}$ = 15.3 ± 2.72 cm; $P < 0.05$; $F_{1,32}$ = 6.88, $P < 0.05$). Thus, caging appears to negatively affect *Spartina* growth and elongation of new shoots.

DISCUSSION

Modification of host plant quality has been implicated as an important factor promoting intra- and interspecific competition among sap-feeding insects (McClure, 1980; Moran and Whitham, 1990; Inbar et al., 1995), but the mechanisms of host plant alteration and how such modifications impact subsequent

herbivores are not clear (Wellings and Dixon, 1987; Meyer, 1993; Denno et al., 1995). In this study, we provide evidence that *Prokelisia dolus* significantly reduces host plant quality by limiting plant growth and decreasing the levels of certain amino acids.

Specifically, we determined that the available surface area of *Spartina* plants was reduced as a consequence of a decrease in culm and tiller elongation, production of new leaves, and an increase in leaf mortality associated with planthopper feeding (Figure 2, Table 3). Three potential consequences of decreased plant surface area include reduced photosynthesis (Fitter and Hay, 1981; Crawley, 1983), a reduction in available nutrients (Schaffer and Mason, 1990), and increased herbivore crowding (Dixon and Logan, 1972, 1973). For many wing-dimorphic herbivores including *P. dolus*, intra- and interspecific crowding can trigger the production of migratory forms, and as a consequence there is an increase in emigration from crowded habitats (Dixon, 1985; Lamb and MacKay, 1987; Denno et al., 1985; Denno and Roderick, 1992; Denno and Peterson, 1995).

Feeding by *P. dolus* also significantly reduced the concentrations of GABA, Ile, Leu, Lys, Thr, and Val (Figure 1). Although some of these amino acids are considered essential for aphid growth and reproduction (Dadd and Krieger, 1968; Leckstein and Llewellyn, 1973; Emden, 1973), it is not clear if reductions in these compounds adversely impact the performance of planthoppers. Koyama (1992) found that no single amino acid is essential for growth of the white-backed planthopper, *Sogatella furcifera* (Horvath) (Hemiptera: Delphacidae), with the possible exception of sulfur-containing compounds (Cys or Met). Furthermore, yeastlike endosymbiotes may provide essential nutrients if specific amino acids are absent from the diets of planthoppers (Houk and Griffiths, 1980; Campbell, 1989). Perhaps more importantly in our study, planthopper feeding reduced the total concentration of amino acids in *Spartina* leaves, thereby reducing the amino nitrogen quality of these host plants for subsequent herbivores.

Feeding by *P. dolus* also increased concentrations of Glu (Figure 1). Glutamic acid is a sucking stimulant for the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae) (Sōgawa, 1982), and increased ingestion may be a compensatory mechanism for accelerating nutrient intake on nitrogen-deficient plants (McNeill and Southwood, 1978; Cook and Denno, 1994). Moreover, increased levels of Glu inhibit feeding altogether in some aphids and may indicate an overall deterioration in nutritional quality of the host plant (Srivastava et al., 1983; Weibull, 1988; Douglas, 1993).

Phloem-feeding insects leave stylet sheaths in plant tissues that disrupt phloem transport above the feeding site. As a consequence, local amino acid concentrations may temporarily increase (Cagampang et al., 1974; Sōgawa, 1982; Bacheller and Romeo, 1992). With the exception of Glu, such dramatic increases in amino acid concentrations did not occur in our study. This may be

due to one or more of the following reasons. First, marked increases in free amino acid concentrations are typically associated with high densities of planthoppers. Although the densities used in our experiment (initially 70 planthoppers/stem) represented the highest densities that occur in natural populations (Denno et al., 1986; Denno and Roderick, 1992), they were lower than those reported to cause the most dramatic increases in amino acid concentrations by Cagampang et al. (1974) and Bacheller and Romeo (1992). In these studies, increases in amino acid concentrations were much less or not observed at lower planthopper densities.

Although planthoppers can increase local amino acid concentrations under high-density conditions, plants may rapidly deteriorate and become unsuitable for planthopper feeding (Cagampang et al., 1974; Cook and Denno, 1994). Thus, mobile planthopper females may take advantage of temporary pulses of induced amino nitrogen, but they may not select such plants for oviposition on the basis of such cues as increased Glu that may signal the onset of plant deterioration (see Cook and Denno, 1994).

Second, some of the differences between our study and those reported for planthoppers feeding on rice (Sōgawa, 1971, 1982; Cagampang et al., 1974) may be due to differences between the physiology of *Spartina* and rice or differences in how these plants respond to phloem feeding. Numerous studies indicate that amino acid concentrations within the phloem vary among plant species and cultivars (Weibull, 1988; Douglas, 1993; Sandstöm and Pettersson, 1994).

Finally, the environment in which plants grow likely impacts the responses of plants to phloem feeders. In a study similar to ours, Bacheller and Romeo (1992) grew *S. alterniflora* under saline conditions and found dramatic increases in plant stress-associated compounds. In our experiment, salt was not added to the plant culturing medium. Thus, we did not observe significant increases in asparagine, an amide associated with plant stress (Brodbeck and Strong, 1987), or proline, which is specifically associated with osmotic stress in *Spartina* (Cavalieri and Huang, 1981).

In this study, the performance of both plant and herbivore was enhanced by increased nitrogen availability. Fertilization dramatically increased concentrations of most of the nitrogen compounds assayed (Figure 1); plant growth and production was enhanced by N fertilization; and planthopper survival was higher on plants in the high-N treatment. These results were anticipated because increased nitrogen availability from fertilization often results in enhanced insect performance or population increase (Waring and Cobb, 1989; Cook and Denno, 1994). Increased nitrogen availability may ameliorate the effects of planthopper feeding because a significant planthopper × fertilizer interaction did exist for culm elongation (Table 3) and two of the nitrogen compounds assayed (Ala and Trp) (Table 1). Thus, both nitrogen availability and planthopper feeding influ-

ence *Spartina* growth and amino acid composition, and these bottom up and lateral forces have potentially interactive effects.

Our results provide a possible explanation for the plant-mediated competitive effects observed in both contemporaneous and delayed interactions of *Prokelisia* planthoppers. In a previous study, neither survivorship, development time, nor body size of *P. dolus* was adversely affected by intra- or interspecific crowding by its congener, *P. marginata* Van Duzee (Denno and Roderick, 1992). However, in another study, the development time of *P. dolus* was significantly extended on plants on which planthoppers had previously fed (Denno et al., 1995; R. Denno and J. Cheng, unpublished data). Thus, *P. dolus* is sensitive to delayed competition resulting from intra- and interspecific interactions, but does not suffer negative effects from contemporaneous interactions with *P. marginata*.

Our results indicate that the concentrations of several amino acids in *Spartina* are reduced by planthopper feeding. Some amino acids (e.g., Ala, Arg, Asx, GABA, Leu) decreased after only nine days of exposure to planthoppers, while decreases in others were more delayed. Furthermore, our study indicated that subsequent generations of planthoppers encounter plants with fewer leaves and smaller culms and tillers. As a result, feeding by planthoppers may induce both qualitative and quantitative changes in *Spartina* plants that could adversely affect the performance of future generations of planthoppers. These impacts may be even more dramatic in nature than we observed in our experiment because our comparisons were made on caged plants in which the concentrations of several amino acids and culm elongation were artificially increased by caging.

In the *Prokelisia/Spartina* system, competitive interactions could be mediated by both reductions in plant growth and surface area as well as diminished plant nutrition. Although reductions in host plant growth associated with sap feeding have been documented for a number of insect species (e.g., Dixon, 1971; Cockfield et al., 1987; Meyer and Root, 1993), our study adds to the comparatively sparse literature detailing how phloem feeders adversely affect both the growth and amino nitrogen profiles of their host plants (see Cagampang et al., 1974; Sōgawa, 1982; Wellings and Dixon, 1987).

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OVIPOSITION STIMULANTS FOR SULFUR BUTTERFLY, *Colias erate poliographys*: CYANOGLUCOSIDES AS SYNERGISTS INVOLVED IN HOST PREFERENCE

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Abstract—Host-plant chemicals stimulating oviposition by a Leguminosae-feeding pierid butterfly, *Colias erate poliographys*, were isolated and identified from one of its primary host plants, white clover (*Trifolium repens*). Females readily deposited eggs in response to methanolic extracts of the plant, and subsequent partition of the extracts with organic solvents revealed that chemical constituents critical for host recognition reside in the water-soluble fraction. Further fractionation of the hydrosoluble fraction by column chromatography led to the separation of an active fraction and two cyanoglucosides, linamarin and lotaustralin. Conspicuous oviposition response was evoked by unidentified polar compound(s), while these cyanoglucosides exerted no stimulatory activity by themselves. However, ovipositing females preferred samples containing either of the two cyanoglucosides. In dual-choice bioassays, significantly more eggs were laid on samples admixed with the cyanoglucosides, suggesting that the cyanoglucosides serve as synergistic oviposition stimulants and could play an important role in host selection.

Key Words—Oviposition stimulants, *Colias erate*, Lepidoptera, Pieridae, *Trifolium repens*, Leguminosae, cyanoglucosides, linamarin, lotaustralin.

INTRODUCTION

Recent progess in studies on chemical interactions between herbivorous insects and plants has shed light on phytochemical mediators involved in oviposition behavior of a number of lepidopterans (Feeny, 1992; Renwick and Chew, 1994;

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Honda, 1995a). It is well established that ovipositing females of butterflies rely, to a large extent, on chemotactile stimuli evoked by plant secondary metabolites as cues to assess the suitability of potential hosts they landed on (Honda, 1995b; Nishida, 1995).

The sulfur butterfly, *Colias erate poliographys* (Pieridae), usually occurs in open grassland and utilizes as host plants a variety of legumes including the genera *Trifolium*, *Lotus*, *Robinia*, and *Vicia* (Endo and Nihira, 1990). Host plants most preferred by ovipositing females in the field are *Trifolium repens* and *Lotus corniculatus*, both of which have been reported to contain potentially toxic compounds, the cyanogenic glycosides (Butler, 1965).

Host plants of most pierid butterflies belong to the family Cruciferae or Leguminosae. Until now, investigations of chemical mechanisms of host selection by adult females of the Pieridae, however, have been concentrated on a couple of species in the genus *Pieris* that are specialists on crucifers (Chew and Renwick, 1995).

One of our interests in the *Colias*–legume association is the strategy of the insect for overcoming the chemical barrier elaborated by the plants. We report here on oviposition stimulants for *C. erate poliographys* present in white clover.

METHODS AND MATERIALS

Insects. Adult females of *C. erate poliographys* were captured in the field at Higashihiroshima in Hiroshima Prefecture. The subjects of the behavioral bioassays were their female offspring that had been reared on potted white clover (*T. repens*) at 25°C with a 16-hr light–8-hr dark regime. Both sexes of newly emerged individuals were kept under quasinatural conditions in a greenhouse (7 × 10 m; height, 3.5 m) stocked with flowering plants until commencement of egg-laying by females. Thereafter, females were transferred to our laboratory and were fed with 15% aqueous sucrose daily during the experiments. Gravid females (5–18 days old) used for the bioassay were confirmed beforehand to respond positively to the foliage of *T. repens*. The two color morphs of females (alba and yellow) were indiscriminately tested, because both proved, by field observations and preliminary experiments, to display similar behavioral responses.

Extraction and Fractionation of Plant Materials. Young foliage of *T. repens* (300 g) collected in the spring was extracted with methanol (1 liter) at room temperature for one month. The methanolic extract was concentrated in vacuo at 40°C, and an aliquot of the whole concentrate, after being dispersed in water, was extracted successively with chloroform (fraction 1) and isobutanol (fraction 2). The postisobutanol fraction (aqueous layer; fraction 3) was further fractionated by column chromatography on porous polymer gel (MCI gel CHP20P,

75–150 μm, Mitsubishi Kasei), and eluted stepwise with H_2O (fraction 3-1), 25% aq. MeOH (fraction 3-2), 50% aq. MeOH (fraction 3-3), and MeOH (fraction 3-4).

Isolation of Compounds A and B. Fraction 3-2, once lyophilized, was redissolved in H_2O and treated successively with cation exchange resin (Accell Plus CM, Waters) and anion exchange resin (Accell Plus QMA, Waters). The neutral fraction was found to comprise a major compound (A) and some other minor substances. Final purification of compound A was achieved by means of preparative TLC (Merck PLC plate Silica gel 60; EtOAc—MeOH—H_2O 8:2:1), yielding pale brown crystals ($Rf = 0.64$). By an analogous procedure, compound B was isolated from fraction 3-3 (this fraction was not contaminated with compound A) as pale brown crystals ($Rf = 0.67$).

Bioassay for Ovipositional Response. Artificial leaves made of squares of yellowish green paper (5.0 × 5.0 cm^2) were used as an ovipositional substrate. Assay trays consisted of a pair of Petri dishes (13 cm ID; height, 3.3 cm), with five paper leaves laid (partially overlapping one another) at the bottom. One tray was treated with 3 ml of 1% solution (50% aq. MeOH) of a test sample. The leaves, after being air-dried, were remoistened by spraying the same quantity of water, while the other tray acting as a control was moistened with water only. Two to four pairs of females in a transparent plastic chamber (33 × 42 cm; height, 24 cm) externally illuminated with an incandescent lamp (3000 lux) were provided with a choice of two trays that were set 5 cm apart from each other at the bottom of the chamber. The stimulatory activity of test materials was evaluated by counting the number of eggs deposited on the substrate during a period of 6 hr. Significance of differences between treatments and controls was assessed by a binominal test, under the null hypothesis that total number of eggs would be distributed evenly over treated and control trays.

Instrumentation for Structural Elucidation. The ^{13}C NMR (CMR) spectra were measured at 100 MHz in D_2O on a Jeol JNM-A400 FT-NMR spectrometer with tetramethylsilane as the external standard. Chemical shifts were represented by δ units. Multiplicity of signals determined by DEPT was abbreviated as follows: singlet, s; doublet, d; triplet, t; and quartet, q.

FAB-mass spectra (FAB-MS) were obtained with a Jeol SX 102A mass spectrometer using glycerine as a matrix, with negative ions being recorded.

The optical rotation was measured with a Jasco DIP-370 digital polarimeter at 20°C in H_2O.

RESULTS

The relative proportions of the dry weights of the methanol extract and fractions 1–3 to the fresh weight of the leaves of *T. repens* were approximately

5.5%, 0.7%, 0.4%, and 4.4%, respectively (an average of three replicates). Since preliminary experiments with a methanol extract suggested that the ovipositional response of *C. erate* females at concentrations below 1% was not high enough to make precise estimation of the stimulatory activity, samples were all tested at a concentration of 1%, unless otherwise noted. Ovipositing females that were permitted free flight in the chamber alighted on assay trays from time to time, followed by drumming on the surface of artificial leaves with foretarsi. If a sample is acceptable, the female lays an egg on the upper surface of the leaves.

As shown in Table 1, the methanol extract of *T. repens* potently induced oviposition by females, indicating that active principle(s) are contained in the extract. Whereas neither the chloroform fraction (fraction 1) nor the isobutanol fraction (fraction 2) exerted any stimulatory action on egg-laying, the water-soluble fraction (fraction 3) was found to be highly active. The activity of a mixture of fractions 1 and 2 appeared very weak, or rather equivocal, because the total number of eggs deposited was extremely low. Therefore, although these results do not rule out the possible implication in oviposition of some substances present in fractions 1 and 2, it is concluded that the phytochemicals most responsible for host recognition are located in fraction 3. This fraction was then further separated by column chromatography into four subfractions (fractions 3-1 to 3-4). The relative proportions of these four fractions to the fresh

TABLE 1. OVIPOSITION RESPONSE OF *Colias erate* TO SAMPLES PREPARED FROM HOST PLANT, *Trifolium repens*

Sample[a]	Eggs laid[b] (*N*)		Females tested (*N*)	*P*[c]
	Treated	Control		
MeOH extract	223 (44.6)	0	5	<0.001
Fraction 1	0	0	16	
Fraction 2	0	0	12	
Fraction 1 + Fraction 2[d]	8	0	12	<0.005
Fraction 3	175 (43.8)	0	4	<0.001
Fraction 3-1	107 (13.4)	9 (1.1)	8	<0.001
Fraction 3-2	13	3	8	<0.05
Fraction 3-3	8	6	12	NS[e]
Fraction 3-4	3	2	16	NS

[a]Concentration: 1%.
[b]Mean number of eggs laid per female is given in parentheses.
[c]Binominal test.
[d]Equivalent mixture containing 0.5% each.
[e]Not significant.

weight of *T. repens* leaves were approximately 3.5%, 0.14%, 0.16%, and 0.18%, respectively. Potent positive response was evoked by fraction 3-1. Fraction 3-2 also exhibited weak activity, although this may possibly be due to contamination. TLC analysis and CMR measurement suggested that fraction 3-1 consists of manifold polyhydroxylated compounds including ubiquitous mono- and disaccharides. On the other hand, fraction 3-2 proved to contain a peculiar compound (A) as a predominant component, while another characteristic compound (B) was present in fraction 3-3. Thus, compounds A and B were isolated from their respective fractions, and elucidation of their structures was attempted on the basis of spectroscopic and physicochemical properties.

Compound A. Mp: 135–140°C. $[\alpha]_D^{20} = -27.6°C$ (H_2O, c0.5). FAB-MS: $(M-H)^-$ at *m/z* 246.0984. CMR, δ(ppm): 26.2(q), 26.8(q), 60.6(t), 69.5(d), 72.0(s), 72.8(d), 75.5(d), 76.0(d), 98.9(d), 121.4(s). The exact mass number obtained by FAB-MS definitely indicated the molecular formula of this compound as $C_{10}H_{17}O_6N$. CMR information revealed the existence of two methyl carbons (26.2 ppm and 26.8 ppm) and a glucosyl moiety in its molecule. A resonance at 72.0 ppm was assignable to a quarternary carbon attached to an oxygen atom, and the presence of a cyano group was suggested by a diagnostic signal at 121.4 ppm. From all these data, this compound was considered to be linamarin (1-cyano-1-methylethyl-β-D-glucopyranoside), a well-known cyanogenic compound already known from *T. repens* (Butler, 1965). The identity was corroborated by comparison with a commercially available authentic sample (Sigma).

Compound B. Mp: 123–129°C, $[\alpha]_D^{20} = -22.7°$ (H_2O, c0.5). FAB-MS: $(M-H)^-$ at *m/z* 260.1140. CMR, δ(ppm): 7.8(q), 23.1(q), 32.9(t), 60.5(t), 69.4(d), 72.7(d), 75.5(d), 75.7(s), 76.0(d), 98.5(d), 120.9(s). This compound, of molecular formula $C_{11}H_{19}O_6N$, was assumed to be a homolog of compound A with an additional methylene unit. The CMR spectrum was suggestive of the presence of an ethyl substituent (7.8 ppm and 32.9 ppm) attached to a quarternary carbon atom at 75.7 ppm. Taking into account the similarity of chemical shift values of other resonances to those of compound A and referring to reported data (Wray et al., 1983; Pitsch et al., 1984), compound B was concluded to be lotaustralin [(1*R*)-1-cyano-1-methylpropyl-β-D-glucopyranoside], which also has previously been identified from *T. repens* (Butler, 1965). The chemical structures of the two compounds have been published on many occasions (see, for example, Nahrstedt, 1988).

The influence of these compounds on oviposition behavior was examined singly and in combination with fraction 3-1 (Table 2). Apparently, neither of the two cyanoglucosides exerted stimulatory activity when bioassayed alone. However, significantly more eggs were laid on binary mixtures comprising both fraction 3-1 and a cyanoglucoside than on fraction 3-1 alone (control). The females clearly show strong affinity or preference for cyanoglucosides in ovi-

TABLE 2. EFFECT OF HOST-PLANT CYANOGLUCOSIDES ON OVIPOSITION BY *Colias erate* FEMALES

Sample[a]	Eggs laid[b] (*N*) Treated	Control[c]	Control[d]	Females tested (*N*)	*P*[e]
A[f]	0	0		8	
B[g]	0	3		4	
Linamarin[h]	0	0		8	
Fraction 3-1 + A	172 (43.0)		62 (15.5)	4	<0.001
Fraction 3-1 + B	167 (41.8)		53 (13.3)	4	<0.001
Fraction 3-1 + linamarin	235 (29.4)		91 (11.4)	8	<0.001

[a]Concentration: 1%. In binary systems, the concentrations of fraction 3-1 and cyanoglucoside are 0.77% and 0.23%, respectively.
[b]Mean number of eggs laid per female is given in parentheses.
[c]Water only.
[d]Fraction 3-1 (1%).
[e]Binominal test.
[f]Linamarin isolated from *T. repens*.
[g]Lotaustralin isolated from *T. repens*.
[h]Commercially purchased.

position. Moreover, cyanoglucosides seem likely to enhance the females' activity of oviposition behavior, for the number of eggs deposited per individual appears to have increased as compared with the case where females were stimulated by fraction 3-1 alone (Tables 1 and 2). Consequently, the two cyanoglucosides, linamarin and lotaustralin, can be regarded as oviposition stimulants acting synergistically together with fraction 3-1 in this experimental situation.

DISCUSSION

The results definitely indicate that recognition of *T. repens* as a host by *C. erate poliographys* could be mediated largely by water-soluble substances. Although unidentified, active constituents crucial for inducing egg-laying are presumably highly polar compounds present in fraction 3-1. However, the two cyanoglucosides, lanamarin and lotaustralin, in the host plant, although being quite inactive by themselves, prominently enhanced the acceptability of fraction 3-1 for females when admixed with them. Accordingly, it is evident that females are able to perceive these cyanogenic glucosides and make use of them as one of the cues in assessing host-plant quality. It is therefore very likely that females can discriminate cyanoglucoside-containing leguminous plants from others. Since *C. erate* is also known to utilize several cyanoglucoside-free legumes as host

plants, the cyanoglucosides are not indispensable for releasing egg-laying. The function of the two cyanoglucosides in oviposition behavior of this butterfly is thus likely to be best interpreted as synergists involved in host preference rather than host recognition. Extreme synergism of diverse compounds in stimulation of oviposition is exemplified by studies on several papilionid butterflies (Nishida et al., 1987; Ohsugi et al., 1991; Honda, 1990; Feeny et al., 1988; Nishida and Fukami, 1989; Sachdev-Gupta et al., 1993), and it also has been demonstrated that oviposition of a danaid butterfly, *Ideopsis similis*, was stimulated by a combination of structurally related phenanthroindolizidine alkaloids (Honda et al., 1995). By contrast, intensive investigations of oviposition stimulants for a few *Pieris* butterflies have shown that sufficient ovipositional responses of females can usually be obtained by a single glucosinolate (Traynier and Truscott, 1991; van Loon et al., 1992; Huang et al., 1993, 1994). In this context, the chemical mechanism of host selection by a legume-feeding *C. erate* appears to be somewhat different from that of crucifer-feeding *Pieris* species.

The cyanogenic glycosides are well-known unique compounds widely distributed in the plant kingdom (Nahrstedt, 1987) and also occur in several taxa of arthropods (Duffey, 1981; Nahrstedt, 1988). Due to their potential toxicity and unpalatability, as well as the release of HCN and aglycones when cyanogenic individuals are damaged by grazing or crushing, these chemicals are thought to be defensive substances against herbivores or predators. In fact, it has been reported that cyanogenic plants or individuals are avoided by some herbivores (Jones, 1962; Compton and Jones, 1985), and the meaning of cyanogenesis as a defensive strategy of plants in plant–herbivore interactions has been fully discussed (Jones, 1988). On the other hand, a number of lepidopterous insects have been shown to store in their body tissues certain cyanoglucosides, which are biosynthesized from amino acid precursors (Wray et al., 1983) and/or sequestered by larvae from host plants (Nahrstedt, 1986). In particular, linamarin and lotaustralin are widespread in *Zygaena* moths (Nahrstedt, 1988). Although it remains unknown whether or not *C. erate* is capable of cyanogenesis or how it manipulates cyanogenic compounds, earlier work has revealed that other *Colias* species seem neither to produce nor to sequester cyanoglucosides (Witthohn and Naumann, 1987). At any rate, it is obvious that *C. erate* larvae can tolerate cyanide ingested from the food plant, and besides, it would be a matter of specific ecological meaning that adult females, far from avoiding cyanoglucosides, show unequivocal preference for them in oviposition. Interestingly enough, larvae of the southern armyworm, *Spodoptera eridiana*, have been reported to use hydrocyanic acid as a feeding stimulant (Brattsten et al., 1983). Willing acceptance of highly toxic substances by ovipositing females has also been reported for *Aristolochia*-feeding troidine butterflies, in which poisonous aristolochic acids accounted, in part, for the stimulus evoked by the host plants (Nishida and Fukami, 1989; Sachdev-Gupta et al., 1993). Another instance

of use of toxic phytochemicals in host-plant recognition by an Apocynaceae-feeding danaid butterfly, *Idea leuconoe*, involved macrocyclic pyrrolizidine alkaloids (Honda et al., unpublished data). Adaptation or preference of mother butterflies for toxic substances, as exemplified by these investigations, would afford potential of strategic advantage to the survival of their progeny by ensuring the larvae protection against predatory or parasitic enemies and/or by acquiring greater resource allocation.

The characterization of other constituents that have a critical effect on regulating egg-laying by this butterfly is now in progress. It is also necessary to determine whether the female butterflies can detect the presence of linamarin and lotaustralin in intact, undamaged leaves of *T. repens*.

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GAZELLE HERBIVORY AND INTERPOPULATION DIFFERENCES IN CALCIUM OXALATE CONTENT OF LEAVES OF A DESERT LILY

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Abstract—We investigated the abundance and distribution of calcium oxalate crystals in the leaves of wild populations of a Negev desert lily, *Pancratium sickenbergeri*, in relation to herbivory. Three species of herbivores are known to eat the leaves of this lily: a small antelope, the dorcas gazelle *Gazella dorcas*, a moth larva *Polytella cliens*, and a land snail *Eremina desertorum*. All three species eat only those parts of the leaves where calcium oxalate raphides are absent, suggesting that it is an effective defensive chemical. We compared the abundance of raphides in three isolated lily populations that differed only in the amount of gazelle herbivory. Within lily populations, we found neither size-related differences in raphide abundance nor differences in raphide abundance between plants that had previously been partially consumed and those that had not. We found significant differences among lily populations in the amount of calcium oxalate crystals in their leaves, with the most raphides being found in the population suffering most herbivory, fewer in a population with intermediate herbivory, and the least in a population without gazelle herbivory. Additionally, sand samples showed no differences among populations in two major nutrients (nitrogen and phosphorus) but significantly more calcium in the sand in the population without herbivory. Thus, calcium oxalate abundance in the leaves of *Pancratium sickenbergeri* is not constrained by resource availability but rather appears to have been selected for by gazelle

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herbivory. This is the first study to show the effects of selection on calcium oxalate production in a wild plant by a wild herbivore.

Key Words—Calcium oxalate, lilies, gazelles, feeding deterrent, plant-animal interactions, herbivory, desert.

INTRODUCTION

It has long been known that many plant species produce raphides of calcium oxalate in their leaves (Franceschi and Horner, 1980). Calcium oxalate is regarded as the most common form of biologically controlled mineral deposition in plants (Arnott, 1983). Indeed, calcium oxalate deposits have been reported in more than 240 families of flowering plants. Calcium oxalate formation may provide a necessary mechanism for regulating calcium in the cytosol, preventing the buildup of excess free calcium that is toxic to cells when found at high concentrations (Webb et al., 1995). Calcium oxalate is also presumed to serve a defensive function against herbivores (Whittaker, 1970), largely because of its effects on the human bladder and urinary tract, resulting in the pathological condition known as oxaluria (Zarembski and Hodgkinson, 1969). Some plants containing calcium oxalate, such as *Dieffenbachia picta*, caused painful edematous swelling of the oral mucous membranes (Gardner, 1994). *Psilocaulon absimile*, which contains large quantities of oxalic acid, together with lesser quantities of malic and tartaric acid, caused death in rabbits within 3 hr (Rimington and Steyn, 1933). However, in spite of the large body of literature on the potential importance of calcium oxalate as a defense chemical, there has been no study of natural variation in calcium oxalate production in wild plants and its effect on wild herbivores.

The purpose of this study is to examine the chemical defenses of the leaves of subpopulations of the geophyte, the lily *Pancratium sickenbergeri* (Amaryllidaceae), with emphasis on the nature of their evolutionary responses to herbivory. *Pancratium sickenbergeri* is commonly found in a number of isolated sand dunes of the Negev desert (Feinbrun-Dothan, 1986).

We place emphasis here on five related aspects of calcium oxalate production by the lilies:

1. Do herbivores respond behaviorally to the presence of calcium oxalate? We have observed over six years that gazelles eat only the last 1 cm at the distal tip of the leaf (Ward and Saltz, 1994), while the snail and the moth larva eat the tip as well as the lateral strip and the epidermis of the strap-shaped leaf. We wished to determine whether these peculiar patterns of feeding behavior avoid the major distribution of calcium oxalate in the leaf.

2. Is calcium oxalate production higher in populations with higher levels

of herbivory? That is, is there any evidence for selection on lilies to produce more calcium oxalate where defense is more necessary?

3. Does the nutrient status of the sand affect the production of calcium oxalate? A number of workers have postulated that resource availability constrains the production of chemical defenses in plants (see, e.g., Coley et al., 1985; Du Toit, 1995). In this desert environment, nutrient resources are typically low (Evenari et al., 1982) and thus expected to be limiting. Furthermore, many plant compounds supposed by ecologists to exist solely as secondary metabolic products for the protection of plants (see, e.g., Whittaker and Feeny, 1971) may also be selected for their role in primary metabolic processes (Seigler and Price 1976). Calcium oxalate may function to sequester excess calcium and remove it from active metabolism, modulating differences between external calcium supply and required levels of free calcium in the cytosol (Webb et al., 1995). Thus, one might predict that populations of lilies growing in sand containing more calcium will have higher levels of calcium oxalate in their leaves.

4. Do young lilies produce more calcium oxalate than old plants? Plants (usually woody dicots) that are browsed at an early stage may show retarded growth and increased time to reach maturity (Bryant et al., 1983, 1991). Hence, selection should favor increased defense of young plants, as has frequently been found to be the case for other chemicals (e.g., Reichardt et al., 1984; Basey et al., 1990) as well as thorn defenses (e.g., Brooks and Owen-Smith, 1994).

5. Is calcium oxalate an inducible or constitutive defense? If defense production is costly, then all plants, regardless of the level of herbivory in the particular subpopulation, may possess the ability to produce defenses, but only do so when they are partially consumed (i.e., defenses are inducible and phenotypically plastic; Vail, 1992). Leaves are known to be functionally plastic and to change the allocation of resources to defense chemicals (Welter, 1989; Baldwin, 1993). This jack-of-all-trades strategy may be sufficient to defend the plants, although it is unlikely to be as effective as either pure strategy (Belsky et al., 1993), i.e., a growth-favored strategy in the absence of herbivory or a defense-favored strategy (i.e., a constitutive defense, sensu Adler and Karban, 1994).

Herbivore Species. We have recorded three important species of animals eating the leaves of this lily, namely the land snail *Eremina desertorum* (Mollusca: Pulmonata), the moth larva *Polytella cliens* (Insecta: Lepidoptera), and the dorcas gazelle *Gazella dorcas* (Mammalia: Bovidae). The distributions of the gazelle and the moth are sympatric with those of the lily in sand dunes throughout the Negev desert of Israel, while the snail is found only in the western Negev desert. Of these herbivores, the gazelle is by far the most important. During the winter of 1991, the gazelles ate leaves from 35–87% of the plants in eight permanent plots in the central Negev (median = 75%, N = 1217 plants

examined; Ward and Saltz, 1994). During the winter of 1995, gazelles ate leaves from 0–75% of the plants per plot (median = 53%, $N = 400$ plants examined). Of those plants selected by the gazelles in 1995, 21–39% (median = 29%) of all leaves were partially eaten. In contrast, the moth larva was found on only 1.8% of plants in the same plots in 1991 and has not been seen there at all in the five years thereafter. We do not have data for the number of plants with snails on them in the western Negev, but casual observations indicate that they are no more abundant than the moth larvae. Laboratory observations showed that the snails ($N = 50$ individuals) were unable to maintain themselves on a pure diet of lily leaves and, when forced to do so, quickly entered estivation (Spiegel, unpublished data).

The lily is a major food item of the dorcas gazelle *G. dorcas* (Lawes and Nanni, 1992; Ward and Saltz, 1994). In addition to feeding on leaves in winter, gazelles dig for underground parts of the lilies in the summer months, completely consuming up to 5–10% of these plants each year. Between 50% and 88% of the lilies in the dunes are partly consumed (Ward and Saltz, 1994). Additionally, the gazelles consume most lily flowers; a lily flower on these sand dunes has less than a 1:30 000 probability of surviving to the seed-producing stage (Saltz and Ward, 1997). For these reasons, we concentrate on the possible selective effects of the gazelle on the chemical defenses of the lily because of its role as the major herbivore.

In this study, we compare lilies in two habitat types: (1) Open dune habitats support a high density of these geophytes, making them attraction points for the dorcas gazelle. (2) Canyon dune habitats present good growing conditions for lilies and have no gazelle herbivory. Gazelles never enter canyons, presumably because of the presence of a major predator, the leopard *Panthera pardus*. The interaction between gazelle herbivory and lilies provide a unique opportunity to examine the role of evolutionary responses of plants to their herbivores because the gazelle is the only significant herbivore on the lilies, the distinctly substructured populations of lilies on sand-dune islands, and the different costs of herbivory to the plants in each subpopulation (Ward and Saltz, 1994).

METHODS AND MATERIALS

Study Area. In many places in the Negev, islands of sand can be found ranging in size from several hundred square meters to several square kilometers. These islands are wind traps for sand carried across the desert. Several such islands can be found in Makhtesh Ramon, our main study site, which is an elongated (40-km-long), heart-shaped erosional cirque in the central Negev. The interaction between the gazelles and lilies in Makhtesh Ramon is the subject of a long-term study (see, e.g., Ward and Saltz, 1994; Saltz and Ward, 1997),

now in its sixth year. Hence, we can classify the different lily populations into those enduring high, low, and no herbivory with a high degree of certainty. Regarding lily populations that are fed upon by gazelles, we concentrated our study in the two isolated, eastern lobes of this cirque, known as Ardon and Machmal valleys, located about 6 km apart at its eastern end and separated by a large hill. The predominantly western winds have created large sand deposits along the eastern walls of these valleys. These sand dunes support unique plant communities (Ward et al., 1993; Ward and Olsvig-Whittaker, 1993), with only one perennial plant species, the lily *P. sickenbergeri*, common to both dunes. In addition, we measured calcium oxalate content of leaves of lilies growing in Neqarot canyon, where there is no herbivory. This canyon population is separated from the other two populations by 7–10 km.

Machmal valley lilies suffer a higher level of herbivory than Ardon lilies (Saltz and Ward, 1997). Hence, we predicted that if calcium oxalate is produced in response to herbivory, the levels of this chemical would be highest in Machmal lilies, intermediate in Ardon, and low or nonexistent in Neqarot.

Natural History of the Plant. The lily blooms in fall, producing 1–15 white flowers on one or two shafts. The flowers are pollinated by syrphid flies (Syrphidae). Each flower produces relatively large seeds that are wind dispersed. The seed germinates after the winter rains, and leaves are produced. A bulb then develops that grows larger and deeper with time (Ward and Saltz, 1994; Saltz and Ward, 1997). In spring, all leaves dry up and fall off, leaving no aboveground material. In subsequent years, leaves appear on the surface after the winter rains and may remain green until late spring, depending on rainfall and temperature. The number of lilies that produce leaves each winter is dependent on the amount of rainfall, which also determines the number of plants that will flower the following fall (Saltz and Ward, 1997). There appears to be no vegetative reproduction in these lilies (Ward and Saltz, 1994).

Calcium Oxalate Measurements. We removed the largest leaf from each of 27 plants in digs (i.e., previously partially consumed by gazelles) and 26 plants out of digs in Ardon valley, 29 plants in digs and 36 plants out of digs in Machmal valley, and 23 plants in Neqarot canyon. We made the comparison between plants in and out of digs because the major removal of plant biomass is when the gazelles dig in the sand in the summer months to remove part or all of the stem and bulb. Hence, if there are inducible defenses in these plants, we might expect them to respond to this major impact of herbivory.

The potassium permanganate titration technique of Lewis and Weinhouse (1957) was initially used to ascertain that the raphides contained oxalic acid. Thereafter, we made fine transverse sections of the leaves and viewed these under 10× magnification under a light microscope. We counted each raphide visible in a single field of view, following Zindler-Frank (1995). These data were $\log_{10}$-transformed for analysis.

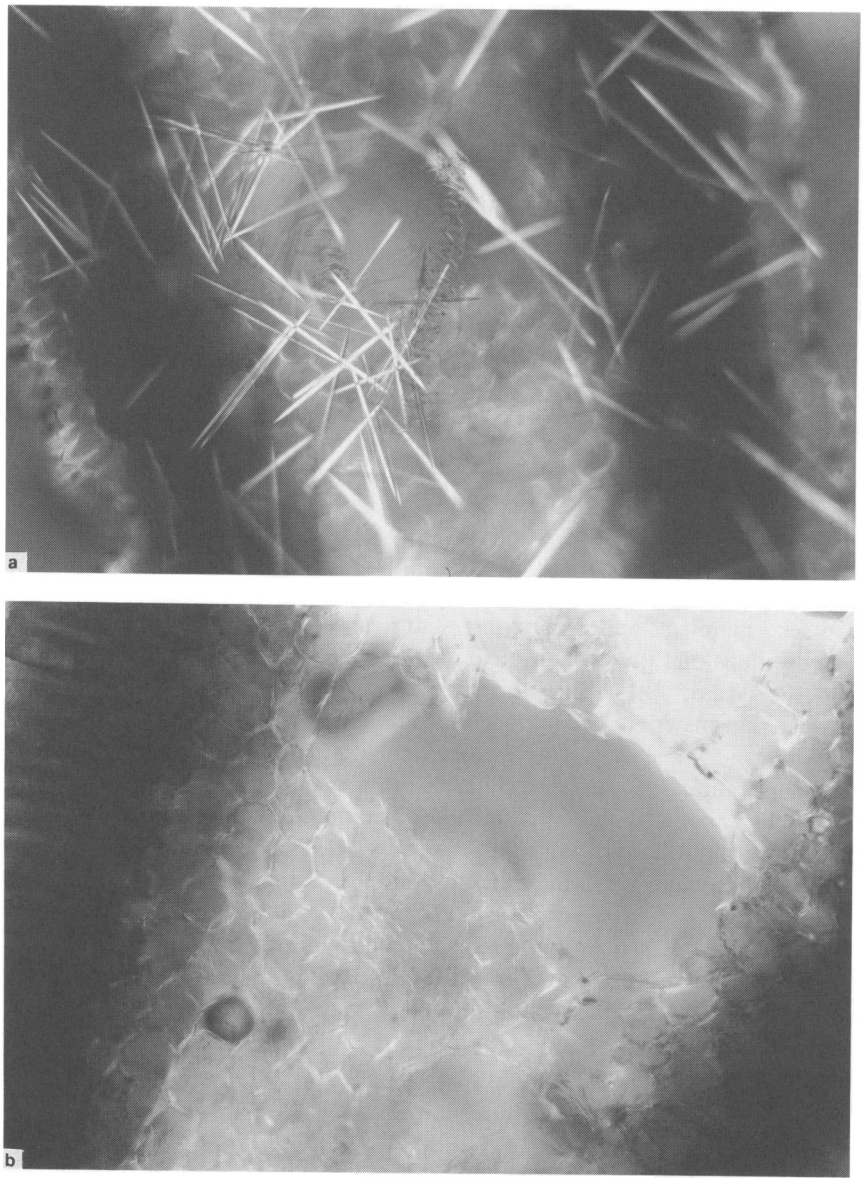

FIG. 1. Transverse section through leaf of lily *Pancratium sickenbergeri*. (a) Raphides of calcium oxalate in the spongy mesophyll in the central part of a leaf. (b) Distal 1-cm tip of the leaf; note that raphides are absent. (c) Lateral edge and epidermis of the leaf; note that raphides are absent. All photographs taken at 10× magnification. Reduced 25% for reproduction.

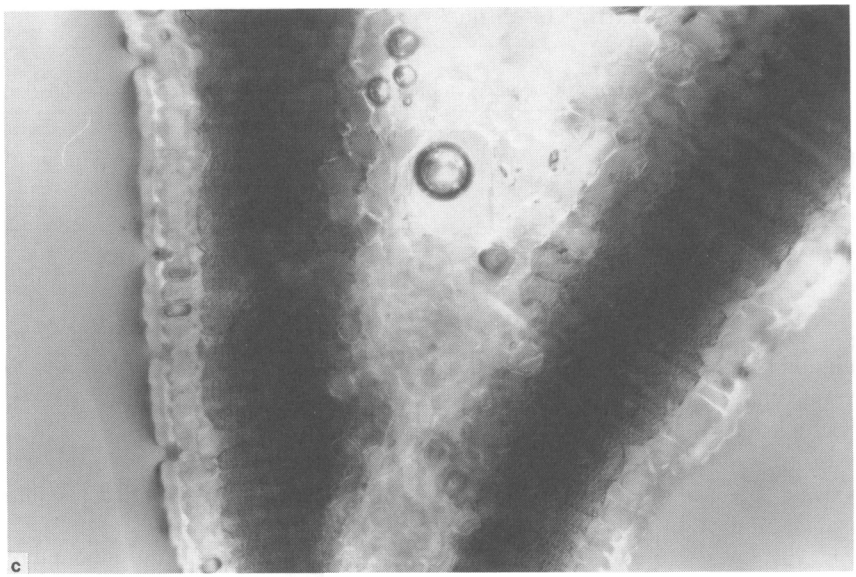

FIG. 1. Continued.

Sand Sampling. Five 1-kg sand samples were taken from 5–30 cm below the surface in each of the three study sites and analyzed for total calcium, nitrate nitrogen, and total phosphorus concentration using standard analytical techniques (Black, 1982).

RESULTS

Distribution of Raphides. Microscope examination showed that raphides were abundant in the spongy mesophyll (as has been found in other plant species; Webb et al., 1995) (Figure 1a), with the exception of the last 1 cm at the tip of the leaf where they were absent in all leaves examined (N = 145 plants; Figure 1b). Raphides were absent from the epidermis and the 1- to 2-mm strip on the lateral edges of the leaf (Figure 1c). No raphides were found in the stem or bulb of the lily. Thus, the three herbivore species eat only those parts that are not defended by calcium oxalate.

Sand Composition. We found no significant differences among sand samples from the three study sites in the amount of nitrate nitrogen (F = 0.45, P = 0.65, df = 2, 12) or total phosphorus (F = 1.22, P = 0.33, df = 2, 12; Table 1). However, there were significant differences in the amount of calcium in the sand at the three sites (F = 7.64, P = 0.007, df = 2, 12; Figure 2).

TABLE 1. QUANTITIES OF NUTRIENTS IN SAND IN THREE STUDY SITES[a]

Site	Nitrate nitrogen (mg/kg)	Phosphorus (mg/kg)
Ardon	1.28 ± 0.477	2.62 ± 0.992
Machmal	1.08 ± 0.426	2.98 ± 0.380
Neqarot	1.18 ± 0.477	3.94 ± 1.107

[a]Sample sizes are 5 per site per analysis.

Calcium Oxalate Concentration in Leaves. There was no significant correlation between the $\log_{10}$ number of oxalate raphides and plant size, as indexed by maximum leaf width (a reliable index of overall size) (Ward and Saltz, 1994). Coefficients of determination (r^2) ranged from 0.34 to 0.40 (P = 0.257–0.569, error df = 14, 42) in the three sites. Because there is a positive correlation between size and age (Ward and Saltz, 1994), these data indicate that calcium oxalate production is not age-dependent.

There was a highly significant difference among sites in the amount of calcium oxalate in the leaves, with the highest amount found in Machmal valley,

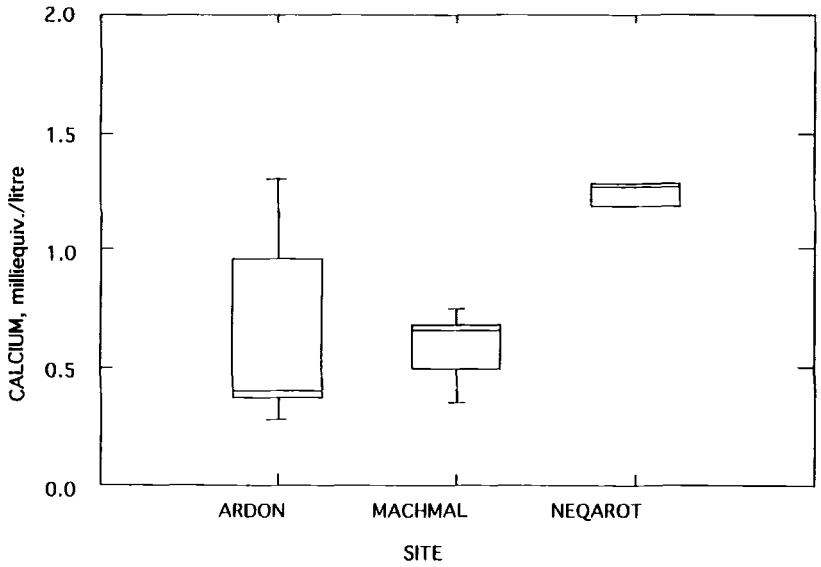

FIG. 2. Box-and-whisker plot of amount of calcium (in milliequivalents per liter) in the sand at the three study sites. Neqarot has no herbivory, Ardon an intermediate level of herbivory, and Machmal the highest level of herbivory.

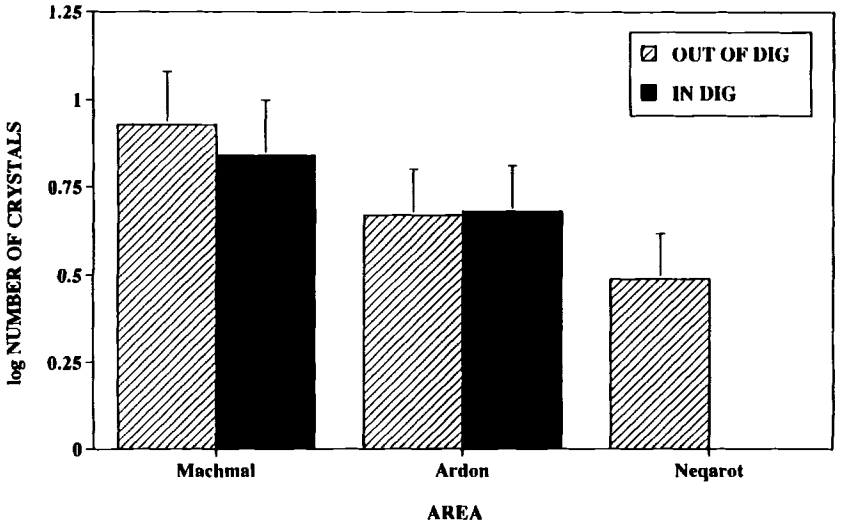

FIG. 3. Means and standard errors of $\log_{10}$ number of crystals in a transverse section of leaf. Hatched bars = leaves taken from plants not previously consumed; dark bars = leaves taken from plants partially consumed in a preceding summer.

lowest in Neqarot canyon, and an intermediate amount in Ardon valley ($F = 7.19$, $P = 0.009$, $df = 2, 62$; Figure 3). This result corresponds with the finding that most herbivory occurs in Machmal valley, followed by Ardon valley, with no herbivory in Neqarot canyon. Contrary to the prediction of the resource availability model of plant defenses, the lowest level of defenses was found in the site with the highest level of calcium in the sand (i.e., Neqarot canyon; Figure 2).

We found no significant difference between the amount of calcium oxalate in digs and outside of them ($F = 0.29$, $P = 0.59$, $df = 1, 118$) and no interaction effect (sites × in/out of digs) ($F = 0.45$, $P = 0.50$, $df = 1, 118$). Thus, this trait does not appear to be inducible, at least with regard to the effect of herbivory on the bulb and stem.

We examined the power of this analysis to reject the null hypothesis that there was no significant difference between the mean number of calcium oxalate raphides in leaves in and out of digs. We found that $\beta = 100\%$ to reject H_0 when effect size (i.e., difference between means) equals 4% or more, and $\beta = 84\%$ to detect an effect size of a 1% difference between means ($\beta = 80\%$ is the conventionally accepted minimum level of power) (Cohen, 1988). Thus, the lack of significant difference in the amount of calcium oxalate in the leaves of plants in and out of digs is not due to a sample size insufficient to reject the null hypothesis.

DISCUSSION

Herbivore Feeding Behavior and Calcium Oxalate Distribution. The behavioral avoidance by the gazelle, moth, and snail of calcium oxalate in the leaves of *P. sickenbergeri* suggests that the chemical is an effective deterrent to these herbivores. Nonetheless, this chemical functions imperfectly because the herbivores are still able to inflict damage on the plant. In the case of the snail and the moth larva, leaves shrivel and dry up after being eaten, presumably because the waxy cuticle is removed, facilitating desiccation. Because these herbivores are rare (and the snail is apparently unable to specialize on the lily), the effect of these herbivores is unlikely to select for calcium oxalate production in parts of the leaf that are currently unprotected. In the case of the moth larva, the possibility of population outbreaks (large increases in population size) cannot be excluded. Such outbreaks may cause intense selection on the plants for appropriate defense responses over brief periods. In the six years of our study, no such outbreaks have been recorded.

When the gazelle feeds on the leaves, the leaves continue to grow (Ward and Saltz, 1994; Saltz and Ward, 1997). Natural history observations have shown that, because the leaves have a basal meristem, leaf damage is minimized and there is no significant difference in photosynthetic area of eaten and uneaten plants at the end of the growing season (Ward and Saltz, 1994). However, gazelles select plants and leaves that are larger than average (Ward and Saltz, 1994). Hence, the above-mentioned result could have been an artifact of this selective herbivory. We performed a clipping experiment to establish whether there was indeed a cost of leaf herbivory by the gazelles. We found that gazelle herbivory on the leaves is costly to these plants, in spite of this chemical defense, because unclipped plants grow significantly faster than clipped plants (Saltz and Ward, 1997).

Influence of Soil Nutrients. It has frequently been shown that the production of calcium oxalate crystals is associated with the increased availability of calcium in the soil (e.g., Frank, 1972; Franceschi and Horner, 1979; Borchert, 1985; Zindler-Frank, 1995). We found the opposite to be true. Our results therefore contrast with the hypothesis that the availability of nutrients in the soil affects the production of chemical defenses (see, e.g., Waterman and Mole, 1989; Herms and Mattson, 1992) because we found no difference in two other major soil nutrients, nitrogen and phosphorus, yet found differences in chemical defenses among populations.

Interpopulation Differences in Calcium Oxalate Production. Our results are consistent only with the hypothesis that selection pressure of gazelle herbivory has resulted in the differences we found. This observation, coupled with the lack of both phenotypic variation in calcium oxalate production within popula-

tions due to herbivory and an age/size effect, suggests that there are genetic differences among populations.

Our results are consistent with the notion that calcium oxalate is a constitutive defense in these lilies (sensu Adler and Karban, 1994). Furthermore, our finding that lilies in the Neqarot population produced CaC_2O_4 shows that these plants maintain defenses even when the probability of being eaten is low (only in one year of six has insect herbivory been detected there). These observations further challenge the previously widely accepted hypothesis that the production of chemical defenses has an energetic cost [see Adler and Karban (1994) for a review of this concept]. We temper our statement here with the observation that lilies may increase calcium oxalate production in response to herbivory on the leaves only (rather than on the plant as a whole). A clipping experiment is required to test this possibility.

Congruent with the above results, the lilies have another form of defense that appears to have been selected by gazelle herbivory (Saltz and Ward, 1997). That is, lilies in Ardon and Machmal valleys grow their bulbs down deeper into the substrate than lilies in Neqarot, in order to minimize the probability of consumption when the gazelles dig in the sand to remove part or all of the bulb in the summer months.

Genotype–Environment Interactions. It has often been asked whether heterogeneous selection in local environments can contribute to the maintenance of genetic diversity in natural populations. Population genetics theory predicts that a genotype × environment (G × E) interaction might maintain genetic variation if the relative fitness of a genotype changes in different environments (Gillespie and Turelli, 1989). For the maintenance of genetic variation in this manner, there must be: (1) genetic variation for components of fitness; (2) environmental heterogeneity, and (3) G × E interaction for components of fitness, such that the fitness ranking of existing genotypes changes among environments (Mitchell-Olds, 1992).

Interpopulation differences in the level of herbivory provide an ideal opportunity to test the existence of G × E interactions (Maddox and Cappuccino, 1986). Herbivores often affect different populations of a plant species to consistently different degrees, due to the relative availability of alternative food sources in each habitat and the structural characteristics of the habitat, among other reasons. Many plant species have evolved various strategies to defend themselves against herbivores, such as the production of chemical defenses and thorn production (see reviews by Karban and Myers, 1989; Owen-Smith, 1993; Herms and Mattson, 1994). Because the production of these defenses is expensive, trade-offs between growth and the production of defenses are frequently detected (Herms and Mattson, 1992).

Additionally, most desert plants lack adaptations for long-range seed dis-

persal due to the high spatial heterogeneity and stochasticity in germination success and subsequent growth and reproductive conditions. This ensures that it is no more advantageous to disperse far than nearby (Ellner and Shmida, 1981). If this is true for these lilies, seeds may not reach one subpopulation from another. The result is a genetically substructured metapopulation. One might reasonably predict that plants in populations with little herbivory (e.g., Neqarot) will be selected for a high growth–low defense strategy, while those in areas of high herbivory (e.g., Ardon and Machmal) may have been selected for a slow growth–high defense strategy (Maddox and Cappuccino, 1986; Herms and Mattson, 1994). These selective differences caused by herbivores set the stage for the evolution of G × E interactions, following Mitchell-Olds' (1992) criteria outlined above. That is, herbivores such as gazelles may directly create environmental heterogeneity and this in turn may result in selection for G × E interaction for two components of fitness—growth and defense production.

It is clear that further experimentation is required to tease out the effects of genotypic and phenotypic factors in determining the level of calcium oxalate in the leaves of this lily. Transplant experiments (of plants among habitats) will be necessary to ascertain whether genotype–environment interactions have evolved.

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BENEFITS OF CITRATE OVER EDTA FOR EXTRACTING PHENOLIC ACIDS FROM SOILS AND PLANT DEBRIS

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Abstract—The effectiveness of various citrate extractions to recover ferulic acid, a phenolic acid, from Cecil A_p and B_t soil and plant surface debris (crimson clover, rye, subterranean clover, and wheat) was compared with that of EDTA extractions. Citrate extractions were equivalent to or better than EDTA in recovering phenolic acids from soil and plant debris. Citrate, unlike EDTA, did not interfere with the Folin & Ciocalteu's phenol reagent when determining total phenolic acid content in soil and plant debris extracts. Care, however, must be taken when using Folin & Ciocalteu's phenol reagent to estimate total phenolic acid content, particularly in the presence of soil organic matter. Citrate was also more effective in extracting phenolic acids from A-horizon soils. Thus, citrate extractions overcome some of the major limitations observed for EDTA extractions.

Key Words—Citrate, EDTA, soil extractions, ferulic acid, phenolic acids, *Trifolium incarnatum* L. Tibbee, *Trifolium subterraneaum* L. Mt. Barker, *Secale cereale* L. Wrens Abruzzi, *Tritium aestivum* L. Coker 983.

INTRODUCTION

A number of researchers have concluded that available (free and reversibly bound) phenolic acids in soils can be determined adequately by extracting soils with water and EDTA (Kaminsky and Muller, 1977; Dalton et al., 1987; Blum et al., 1994). Water extractions provide an estimate of the free phenolic acids in the soil, while the difference between EDTA and water extractions provides an estimate of the reversibly bound phenolic acids in the soil. EDTA extractions of soils, however, have some limitations. For example: (1) EDTA is at times

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ineffective in extracting phenolic acids bound to plant debris or soil organic matter, a rich source of phenolic acids (Blum et al., 1992); (2) Folin & Ciocalteu's phenol reagent for estimating total phenolic acids cannot be used with EDTA extracts because the phenol reagent is immediately reduced upon contact with EDTA (Blum et al., 1992); and (3) recovery of phenolic acids immediately after addition to Cecil A_p soil materials was at times greater for water extractions than for EDTA extractions (Blum, unpublished data). This led to a search for an alternative extractant to EDTA. Here we compare various citrate extractants with EDTA.

METHODS AND MATERIALS

One-Gram Soil Samples. Cecil A_p- and B_t-horizon soil materials (2.42% and 0.15% total carbon, respectively) from the same pedon (Typic Kanhapludults, clayey, kaolinitic, thermic) were obtained from the North Carolina State University Campus, sieved (4 mm), air-dried, and stored at room temperature in the laboratory (Dalton et al., 1983, 1987). One gram of sieved (0.25 mm) air-dried Cecil soil material was added to 15-ml Corex tubes (Corning Inc., Rochester, New York). These tubes were capped (Bacti-Capall, Sherwood Medical Industries) and used directly or autoclaved (15 min at 121°C and 1.2 kg/cm^2) three times at two-day intervals prior to use. Two milliliters of filter-sterilized (0.2-μm membrane filter) aqueous ferulic acid solution (97 μg/ml) were added to tubes just before extraction or to tubes that were then stored in the dark for four days before extraction.

Soil samples were extracted with 0.25 M EDTA (ethylenediamine tetraacetic acid, pH 7; for preparation of solution see Blum et al., 1994), water, or various concentrations (2–250 mM) of citric acid/citrate with or without KCl, imidazole, or dibasic sodium phosphate and pH values ranging from 3.4 to 7 (pH was adjusted with NaOH). All tubes were vortexed after addition of the extractant. At this stage each tube contained 2 ml ferulic acid solution and 2 ml of the double-strength extractant. Tubes were stored in the dark for 0, 2.5, or 5 hr and then vortexed again just before centrifugation (10 min at 12,000*g*). The resulting supernatants were filter sterilized (0.2-μm membrane filter) for quantification of ferulic acid by HPLC analysis (Blum et al., 1994).

Microbial Depletion Experiment—1-g Soil Samples. Corex tubes containing autoclaved soil (1 g) plus filter-sterilized ferulic acid solution or water (2 ml) were stored in the dark for two weeks. Each tube thereafter received 0.5 ml double-strength Hoagland's nutrient solution and 0.5 ml of soil extract (Blum et al., 1994). Soil extract from soils pretreated with ferulic acid was added to reinfest the soil with microbes. Depletion of ferulic acid by microbes was monitored until no ferulic acid could be recovered by a 2.5-hr extraction with 0.25

M citrate, pH 7. At this point, the remaining soil samples were extracted with 1 N NaOH (2.5 hr). Humic acids were precipitated by adjusting the NaOH extracts to approximately pH 2.5 with HCl. After centrifugation (10 min, 12,000*g*), the resulting supernatants were adjusted to pH 5 with NaOH and filter sterilized (0.2-μm membrane filters) for quantification of ferulic acid by HPLC analysis (Blum et al., 1994).

Twenty-Five-Gram Soil Samples. Cecil A_p soil from the North Carolina State University Campus and from Lake Wheeler Road Field Laboratory 5 km south of North Carolina State University were air-dried and stored at room temperature in the laboratory. Twenty-five grams (2.42% and 1.01% carbon, respectively) of sieved (0.25 mm) air-dried soil material were added to 250-ml Erlenmeyer flasks. Flasks were capped with aluminum foil and autoclaved three times at two-day intervals prior to use. Five milliliters of filter-sterilized (0.2-μm membrane filter) aqueous ferulic acid solution (970 μg/ml) or water was added to the flasks and flasks were stored in the dark for four days before extraction. Soils were extracted (2.5 hr) using either 100 ml of 0.25 M citrate, 0.25 M citrate plus 100 mM imidazole, 0.25 M citrate plus 10 mM KCl, 0.25 M citric acid adjusted to pH 7 with 0.5 M dibasic sodium phosphate, 0.25 M EDTA, or water. Ferulic acid and total phenolic acids in extracts were determined by HPLC and Folin & Ciocalteu's reagent analyses, respectively (Blum et al., 1991).

Plant Surface Debris. Crimson clover (*Trifolium incarnatum* L. Tibbee), subterranean clover (*Trifolium subterraneaum* L. Mt. Barker), rye (*Secale cereale* L. Wrens Abruzzi), and wheat (*Tritium aestivum* L. Coker 983) surface debris were collected from no-till cover plots located at the Lake Wheeler Road Field Laboratory one month after glyphosate desiccation. Samples were ground in a mill (20 mesh). Two milliliters of filter-sterilized ferulic acid (32 μg/ml) or water was added to 200 mg autoclaved debris samples in Corex tubes. Tubes were stored in the dark for four days before debris samples were extracted for 2.5 hr with 0.25 M citrate, 0.25 M citrate plus 100 mM imidazole, 0.25 M citric acid adjusted to pH 7 with 0.5 M dibasic sodium phosphate, or water. Procedures were identical to 1-g soil extractions. Ferulic acid and total phenolic acids in the extracts were determined by HPLC and Folin & Ciocalteu's reagent analyses, respectively (Blum et al., 1991).

In a second experiment, debris (200 mg, not autoclaved) was extracted for 2.5 hr with 50 ml of 0.25 M citrate or EDTA and the resultant extracts were filter sterilized (0.2-μm membrane filter) before HPLC analysis (Blum et al., 1991). In addition, debris was extracted for 2.5 hr with 1 N NaOH. The NaOH extracts were adjusted to pH 2.5 with HCl and centrifuged (10 min at 12,000*g*). The resulting supernatant was adjusted to pH 5.0 with NaOH, filter sterilized, and analyzed for ferulic acid by HPLC (Blum et al., 1991).

RESULTS

EDTA Extraction Procedures—1-g Samples. For Cecil A_p soil, to which 194 μg/g ferulic acid had been added four days earlier, recoveries for immediate and 2.5- and 5-hr 0.25 M EDTA extractions were 139 ± 2.5 (mean ± standard error), 156 ± 1.5, and 159 ± 0.5 μg/g soil, respectively. Recovery from Cecil B_t averaged 155 ± 1.5 μg/g soil for all three extraction times. Thus a 2.5-hr extraction with 0.25 M EDTA was adequate for both types of Cecil soil.

Extractions immediately after addition of 194 μg ferulic acid to Cecil A_p soil material resulted in a recovery of 172 ± 1.7 μg by a 2.5-hr water extraction and 156 ± 1.4 μg by a 2.5-hr EDTA extraction. If the extraction procedures were conducted 3 hr after the addition of ferulic acid, the recovery rates were essentially identical (i.e., 157 ± 3.4 and 155 ± 1.3, using water and EDTA, respectively). Four days after ferulic acid addition, the recovery of ferulic acid was higher for EDTA (156 ± 1.5 μg) than water (83 ± 2.5 μg). Thus the effectiveness of EDTA extractions varied with the time of ferulic acid–Cecil A_p soil contact.

Citrate plus KCl—1-g Samples. For initial development of the extraction procedure, I tested a range of citric acid/citrate concentrations (0–14 mM) plus 10 mM KCl, extractant pH values (3.4 to 7.0), and a 2.5-h extraction time. KCl was included in the extraction mixture to maintain ionic strength and aid in soil deflocculation. For the 1-g soil samples, 10 mM (pH 7) citrate plus KCl with a 2.5-hr extraction time appeared to be adequate for the recovery of ferulic acid (Figure 1A). That the citrate extraction of phenolic acids was best at pH 7 (Figure 1B; maximum pH value tested) was not surprising since the pK values for citric acid are: $pK_1 = 3.128$, $pK_2 = 4.761$ and $pK_3 = 6.396$ (Windholz and Budavari, 1983). To have all three carboxylic acid groups of citric acid in the anionic state (i.e., citrate) would require a pH above 8. However, to minimize the extraction of humic acids from soils, a maximum pH of 7 was used. Furthermore, since the buffering capacity of citrate plus KCl is limited, care was subsequently taken to make certain that the pH of the extraction solution was near 7. To verify that the 2.5-hr extraction time was the most appropriate for citrate plus KCl extractions, ferulic acid was extracted immediately, 2.5, or 5 hr after addition of citrate plus KCl. The 2.5-hr extraction procedure clearly was the best compromise (Figure 2A and B). Increasing the concentration of KCl above 10 mM was not advisable, since concentrations much above this resulted in the formation of a white precipitate in the presence of Folin & Ciocalteu's phenol reagent.

Comparison of 10 mM citrate plus 10 mM KCl and 0.25 M EDTA extractions of ferulic acid 1 hr and four days after the addition of ferulic acid to Cecil A_p and B_t soils demonstrated that citrate extractions were better than EDTA. Recoveries of ferulic acid were 168 ± 2 and 158 ± 1.6 μg/g for citrate and

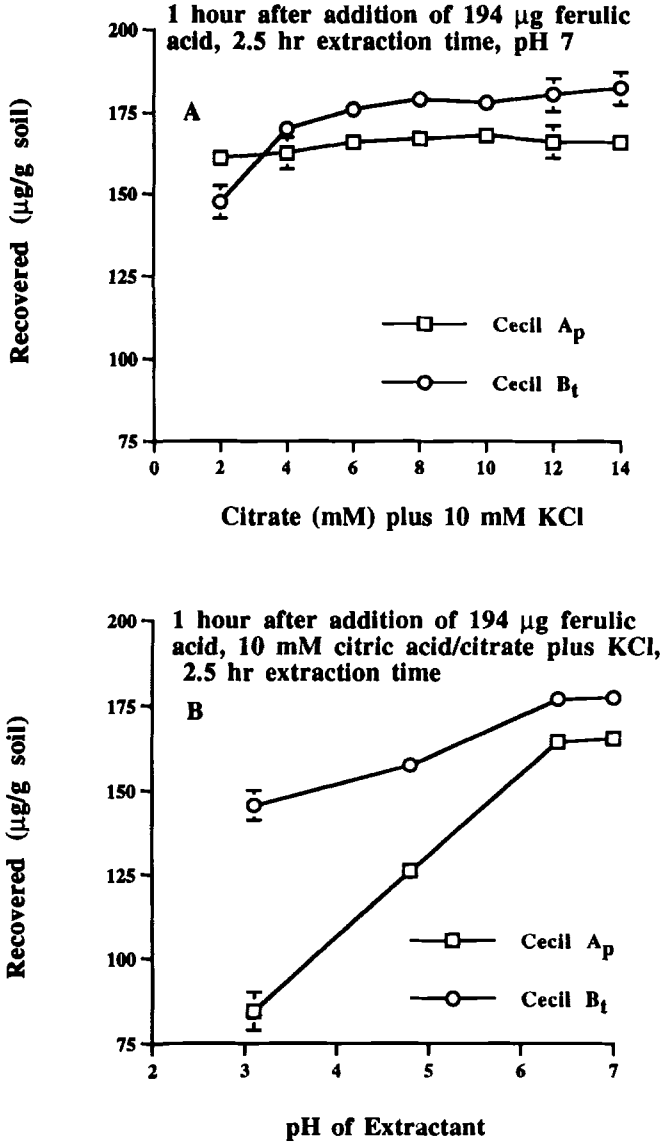

FIG. 1. Recovery of ferulic acid from Cecil A_p and B_t soil materials by solutions of citric acid/citrate varying in concentration plus 10 mM KCl (A) and varying in pH values (B). Means ± standard errors (N = 2). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

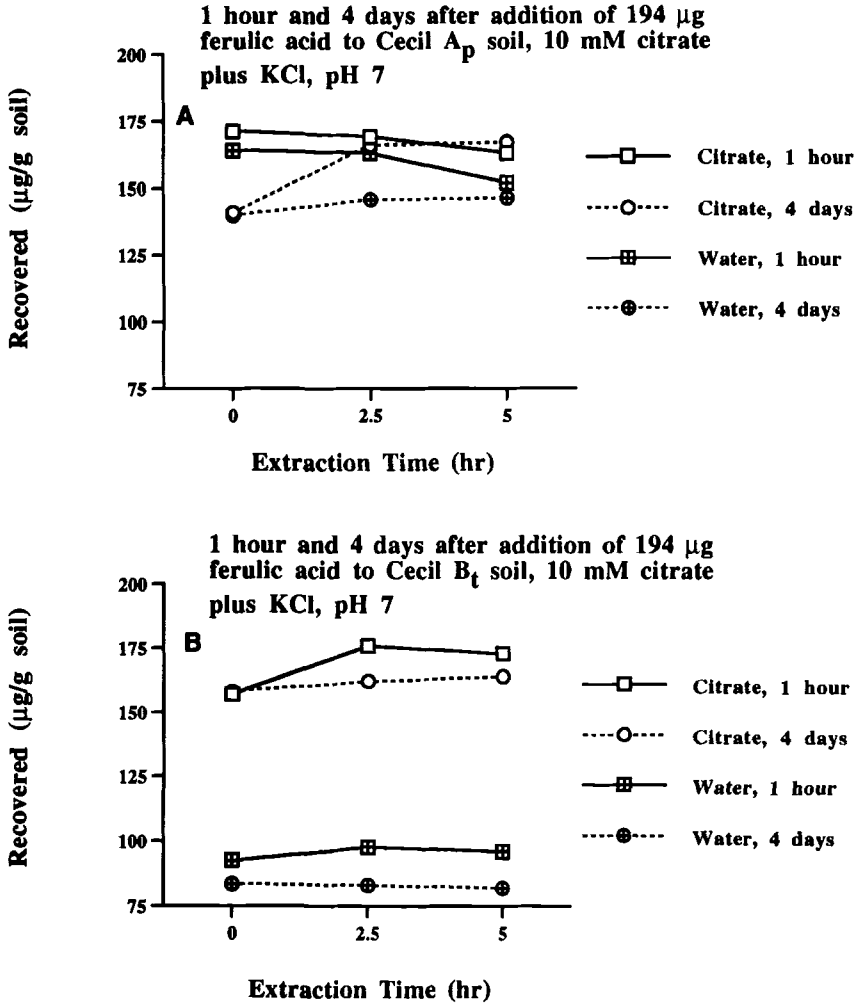

FIG. 2. Recovery of ferulic acid from Cecil A_p (A) and B_t (B) soil materials by 10 mM citrate plus 10 mM KCl (pH 7) or water 1 hr or four days after the addition of ferulic acid. Immediate and 2.5- and 5-hr extraction times were used. A_p and B_t soil samples were analyzed separately ($N = 2$). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

EDTA, respectively. There was also a significant soil × time interaction. Recoveries were as follows: 1 hr after ferulic acid addition—164 ± 2.9 and 170 ± 3.7 μg/g for Cecil A_p and B_t, respectively; four days after ferulic acid addition—161 ± 2.8 and 158 ± 2.1 μg/g for Cecil A_p and B_t, respectively.

Citrate—1-g Samples. Recovery of ferulic acid by citrate (without KCl) required a concentration > 125 mM (Figure 3A). Comparison of 125 mM citrate and 0.25 M EDTA extractions of ferulic acid four days after the addition of ferulic acid to soil demonstrated that 125 mM citrate was better than EDTA for the Cecil A_p but not for the B_t soil materials (Figure 3B).

Stability of Phenolic Acids in Citrate. Since extracts frequently must be stored before they can be analyzed, the stability of representative phenolic acids in citrate was determined. Filter-sterilized ferulic, *p*-coumaric, vanillic or *p*-hydroxybenzoic acid solutions (0.5 mM) stored in the refrigerator at 9°C were stable (no statistical significant changes in phenolic acid concentrations were observed for citrate or water solutions) in 0.125 or 0.25 M citrate solutions (pH 7) for at least six days, the maximum time tested.

Buffered Citrate—1-g Samples. A buffered citrate solution that will not react with Folin & Ciocalteu's phenol reagent could aid considerably in the effective recovery of phenolic acids from soils, since the recovery of phenolic acids from soils is a function of soil-extract pH (Dalton et al., 1983). Of the biological buffers tested [i.e., MOPS (pK_a = 7.2; range 6.5–8.2), HEPES (pK_a = 8; range 6.8–8.2], TES (pK_a = 7.5; range 6.8–8.2), imidazole (pK_a = 7; range 6.2–7.8), and citrate–phosphate buffer (range 2.2–8.0)], only HEPES was unsatisfactory because it reduced Folin & Ciocalteu's phenol reagent. With the exception of citrate–phosphate buffer (30 mM citrate), 50 mM concentrations were used. Citrate–imidazole and citrate–phosphate solutions were chosen for further testing.

Initially we tested a range of 0–100 mM imidazole in the presence of 10 mM citrate. Maximum recovery occurred at >25 mM for the B_t soil and 100 mM (maximum tested) for the A_t soil (Figure 4A). Concentrations greater than 100 mM were not desirable because of the increasing reduction (i.e., blue color development) of the Folin & Ciocalteu's phenol reagent by imidazole. Imidazole (100 mM) was tested with increasing concentrations of citrate. Citrate concentrations between 125 and 250 mM were adequate (Figure 4B). Above 250 mM, recovery of ferulic acid declined significantly for the Cecil A_p soil.

Citric acid solutions ranging from 0.03 M to 0.25 M were adjusted to pH 7 with dibasic sodium phosphate to produce solutions with citrate concentrations ranging from 10 to 30 mM. Four days after addition of 194 μg/g ferulic acid, 10–30 mM citrate–phosphate buffer recovered 169 ± 0.3 and 174 ± 1.15 μg/g ferulic acid for the Cecil A_p and B_t soil samples, respectively. By comparison, water recovered 152 ± 0.5 and 80 ± 2 μg/g, respectively. No significant differences in recovery of ferulic acid were observed for 0.25 M citrate and the 10 or 30 mM citrate–phosphate solutions.

Microbial Depletion. To determine how closely the recovery of ferulic acid by citrate corresponded to microbially available ferulic acid in soil, ferulic acid or water was allowed to interact with sterile soil for two weeks before microbes

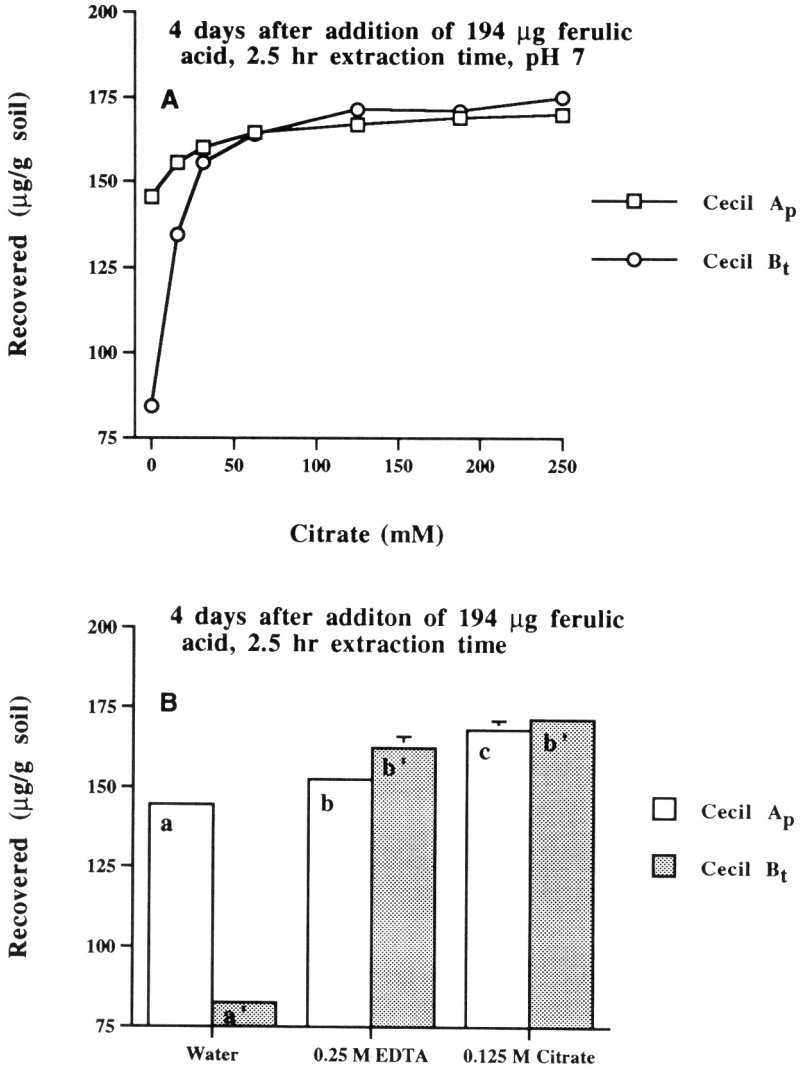

FIG. 3. Recovery of ferulic acid from Cecil A_p and B_t soil materials by varying concentrations of citrate (pH 7) (A) and 0.125 M citrate (pH 7), 0.25 M EDTA, or water (B) four days after the addition of ferulic acid. Means ± standard errors ($N = 2$). Bars with identical letters are not significantly different. Cecil A_p (a, b, c) and B_t (a′, b′, c′) were analyzed separately. Absence of error bars indicates that error bars were too small to be detected.

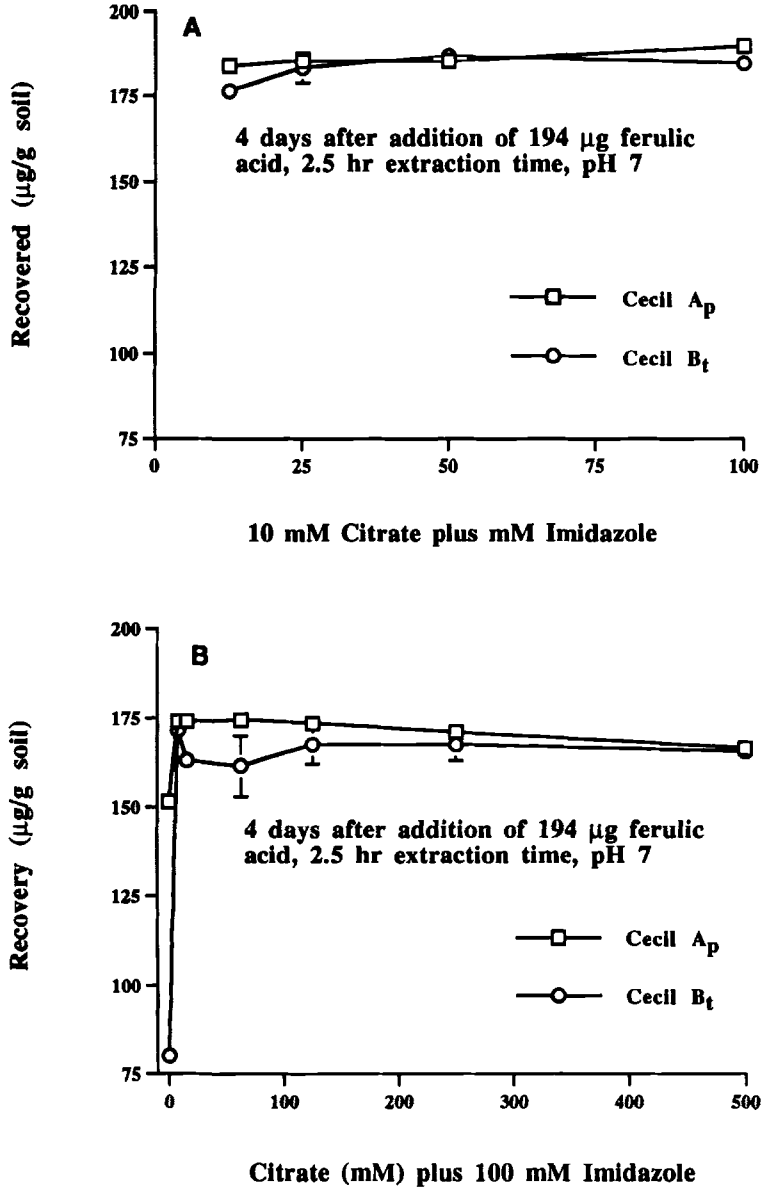

FIG. 4. Recovery of ferulic acid from Cecil A_p and B_t soil materials by 10 mM citrate plus varying concentrations of imidazole (A) and 100 mM imidazole plus varying concentrations of citrate (B) Means ± standard errors (N = 2). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

were introduced. Soil samples were extracted with 1 N NaOH, a more rigorous extractant than either EDTA or citrate, as soon as ferulic acid could no longer be recovered by citrate extractions from ferulic acid-amended soil samples. Different soil samples were used for the citrate and NaOH extractions. NaOH recovered 3.46 ± 0.68 and 2.95 ± 0.57 μg/g soil from the amended and unamended Cecil A_p soils, respectively. For Cecil B_t soils the recoveries were 2.25 ± 0.23 and 0 μg/g, respectively. The difference in the amount recovered from amended and unamended ferulic acid soils represented sorbed ferulic acid that could not be recovered by citrate and was not available to microbes. These results suggested that 0.25 M citrate (pH 7) extractions closely estimated the microbially available ferulic acid (i.e., phenolic acids) in these soils.

Extractions—25-g Samples. As a first step, the pH values of Cecil A_p soil–extract (1 soil–4 extract, w/v) solutions were determined after 2.5 hr. The pH values were as follows: water, pH 4.1 ± 0.36; 0.25 mM citrate plus 10 mM KCl, pH 7.0 ± 0.004, or 100 mM imidazole, pH 7.0 ± 0.01; 0.25 M citrate adjusted to pH 7 with 0.5 M dibasic sodium phosphate, pH 7.0 ± 0.01; and 0.25 M EDTA, pH 7.0 ± 0.01. The pH values for lower concentrations of citrate with or without KCl or imidazole ranged from 6.13 to 6.93.

When HPLC is used to quantify ferulic acid, any ferulic acid recovered by water extraction estimates the amount of ferulic acid in the soil solution. Recovery of ferulic acid using the various citrate and EDTA extractions estimates the amount of ferulic acid in the soil solution plus what is reversibly sorbed by the soil. When the Folin & Ciocalteu's reagent is used, resulting values for the respective extracts represent the recovery of all reactive substances (e.g., phenolic acids, humic acids, fulvic acids, some cyclic amino acids) (Box, 1983). Ferulic acid recovered was thus estimated by calculating the difference between ferulic acid amended and unamended soils. The HPLC and Folin & Ciocalteu's reagent values were related (r = 0.81), but the Folin & Ciocalteu's reagent tended to overestimate the ferulic acid values, particularly in the presence of higher soil organic matter content (Table 1). The difference in recovery between the water and citrate extractions would represent the amount reversibly sorbed by the soil. The amount of ferulic acid reversibly sorbed to the 1.01% carbon Cecil A_p soil after four days was 38 μg (20%) for the HPLC and 31 μg (16%) for the Folin & Ciocalteu's reagent analysis. The amount of ferulic acid reversibly sorbed to the 2.42% carbon Cecil A_p soil after four days was 39 μg (20%) for the HPLC and 42 μg (22%) for the Folin & Ciocalteu's reagent analysis.

Based on the similarity in recovery of ferulic acid by citrate ± KCl or imidazole and citrate–phosphate buffer, I recommend the use of 0.25 M citrate for its simplicity as long as soil–extract solutions can be maintained at pH 7. The similarity in estimating ferulic acid in Cecil A_p soil by HPLC and Folin & Ciocalteu's reagent analysis suggests that citrate extractions can be used to estimate relative total phenolic acid content of soils, something that cannot be

TABLE 1. RECOVERY (μg/g SOIL) OF FERULIC ACID FROM 25-g AUTOCLAVED CECIL A_p SOIL SAMPLES AMENDED WITH 194 μg/g FERULIC ACID OR WATER

Extractant	Method of quantification			
	HPLC[a]	Folin & Ciocalteu reagent		
	amended	Unamended	Amended	Amended − unamended
1.01% carbon				
0.25 M citrate	147 ± 0[ab]	58 ± 7[a]	205 ± 0[a]	147 ± 7[a]
0.25 M citrate + 10 mM KCl	147 ± 3[a]	49 ± 2[a]	210 ± 13[a]	161 ± 11[a]
0.25 M citrate + 100 mM imidazole	148 ± 11[a]	68 ± 6[a]	210 ± 5[a]	142 ± 11[a]
0.25 M EDTA	146 ± 2[a]			
Water	109 ± 14[a]	0[b]	119 ± 18[b]	119 ± 18[a]
2.42% carbon				
0.25 M citrate[c]	154 ± 4[a]	49 ± 1[b]	240 ± 12[b]	191 ± 14[a]
0.25 M citrate + 0.5 M disodium phosphate	164 ± 0[a]	113 ± 5[a]	300 ± 7[a]	189 ± 12[a]
0.25 M EDTA	162 ± 2[a]			
Water	121 ± 6[b]	5 ± 1[c]	154 ± 7[c]	148 ± 7[a]

[a] No ferulic acid was recovered from unamended soils.
[b] Data for the 1.01 and 2.42% carbon soils were analyzed separately. Identical letters within a columns for each soil indicate a lack of significant difference ($P = 0.05$).
[c] pH of soil-extract mixtures were 5.72 ± 0.01, 6.92 ± 0.005, 6.95 ± 0.0, and 4.5 ± 0.3, respectively.

done with EDTA extracts. Folin & Ciocalteu's reagent estimates relative instead of absolute available total phenolic acid because the values generated by this reagent will include other reactive substances besides phenolic acids (Box, 1983), and reduction of this reagent varies with phenolic acid (Blum et al., 1991).

Surface Debris. Crimson clover, rye, subterranean clover, and wheat surface debris from no-till plots, which had been desiccated by glyphosate and weathered for a month in the field, were ground to a size that approximated debris fragments on the plot soil surface. Autoclaved samples of this debris, amended or unamended with ferulic acid, demonstrated (Table 2) that: (1) 0.25 M citrate, 0.25 M citrate plus 100 mM imidazole, or 0.25 M citrate adjusted to pH 7 with 0.5 M dibasic sodium phosphate were equally efficient in recovering ferulic acid from this debris; (2) that reversible sorption (difference between that recovered by water and citrate extractant) by the debris varied with species and ranged from 0% for subterranean clover to 33% for wheat after four days; and (3) that an amendment of 64 μg of ferulic acid to 200 mg of debris was not detected in debris extracts by the Folin & Ciocalteu's reagent.

TABLE 2. RECOVERY (μg/200 mg) OF FERULIC ACID FROM 200 mg STERILE PLANT DEBRIS AMENDED WITH 64 μg FERULIC ACID OR WATER

Extractant[a]	Method of quantification: HPLC[b] Amended	HPLC[b] Unamended	HPLC[b] Difference	Folin & Ciocalteu reagent ($\times 10^3$)[c]
Crimson clover surface debris				
0.25 M citrate	81 ± 5.0[a]	20 ± 1.5[a]	60 ± 6.5[a]	1.74 ± 0.11[a]
0.25 M citrate + 100 mM imidazole	100 ± 3.0[a]	22 ± 0.5[a]	78 ± 2.5[a]	1.81 ± 0.08[a]
Citrate-phosphate buffer	94 ± 0.5[a]	26 ± 4.5[a]	69 ± 5.0[a]	1.84 ± 0.07[a]
Water	69 ± 14[b]	18 ± 1.5[a]	52 ± 1.5[b]	1.15 ± 0.08[b]
Rye surface debris				
0.25 M citrate	53 ± 0.5[a]	8 ± 0.0[a]	46 ± 0.5[a]	1.01 ± 0.03[a]
0.25 M citrate + 100 mM imidazole	55 ± 2.0[a]	8 ± 2.5[a]	46 ± 0.5[a]	1.10 ± 0.05[a]
Citrate-phosphate buffer	58 ± 2.5[a]	6 ± 3.0[a]	52 ± 0.5[a]	1.14 ± 0.06[a]
Water	38 ± 1.5[b]	6 ± 3.5[a]	33 ± 2.0[b]	0.76 ± 0.08[b]
Subterranean Clover surface debris				
0.25 M citrate	50 ± 0.5[a]	4 ± 0.5[a]	46 ± 0.0[a]	0.98 ± 0.03[a]
0.25 M citrate + 100 mM imidazole	53 ± 1.0[a]	6 ± 0.5[a]	48 ± 0.5[a]	1.06 ± 0.02[a]
Citrate-phosphate buffer	53 ± 0.0[a]	6 ± 0.5[a]	46 ± 0.5[a]	1.03 ± 0.02[a]
Water	55 ± 0.0[a]	7 ± 1.0[a]	48 ± 1.0[a]	0.87 ± 0.04[a]
Wheat surface debris				
0.25 M citrate	54 ± 1.5[a]	5 ± 0.0[a]	50 ± 1.5[a]	1.57 ± 0.07[a]
0.25 M citrate + 100 mM imidazole	54 ± 0.0[a]	4 ± 0.5[a]	50 ± 0.5[a]	1.56 ± 0.04[a]
Citrate-phosphate buffer	55 ± 2.0[a]	2 ± 0.0[a]	53 ± 2.0[a]	1.59 ± 0.02[a]
Water	36 ± 0.5[a]	6 ± 0.0[a]	30 ± 0.5[b]	1.28 ± 0.07[a]

[a] Citrate-phosphate buffer = 0.5 M citrate adjusted to pH 7 with 0.5 M dibasic sodium phosphate.
[b] All main treatment effects and interactions were significant. Identical letters within columns for each debris indicate a lack of significant difference ($P = 0.05$).
[c] Debris species, extractant, and debris species × extractant were significant. Identical letters within columns for each debris indicate a lack of significant difference ($P = 0.05$).

Although it has been demonstrated that the sorption of autoclaved Cecil soils were similar to Cecil soils that had not been autoclaved (Dalton et al., 1989), the validity of this relationship had not been determined for plant debris. Recovery of ferulic acid from surface debris (unamended) that had not been autoclaved was 2, 2, 0, and 8 times higher than autoclaved crimson clover, rye, subterranean clover, and wheat debris, respectively (Table 2 and 3). Irreversible sorption of ferulic acid by debris was thus enhanced by autoclaving for some of the debris samples.

With the exception of crimson clover, where recovery by EDTA was 70% lower, EDTA and citrate extracted identical amounts of ferulic acid from debris

TABLE 3. RECOVERY (μg/200 mg) OF FERULIC ACID FROM PLANT DEBRIS (NOT AUTOCLAVED)[a]

Extractant	Crimson clover	Rye	Subterranean clover	Wheat
0.25 M citrate	42 ± 5.4[ab]	17 ± 1.7[b]	5 ± 1.2[b]	40 ± 3.6[b]
0.25 M EDTA	13 ± 0.5[b]	15 ± 1.4[b]	6 ± 1.9[b]	39 ± 5.3[b]
1 N NaOH	59 ± 1.4[a]	732 ± 8.7[a]	51 ± 1.6[a]	550 ± 22[a]

[a]All main treatment effects and interactions were significant. Identical letters within a column indicate a lack of significant difference ($P = 0.05$).

(Table 3). Sodium hydroxide, a much more rigorous extraction procedure than EDTA or citrate, recovered approximately 1–4, 46, 8, and 14 times more ferulic acid from crimson clover, rye, subterranean clover, and wheat debris, respectively, than did EDTA or citrate (Table 3). Sodium hydroxide extractions are known to release phenolic acids that are not directly available to microorganisms or roots (Kaminsky and Muller, 1978; Dalton et al., 1987).

DISCUSSION

Definitive insight about the role of phenolic acids in allelopathic interactions requires numerous considerations, including data on the rates of phenolic acid input and generation, turnover rates, and available concentrations in soils. In addition, it is important to know how uptake of phenolic acids by various seeds or roots is modified by various soil sinks (e.g., clays, organic matter, and microbes) as well as how the function of these sinks are modified by various biotic and physicochemical processes in the soil. However, no matter what approach is taken in collecting such data, some type of soil extraction will be required. It is not surprising, therefore, that considerable effort has been made to identify useful and effective soil extraction procedures (e.g., see Dalton et al., 1987; Siqueira et al., 1991). Although a variety of extractants have been used (e.g., water, sodium hydroxide, calcium hydroxide, methanol, EDTA), water and neutral EDTA extractions have provided the most useful estimates of available (free and reversibly sorbed) phenolic acids in soils (Kaminsky and Muller, 1977; Blum et al., 1994). Water extractions provide an estimate of free phenolic acids in soils, while the difference between water and neutral EDTA extractions provide an estimate of reversibly bound phenolic acids. Neutral EDTA extractions, however, are not completely without problems. For example, neutral EDTA extractions are frequently ineffective in extracting phenolic

acids bound to plant debris or soil organic matter, a rich source of phenolic acids (Blum et al., 1992). Furthermore, EDTA itself reduces the Folin & Ciocalteu's phenol reagent used to estimate total phenolic acid content in soil and organic matter extracts.

Reversible sorption of the anionic and protonated phenolic acids (pK_a of approximately 4.5) by soils can occur by a variety of processes. The anionic forms, for example, can bind to positively charged sites on soil surfaces (Watson et al., 1973) and indirectly to negative charged sites by way of multivalent cation bridges (Greenland, 1965, 1971). The protonated forms can be sorbed by the soil organic matter (Chiou, 1989; Hasset and Banwart, 1989) and/or polymerized into humic substances in the soil (Martin et al., 1972; Martin and Haider, 1976; Haider et al., 1977; Wang et al., 1986). Chelates such as EDTA appear to easily disrupt complexes resulting from anion exchange or cation binding (primarily comprising the reversible phenolic acid fraction). Under the right conditions, organic acids, such as citric acid, released by microorganisms into the soil (Duff et al., 1963) could also disrupt such complexes and possibly do so more effectively than EDTA.

The effectiveness of EDTA extracts to recover reversibly bound phenolic acids from soils is largely a function of pH (most useful at pH 7), although EDTA concentration is also important (Blum et al., 1994). Here citrate extraction of phenolic acids from Cecil soils proved to be most useful at pH 7 and at > 1.25 M. The citrate concentration, however, could be reduced in the presence of a deflocculent or buffering agent (e.g., KCl, imidazole, dibasic sodium phosphate). Concentrations above a minimum (not defined) for both EDTA and citrate were important primarily in overcoming the buffering capacity of the soil or organic material; a pH of 7 must be maintained during the extraction process. Comparisons of ferulic acid concentrations extracted from soil and plant debris by citrate and EDTA suggested that citrate extractions: (1) were equivalent or better than EDTA in recovering phenolic acids, (2) were equivalent and, at times, more effective in extracting phenolic acids from A-horizon soils and plant debris, and (3) were more effective in recovering phenolic acid immediately after addition to soil. Furthermore, citrate, unlike EDTA, did not interfere with the Folin & Ciocalteu's phenol reagent when determining total phenolic acid content in soil and plant debris extracts. Thus, citrate extractions overcame some of the major limitations observed for EDTA extractions. However, both neutral 0.25 M EDTA and neutral 0.25 M citrate apparently recovered primarily from soil phenolic acids that are available to microbes and thus potentially to seed and roots.

A major concern regarding allelopathic interactions involving phenolic acids pertains to the fact that concentrations of individual phenolic acids recoverable from field soils are well below the levels required for inhibition of germination and seedling growth in laboratory bioassays. Research has suggested that alle-

lopathic interactions involving phenolic acids under field conditions are most likely a result of mixtures of phenolic acids and other organic compounds (Blum, 1996). Since the isolation of a large number of individual compounds in soil extracts is difficult and tedious, reseachers have been looking for ways to estimate total phenolic acid content of soil or plant debris extracts. The Folin & Ciocalteu's phenol (FC) reagent has been used to make such estimates (e.g., see Blum et al., 1991). Although Blum et al. (1991) observed that individual phenolic acids in soil extracts were highly correlated to total phenolic acid content of the extract determined by the FC reagent, the connection between total phenolic acid content of extracts and biotic responses (e.g., germination, or radicle and seedling growth) has been much more difficult to characterize (Blum et al., 1992). In part, this difficulty results from the fact that total phenolic acid estimated by the FC test are not very sensitive to changes in individual phenolic acids. For example, the addition of 64 μg of ferulic acid to 200 mg of plant debris was not detectable. In addition, the FC test overestimated the amount of ferulic acid added to soil in the presence of higher organic matter content of soil. Both of these observations led to the suggestion that total phenolic acid content determined by the FC reagent may be confounded by the levels of organic matter in the soil.

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SPECIES- AND CASTE-DETERMINED MANDIBULAR GLAND SIGNALS IN HONEYBEES (*Apis*)

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Abstract—Queens and workers of five honeybee species (*Apis mellifera*, *A. cerana*, *A. dorsata*, *A. florea*, and *A. andreniformis*) were analyzed for their mandibular gland components. In *A. mellifera*, the queen mandibular pheromone consists of 9-hydroxy- and 9-keto-2(*E*)-decenoic acids (9-HDA and ODA), methyl *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA), and is responsible for retinue attraction, among other functions. In retinue bioassays with workers of *A. cerana* (whose queens lack HVA), ODA, 9-HDA, and HOB were sufficient to elicit maximal retinue behavior. This suggests that the known queen mandibular pheromone components detected in mandibular glands of *A. cerana* queens constitute the functional queen mandibular pheromone in this species. Both castes of *A. mellifera* produce 10- and 8-carbon acids that are functionalized at the last position in the chain, and these are the predominant compounds found in worker mandibular glands. Workers of the other species also had these compounds, along with 9-HDA and ODA that are normally not present in *A. mellifera* worker glands. Queens and workers of each species had a unique

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combination of mandibular compounds. The aromatic compounds were characteristic of queens from the cavity-nesting species, *A. mellifera* (HOB and HVA) and *A. cerana* (HOB). These two species also had more pronounced differences in the mandibular blends of queens and workers than the open-nesting species, *A. dorsata*, *A. florea*, and *A. andreniformis*. Our results indicate that the more derived cavity-nesting species of *Apis* have evolved greater caste-specific differences between queens and workers and a higher number of queen pheromone components, compared to the open-nesting species.

Key Words—Honeybee, mandibular gland, chemical signature, pheromone, queen, worker, *Apis*.

INTRODUCTION

Honeybees (*Apis*) have adapted to many conditions by varying their nesting habits and the underlying behavior and physiology required to nest in different locations. The open-nesting species, *A. dorsata*, *A. florea*, and *A. andreniformis*, all of which are native to Asia, construct a single comb suspended from a branch or rock and protect themselves with a dense curtain of workers around the comb. The cavity-nesting species, *A. cerana*, an Asian species, and *A. mellifera*, a European/African species, construct multiple parallel combs in cavities (Otis, 1991). Open- and cavity-nesting species differ in their defense strategy (Seeley et al., 1982), absconding behavior, and physiological aspects such as worker tempo, metabolic rate, and longevity (Dyer and Seeley, 1987, 1991). The various honeybee species might also differ in the chemical signatures of queens and workers, and the patterns of these differences may prove informative with respect to the evolutionary history of the genus. In this paper we describe the chemical signals produced in the mandibular glands of queens and workers of three open-nesting and two cavity-nesting species.

Both female castes in *A. mellifera* produce a blend of several compounds in their mandibular glands. Mated queens have two aliphatic compounds, 9-hydroxy-2(*E*)-decenoic acid (9-HDA) and 9-keto-2(*E*)-decenoic acid (ODA) and two aromatic ones, methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Winston and Slessor, 1992). The two aliphatic components in queens have the functional group at the penultimate (ω-1) position in the chain. Workers have 10-hydroxy-2(*E*)-decenoic acid (10-HDA), 10-hydroxydecanoic acid (10-HDAA), and their respective diacids, 2(*E*)-decenedioic ($C_{10:1}$ DA) and decanedioic ($C_{10:0}$ DA) acids. These acids are functionalized at the last (ω) position in the chain. Both castes are able to produce the other caste's aliphatic compounds in small quantities. Queens have some 10-HDA and 10-HDAA (Crewe, 1982), and workers have a trace of 9-HDA (Plettner et al., 1995). Occasionally, traces of ODA can be found in the glands of

queenless workers (Plettner et al., 1993), more frequently in African than European subspecies (Crewe and Velthius, 1980). Both castes bias the biosynthesis of the aliphatic mandibular components towards their major compound: queens produce more of the 10-carbon ω-1-functionalized acids and workers produce more of the ω-functionalized ones (Plettner et al., 1996). In contrast, the aromatic components appear to be characteristic only of queens.

Caste-specific chemical signatures are important for various colony functions. Workers sense the presence of their queen and form a retinue around her because of her queen-specific signal. Workers form a retinue around a dead queen or a lure impregnated with a solvent extract of a queen, indicating that this behavior is mediated by a semiochemical. The pheromone largely responsible for retinue formation in *A. mellifera* consists of ODA, 9-HDA (70% *R* and 30% *S*), HOB, and HVA (Slessor et al., 1988). This queen mandibular pheromone (QMP) not only elicits short-term behaviors such as retinue formation, swarm stabilization (Winston et al., 1982), and drone attraction (only ODA; Butler and Fairey, 1964), but also has long-term physiological effects such as the inhibition of queen rearing (Winston et al., 1990, 1991). In addition, ODA inhibits the synthesis of juvenile hormone III in workers of *A. mellifera* (Kaatz et al., 1992) and, through this effect, delays the onset of foraging (Pankiw, 1995).

Workers form a retinue around the queen in all honeybee species (Free, 1987). However, bioassay-guided identification of retinue-active compounds has only been done in *A. mellifera* (Kaminski et al., 1990), so compounds responsible for retinue behavior are unknown for the other species. Both ODA and 9-HDA have been detected and identified in several studies of Asian species (Shearer et al., 1970; Free, 1987), but the aromatic compounds have not been investigated. Moreover, mandibular glands from workers of Asian honey bee species have not been analyzed. It is therefore not known whether the mandibular glands in queens and workers of Asian honeybee species produce distinct chemical signals and whether the queen-produced compounds can elicit retinue formation.

The compounds produced in the mandibular glands of *A. mellifera* workers appear to be involved in food preservation and larval nutrition. The ω-hydroxy acids and the corresponding diacids are found in royal jelly (Barker et al., 1959; Weaver et al., 1968), where they may function as antiseptics (Blum et al., 1959). Moreover, 10-HDA inhibits the germination of pollen, which is important for pollen storage (Lukoschus and Keularts, 1968), and is an important larval nutrient that prevents larvae from pupating precociously (Kinoshita and Shuel, 1975).

The objective of this study was to explore differences between *A. mellifera* and four Asian species (*A. dorsata*, *A. florea*, *A. andreniformis*, and *A. cerana*) in the compounds found in the mandibular glands of queens and workers. The second objective was to determine whether the known QMP compounds detected

in mandibular glands of *A. cerana* queens elicit retinue formation in workers of that species.

METHODS AND MATERIALS

Collection of Specimens. All but two of the queens for this study were collected in 1989 in the following localities: one *A. dorsata*, five mated and all virgin *A. cerana* near Kuala Lumpur, Malaysia; the second *A. dorsata* and two *A. andreniformis* in Tenom, Sabah, Malaysia; five *A. andreniformis* on the southwest coast of Malaysia between Muar and Batu Pahat; and *A. florea* in Bangkok, Thailand. Foraging workers of *A. dorsata* and *A. andreniformis* were collected in 1995 in Kedah (Pedu Lake) and Melakka, Malaysia, respectively. The bees were stored at −30°C for up to five months, shipped in liquid nitrogen (dry shipping tank), and stored at −70°C until dissection of their mandibular glands. Foraging workers of *A. cerana* and *A. florea* and the remaining three mated queens of *A. cerana* were collected in Sri Lanka at the Horticultural Research Station in Kananwila, Horana. The heads of these bees were shipped in sealed ampoules in 100 μl of methanol. Workers of *A. mellifera* were collected in March 1994 at Simon Fraser University (British Columbia, Canada) and stored at −30°C until dissection.

Status of Bees. The queens of *A. dorsata* and *A. andreniformis* were collected from functioning colonies with brood, indicating that they were mated and laying. The virgin queens of *A. florea* were obtained by N. and G. Koeniger, who removed queens from colonies and caged the queen cells. They also obtained the mated queen of *A. florea* by removing her from a functioning colony with brood (Koeniger et al., 1989). The queens of *A. cerana* were reared at the Universiti Pertanian Malaysia and those of *A. mellifera* were reared at Simon Fraser University.

The workers of *A. dorsata* were collected while foraging for pollen on flowers of sensitive plants (*Mimosa* sp.) The *A. andreniformis* workers were foraging for pollen at palm infloresences when they were collected. Foraging workers of *A. cerana* and *A. florea* were attracted to a cup with sugar water, where they were collected. Workers of *A. mellifera* were overwintered bees.

Extraction and Analysis. The mandibular glands were extracted with methanol, and a small portion of this extract was derivatized with bistrimethylsilyl trifluoroacetamide (BSTFA) and analyzed by splitless capillary gas chromatography on a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector and a 30-m fused silica (DB-1) column (Slessor et al., 1990) The GC was programmed 100°C (1 min), 10°/min to 185°C (4 min), 3°/min to 200°C (0 min), and 25°/min to 260°C (20 min), flow 40 ml/min, head pressure 125 kPa. Standard solutions of ODA, HOB, HVA, 9-HDA, 10-HDAA, and 10-HDA were used to calibrate the response of the instrument. To ensure

peak authenticity, all samples were examined by splitless GC–mass spectrometry on a Varian 3400 GC with a 30-m fused silica (DB-5) column interfaced with a Varian Saturn ion trap mass spectrometer operated in electron impact mode, set at a target value of ca. 5000 and an ionization current of 10 μA.

Most of the mandibular gland contents remained with the mandibular glands upon dissection. Only 22 $\pm$ 6% (N = 9) of the total material remained behind in the head of a worker after dissection. In the case of the Sri Lankan bees, the shipping solvent also was analyzed because some material was extracted from the intact head during shipping. Approximately 45 $\pm$ 5% of the total material was in the solvent, 30 $\pm$ 4% was in the mandibular glands, and the remaining 24 $\pm$ 4% was contained in the rest of the head (N = 10). The amounts found in the shipping solvent and the mandibular glands were summed.

Bioassay of Synthetic Queen Mandibular Pheromone with Workers of A. cerana. Two synthetic QMP blends (supplied by Phero Tech, Inc.) were tested on workers of *A. cerana* as described by Kaminski et al. (1990). The first blend consisted of ODA (46.4%), 9-HDA (46.0%), HOB (7.0%), and HVA (0.6%); the second blend lacked HVA but was otherwise identical to the first blend. The objective of this bioassay was to determine whether HVA is necessary to elicit a maximal retinue response in *A. cerana*.

RESULTS

Queens. Queens of all the species analyzed contained ODA and 9-HDA (Table 1). The amount of ODA is within the same range as reported for *A. dorsata* and *A. mellifera* by Shearer et al. (1970). Previously reported quantities of ODA in mated *A. cerana* queens are higher than the ones found in this study. Free (1987) reported around 200 μg of ODA for mated *A. cerana* queens. In *A. andreniformis*, *A. cerana*, and *A. mellifera* there was more ODA than 9-HDA (Kruskal-Wallis, $P < 0.05$), while in *A. florea* there was no significant difference in the quantities of these acids (pooled virgin and mated, Kruskal-Wallis, $\chi^2 = 1.6$, $df = 1$, $P = 0.2$). *A. mellifera* and *A. cerana* queens had easily detectable quantities of HOB, which was not detectable in any of the *A. florea*, *A. andreniformis*, and *A. dorsata* queens. HVA was only detected in mated *A. mellifera* queens. The worker acid 10-HDA was found in variable quantities in all the queens, while 10-HDAA was found at readily detectable levels in *A. mellifera* and *A. cerana* and in trace amounts in *A. florea*. Queens of *A. dorsata* and *A. andreniformis* did not contain detectable 10-HDAA. Further, queens of *A. mellifera* had more 10-HDA than 10-HDAA (Kruskal-Wallis, $\chi^2 = 37$, $df = 1$, $P = 0.0001$), while in queens of *A. cerana* there was no significant difference in the quantities (Kruskal-Wallis, $\chi^2 = 0.6$, $df = 1$, $P = 0.4$).

We compared quantities of the QMP components and worker acids for

TABLE 1. AMOUNTS OF VARIOUS MANDIBULAR GLAND COMPONENTS IN QUEENS OF FOUR ASIAN HONEYBEE SPECIES AND *A. mellifera*, A EUROPEAN/AFRICAN SPECIES

Species	Age (days)	*N*	Amount (μg), mean ± SE[a]					
			ODA	9-HDA[b]	HOB	HVA	10-HDA	10-HDAA
A andreniformis (mated)	unk.	7	10.3 ± 3.6a	0.3 ± 0.1a			0.2 ± 0.1a	
A. florea (virgin)	unk.	4	1.5 ± 0.5a	0.7 ± 0.2ab			7.6 ± 0.7b	0.10 ± 0.03a
A. florea (mated)[c]	unk.	1	0.8	1.4			8.1[d]	0.04[d]
A. dorsata (mated)[c]	unk.	2	103 ± 41	34.0 ± 0.0			0.4 ± 0.2	
A. cerana (virgin)	6	8	40.1 ± 12.0b	2.4 ± 0.5b	0.9 ± 0.5ab		0.8 ± 0.3a	0.9 ± 0.5a
A. cerana (mated)	minimum 14	7	28.8 ± 6.5b	18.1 ± 11.2b	6.8 ± 4.5a		1.8 ± 0.7a	1.1 ± 0.4a (N = 6)
A. mellifera (virgin)	6	10	70.4 ± 8.8c	12.6 ± 2.0b	0.3 ± 0.03b	0.0 ± 0.0a	76.7 ± 13.9c	1.5 ± 0.3a
A. mellifera (mated)	1 year	29	231 ± 17d	164 ± 15c	27.7 ± 2.7c	4.2 ± 0.4b	27.3 ± 2.4d	8.1 ± 0.8b

[a] Means within one column followed by the same letter are not significantly different ($P < 0.05$) by pairwise comparisons (Kruskal-Wallis test). A blank indicates that the compound was not detected in the GC-MS analysis and, therefore, no mean could be calculated. Range for *A. dorsata*. ODA = 9-keto-2(*E*)-decenoic acid; 9-HDA = 9-hydroxy-2(*E*)-decenoic acid; HOB = methyl *p*-hydroxybenzoate; HVA = 4-hydroxy-3-methoxyphenylethanol (homovanillic alcohol); 10-HDA = 10-hydroxy-2(*E*)-decenoic acid; 10-HDAA = 10-hydroxydecanoic acid; unk. = unknown.

[b] Total R + S 9-HDA.

[c] These bees were not included in the statistical analysis.

[d] Amount determined from GC-MS traces.

virgin and mated queens where both types were available. *A. mellifera* stood out as having the most pronounced differences between virgin and mated states (Table 1). All the components of the mandibular blend increased significantly from young virgins to 1-year-old mated queens, except for 10-HDA, which decreased significantly. *A. mellifera* virgin queens showed no significant difference in the quantities of 10-HDA and ODA (Kruskal-Wallis, $\chi^2 = 0.006$, $df = 1$, $P = 0.94$). In mated, laying queens, the 10-HDA content had decreased and the ODA content increased to a significantly higher level than 10-HDA (Kruskal-Wallis, $\chi^2 = 43$, $df = 1$, $P = 0.0001$). *A. cerana* did not show a significant change in the level of any mandibular component. In *A. florea*, there may have been little change, although more data are required for a clear picture.

Workers. Workers of all species analyzed had ω-hydroxy acids (10-HDA, 10-HDAA, and 8-HOAA), $C_{10:0}$ DA, and 9-HDA in their mandibular glands (Table 2). In *A. dorsata* and *A. mellifera*, 10-HDA was present at significantly higher quantities than the other ω-hydroxy acids (Kruskal-Wallis, $\chi^2 > 19$, pairwise comparisons, $P < 0.05$) and was overall the major component. In *A. florea*, 10-HDA and 8-HOAA were present in equal quantities (Kruskal-Wallis, $\chi^2 = 0.3$, $df = 1$, $P = 0.6$) and were the major components. Workers of *A. andreniformis* had the same pattern as *A. florea* with respect to 10-HDA and 8-HOAA, but the major component in the mandibular glands was ODA. In *A. cerana* there were no significant differences in the quantities of the three ω-hydroxy acids (Kruskal-Wallis, $\chi^2 < 2$, pairwise comparisons, $P > 0.05$). Workers of open-nesting species had ODA, while workers of cavity-nesting species had no detectable ODA. Further, workers of *A. dorsata* and *A. florea* had more 9-HDA than ODA (Kruskal-Wallis, $P < 0.05$), while for *A. andreniformis* the pattern was reversed (Kruskal-Wallis, $\chi^2 = 12$, $df = 1$, $P = 0.0007$).

Workers of all species had $C_{10:0}$ DA and all, except *A. cerana*, had $C_{10:1}$ DA. The hydroxy acids 10-HDAA and 10-HDA were present in higher quantities than the corresponding diacids in all species except *A. andreniformis*, for which there was no significant difference in the quantities of 10-HDAA and $C_{10:0}$ DA (Kruskal-Wallis, $\chi^2 = 2.3$, $df = 1$, $P = 0.13$). The open-nesting bees had more diacids relative to the corresponding hydroxy acids than the cavity-nesting species (compared pairwise between open- and cavity-nesting species as ratios of quantities, Kruskal-Wallis, $P < 0.05$).

Differences between Queens and Workers in Mandibular Components. Only the queens of cavity-nesting species had detectable levels of the aromatic mandibular components. In workers of cavity-nesting species, ODA was not detected. Neither workers nor queens of open-nesting species contained HOB or HVA, but both castes had ODA. In *A. dorsata*, queens had more ODA than the workers; the same was true in *A. florea* (pooled mated and virgin queens, Kruskal-Wallis, $\chi^2 = 6.4$, $df = 1$, $P = 0.01$) and in *A. andreniformis*, queens and workers did not differ significantly in their ODA content (Kruskal-Wallis, $\chi^2 = 0.23$, $df = 1$, $P = 0.63$).

TABLE 2. AMOUNTS OF VARIOUS MANDIBULAR GLAND COMPONENTS IN WORKERS OF FOUR ASIAN HONEYBEE SPECIES AND *A. mellifera*, A EUROPEAN/AFRICAN SPECIES

Species	*N*	Amount (μg), mean ± SE[a]						
		ODA	9-HDA	10-HDA	10-HDAA	8-HOAA	C10:0 DA	C10:1 DA
A. andreniformis	9	5.4 ± 0.5a	1.5 ± 0.3ac	3.2 ± 0.5a	0.8 ± 0.1a	2.5 ± 0.2a	0.6 ± 0.1a	0.9 ± 0.2a
A. florea	22	0.5 ± 0.2b	2.9 ± 0.3b	11 ± 1b	0.8 ± 0.1a	9.6 ± 0.8b	0.28 ± 0.03b	2.6 ± 0.4b
A. dorsata	22	0.14 ± 0.01b	1.9 ± 0.3a	56 ± 5c	3.9 ± 0.4b	6.5 ± 0.7c	1.5 ± 0.2c	6.8 ± 0.7c
A. cerana	22	n.d.	0.9 ± 0.1c	0.8 ± 0.2d	1.5 ± 0.3a	0.8 ± 0.1d	0.14 ± 0.03d	n.d.
A. mellifera	22	n.d.	1.4 ± 0.2a	95 ± 15e	23 ± 3c	2.7 ± 0.4a	2.2 ± 0.3c	1.1 ± 0.3a

[a]Means within one column followed by the same letter are not significantly different ($P < 0.05$, Kruskal-Wallis). n.d. = not detected.

The ratio of the two isomeric compounds 10-HDA and 9-HDA is an indicator of the tendency to bias the biosynthesis of the aliphatic mandibular acids towards the ω- or ω-1-functionalization pattern. Workers of *A. mellifera* have a ratio much larger than 1, indicating a strong tendency to produce 10-carbon ω-functionalized acids, and queens have a ratio smaller than 1, reflecting a tendency to produce more 10-carbon ω-1-functionalized acids. In all cases except *A. florea*, queens had a ratio of 10-HDA to 9-HDA less than 1 (Table 3). Workers of *A. mellifera* and *A. dorsata* had ratios much greater than 1; workers of *A. florea* and *A. andreniformis* had ratios only slightly higher than 1; and workers of *A. cerana* had nearly equal quantities of 10-HDA and 9-HDA. All species exhibited significant caste-related differences in the 10-HDA:9-HDA ratio.

Bioassay of Synthetic QMP with Workers of A. cerana. The number of times workers contact the QMP-impregnated glass lure and the number of workers that enter the elliptical space around the lure are a measure of the retinue response to the QMP sample (Kaminski et al., 1990). The retinue responses to QMP blends with and without HVA did not differ significantly, suggesting that HVA is not required for maximal worker attraction in *A. cerana* (Table 4). This result is consistent with the mandibular components detected in queens of this species.

TABLE 3. DIFFERENCES BETWEEN QUEENS AND WORKERS IN AMOUNTS OF 9-HDA AND 10-HDA AND IN RATIO OF 10-HDA TO 9-HDA FOR FIVE SPECIES OF HONEYBEE

Species	Caste	*N*	Amount (μg)[a] 9-HDA	Amount (μg)[a] 10-HDA	Ratio of 10-HDA to 9-HDA
A. andreniformis	Queen	7	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.2
	Worker	9	**1.5 ± 0.3**	**3.2 ± 0.5**	**2.3 ± 0.3**
A. florea	Queen	5[b]	0.9 ± 0.2	7.7 ± 0.6	11 ± 2
	Worker	22	**2.9 ± 0.3**	11 ± 1	**3.9 ± 0.3**
A. dorsata	Queen	2[c]	34 ± 0	0.4 ± 0.2	0.01 ± 0.01
	Worker	22	1.9 ± 0.3	56 ± 5	35 ± 3
A. cerana	Queen	7[d]	18 ± 11	1.8 ± 0.7	0.2 ± 0.1
	Worker	22	**0.9 ± 0.1**	0.8 ± 0.2	**0.8 ± 0.1**
A. mellifera	Queen	29[d]	164 ± 15	27 ± 2	0.2 ± 0.03
	Worker	22	**1.4 ± 0.2**	**95 ± 15**	**110 ± 45**

[a]Mean ± SE. Bold entries indicate a significant difference between queens and workers (pairwise comparison, Kruskal-Wallis, $P < 0.05$).
[b]The mated and virgin queens were pooled.
[c]Because there were only two *A. dorsata* queens, no statistical analysis could be performed for this species.
[d]Only data for the mated queens were used.

TABLE 4. RETINUE BIOASSAY OF SYNTHETIC QUEEN MANDIBULAR PHEROMONE (QMP) ON WORKERS OF *A. cerana*

Treatment	Dose (Qeq)[a]	Contacts (mean ± SE)[b]	Workers in ellipse (*N*)[c]
Full QMP[d]	10^{-6}	0.5 ± 0.2	7 ± 1
	10^{-4}	**2.5 ± 0.6**[f]	**13 ± 2**
	10^{-2}	**4.6 ± 0.6**	**25 ± 3**
QMP without HVA[e]	10^{-6}	1.5 ± 0.6	**9 ± 2**
	10^{-4}	**1.6 ± 0.4**	**11 ± 1**
	10^{-2}	**4.1 ± 0.4**	**26 ± 3**
solvent	(control)	0.6 ± 0.2	5 ± 1

[a]Qeq = queen equivalent, the average amount found in a queen of *A. mellifera* [200 μg ODA, 80 μg 9-HDA, 4 μg HOB, and 0.4 μg HVA, see Pankiw *et al.* (1996)].
[b]Contacts = the number of times a lure is contacted by workers during a 30-sec interval.
[c]Workers in the ellipse = the number of workers entering the elliptical space one bee length around the lure.
[d]Full QMP is the five-component blend found in *A. mellifera*.
[e]QMP without HVA mimics the four-component blend found in *A. cerana*.
[f]Values in bold type are significantly different from the solvent control (Tukey, $P < 0.05$). There are no significant differences in pairwise comparisons between full QMP and QMP without HVA at the same dose (Tukey, $P < 0.05$).

DISCUSSION

Species Specificity in Mandibular Blends. Our analysis revealed that queens and workers of each honeybee species have a unique multicomponent blend in their mandibular glands. Several characteristics set the open- and cavity-nesting species apart. Queens of open-nesting species do not contain aromatic compounds or 10-HDAA, while queens of cavity-nesting species do. Workers of open-nesting species contain ODA, which is not present in workers of the cavity-nesting species. Similarly, workers of the open-nesting species have more diacids relative to the corresponding hydroxy acids than workers of cavity-nesting species.

Some characteristics of the mandibular gland signatures differentiate species within the open-nesting group. The dwarf bees, *A. florea* and *A. andreniformis*, differ in their 10-HDAA/$C_{10:0}$ DA pattern in workers and in their 9-HDA/ODA profiles in both castes. Workers of *A. florea* have more 10-HDAA than $C_{10:0}$ DA and more 9-HDA than ODA, while workers of *A. andreniformis* have equal quantities of 10-HDAA and $C_{10:0}$ DA and less 9-HDA than ODA. Thus, the dwarf bees show a few differences in their mandibular blends, in spite of being close phylogenetically (Alexander, 1991). The only characteristic that differs between both dwarf species and *A. dorsata* is the ω-hydroxy acid pattern in

workers. In *A. florea* and *A. andreniformis* 10-HDA and 8-HOAA are the major ω-hydroxy acids, while in *A. dorsata* 10-HDA is the major ω-hydroxy acid. Interestingly, the ω-hydroxy acid pattern in *A. dorsata* and *A. mellifera* workers is very similar, even though these species are not close phylogenetically (Alexander, 1991).

The cavity-nesting species differ in several mandibular gland characteristics, in spite of being close phylogenetically (Alexander, 1991). While workers of *A. cerana* have equal quantities of all three ω-hydroxy acids, 10-HDA is the major one in *A. mellifera*. However, the most important difference between the two cavity-nesting species is in the aromatic components in queens. Mated queens of *A. mellifera* have HVA, while those of *A. cerana* do not have detectable amounts. Further, HVA is necessary in *A. mellifera* for a full retinue response to QMP, while in *A. cerana* it is not. The compounds detected in queen mandibular glands of *A. cerana* are sufficient to elicit maximal QMP-mediated retinue response, and therefore constitute the functional QMP in this species.

The ω- and ω-1-functionalized acids were present in all the species analyzed in different total amounts and in different ratios. The ratio of components may reflect underlying differences in the biosynthetic pathway. Mandibular acid biosynthesis in *A. mellifera* is a three-step process that starts with the hydroxylation of stearic acid at the last or penultimate position to give 18- and 17-hydroxyoctadecanoic acids, respectively. The 18-carbon hydroxy acids are then chain shortened by β-oxidation, mainly to the 10-carbon length. The resulting chain shortened ω- and ω-1-hydroxy acids are oxidized to the corresponding diacids and keto acid, respectively (Plettner 1995; Plettner et al., 1995, 1996). Mandibular acid biosynthesis has not been studied in the other species, but, given that the same set of compounds is present, it is likely that the other species have a similar pathway. However, differences in the chain length distribution may arise by different chain length specificities in β-oxidation, and differences in the proportion of diacids and keto acids relative to the hydroxy acids could arise by different tendencies to oxidize the hydroxy acids.

Caste Selectivity in Mandibular Blends. Comparison between castes of each species with respect to aromatic components, the level of ODA, and the 10-HDA:9-HDA ratio revealed that queens always had a different mandibular blend than the workers. All three aspects differed in workers and queens of the cavity-nesting species: only the queens had aromatic components and ODA, and queens had a lower 10-HDA:9-HDA ratio than workers. In *A. dorsata* and *A. florea*, neither caste had aromatic components, and queens of both species had more ODA than workers. Queens of *A. dorsata* had a lower 10-HDA:9-HDA ratio than workers, and the opposite was true in *A. florea*. Only the 10-HDA:9-HDA ratio differed between the castes in *A. andreniformis*, queens having a lower ratio than workers. In general, the cavity-nesting species showed the strongest

caste differences in the mandibular blend, and the open-nesting species the smallest. Further, *A. andreniformis* had queen and worker blends that overall were most similar of all the species examined.

Since the open-nesting species had a smaller chemical queen/worker difference than the cavity-nesting ones, it is possible that they require other chemical and/or nonchemical cues for caste recognition in addition to mandibular gland semiochemicals. Even in *A. mellifera* there is considerable variation in worker retinue response to the queen mandibular pheromone (Pankiw et al., 1994). Strains of *A. mellifera* that exhibit very low response to QMP in laboratory bioassays exhibit normal retinue behavior in their colony. These strains may be responding to another queen-produced retinue releaser (Slessor et al., 1996).

Queen Ontogeny. In *A. mellifera* queens, the mandibular gland content changed from a blend with equal quantities of ODA and 10-HDA to a blend with more ODA than 10-HDA following mating. A similar change in the aliphatic compounds was observed in African races of *A. mellifera* (Crewe, 1982). The aromatic components characteristic of queens also increased, as was observed before by Slessor et al. (1990). In *A. cerana*, queen ontogeny appeared to be much less pronounced than in *A. mellifera*, but additional data from older queens of *A. cerana* are needed to confirm this.

A. mellifera, the species with the most pronounced queen/worker differences in the mandibular blend also is the species with the largest ontogenic change in the mandibular glands of queens. Young virgin *A. mellifera* queens may chemically mimic workers by having a high proportion of worker acids and traces or nondetectable levels of the aromatic components. This chemical camouflage may enable queens to minimize aggression by the workers until they have mated. The ODA level in virgin queens increases from trace levels at emergence to nearly one third queen equivalent prior to mating (Slessor et al., 1990; Pankiw et al., 1996), possibly because this compound functions as a drone attractant (Gary, 1962; Butler and Fairey, 1964). The aromatic components increase sharply after mating (Slessor et al., 1990; Pankiw et al., 1996) to give a blend with maximal retinue attractancy (Slessor et al., 1988) once the queen is fully functional.

CONCLUSIONS

The five honeybee species analyzed have distinct blends of compounds in the mandibular glands of both female castes. Furthermore, within each species, queens and workers have different blends. These species and caste differences are determined by combination of a few characters in distinct ways, including: (1) the presence or absence of aromatic components, (2) the level of ω-func-

tionalized acids relative to the level of ω-1-functionalized ones, (3) the chain length profiles among acids with the same functionalization, and (4) the level of hydroxy acids relative to the corresponding keto- or diacids. Species- and caste-related variation in the latter three characters may reflect underlying differences in the biosynthesis of the aliphatic mandibular components.

New analyses have confirmed that the cavity-nesting species are more recently derived than the open-nesting species (Alexander, 1991; Dyer, 1991). Our results demonstrate that the ancestral bees had a simpler queen mandibular blend that contained only aliphatic components and that the aromatic components were added later in the evolutionary history of the genus. Furthermore, the cavity-nesting species have larger queen/worker differences than the open-nesting ones, suggesting that increased queen/worker differentiation in mandibular gland components and their corresponding functions in the colony have been a significant aspect of evolution within the genus *Apis*. While the mandibular gland characters described in this paper are not sufficient for a cladistic analysis, they should be included with other independent characters in further studies of *Apis* phylogeny.

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DO SCLERACTINIAN CORALS ENGAGE IN CHEMICAL WARFARE AGAINST MICROBES?

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Abstract—Corals are constantly exposed to ubiquitous microbes. Detrimental effects of microbes on corals include surface fouling and disease. To prevent fouling and disease, corals need to resist microbial colonization and invasion. One way that this could be achieved is by chemical defense. Extracts from 100 scleractinian coral species (44 genera and 13 families) were screened for antimicrobial activity against seven microbe species (*Alteromonas rubra, Photobacterium damsela, Vibrio harveyi, Vibrio alginolyticus, Vibrio parahaemolyticus, Synechococcus* sp., and *Staphylococcus aureus*). Activity against *Synechococcus* sp. (a marine cyanobacterium) was recorded in 100 coral species, and eight of these coral species also inhibited the growth of marine bacteria. The extent of microbial colonization on coral surfaces was assessed in 20 scleractinian species to test the hypothesis that fewer microbes occur on corals that have antimicrobial compounds. Bacterial counts exceeded cyanobacterial counts on coral surfaces, and coral species with antibacterial activity had the fewest bacteria on their surfaces. Thus, corals with less heavily colonized surfaces chemically inhibit microbial colonization.

Key Words—Antimicrobial activity, chemical defense, coral extracts, cyanobacterium, marine bacteria, scleractinian.

INTRODUCTION

Corals interact with microbes that impact them in various ways, such as surface fouling, infection, and disease. The first stage of surface fouling begins with microbial colonization of exposed surfaces (Zobell and Allen, 1935; Wahl, 1989). A cyanobacterium, *Phormidium corallyticum*, has been implicated in the

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black band disease of corals (Rützler and Santavy, 1983; Antonius, 1985, 1988). In addition, microbes involved in tissue necrosis reduce the ability of corals to survive other stresses such as sedimentation (Hodgson, 1990). The survival of corals exposed to sedimentation and chemical pollutants is improved when microbial growth on the coral surface is inhibited by antibiotics (Mitchell and Chet, 1975; Hodgson, 1990). This means that the outcome of coral–microbe interactions affects the health and survival of corals. Bacteria and other marine microbes aggregate in coral mucus (Coffroth, 1985, 1990) and are removed when the mucus is shed. Thus mucus production is one way that corals may resist microbial colonization and attack. Another possible mechanism is chemical defense, which involves the use of toxic or biologically active chemicals that either kill or inhibit microbial growth.

There have been numerous studies of the chemical defenses of marine algae, sponges, ascidians, marine mollusks, gorgonians and soft corals (see reviews by Bakus et al., 1986; Coll, 1992; Paul, 1992; Pawlik, 1993), but there are few equivalent studies on hard corals (Order: Scleractinia). Aggressive competitive interactions occur commonly among scleractinians. These may involve greatly elongated sweeper tentacles and polyps, extracoelenteric digestion by mesenterial filaments, and smothering by mucus secretions containing large quantities of nematocysts (Lang and Chornesky, 1990). These defenses are generally classified as structural because the interactions are mediated by structures (mucus secretion excepted), but they also involve chemical mechanisms (e.g., digestion by enzymes, toxins from nematocysts). Indeed, the possibility that hard corals engage in chemically mediated interactions either among themselves or towards other reef organisms has been proposed by several authors (Hildemann et al., 1977; Sheppard, 1982; Rinkevich and Loya, 1983; De Ruyter van Steveninck et al., 1988), and there is preliminary chemical evidence for the secretion of bioactive compounds by a scleractinian coral (Koh, 1996). Biologically active compounds present in scleractinians can affect the cardiovascular and central nervous systems of terrestrial mammals (e.g., Kaul et al., 1977; Fujiwara et al., 1982; Gonoi et al., 1986). However, the activity of these compounds against marine organisms and its ecological significance is unknown. Gunthorpe and Cameron (1990a,b) tested extracts from scleractinians for toxicity against a coral, a hydroid, and sea urchin eggs, but did not include marine microbes in their study.

Apart from the study by Burkholder and Burkholder (1985), where an unknown number of scleractinians were tested for activity against marine bacteria (only the identities of three inactive species were reported), marine microbes have not been used in the study of bioactive compounds in scleractinians. Since microbes are ubiquitous in the marine environment, and can have detrimental effects on corals (Zobell and Allen, 1935; Rützler and Santavy, 1983; Antonius, 1988; Wahl, 1989), it is important to understand the interactions between corals

and marine microbes. The aim of this study was to determine whether scleractinian corals can employ bioactive chemicals in their interactions with marine microbes by assessing the antimicrobial properties of coral extracts. To do so, I carried out a large-scale survey of scleractinian corals for chemicals that inhibit the growth of marine microbes. Antimicrobial activity levels were then compared with the degree of microbial fouling on coral surfaces. I predicted that coral species with antimicrobial properties would have fewer microbes on their surfaces.

METHODS AND MATERIALS

Coral Extract Preparation. Coral samples were collected from three locations (Davies Reef, Orpheus Island, and Lizard Island) on the Great Barrier Reef, Australia, between May 1993 and March 1994. The corals were identified according to taxonomic descriptions of the scleractinian corals in the geographic region (Veron and Pichon, 1976, 1980, 1982; Veron et al., 1977; Veron and Wallace, 1984; Veron, 1986). One hundred species of corals from 44 genera and 13 families were sampled. For each species, five specimens were collected, each from a different colony. Specimens (approximately 10–60 g each depending on degree of calcification) were frozen immediately after collection and thawed prior to extraction in ethanol. (Ethanol has low toxicity and is routinely used for preparing extracts in large-scale screening for bioactive compounds.) Extraction was carried out overnight under refrigeration to minimize denaturation of chemicals. The extracts were then filtered, the solvent was removed under vacuum, and the extracts freeze-dried. Each specimen was extracted separately, and all the extracts were screened individually against seven microbes.

Bioassay Organisms. The seven microbes selected as bioassay organisms were *Alteromonas rubra, Photobacterium damsela, Vibrio harveyi, V. alginolyticus, V. parahaemolyticus, Synechococcus* sp., and *Staphylococcus aureus.* The first six species are marine (all isolated from Australian waters) and are therefore likely to be encountered by the corals screened in this study. The seventh, *S. aureus*, is terrestrial and was included to provide a basis for comparison with the other major work on bioactivity in scleractinian corals (Gunthorpe, 1990), which did not test any marine microbes. *Alteromonas rubra* was isolated previously from a coral (Streiner, 1990), but its pathological significance is unknown. *Photobacterium damsela* and *V. harveyi* are both pathogens of fish, crustaceans, and shellfish (Fouz et al., 1992; Muir, 1991; Sutton and Garrick, 1993) and are frequently found in wounds of marine organisms. *Vibrio alginolyticus* and *V. parahaemolyticus* occur in bacterial films that develop on marine substrates (Kaneko and Colwell, 1975; Belas and Colwell, 1982), and they may play a role in the initial stages of surface fouling. *Synechococcus* spp. are

common cyanobacteria found in seawater and marine sediments (Glover, 1985). They are not known to be pathogenic to marine organisms, but a species with hemolytic toxins was isolated from a site of mass fish mortality (Mitsui et al., 1989).

Measurement of Bioactivity. The standard disc diffusion assay (Acar, 1980) was used for assessing antimicrobial activity of coral extracts. To survey a large number of coral species, screening was limited to one concentration per extract. Appropriate amounts of extracts were weighed and redissolved in 80% ethanol for incorporation into paper discs at 500 μg/disc (Whatman, 6 mm diameter) which were then air-dried. The dosage of 500 μg/disc was also employed in Gunthorpe's (1990) work on scleractinian antimicrobial activity and was used here to facilitate comparison of our results. Ongoing work indicates that the natural concentration of ethanol-soluble compounds in coral tissue is greater than the amount tested in this study (unpublished data). Controls for the effects of solvent (80% ethanol) and salt residues from seawater were included. Solvent control discs contained only the equivalent amount of solvent; salt control discs contained 500 μg of the salt residue of freeze-dried seawater dissolved in 80% ethanol. Both types of control discs were air-dried. The discs were placed on agar media that had been seeded with monocultures of microbes. The following culture media were used: modified luminous agar medium (Streiner, 1990) in the bioassays involving *V. parahaemolyticus, V. alginolyticus, V. harveyi*, and *P. damsela; Alteromonas* agar medium (Streiner, 1990) for *A. rubra* bioassays; a cyanobacterial medium, MN + B_{12} agar medium (Waterbury and Stanier, 1978), for *Synechococcus* sp. bioassays; nutrient agar (Oxoid, Unipath Ltd., Hampshire, England) for *S. aureus* bioassays. All the microbes were incubated at room temperature (approximately 25°C), the recommended temperature for the marine microbes (Streiner, 1990). In addition, *Synechococcus* (an autotrophic cyanobacterium) was incubated under continuous light. Activity was measured by the width of the zone around each disc where microbial growth was inhibited. Measurements were taken as soon as the microbes had formed a confluent lawn on the medium. Activities of coral extracts were compared to activities of standard antibiotics by testing the marine microbes (all gram negative), and *S. aureus* (gram positive) against suites of standard antibiotics suitable for the respective groupings (Table 3 below). Additional extracts from other marine invertebrates belonging to five phyla were screened against *Synechococcus* (at 500 μg/disc) to verify that this species of microbe was not a hypersensitive organism, even though it seemed particularly sensitive to coral extracts. None of the bioassay organisms were inhibited by the control discs.

Enumeration of Microbes on Coral Surfaces. To test the hypothesis that microbes are less numerous on the surfaces of corals with antimicrobial activity, I counted the number of bacteria and cyanobacteria on the surfaces of 20 species of corals, each represented by five specimens from different colonies. Specimens

were rinsed with sterile seawater and surface microbes were dislodged by scraping with a sterile scalpel blade. The microbes were suspended in 10 ml of sterile seawater and counted using a modification of the standard spread plate method (US EPA, 1987). Suspensions of microbes were thoroughly mixed, serially diluted, and known aliquots of each dilution were spread evenly on plates of agar media. Modified luminous agar (Streiner, 1990) was used for counting bacteria. The amount of agar in the medium was increased to 3.5% to restrict the coalescence of swarming colonies of bacteria so that individual colonies could be easily distinguished. MN+B_{12} agar medium (Streiner, 1990) was used for counting cyanobacteria. The plates were incubated at room temperature (approximately 25°C). Bacterial colonies on each plate were counted daily until no more colonies appeared. Cyanobacterial colonies grew more slowly and were counted weekly until no new colonies appeared. The surface areas of each coral specimen was calculated as the sum or surface areas of geometrical shapes that made up the specimen. The microbes on five pieces of rubble were also enumerated for comparison with the coral species.

Statistical Analyses. Confidence intervals ($\alpha = 0.05$) were calculated for all the inhibition zones to determine whether they were significantly greater than zero. Data from the *Synechococcus* sp. and *S. aureus* bioassays were further analyzed statistically. For these two species, a nested ANOVA (specimens nested within coral species) was carried out to determine the amount of variability at each level. Variance components were calculated for each level. For comparison purposes, these variance components can be expressed as percentages of the sum of the total variance (Sokal and Rohlf, 1981). Regression analysis was used to determine if activity against *Synechococcus* was related to the density of cyanobacteria on coral surfaces.

RESULTS

There were consistent taxonomic patterns in the bioactive responses of the 100 species of scleractinians. Most did not show activity against bacteria, but all 100 coral species demonstrated some level of antimicrobial activity against the cyanobacterium *Synechococcus*, with a mean inhibition zone of 7.7 ± 0.4 mm (Table 1). Some coral species also showed activity against the other microbes. Overall, the magnitude of activity against most of the microbes was low, with mean inhibition zones of 1–2 mm. The number of coral species that were able to inhibit bacterial growth varied from zero for *V. parahaemolyticus* to 27 for *S. aureus* (Table 1).

Activity against Marine Bacteria. Extracts from most of the coral species showed no activity against the marine bacteria. Significant activity was detected in six coral species (*Tubastraea faulkneri, T. micrantha, T. diaphana, Tubas-*

TABLE 1. INHIBITION OF MICROBIAL GROWTH BY EXTRACTS OF CORALS[a]

Bioassay organisms	Number of active coral species (out of 100)	Mean width of inhibition zone ± 1 standard error (mm)
Vibrio parahaemolyticus	0	0
Vibrio alginolyticus	2	1.5 ± 0.5
Vibrio harveyi	2	1.5 ± 0.1
Photobacterium damsela	5	1.3 ± 0.2
Alteromonas rubra	5	1.0 ± 0.2
Synechococcus sp.	100	7.7 ± 0.4
Staphylococcus aureus	27	1.8 ± 0.2

[a] Active coral species were those with at least one extract that inhibited microbial growth. Controls had zero inhibition.

traea sp., *Turbinaria peltata*, and *Porites lichen*, Figure 1). Five of these (*Tubastraea* spp. and *T. peltata*) belong to the family Dendrophylliidae. These five were all active against *P. damsela*. Activity against *A. rubra* was significantly greater than zero in three species, including two dendrophylliids (*T. faulkneri*, *Tubastraea* sp., and *P. lichen*). *Tubastraea faulkneri* and *P. lichen* extracts were active against two other species of marine bacteria, *V. alginolyticus* and *V. harveyi*, but activity of *P. lichen* against *V. alginolyticus* was marginal. The activity of extracts from *T. faulkneri* was consistently higher than that of the other coral species (Figure 1). *Tubastraea faulkneri* extracts also inhibited more microbes than any other coral species (six of seven). Extracts of *P. lichen* inhibited four of the seven microbes. None of the coral extracts showed any measurable activity against *V. parahaemolyticus*. Activities of coral extracts against marine microbes were less than half the activities demonstrated by the most potent antibiotics but were comparable to or exceeded the activities of the other antibiotics (Table 3 below).

Activity against Cyanobacterium Synechococcus *sp.* Of the 100 species that inhibited the growth of *Synechococcus* sp., the inhibition zones of 93 were significantly different from zero. The most active species were *Acropora verweyi* and *Astreopora myriophthalma* with mean inhibition zones of 37.2 ± 0.2 mm and 36.7 ± 0.8 mm respectively (Table 2). At the family level, activity (calculated as the mean of all the species in a family) was highest in Acroporidae (15.9 ± 0.5 mm), and lowest in Mussidae (1.7 ± 0.1 mm, Figure 2). The range of activities in the coral extracts was comparable to that of all but two standard antibiotics (Table 3). Interspecific differences in activity accounted for 94.46% of the total variation, whereas the variation within each coral species contributed only 3.21% (Table 4), i.e., the level of activity varied widely among

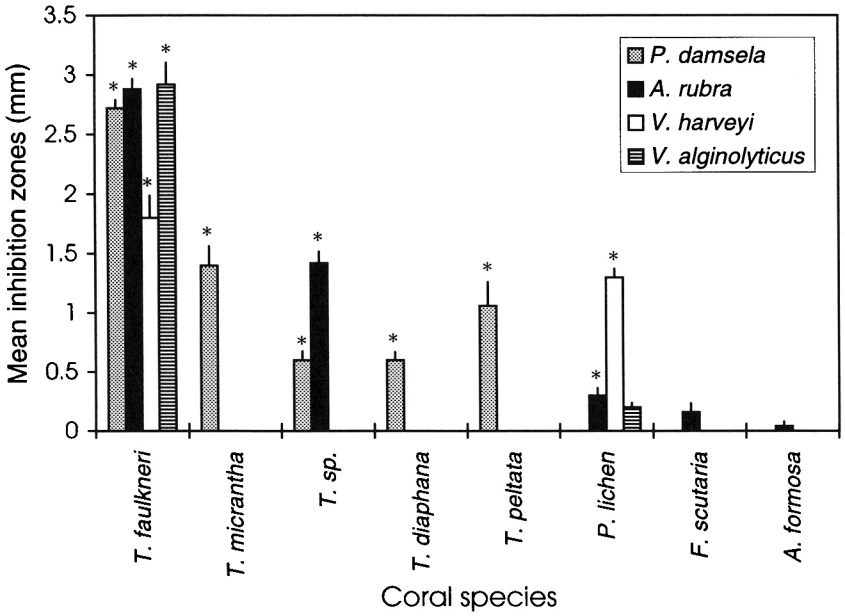

FIG. 1. Mean antimicrobial activity of extracts from corals against four species of marine bacteria, measured as the width of a zone devoid of microbial growth (inhibition zone) around a paper disc containing 500 μg of coral extract. Error bars represent standard errors (N = 5 colonies per coral species). *Widths significantly different from zero.

species but was consistent within each species. To rule out the possibility that the ubiquitous response of corals against *Synechococcus* was the effect of a hypersensitive bioassay organism, extracts from other marine invertebrates were tested against it. Activity of extracts from two species each of ascidians, bryozoans, gorgonians, sponges, and gastropods ranged from zero (gastropods and ascidians) to a maximum of a 5.0-mm inhibition zone in a gorgonian *Briarium* sp. (Table 5). This was much lower than the range of activities demonstrated by coral extracts (Table 2) and indicates that *Synechococcus* is not hypersensitive.

Activity Against Staphylococcus aureus. Significant activity against the terrestrial bacterium *S. aureus* was detected in extracts from 11 of the 100 coral species (Figure 3). There was a wide range of responses among the corals. The largest inhibition zone was 10.8 $\pm$ 0.3 mm for *Acropora microphthalma* but most were less than 5 mm (Figure 3). Variation in activity between coral species accounted for 73.59% of the total variation, and variation within coral species was 21.20% (Table 6).

TABLE 2. ANTIMICROBIAL ACTIVITY OF 100 CORAL SPECIES AGAINST MARINE CYANOBACTERIUM *Synechococcus* sp.[a]

Species	Mean	SE	Species	Mean	SE
Family Acroporidae			**Family Caryophylliidae**		
Acropora aspera	18.8	2.3	*Euphyllia divisa*	5.4	0.7
Acropora austera	16.5	1.2	*Euphyllia glabrescens*	2.4	0.2
Acropora cuneata	2.7	0.8	*Physogyra lichtensteini*	2.8[b]	1.1
Acropora cytherea	28.0	1.0	**Family Dendrophylliidae**		
Acropora digitifera	28.6	1.3	*Tubastraea diaphana*	8.3	0.7
Acropora elseyi	31.1	0.9	*Tubastraea faulkneri*	9.3	0.2
Acropora florida	1.9	0.3	*Tubastraea micrantha*	10.6	0.9
Acropora formosa	0.8[b]	0.3	*Tubastraea* sp.	4.7	0.5
Acropora gemmifera	28.8	1.1	*Turbinaria peltata*	10.6	0.9
Acropora grandis	32.5	1.5	*Turbinaria reniformis*	0.8[b]	0.4
Acropora humilis	12.1	0.4	**Family Faviidae**		
Acropora hyacinthus	30.5	1.3	*Caulastrea furcata*	1.3	0.1
Acropora longicyathus	17.3	1.9	*Diploastrea heliopora*	2.3	0.3
Acropora loripes	2.4	0.7	*Echinopora gemmacea*	3.8	0.5
Acropora microclados	4.7	0.8	*Echinopora horrida*	1.0	0.3
Acropora microphthalma	24.9	2.4	*Echinopora lamellosa*	3.1	0.6
Acropora millepora	23.4	1.3	*Echinopora mammiformis*	1.5	0.2
Acropora nana	27.0	2.8	*Favia lizardensis*	3.8	0.9
Acropora palifera	2.8	0.7	*Favia pallida*	3.0	0.7
Acropora robusta	3.0	0.6	*Favia stelligera*	3.4	0.1
Acropora samoensis	32.4	1.0	*Favites abdita*	1.7	0.3
Acropora sarmentosa	3.0	0.4	*Favites halicora*	3.7	0.2
Acropora tenuis	28.6	2.4	*Goniastrea palauensis*	3.6	0.4
Acropora valida	4.3	0.2	*Goniastrea retiformis*	6.2	1.8
Acropora verweyi	37.2	0.2	*Leptoria phrygia*	1.2	0.2
Anacropora puertogalerae	6.0	0.4	*Oulophyllia crispa*	0.8[b]	0.3
Astreopora myriophthalma	36.7	0.8	**Family Fungiidae**		
Montipora aequituberculata	7.2	0.7	*Fungia echinata*	1.5	0.2
Montipora foliosa	7.1	1.0	*Fungia paumotensis*	1.5	0.1
Montipora hispida	2.8	0.3	*Fungia scutaria*	2.8	0.6
Montipora undata	2.8	0.7	*Heliofungia actiniformis*	1.2	0.2
Montipora verrucosa	2.8	0.6	*Herpolitha limax*	5.9	1.2
Family Agariciidae			*Podabacia crustacea*	3.9	0.2
Leptoseris yabei	4.8	0.8	*Polyphyllia talpina*	2.7	0.2
Pachyseris rugosa	3.5	0.3	*Sandalolitha robusta*	1.4	0.2
Pachyseris speciosa	6.1	0.7	**Family Merulinidae**		
Pavona cactus	1.1	0.02	*Hydnophora microconos*	1.8	0.3
Pavona decussata	3.5	0.2	*Hydnophora rigida*	3.4	0.1
			Merulina ampliata	0.7	0.1
			Family Mussidae		
			Cynarina lacrymalis	3.2	0.2

TABLE 2. Continued

Species	Mean	SE	Species	Mean	SE
Family Mussidae			*Pocillopora eydouxi*	6.4	0.6
Lobophyllia corymbosa	2.6	0.3	*Pocillopora verrucosa*	7.3	1.0
Lobophyllia hemprichii	1.3	0.2	*Seriatopora hystrix*	5.1	0.3
Lobophyllia pachysepta	0.2[b]	0.1	*Stylophora pistillata*	1.1	0.3
Scolymia vitiensis	0.6[b]	0.2	**Family Poritidae**		
Symphyllia recta	2.4	0.2	*Alveopora catalai*	15.6	0.7
Family Oculinidae			*Alveopora verrilliana*	3.4	0.2
Acrhelia horrescens	3.9	0.1	*Goniopora columna*	3.0	0.3
Galaxea astreata	2.7	0.4	*Goniopora djiboutiensis*	0.1[b]	0.1
Family Pectiniidae			*Goniopora lobata*	1.1	0.3
Echinophyllia orpheensis	10.6	1.4	*Goniopora tenuidens*	2.6	0.4
Mycedium elephantotus	5.2	0.8	*Porites cylindrica*	1.5	0.2
Oxypora lacera	10.6	1.1	*Porites lichen*	9.3	1.6
Pectinia alcicornis	8.0	0.2	*Porites rus*	2.9	0.3
Pectinia lactuca	5.5	0.5			
Family Pocilloporidae			**Family Siderastreidae**		
Pocillopora damicornis	1.9	0.5	*Coscinaraea exesa*	7.0	1.3

[a] Measured as the mean width (in mm) of the inhibition zone in five samples. SE = standard error.
[b] Widths not significantly different from zero.

Activity of Coral Extracts versus Standard Antibiotics. The standard antibiotics showed varying levels of activity against all seven microbes (Table 3). The maximum levels of activity exhibited by the antibiotics against bacteria were higher than those of coral extracts, but some of the standard antibiotics did not inhibit microbial growth. Generally, antibacterial activities of coral extracts were comparable to standard antibiotics with mid to lower levels of activity. Activities of standard antibiotics and coral extracts against the cyanobacterium were relatively similar.

Microbial Counts on Coral Surfaces. There were always more culturable bacteria than cyanobacteria on the surfaces of corals (Figure 4). The mean number of bacteria on coral surfaces (5141 $\pm$ 1360 bacteria per cm^2, N = 20) exceeded the mean number of cyanobacteria (13 $\pm$ 5 cyanobacteria per cm^2, N = 20). In general, coral species with antibacterial activity (*T. micrantha, T. faulkneri, P. lichen,* and *Acropora formosa*) had the fewest bacteria (Figure 4), as predicted. The exception was *Pocillopora damicornis*, which did not show antibacterial activity in the bioassays, but had low bacterial counts similar to

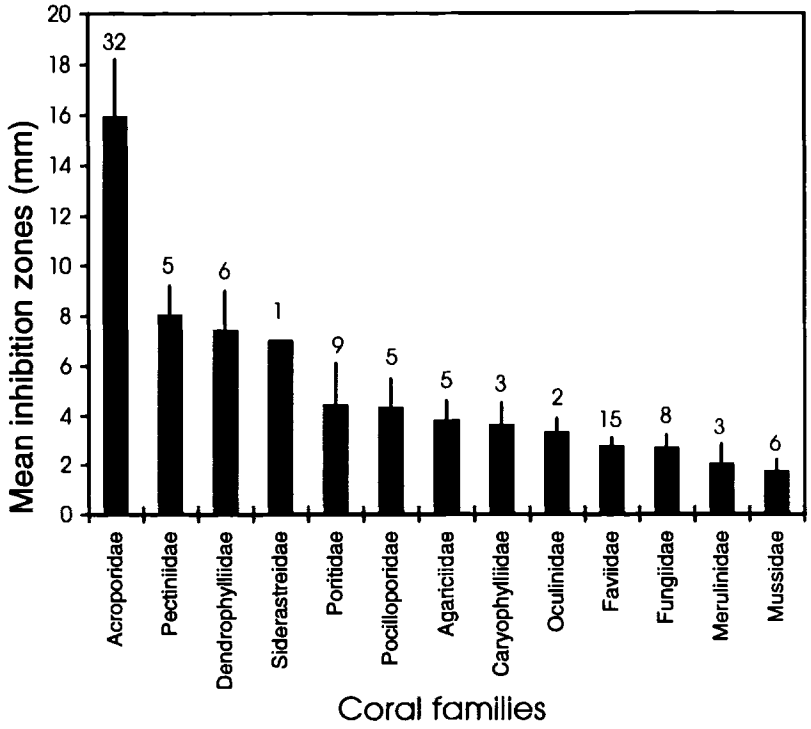

FIG. 2. Mean antimicrobial activity of extracts from corals against the marine cyanobacterium *Synechococcus* sp., expressed as the width of a zone devoid of microbial growth (inhibition zone) around a paper disc containing 500 μg of coral extract. Error bars represent standard errors. Values above each bar are numbers of species for that family.

the above four coral species. Both bacteria and cyanobacteria were most abundant on the surface of rubble. There was no correlation between the levels of activity against *Synechococcus* and the amount of cyanobacteria on coral surfaces ($r^2 = 0.038$, $N = 20$).

DISCUSSION

This study shows that scleractinian corals can use chemical defenses. Extracts from these corals inhibited the growth of a number of microbes and activity against microbes varied tremendously between coral species. *Tubastraea faulkneri* inhibited all but one species of microbe tested. Extracts from all coral

TABLE 3. ACTIVITY OF STANDARD ANTIBIOTICS AGAINST BIOASSAY ORGANISMS[a]

	Gram negative						Gram positive	
Antibiotics	A. r.	P. d.	V. a.	V. h.	V. p.	S. sp.	Antibiotics	S. a.
Amikacin 30	2.0	3.0	3.0	2.0	3.0	1.0	Erythromycin 15	10.0
Ticarcillin 75	5.0	8.5	0.0	0.0	0.0	40.0	Framycetin 100	7.0
Cephalothin 30	0.0	7.5	1.0	4.0	3.5	55.0	Ampicillin 10	9.0
Sulphamethoxazole 25	1.5	4.0	6.0	7.0	6.0	22.0	Penicillin 10	11.0
Neomycin 30	2.5	4.0	4.0	3.5	2.5	2.5	Cloxacillin 5	8.0
Streptomycin 10	0.0	1.0	0.5	0.5	0.5	3.0	Tobramycin 10	5.0
Gentamicin 10	1.5	4.0	3.0	2.5	2.0	1.0	Gentamicin 10	5.5
Tetracyclin 30	6.0	6.5	2.0	3.0	2.0	10.0	Tetracyclin 30	8.5

[a]Expressed as the width (in mm) of a zone devoid of microbial growth (inhibition zone) around each antibiotic disc. The names of the microbes are abbreviated as follows: A. r., *Alteromonas rubra*; P. d., *Photobacterium damsela*; V. a., *Vibrio alginolyticus*; V. h., *Vibrio harveyi*; V. p., *Vibrio parahaemolyticus*; S. sp. *Synechococcus* sp.; S. a., *Staphylococcus aureus*.

species sampled inhibited growth of the cyanobacterium *Synechococcus* sp. and there were fewer cyanobacteria than bacteria on the surfaces of live corals. Corals with bioactive compounds capable of inhibiting bacterial growth also had fewer bacteria on their surfaces than those without antibacterial activity.

The levels of antimicrobial activity reported here are comparable with those in a previous study of the toxicity of scleractinian corals (Gunthorpe, 1990). Both studies used comparable techniques and found that inhibition zones rarely exceeded 5 mm for *S. aureus*, which was the only bioassay organism in common. In the previous study, a coral species was active if it had at least one extract that inhibited any bacteria. The data were not statistically analyzed to determine whether the amount of inhibition was significantly different from zero. By that definition of activity, a greater proportion (27%) of the coral species

TABLE 4. VARIANCE COMPONENTS FOR ACTIVITY OF CORAL EXTRACTS AGAINST *Synechococcus* sp.

Variance source	Mean square	Variance component	Percent of total
Species	2289.17	90.86	94.46
Specimens	17.67	3.09	3.21
Error	2.24	2.24	2.33
Total	95.31	96.19	100.00

TABLE 5. ACTIVITY OF EXTRACTS FROM FIVE INVERTEBRATE PHYLA TESTED AGAINST *Synechococcus* sp.

Phylum	Test organism	Width of inhibition zone (mm)
Mollusca	dorid nudibranch sp. 1	0.0
	Pseudovertagus aluco	0.0
Chordata	*Polycarpa* sp.	0.0
	Hypodistoma deerata	0.0
Coelenterata	*Briarium* sp.	5.0
	gorgonian sp. 1	2.0
Porifera	*Stelletta clavosa*	0.5
	sponge sp. 1	2.5
Ectoprocta	bryozoan sp. 1	2.0
	bryozoan sp. 2	2.5

that were screened in this study were active against *S. aureus* compared to the proportion of active species (15%) in the previous study (Gunthorpe, 1990). The prevalence of coral activity was possibly underestimated in the earlier study, as only 20 species from four families (Mussidae, Faviidae, Poritidae, Merulinidae) were sampled, compared to a larger sample of 100 species from 13 families (see Table 2 for list of species and families). However, our findings were not significantly different from each other (χ^2 with Yates' correction = 0.72, P = 0.40). The two studies had eight scleractinian species in common. Three of these were active by the criterion in the earlier study, but did not show any activity in the present one. This could be the result of spatial and/or temporal variation. Both forms of variation in antimicrobial activity have been reported previously in scleractinians (Gunthorpe and Cameron, 1990a).

Interpretation of Bioassay Results. Since neither solvent nor salt controls inhibited microbial growth, all levels of inhibition measured in the coral extracts indicate the presence of antimicrobial activity. The questions that arise are whether the activity is biologically significant and whether "active" corals are able to inhibit microbial growth in situ. The level of activity that is measured in the disc diffusion bioassay is dependent on both the rate of diffusion of extract into agar and the potency of the extract. An extremely potent extract with a slow diffusion rate will appear to have a low level of activity in the bioassay. Diffusion of antimicrobial compounds may not be necessary in the field. The most efficient location for active compounds that resist microbial fouling and infection is on the surface of the coral. If so, then assuming that the natural concentration of the inhibitory or bioactive compounds at the surface are at least equal to the amount found in 500 μg of coral extract, any amount of inhibition would be significant, as the microbes that are in the direct contact with the coral

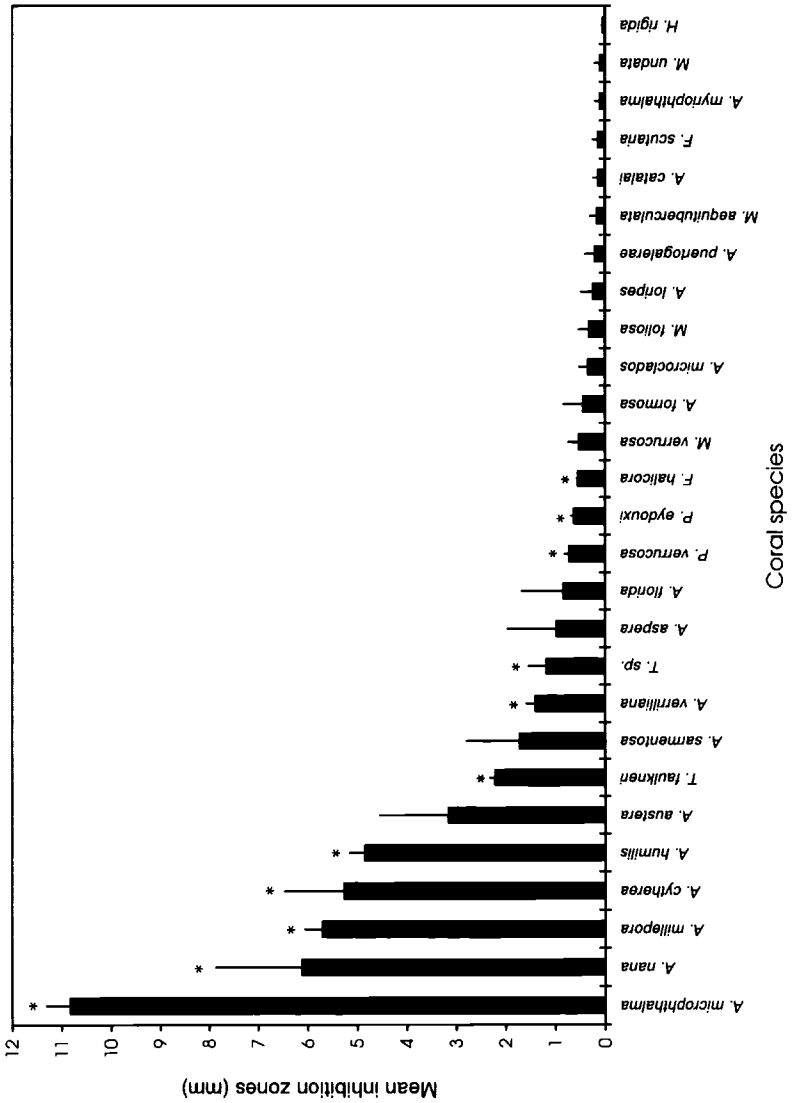

FIG. 3. Mean activity of extracts from corals against the terrestrial bacterium *Staphylococcus aureus*, expressed as the width of a zone devoid of microbial growth (inhibition zone) around a paper disc containing 500 μg of coral extract. Error bars represent standard errors ($N = 5$ colonies for each coral species). *Widths significantly different from zero.

TABLE 6. VARIANCE COMPONENTS FOR ACTIVITY OF CORAL EXTRACTS AGAINST *Staphylococcus aureus*

Variance source	Mean square	Variance component	Percent of total
Species	167.93	6.33	73.59
Specimens	9.57	1.82	21.20
Error	0.45	0.45	5.21
Total	8.37	8.61	100.00

surface would be inhibited. Microbial colonization of a surface begins with an attachment phase (Wahl, 1989), which may be influenced by bioactive compounds in some marine organisms (e.g., Slattery, et al., 1995). It would be of interest to investigate the effectiveness of scleractinian extracts in preventing attachment of microbes. Since attachment is dependent on physical contact with the coral surface, inhibition of attachment is another way that bioactive compounds can limit microbial fouling without diffusing away from the surface.

Implications of Bioactivity in Corals. There were large differences in activity across the coral species. The wide range of interspecific variation in antimicrobial activity shown by the coral species against a particular microbe (94.46% for *Synechococcus* sp. and 73.59% for *S. aureus*, Tables 4 and 6, respectively) may reflect differences in chemical concentration and composition among coral species. Gunthorpe and Cameron (1990a,b), also found that toxicity in scleractinian corals varied greatly among coral species.

The activity of extracts from five species of dendrophylliid corals against *P. damsela* and, especially, the broader spectrum of activity in *T. faulkneri* extracts is significant because bioactive chemicals (aplysinopsin-type compounds) have been isolated previously from dendrophylliid corals, in particular *Tubastraea* and *Dendrophyllia* spp. (Fusetani et al., 1986; Guella et al., 1988). The ecological significance of these bioactive compounds is not known, but Fusetani et al. (1986) postulate that they are a defense against microorganisms. This hypothesis is supported by the antimicrobial activity of *T. faulkneri* extracts in this study. The antimicrobial activity may also confer some advantages in resisting fouling, although bacterial films (microbial fouling) do not necessarily lead to subsequent macrofouling (Maki et al., 1988).

The high prevalence of coral activity against the cyanobacterium *Synechococcus* was striking and cannot be explained by hypersensitivity of the bioassay organism, since other marine invertebrates show either no activity (four of 10 species) or activity well below the maximum shown by scleractinians (compare Tables 2 and 5). This implies that activity against *Synechococcus* may reflect the importance of competitive and pathological interactions between corals and

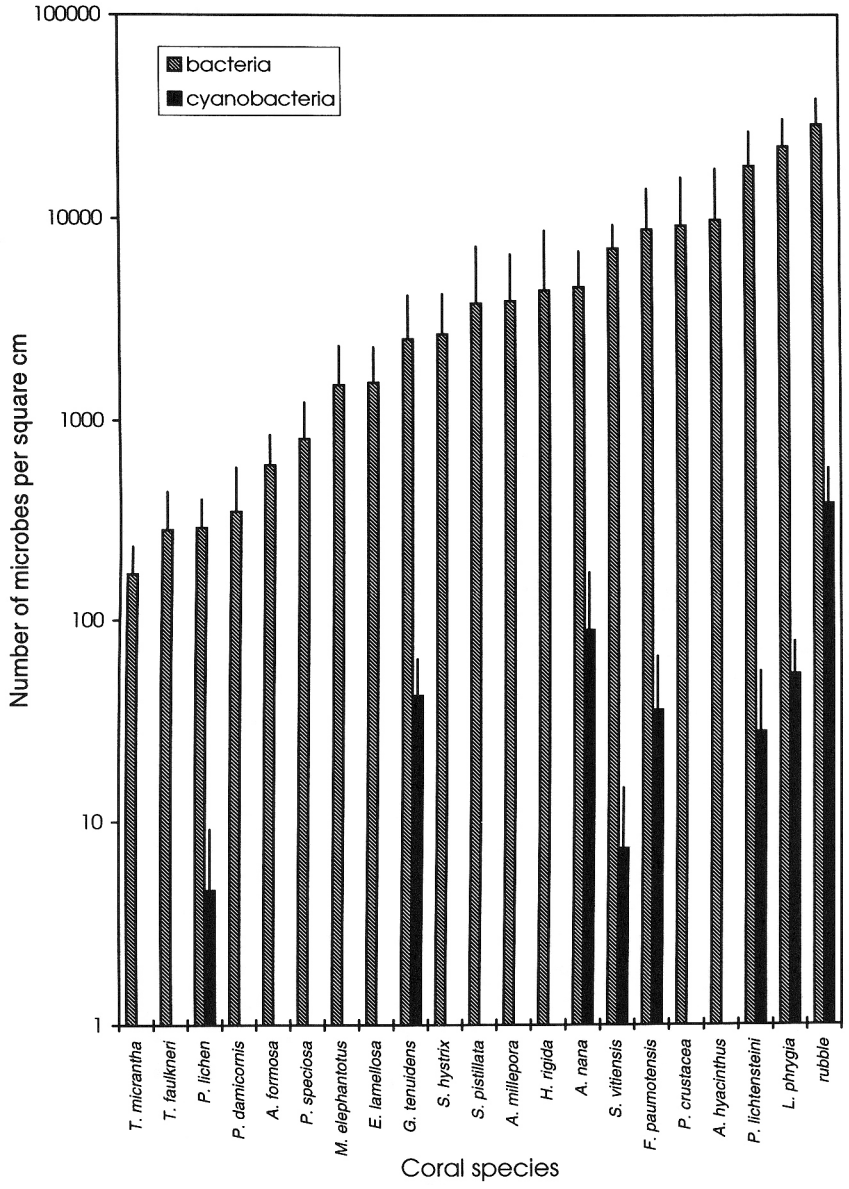

FIG. 4. Counts of bacteria and cyanobacteria on coral surfaces, expressed as mean number of microbes per cm^2 + 1 standard error ($N = 5$ for each coral species and for the rubble).

cyanobacteria. *Synechococcus* species are toxic (Mitsui et al., 1989; D. Griffiths, personal communication), and they have the same cell-wall characteristics as another cyanobacterium, *P. corallyticum*, which is an integral part of the microbial community that causes black band disease in corals (Rützler and Santavy, 1983; Antonius, 1985, 1988). Susceptibility to the disease varies among coral species (Antonius, 1988), which suggests that varying levels of activity against *P. corallyticum* may occur, similar to those against *Synechococcus* sp. *Synechococcus* is autotrophic and its growth on coral surfaces might diminish the photosynthetic resources available to the symbiotic zooxanthellae within corals. If this is true, the widespread occurrence of activity in coral extracts against *Synechococcus* may be a competitive response.

The variability of activity against *Synechococcus* sp. within each species of coral (3.21% of total variation, Table 4) is much lower than the corresponding amount of variation in activity against *S. aureus* (21.20%, Table 6). Assuming that corals produce bacteria-specific rather than general broad-spectrum antibiotics and that the presence and concentrations of these compounds may vary within each species, the higher intraspecific variation in activity against *S. aureus* could be because of a lack of selection for activity against terrestrial microbes in the marine environment. Activity against terrestrial microbes would then be the result of similarities between marine and terrestrial microbes rather than a consistent characteristic of the coral. Only one (*T. faulkneri*) of 100 coral species tested in this study had broad-spectrum activity against microbes (Figure 1). Kim (1994) found that nonmarine bacteria were more sensitive to gorgonian extracts than marine bacteria. Similarly, gram positive bacteria were particularly sensitive to sponge extracts, but little activity was recorded against gram negative bacteria, two of which were unidentified marine species (McCaffrey and Endean, 1985). Even taxa that exhibited "broad-spectrum activity" against a number of microbes did not consistently inhibit the same suite of microbes (Gunthorpe and Cameron, 1990a,b; Amade et al., 1982). Taken together, the results of these other workers and of this study imply that general broad-spectrum antibiotics are the exception rather than the rule in marine invertebrates.

Results of the large-scale survey showed that few coral species possessed antibacterial activity but most had anticyanobacterial activity (Table 1). Therefore, one would expect to find more bacteria than cyanobacteria on the coral surfaces, which was indeed the case (Figure 4). The general rarity and low level of activity in scleractinian corals against marine bacteria, together with the high counts of bacteria on the surfaces of coral species that did not demonstrate antibacterial activity, imply that these species do not employ chemical defenses against marine bacteria. The density of bacteria attached to the surface of corals sampled in this study ($0.17–28.95 \times 10^3$ cells/cm^2) is substantially lower than the counts of bacteria in the mucus of two Great Barrier Reef scleractinians,

which ranged from 5.7 to 12.4 $\times$ 10^5 cells/cm^2 (Coffroth, 1990). Mucus on corals may serve as a barrier that bacteria have to penetrate in order to colonize the coral surface. *Vibrio alginolyticus* grows rapidly on the mucus of a soft coral (*Heteroxenia fuscesens*) and may be able to maintain itself on the surface of the soft coral (Ducklow and Mitchell, 1979). Since the growth of *V. alginolyticus* was not inhibited by most of the coral extracts tested, this could be an indication that it is also able to flourish in the mucus of hard corals. Little is known of the significance of bacteria that occur in coral mucus. The bacteria could be commensals whose presence is tolerated by their coral hosts, or they could be a source of food for the corals (Sorokin, 1973).

Assuming that no two corals have identical suites of active chemical components, testing all coral extracts at a fixed dose of 500 μg/disc provided a standardized means of comparing the bioactivity of the various chemical compounds in corals. It showed which corals have the most active combination of compounds. To determine which corals have the best chemical defenses (i.e., which are the most able to inhibit microbial growth) in the field, the natural concentrations of these compounds need to be determined. I have found that overnight extraction in ethanol removes 25–45% of all compounds extractable by that solvent. The concentration of extractable compounds in coral tissue is half to one order of magnitude higher than the dose used in this study (unpublished data). Thus, the results presented here may represent a conservative estimate of the bioactivity in scleractinians. The actual ability of a coral to prevent microbial growth and infection depends on the potency of its chemicals and their availability. Corals with highly active compounds may only have small amounts of these compounds and consequently be no better at antimicrobial warfare than corals with enormous amounts of less potent compounds. Determining the natural concentrations of these compounds would give a better indication of the chemical arsenal that coral species have at their disposal.

Having shown that scleractinian corals can use chemical defenses against certain microbes and that coral surfaces have fewer cyanobacteria than bacteria, this study poses several important questions. How do the corals use their bioactive compounds to prevent microbial growth and infection? Do the compounds inhibit microbial attachment? Are the compounds released into the mucus or are they sequestered in special cells in the epidermis? What are the actual concentrations of these compounds? Does the widespread activity against *Synechococcus* sp. extend to other species of cyanobacteria? The species that has been implicated in black band disease of corals was not available at the time of study, but has since been isolated from diseased corals of the Great Barrier Reef. Corals are being screened for activity against this microbe. As the number of microbes tested increases, so will our knowledge of the extent and importance of antimicrobial activity in corals.

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ALLELOCHEMICAL ACTIVITIES OF PYRROLIZIDINE ALKALOIDS: INTERACTIONS WITH NEURORECEPTORS AND ACETYLCHOLINE RELATED ENZYMES

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Abstract—Thirteen pyrrolizidine alkaloids (PAs) 3′-acetylheliosupine, echihumiline, echihumiline *N*-oxide, echimidine, heliosupine, heliosupine *N*-oxide, heliotrine, monocrotaline, pycnanthine, retronecine, riddeline, senecionine, and seneciphylline) were analyzed for their interactions with acetylcholine-related enzymes, such as acetylcholine esterase (AChE), butyrylcholinesterase (BChE), choline acetyl transferase (ChAT), and neuroreceptors, such as α_1- and α_2-adrenergic, nicotinergic (nACh), muscarinergic (mACh) and serotonin$_2$ (5-HT$_2$) receptors. Whereas most PAs did not affect the enzymes, they show significant binding activities to mACh and 5-HT$_2$ receptors: Twelve PAs exhibited a 50% inhibition of the specific binding of the radioligand [^{3}H]quinuclidinyl benzilate (QNB) at the mAChR, i.e., IC_{50} values were between 8.7 μM and 512.5 μM, and 10 PAs exerted a 50% inhibition of the specific binding of the radioligand [^{3}H]ketanserine at the 5-HT$_2$R with IC_{50} values between 23.2 μM and 608.6 μM. The most active compound was 3′-acetylheliosupine, which was able to bind to all of the studied receptors with IC_{50} values in the range between 2.9 μM and 159.7 μM. The data imply that free PAs and PA *N*-oxides can affect several molecular targets: Besides long-term toxicity through DNA alkylation (by PA metabolites generated in the liver), liver and pneumotoxicity, neuroreceptors (among other molecular targets) may be modulated. The interference of PAs with neuronal signal transduction could mediate adverse physiological responses in herbivores and could thus contribute to chemical defense in plants and animals against herbivores and predators.

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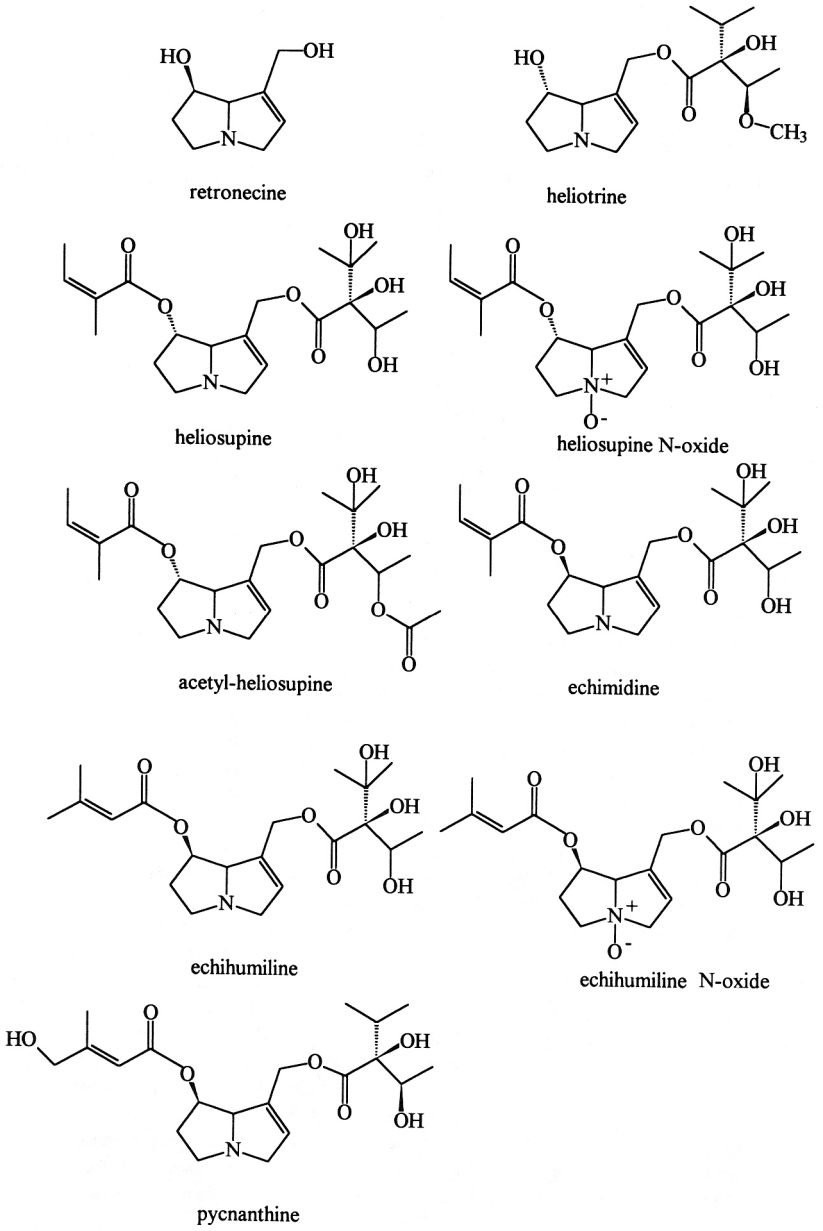

FIG. 1. Structures of pyrrolizidine alkaloids employed in the receptor study.

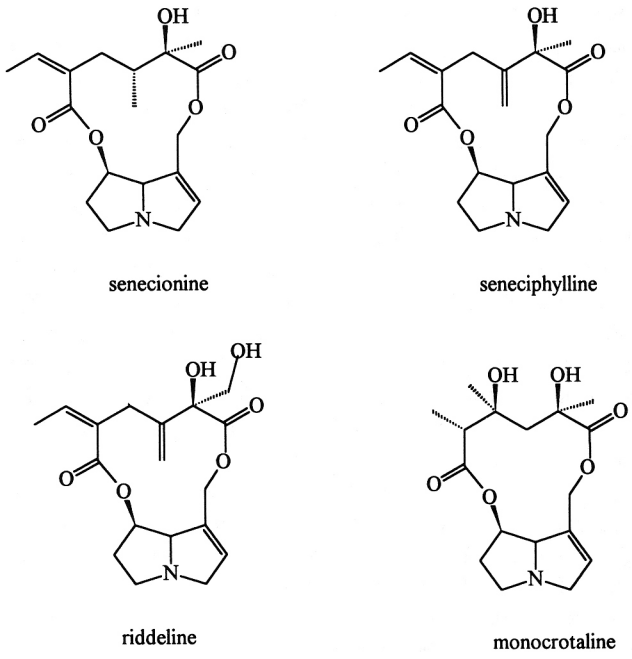

FIG. 1. Continued.

Key Words—Pyrrolizidine alkaloids, pyrrolizidine *N*-oxides, cholinergic, adrenergic, serotonin receptors, BChE, AChE, ChAT, toxicity, pharmacology, chemical defense, radioreceptor assay, neuroreceptors.

INTRODUCTION

Pyrrolizidine alkaloids are widely distributed within four plant families, especially the Boraginaceae, Asteraceae, Orchidaceae but also the Leguminosae. In addition, they occur sporadically in other families, such as Apocynaceae, Poaceae, and Ranunculaceae (Hartmann and Witte, 1995; Mattocks, 1986). More than 370 structures have been reported from about 560 plants in which they are usually accumulated as *N*-oxides (Hartmann and Witte, 1995; Roeder, 1995). PAs are ester alkaloids, consisting of a necine base (i.e., amino alcohols, mostly 1,7-diols) and also one or two acids, the so-called necine acids, resulting in circular (see senecionine) or open-chain (see heliosupine) structures (Figure 1). Because of many combinations of differing necine acids, a large number of pyrrolizidine structures are possible.

PAs are toxic to man and livestock. The most important toxicological feature is their hepatotoxicity. Symptoms of the so-called venoocclusive disease include weakness, increase of pulse and breathing frequency, colic, and swelling of the liver (Mattocks, 1986; Roeder, 1995). A pronounced pneumotoxicity also has been reported (Schultze and Roth, 1993; Wilson et al., 1992). Furthermore, PAs exhibit substantial mutagenicity, carcinogenicity, embryotoxicity, and a weak virustatic and antileukemic activity. These effects are results of a toxification reaction in the liver. Liver enzymes convert PAs to reactive pyrrols with exomethylene groups, which are able to bind at several cellular targets such as DNA (Habs et al., 1982; Hincks et al., 1990; Miser et al., 1992; Peterka et al., 1994; Pool, 1982; Taylor et al., 1992; Wagner et al., 1993; Wiessler, 1994). In addition, some derivatives are used pharmaceutically. Platynecine has been employed as an antimuscarinic agent (Atal, 1978) and semisynthetic derivatives of PAs exhibit hypotensive, local anesthetic, ganglionic blocking, neuromuscular blocking, and antispasmodic activities (Atal, 1978).

Since grazing cattle and sheep usually avoid *Senecio* and other PA-rich plants and because of the well-documented toxicological phenomena, it has been argued frequently that PAs serve as chemical defense compounds for plants producing them (Boppré, 1995; Brown and Trigo, 1995; Wink, 1993). In addition, there is good experimental evidence that PAs are feeding deterrents for many nonspecialized insect herbivores (Detzel and Wink, 1993; Brown and Trigo, 1995; Masters, 1991; Wink, 1993).

Some insects, especially moths, butterflies, a few leaf beetles, locusts, and aphids sequester the defense compounds of their host plants and use them for their own protection against predators (reviews Bernays and Chapman, 1994; Brown and Trigo, 1995; Harborne, 1993; Rosenthal and Berenbaum, 1991, 1992; Wink, 1993). Due to the pioneering work of M. Rothschild, T. Eisner, K. Brown, and D. Schneider and coworkers, the best studied group of secondary metabolites that are widely used as acquired defense compounds in insects are pyrrolizidine alkaloids (for reviews see Boppré, 1990; Brown and Trigo, 1995; Harborne, 1993; Hartmann and Witte, 1995; Rosenthal and Berenbaum, 1991; Rothschild, 1972; Rothschild et al., 1972; Schneider, 1986).

Many PA insects can tolerate lage amounts of PAs without damage (Boppré, 1990, 1995; Brown and Trigo, 1995; Schneider, 1986; Hartmann and Witte, 1995). Most of them store the acquired defense substances and some of them even use PAs as precursors for the biosynthesis of pheromones. In arctiids of the genus *Creatonotos*, PAs influence the development of the coremata (Schneider et al., 1982).

In order to evaluate the molecular activities of alkaloids and their evolutionary and ecological roles, we have established a series of in vitro assays addressing a number of molecular targets such as DNA, RNA and related enzymes, protein biosynthesis, membrane stability, inhibition of acetylcholine-

converting enzymes and neuroreceptors, such as nicotinic and muscarinic acetylcholine receptors (AChR), α_1- and α_2-receptors, and serotonin receptors (Schmeller et al., 1994, 1995, 1997; Wink and Latz-Brüning, 1995; Wink and Twardowski, 1992). In this study we investigated whether a series of 13 PAs (Figure 1) isolated from *Senecio, Echium*, and *Cynoglossum* affect molecular targets such as ACh-converting enzymes and neuroreceptors, which are important in the neuronal signal transduction of animals.

METHODS AND MATERIALS

Alkaloids. 3′-Acetylheliosupine, echihumiline, echihumiline *N*-oxide, echimidine, heliosupine, heliosupine *N*-oxide, pycnanthine, and retronecine were isolated from species of *Cynoglossum, Echium* and *Senecio* (El-Shazly et al., 1996a–d). Riddeline, senecionine, and seneciphylline were kindly provided by R. J. Molyneux; heliotrine and monocrotaline were obtained commercially. The identity of the PAs analyzed was checked with GC, GC-MS, and NMR. The purity was greater than 95% for all compounds.

Membrane Preparation for Receptor Binding Studies. Porcine brains obtained within 30 min after the death of the animals from a local slaughterhouse were used to prepare receptor-rich membranes. The brains were immediately frozen in liquid nitrogen, and 50 g brain tissue per 200 ml ice-cold buffer (0.32 M sucrose, 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA) were homogenized twice for 15 sec in a blender and then for 1 min with an ultraturrax. The homogenate was centrifuged three times for 15 min at 1400*g* and 4°C to separate cellular debris. The supernatant was spun down at 100,000*g* for 60 min. The resulting pellet was resuspended in buffer (as above but without sucrose). Aliquots were stored frozen at −80°C. Protein content was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard (Schmeller et al., 1994, 1995).

Binding Assays. Binding assays (in triplicate) were performed using a rapid filtration technique essentially as described by Yamamura and Snyder (1974).

Muscarinic Receptor (mAChR). Brain membrane preparations, adjusted to 500 μg protein in a final volume of 500 μl buffer, were incubated with [^{3}H]quinuclidinyl benzilate (QNB) (52.3 Ci/mmol; Dupont NEN) for 1 hr at 20°C in the absence and presence of pyrrolizidine alkaloids. Atropine (20 μM) served as a positive control substance. The incubation was stopped with 3 ml ice cold 0.9% NaCl solution and filtered by suction through Whatman GF/C glass fiber filters. The filters were washed three times with 3 ml 0.9% NaCl, placed in vials, and dried for 30 min at 60°C. Their radioactivity was measured in a liquid scintillation counter (RackBeta, Pharmacia) using Ultima-Gold (Packard) as scintillation cocktail.

Nicotinic Receptor (nAChR). [^{3}H]Nicotine (85 Ci/mmol; Amersham) was used to assay specific binding of PA to the nicotinic ACh receptor (nAChR). The membrane preparation was incubated for 40 min with differing concentrations of PA or 1 mM nicotine as a positive control. The GF/C filters were presoaked with polyethylene glycol 8000 (5% in water) for 3 hr to reduce nonspecific binding of [^{3}H]nicotine. Further procedures were the same as described above for mAChR.

α_1-Receptor. [^{3}H]Prazosine (78 Ci/mmol; DuPont NEN) was used to assay specific binding of PA to the α_1-receptor. The membrane preparation was adjusted to 400 μg in a final volume of 500 μl and incubated for 45 min at 20°C with differing concentrations of PA or 400 μM phentolamine as a positive control. Further procedures were the same as described above for mAChR.

α_2-Receptor. [^{3}H]Yohimbine (81 Ci/mmol; DuPont NEN) was used to assay specific binding of PA to the α_2-receptor. The membrane preparation was adjusted to 400 μg in a final volume of 500 μl and incubated for 30 min at 20°C with differing concentrations of PA or 400 μM phentolamine as a positive control. Further procedures were the same as described above for mAChR.

Serotonin Receptor(5-HT$_2$R). [^{3}H]Ketanserine (85.1 Ci/mmol; DuPont NEN) was used to assay specific binding of PA to the serotonin$_2$ receptor (5-HT$_2$R). The membrane preparation was adjusted to 400 μg in a final volume of 500 μl and incubated for 40 min at 20°C with differing concentrations of PA or 100 μM mianserine as a positive control. Further procedures were the same as described above for mAChR.

Enzyme Assays. The assays (in triplicate) of the esterases were based on a photometric method. Choline acetyltransferase activity was assayed essentially as described by Fonnum (1966, 1975).

Acetylcholine Esterase (AChE). The activity of acetylcholinesterase was measured according to the method of Ellman et al. (1961). We used AChE type VI-S from the electric eel (400 units/mg, Sigma). Different concentrations of PA were incubated at room temperature with butyrylthiocholine iodide (BTC, 18 mM) and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB, 0.75 mM) with 50 μl enzyme solution in a final volume of 300 μl in microtiter plates. The enzyme reaction was stopped with 30 μl physostigmine (20 mM) after about 10 min. The colored product produced was measured at 405 nm in an autoreader (Virion). All solutions were prepared in potassium phosphate buffer (pH 7.2, 0.5 mM).

Butyrylcholine Esterase (BChE). Procedures were the same as described for AChE but butyrylcholine esterase from horse serum (11.6 units/mg, Sigma) was used in the assays.

Choline Acetyltransferase (ChAT). We used choline acetyltransferase from bovine brain (12 units/mg, Sigma) to assay the inhibitory effects of the PAs. Different concentrations of PAs were incubated at 37°C in a waterbath with [^{3}H]acetyl coenzyme A (1 Ci/mmol, DuPont NEN), 25 μl choline (100 mM),

and 50 μl enzyme solution in a final volume of 500 μl. The incubation was stopped with 300 μl trifluoroacetic acid. The resulting [^{3}H]acetylcholine was precipitated with the addition of 1 ml hydroxylamine hydrochloride (1 mM), 1 ml acetylcholine (10 mg/ml), and 1 ml sodium tetraphenyl borate (50 mM) after standing overnight at 4°C. The precipitate was collected on Whatman GF/C glass filters with suction, washed three times with 2 ml ethanol, dried for 30 min at 60°C, and dissolved in 4 ml of a mixture of benzylalcohol and acetonitrile (1 : 1). Radioactivity was measured in a liquid scintillation counter (RackBeta, Pharmacia) with Ultima-Gold as the scintillation cocktail.

RESULTS

Binding of PAs to Neuroreceptors. The affinity of 13 pyrrolizidine alkaloids to neuroreceptors from porcine brain was determined in radioligand assays, i.e., we tested whether a radioligand that specifically binds to a certain neuroreceptor (muscarinic and nicotinic acetylcholine receptor, serotonin receptor, and α_1- and α_2-receptor, respectively) can be displaced by an alkaloid added to the assay mixture. Nearly all PAs evaluated in this study showed affinity to the muscarinic acetylcholine receptor (Table 1, Figure 2) and inhibited specific binding of the radioligand QMB. Echihumiline *N*-oxide was most active and 8.7 μM replaced 50% (= IC_{50} value) of the specifically bound QMB, followed by senecionine (43.0 μM), heliotrine (52.2 μM), and seneciphylline (52.5 μM). PAs were inactive (except 3′-acetylheliosupine) at the nicotinic acetylcholine receptor (Table 1, Figure 2). Another neuroreceptor affected by PAs was the serotonin receptor (5-HT_2R). Ten of the 13 PAs tested inhibited the specific binding of the radioligand [^{3}H]ketanserine with IC_{50} values between 23.2 and 609 μM (Table 1, Figure 2); 3′-acetylheliosupine and heliosupine were the most active alkaloids. Although only 3′-acetylheliosupine and heliosupine affected the α_1-receptor, these two PAs and also echihumiline, echihumiline *N*-oxide, and seneciphylline were active at the α_2-receptor (Table 1, Figure 2).

Inhibition of Acetylcholine-Related Enzymes by PAs. The enzymes involved in the metabolism or synthesis of acetylcholine or butyrylcholine (acetylcholine esterase, butyrylcholine esterase, and choline acetyl transferase) were hardly affected by the PAs tested. Only echihumiline and pycnanthine inhibited the butyrylcholine esterase (Table 1, Figure 2).

DISCUSSION

Pharmacological and Molecular Activities of PAs. In this investigation we have provided evidence that some PAs can displace specific ligands of muscarinic acetylcholine, serotonin, and, to a lesser degree, adrenergic receptors. It is

TABLE 1. INHIBITION OF SPECIFIC BINDING OF RADIOLIGANDS TO NEURORECEPTORS BY PAS OR PA *N*-OXIDES AND INHIBITION OF ENZYMES INVOLVED IN ACETYLCHOLINE METABOLISM[a]

Alkaloid	$Alpha_1$	$Alpha_2$	$5\text{-}HT_2$	mAChR	nAChR	AChE	BChE	ChAT
3′-Acetylheliosupine	39.1	2.9	23.2	71.3	159.7	n.a.	n.a.	n.a.
Echihumiline	n.a.[b]	358.8	549.0	89.2	n.a.	n.a.	314.4	n.a.
Echihumiline *N*-oxide	n.a.	>50	182.0	8.7	n.a.	n.d.[c]	n.d.[c]	n.a.
Echimidine	n.a.	900.6	257.6	512.5	n.a.	n.a.	n.a.	n.a.
Heliosupine	148.1	15.0	77.1	392.0	n.a.	n.a.	n.a.	n.a.
Heliosupine *N*-oxide	n.a.	n.a.	n.a.	350.0	n.a.	n.a.	n.a.	n.a.
Heliotrine	n.a.	n.a.	535.4	52.2	n.a.	n.a.	n.a.	n.a.
Monocrotaline	n.a.	n.a.	203.4	n.a.	n.a.	n.a.	n.a.	n.a.
Pycnanthine	n.a.	n.a.	407.6	177.2	n.a.	n.a.	462.6	n.a.
Retronecine	n.a.	n.a.	n.a.	127.9	n.a.	n.a.	n.a.	n.a.
Riddelline	n.a.	n.a.	n.a.	208.7	n.a.	n.a.	n.a.	n.a.
Senecionine	n.a.	n.a.	249.4	43.0	n.a.	n.a.	n.a.	n.a.
Seneciphylline	n.a.	341.4	608.6	52.5	n.a.	n.a.	n.a.	n.a.

[a] IC_{50} values in μM (concentration of PA that inhibits the specific binding by 50%); nAChR = nicotinic acetylcholine receptor, mAChR = muscarinic acetylcholine receptor, $alpha_1$, $alpha_2$ = adrenergic receptor, $5\text{-}HT_2$ = serotonin receptor; acetylcholine esterase = AChE, butyrylcholine esterase = BChE, choline acetyl transferase = ChAT.

[b] n.a. = not active.

[c] The inhibition of esterase activity with heliosupine *N*-oxide was not determinable, because it caused a chemical reaction with the reagents.

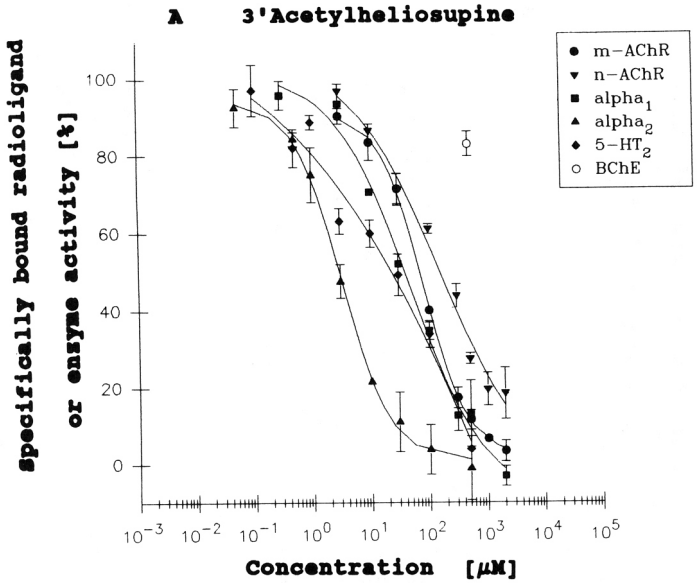

FIG. 2. Dose-response curves for displacement of specifically bound radioligands by PAs at neuroreceptors and inhibition of butyrylcholine esterase. 3′-Acetylheliosupine (A), heliosupine (B), echihumiline (C), echihumiline *N*-oxide (D), and pycnanthine (E) were added to the assay at different concentrations, and it was determined whether and how much the radiolabeled specific ligand was replaced (no displacement = 100%). mAChR = muscarinic acetylcholine receptor; nAChR = nicotinic acetylcholine receptor, $alpha_1$, $alpha_2$ = adrenergic receptor, 5-HT_2 = serotonin receptor. Of the enzymes tested, only butyrylcholine esterase (BChE) was affected by PAs; therefore, inhibition curves are shown for this enzyme only (no inhibition = 100%). Values are means ± SD (N = 3).

remarkable that echihumiline *N*-oxide showed the highest affinity of all PAs tested at the mAChR, whereas the free base was 10 times less active. This suggests that *N*-oxidation creates a quarternary nitrogen as in acetylcholine. Since the pair heliosupine/heliosupine *N*-oxide did not reveal a similar relationship, our evidence for higher activity of PA *N*-oxides is not conclusive at present. However, it is remarkable that PA *N*-oxides, which are the common storage form of PAs in plants and animals, are not inactive (as postulated by Hartmann and Witte, 1995); in contrast, they are pharmacologically active and have been shown to be feeding deterrents in a number of insect predator interactions (Brown and Trigo, 1995; Dam et al., 1995). 3′-Acetylheliosupine had the broadest activity and replaced specifically bound radioligands at all receptors studied. It was remarkable that its affinity was always five times higher than that of the

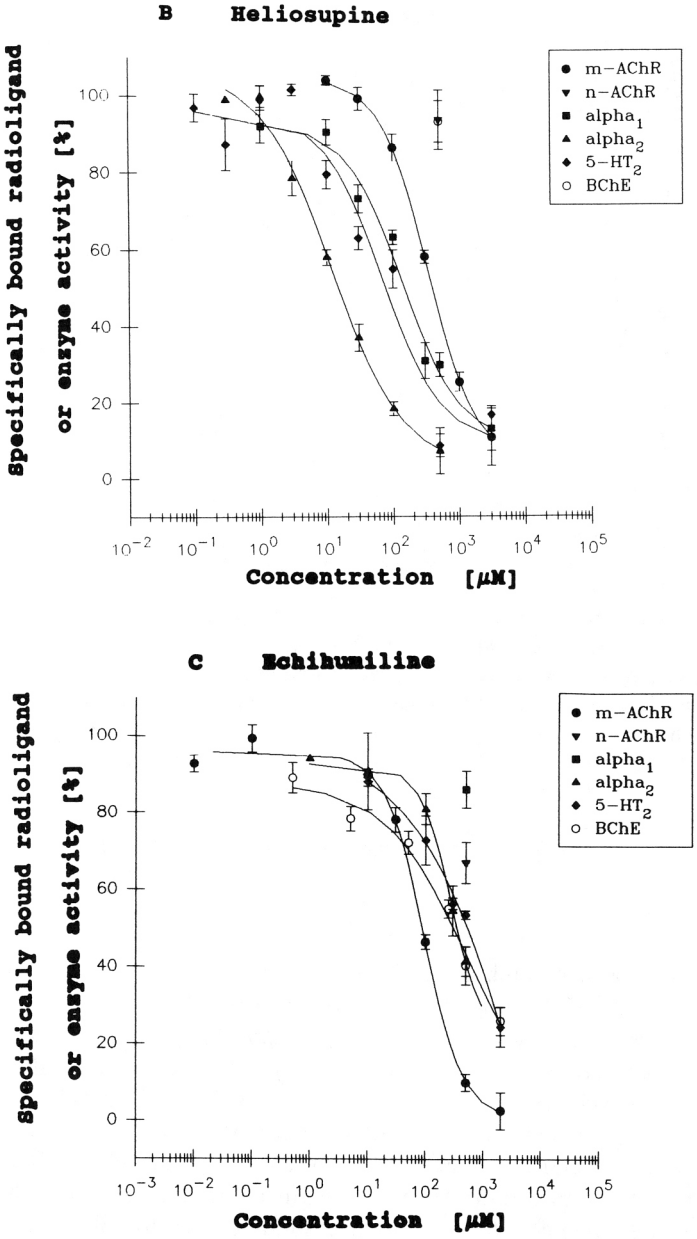

FIG. 2. Continued.

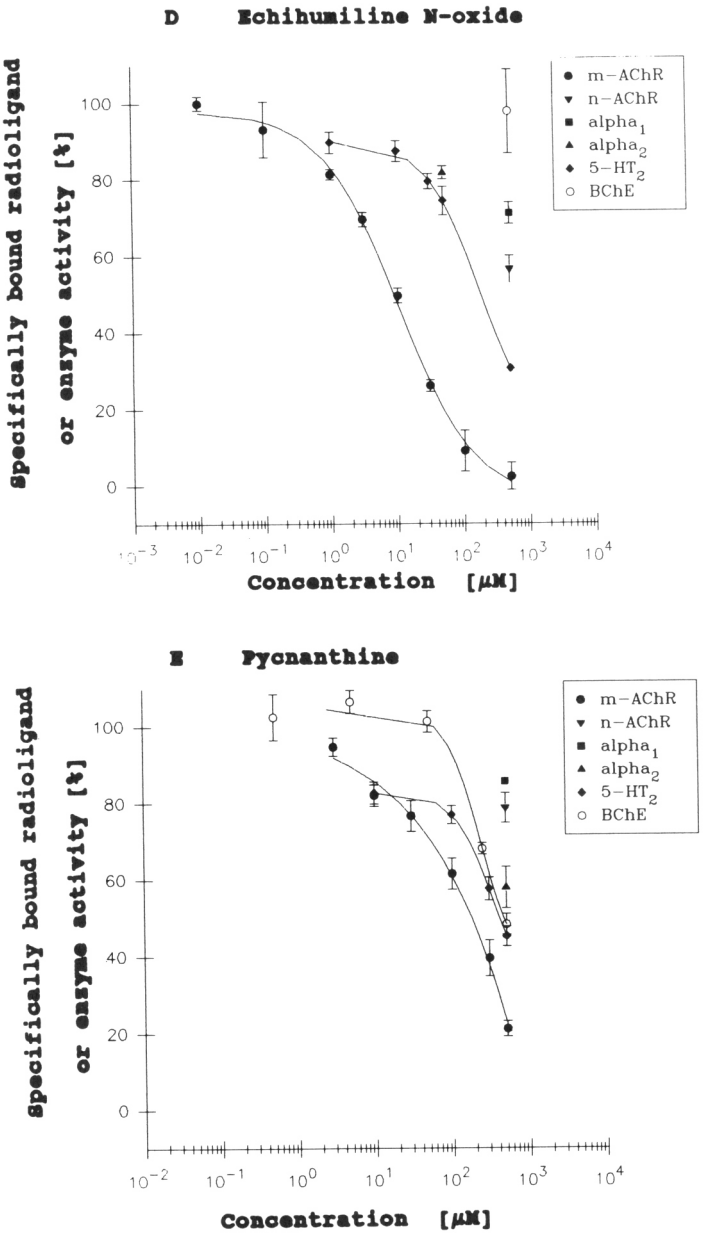

FIG. 2. Continued.

chemically closely related heliosupine at the same receptor. In a previous study we had tested whether the PAs senecionine, riddeline, and heliotrine affect DNA (for example, by intercalation), DNA- and RNA-related polymerases, protein biosynthesis, and membrane stability, but we could not detect any significant activity at these molecular targets (B. Latz-Brüning and M. Wink, unpublished). As had been demonstrated earlier (reviewed in Mattocks, 1986), only the metabolites generated from PAs in the liver have alkylating properties.

How does one correlate the PA binding data with neuropharmacological activities of known alkaloids? Whereas tropane alkaloids are extremely active mAChR antagonists (IC_{50} for atropine or scopolamine binding was 0.005 or 0.002 μM, respectively; Schmeller et al., 1995), other alkaloids with known mAChR activity, such as pilocarpine or arecoline, have binding affinities of IC_{50} 11 or 32 μM, respectively (T. Schmeller and M. Wink, in preparation), which are in the range of the most active PAs, e.g., echihumiline *N*-oxide. Alkaloids with known serotonin receptor activity include ergot and β-carboline alkaloids, such as ergometrine or harmaline. Ergometrine, harmaline, and harmalol have IC_{50} values of 1.5, 14.6 and 30.6 μM, respectively (Schmeller and Wink, in preparation), which are again in the same range as the most active PA studied, 3′acetylheliosupine.

Our receptor assays do not indicate whether the alkaloid–receptor interaction is agonistic or antagonistic. In case of the mAChR interactions, it is likely that PAs are antagonistic, since platynecine (the 1,2-dihydroderivative of senecionine) exhibits an atropine-like spasmolytic activity in animals (Atal, 1978). Furthermore, heliotrine and monocrotaline, which are representative for most PAs studied (Figure 1), were acetylcholine antagonists in assays with guinea pig ileum (Pomeroy and Raper, 1971a,b). Ten of the 13 PAs examined inhibited the specific binding of ketanserine at the serotonin receptor. The observation that lung thrombus occurs in animals after treatment with monocrotaline or its metabolite monocrotaline pyrrole (Chesney et al., 1974; Schultze and Roth, 1993; Turner and Lalich, 1965; Valdivia et al., 1967) suggests that the PA action might be agonistic at 5-HT_2 receptors of thrombocytes. The agonistic action would explain the pneumotoxicity and the increase of arterial pressure in the lung, which was described by several authors (Ghodsi and Will, 1981; Hilliker et al., 1982; Kay et al., 1982; Huxtable, 1979, 1990; Meyrick et al., 1980; Wagner et al., 1993; Wilson et al., 1992). This pharmacological property may be the base for the pharmaceutical use of *Senecio* species as sources of hemostatic drugs (Manstein, 1953, 1959).

Function of PAs as Defense Compounds. Since the majority of 1,2-unsaturated PAs are toxic to man and domestic animals and act as feeding deterrents to many insect herbivores, it has been suggested that PAs serve as defense chemicals for the plants producing them (Boppré, 1990; Brown and Trigo, 1995; Wink, 1993). However, Hartmann and Wite (1995) have argued that PAs do

not have a short-term toxicity and that "it is difficult to see any benefit to a plant damaged by a mammalian herbivore that suffers from the defence compounds only weeks later." Although PAs seem to be a feeding deterrent to grazing mammals and insects, "the complexity of the field situation often precludes a clear demonstration of effectiveness of PAs in plant defence against insects" (Hartmann and Witte, 1995).

The displacement of specifically bound radioligands at the mACh and serotonin receptors implies that short-term effects should exist. The question is whether the inhibitor concentrations determined in our in vitro experiments (Table 1) relate in any way to in vivo conditions. A simple calculation may help to assess this problem. PAs are stored in high concentrations in plants at sites that are important for growth and reproduction and can reach 1–2% of the dry weight (Biller et al., 1994; Dam et al., 1994, 1995; Meier, 1994). For example, suppose that a plant has a PA concentration of 100 mg/100 g fresh weight and that a small herbivore feeding at the plant has a body weight of 1000 g. If this animal ingests 100 g of the plant it would take up 100 mg PAs. If we assume that the alkaloids are completely absorbed and equally distributed in the body of the animal, it would have a concentration of 100 mg PA/kg body weight. If one takes a mean molecular weight of 400 for PAs (e.g., heliosupine has a molecular weight of 397), the PA concentration in the herbivore would be 250 μM, which would be high enough to partially or completely block the binding of acetylcholine or serotonin at their receptors. Further experiments with different herbivore species (which can substantially vary in their susceptibility; Mattocks, 1986) are needed to better understand the dose dependence, kinetics, and short-term toxicity of PAs and PA *N*-oxides.

Since any substantial interference at the muscarinic acetylcholine and serotonin receptors (e.g., when natural ligands cannot bind to their receptors any longer because they are blocked by PAs) will influence neuronal signal transduction, CNS activity, and possibly muscular activity, the intake of a large dose of PAs should lead to short-term (within several hours after ingestion) physiological disturbances. Such rapid effects have been seen in pharmacological experiments with intact animals, as mentioned above. These adverse effects and the bitter taste of PAs should provide a clue to herbivores to avoid these plants in the future through associative learning. The long-term hepatotoxic and mutagenic effects might have an impact as well. Herbivores that feed on PA-rich plants may be less successful in reproduction since they may die prematurely or have offspring with deformations, whereas more "prudent" grazers that have learned to avoid PA-rich diets may have more offspring.

Although it is normally emphasized in textbooks of pharmacology that a certain alkaloid specifically interacts with a particular molecular target, a comparative study showed that usually not just one but several molecular targets are affected by a single alkaloid (Wink, 1993; Wink et al., 1997). For example,

the quinolizidine alkaloids lupanine, sparteine, or cytisine affect either the nAChR and/or the mAChR (Schmeller et al., 1994), but they concomitantly inhibit sodium and potassium channels and may even interfere with protein biosynthesis (Wink and Twardowski, 1992; Wink, 1992, 1993). Since plants accumulate mixtures of QA (Wink et al., 1995), even more targets may be affected, which would explain their general toxicity (Wink, 1992). We have argued that this strategy (i.e., to produce mixtures of natural products and secondary metabolites that affect several targets concomitantly) is advantageous for the plant, since it will be difficult for a herbivore to adapt to this diet, especially if this adaption would require a target site modification at each molecular target that is affected by the secondary compounds in question (Schmeller et al., 1994; Wink, 1993).

PAs could be another example for this phenomenon. Besides an interaction with muscarinic and serotonin receptors present in the brain and other organs, PA metabolites that are generated in vertebrate herbivores in a detoxification reaction (Mattocks, 1986) exhibit mutagenic and carcinogenic properties by affecting targets that are not related and that produce a number of different effects. We have not yet assayed all molecular targets, and it might be possible that additional targets are also involved. Nevertheless, it is remarkable that a number of insects sequester PAs from their food plants and exploit them for their own defense (Boppré, 1990; Brown and Trigo, 1995; Hartmann and Witte, 1995; Rothschild, 1972). How these insects avoid self-intoxication has not been elucidated in detail. It may be that insects avoid the mutagenic effects because they do not convert PAs into the same metabolites as vertebrates do in their liver. In the case of the monarch butterfly, *Danaus plexippus*, which sequesters cardiac glycosides, we have recently shown that their target Na^+/K^+-ATPase has been modified by point mutation, so that cardiac glycosides do not bind (Holzinger et al., 1992; Holzinger and Wink, 1996). Since acetylcholine and serotonin receptors are also important for insects, it could be possible that PA-storing insects have acquired a PA insensitivity through modification of their mAChR or 5-HT_2R. Sequestration of PAs in the integument, as described for *Creatonotos transiens* and other PA-storing moths (Nickisch-Rosenegk and Wink, 1993, 1995), would also help to separate the toxin from neuroreceptors.

In conclusion it is likely that PAs (both free bases and PA *N*-oxides) function as potent chemical defence compounds that directly affect neuroreceptors, whereas their pyrrol metabolites (generated in the vertebrate herbivore) interact with other proteins and DNA. Since PAs are present at the appropriate times, sites, and concentrations in plants, they can thus exhibit an important ecological function for the plants producing them. A parallel can be seen in insects that have specialized on PA-rich food plants and that sequester PAs and use them for their own defense against predators.

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ELECTROPHYSIOLOGICAL RESPONSES OF MALE POTATO CYST NEMATODES, *Globodera rostochiensis* AND *G. pallida*, TO SOME CHEMICALS

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Abstract—Electrophysiological techniques and behavioral bioassays were used to evaluate the responses of males of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, to various chemicals. Exposure of all males of both species to acetylcholine elicited strong electrophysiological responses and positive movement towards the source. By contrast, neither glycine nor citric acid induced any marked electrophysiological responses and only males of *G. pallida* showed significant behavioral responses, moving toward glycine and away from citric acid. The electrophysiological and behavioral responses to GABA and α-aminobutyric acid were complementary, with *G. rostochiensis* showing significant response only to the latter and *G. pallida* responding significantly only to the former. The electrophysiological response of individuals of both species to L- and D-tryptophan varied from no response to a strong response with a significant mean increase in spike activity of *G. rostochiensis* to D-tryptophan and of *G. pallida* to L-tryptophan; behavioral bioassays showed L-tryptophan was a deterrent for *G. pallida*. Males of both species responded significantly in both electrophysiological and behavioral assays to L-glutamic acid but not to D-glutamic acid.

Key Words—Behavior, electrophysiology, *Globodera rostochiensis*, *Globodera pallida*, potato cyst nematodes, GABA, α-aminobutyric acid, L-tryptophan, D-tryptophan.

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INTRODUCTION

Orientation and chemical attraction of plant parasitic nematodes to each other, to plant roots, and to root diffusates have been demonstrated in many species using behavioral bioassays (Webster, 1969; Dusenbery, 1987; Riga and Webster, 1992). However, there is little evidence of the specificity of the nematode responses to chemicals. Only a few specific substances involved in chemotaxis of plant parasitic nematodes have been investigated (Ishikawa et al., 1986), including various inorganic salts (Castro et al., 1990). So far, only one compound with nematode pheromone properties has been isolated and identified (Jaffe et al., 1989).

The microscopic size of most species of plant parasitic nematodes makes physiological and biochemical investigations difficult, and there is a paucity of basic information on chemoreception (Perry, 1996). The most frequently used behavioral bioassays to investigate chemotaxis of plant parasitic nematodes are based on radial two dimensional attraction gradients established in thin layers of agar. Several modifications of the basic technique have been used, ranging from photographing nematode tracks (Riddle and Bird, 1985; Riga and Webster, 1992) to a video monitoring of nematode movement (Dusenberry, 1983; Pine and Dusenberry, 1987) and to an assay based on narrow agarose tracks that allows nematodes to move only in a linear direction (Castro et al., 1989). However, methods based on movement bioassays have several disadvantages and provide only limited information (Perry and Riga, 1995).

Chemicals that influence the behavior of insects have been studied for many years and, for example, alarm pheromones (Bowers et al., 1972; Edwards et al., 1973; Weintjens et al., 1973) and sex pheromones (Dawson et al., 1990) of aphid species have been identified. Electrophysiological recordings from the aphid antenna, coupled with high-resolution gas chromatography, have enabled the identification of synergists for the alarm pheromone of the turnip aphid *Lipaphis erysimi* (Dawson et al., 1987). Use of the electrophysiological technique on individual, intact nematodes will considerably extend knowledge of nematode sensory perception (Perry and Riga, 1995). It has already been used to demonstrate changes in neurophysiological activity in response to chemical stimulation of two of the anterior sensillae, the cephalic papillae (Jones et al., 1991) and the amphid (Riga et al., 1995) of an avian parasitic nematode, *Syngamus trachea.* Electrophysiological examination of the responses of individual adult males of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* to sex pheromones from adult females showed that only *G. rostochiensis* exhibited specific mate recognition (Riga et al., 1996).

The objective of the present study was to use electrophysiological techniques and behavioral bioassays to investigate responses of adult males of *G.*

rostochiensis and *G. pallida* to various chemicals, similar to those inducing gustatory responses by insects (Mullin et al., 1994).

METHODS AND MATERIALS

Source of Experimental Material. Adult males of *G. rostochiensis* pathotype Ro1 and *G. pallida* pathotype Pa2/3 were obtained from separate plant cultures. Cysts of each species were introduced into pots containing 2-week-old potato plants, cv. Désirée or cv. Maris Piper, growing in sand/loam that had been steam sterilized prior to planting. After three weeks, plants were removed, soil was washed from the roots, and the plants were placed in supports with the roots in a plastic bowl containing continuously aerated water. Males were siphoned from the bottom of the bowl and kept in artificial tap water (ATW) (Greenaway, 1970) at 4°C until required; males were used within 72 hr of collection.

Electrophysiological Assay. The electrophysiological assay was used as described by Riga et al. (1996). In summary, a male nematode was placed in a plastic well that was filled with ATW and was tethered by sucking its tail and mid-body region into a drawn capillary connected to a vacuum pump. The recording electrode was inserted as closely as possible into the same area of the cephalic region of each nematode tested. The indifferent electrode was placed in the ATW close to the cephalic region of the nematode. The indifferent and recording electrodes were pulled on a Narishige electrode puller and trimmed as required; they were filled with 0.5 M KCl and had Ag–AgCl contacts (Clark, Pangbourne, UK). The recording electrode had a resistance of 120–150 MΩ. Resulting electrical signals were amplified 500 times on a Universal AC/DC amplifier (Syntech, Type UN-06, Hilversum, The Netherlands). The signals were displayed on an oscilloscope and stored on a digital audio tape recorder (Sony, Itakura, Japan). Signals were subsequently analyzed using the IDAC interface board and AutoSpike program (Syntech).

Each male nematode was stimulated with each of the following chemicals (Sigma, Poole, UK) at 10 mM concentration: acetylcholine, glycine, L- and D-tryptophan, γ- and α-aminobutyric acids (GABA and AABA, respectively), D- and L-glutamic acid, and citric acid. The ATW was removed from the well where the nematode was immobolized and the test solution (stimulus) was immediately pipetted onto the nematode. The stimulus could be removed by perfusing the well with ATW and then restarting the recording in the presence of ATW only. Electrical activity was recorded from each nematode before, during, and after stimulation for up to 20–30 min at 20°C. Five different nematodes of each species were used to test each of the above chemicals. Student's *t* test (Sokal

and Rohlf, 1981) was performed on the data and results are reported as significant at $P < 0.05$.

Petri Dish Behavioral Bioassays. Behavioral bioassays (Riga and Webster, 1992) were performed on 1.5% (w/v) freshly prepared water agar (depth 1.0–1.5 mm) at 20°C under low light intensity, using all the above chemicals. Briefly, 10 μl of each chemical were introduced onto the center of the agar in a 6-cm-diameter Petri dish, which was then allowed to stand for 2 hr to establish a concentration gradient before a single male nematode of *G. rostochiensis* or *G. pallida* was placed at a known point 0.5 cm from the edge of the Petri dish. The position of the male nematode was noted after 1 hr, and the distance between the final position and the origin was recorded (Riga and Webster, 1992). Experience indicates that in this time period, a strong response would be demonstrated by movement of 1.0–2.0 cm. Control treatments used ATW as the target solution. Thirty males were used for each treatment/nematode combination. Paired *t* tests were performed on the data as above.

RESULTS

Electrophysiological Assay. Cellular activity in the form of action potentials was recorded as spike activity. Figure 1 shows a sample computer record of

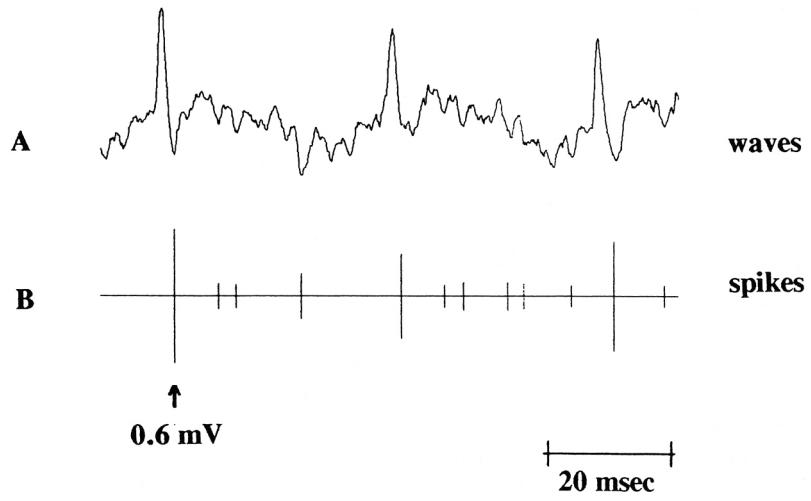

FIG. 1. Sample computer record showing extracellular recordings from the cephalic region of an adult male *Globodera rostochiensis* in the form of waves and spikes in response to acetylcholine. (A) The original action potential recordings; (B) the computer representation of time and amplitude of recognized spikes. The time-scale bar represents 20 msec and the amplitude of an average spike is 0.6 mV.

extracellular recordings from the cephalic region of an adult male *G. rostochiensis* in the form of waves and spikes in response to acetylcholine. The magnitude of the response of individual male *G. rostochiensis* and *G. pallida* to the chemicals and the mean number of spikes per second before, during, and after exposure to each chemical are given in Table 1. Acetylcholine elicited a strong response in all males, and the increase in mean spike activity during exposure was significant for both species. Similarly, there was a significant increase in mean spike activity from males of both species during exposure to L-glutamic acid and all individuals responded. In contrast, exposure to D-glutamic acid elicited no significant increase in mean spike activity and the majority of individuals gave no response. There was no change in mean spike activity on exposure to citric acid or glycine and only four individuals in total showed a slight response.

Globodera rostochiensis and *G. pallida* responded differently to L- and D-

TABLE 1. RESPONSES OF FIVE *Globodera rostochiensis* AND FIVE *G. pallida* MALES TO A RANGE OF CHEMICALS[a]

	G. rostochiensis					*G. pallida*				
	1	2	3	4	5	1	2	3	4	5
Acetylcholine	0	0	0	0	0	0	0	0	0	0
	(38 ± 10; 195* ± 26; 31 ± 3)					(16 ± 3; 176* ± 24; 37 ± 8)				
Glycine	°	–	–	°	–	°	–	–	–	–
	(36 ± 10; 64 ± 13; 42 ± 6)					(54 ± 15; 75 ± 8; 72 ± 11)				
L-Tryptophan	0	°	–	°	–	0	0	°	°	–
	(36 ± 8; 75 ± 19; 30 ± 5)					(30 ± 5; 127* ± 35; 35 ± 7)				
D-Tryptophan	0	0	°	0	°	–	–	0	°	0
	(46 ± 13; 193* ± 61; 61 ± 19)					(31 ± 10; 93 ± 28; 27 ± 6)				
GABA	–	°	0	–	0	°	°	0	°	–
	(45 ± 6; 83 ± 25; 36 ± 6)					(44 ± 10; 139* ± 42; 65 ± 19)				
AABA	0	0	°	°	0	–	°	–	°	–
	(33 ± 13; 122* ± 18; 60 ± 12)					(23 ± 6; 48 ± 13; 37 ± 11)				
D-Glutamic	–	°	°	–	–	–	°	–	–	–
	(38 ± 9; 54 ± 6; 43 ± 7)					(33 ± 12; 49 ± 13; 39 ± 15)				
L-Glutamic	0	°	°	0	°	0	0	0	°	°
	(42 ± 13; 85 ± 11; 39 ± 7)					(32 ± 7; 146* ± 36; 32 ± 9)				
Citric acid	–	–	°	–	–	–	–	–	–	–
	(31 ± 5; 42 ± 7; 47 ± 15)					(36 ± 8; 38 ± 5; 45 ± 9)				

[a]The response of each nematode is indicated as a marked response (0), a slight response (°), or no response (–). The figures in parentheses are the mean number of spikes per second produced by the nematodes before, during, and after exposure to each chemical. A significant increase in spike activity during exposure is indicated by an asterisk ($P < 0.05$); each value is the mean ± standard error for five individuals.

tryptophan and to GABA and AABA (Table 1). In general, individuals of *G. pallida* showed a greater response than *G. rostochiensis* to L-tryptophan and the mean number of spikes per second from *G. pallida* increased significantly during exposure, whereas there was no increase with *G. rostochiensis*. The converse occurred when D-tryptophan was tested; in general, individuals of *G. rostochiensis* showed the greater response and the mean spike activity increased significantly only with *G. rostochiensis*. Exposure of *G. pallida* to GABA elicited a response in all individuals and a significant increase in mean spike activity, whereas there was either no response or only a slight response of individuals to AABA and no significant increase in mean spike activity. In contrast, all *G. rostochiensis* males responded to AABA and mean spike activity increased significantly, whereas the response to GABA was inconsistent and the increase in spike activity was not significant.

An example of a typical positive response and no response of an adult male *G. pallida* is shown in Figure 2. When a male of *G. pallida* was stimulated with L-glutamic acid 85 sec into the recording (arrow A), the frequency of the spikes produced by the cells of the cephalic region increased markedly from 17 spikes/sec to 266 spikes/sec during stimulation and then decreased to 12 spikes/sec after the stimulus was removed (arrow B) and replaced with ATW (Figure 2, I). When a male of *G. pallida* was stimulated with D-glutamic acid 40 sec into the recording (arrow A), the frequency of the spikes produced by the cells of the cephalic region were 30 spikes/sec before stimulation, 36 spikes/sec during stimulation, and 30 spikes/sec after the stimulus was removed (arrow B) and replaced with ATW (Figure 2, II).

Control tests, where nematodes were exposed to fresh ATW, elicited no significant change in spike frequency (results not shown). This indicates that the responses to the test chemicals were to the stimulus and not to slight variations in temperature or oxygen content, for example, associated with exposure to a fresh solution.

Petri Dish Bioassays. The mean distances, in centimeters, traveled in 1 hr by males of *G. rostochiensis* and *G. pallida* in response to each chemical and ATW controls are summarized in Table 2. Males of both species moved a significant distance towards acetylcholine and L-glutamic acid, but D-glutamic acid was not an attractant or repellent. Males of *G. rostochiensis* showed no significant response to citric acid or glycine but *G. pallida* males responded significantly to both, being repelled by citric acid and attracted by glucine. D-Tryptophan elicited no significant movement response from males of either species, and *G. rostochiensis* also did not respond to L-tryptophan; however, *G. pallida* males showed significant movement away from this chemical. Males of *G. pallida* moved a significant distance towards GABA but not to AABA, whereas the converse responses occurred with *G. rostochiensis* males.

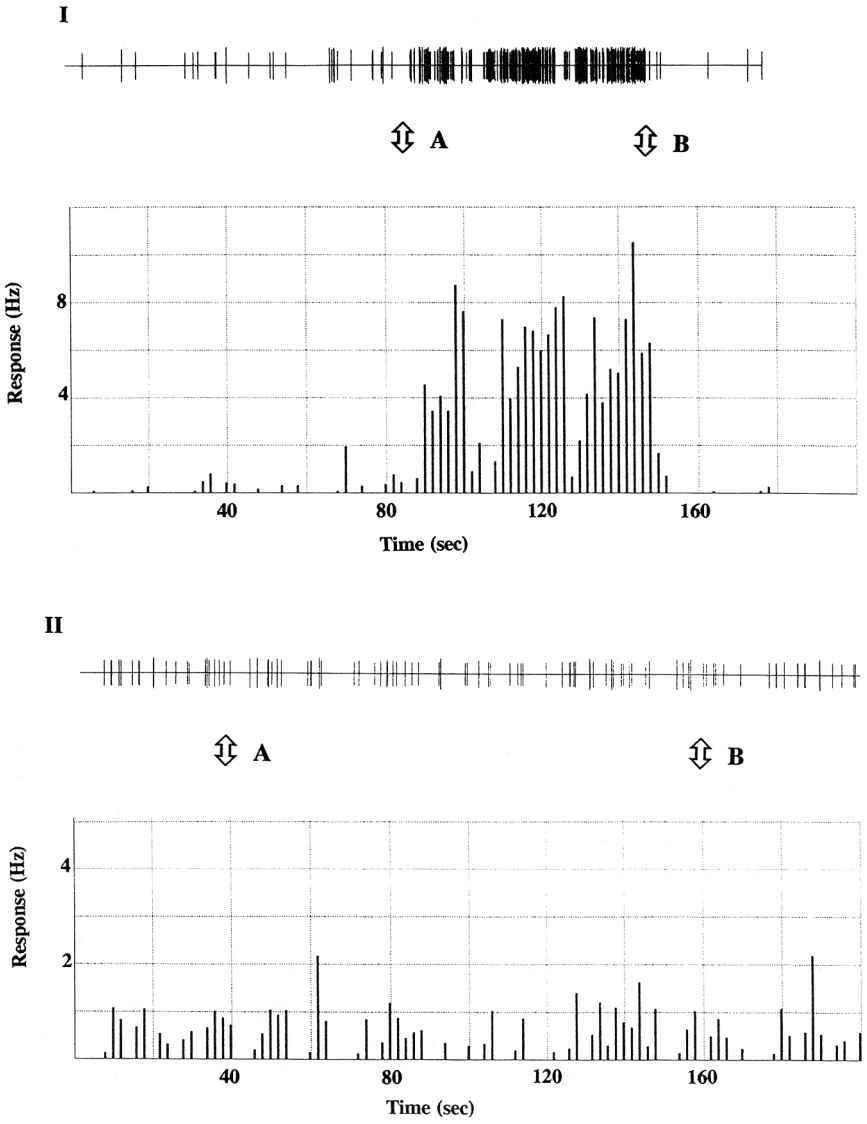

FIG. 2. Extracellular recordings from the cephalic region of an adult male *Globodera pallida* in response to 10 mM L-glutamic acid (I) and 10 mM D-glutamic acid (II). The upper part of both figures shows actual spike events, while the histograms give the accurate total spike frequency. Arrow A indicates when the chemical was introduced and arrow B indicates when it was removed by perfusion with ATW. Note scale differences for response.

TABLE 2. DISTANCE TRAVELED BY ADULT MALES OF *Globodera rostochiensis* AND *G. pallida* IN RESPONSE TO VARIOUS CHEMICALS AND ARTIFICIAL TAP WATER (ATW) CONTROL[a]

	Distance (cm/hr)	
	G. rostochiensis	*G. pallida*
Acetylcholine	0.86* ± 0.20	1.30* ± 0.20
Glycine	0.21 ± 0.10	0.55* ± 0.12
L-Tryptophan	0.23 ± 0.10	−0.17* ± 0.08
D-Tryptophan	0.07 ± 0.03	0.13 ± 0.19
GABA	0*	0.43* ± 0.16
AABA	0.40* ± 0.10	0.28 ± 0.10
D-Glutamic	0.35 ± 0.12	−0.03 ± 0.10
L-Glutamic	0.60* ± 0.21	0.30* ± 0.10
Citric acid	0.20 ± 0.10	−0.15* ± 0.05
ATW	0.08 ± 0.03	0.08 ± 0.03

[a]Each value is the mean ± standard error based on 20 individuals. A significant difference ($P < 0.05$) from the control is indicated by an asterisk.

DISCUSSION

Plant parasitic nematodes range in length from about 0.3 to 10.0 mm, with *G. rostochiensis* and *G. pallida* males being approximately 1.2 mm long and 0.03 mm in maximum diameter. This small size makes intracellular recordings (Davis and Stretton, 1989a,b) and direct recordings from individual sense organs extremely difficult. The first electrophysiological recordings of responses from live, intact nematodes were made by Jones et al. (1991); extracellular recordings of changes in electrical activity inside the body of male *G. rostochiensis* were recorded in response to the sex pheromone from adult females and to D-tryptophan. Recent work (Riga et al., 1995, 1996) and the present study on sensory responses of intact nematodes used a modified electrophysiological assay, enabling more effective movement restriction and more detailed analysis of the extracellular recordings.

Extracellular electrophysiological recordings from the cephalic region of adult males of *G. rostochiensis* and *G. pallida* showed that the nematodes can perceive the presence of various chemicals and demonstrated differences in response. Variation in response of individuals to some of the chemicals may reflect the fact that it is not possible to ensure that recordings are from the same cell each time. In general, the behavioral bioassays supported the electrophysiological data, although results from the two experimental approaches are not

strictly comparable because the concentrations of the chemicals to which the nematodes initially respond differ. In the electrophysiology tests, the concentration of each chemical was 10 mM, whereas in the behavioral bioassays this concentration was placed at the center of the agar and the chemical was allowed to diffuse for 2 hr and establish a concentration gradient; males were then placed 2.5 cm away from the center at a point where the concentration must have been much less than 10 mM. A variety of low-molecular-weight compounds was used in this study, similar to those used to study insect gustatory chemoreceptors (Mullin et al., 1994), and it is likely that their diffusion rate through agar and the concentration gradient established was similar.

Given the involvement of acetylcholine with transmission of nerve impulses, it is not surprising that exposure of all males of both species to acetylcholine elicited strong electrophysiological responses; behavioral bioassays showed enhanced movement with a positive orientation towards the source. By contrast, neither glycine nor citric acid induced any marked electrophysiological responses. Glycine is an alpha amino acid usually giving phagostimulatory responses in insects (Mullin et al., 1994), and males of both species moved towards the source, with males of *G. pallida* being significantly attracted. Citric acid elicited no marked electrophysiological responses and was a repellent for males of *G. pallida* in behavioral bioassays.

Behavioral bioassays were conducted at 20°C and controls showed no differences between species in the distance moved; previous work (Robinson et al., 1987) found no differences between *G. rostochiensis* and *G. pallida* in the activity of second-stage juveniles (J2) at this temperature. In general, in the present work, males of *G. pallida* moved further in response to test chemicals than males of *G. rostochiensis*, and this seems to be a facet of the responses to stimuli rather than intrinsically greater activity of *G. pallida*. One exception was the response to AABA, where only males of *G. rostochiensis* moved a significant distance towards the source.

The data for mean spike activity and behavioral responses to GABA and AABA were complementary, with *G. rostochiensis* showing significant response only to the latter and *G. pallida* responding significantly only to the former. However, examination of the electrophysiological responses of individuals shows that the difference was not as unequivocal as analysis of mean spike activity and behavioral bioassays indicated. In insects, GABA and AABA usually give phagostimulatory responses (Mullin et al., 1994). GABA is also an inhibitory neuromuscular transmitter in nematodes, exerting a hyperpolarizing action on the muscle cells of *Ascaris suum* by opening chloride ion channels (del Castillo et al., 1964; Martin, 1980). Application of GABA to cut individuals of *Caenorhabditis elegans* caused a flaccid paralysis (Lewis et al., 1980), but recent evidence indicates that GABA is also an excitatory neurotransmitter in this species (McIntire et al., 1993). GABA-like immunoreactivity has been detected

in neuronal processes, probably the inhibitory motor neurons, in both the dorsal and ventral nerve cords of *G. rostochiensis* J2; in the nerve ring, immunoreactivity was observed in neuronal processes and in a cell body, probably the ventral ring motor neuron (Stewart et al., 1994).

The electrophysiological responses of individuals of both species to L- and D-tryptophan varied from no response to a strong response with a significant mean increase in spike activity of *G. rostochiensis* to D-tryptophan and of *G. pallida* to L-tryptophan. Behavioral bioassays showed L-tryptophan was a deterrent for *G. pallida.* Previous studies have demonstrated changes in electrical activity of the anterior end of *G. rostochiensis* males to 1 mM D-tryptophan (Jones et al., 1991), and behavioral studies demonstrated that *C. elegans* avoided 1 mM D-tryptophan (Dusenbury, 1975). When amphids of *S. trachea* were stimulated with 100 mM D-tryptophan, the two responding neurons started to show adaptation approximately 17 sec after application of the chemical (Riga et al., 1995). In the present work, adaptation was not evident in the electrophysiological responses of males of either species of *Globodera* to any of the chemicals, used at 10 mM concentration; adaptation was also not evident when 1 mM D-tryptophan was used with *G. rostochiensis* (Jones et al., 1991).

There was an interesting contrast in the response of *G. rostochiensis* and *G. pallida* to glutamic acid. Males of both species responded significantly in both electrophysiological and behavioral assays to L-glutamic acid but not to D-glutamic acid. This is the converse of the response found in insects, where the D isomer of many amino acids usually elicits a phagostimulatory response while many L-amino acids are feeding deterrents (Mullin et al., 1994).

Chemoreception is essential for suitable food selection, and the electrophysiological and behavioral analyses of responses of various species of ecto- and endoparasitic nematodes to specific plant compounds will provide information about feeding deterrents and stimulants that may relate to plant host suitability and the biochemical nature of the resistant response. A comparison of different life-cycle stages of individual species is often instructive. For example, in *Globodera* species the J2 is the infective stage that establishes a feeding site within the host roots, whereas adult males exit from the root to mate with the females; the males may have no need for food (Evans, 1970), but the stylet and pharyngeal glands appear functional, and it is possible that alternative food sources are used. Overall differences in the response of *G. rostochiensis* and *G. pallida* may indicate subtle differences in food preference. The electrophysiological assays have the advantage of demonstrating that the difference in recognition of a stimulus is due to direct differences in sensory responses rather than other factors, such as differences in mobility or activity of the nematodes, which can complicate interpretation of agar plate bioassays.

The electrophysiological technique offers exciting potential to analyze the responses to fractions of compounds responsible for attracting nematodes to plant

roots, and the technique will also enhance understanding of the mode of action of control agents by determining whether the lack of response is due to inhibition of neurotransmission. Behavior modifying chemicals could achieve commercial use in crop protection by incorporation into integrated control methods. For example, Castro *et al.* (1991) reported that salts of specific ions repelled *Meloidogyne incognita* and protected tomato roots.

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BIOSYNTHESIS OF DEFENSIVE ALLOMONES IN LEAF BEETLE LARVAE: STEREOCHEMISTRY OF SALICYLALCOHOL OXIDATION IN *Phratora vitellinae* AND COMPARISON OF ENZYME SUBSTRATE AND STEREOSPECIFICITY WITH ALCOHOL OXIDASES FROM SEVERAL IRIDOID PRODUCING LEAF BEETLES

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Abstract—(7*S*)-[$^2H_5$]-Salicylalcohol (**3**) and (7*R*)-[$^2H_1$]-salicylalcohol (**5**) have been synthesized in order to examine the stereospecificity of salicylalcohol oxidase from the defensive secretion of the salicylaldehyde-producing leaf beetle *Phratora vitellinae*. Oxidation was found to proceed by selective removal of the C(7)-H_R hydrogen atom (*Re*-specificity) to yield salicylaldehyde. (7*S*)-[$^2H_6$]-Benzylalcohol (**9**) was also oxidized *Re*-specifically to benzaldehyde, but in much lower yield, indicating the importance of the orthohydroxy group of salicylalcohol in substrate enzyme binding. The stereospecificities of terpenoid oxidases from six species of iridoid-producing leaf beetle were examined using (1*R*,8*R*)-[$^2H_2$]-8-hydroxygeraniol (**10**), and were all found to oxidize the substrate *Re*-specifically. Cross-activity of oxidation was found in a number of species, with *P. vitellinae* able to oxidize terpenoid (**10**) and two of the iridoid-producing species able to oxidize salicylalcohol analogue (**3**), again with *Re*-specificity. However, when the two substrate analogs were presented together, in equal concentrations, preferential oxidation of the natural analog

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was observed in each case. The kinetics of oxidation for a number of terpenoid and aromatic alcohols by the defensive secretion of the iridoid-producing leaf beetle *Phaedon armoraciae* have been studied, revealing a large difference between the rate of (primary, allylic) terpenoid alcohol oxidation and the rate of salicylalcohol oxidation, thus accounting for the observed selectivity.

Key Words—Salicylalcohol, salicylaldehyde, oxidase, *Re*-specificity, iridoids, leaf beetle, *Phratora*, Chrysomelidae.

INTRODUCTION

Leaf beetles of the family Chrysomelidae are phytophagous insects that form dense, conspicuous aggregates on their host plants, making them particularly vulnerable to predators. In order to counteract this susceptibility, several spectacular defense mechanisms have evolved in the Chrysomelidae, including mechanical, behavioral, and chemical responses (Deroe and Pasteels, 1982). Chemical defense plays a significant role in the protection of both larvae and adults in many leaf beetle species (Pasteels et al., 1982). Larvae of most species in the subtribe Chrysomelina possess nine pairs of defensive glands, located dorsally in the thorax and the first seven abdominal segments (Garb, 1915). The glands are eversible and, when the larva is in a passive state, form reservoirs for the defensive secretion. Upon molestation, however, the everted glands protrude from the body of the insect, exposing a droplet of defensive secretion at their tip.

The chemical composition of larval defensive secretions from several leaf beetle species has been investigated, revealing a range of simple aliphatic and aromatic compounds (Pasteels et al., 1984). Of the 24 species examined to date, 14 contain methylcyclopentanoid monoterpenes (iridoids); seven contain salicylaldehyde and, in some cases, benzaldehyde; one contains a mixture of phenylethyl esters; one possesses a host-plant-dependent mixture of butanoic acid and derivatives (Hilker and Schulz 1994); and one contains the hydroxynaphthoquinone juglone.

The species of leaf beetle that contain iridoids are assumed to synthesize them de novo and, indeed, we now have direct evidence for this (Oldham et al., in preparation). Furthermore, in a recent investigation into iridoid biosynthesis in chrysomelids, we have demonstrated that the methylcyclopentanoids are produced from geraniol, via 8-hydroxygeraniol and 8-oxogeranial, using a pathway similar to that found in plants (Lorenz et al., 1993; Veith et al. 1994). In contrast, those species of leaf beetle that contain salicylaldehyde do not biosynthesize the compound de novo, but obtain it from their host plants (*Populus* or *Salix*) in the form of salicin, a phenolic glycoside, which they cleave and oxidize to give salicylaldehyde (Pasteels et al., 1983). The juglone-producing

species probably also derives its defensive substance from the host plant (*Juglans*), which contains a structurally related phenolic glycoside.

The recent discovery of 8-hydroxygeraniol-8-*O*-β-glycoside in an iridoid-producing leaf beetle (Daloze and Pasteels, 1994) demonstrates that the production of iridoids and salicylaldehyde (and possibly juglone) by leaf beetles, shares two common transformations, namely glucosidation and oxidation. In salicylaldehyde-producing species, a β-glucosidase cleaves salicin to give salicylalcohol (Soetens et al., 1993) and an oxidase converts the diol to salicylaldehyde. Similarly, in the iridoid-producing species, a β-glucosidase is required to hydrolyze 8-hydroxygeraniol-8-*O*-β-glycoside to 8-hydroxygeraniol, which is then oxidized to 8-oxogeranial.

In a previous study, the stereochemical course of 8-hydroxygeraniol oxidation by *Phaedon armoraciae* was investigated, and the oxidation at both C_1 and C_8 was found to be *Re*-specific (Veith et al., submitted). Here we describe an investigation into the stereochemistry of the oxidase in a salicylaldehyde-producing leaf beetle, *Phratora vitellinae*, and compare its stereo- and substrate specificity with those of several leaf beetle iridoid oxidases.

METHODS AND MATERIALS

General

Horse liver alcohol dehydrogenase (HLADH) was purchased from Boehringer (Mannheim, Germany) as a solution (100 mg/10 ml) in KH_2PO_4 buffer (0.02 M, pH 7.0). ^{1}H- and ^{13}C-NMR spectroscopy was conducted on Bruker Cryospec WM 250 and Bruker WM 400 spectrometers. Infrared spectra were measured on a Bruker IFS 88 spectrometer. Gas chromatography–mass spectrometry (GC-MS) was performed on a Carlo-Erba Vega gas chromatograph combined with a Finnigan ITD 800 (ion trap) mass spectrometer in the EI mode (70 eV). CP-SIL-8-CB (Chrompack, 30 m × 0.25 mm) and SE-30 (10 m × 0.32 mm) fused-silica capillary columns were used with helium as the carrier gas (30 cm/sec). Gas chromatography was conducted on a Carlo-Erba HRGC 5300 Mega series gas chromatograph equipped with a SE-30 (10 m × 0.32 mm) fused silica capillary column, with hydrogen as the carrier gas (30 cm/sec), and a FID detector. High-resolution mass spectra (HR-MS) were measured on Finnigan MAT 90 and Kratos MS50 mass spectrometers. Liquid column chromatography was performed on Si 60 (0.040–0.063 mm, E. Merck, Darmstadt, Germany) silica gel.

8-Hydroxygeraniol and (±)-linalool were purchased from Aldrich, geraniol was obtained from Fluka and (±)-citronellol was produced by reduction of (±)-citronellal (Fluka) with $NaBH_4$.

Syntheses

[$^2H_5$]-Salicylaldehyde (2). Sodium (3.5 g, 152 mmol) was slowly added to ice cooled D_2O (8.2 ml) (**caution**) in a 50-ml round-bottomed flask equipped with a reflux condenser. On completion of the reaction, [$^2H_5$]-phenol (**1**) (1 g, 10-mmol) was added to the solution and the reaction mixture heated to 80°C, in order to facilitate the formation of sodium [$^3H_5$]-phenolate. The reaction mixture was then cooled to 65°C, and $CHCl_3$ (1.7 ml, 20 mmol) was added. After 90 min at 65°C, the solution was allowed to cool to room temperature and acidified to pH 1 by addition of 5 N H_2SO_4. The aqueous phase was extracted with diethylether (3 × 20 ml) and the combined organic phase washed with brine (2 × 15 ml) and dried over anhyd. $MgSO_4$. Purification of the crude material was achieved by column chromatography (eluent pentane:diethylether 95:5) to give 400 mg of [$^2H_5$]-salicylaldehyde (**2**) (31% yield). ^1H NMR ($CDCl_3$, ppm): 10.95 (1 H, s); ^{13}C NMR ($CDCl_3$, ppm): 117.24 (t, J = 25 Hz), 119.36 (t, J = 25 Hz), 120.52 (s), 133.30 (t, 2J = 25 Hz), 136.55 (t, J = 24 Hz), 161.66 (s), 196.29 (t, J = 27 Hz); IR (film): 3142, 2123, 1643, 1555, 1394, 1301, 1216, 1162, 1011, 884, 828, 806, 706, 635 cm^{-1}; EI-MS (relative intensity): 127 (M^+, 100), 126 (47), 125 (67), 120 (8), 108 (17), 82 (5), 80 (27), 73 (6), 69 (15), 66 (8), 54 (6), 52 (11), 49 (5), 45 (5); HR-MS: found 127.0682, calculated for $C_7HD_5O_2$ 127.0681.

(7S)-[$^2H_5$]-Salicylalcohol (3). To a solution of [$^2H_5$]-salicylaldehyde (**2**) (50 mg, 0.39 mmol) in 0.1 M sodium phosphate buffer (5 ml, pH 7.5), ethanol (2 ml, 34 mmol), NAD^+ (25 mg, 0.03 mmol), and HLADH solution (500 μl, see General section for details) were added. The reaction was shaken at room temperature for 16 hr before the addition of further 0.1 M sodium phosphate buffer (15 ml, pH 7.5), ethanol (1.5 ml, 25 mmol), NAD^+ (25 mg, 0.03 mmol), and HLADH solution (500 μl). After an additional 12 hr, the characteristic disappearance of color indicated the reduction was complete. The solution was extracted with diethylether (3 × 10 ml), the organic phase washed with brine (15 ml) and dried (anhyd. $MgSO_4$). Column chromatography on silica gel (eluent pentane–diethylether 30:70) afforded 32.7 mg of (7*S*)-[$^2H_5$]-salicylalcohol (**3**) (65% yield). ^1H NMR ($CDCl_3$, ppm): 1.2 (1 H, s), 1.6 (1 H, bs), 4.8 (1 H, s); IR (KBr disk): 3445, 3154, 2928, 2360, 1733, 1599, 1573, 1393, 1250, 1191, 1147, 1006, 934, 884, 799, 722 cm^{-1}; EI-MS (relative intensity): 130 (6), 129 (M^+, 67), 128 (5), 112 (13), 111 (100), 109 (10), 84 (10), 83 (90), 82 (16), 81 (24), 80 (10), 69 (5), 56 (5), 55 (9), 54 (10), 53 (8), 52 (7): HR-MS: found 129.0835, calculated for $C_7H_3D_5O_2$ 129.0838.

(7R)-[$^2H_1$]-Salicylalcohol (5). To a solution of salicylaldehyde (**4**) (35 mg, 0.28 mmol) in 0.1 M sodium phosphate buffer (12 ml, pH 7.5), [$^2H_6$]-ethanol (1.5 ml, 25 mmol), NAD^+ (36 mg, 0.054 mmol), and HLADH solution (600 μl) were added. The reaction was shaken at room temperature for 14 hr before

the addition of further 0.1 M sodium phosphate buffer (1.2 ml, pH 7.5), [$^2H_6$]-ethanol (150 μl, 2.5 mmol), NAD^+ (5 mg, 0.006 mmol), and HLADH solution (70 μl). After 24 hr, additional NAD^+ (5 mg, 7.5 mmol), [$^2H_6$]-ethanol (250 μl, 4.2 mmol), and HLADH (100 μl) were added, and the reaction was allowed to proceed for a further 40 hr. The aqueous phase was extracted with diethylether (3 × 10 ml) and the combined extracts washed with brine (15 ml) and dried (anhyd. $MgSO_4$). Silica column chromatography (eluent pentane–diethylether 30:70) yielded 26.3 mg of (7*R*)-[$^2H_1$]-salicylalcohol (**5**) (75% yield). ^1H NMR ($CDCl_3$, ppm): 2.1 (2 H, s), 4.78 (1 H, s), 6.7–7.2 (4 H, m); ^{13}C NMR ($CDCl_3$, ppm): 64.30 (t, J = 23 Hz), 116.54 (s), 120.09 (s), 124.63 (s), 127.85 (s), 129.52 (s), 156.10 (s); IR (KBr disk): 3448, 3160, 2162, 1615, 1595, 1459, 1387, 1308, 1238, 1191, 1111, 1043, 1008, 939, 898, 864, 847, 750, 727 cm^{-1}; EI-MS (relative intensity): 126 (5), 125 (M^+, 67), 124 (5), 108 (12), 107 (98), 106 (10), 80 (9), 79, (100), 78 (35), 77 (18), 65 (6), 63 (5), 53, (10), 52 (15), 51 (15), 50 (7); HR-MS: found 125.0584, calculated for $C_7H_7DO_2$ 125.0587.

[$^2H_7$]-Benzylalcohol (7). To a stirred suspension of lithium aluminum deuteride (1 g, 23.8 mmol) in diethylether (40 ml), a solution of [$^2H_5$]-benzoic acid (**6**) (2.5 g, 19.5 mmol) in diethylether (30 ml) was slowly added. After 15 min the reaction mixture was hydrolyzed by the addition of water (30 ml) and H_2SO_4 (30 ml, 10%). The phases were separated and the aqueous phase was extracted with diethylether (3 × 20 ml). The combined ether extracts were washed with brine (2 × 30 ml) and dried (anhyd. $MgSO_4$) to give, after evaporation of the solvent, 2.23 g of pure [$^2H_7$]-benzylalcohol (**7**) (99.5% yield). ^1H NMR ($CDCl_3$, ppm): 2.28 (1 H, bs); IR (film): 3344, 2956, 2926, 2854, 2276, 2202, 2113, 2095, 2073, 1698, 1382, 1328, 1250, 1186, 1094, 1051, 1029, 960, 840, 820, 722, 638 cm^{-1}; EI-MS (relative intensity): 116 (7), 115 (M^+, 100), 114 (10), 113 (56), 98 (20), 96 (6), 94 (5), 86 (9), 85 (93), 84 (8), 83 (7), 82 (29), 81 (20), 70 (7), 66 (5), 54 (21), 52 (9), 42 (9); HR-MS: found 115.1006, calculated for C_7HD_7O 115.1015.

[$^2H_6$]-Benzaldehyde (8). To a solution of [$^2H_7$]-benzylalcohol (**7**) (2 g, 17.3 mmol) in dichloromethane (100 ml), manganese dioxide (15.1 g, 173 mmol) was added in portions. After stirring for 24 hr at room temperature, the suspension was filtered through a sinter and the filtrate dried (anhyd. $MgSO_4$). The solvent was removed and the residue purified by silica column chromatography (eluent pentane–diethylether 90:10) to give 1 g of [$^2H_6$]-benzaldehyde (**8**) (50% yield). IR (film): 2281, 2103, 2090, 2066, 2041, 1682, 1630, 1562, 1545, 1376, 1330, 1300, 1179, 1048, 958, 868, 844, 834, 816, 750, 624 cm^{-1}; EI-MS (relative intensity): 113 (7), 112 (M^+, 99), 111 (11), 110 (100), 84 (19), 83 (5), 82 (79), 76 (7), 56 (10), 54 (53), 52 (29), 42 (5); HR-MS: found 112.0777, calculated for C_7D_6O 112.0795.

(7S)-[$^2H_6$]-Benzylalcohol (9). To a solution of [$^2H_6$]-benzaldehyde (**8**)

(1 g, 8.9 mmol) in 0.1 M sodium phosphate buffer (130 ml, pH 7.5), ethanol (40 ml, 675 mmol), NAD^+ (160 mg, 0.25 mmol) and HLADH solution (2 ml) were added. The reaction mixture was shaken at room temperature for 75 min and then extracted with diethylether (3 × 50 ml). The combined ether layers were dried (anhyd. $MgSO_4$). Removal of the solvent gave 770 mg of pure (7*S*)-[$^2H_6$]-benzylalcohol (**9**) (75% yield). ^1H NMR ($CDCl_3$, ppm): 2.02 (1 H, bs), 4.62 (1 H, s): ^{13}C NMR ($CDCl_3$, ppm): 64.8 (t, J = 28 Hz), 126.1–128.4 (5 × s), 140.6 (s); IR (film): 3340, 2900, 2276, 2141, 1415, 1376, 1346, 1306, 1274, 1152, 1057, 1025, 940, 840, 820, 733, 649 cm^{-1}; EI-MS (relative intensity): 115 (6), 114 (M^+, 100), 113 (22), 112 (46), 97 (15), 85 (25), 84 (56), 83 (5), 82 (16), 81 (16), 80 (5), 54 (9); HR-MS: found 114.0936, calculated for $C_7H_2D_6O_2$ 114.0952. $[\alpha]_{546}^{22}$ = + 1.41 (c = 1.0, $CHCl_3$)

Incubation Experiments

Solutions of deuterium-labeled aromatic alcohols **3, 5,** and **9** and labeled 8-hydroxygeraniol (**10**) (100 μl, 0.1% in 0.1 M K_2HPO_4/KH_2PO_4 phosphate buffer, pH 7) were incubated with stock solutions of leaf beetle defensive secretions (100 μl phosphate buffer containing 2–3 insect equivalents per test) overnight. The secretion was collected as described previously (Veith et al., 1996). The aqueous solutions were extracted with ether (200 μl), and the ether layer analyzed by GC and GC-MS (see General section for details) to determine the extent of oxidation of the metabolites. Each incubation was repeated two or three times, depending on the availability of the insects.

Kinetic Experiments with P. armoraciae *Secretion*

A stock solution of *Phaedon* defensive secretion was prepared by collecting the secretions from 24 larvae in sodium phosphate buffer (0.1 M, pH 7, 2 ml). Aliquots of the stock solution (150 μl) were incubated with an equal volume of either 8-hydroxygeraniol, salicylalcohol, geraniol, or citronellol solution (10 mM, in 0.1 M phosphate buffer pH 7 with 1% DMSO). The progress of oxidation was monitored by UV spectroscopy (Perkin Elmer Lambda 2S UV/VIS spectrometer) against the stock solution (150 μl) and phosphate buffer (0.1 M, pH 7, 1% DMSO, 150 μl) at 23°C. Oxidation of 8-hydroxygeraniol and geraniol to 8-oxogeranial and geranial, respectively, was detected at 240 nm, salicylalcohol oxidation to salicylaldehyde was detected at 250 nm, and citronellol oxidation to citronellal was monitored at 300 nm. After 16 hr, incubation was stopped by addition of methanol (50 μl), the products extracted with ethyl acetate (200 μl), and the ratio of alcohol to aldehyde determined by GC analysis. 8-Hydroxygeraniol was incubated with the stock solution of secretion at the beginning of the experiments and at the end in order to account for any decrease in activity of the oxidase over the course of the tests. A repeat experiment, using

an extract of the secretion from an additional 24 larvae (of similar size to the first) gave almost identical results, and the two sets of data were pooled.

Collection and Rearing of Leaf Beetles

Phratora vitellinae, *Gastrophysa viridula*, and *Plagiodera versicolora* were collected near Brussels, Belgium, and near Bonn, Germany; *Phratora laticollis* were collected near Brussels; and *Hydrothassa glabra* and *Prasocuris phellandrii* were collected in Bavaria, Germany. *Phaedon armoraciae* were maintained as a year-round laboratory culture at 18°C, with a 12-hr light–dark schedule, and fed on Chinese cabbage.

RESULTS AND DISCUSSION

Stereochemistry of salicylalcohol oxidation by P. vitellinae. (7*S*)-[$^2H_5$]-Salicylalcohol (**3**) and (7*R*)-[$^2H_1$]-salicylalcohol (**5**) were synthesized enantiospecifically in order to study the stereochemistry of salicylalcohol oxidation by *P. vitellinae*. (7*S*)-[$^2H_5$]-Salicylalcohol (**3**) was synthesized from [$^2H_5$]-phenol (**1**) in two steps (see Figure 1). A Reimer-Tiemann reaction (using NaOD as a base) was employed to convert the phenol (**1**) into [$^2H_5$]-salicylaldehyde (**2**). Reduction of the aldehyde (**2**) to (7*S*)-**3** was achieved using horse liver alcohol dehydrogenase (HLADH) in the presence of ethanol and NAD^+ (for a review of HLADH-catalyzed transformations see Wong and Whitesides, 1994). The enzyme is known to deliver a hydride ion to the *Re*-face of a prochiral aldehyde exclusively, resulting in an (*S*)-absolute configuration at C-7 of alcohol **3.**

(7*R*)-[$^2H_1$]-Salicylalcohol (**5**) was synthesized in one step from salicylaldehyde (**4**) using HLADH in the presence of [$^2H_5$]-ethanol and NAD^+ (see Figure 2). Here, a deuteride ion was delivered to the *Re*-face of aldehyde **4** resulting in the formation of alcohol **5** with (*R*)-absolute configuration at the chiral center.

Incubation experiments, carried out using 0.1% solutions of the chiral salicylalcohols **3** and **5** in phosphate buffer (pH 7.5) together with aliquots of a stock solution of *P. vitellinae* defensive secretion (approximately 2–3 insect equivalents per test), unambiguously demonstrated the stereospecificity of salicylalcohol oxidase in *P. vitellinae*. Salicylalcohol analog **3** was oxidized to salicylaldehyde analog **2** in vitro (see Figure 1) in 91% yield, while alcohol **5** was oxidized to salicylaldehyde **4** (see Figure 2) in 86% yield (due to the large amount of material produced, natural salicylaldehyde from the secretion did not interfere with that formed from in vitro incubation). The identities of the oxidation products were easily confirmed as **2** and **4,** from their mass spectra and GC retention times, since these aldehydes were used as intermediates in the syntheses of the chiral salicylalcohols (see Figures 1 and 2) and were, therefore,

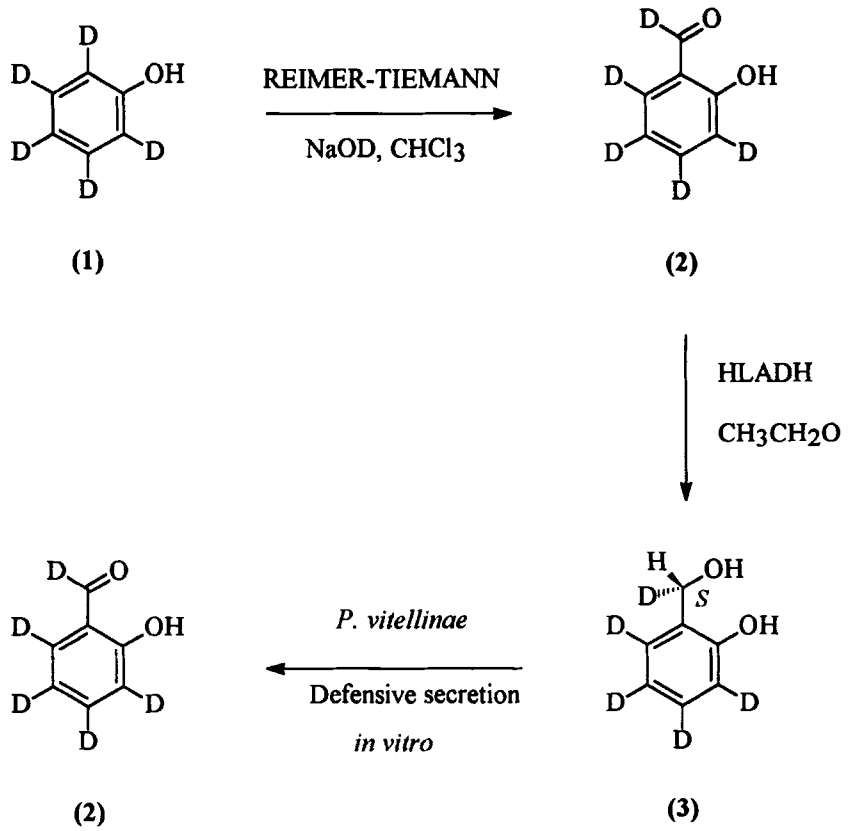


FIG. 1. Synthesis of (7*S*)-[$^2H_5$]-salicylalcohol (**3**), from [$^2H_5$]-phenol (**1**), and its subsequent *Re*-specific oxidation to [$^2H_5$]-salicylaldehyde (**2**) by in vitro incubation with *P. vitellinae* defensive secretion.

already available as standards. Thus, the oxidase in the defensive secretion of *P. vitellinae* has been shown to remove selectively the C-7H_R hydrogen atom of salicylalcohol (*Re*-specificity) during oxidation of the diol to salicylaldehyde.

(7*S*)-[$^2H_6$]-Benzylalcohol (**9**) was synthesized in three steps from [$^2H_5$]-benzoic acid (**6**) (see Figure 3) to provide a metabolic probe that would evaluate the importance of the orthohydroxy group, in salicylalcohol, for substrate-enzyme binding. [$^2H_5$]-Benzoic acid (**6**) was reduced with lithium aluminum deuteride to give [$^2H_7$]-benzylalcohol (**7**). Oxidation of **7** with manganese dioxide afforded [$^2H_6$]-benzaldehyde (**8**), which was reduced with HLADH in the presence of ethanol and NAD^+ to give (7*S*)-[$^2H_6$]-benzylalcohol (**9**) (see Figure 3). In vitro incubation of **9** with *P. vitellinae* defensive secretion gave [$^2H_6$]-

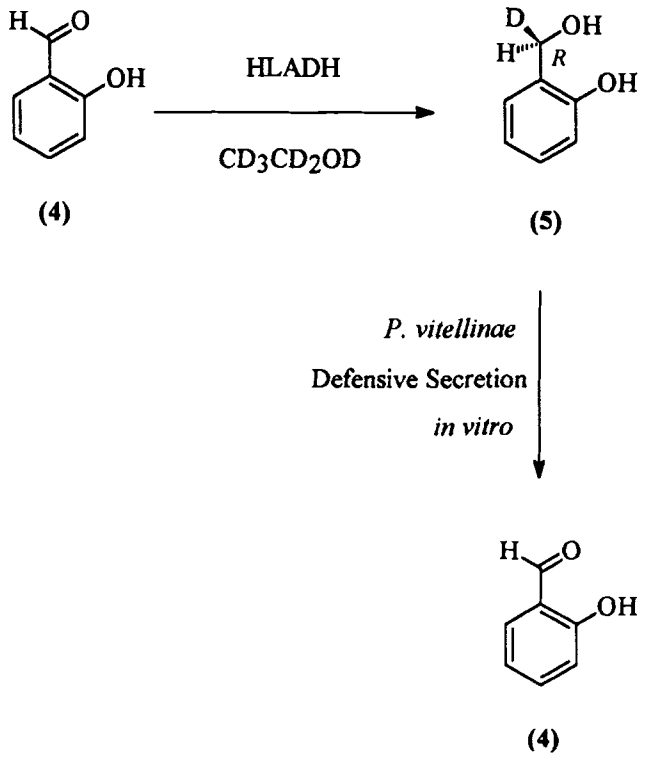


FIG. 2. Synthesis of (7*R*)-[$^2H_1$]-salicylalcohol (**5**) and its *Re*-specific oxidation to salicylaldehyde (**4**) by *P. vitellinae* defensive secretion.

benzaldehyde (**8**) in very low yield (< 1%), identified by comparison of its mass spectrum and GC retention time with those of an authentic sample (used as an intermediate in the synthesis of **9**, see Figure 3). The small degree of oxidation clearly demonstrated that the presence of an orthohydroxy group is important for the interaction of salicylalcohol with the *P. vitellinae* enzyme. However, the absence of a hydroxy group had no effect on the stereospecificity since, as with the salicylalcohols, *Re*-specificity was observed in the oxidation of benzylalcohol (**9**) to benzaldehyde (**8**).

Substrate and Stereospecificity of Oxidases from Chrysomelid Larval Defensive Secretions. In order to compare the stereochemistry of salicylalcohol oxidase from *P. vitellinae* with the terpenoid alcohol oxidases from iridoid-producing leaf beetles, the chiral deuterated salicyl- and benzylalcohols **3**, **5**, and **9**, and chiral deuterated 8-hydroxygeraniol (**10**) (prepared previously, Veith et al., 1996) were incubated with defensive secretions from a number of chry-

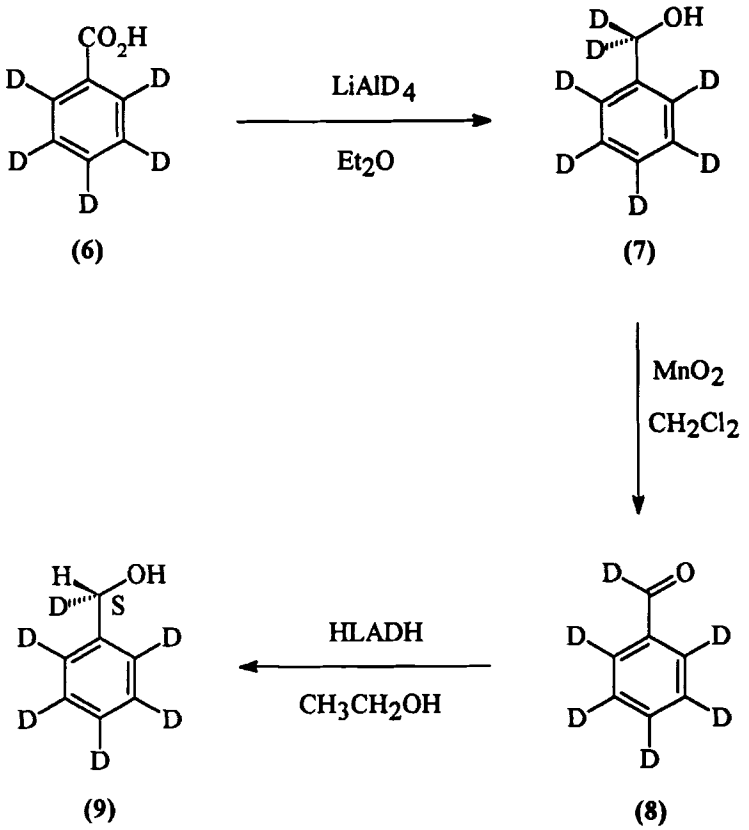
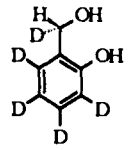
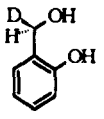
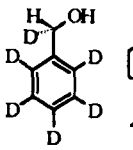
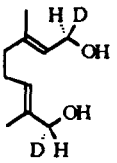


FIG. 3. Synthesis of (*S*)-[$^2H_6$]-benzylalcohol (**9**) from [$^2H_5$]-benzoic acid (**6**) via [$^2H_7$]-benzylalcohol (**7**) and [$^2H_6$]-benzaldehyde (**8**).

somelid species. The results are summarized in Table 1 (due to the limited availability of some species, not all compounds were tested on all species). The observations that the *P. vitellinae* enzyme oxidized **3, 5,** and **9** with *Re*-specificity are reported above. In addition, the secretion from this salicylaldehyde producing species was also found to oxidize [$^2H_2$]-8-hydroxygeraniol (**10**) to 8-oxocitral, again with *Re*-specificity (see Table 1). When (7*S*)-[$^2H_5$]-Salicylalcohol (**3**) and [$^2H_2$]-8-hydroxygeraniol (**10**) were incubated together with the *P. vitellinae* defensive secretion, preferential oxidation of the natural substrate analog **3** was observed (95% oxidation of **3** to **2,** 18% oxidation of **10** to 8-oxocitral, see Table 1). This substrate specificity, together with the identical stereochemistry of both oxidations, suggested that a single enzyme may have been responsible for the oxidation of both alcohols, since a mixture of two oxidases (an

TABLE 1. SUBSTRATE AND STEREOSPECIFICITY FOR OXIDATION OF AROMATIC AND TERPENOID ALCOHOLS BY DEFENSIVE SECRETIONS OF SEVERAL LEAF BEETLE LARVAE

	Substrate				
					
Species of leaf beetle (defensive substance)[a]	(3)	(5)	(9)	(10)	3 + 10
P. vitellinae (SA)	*Re*	*Re*	*Re* (<1%)[b]	*Re*	*Re* (95%) + (18%)[c]
P. armoraciae (IM)	*Re*	*Re*	0	*Re*	*Re* (<1%) + (14%)
P. laticollis (IM)	NT[d]	NT	NT	NT	*Re* (<1%) + (44%)
G. viridula (IM)	0[e]	0	0	*Re*	NT
P. versicolora (IM)	0	0	0	*Re*	*Re* (0%) + (51%)
H. glabra (IM)	NT	NT	NT	*Re*	NT
P. phellandrii (IM)	NT	NT	NT	*Re*	NT

[a]SA = salicylaldehyde, IM = iridoid monoterpene.
[b]Percentage conversion of alcohol to aldehyde.
[c]Individual percentage conversions of alcohols **3** and **10** to their respective aldehydes.
[d]NT = not tested.
[e]0 = no oxidation.

aromatic alcohol oxidase and a terpenoid alcohol oxidase) would be expected to produce a more equal mixture of the two aldehydes. Nevertheless, it was impossible to rule out the presence of two enzymes in very different concentrations (e.g., a high concentration of aromatic alcohol oxidase and a low concentration of terpenoid alcohol oxidase). At this point we do not have sufficient evidence to determine if there are one or several enzymes.

Phaedon armoraciae, an iridoid-producing leaf beetle, possessed a secretion that readily oxidized the natural terpene substrate analog **10** (the resulting acyclic dialdehyde was readily detected and identified by GC-MS as it accumulates in vitro, but is not present in the natural secretion) and the unnatural aromatic alcohol analogs **3** and **5** *Re*-specifically, but which preferentially oxi-

dized the terpene **10** when incubated in the presence of **3** (see Table 1). As with *P. vitellinae*, this suggested a single enzyme was responsible for the oxidation of both alcohols. Larvae of the iridoid-producing leaf beetle *Phratora laticollis* were only available in limited numbers, and their defensive secretion was only incubated with the mixture of **3** and **10.** However, the result of *Re*-specific oxidation of both alcohols, with preference for **10,** demonstrated a similarity of enzyme specificity to that in *P. armoraciae*.

In contrast, the defensive secretions of the iridoid-producing species *Gastrophysa viridula* and *Plagiodera versicolora* only oxidized their natural substrate analog **10** and not the aromatic alcohols **3, 5,** and **9** (see Table 1). However, like all the oxidations, *Re*-specificity was observed, selectively removing H_R from C-1 and C-8 of the substrate. The defensive secretions of *Hydrothassa glabra* and *Prasocuris phellandrii*, two more iridoid-producing leaf beetles, were only incubated with the natural metabolite analog 8-hydroxygeraniol (**10**). *Re*-specific oxidation was also observed in these species.

From the results, it was clear that there were a number of similarities and differences between oxidations carried out by the secretions of various leaf beetle larvae. First, all the leaf beetle secretions oxidized the substrates with *Re*-specificity, demonstrating an identical stereochemical course of oxidation in all species. Secondly, it has been shown that both salicylalcohol oxidase and the various 8-hydroxygeraniol oxidases are oxygen-dependent, $NADP^+$-independent enzymes (Veith et al., 1996; Duffey and Pasteels, in preparation). A major difference between the oxidative capabilities of the secretions was substrate specificity; defensive secretions from *P. vitellinae*, *P. armoraciae*, and *P. laticollis* were all able to oxidize salicyl- and terpenoid alcohols, whereas secretions from *G. viridula* and *P. versicolora* only oxidized their natural terpenoid substrate. Whether this indicates that the former group of species possess two enzymes (a terpenoid alcohol oxidase and an aromatic alcohol oxidase) or whether they each utilize a singe enzyme with relatively low substrate specificity is as yet unclear.

Since the *Phaedon armoraciae* secretion showed interesting substrate tolerance and because it was readily available from our laboratory culture, we studied its ability to oxidize a range of alcohols. A standard stock solution of secretion was prepared by dissolving the defensive secretions from 24 larvae in sodium phosphate buffer (2 ml). Aliquots (150 μl) of the solution were incubated with solutions of 8-hydroxygeraniol, geraniol, citronellol, and salicylalcohol (150 μl, 10 mM). The time course of the oxidations was monitored by UV spectroscopy over 16 hr, and the final concentrations of oxidized product were determined by gas chromatography. In order to account for any deterioration of the oxidase between incubations, 8-hydroxygeraniol oxidation was examined at the beginning of the experiments and again at the end. A reduction in the rate of oxidation (23%) was observed over the six days of measurements, and each

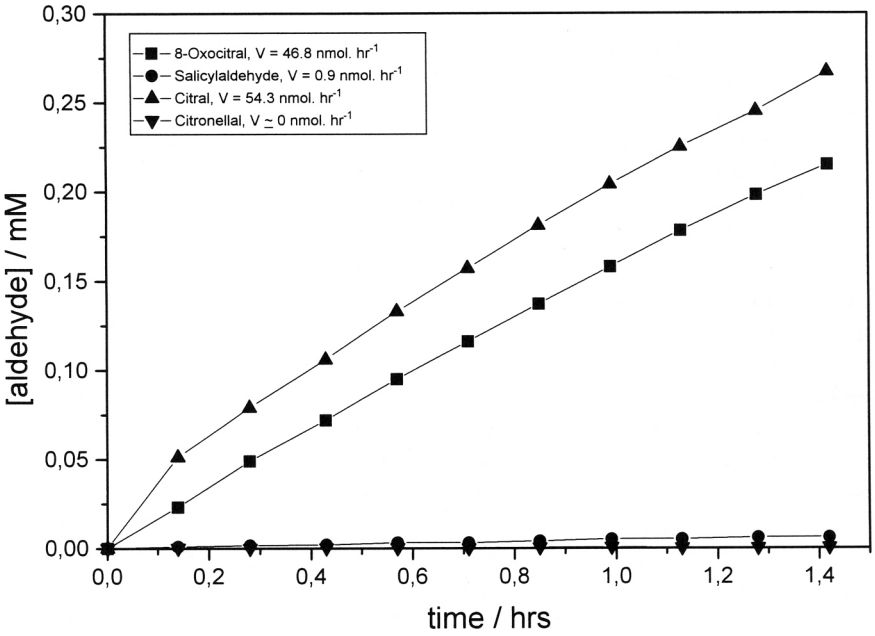


FIG. 4. Plot of aldehyde concentration against time for the oxidation of a number of terpenoid and aromatic alcohols by *Phaedon armoraciae* defensive secretion. Initial reaction velocities (V) are shown in the legend. The data result from the average of two separate sets of incubations.

incubation was corrected accordingly. Figure 4 shows curves of aldehyde (product) concentration vs. time for oxidation of the four alcohols by *Phaedon* defensive secretion (only the initial, largely linear period of the reaction is presented). The curves clearly illustrate that oxidation of 8-hydroxygeraniol and geraniol occurred smoothly. Interestingly, the initial velocity (V) of geraniol oxidation was determined to be slightly greater than that of the natural substrate 8-hydroxygeraniol (V = 54.3 and 46.8 nmol/hr, respectively). Oxidation of salicylalcohol was found to be very slow, with the low concentration of oxidase used (V = 0.9 nmol/hr). This large difference in velocity between 8-hydroxygeraniol and salicylalcohol oxidation explained the substrate specificity observed when the two compounds were incubated together with *Phaedon* defensive secretion (see Table 1). The absence of any detectable citronellol oxidation showed that the *Phaedon* oxidase(s) required an allylic or benzylic alcohol substrate, a result that was also found with 8-hydroxygeraniol oxidase from plants (Ikeda et al., 1991), although the plant enzyme is an $NADP^+$-dependent oxidoreductase, unlike the insect enzyme which is an oxygen dependent oxidase.

In a separate experiment, (±)-linalool (10 mM, 150 μl in buffer/1% DMSO, pH 7) was incubated with the defensive secretion from *Phaedon* (secretion from 2 insects in 150 μl buffer, pH 7) in order to establish whether the oxidase was capable of oxidatively rearranging the tertiary alcohol to geranial. However, no geranial was visible by GC. A positive result may have been significant, since almost all plants contain linalool (normally as a glycoside) and the ability of iridoid-producing leaf beetles to incorporate this substance into the iridoid pathway would have been advantageous to them.

To summarize, salicylalcohol oxidase from *P. vitellinae* is a *Re*-specific oxidase for which the orthohydroxy group of the substrate appears to be important in enzyme-substrate binding. This stereospecificity is identical to that of terpenoid oxidases from a range of iridoid-producing leaf beetle species. The defensive secretion of *P. vitellinae* is capable of oxidizing 8-hydroxygeraniol to 8-oxocitral, just as some of the secretions of the iridoid producers are able to oxidize salicylalcohol. The kinetics of oxidation by the secretion of one such species (*P. armoraciae*) reveal a large difference in reaction velocity between (allylic) terpenoid and aromatic alcohols.

These results suggest either that the oxidases employed by iridoid-producing leaf beetles and by the salicylaldehyde producing species, *P. vitellinae*, are very similar and may share a common origin or that some of the leaf beetles possess both a terpenoid alcohol and aromatic alcohol oxidase. In either case the enzymes link the species. Since it is believed that de novo iridoid production is the more primitive trait (Pasteels et al., 1990), it appears that the salicylalcohol oxidase from *P. vitellinae* may have evolved from an oxidase produced by an iridoid synthesizing ancestor.

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PROLONGED ADAPTATION AS POSSIBLE MATING DISRUPTION MECHANISM IN ORIENTAL FRUIT MOTH, *Cydia* (=*Grapholita*) *molesta*

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Abstract—The effects of pheromone preexposure on flight behavior of male Oriental fruit moth, *Cydia molesta*, were investigated in laboratory experiments using a wind-tunnel and in an orchard through insect releases at the center of a grid of pheromone traps. In the wind-tunnel experiments, the proportion of insects performing various behavioral phases was found to depend on the intensity and duration of the adapting stimulus and the recovery time allowed between exposure and measurement, indicating adaptation of at least some elements of the sensory system. The orchard experiments exhibited similar adaptation effects at similar exposure levels, causing the entry of treated insects into the traps to be delayed compared to controls and a reduction in insects caught for the highest preexposure concentration. The levels of pheromone exposure required to produce significant adaptation were, however, found to be much higher than could be expected under conditions of release of synthetic pheromone for insect control through mating disruption.

Key Words—Oriental fruit moth, *Cydia molesta*, *Grapholita molesta*, mating disruption, pheromone, adaptation, habituation.

INTRODUCTION

Certain pest Lepidoptera can be controlled by releasing synthetic sex pheromones to disrupt mating (Henneberry et al., 1982; Plimmer, 1982; Vickers et al., 1985; Cardé, 1990), but practical implementation requires decisions regard-

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ing the blend to be released, the rate of release, and the density of dispensers. Up to now these parameters have been determined empirically (Charlton and Cardé, 1982) and few laboratory procedures are available to speed up the process (Minks and Cardé, 1988). One reason for the absence of appropriate laboratory tests is that mechanisms of disruption are not well understood (Sanders, 1985; Weatherston, 1990; Liu and Haynes, 1993). Bartell (1982) and Cardé (1990) listed possible mechanisms that included adaptation of peripheral sensors, habituation of the central nervous system, confusion leading to males following false trails, and masking of the pheromone plumes from the females. Further development and fine-tuning of the mating-disruption technique would benefit greatly if the precise disruption mechanisms could be identified and those not contributing eliminated from design considerations. Accounts of mating disruption usually focus on confusion and false-trail following, for which there is anecdotal evidence but little quantitative data. Concerted attempts to better understand mating disruption mechanisms are long overdue.

The experiments reported here are directed towards establishing whether adaptation, with a time scale of an hour or so, is an active mechanism for mating disruption in the Oriental fruit moth, *Cydia* (=*Grapholita*) *molesta*. Such an effect can operate at the level of the peripheral or central nervous system. Bartell (1982) differentiated between these two possibilities by calling the first "adaptation" and the second "habituation". The methods used in this paper do not allow for any distinction to be made between these two possibilities and either or both effects could be present. Thus, the term "adaptation" has been used in a generic sense to indicate the possibility that the observed effects could be peripheral and/or central in nature.

Whether the sensory apparatus of an organism can be adapted may be established by applying an adapting stimulus, allowing some recovery time, and then assaying the behavior in response to the stimulus of interest. A reduced response to the stimulus would be an indication of adaptation. Increasing the strength and duration of the adapting stimulus should increase adaptation, whereas increasing the recovery period should allow the organism to recover and the behavioral response to be reestablished. The series of experiments carried out by Bartell and Lawrence (1973, 1976, 1977) on *Epiphyas postvittana* served as a starting point. These were performed in a bioassay apparatus (Bartell and Shorey, 1969) that constrained the insects inside small cages, allowing for only movement and wing flutter as an indication of excitation. We substituted a wind-tunnel, which, since it allows the insect to fly to the pheromone source, more closely resembles natural conditions (Linn and Roelofs, 1981; Kuenen and Baker, 1981; Sanders, 1982, 1985). Nevertheless the wind-tunnel still represents a highly artificial environment for the insect, so it was decided to develop an experimental procedure that would allow parallel experiments to be conducted with free-flying insects in an orchard.

METHODS AND MATERIALS

Insects. Oriental fruit moth males were taken from a laboratory culture that had been established from insects collected as pupae from an orchard two years earlier. They were reared on small apples and kept at 27°C with 14:10 day–night cycle. The larvae pupated in the hollows of corrugated cardboard placed inside the rearing boxes, and the sexes were separated as pupae. For the wind-tunnel experiments, 1- to 3-day-old male pupae were collected and the adults allowed to emerge, and kept till required, inside a lightbox with lights out set to midday. For orchard experiments pupae were synchronized to natural light cycles.

Adaptation Exposures—Wind-Tunnel and Orchard Experiments. The requirement for a simple system to preexpose the insects that could be used both in the laboratory and the field largely determined the choice of method. For the wind-tunnel experiments insects were placed in a 2.5-liter glass jar with a stainless steel wiremesh cage (5 cm tall, 5 cm diameter) suspended from the metal lid via a glass tube. The pheromone source was then lowered down the tube into the cage using a thread and a stopper clamped the thread and sealed the tube. This arrangement prevented the insects from coming into direct contact with the pheromone source. The insects were exposed in a fume cupboard, and as soon as the required period was over, they were removed from the jars and placed in small stainless steel mesh cages and moved to the wind-tunnel room. Control insects were placed in a similar jar but with no cage. The jars, lids, cage, and tube were heated to 120°C between experiments to remove any pheromone, and they were also liberally washed with acetone whenever a change in pheromone concentration was effected.

An identical arrangement to the above was tried for the orchard experiments with release of the insects effected by removing the lids together with the pheromone and separator cages from the jars. It was found, however, that the insects experienced some difficulties in flying out of the jars. Attempted flights usually saw them landing somewhere else inside the jar, indicating that they probably could not differentiate between the clear glass sides and the open top. A slightly different exposure and release arrangement was therefore used. The insects were placed in a wire mesh cage as wide as would fit through the neck of a jar and the pheromone source lowered into the jar via a hole in the lid and suspended just above the top of the cage. Control insects were treated similarly but no sources was placed in the jar. Release strategies are further discussed below under Orchard Experiments.

The sources of pheromone used to deliver the adapting stimulus were made from 15-mm lengths of 0.5-mm-OD surgical rubber tubing loaded with pheromone by dipping them into a three-component solution in cyclohexane, (*Z*)8-decadienyl acetate (*Z*8–12:OAc), (*E*)-8-decadienyl acetate (*E*8–12:OAc) and (*Z*)-8-decadienyl alcohol (*Z*8–12:OH)], in a 20:1:1 ratio (Cardé et al., 1979).

Sources with three different rates of release were produced approximating 32, 320, and 3200 times the rate of a female moth. The 1 female equivalent (FE) standard, used throughout this document, was the average release rate during the calling period of 8.5 ng/hr measured by Lacey and Sanders (1992). The release rates from the sources were checked using a gas chromatograph.

The concentration of pheromone in the jars was measured by sampling the air inside a round chemical flask of similar volume. The flask was substituted for the jars because less then perfect sealing of the lids on to the jars made it impossible to force air to flow through the jars at the slow rates required for sampling without causing a significant fall of concentration inside the jar. The flasks had three separate necks. The source was inserted through the central neck, dry air introduced through a side neck at a rate of 10 ml/min (nominal), but measured each time, and the air leaving the flask through the third neck made to pass through a 30-cm-long × 1-mm-diameter glass tube cooled with Dry Ice for 50 mm at the center. The pheromone deposited on the surface of the glass tube was measured by running two separate 5-μl beads of hexane along its length, collecting the material with a syringe, and injecting it into a gas chromatograph. A measured amount of a Z11–12:OAc solution of known concentration was added to calibrate the instrument.

Wind-Tunnel Measurements. The Perspex wind-tunnel measured 0.43 m × 0.43 m × 1.8 m. Air was driven through it using a household fan at approximately 0.35 m/sec and exhausted to the outside by a fan placed 0.5 m from the end. A personal computer connected to series of buttons mounted along the length of the tunnel was used to record takeoffs, sustained flights, approaches to source, and landings on the source.

Insect performance in the wind-tunnel was tested with a 2.4-FE source, which elicited the best flying and landing responses. Measurements of responses in the wind-tunnel commenced as soon as the designated recovery period after adaptation was over and continued over the next 30 min or until the supply of insects was exhausted. Insects were removed from the tunnel when they landed on the source or 2 min had elapsed since their introduction into the tunnel, whichever was first. Introductions of three exposed insects into the tunnel were alternated with three unexposed controls.

Orchard Experiments. The experiments were carried out in a small experimental apple orchard at the northern edge of the city of Canberra in which a 5 × 5 grid of Pherocon 1CP-type traps was set up. The traps, each baited with a 32-FE pheromone source, were hung from tree branches at a height of 2 m. Their separation, dictated by the orchard layout, was 7 m in a N–S and 10 m in an E–W direction. The central trap position from which insects were released was left vacant. Similar numbers of insects were placed in the treatment and control jars in the laboratory and dusted with acetone-soluble dye powders of different colors by dropping a small quantity of the dye in the jars and shaking

them gently. The jars were then transported to the orchards and placed in a shaded location 50 m from the grid of traps. At the appropriate time the pheromone source to be used to adapt the treated insects was lowered into its jar. Once the exposure period was over, the adapting source was removed and the jars taken to the center of the grid and the insects released.

Two different release strategies were tried. Either the cages were removed from the jars, the two ends removed, and the cage placed in the foliage so that the insects were free to fly or the exposed and control insects were tapped out of the cages onto separate wooden platforms. We were interested to discover whether residual pheromone on the cage of the exposed insects might keep them longer inside the cage or whether disturbing them by forcing them out of the cages would affect their subsequent behavior. We were unable to detect any difference between the two methods, and the results have been combined. Once the insects had been released, the pheromone traps were checked at 5-min intervals and insects found in the traps transferred to filter papers and the time of the removal recorded. A drop of acetone added to each insect diffused the dye over the filter paper and allowed treated, control, and wild males to be differentiated.

The activity period of Oriental fruit moth is strongly influenced by temperature and light conditions, which varied considerably from day to day in the area where the experiments were carried out. On warm days, 25°C and above, activity started about 90 min before dark, but it commenced earlier and the activity period was shorter on cooler days. Because the treatments had to begin an hour before the release time, we had to try to predict the onset of flight activity. On some nights when the temperature fell more rapidly than expected the insects were released too late and none or very few were recaptured. We discarded such data sets and retained only those for which at least 20% of the released insects were recaptured.

RESULTS

Wind-Tunnel Measurements. The percentage of insects progressing through the various behavioral stages, from takeoff to landing on the source, following different treatments are shown in Figures 1a–f. Exposures to 32 or 320 FE for 10 min with 20 min of recovery time or for 60 min with 30 min of recovery produced no reduction in the number of insects reaching the source (Figure 1a–c). Treated insects were consistently found to perform slightly better than the controls, although pairwise comparisons of percentages at each behavioral stage indicated no significant difference at a 95% level of confidence. Reductions were not observed until the adapting source was increased to 3200 FE. A 10-min exposure with a 20-min recovery reduced performance by approximately

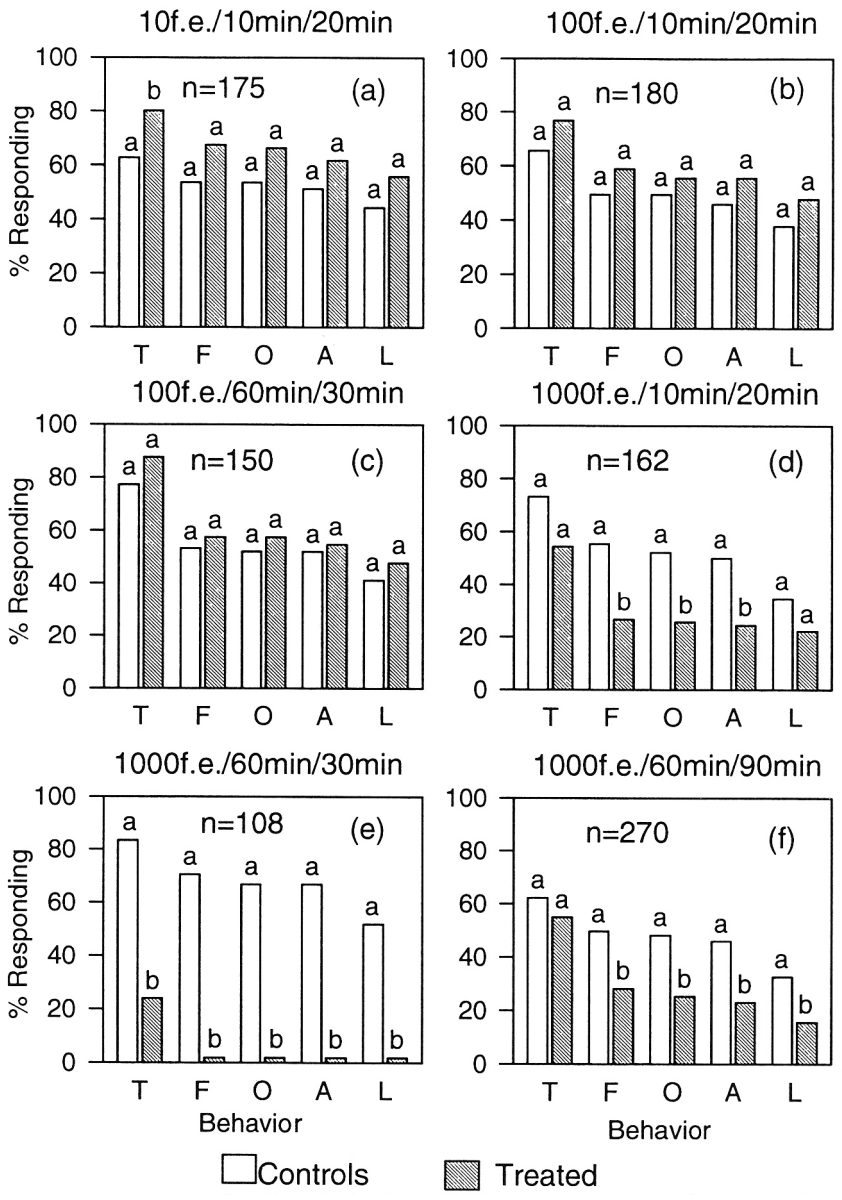

FIG. 1. Takeoffs (T), flights (F), oriented flights (O), approaches (A), and landings (L) on a 2.4-FE source in the wind-tunnel. Plot headings indicate: source strength/length of exposure/recovery time before start of measurement period, which extended over a further 30 min. (Column pairs with the same letter are not significant at 1% probability level, χ^2 test.)

half (Figure 1d), and all except one of the treated insects were inhibited from oriented flight when the exposure was increased to 60 min with 30 min allowed for recovery, (Figure 1e). Increasing the recovery time to 90 min restored the performance of approximately half of the treated insects (Figure 1f).

Orchard Measurements. The proportions of control and treated insects recaptured for the three treatments are shown in Figure 2. At 32 and 320 FE, there was no difference between control and treated insects but a significant reduction in the proportion of treated insects recaptured was found with exposure to 3200 FE. The mean times from release to recapture are shown in Figure 3. Recapture times for each treatment were compared using the Mann-Whitney U test. A nonparametric method was used because considerable divergence from a normal distribution precluded a test based on comparison of the means. There was no significant difference between a 32-FE source and controls, but the mean recapture times were significantly longer ($P < 1\%$ probability of a null result) for the 320- and 3200-FE sources than for the controls.

Preexposure Concentrations. The average concentration of pheromone in the round flasks averaged over a 1-hr period following introduction of the source was 1.2 ± 0.4 $\mu g\ m^3$ ($N = 3$) for the 32-FE source and 65 ± 17 $\mu g\ m^3$ ($N = 6$) for the 3200-FE source.

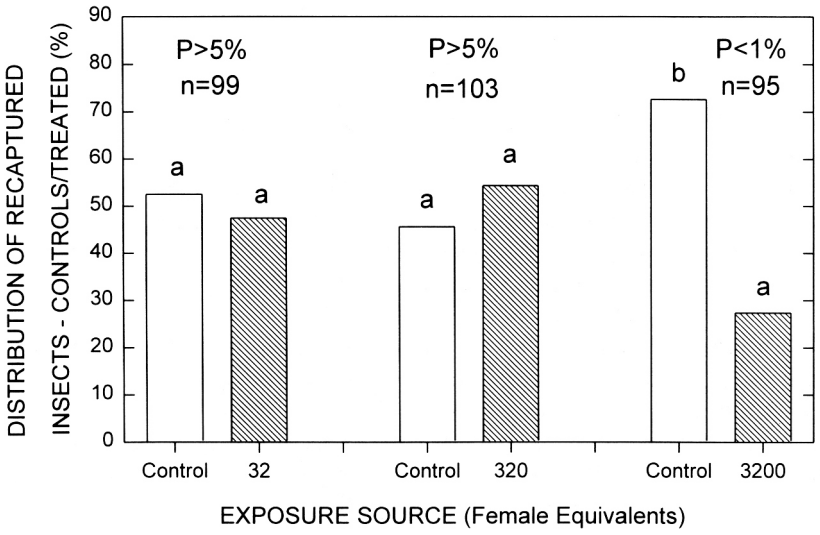

FIG. 2. Control and treated (60 min exposure to 32-, 320-, and 3200-FE sources) insects expressed as percentages of total number recaptured (n) after release in an orchard. (Column pairs with the same letter are not significantly different at indicated probability levels, χ^2 test.)

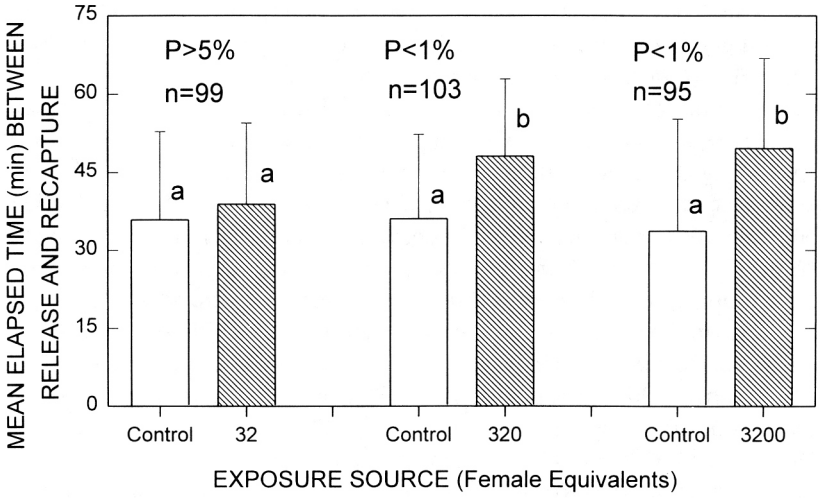

FIG. 3. Mean times from release to recapture for control and treated (60 min exposure to 32-, 320-, and 3200-FE sources) insects. [Column pairs with the same letter are not significantly different at indicated probabilities. Mann-Whitney U test. The means and error bars were calculated by bootstrap analysis (Efron, 1979) for presentation purposes only, they were not used in the estimation of significance].

DISCUSSION

The wind-tunnel results illustrate a classic adaptation–disadaptation effect. Adapting stimuli of low intensity and short duration have no observable detrimental effect on behavior. As stimulus intensity and exposure times are increased, there is then an intermediate region in which partial adaptation is apparent, culminating in high levels of adaptation when a high enough dose and exposure are used. If the recovery period is then extended, the organisms recover and start to respond once again.

We interpret the data collected in the orchard (Figure 4) as an indication of the period required by the treated insects to recover from adaptation caused by exposure to the pheromone. The arrival of Oriental fruit moth males at pheromone traps occurs within a window of approximately 60–90 min (Rothschild and Minks, 1974). The limits of this window are determined by light and temperature conditions, with darkness always being a terminating factor. Insects were released as close as possible to the start of this window, and the frequency at which they entered the pheromone-baited traps was observed to reach a peak and then decline, represented schematically by normal curves in Figure 4. With the 32-FE exposure (Figure 4a), no effective adaptation takes place, and the

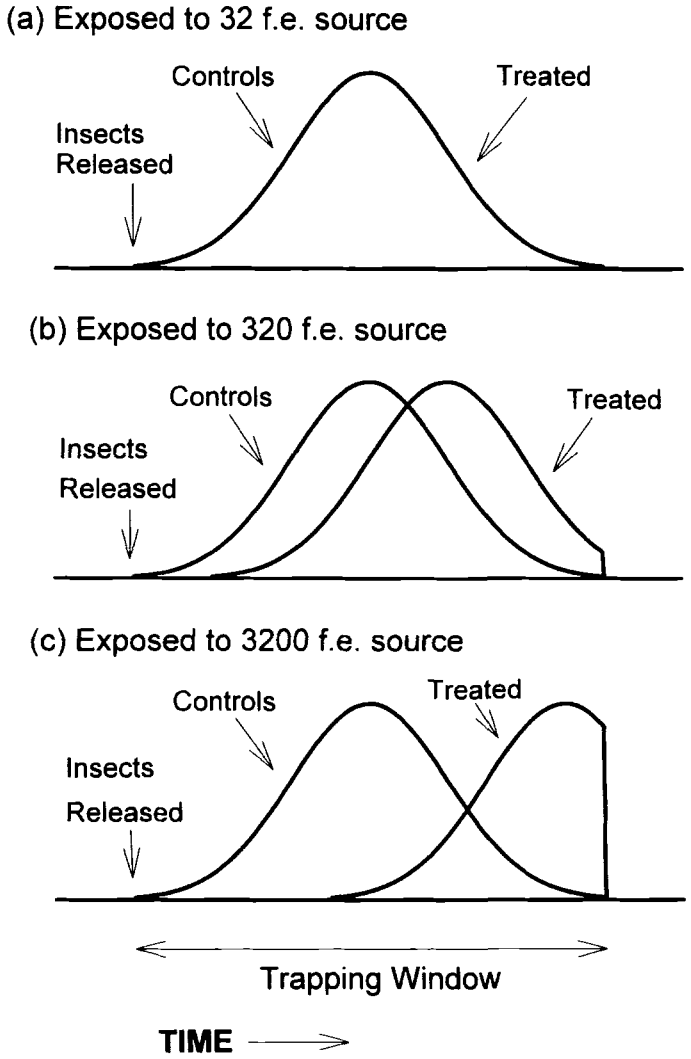

FIG. 4. Hypothesized recapture frequency shift due to adaptation, after exposure to (a) 32-, (b) 320-, and (c) 3200-FE sources.

number of controls and treated insects reaching the traps are the same with similar frequency distributions. For the 320- and 3200-FE exposures, we postulate that adaptation causes a delay in the movement of the treated insects to the traps, resulting in an observable increase in the average time of arrival at the traps. The delay is small with the 320-FE exposure, so that there is no

effective reduction in the numbers recaptured (Figure 4b), because they all recover before the close of the flight window. However, with the 3200-FE source (Figure 4c), the delay is long enough to cause the trailing edge of the flight window to cut off a significant part of the distribution, causing a drop in the number of insects entering the traps.

Very few direct quantitative measurements of pheromone in the air under mating disruptive conditions have been carried out. There are none available for the Oriental fruit moth, but Flint et al. (1990, 1993), using aerial collection with charcoal cartridges and GC-MS instrumentation, obtained measurements in the range of 1–5 ng/m^3 for Gossyplure (used to control pink bollworm), dispensed from 1000 dispensers per hectare and releasing 25–80 mg/dispenser/hr. Under a rather different canopy, a pea crop, Witzgall et al. (1996) measured concentrations of 2 and 3 ng/m^3 using EAG techniques. Calculations based on rates of release of pheromone from dispensers, [e.g., Sauer et al. (1992)], indicate that these values are within the expected range and would not be very different from mating disruption applications against Oriental fruit moth.

The dynamics of pheromone distribution in an orchard are, however, much more complex than suggested by the measurements described above. They include absorption by leaves, etc. (Suckling et al., 1996), which could lead to sheltering of an insect and exposure to concentrations lower than the average, but also the possibility that an insect may also perceive much higher pheromone levels if sitting close to a dispenser. Pheromone concentrations in an orchard also fluctuate due to air movements, as observed by Suckling and Angerilli (1996) using EAG techniques. The exposure to pheromone of the insects in the jars would not be constant but would have increased from the time the source was introduced up to a point of equilibrium, although the insects would not have experienced high-frequency fluctuations in pheromone concentration. How comparable the two exposures regimes are may depend on the averaging of such fluctuations by the sensory apparatus, CNS processing by the insect itself, and the ratio of the peaks of concentration to the average. Bartell and Lawrence (1977) observed that pulsed pheromone was more effective in disrupting behavior than constant levels. Suckling and Angerilli (1996) used a dispenser density lower than is normally used in mating disruption, 100 distributed singly in a 10 $\times$ 10 array or grouped at one (all 100 dispensers) or nine (11 dispensers each) sites in a single 0.5-ha plot. This compares with the mating disruption density of 1000 dispensers/ha in larger plots recommended for most insects. They also could not measure any nonfluctuating background levels due to interference with EAG signals from other sources such as plant odors (Rumbo et al., 1995). The ratio of the peak pheromone levels to the average could therefore not be determined. Suckling and Angerilli (1996) found an inverse relationship between pheromone pulse frequency and trap catch with the moth *Epiphyas postvittana* but, with their lower density of dispensers, areas of the orchard may have

experienced dropouts in pheromone concentration, especially when their dispensers were concentrated at only one or nine sites. From their data it is difficult to positively establish that the sharp pulses of higher pheromone concentration have any effect on mating disruption. The application of EAGs in the field is relatively new and such information may eventually become available as measurement techniques are refined. What is clear is that for those few cases for which the average concentration of pheromone has been measured, it is much lower than the 65 $\mu l/m^3$ found to be required in the jars to achieve worthwhile long-term adaptation. One must therefore conclude that long-term adaptation arising from nonfluctuating concentrations of pheromone is unlikely to be a mechanism involved in mating disruption in the Oriental fruit moth. Any long-term adaptation arising from fluctuations in pheromone concentrations remains to be determined.

These observations do not prove that adaptation is absent when moths are continuously surrounded by a pheromone-permeated atmosphere at concentration levels used for mating disruption. However, they do indicate that recovery from exposure to such levels of pheromones in the air is quite rapid, of the order of minutes, when the pheromone is removed. With most insect species mating at dusk or later, the critical period for mating disruption usually coincides with falling temperature, which results in a reduction in the amount of pheromone released from the dispensers compared to earlier in the day (Sexton, 1989; Bradley et al., 1995). Apart from some buffering action due to absorption in biological structures and rerelease later on (Suckling et al., 1996), any adaptation caused by high amounts of pheromone released during the warmer parts of the day will not contribute to mating disruption. It is essential that dispensers be designed to release enough pheromone during the active period of the moth, but with current designs this implies that most of the active material is wasted by being released at the wrong time.

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SEMIOCHEMICAL-MEDIATED LOCATION OF HOST HABITAT BY *Apanteles carpatus* (SAY) (HYMENOPTERA: BRACONIDAE), A PARASITOID OF CLOTHES MOTH LARVAE

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Abstract—In Y-tube olfactometer bioassays, adult *Apanteles carpatus* (Say), were attracted to beaver or rabbit pelts infested with larvae of the casemaking clothes moth (CCM), *Tinea pellionella* L. Porapak Q-captured volatiles from a CCM-infested beaver pelt were also very attractive, whereas isolated CCM larvae or larval feces were not. Coupled gas chromatographic-electroantennographic detection (GC-EAD) analysis of the Porapak Q volatile extract revealed two compounds that elicited responses by *A. carpatus* antennae. Coupled GC–mass spectrometry (MS) in electron impact and chemical ionization modes of these compounds indicated, and GC-MS and GC-EAD of authentic standards confirmed, that they were nonanal and geranylacetone. While each compound singly did not attract *A. carpatus*, a 1 : 1 blend of both compounds was as attractive as the volatile extract. Because these compounds are host habitat-derived, *A. carpatus* must be a habitat rather than host specialist, responding to kairomonal indicators of localized and specific habitats such as animal hair or feather. The tritrophic interaction between *A. carpatus*, its clothes moth hosts and their animal-derived habitats is similar to the well-studied relationship between parasitoids of insect herbivores and their host plant habitats.

Key Words—*Apanteles carpatus, Tinea pellionella*, Hymenoptera, Braconidae, Lepidoptera, Tineidae, semiochemicals, coupled gas chromatographic-electroantennographic detection, host-habitat location, tritrophic interactions, geranylacetone, nonanal.

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INTRODUCTION

Host location by hymenopterous parasitoids is commonly mediated by semiochemical stimuli (Vinson, 1984). These are often produced by the insect host itself or its products, such as larval frass or silk (Lewis and Jones, 1971; Wesoloh, 1976). In well-studied tritrophic communication systems, host-finding by parasitoids is mediated by semiochemicals emitted by the host habitat (Dicke et al., 1990; Turlings et al., 1990; Steinberg et al., 1992), including host plants (Takabayashi et al., 1991; Turlings et al., 1991) or fungi (Vet, 1983; Vet and Van Opzeeland, 1985) and yeasts (Dicke et al., 1984) growing on (dead) plants.

Apanteles carpatus (Say) (Hymenoptera: Braconidae) is a parthenogenetic parasitoid of larval clothes moths, such as the webbing clothes moth (WCM), *Tineola bisselliella* (Hum.) and the casemaking clothes moth (CCM) (Lepidoptera: Tineidae) (Figure 1)(Fallis, 1942; Rutz and Scoles, 1989). The natural environment of the CCM includes well-sheltered nests (Woodroffe, 1953), dry corpses (Bornemissza, 1957), or animal lairs (Mallis, 1969; Hill, 1990) that are not exposed to direct light. CCM larvae feed on and digest animal-derived

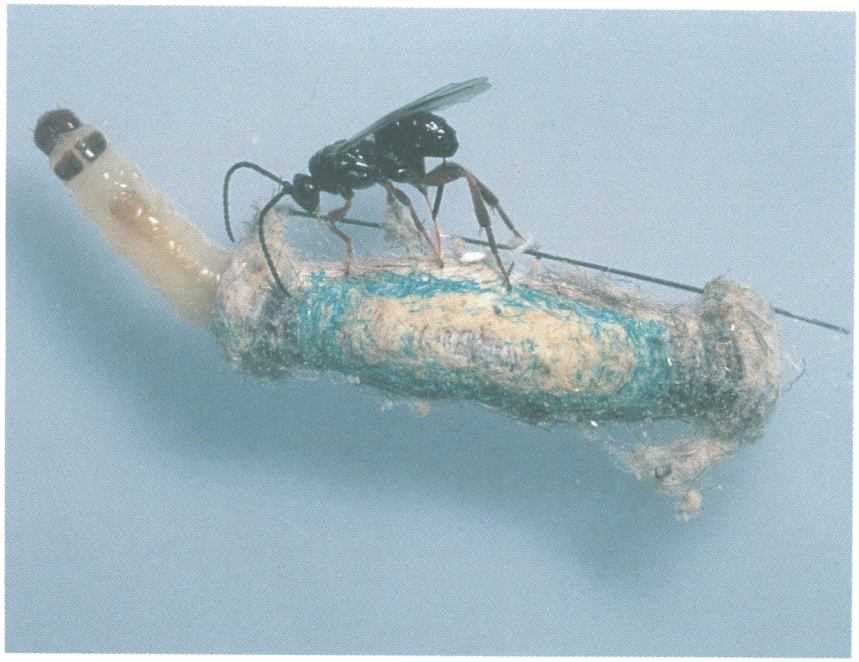

FIG. 1. Female *Apanteles carpatus* (Say) ovipositing in a larva of the casemaking clothes moth, *Tinea pellionella* L. Note the green wool in the casing of the larva.

fabric, feather, hide, horn, hair, and fur, causing substantial damage in textile and fur warehouses, museums, and households (Mallis, 1969; Hill, 1990).

Our objectives were to determine qualitatively if host location by *A. carpatus* were semiochemical-mediated, locate the source of and identify the semiochemicals, and characterize their attractiveness to *A. carpatus*.

METHODS AND MATERIALS

Insect Cultures

Adult *A. carpatus* emerged from CCM larvae that had infested a 20-month-old, untanned beaver pelt in a museum storage area. The pelt originated from British Columbia's interior. It was kept at 20–25°C, 40–70% relative humidity, and a 10L:14D photoperiod in a 40-liter glass container with a mesh lid. Second- and third-generation parasitoids were reared on larvae of WCM (Insect Control and Research, Baltimore, Maryland 21228) and larvae of CCM, respectively, feeding on discarded wool clothing (WCM) and beaver pelt (CCM), both supplemented with brewer's yeast. When beaver pelts became unavailable, they were replaced by alum-tanned rabbit pelts (Pacific Leather & Fur Dressers, Vancouver, British Columbia.)

For two experiments (Table 1; experiments 2 and 3), adult *A. carpatus* eclosed from cocoons removed from CCM casings and placed individually in 35-mm Petri dishes. Parasitoids for all other experiments (experiments 1, 4–14) emerged naturally from CCM larvae on rabbit (experiments 4, 5, and 7) or beaver pelts (experiments 3, 6, 8–14). Groups of about 20 *A. carpatus* adults were maintained in wooden cages (15 × 15 × 12 cm) with mesh backs and Plexiglas fronts and were provided daily with fresh sliced apple, sucrose, and a 10% honey–water solution.

Capture and Extraction of Volatiles

A CCM-infested beaver pelt (900 cm^2) was aerated for one week in a cylindrical Pyrex glass chamber. A water-aspirator drew charcoal-filtered, humidified air at 2 liters/min through the chamber and a glass column (14 cm × 13 mm OD) filled with Porapak Q (50–80 mesh, Waters Associates, Inc., Milford, Massachusetts 01757). Volatiles were eluted from the Porapak Q with 5 ml of redistilled pentane. The eluent was concentrated to 2 ml by distillation in a 30-cm Dufton column, adjusting the volatile extract so that 2 μl equaled 5 pelt-min of volatile collection. Uninfested, salted, air-dried deer pelt (500 cm^2) and 25 pieces (2.3 cm^2 each) of "cut" and "intact" rabbit pelt were also aerated and volatiles eluted and concentrated as above. Fur of cut rabbit pelt was shortened to 5 mm with blunt scissors and the cut hairs were included in the aerated

TABLE 1. NUMBERS OF HOST-NAIVE OR HOST-EXPERIENCED *Apanteles carpatus* AND DETAILS ON STIMULI TESTED IN Y-TUBE OLFACTOMETER OR WIND-TUNNEL BIOASSAYS

Exp.	Parasitoids tested (*N*)	Disposition of parasitoids[a]	Y-tube Olfactometer	
			Arm1	Arm 2
1	20	Experienced	2.3-cm^2 piece of beaver pelt infested ≥5 days with 5 CCM larvae (fourth instar)	Blank
2	50	Naive	2.3-cm^2 piece of rabbit pelt infested ≥3 days with 5 CCM larvae (fourth instar)	2.3-cm^2 piece of uninfested rabbit pelt
3	20	Experienced	2.3-cm^2 piece of rabbit pelt infested ≥3 days with 5 CCM larvae (fourth instar)	2.3-cm^2 piece of uninfested rabbit pelt
4	20	Experienced	larval feces (10 mg) collected within 24 hr of production by 200 CCM larvae (third–fourth instar) feeding on rabbit pelt; stored at −2°C until 1 hr before testing	Blank
5	40	Experienced	5 CCM free-moving larvae (fourth instar) with casing	2.3-cm^2 piece of rabbit pelt infested 5 days with 5 CCM larvae (fourth instar)
6	30	Experienced	2.3-cm^2 piece of beaver pelt infested ≥5 days with 5 CCM larvae (fourth instar)	2.3-cm^2 piece of beaver pelt infested ≥5 days with 5 CCM larvae (fourth instar) removed just prior to bioassay
7	40	Experienced	2.3-cm^2 piece of rabbit pelt with scissors-cut hair	2.3-cm^2 piece of rabbit pelt with intact hair
8	20	Experienced	Porapak Q-captured volatiles from beaver pelt infested ≥5 days with	Pentane (2 μl) control on Whatman no. 1 filter paper

			CCM larvae (5 pelt-min in 2 μl of pentane on Whatman no. 1 filter paper)	
9	20	Experienced	Nonanal (16 ng) in hexane (2 μl) on Whatman no. 1 filter paper	Hexane (2 μl) control on Whatman no. 1 filter paper
10	20	Experienced	Geranylacetone (16 ng) in hexane (2 μl) on Whatman no. 1 filter paper	Hexane (2 μl) control on Whatman no. 1 filter paper
11	20	Experienced	Geranylacetone (16 ng) in pentane (2 μl) on Whatman no. 1 filter paper	Porapak Q-captured volatiles from beaver pelt infested ≥5 days with CCM larvae (5 pelt-min in 2 μl of pentane on Whatman no. 1 filter paper)
12	30	Experienced	Geranylacetone (16 ng) and nonanal (16 ng) in pentane (2 μl) on Whatman no. 1 filter paper	Porapak Q-captured volatiles from beaver pelt infested ≥5 days with CCM larvae (5 pelt-min in 2 μl of pentane on Whatman no. 1 filter paper)
13	30	Experienced	Geranylacetone (8 ng) and nonanal (8 ng) in hexane (2 μl) on Whatman no. 1 filter paper	Geranylacetone (8 ng) in hexane (2 μl) on Whatman no. 1 filter paper
			Windtunnel	
			Tube 1	Tube 2
14	60	Experienced	Geranylacetone (8 ng) and nonanal (8 ng) in hexane (2 μl) on Whatman no. 1 filter paper	Hexane (2 μl) on Whatman no. 1 filter paper

[a]For experiments 2–5 and 7, exposure (>24 hr) of emergent *A. carpatus* to rabbit pelt (2.3 cm^2) infested with two fourth-instar CCM feeding >3 days, produced host-experienced parasitoids. Naive parasitoids had no exposure to host-infested pelt. Host-experienced *A. carpatus* for experiments 1, 6, and 8–14 were produced by exposing (>24 hr) emergent adults to fourth-instar CCM feeding >5 days on beaver pelt.

material. Separated casings and larvae from 150 CCM third to fifth instars and 150 WCM fourth and fifth instars were extracted for 5 min in hexane. The extracts were concentrated by a gentle nitrogen stream so that 1 μl equaled 10 larval equivalents.

Volatile Analyses

Aliquots of 2.5 pelt-min equivalents of Porapak Q-captured volatiles or 10 larvae equivalents of hexane extracts were subjected to analysis by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-210, or DB-23 (J & W Scientific, Folsom, California 95630). Full-scan electron-impact (EI) and chemical-ionization (isobutane) (CI) mass spectra of EAD-active compounds were obtained by coupled GC–mass spectrometry (MS), using a Varian Saturn II Ion Trap GC-MS and a HP 5985B GC-MS, respectively, each fitted with the DB-210 or DB-5 column referred to above.

Laboratory Bioassays

Y-tube Olfactometer Bioassay. Anemotactic responses of 20–50 walking *A. carpatus* parasitoids to odor sources were assessed per experiment in a Y-shaped Pyrex glass olfactometer (ID 23 mm; stem 25 cm; arms 20 cm at a 120° angle) at 22–27°C and 40–70% relative humidity. Air drawn through the apparatus at 5 liters/min with a water aspirator carried volatiles from odor sources inside the arms toward parasitoids released individually into the stem of the Y-tube. A parasitoid that penetrated ≥ 10 cm into a side arm within 15 min was classified as a responder. All others were classified nonresponders and were not included in statistical analyses. Each replicate employed a new odor source, Y-tube, and parasitoid. For each replicate, odor sources were randomly assigned to, and placed near, the orifice of side arms. During bioassays, a spotlight at the Y-junction served as a light source, and filter paper barriers placed in each arm downwind of test stimuli standardized visual cues. Y-tubes were washed between replicates with Sparkleen and dried at 125°C for > 15 min.

Wind-Tunnel Bioassay. Upwind flight by *A. carpatus* in response to volatile stimuli was assessed in a Plexiglas wind tunnel (0.5 × 0.5 × 1.0 m) illuminated with a diffused, broad-spectrum 40-W fluorescent light, through which air was drawn at 8 cm/sec in a laminar flow (McDonald, 1995). Upwind, paired glass tubes (11 cm OD, 25 cm apart, 3 cm above the wind-tunnel floor) were baited with Whatman no. 1 filter paper impregnated with volatile stimuli. Five parasitoids in each of 12 replicates were introduced 1 m downwind of the volatile sources and their flight behavior was observed for up to 30 min. A parasitoid entering a glass tube was recorded as a responder and was immediately removed.

All others were classed as nonresponders and were not included in statistical analyses.

Bioassay Experiments. Fourteen binary choice experiments were conducted (Table 1). The first experiment tested the response of host-experienced parasitoids to odors of CCM-infested beaver pelt *versus* an unbaited arm. The second and third experiments tested the response of host-naive (experiment 2) and host-experienced (experiment 3) parasitoids given a choice between uninfested and CCM-infested rabbit pelt. To locate the source of semiochemicals, experiments 4–7 tested the response of experienced parasitoids (first generation) to various odor sources, as follows: CCM larval feces *versus* blank (experiment 4); rabbit pelt with CCM larvae *versus* CCM larvae (experiment 5); beaver pelt with or without CCM larvae (experiment 6); and intact *versus* scissors-cut rabbit pelt (experiment 7).

Experiments 8–13 tested the response of parasitoids (third generation) to volatile extracts and to EAD-active, synthetic volatiles, as follows: volatiles from CCM-infested beaver pelt *versus* control (experiment 8); geranylacetone (experiment 9) and nonanal (experiment 10) (both Aldrich Chemical Company, Milwaukee, Wisconsin 53233) *versus* control; volatiles from CCM-infested beaver pelt *versus* geranylacetone (experiment 11) and *versus* geranylacetone plus nonanal (experiment 12); and geranylacetone *versus* geranylacetone plus nonanal (experiment 13). A final experiment (experiment 14) tested upwind flight of host-experienced *A. carpatus* (third generation) in response to geranylacetone plus nonanal or to a solvent control.

Statistical Analysis

The numbers of parasitoids responding to stimuli in the laboratory bioassays were compared with the χ^2 goodness-of-fit test, using Yates' correction for continuity ($\alpha = 0.05$) (Zar, 1984).

RESULTS

Experienced *A. carpatus* were strongly attracted to CCM larvae feeding on beaver pelt (Figure 2, experiment 1). Similarly, both experienced and naive parasitoids responded to CCM larvae feeding on rabbit pelt (Figure 2, experiments 2 and 3). In various experiments designed to identify the source of the attraction of *A. carpatus*, feces from CCM larvae did not elicit a response (Figure 3, experiment 4); rabbit pelt with feeding CCM larvae was preferred over isolated CCM larvae and casings (Figure 3, experiment 5); and pieces of infested beaver pelt with or without feeding larvae were equally attractive (χ^2 test, $P = 0.36$) (Figure 3, experiment 6). Scissors-cut rabbit pelt was more attractive than intact rabbit pelt (Figure 3, experiment 7). The low responsive-

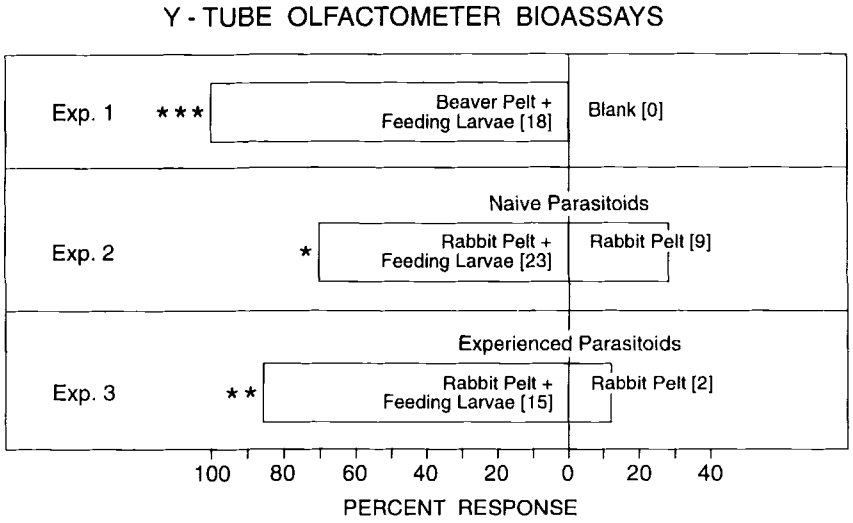

FIG. 2. Responses in experiment 1 (Table 1) of walking host-experienced *Apanteles carpatus* in a Y-tube olfactometer to beaver pelt infested with third- to fifth-instar case-making clothes moth (CCM), *Tinea pellionella*, or to a blank; and responses in experiments 2 and 3 (Table 1) of host-naive and host-experienced *A. carpatus* to uninfested rabbit pelt or to pelt infested with third- to fifth-instar CCM. Numbers of parasitoids responding to each stimulus are given in parentheses. Bars with asterisks indicate a significant preference for a particular treatment (χ^2 test; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

ness of parasitoids in experiments 2 and 5 may be attributed to low barometric pressure (Steinberg et al., 1992).

Extracts of Porapak Q-captured volatiles from infested beaver pelt were highly attractive (Figure 4, experiment 8). Analysis of these volatiles by GC-EAD revealed two consistently EAD-active compounds (Figure 5), with both EI and CI mass spectra very similar to those previously reported for nonanal and geranylacetone (Adams, 1989). Identical retention and mass spectral characteristics and comparable EAD activity of authentic nonanal and geranylacetone and corresponding compounds in volatile extracts confirmed these assignments. GC-EAD analyses also qualitatively confirmed the presence of these two compounds in volatiles emanating from deer and rabbit pelts but failed to detect them in hexane extracts of CCM larvae or their casings or in WCM larvae. Geranylacetone, but not nonanal (χ^2 test, $P = 0.17$), singly attracted *A. carpatus* (Figure 4, experiments 9 and 10).

Volatiles from infested beaver pelt were more attractive than geranylacetone alone (Figure 4, experiment 11) and as attractive (χ^2 test, $P = 0.09$) as ger-

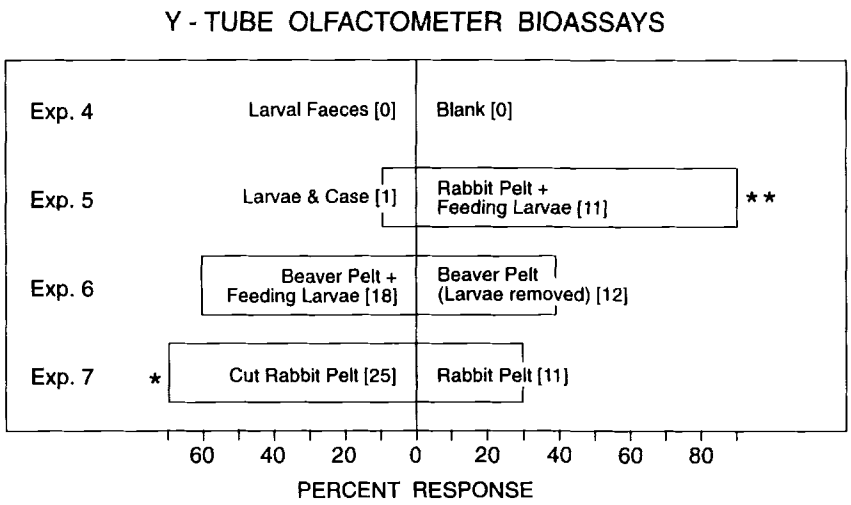

FIG. 3. Responses in experiments 4–7 (Table 1) of walking host-experienced *Apanteles carpatus* in a Y-tube olfactometer to various stimuli from larvae of the casemaking clothes moth, *Tinea pellionella*, or to stimuli from host habitat. Numbers of parasitoids responding to each stimulus are given in parentheses. Bars with asterisks indicate a significant preference for a particular treatment (χ^2 test; $*P < 0.05$; $**P < 0.01$).

anylacetone plus nonanal (Figure 4, experiment 12). These two compounds combined were significantly more attractive than geranylacetone alone (Figure 4, experiment 13). In the wind-tunnel bioassay, attractiveness of these two compounds exceeded that of a solvent control (Figure 4, experiment 14).

DISCUSSION

Orientation of *A. carpatus* to CCM-infested beaver pelt (Figure 2, experiment 1) or to extracts of Porapak Q-captured volatiles from infested beaver pelt (Figure 4, experiment 8) and antennal responses to compounds in the captured volatiles (Figure 5) indicate olfactory recognition of, and long range attraction to, the host and/or host habitat. While further experiments are necessary to fully explore the phenomenon of "learning" (Vet, 1983; Steinberg et al., 1993; Turlings et al., 1993) in *A. carpatus*, similar attraction of host-naive and host-experienced parasitoids to CCM-infested rabbit pelt (Figure 2, experiments 2 and 3) suggests that the foraging response is unconditioned. Lack of parasitoid attraction to isolated host larvae plus casing (Figure 3, experiment 5) or to larval feces (Figure 3, experiment 4) and equal attraction to beaver pelt with or without host larvae (Figure 3, experiment 6) suggests that foraging behavior is mediated

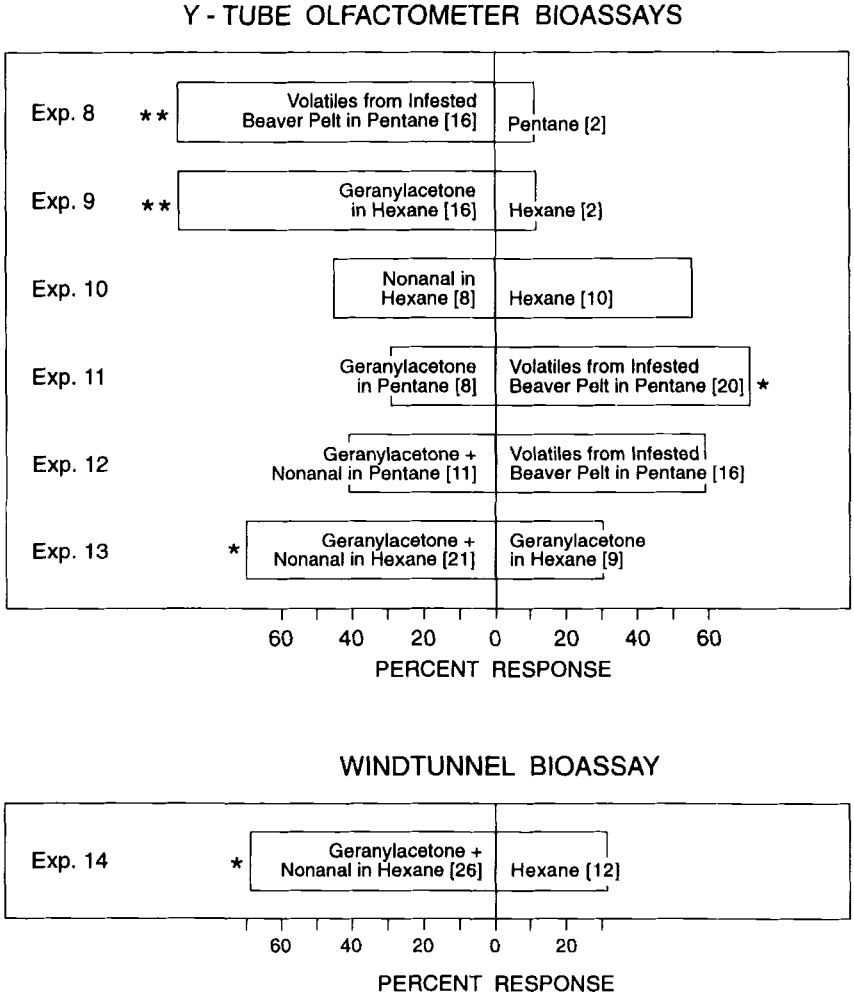

FIG. 4. Responses in experiments 8–13 (Table 1) of walking host-experienced *Apanteles carpatus* in a Y-tube olfactometer to volatiles from beaver pelt infested with larvae of the casemaking clothes moth, *Tinea pellionella*, or to synthetic candidate semiochemicals, and responses in experiment 14 of flying host-experienced *A. carpatus* in a wind tunnel to synthetic semiochemicals and to a solvent control. Numbers of parasitoids responding to each stimulus are given in parentheses. Bars with asterisks indicate a significant preference for a particular treatment (χ^2 test; $*P < 0.05$; $**P < 0.01$).

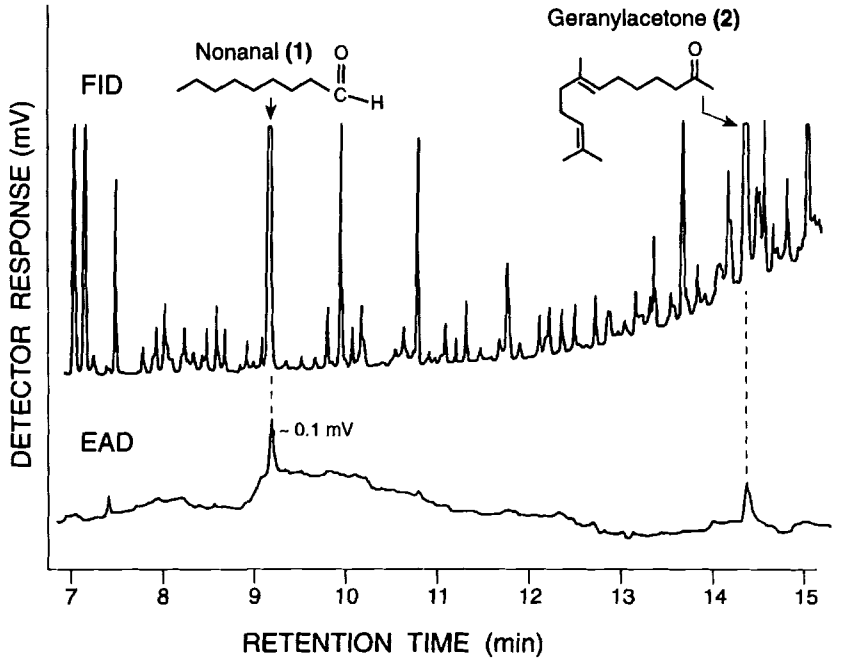

FIG. 5. Flame ionization detector (FID) and electroantennographic detector (EAD: *Apanteles carpatus* antenna) responses to aliquots of 2.5 pelt-min equivalents of volatiles from beaver pelt infested with larvae of the casemaking clothes moth, *Tinea pellionella*. Chromatography: Hewlett Packard 5890A equipped with DB-5 coated column; linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 240°C; temperature program: 1 min at 50°C, 10°C/min to 240°C.

by semiochemicals originating from the host habitat but not the host larva. However, larval secretions on the fur as a possible source of parasitoid attraction cannot yet be excluded. Because synthetic nonanal and geranylacetone at equivalent quantities were as attractive as volatiles from infested beaver pelt (Figure 4, experiment 12), foraging response of parasitoids is obviously associated with these two compounds. In a synergistic manner, both compounds attract walking (Figure 4, experiments 12 and 13) and flying (Figure 4, experiment 14) parasitoids.

While nonanal and geranylacetone mediate long-range attraction of *A. carpatus* to host habitat, tactile and vibrational cues may facilitate detection of host larvae. In the presence of host larvae, *A. carpatus* parasitoids probe any fibrous substrate such as cotton balls and wool fabric (personal observation). Although CCM feces do not contribute to long-range attraction (Figure 3, experiment 4),

their presence in or on larval tubes of WCM increase oviposition rates of the parasitoids (Fallis, 1942).

Well-studied tritrophic communication systems involve plants, herbivores, and their parasitoids (Strand and Vinson, 1982; Dicke et al., 1990; Turlings et al., 1990; Steinberg et al., 1992; McCall et al., 1993). Feeding by a herbivore changes the quantity and quality of the plant volatile blend (Turlings et al., 1991; Steinberg et al., 1993), which in turn induces attraction of parasitoids or predators to the herbivore habitat. Because the keratinaceous food source of clothes moth larvae in the *A. carpatus* tritrophic system is no longer alive, it is questionable whether the semiochemical-based interaction between the first and third trophic level can be classed as communication. Superior attractiveness of scissors-cut rather than intact rabbit pelt (Figure 3, experiment 7), however, suggests that mechanical damage to fur induced a foraging response analogous to that of a caterpillar feeding on a plant leaf (Mattiacci et al., 1994). While scissors-cutting of fur resulted in only slight quantitative increase of released nonanal and geranylacetone, multiple hair cuttings by larval mouthparts may effectively enhance release of these compounds. If this hypothesis were confirmed in quantitative studies, *A. carpatus*, like herbivore parasitoids, would respond to semiochemicals provided by the first trophic level but induced and/or enhanced by organisms in the second trophic level.

Hosts of *A. carpatus* are invariably keratinophagous caterpillars. Natural host habitats, such as animal cadavers, animal lairs, or bird nests (Mallis, 1969; Hill, 1990) are highly specific and localized. Both olfactory recognition of (Figure 5), and orientation toward, host-habitat volatiles (Figure 4, experiments 8 and 12–14) suggest that *A. carpatus* is a habitat rather than host specialist (Vet and Dicke, 1992).

Because geranylacetone and nonanal are common in volatile blends from plants (Borg-Karlson, 1987; Chinta et al., 1994), animals (Chung and Cadwaller, 1993), and stored products (Pierce et al., 1990; Mushobozy et al., 1993), one might doubt whether they are specific enough to serve as reliable indicators of host-infested habitat. However, relative concentrations of geranylacetone in plant volatiles are low (Borg-Karlson, 1987), and although nonanal may be abundant (Borg-Karlson, 1987; Chinta et al., 1994), it alone is not attractive to foraging parasitoids (Figure 4, experiment 9).

High concentrations both of nonanal and geranylacetone in volatile extracts of infested beaver pelt (Figure 5), accounting for 15% of all compounds, may be sufficient to provide reliable host-habitat stimuli, analogous to herbivore-induced synomones that guide certain parasitoids to their herbivorous hosts. Further studies are needed to fully characterize differences in the quantity and quality of volatiles from animal-derived sources when being colonized by CCM. Because habitat but not host volatiles mediate foraging behavior by *A. carpatus* (Figure 3, experiments 4–7), periodic departure of third- to fifth-instar CCM

from their feeding habitat (personal observation) may represent a strategy of parasitoid avoidance (Vet and Dicke, 1992), as reported for caterpillars of the cabbageworm, *Pieris rapae* (L.) (Mauricio and Bowers, 1990).

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IDENTIFICATION OF SEX PHEROMONES OF *Leucania anteoclara* SM. AND *Leucania commoides* GN. (LEPIDOPTERA: NOCTUIDAE: HADENINAE)[1]

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Abstract—The sex pheromone components of two species of *Leucania* that occur sympatrically in western Canada were identified in abdomen-tip extracts from calling female moths. (Z)-11-hexadecenyl acetate was the main component and (Z)-9-tetradecenyl acetate the second component in both species. The third component necessary for specific attractancy was (Z)-11-hexadecenyl aldehyde for *L. anteoclara* and (Z)-11-hexadecenyl alcohol for *L. commoides*. The third component for each species was an attractant inhibitor when added as a fourth component to the attractant blend for the reciprocal species. The most effective synthetic blend for the attraction of males in the field was Z9-14:Ac/Z11-16:Ac/Z11-16:Ald in a ratio of 1:10:4 for *L. anteoclara* and Z9-14:Ac/Z11-16:Ac/Z11-16:OH in a ratio of 5:4:1 for *L. commoides*.

Key Words—*Leucania anteoclara, Leucania commoides*, Lepidoptera, Noctuidae, sex pheromone, (Z)-11-hexadecenyl acetate, (Z)-9-tetradecenyl acetate, (Z)-11-hexadecenyl aldehyde, (Z)-11-hexadecenyl alcohol, seasonal flight period, abundance.

INTRODUCTION

The genus *Leucania* is represented in North America by 26 species (Hodges et al., 1983) and although some of the species are widespread and common, the

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larvae that feed on grasses and sedges (Forbes, 1954; Crumb, 1956; Godfrey, 1972) are rarely noticed. During a program to develop sex attractants for monitoring the abundance of pest species of noctuids in western Canada, two species of *Leucania* commonly occurred as contaminants in pheromone traps baited with attractants developed for two of the pest species (Byers and Struble, 1987). *Leucania anteoclara* Sm. was a major contaminant (17%) of catches in traps baited for the bertha armyworm, *Mamestra configurata* Wlk., and *L. commoides* Gn. was a minor contaminant (2%) of catches in traps baited for the variegated cutworm, *Peridroma saucia* (Hbn.). Investigation of the pheromones of the two *Leucania* species was undertaken in an attempt to identify inhibitory sex pheromone compounds whose inclusion in the sex attractant for the respective target pest species would enhance specificity.

L. anteoclara is a western species occurring on the northern Great Plains and intermountain areas of southern British Columbia and the northwestern United States (J. D. Lafontaine, personal communication). *L. commoides* is more widespread, occurring across southern Canada from Nova Scotia to British Columbia and south to Florida and New Mexico (Forbes, 1954). Color depictions of the moth of *L. commoides* are shown in Holland (1968) and Rockburne and Lafontaine (1976), and a description of the larva is given in Godfrey (1972).

METHODS AND MATERIALS

The moths used were from isofemale lines originating from gravid female moths collected in 1992 and 1993 in a light trap at Lethbridge, Alberta. Larvae were reared on densely planted wheat seedlings grown in 15.5-cm-diameter plastic pots in a greenhouse at 22 ± 3°C and 16:8D photoperiod. The larvae were confined on the plants by a 15.2-cm-diameter × 45-cm-high clear plastic cylinder with a screened cap and side vents. Attempts to rear the larvae on an artificial diet (Hinks and Byers, 1976) produced few mature larvae. *L. anteoclara* required about 45 days from hatching to pupation and *L. commoides* about 60 days. Both species had a preoviposition period of 10–14 days and the eggs hatched in about seven days.

Pheromone gland extracts were obtained from calling females kept at 17 ± 2°C, ca. 40% relative humidity, and 16:18-D photoperiod. Calling by females peaked between 3 and 4 hr into scotophase for *L. anteoclara* and 1 and 3 hr for *L. commoides*. Ovipositors were everted by gently squeezing the moths and the abdomen tip removed under 10× magnification with fine forceps. Adherent abdominal tissue was removed and the tips soaked in *n*-hexane for about 2 hr. Extracts were filtered and reduced to 0.5 female equivalents (FE)/μl under N_2 without further cleanup. Extracts contained material from 30 to 75 moths. Sep-

arate extracts were obtained from three isofemale lines of *L. anteoclara* and from two isofemale lines of *L. commoides*.

Electroantennographic responses to candidate pheromone components were determined by gas chromatography (GC) with a Varian 2700 modified to enable simultaneous use of an electroantennographic detector (EAD) and a flame ionization detector (FID) (Struble and Arn, 1984). The effluent was split in a 1:2 ratio between the EAD and the FID. Authentic standards of all even-number C_{10} to C_{16} acetates and alcohols, and the corresponding *Z* and *E* monounsaturated acetates and *Z* monounsaturated alcohols and aldehydes with the double bond at odd-number position 5–11, were screened for their ability to elicit an antennal response.

GC analysis of the abdomen-tip extracts was done using a Hewlett-Packard 5830 equipped with a FID. Supelcowax-10 capillary columns (30 m × 0.32 mm ID, 0.25-μm film thickness) were used for most analyses, but some were done using a J&W DB-17 capillary column (30 m × 0.32 mm ID, 0.25-μm film thickness). Helium carrier gas velocity was 30 cm/sec at 50°C; various temperature programs were used with splitless injection. The initial identification of peaks was by comparison of retention time with those of the standards. Further support for the GC-based assignments were provided by mass spectra and field testing of test blends for attractancy.

Mass spectra of the components in the abdomen-tip extracts were obtained with a Hewlett-Packard 5989A mass spectrometer interfaced to a Hewlett-Packard 5890 Series II gas chromatograph. Analyses were conducted in the electron impact mode with a high energy dynode. Chromatographic separations were achieved using a Supelcowax-10 capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness). Typical conditions for the splitless injections were: injector, 225°C; transfer line, 225°C; helium carrier gas using constant flow mode; temperature program, 50°C (1 min), 50–140°C at 25°C/min, 140–200°C at 10°C/min, and hold (10 min). Mass spectral conditions were: electron impact mode at 70 eV, full scan with high-energy dynode on, ion source at 275°C, quadrupoles at 100°C. All mass spectra were compared with those of authentic synthetic standards.

The synthetic pheromone components were from a collection of chemicals that had either been purchased from various suppliers or synthesized by our former colleague, Dr. D. L. Struble. Acetates were purified by argentation liquid chromatography (Houx et al., 1974) to chemical purity of >99% and isomeric purity >99.8%. Alcohols of similar purity were obtained by treatment of the corresponding acetate with methanolic potassium hydroxide. Aldehydes were prepared from the corresponding alcohols by the procedure of Corey and Suggs (1975). Synthetic blends were formulated in *n*-hexane. Red rubber septa (Thomas Scientific, Catalog No. 1780-J07) were used as dispensers and traps were of the

cone-orifice type (Struble, 1983) or all-green Unitraps (Phero Tech Inc., Delta, British Columbia). Treatments were replicated four times in a randomized block design within a single row in the north–south direction along the edge of cultivated or native grass pastures. Traps were positioned at a height of 1 m and spaced 15 m apart with a guard trap, baited with the best attractant known at the time, at each end of the row to reduce the end effect. Catches in the guard traps were not included in the experimental data. Traps were emptied and catches recorded at two- or three-day intervals. Analysis of variance was done on transformed ($\sqrt{x}$ + 1) data, and treatment means compared by Duncan's multiple range test.

In 1993–1995 the seasonal flight periods of the two species and the specificity of the optimized attractants were determined from daily catches in two pairs of cone-orifice pheromone traps located 1.5 km apart on the Lethbridge Research Centre. These traps were operated from the last week in May to mid September and the lures replaced every four weeks. All moths were identified and the numbers of *Leucania* caught were plotted against time with smoothing of the points by spline interpolation (SAS/Graph). The large numbers of moths that were caught in the various experiments conducted on or near the Lethbridge Research Centre indicated that both species were abundant. To test the possibility that this might be a local pheromonen, pairs of Unitraps for each species were placed 100 m apart at three sites spaced about equidistantly along a 160-km transect across southern Alberta.

Identification of representative pinned specimens of reared and field-captured moths was confirmed by J. D. Lafontaine (Biological Resources Division, Centre for Land and Biological Resources Research, Agriculture and Agri-Food Canada, Ottawa).

RESULTS AND DISCUSSION

Seven and eight potential pheromone compounds were identified by GC retention times and GC-MS mass spectra in the abdomen-tip extracts of *L. anteoclara* and *L. commoides*, respectively (Table 1). Screened compounds that elicited an unequivocal male antennal response, but were not detected in the gland extracts, are also shown in Table 1. GC-EAD-FID analyses of *L. anteoclara* extracts consistently yielded coincident EAD and FID responses for Z9–14:Ac, Z11–16:Ac, and Z11–16:Ald, indicating that these were likely to be the pheromonally active components. Similarly, analysis of *L. commoides* extracts yielded consistent coincident responses for Z9–14:Ac, Z11–16:Ac, Z9–14:OH, and Z11–16:OH.

Field testing showed that all three of the "pheromonally active" compounds detected by GC-EAD-FID in the *L. anteoclara* extracts were essential

TABLE 1. POTENTIAL PHEROMONE COMPONENTS PRESENT IN ABDOMEN-TIP EXTRACTS OF FEMALES OR ELICITING A MALE ANTENNAL RESPONSE

	L. anteoclara			*L. commoides*		
	Female extract		Male	Female extract		Male
Component	GC-MS	Relative amount[a]	antennal response[b]	GC-MS	Relative amount[a]	antennal response[b]
12:Ac	ND[c]		0	ND		0.04
Z9-12:Ac	ND		0.04	ND		0.21
14:Ac	ND		0.01	ND		0.04
Z9-14:Ac	+[d]	13.1	0.10	+	60.4	0.47
Z11-14:Ac	ND		0.10	ND		0.12
16:Ac	+	3.2	0	ND		0.01
Z9-16:Ac	+	1.7	0.01	+	3.5	0.04
Z11-16:Ac	+	100	0.29	+	100	0.20
14:OH	ND		0	+	16.5	0
Z9-14:OH	ND		0.08	+	3.1	0.18
16:OH	+	1.1	0.01	+	5.9	0.01
Z11-16:OH	ND		0.10	+	6.1	0.08
Z9-14:Ald	ND		0.11	ND		0.06
Z11-14:Ald	ND		0.05	ND		0.03
Z11-16:Ald	+	8.7	0.09	ND		0.05
18:OH	+	6.0	0	+	11.3	0
Main component (ng/FE)		50–80			10–20	

[a] Amount relative to Z11-16:Ac based on GC-MS analyses of hexane extracts of female abdomen-tips.
[b] Typical EAD response (millivolts) of male antennae to 5 ng/μl of synthetic in hexane, 2 μl split injected (25:1).
[c] ND, not detected.
[d] +, complete spectra obtained.

for the attraction of conspecific males (Table 2) and that the optimal ratio of Z9-14:Ac, Z11-16:Ac, and Z11-16:Ald was 1:10:4 (Table 3). In the test providing the data for Table 2, the blend ratios 1:10:1, 1:10:2 and 1:10:4 were about equally attractive for the first few days, but after about one week the 1:10:4 ratio was clearly the most attractive. Presumably the blends with the lower amounts of aldehyde became less attractive over time because of degradation of the aldehyde; however, the presence of the antioxidant, butylated hydroxytoluene (BHT), had little or no effect (Table 3). Preliminary short-term experiments had shown that a blend ratio of 1:10:4 was much more attractive than a ratio of 1:10:10. Over the seasonal flight period of *L. anteoclara* in 1993 to 1995 the 1:10:4 blend was >99% specific.

TABLE 2. NUMBER OF *Leucania anteoclara* MALE MOTHS CAUGHT IN TRAPS BAITED WITH BLENDS OF CANDIDATE PHEROMONE COMPONENTS

Components					Mean number of moths per trap[a]	
Z9–14:Ac	Z11–16:Ac	Z11–16:Ald	Z11–14:Ac	Z11–16:OH	*L. anteoclara*	Nontargets
1	9	1			897	5
	9	1			10	2
1		1			0	30
1	9				18	58
1	9	1		1	1	919[b]
	9	1	1		10	2
	9	1		1	0	485[c]

[a]Four replications, July 12–August 8, 1988; 500 μg/septum, except last treatment which was 100 μg/septum; plus 5 μg BHT/septum.
[b]90% *L. commoides*.
[c]99% clover cutworm [*Discestra trifolii* (Hfn.)].

TABLE 3. NUMBER OF *L. anteoclara* MALE MOTHS CAUGHT AT VARIOUS RATIOS[a] OF ATTRACTANT PHEROMONE COMPONENTS

Components			Mean number of *L. anteoclara* males per trap[b]	
Z9–14:Ac	Z11–16:Ac	Z11–16:Ald		
1992			*June 25–July 13*	
1	10	1	22.8a	
1	10	2	35.3b	
1	10	4	47.3c	
2	10	2	19.8a	
2	10	1	7.3d	
4	10	1	2.0e	
1995			*June 26–July 13*	*July 14–August 22*
1	10	1	63.5a	120.3a
1	10	1[c]	61.3a	111.0a
1	10	4	112.5b	247.3b
1	10	4[c]	109.0b	217.3b

[a]500 μg of main component/septum plus 5 μg BHT.
[b]Four replications, June 25–July 13, 1992. Means within a test followed by the same letter are not significantly different, DMRT ($P > 0.05$).
[c]Without BHT.

Three of the four putative pheromonally active compounds detected by GC-EAD-FID in the *L. commoides* abdomen-tip extracts had previously been shown by empirical testing to be necessary for attraction of conspecific male moths and the fourth, Z9–14:OH, was known to be a potent attractant inhibitor (Struble et al., 1977; Steck et al., 1977). Various ratios of the three attraction components, including the 6:10:1 ratio present in the extracts, were tested, and the 5:4:1 ratio was found to be consistently highly attractive, although as reported by Struble et al. (1977) a wide range of ratios were moderately to highly attractive. Over the seasonal flight period of *L. commoides* in 1993–1995 the 5:4:1 blend was about 98% specific, with *P. saucia* being the main contaminant species.

For both species the active pheromone components were the three monounsaturated compounds present in the highest concentration in the respective extracts, but were not necessarily those that produced the strongest male antennal response (Table 1).

Once an optimized blend for each species had been identified, the remaining potential pheromone components that had been detected in female abdomen-tip extracts of either species or which evoked a definite male antennal response (Table 1) were added singly at a concentration equal to the lowest attractant component (Table 4), except for Z11–16:OH and Z9–14:OH, which were known to be potent inhibitors for *L. anteoclara* and *L. commoides*, respectively, and were therefore tested at 10% of the lowest attractant component. Of the compounds added as fourth components to the *L. anteoclara* attractant, only the attraction inhibitor, Z11–16:OH, had an obvious effect. When added as fourth components to the *L. commoides* attractant, Z11–14:Ald and Z11–16:Ald, in addition to the known potent inhibitor Z9–14:OH, significantly reduced attractancy. It is of interest, and perhaps evolutionary significance, that the third component in both species, Z11–16:Ald for *L. anteoclara* and Z11–16:OH for *L. commoides* inhibits attraction of the other species. Although Z7–12:Ac was also known to be a potent attraction inhibitor of *L. commoides* (Steck et al., 1977), it was not included in this study because it evoked only weak male antennal responses and was not detected in the female extracts.

The potent inhibitor of attraction that was identified for *L. anteoclara*, Z11–16:OH, will not be of any use for enhancing the specificity of attractant lures for the bertha armyworm, for which *L. anteoclara* is sometimes a significant contaminant, because it is also a potent inhibitor of attraction for the bertha armyworm (Underhill et al., 1977; Steck et al., 1984; Struble et al., 1984). The sex pheromone of *P. saucia*, for which *L. commoides* is a minor contaminant species, has been less intensively studied (Struble et al., 1976; Descoins et al., 1978). However, the potent inhibitor of attraction for *L. commoides*, Z9–14:OH, may have potential for enhancing specificity of the *P. saucia* attractant, espe-

TABLE 4. EFFECT OF ADDITION OF FOURTH COMPONENT TO OPTIMIZED THREE-COMPONENT BLENDS[a]

Fourth component	*L. anteoclara*		*L. commoides*	
	Blend ratio[b]	Number of moths/trap[c]	Blend ratio[b]	Number of moths/trap[d]
None		41.8a		128.8a
Z9-12:Ac	1	38.0a	1	113.8ab
Z11-14:Ac	1	28.0ab	1	116.5a
Z9-16:Ac	1	39.3a	1	123.3a
Z9-14:OH	1	37.5a	0.1	55.5c
Z11-16:OH	0.1	15.5b		
Z9-14:Ald	1	34.0a	1	111.5ab
Z11-14:Ald	1	30.3ab	1	60.8c
Z11-16:Ald			1	74.5bc

[a] *L. anteoclara:* Z9-14:Ac/Z11-16:Ac/Z11-16:Ald, 1:10:4; *L. commoides:* Z9-14:Ac/Z11-16:Ac/Z11-16:OH, 5:4:1.

[b] Amounts to three-component blend at a concentration either equal to that of the third component (1) or at 10% of it (0.1).

[c] June 29–July 13, 1995; Unitraps, four replicates. Means within a test followed by the same letter are not significantly different, DMRT ($P > 0.05$).

[d] July 18–August 3, 1995; Unitraps, four replicates.

cially as there is some evidence that it might also enhance attractancy (Struble et al., 1976). Of the two aldehydes that were found to be moderate inhibitors of attraction for *L. commoides*, Z11-16:Ald has been reported to inhibit attraction of *P. saucia*, whereas Z11-14:Ald has little or no effect (Steck et al., 1979). Elucidation of the effect of adding Z9-14:OH or Z11-14:Ald to the sex attractant of *P. saucia* will require field testing when this species is abundant. This occurs infrequently at Lethbridge because an abundance of *P. saucia* is dependent on an influx of immigrant moths (Ayre, 1985).

To determine the quantity of attractant required to provide a consistent catch over the flight period for each species, the optimized attractant blends were tested at 100, 200, 500, 1000, and 2000 μg/septa (four replications, June 26–September 11, 1995). For both species the 2000-μg/septum load was the most attractive (Figure 1), but either a 1000- or 500-μg load would be adequate for survey or monitoring purposes.

The seasonal flight period of the two species largely overlap (Figure 2), although the peak catch for *L. anteoclara* during the second week of July, is about two weeks earlier than that for *L. commoides*. The seasonal flight period observed for *L. commoides* is similar to that reported by Knutson (1944) in

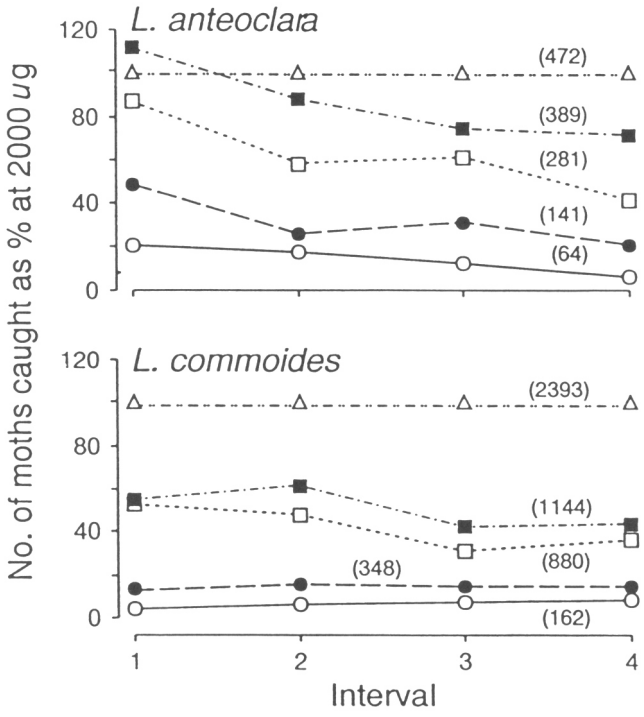

FIG. 1. Effect of septum load on catches of *L. anteoclara* and *L. commoides*. Intervals 1–4 are about equal periods of time between June 26 and September 11, 1995. The numbers in parentheses are the mean number of moths caught per trap with each septum load: Δ, 2000 μg; ■, 1000 μg; □, 500 μg; ●, 200 μg; ○, 100 μg.

Minnesota. The bimodal seasonal flight pattern observed in 1993 was the consequence of a period of cool inclement weather. Little is known about the life histories of the immature stages of either *L. commoides* or *L. anteoclara*. Ayre et al. (1983) speculated that *L. commoides* overwinters in the pupal stage. In the rearings conducted during this study, there was no indication of a pupal, or egg, diapause in either species. Larval development was relatively slow, and it seems unlikely that the larvae could reliably reach maturity before winter, especially as food quality of the host grasses would be low during late summer and fall. It is probable that both species overwinter as early- to mid-instars as do those of *Feltia jaculifera* (Gn.), which has a similar seasonal flight period (Byers et al., 1990).

At the three sites along a transect across southern Alberta, the mean moth

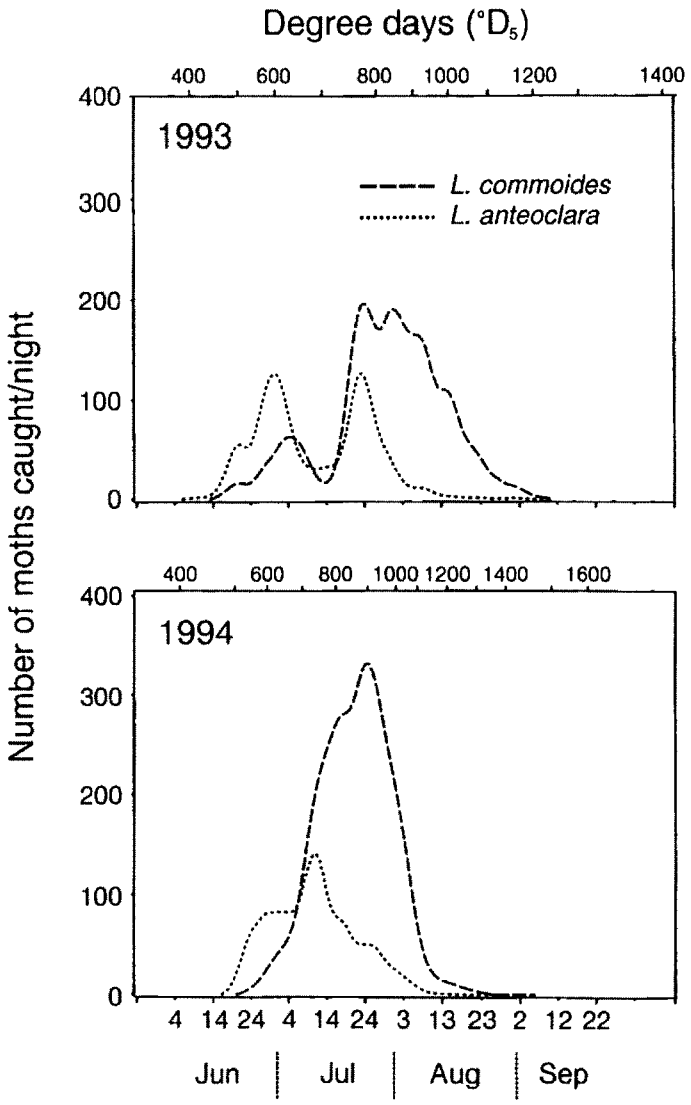

FIG. 2. Seasonal flight periods of *L. anteoclara* and *L. commoides* at Lethbridge, Alberta, in 1993 and 1994 based on catches of male moths in traps baited with the respective specific sex attractant.

TABLE 5. NUMBERS OF MOTHS[a] OF *Leucania anteoclara* AND *Leucania commoides* CAPTURED IN SEX PHEROMONE TRAPS AT SITES ACROSS SOUTHERN ALBERTA, 1993–1995

	L. anteoclara				*L. commoides*			
Site[b]	1993	1994	1995	Mean	1993	1994	1995	Mean
1	844	1546	839	1076	595	2234	1051	1293
2	666	1761	854	1094	604	2262	1430	1432
LRC	1649	1620	1204	1491	3277	4254	2276	3269
3	312	754	286	451	395	1198	407	667
Mean	868	1420	796		1218	2487	1291	

[a]Mean of two traps at each site; Unitraps at sites 1–3 and cone-orifice traps at the LRC site, which were the traps on the Lethbridge Research Centre used to determine seasonal flight periods and attractant specificity.

[b]Sites 1–3 were located about equidistantly across southern Alberta along Highway 3. Site 2 was about 10 km from the LRC site.

catch for 1993–1995 was 874/trap/year for *L. anteoclara* and 1131/trap/year for *L. commoides* (Table 5). The lowest catch for a site-year combination was 286/trap for *L. anteoclara* and 395/trap for *L. commoides*. The variation in catch among sites and years was similar for both species, indicating that the factors determining abundance were the same for both.

The large numbers of moths of both species caught in pheromone traps during this study indicate that both species are unexpectedly abundant. In an early study of noctuids in Montana, based largely on light trap catches, Cook (1930) reported that although *L. commoides* occurred throughout the state, it was rare, whereas *L. anteoclara* was common only in the western part of the state. Although the moths of both *Leucania* species do come to light, the numbers caught in a light trap located on the Lethbridge Research Centre about 50 m from one set of the pheromone traps used to monitor seasonal flight periods were usually much less than 10% of the catches in the pheromone traps, which is in marked contrast to species such as *F. jaculifera*, which come readily to light and for which the catches at light are usually much greater than in traps baited with pheromone lures (Byers and Struble, 1990).

The three-component blends for these two species of *Leucania* at a load of 1000 μg/septum, are highly specific and remain attractive for at least six weeks. They will be useful in studies of the distribution and ecology of these species, which, in view of their evident abundance, may play a significant ecological role in some habitats.

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SEASONAL VARIATION OF PHEROMONE CONCENTRATION IN MATING DISRUPTION TRIALS AGAINST EUROPEAN GRAPE VINE MOTH *Lobesia botrana* (LEPIDOPTERA: TORTRICIDAE) MEASURED BY EAG

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Abstract—Spatial and temporal distributions and relative concentrations of the pheromone of the European grape vine moth *Lobesia botrana* (Lepidoptera: Tortricidae) were measured in mating disruption trials with electroantennograms. Measurements were carried out over several years during the flight season of the two generations of this pest insect. In three consecutive years significantly higher mean relative pheromone concentrations were measured in summer during the flight of the second generation of *L. botrana* than in spring during the flight of the first generation ($P < 0.001$). The relative pheromone concentrations in 1989 reached a maximum between late July and early August, when the highest mean daily temperatures were registered. In addition to the differences in mean relative pheromone concentrations, the spatial and temporal distributions of the pheromone differed significantly between spring and summer. EAG recordings taken in summer showed high, uniform pheromone concentrations in the treated plots, whereas in spring strong temporal and spatial fluctuations were recorded. In a vineyard defoliated by a hailstorm, the mean relative pheromone concentrations measured in summer were not significantly different from those measured in spring ($P > 0.05$), but were significantly lower than those of a nearby intact vineyard ($P < 0.001$). The results provided additional evidence that foliage is an important parameter determining mean pheromone concentrations and temporal and spatial distribution of pheromone in mating disruption trials.

Key Words—*Lobesia botrana*, Lepidoptera, Tortricidae, pheromone concentration, mating disruption, electroantennogram, vineyard, plume structure, fluctuations, plant canopy.

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INTRODUCTION

The European grape vine moth, *Lobesia botrana* (Lepidoptera: Tortricidae), is a serious pest of grape culture in Europe that generally requires some measure of control. Public concern continues to grow about the widespread use of insecticides due to their negative side effects on the environment and residues in crops. Environmentally safe control of pest insects has become an important field of research. One successful method of pest control is the application of sex pheromones in mating disruption (e.g., Jutsum and Gordon, 1989; Ridgway, et al., 1990; Charmillot and Vickers, 1991; Suckling, 1993). The advantage of this method is that pheromones are generally nontoxic and selective in their action compared to insecticides. For a number of pest insects, including *L. botrana*, pheromones have been applied successfully using both sticky traps to monitor flight activity and population dynamics as well as in mating disruption (reviewed in Jutsum and Gordon, 1989; Cardé, 1990; Cardé and Minks, 1995). Despite considerable progress, mating disruption still cannot be universally applied as a pest control method. This may be due to specific insect characteristics, but abiotic factors including temperature, wind conditions, and characteristics of the plant canopy and leaf density, which vary between different plots and crops (Aylor et al., 1976; Uchijima, 1988; Raupach, 1988), may also hamper the effectiveness of mating disruption. In addition, daily and seasonal changes can affect pheromone concentrations and their temporal and spatial distributions in and around the habitat of the pest insect. These differences can require adjustments to the application of the control program in mid-season or necessitate different approaches depending on the pest generation. This is especially the case for insects that have more than one generation per year (e.g., *Lobesia botrana*, *Cydia pomonella*, *Eupoecilia ambiguella*). Mating disruption of *E. ambiguella*, for example, has been demonstrated for the first and second generation, but failure is more likely to occur during the second generation flight (Vogt, 1993). Moreover, mating disruption of *L. botrana* is usually more efficient in the second and third generations (Netter, 1993; Cardé and Minks, 1995).

Our supposition is that the differences in the effectiveness of mating disruption between generations of *L. botrana* are correlated to differences in mean pheromone concentrations and/or temporal and spatial pheromone distributions. To explore this we used an EAG method to describe and compare the structure of the pheromone plume and mean relative pheromone concentrations present in mating disruption plots during the flight peaks of the two generations of *L. botrana*.

METHODS AND MATERIALS

Insects. Pupae of *L. botrana* were supplied weekly by the Landwirtschaftliche Versuchsanstalt of BASF, Limburgerhof, Germany. They were sexed and

kept separately at room temperature until eclosion. After eclosion the males were kept in a refrigerator at 9°C until used. Only antennae of 2- to 4-day-old males were used for the EAG recordings.

Pheromones. The main component of the sex pheromone of *L. botrana* has been identified as (*E*, *Z*)-7,9-dodecadien-1-yl acetate (*EZ*-7,9–12:OAc) (Buser et al., 1974). Pheromone dispensers in the field were of the standard type produced by BASF, each containing 0.1 g *EZ*-7,9–12:OAc.

EAG Apparatus and Measurement of Relative Pheromone Concentrations. The dispersion and relative concentration of pheromones were measured using field electroantennograms (EAG). The measuring system and evaluation of the data were described in detail in Sauer et al. (1992) and Karg et al. (1994). The EAG-amplitude responses of the antennae of male *L. botrana* elicited by known pheromone concentrations (10^{-2}–10^{-7} pheromone in paraffin oil) were measured in the laboratory. The EAG amplitudes were normalized by dividing the antennal responses to a given stimulus by the EAG response to a calibration pulse of 10^{-4} pheromone in paraffin oil, which gave the normal dose–response curve shown in Figure 1C (Sauer et al., 1992; Karg and Sauer, 1995).

Antennal preparations carried into the field were protected by a charcoal filter cover that could be removed for recording the antennal response. Each

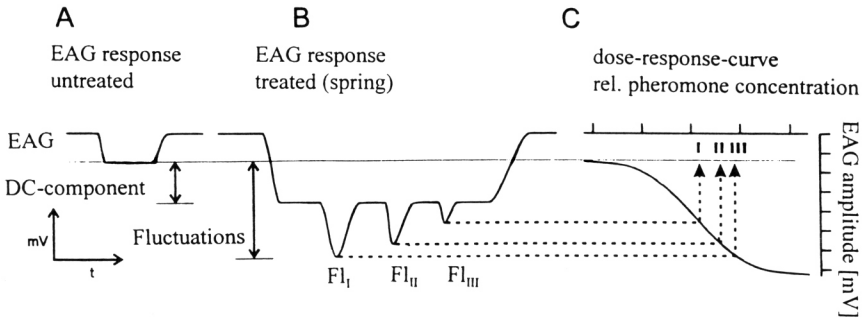

FIG. 1. Schematic drawing of EAG recordings taken in an untreated vineyard (A), a pheromone-treated vineyard in spring (B), and the dose–response-curve (C) established in the laboratory. The EAG response is a depolarization of the antennal potential. In order to calculate relative pheromone concentrations, the EAG measured in an untreated control vineyard (B) is subtracted from the EAG measured in a treated vineyard. This differential EAG consists of two components, a DC component and additional fluctuations. The amplitude of the fluctuations in the pheromone concentrations (Fl_I to Fl_{III}) is measured from the baseline of the differential EAG (horizontal line) to the maximum decline. The transformation of EAG amplitudes into relative pheromone concentrations uses the previously established normalized dose–response curve. The classifications of the fluctuations are show at the right side of the graph (C). The height of the normalized EAG (*y* axis) corresponds to a relative pheromone concentration on the *x* axis.

pheromone measurement in the field began with at least three calibration pulses, followed by three measurements of the airborne pheromone concentration in the field after removal of the charcoal filter from the antennal preparation. The mean response measurements to airborne pheromone concentrations were divided by the mean response of the associated calibration pulses to 10^{-4} pheromone in paraffin oil, giving one value for mean normalized EAG amplitudes. With the help of the normalized dose–response curve generated in the laboratory (Figure 1C), the relative pheromone concentration of the measurements taken in the field could be derived by transferring the mean normalized EAG amplitudes (Figure 1C, *y* axis) to the corresponding relative pheromone concentration (Figure 1C, *x* axis). Measurements of the mean relative pheromone concentration were repeated between 5 and 15 times at every location. A single antenna was used for all related measurements.

Contribution of Plant Volatiles to Overall EAG Response. In pheromone-treated fields, the volatiles that were responsible for the EAG consisted of two parts. One contribution to the overall response of the antenna was generated by the environmental odors present in the ambient air (EAG response in an untreated vineyard), and a second contribution was made by the applied pheromone (Figure 1B). Previous experiments with *L. botrana* (Sauer et al., 1992) have shown that the EAG response elicited by environmental volatiles (nonpheromones) can be neglected due to their small contribution to the overall EAG response. Nevertheless, unlike previous work (Sauer et al., 1992; Karg and Sauer, 1995), the contribution of environmental volatiles was taken into account in the experiments reported here. To make this correction, the contribution of pheromone to the overall antennal response was measured by subtracting the normalized EAG amplitude measured in the untreated control plot from the normalized EAG amplitude measured in the pheromone-treated area. This approach has been used in prior publications (Karg et al., 1994; Suckling et al., 1994) and is called the differential EAG (Figure 1B).

Measurement of Pheromone Fluctuations. Measurements taken in the spring and in defoliated plots in summer showed strong fluctuations in the EAG signal. These strong EAG fluctuations were not present in the summer measurements taken in a vineyard with fully developed foliage. The EAG signal consisted of two components, a depolarization elicited by a rather constant background of pheromone (Figure 1, DC-component) and additional rapid changes/fluctuations (Figure 1, Fl_I to Fl_{III}) not present in a vineyard with fully developed foliage in summer. The mean relative pheromone concentrations were estimated as described above by using the DC component only. The additional fluctuations of pheromone concentration were not taken into account for the mean relative pheromone concentration, but they were used for the description of the fine structure of the pheromone plume. The height of the fluctuations was measured and normalized by dividing by the EAG responses to calibration pulses. Because

the height of the normalized EAG (Figure 1, y axis) corresponds to a relative pheromone concentration on the x axis, the height of the fluctuations can easily be transferred to fluctuations in the relative pheromone concentrations. The EAG response follows a log-linear relationship and the amplitude classes were chosen as A = $<9.9 \times 10^{-7}$; B = 1×10^{-6} to 3.3×10^{-6}; C = 3.4×10^{-6} to 9.9×10^{-6}; D = 1×10^{-5} to 3.3×10^{-5}; and E = 3.4×10^{-5} to 9.9×10^{-5}.

Wind and Temperature. Wind was monitored with a hot wire anemometer (Fa. Heinecken, Starnberg, Germany), and the temperatures were measured using a digital thermometer (ER 110, Conrad Electronic, Germany).

Field Sites. All experiments were carried out between summer 1988 and autumn 1990 in vineyards in Rhineland–Palatinate, Germany (Billigheim, Wachenheim, Friedelsheim, Essingen). Plot sizes (N = 3 replicate plots at each site) were between 0.8 and 5 ha. In the treated plots, 500 dispensers/ha were applied. Antennal responses to environmental volatiles (e.g., from host plants) were measured in untreated control vineyards of the same grape variety when possible, and upwind from the pheromone-treated trials. The EAG system was then moved to the physical center of the pheromone treated fields for subsequent measurements.

Experiment 1. Changes of Mean Relative Pheromone Concentrations in One Year. Mean relative pheromone concentrations were measured in the center of pheromone-treated plots in Billigheim in 1989 between May 2 and August 9, 1989, in order to monitor the changes of the mean relative pheromone concentration during the flight activity of *L. botrana*.

Experiment 2. Changes of Mean Relative Pheromone Concentrations in Consecutive Years. The mean relative pheromone concentrations measured in the center of the mating disruption trials were measured in Billigheim over three consecutive years (1988–1990) (N = 10). The measurement were carried out during May and June and again between the end of July and the end of August during the flight peaks of the two generations of the pest.

Comparison of Mean Relative Pheromone Concentrations Measured in Summer in a Vineyard with Fully Developed Foliage, a Defoliated Vineyard, and in Spring. In summer 1989 several hectares of vineyards near Essingen were defoliated by a hailstorm. The area was near the site where all other experiments had been conducted. Nine new plots of approximately 300 m^2 each were established in this area in order to compare differences in mean pheromone concentration and distribution under similar climatic conditions, but with differences in plant canopy configurations. We established (1) three replicate plots in an unaffected (intact) vineyard with fully developed leaves (stages 27–35; Eichhorm and Lorenz, 1977), (2) three replicate plots approximately 120 m east of the first set, but inside the defoliated area, and (3) three untreated control plots approximately 100 m upwind from the pheromone treated plots. Dispensers (9 weeks old) were taken from a plot in Billigheim and applied in these plots

at the usual density of 500/ha. Five days after application of the dispensers in the plots, the mean relative pheromone concentration present in the two treated types of plots and the EAG response from the untreated control blocks were measured. Measurements were taken repeatedly ($N > 10$) at three locations inside the plots at intervals of less than ten min on the same day under very similar climatic conditions.

Measurement of Fine Structure of Pheromone Plumes. In order to measure the temporal distribution of the pheromone and possible fluctuations of pheromone concentration, the time of sampling ambient air in the pheromone-treated area with the charcoal filter removed was extended for up to 2 min. In the spring 19 recordings were taken, and 21 recordings were taken in the summer in the defoliated plots. Each recording had a duration ranging from 30 sec to 2 min.

RESULTS

Comparison of Mean Relative Pheromone Concentration Changes in One Year. The results of the measurements of the mean relative pheromone concentrations in Billigheim in 1989 between May 2 and August 9, 1989, are shown in Figure 2. The mean relative pheromone concentrations were 1.54×10^{-6} on May 2 and 9.21×10^{-7} on May 24, 1989 ($N > 10$). The mean relative pheromone concentration increased to 1.54×10^{-5} on June 10 and reached a maximum of 2.15×10^{-4} measured on June 22. Thereafter the mean relative pheromone concentration in treated plots slowly declined to 2.54×10^{-5} on June 22. The mean relative pheromone concentrations in 1989 reached a maximum at the end of July and the beginning of August, when the highest mean daily temperatures also were registered.

Mean Relative Pheromone Concentration in Consecutive Years. Figure 3 shows a comparison of the mean relative pheromone concentration measured in the center of the mating disruption trials in Billigheim from 1988 through 1990. The measurements were carried out in May and June and again between the end of July and the end of August during the flight peaks of the two generations of the pest (Netter, 1993). Only data sampled under similar environmental conditions, with mean windspeeds <2.5 m/sec were summarized in this comparison. There were no significant differences in the mean relative pheromone concentrations between the three replicate plots in each year ($P > 0.05$), and the data were pooled. The mean relative pheromone concentration measured in spring 1989 (May 9 and 16) was 9.13×10^{-7} ($N = 18$), and 7.51×10^{-7} ($N = 22$) in 1990 (May 3 and 8). No data are available for spring 1988. The mean relative pheromone concentrations in the same plots in summer were significantly higher compared to the concentrations measured in the corresponding year ($P < 0.001$) and reached values of 2.37×10^{-4} ($N = 21$) in 1988 (August

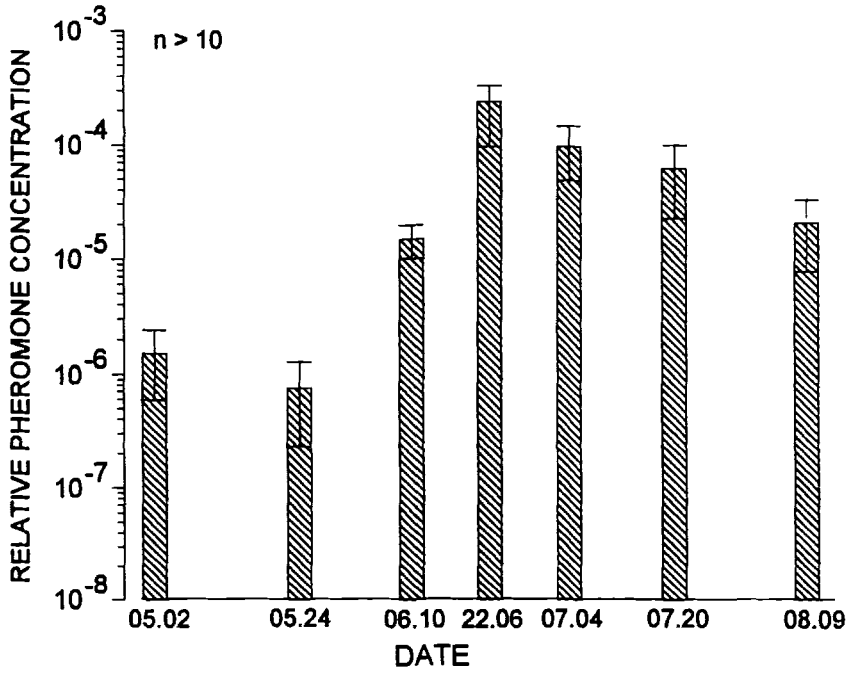

FIG. 2. Mean relative pheromone concentrations measured in Billigheim in 1989. The bars indicate the range of the measured pheromone concentrations. The mean daily temperatures in the center of pheromone treated plots are also shown on the x axis.

12 and 16), 1.35×10^{-4} ($N = 22$) in 1989 (August 9, 10, and 17), and 2.36×10^{-5} ($N = 17$) in 1990 (August 2 and 29). There were no significant differences in the values measured in the plots in the summer of one year, and the data were pooled. Comparison of the mean relative pheromone concentrations of different years showed a significantly lower pheromone concentration in 1990 ($P < 0.001$). However, the mean pheromone concentration in summer was still significantly higher than the value measured in spring in the same year ($P < 0.001$).

Comparison of Mean Relative Pheromone Concentrations Measured in Summer in Vineyard with Fully Developed Foliage, Defoliated Vineyard, and in Spring. The mean relative pheromone concentration measured in summer in the replicate plots was 2.21×10^{-4}. The mean relative concentration in the nearby defoliated plots was significantly lower at 1.89×10^{-6} than that in the vineyard with an intact canopy ($P < 0.001$). The mean relative pheromone concentration measured in the spring (8.29×10^{-7}) also was significantly lower

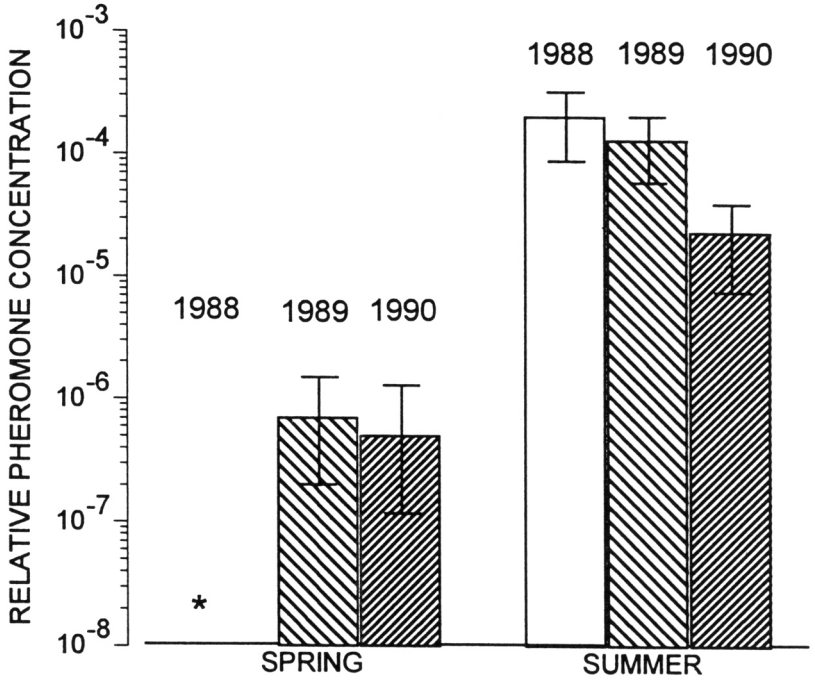

FIG. 3. Mean relative pheromone concentrations measured in spring and summer 1988–1990 in Billigheim, Germany. The measurements taken in the same plots in summer were significantly higher compared to the measurements taken in spring ($P < 0.001$). The bars indicate the range of the measured values. *No measurements were taken in spring 1988.

than in the summer ($P < 0.001$), but not significantly different from the concentration measured in the defoliated plots ($P > 0.05$). The data are presented in Figure 4.

Measurement of Fine Structure of Pheromone Plumes. Figure 5 shows typical examples of the measurement of the fine structure of the pheromone plumes measured in the center of a pheromone treated plot in summer 1989, in the center of the same pheromone treated plot in spring 1989, and in the center of a nearby pheromone treated and defoliated plot in summer 1989. The bars in Figure 5 indicate the time when the charcoal filter was placed over the inlet of the glass chambers containing the antenna holder. After removal of the charcoal filter, ambient air containing pheromone reached the antenna and elicited an EAG response. When the charcoal filter was replaced, the antennal response rose again. The EAG was rather constant (compared to the EAG measured in the spring, see Figure 5c).

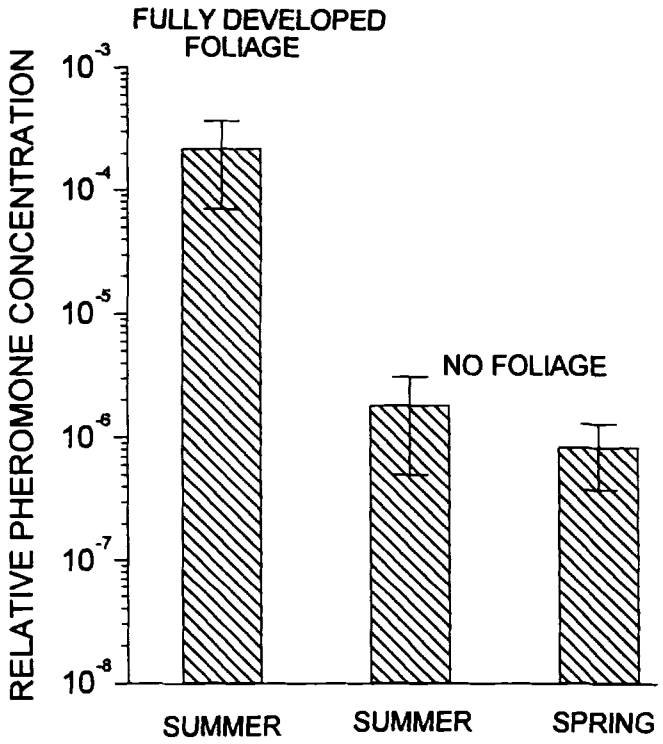

FIG. 4. Comparison of the mean relative pheromone concentrations. Measurements were taken in three replicate plots in summer, with fully developed foliage, in a nearby defoliated vineyard, and in spring. The mean relative pheromone concentrations measured in summer in the plots with fully developed foliage were significantly higher compared to the measurements taken in defoliated plots and in spring ($P < 0.001$). The bars indicate the range of the measured values.

A typical EAG recording taken on May 18, 1989 is shown in Figure 5c. Removal of the charcoal filter caused decline of the EAG. Strong fluctuations of the EAG indicated the presence of strong changes in airborne pheromone concentrations reaching the antenna. No fluctuations exceeding the threshold were measured in the nearby control plots. EAG measurements taken in the summer in plots in the defoliated area again showed strong fluctuations in the pheromone concentration comparable to those recorded in the spring. A typical recording taken on August 6 at 25°C is shown in Figure 5B.

The distribution of the fluctuations of the relative pheromone concentrations shown in Table 1 were very similar in spring and summer in the defoliated

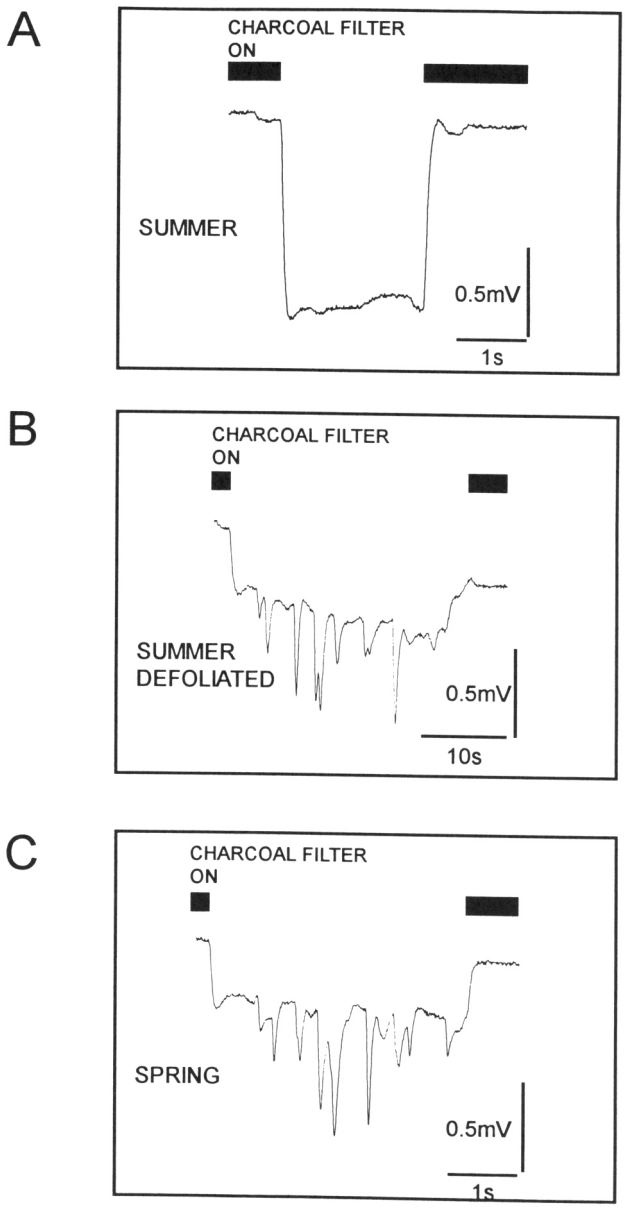

FIG. 5. Original EAG recording taken in the center of a pheromone-treated plot in summer 1989 (A), in the center of a defoliated plot (B) in summer 1989, and in spring 1989 (C) (same location as in A). The heavy black bars (labeled "on" at left) indicate when the charcoal filter was placed on the inlet of the EAG device, thus preventing an EAG response. Lack of a black bar indicates period when the charcoal filter was removed, and the EAG response was obtained.

TABLE 1. DISTRIBUTION OF PHEROMONE CONCENTRATION FLUCTUATIONS MEASURED IN SPRING AND DEFOLIATED VINEYARD IN SUMMER

Amplitude class	Relative pheromone concentration	Number of fluctuations in this amplitude class in 10 min	
		Spring	Summer
A	$<9.9 \times 10^{-7}$	21	18
B	1×10^{-6} to 3.3×10^{-6}	36	16
C	3.4×10^{-6} to 9.9×10^{-6}	48	28
D	1×10^{-5} to 3.3×10^{-5}	69	83
E	3.4×10^{-5} to 9.9×10^{-5}	24	36

plots. The highest number of fluctuations were in amplitude class D between 1×10^{-5} to 3.3×10^{-5} relative pheromone concentrations. In summer a larger number of fluctuations exceeding 3.4×10^{-5} were present. However, the distributions were not significantly different from each other [Wilcox, Mann and Whitney U test, $\alpha = 0.47$ (Sachs, 1973)]

DISCUSSION

Usually two consecutive generations of *L. botrana* occur in the grape-growing areas in Rhineland-Palatinate. While the flight of the first generation peaks in spring in vineyards with very little foliage or when foliage in the first stages is present only (stages 07–12; Eichhorn and Lorenz, 1977), the second generation flies when the grape vines consist of dense rows with fully developed leaves (stages 27–35; Eichhorn and Lorenz, 1977). Mating disruption is applied against both generations but it generally is more efficient for the control of the second generation (Cardé and Minks, 1995).

One possible explanation for the increased risk of failure in the first generation is the higher immigration of mated females from untreated vineyards into the pheromone-treated blocks (Cardé and Minks, 1995). However, the significantly lower mean relative pheromone concentrations we have measured in spring compared to summer (Figures 2 and 3) are likely to be a second factor. These significant differences may be explained by two processes, namely, higher temperatures in summer that lead to higher pheromone release rates from the dispensers and the establishment of higher mean pheromone concentrations that are supported by the presence of a dense leaf canopy. Contradictory to the first process, the pheromone dispensers are usually applied only once per season and

the release rates decline exponentially with time. It might be assumed that the higher release rate in spring, when the dispensers are first set out, should lead to higher mean pheromone concentrations in the field, but our results show that this is not the case because the foliage is thin and does not help maintain the pheromone in the immediate area of the vines.

Later in the season, the dense leaf canopy decreases the average windspeed in the canopy (Uchijiama, 1988) and adsorbs and rereleases pheromone (Wall et al., 1981; Noldus et al., 1991). The leaves function as secondary pheromone dispensers (Karg et al., 1990, 1994; Suckling et al., 1996). These factors support the establishment of increased mean relative pheromone concentrations when foliage is present. Our data corroborate earlier results reported by Karg and Sauer (1992) and Bengtsson et al. (1994), who showed that pheromone concentration was lower above a pea canopy than within the canopy.

Pheromone release and EAG recording can be affected by temperature (Bestmann and Dippold, 1989). However, the comparison of measurements taken in summer in vineyards with fully developed leaves with those taken in the nearby defoliated plots under the same climatic conditions shows a significant difference in mean relative pheromone concentrations, indicating the importance of the foliage. The higher temperatures in summer that induce higher pheromone release rates from the dispensers are not the only factors causing higher mean pheromone concentrations in summer as compared to spring.

Using the field EAG technique with a high temporal resolution, we showed a very different temporal and spatial distribution of the pheromone concentration in vineyards with a dense plant canopy compared to vineyards without foliage. Bengtsson et al. (1994) showed that pheromone concentrations above a pea canopy were characterized by an increased number of fluctuations compared to the measurements taken within the canopy. The ability of foliage to adsorb and release pheromone (Wall et al., 1981; Noldus et al., 1991; Suckling et al., 1996) causes a more even temporal and spatial distribution of the pheromone in the vineyard (Karg et al., 1990, 1994). The lack of a significant difference in distribution of fluctuations in the pheromone concentration in spring compared to defoliated vineyard in summer suggest that the canopy is the main parameter affecting the temporal and spatial distribution of the pheromone in vineyards.

Can the differences in the mean pheromone concentrations and the spatial and temporal distribution of the pheromone help to explain the differences in efficacy of mating disruption of the two consecutive generations of *L. botrana*?

While dose–response relationships between mating disruption and amount of applied pheromone have been shown in a number of cases (e.g., Flint et al., 1990), the importance of the structure of pheromone plumes in mating disruption trials has not been intensively studied. In a vineyard in summer with a dense canopy, adaptation of the moth sensory system and/or habituation of the behavioral response are likely to take place. Both mechanisms require sustained and/

or repetitive stimulation of the olfactory system (Bartell, 1982; Cardé, 1990). During the spring, and in a defoliated vineyard, lower mean pheromone concentrations and fluctuations of the pheromone concentration may allow sensory neurons time to recover. Receptor neurons have been shown to regain their initial senitivity to pheromone within less than a second if returned to clean air (Rumbo, 1981). Thus, in the spring, or in a defoliated vineyard, sensory adaptation is less likely to occur, and consequently disruption may fail (Cardé, 1990).

Pheromone fluctuations were shown to be important, however, in eliciting male upwind flight in wind-tunnel experiments (Kennedy et al., 1980). Male moths of *Adoxophyes orana* released in a wind tunnel did not fly upwind when there was a uniform cloud of pheromone, but required an intermittent signal for sustained upwind flight. These results were supported by studies with *Grapholita molesta* (Willis and Baker, 1984, Baker et al., 1985). Adaptation and/or habituation have been proposed as the cause of this cessation of flight (Baker et al., 1988, Baker and Haynes, 1989; Hansson and Baker, 1991). The importance of intermittent stimulation was corroborated under field conditions by Farkas et al. (1975), who showed that pheromone induced activity of male *Trichoplusia ni* (Huber) was reduced when males were exposed to a continuous pheromone cloud compared to males exposed to pulsed preexposure. Further work is needed to determine if pheromone fluctuations measured in the field are important for mating disruption of *L. botrana*, but our results support the hypothesis that adaptation and/or habituation are the mechanisms on which the success of mating disruption of *L. botrana* is based, and the conditions favoring adaptation and habituation are more favorable in summer with a fully developed plant canopy.

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RESPONSE OF INFECTIVE STAGE PARASITES (NEMATODA: STEINERNEMATIDAE) TO VOLATILE CUES FROM INFECTED HOSTS

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Abstract—Volatile infochemicals play a significant role in the interactions between trophic levels. Volatile infochemicals may allow species within the third trophic level to avoid patches where conspecifics or heterospecifics are present. We show odor-mediated resource assessment by entomopathogenic nematodes in the family Steinernematidae. We hypothesized that the infective juvenile nematodes may reduce inter- and intraspecific competition by responding differently to unparasitized hosts vs. hosts parasitized by conspecific or heterospecific nematodes. All *Steinernema* spp. except *S. carpocapsae* were attracted to hosts that were not parasitized. *Steinernema carpocapsae* infective juveniles were repelled from hosts infected for 4 hr with all heterospecific infections except *S. anomali*, whereas *S. glaseri* were repelled only from *S. riobravis*-infected hosts. *Steinernema feltiae* did not differentiate any heterospecific or heterogeneric infections. *Steinernema glaseri* were attracted to four of five heterospecific infections and *S. anomali* and *S. riobravis* were attracted to two of five heterospecific infections. Both *S. anomali* and *S. glaseri* were more attracted to hosts infected with the out-group *Heterorhabditis bacteriophora* than those infected by conspecific nematodes. Infective juvenile *S. carpocapsae*, *S. anomali*, and *S. glaseri* were more attracted to insects colonized by conspecific nematodes than to uninfested insects. Infective juvenile *S. carpocapsae* were repelled from the 24-hr-old conspecific infections, whereas *S. glaseri* were less attracted to 24- than to 4-hr-old conspecific infections. Experiments with insects injected with bacteria from the

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nematodes suggested the latter as the source of active volatiles. We suggest that odor-mediated host recognition by infective juveniles may reduce inter- and intraspecific competition among Steinernematidae.

Key Words—Foraging strategy, host finding, host recognition, insect-pathogenic nematodes, mate recognition, reproductive isolation, resource assessment, *Steinernema*, *Xenorhabdus*.

INTRODUCTION

Competitive interactions between species impact both competitors negatively (Price, 1984). Therefore, aversion to resources already lost to competitors should be favored by selection. On the other hand, when cooperation among individuals is required for resource procurement (i.e., mass-attack strategies), invasion of a resource by conspecifics should heighten its value, and therefore its attractiveness. Sympatric parasite species with overlapping host ranges are likely to be subject to both of these scenarios simultaneously. The evolution of coexistence of more than one species in the same host is unlikely because parasitoids finally kill and consume it (Strand et al., 1995). Entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) possess overlapping host ranges (Poinar, 1979) and more than one species has often been isolated from the same soil sample (Beavers et al., 1983, Akhurst and Brooks, 1984; Akhurst and Bedding, 1986; Ehlers et al., 1991; Akhurst et al., 1992; Campbell et al., 1995). Although interactions among species have not been studied in the field, differences in foraging strategies (Lewis et al., 1992, 1993; Campbell and Gaugler, 1993; Grewal et al., 1994a), thermal niches (Grewal et al., 1994b), and host affinities (Grewal et al., 1993a,b; Lewis et al., 1996) have been suggested as potential mechanisms reducing competition.

Entomopathogenic nematodes are lethal insect parasites that have a mutualistic association with *Xenorhabdus* and *Photorhabdus* bacteria (Boemare et al., 1993). Each nematode species is specifically associated with only one bacterial symbiont, except that some *Steinernema* species share the same *Xenorhabdus* species (Akhurst, 1993). The only free-living stage, the infective third-stage juvenile, carries bacteria in its intestinal tract (Poinar, 1979). After gaining access to the host hemocoel, the nematodes release bacteria that multiply rapidly, killing the host within 24–48 hr. The bacteria create optimal conditions for growth and sexual reproduction of nematodes. Depending upon the size of the host, one to three nematode generations are completed within the cadaver. When food resources are depleted, the infective juveniles are produced facultatively and leave the cadaver to initiate new infections.

Development and mating of these nematodes occurs only within hosts. These insect parasites have evolved two distinct reproductive strategies. Infec-

tive juveniles of all known species of *Heterorhabditis* develop into hermaphrodites in the first generation, the progeny of which consist of male, female, and hermaphroditic individuals (Zioni et al., 1992). Steinernematid nematodes are dioecious, and male infective juveniles may take a more active role in initiating infections (Grewal et al., 1993c). Following emergence from cadavers, the male infective juvenile *Steinernema* spp. disperse, locate, and establish infections in suitable live hosts (Grewal et al., 1993c). Early infection prepares hosts for nematode development and renders them more attractive to the following conspecific infective juveniles (Grewal et al., 1993c).

We hypothesize that entomopathogenic nematodes may decrease inter- and intraspecific competition by recognizing hosts infected by conspecific and heterospecific nematodes. We tested this hypothesis by recording behavioral responses of infective juvenile entomopathogenic nematodes to hosts that were unparasitized or parasitized by conspecific nematodes or by heterospecific nematodes. The three possible outcomes of these tests may reveal interactions of these extremely common, yet poorly known, parasite communities. Enhanced attraction to parasitized hosts relative to healthy ones would suggest some adaptive advantage for invading such a host; for example, compromised hosts offer reduced risk to invaders, or in the case of a conspecific infection, the certainty of potential mates may select for enhanced attraction (recruitment). Depressed attraction or repellence from parasitized hosts would indicate that selection favors individuals able to recognize competitive situations where it would either lose (in heterospecific infections) or develop asynchronously with potential mates (in conspecific infections). If no differences in the response to parasitized vs. unparasitized hosts occurs, we would expect no positive or negative impact to the individual invading a parasitized host.

METHODS AND MATERIALS

Nematode Strains and Their Maintenance. Five species of *Steinernema* were used: *S. anomali* Kozodoi (Ryazan strain), *S. carpocapsae* Weiser (All strain), *S. feltiae* (Filipjev) (SN strain), *S. glaseri* Steiner (NC strain), and *S. riobravis* Cabanillas, Poinar & Raulston (RGV strain). We also included as an out-group, *Heterorhabditis bacteriophora* Poinar (HP88 strain) for comparison. All nematodes were cultured in greater wax moth *Galleria mellonella* L. larvae at 25°C following methods described by Dutky et al. (1964). Infective juveniles were held in tap water for two to three weeks after their emergence from host cadavers.

Preparation of Hosts. Galleria mellonella larvae were exposed to nematodes in 24-well plates containing filter paper discs. Each larva (170–200 mg) was exposed to 200 infective juveniles of a nematode species in 80 μl of water.

After 4 hr, nematodes that had not penetrated were washed from the host cuticle. Noninfected wax moth larvae were treated with distilled water for 4 hr.

Response of Nematodes to Volatiles. The response of nematodes to volatiles from infected or uninfected hosts was measured using the quadrant plate bioassay described by Grewal and Wright (1992) and modified by Grewal et al. (1994a). Briefly, a 9-cm-diam. Petri dish was quartered and marked with concentric rings at 1, 2, and 3 cm from the center. The dishes were half filled with 2% agar, cooled and air-dried for 1 hr, and sealed with paraffin film. The cover had a 3-mm hole at the edge of one quarter to accommodate a pipet tip with two live infected or noninfected wax moth larvae. An inoculation port in the center allowed nematode introduction. Control treatments were run by the same procedure without *G. mellonella* larvae. The pipet tip with larvae was placed in the dish for 1.5 hr to allow a volatile gradient to form. Approximately 60 washed infective juveniles were then transferred to the center of the assay dish with a metal probe.

Nematodes located in each section of each quadrant of the assay dish were counted 5, 10, 20, and 30 min after inoculation (data for only 30 min duration are presented). An attraction index, defined as the mean distance (millimeters) traveled per nematode toward the treatment, was calculated by equation 1.

$$X = [10T1 + 20T2 + 30T3 - (10C1 + 20C2 + 30C3)] * N^{-1} \quad (1)$$

The number of nematodes in the first, second, and third arcs of the treatment quadrant and in the control quadrant are represented by T1, T2, and T3 and C1, C2, and C3, respectively. N represents the total number of nematodes in the plate. All tests were conducted at 22 ± 1°C. Treatments and controls were replicated three times for each species. After 48 hr, the test larvae were removed from the pipet tips and nematodes established in each larva were counted after dissection.

Response of Nematodes to Volatiles from Insects Injected with Symbiotic Bacteria from Nematodes. We studied the response of selected nematode species to volatiles of *G. mellonella* injected with *Xenorhabdus* bacteria. These were isolated from the infective juvenile nematodes following methods described by Akhurst (1980) and cultured in nutrient broth for 48 hr at 25°C. A bacterial suspension (1.5 μl) was injected into each larva through a proleg. The larvae were then incubated at 25°C for 4 hr. The response of infective juveniles to volatiles from the larvae was then studied using the quadrant plate bioassay as described above. Responses of the nematodes *S. glaseri* and *S. carpocapsae* to their natural symbiotic bacteria *Xenorhabdus poinari* and *X. nematophilus*, respectively, and to *Xenorhabdus* sp. from *S. riobravis*, were studied. Larvae for control treatments were injected with 1.5 μl of sterile nutrient broth. The growth of bacteria in the insects was confirmed by the mortality of insects within 24–48 hr following injection.

Release of Bacteria into Insect Hemolymph by Infective Juveniles. To determine how rapidly the bacteria are released into the host hemocoel by nematodes following penetration, we exposed each *G. mellonella* larva to 200 infective juveniles of either *S. glaseri* or *S. carpocapsae*. Ten larvae were used for each species. After 6 hr, larvae were washed with sterile distilled water to remove nematodes from the cuticle, and the surface was sterilized with 0.2% hyamine for 2 min. One microliter of hemolymph was then drawn out and plated on Trypto soy agar (TSYA) and an indicator medium T7 (Woodring and Kaya, 1988). After three days of incubation at 25°C, the plates were examined for the presence of *Xenorhabdus* bacteria.

Response to Insects Colonized by Nematodes for Different Durations. Wax moth larvae were exposed to 200 infective juveniles of *S. carpocapsae* or *S. glaseri* on filter papers as described above. After 4 or 24 hr of incubation, larvae were washed with distilled water to remove nematodes from the cuticle. Responses of infective juveniles to volatiles from the larvae were then measured using the quadrant plate bioassay.

Statistical Analyses. Nematode responses were analyzed using analysis of variance program (Anonymous, 1987). All comparisons were at 5% significance level.

RESULTS

All *Steinernema* spp. except *S. carpapsae* were attracted to nonparasitized host larvae (Figures 1–5). *S. carpocapsae* were repelled from hosts containing *S. riobravis*, *S. glaseri*, *S. feltiae*, and *H. bacteriophora* infections and showed no response to hosts infected with *S. anomali* (Figure 1). *S. glaseri* infective juveniles were repelled only from insects containing *S. riobravis* infections (Figure 2). Infective juvenile *S. feltiae* were not attracted to hosts containing any heterospecific or heterogeneric infections (Figure 3). Infective juvenile *S. anomali* and *S. riobravis* were not attracted to hosts with heterospecific or heterogeneric infections except *S. feltiae* and *H. bacteriophora* (Figures 4 and 5).

Infective juvenile *S. carpocapsae*, *S. glaseri*, and *S. anomali* showed greater attraction to hosts infected with conspecific nematodes than uninfected hosts ($P < 0.001$, Figure 1; $P < 0.03$, Figure 2; and $P < 0.043$, Figure 3, respectively). Such a response was not observed for *S. feltiae*, and *S. riobravis*. Mean numbers of nematodes established per host following a 4-hr exposure were 33 $\pm$ 3.5, 76 $\pm$ 10.5, 94.5 $\pm$ 7.0, 24.6 $\pm$ 1.1, 15 $\pm$ 3.5, and 12 $\pm$ 2.5 for *S. anomali*, *S. carpocapsae*, *S. feltiae*, *S. glaseri*, *S. riobravis*, and *H. bacteriophora*, respectively.

Responses of infective juveniles to infected hosts were not entirely due to symbiotic bacteria as they were not always detected in hosts following 6 hr of

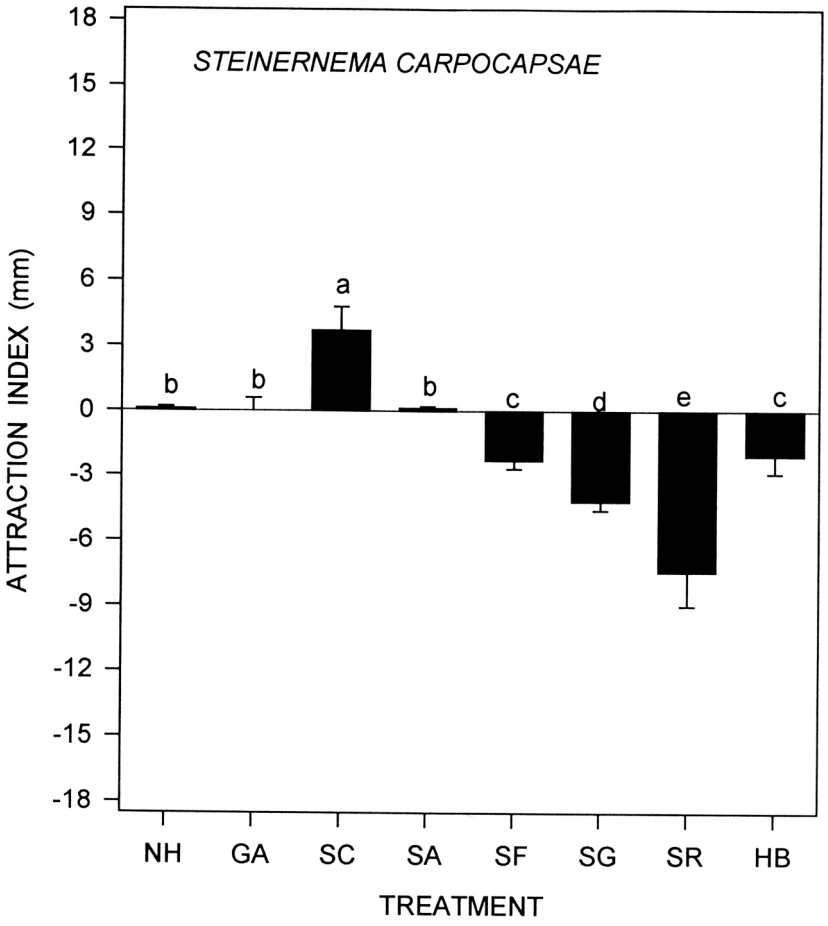

FIG. 1. Attraction or repulsion of infective juvenile *Steinernema carpocapsae* to uninfected or infected hosts. Treatments are: NH = no host; GA = uninfected host; SC = *S. carpocapsae*; SA = *S. anomali*; SF = *S. feltiae*; SG = *S. glaseri*; SR = *S. riobravis*; HB = *Heterorhabditis bacteriophora*. Bars with the same letters are not significantly different at $P > 0.05$.

exposure to the nematodes. Hemolymph from larvae exposed to *S. carpocapsae* infective juveniles for 6 hr contained *X. nematophilus*, whereas those exposed to *S. glaseri* did not. Furthermore, infective juvenile *S. carpocapsae* were repelled from insects injected with their natural symbiont *X. nematophilus* and *Xenorhabdus* bacteria from *S. riobravis* (attraction indices of -0.9 ± 0.3 and -0.7 ± 0.2, respectively). In contrast, infective juvenile *S. glaseri* were attracted

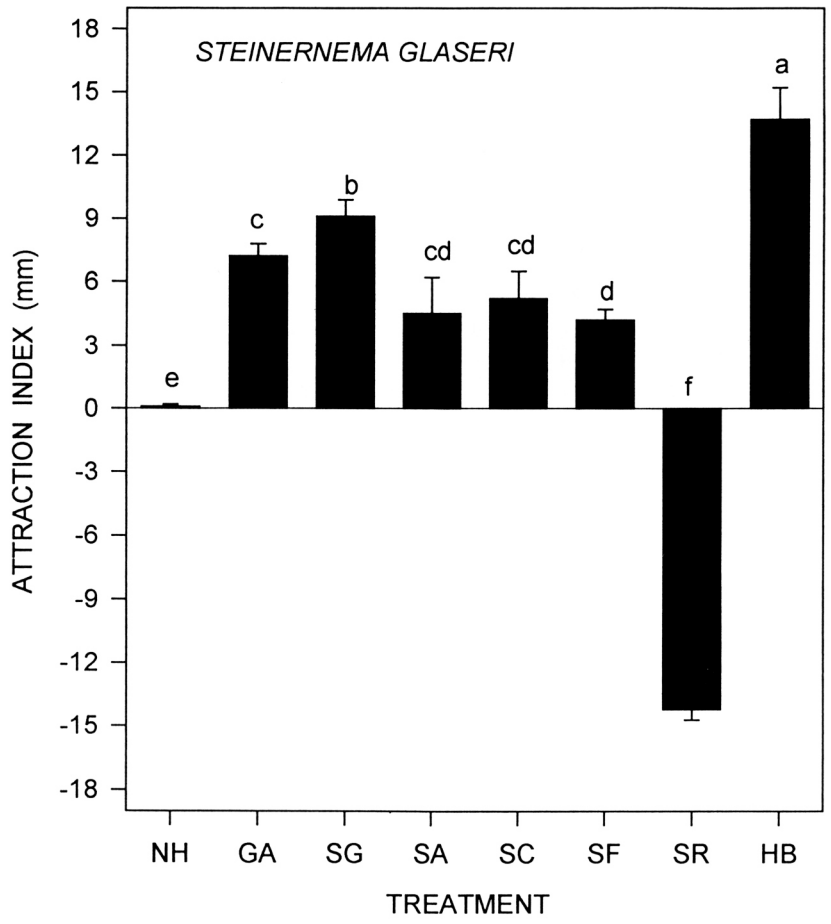

FIG. 2. Attraction or repulsion of infective juvenile *Steinernema glaseri* to uninfected or infected host. Treatments are: NH = no host; GA = unifected host; SG = *S. glaseri*; SA = *S. anomali*; SC = *S. carpocapsae*; SF = *S. feltiae*; SR = *S. riobravis*; HB = *Heterorhabditis bacteriophora*. Bars with the same letters are not significantly different at $P > 0.05$.

to larvae injected with their natural symbiont *X. poinari* and bacteria from *S. riobravis* (attraction indices of 3.5 ± 0.7 and 2.4 ± 0.3, respectively).

Infective juveniles also responded differently to hosts exposed to conspecific nematodes for different durations. Infective juvenile *S. carpocapsae* were attracted to hosts colonized by conspecific nematodes for 4 hr but were repelled from hosts possessing 24-hr-old infection (attraction indices 3.8 ± 0.5 and -8.3

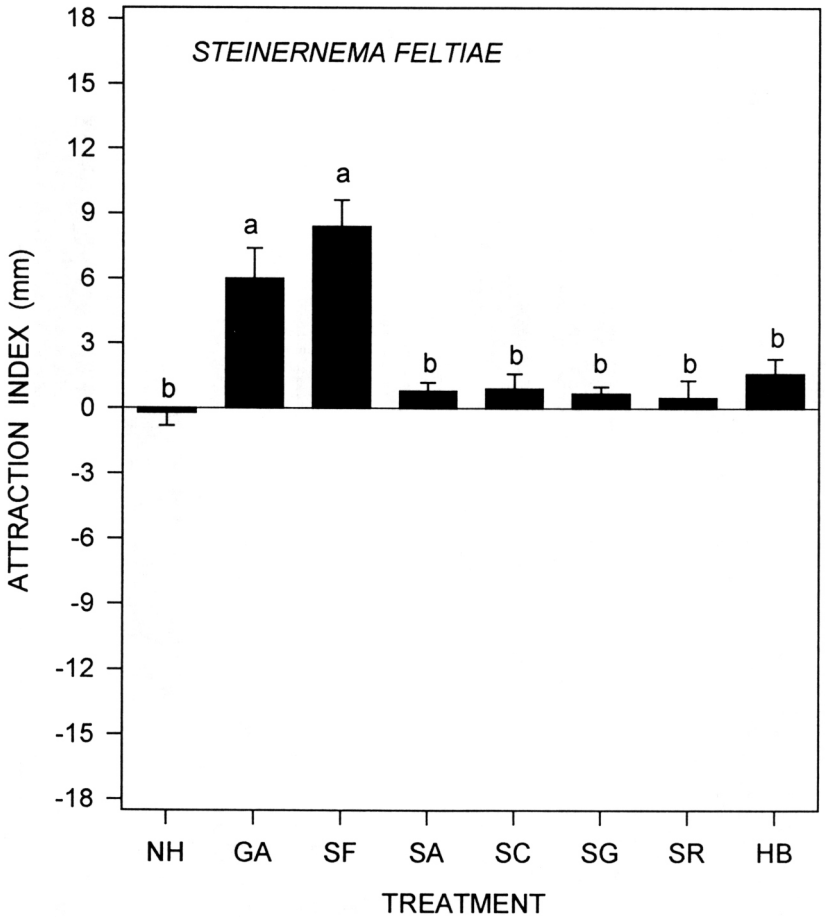

FIG. 3. Attraction or repulsion of infective juvenile *Steinernema feltiae* to uninfected or infected hosts. Treatments are: NH = no host; GA = uninfected host; SF = *S. feltiae*; SA = *S. anomali*; SC = *S. carpocapsae*; SG = *S. glaseri*; SR = *S. riobravis*; HB = *Heterorhabditis bacteriophora*. Bars with the same letters are not significantly different at $P > 0.05$.

± 1.2, respectively, $P < 0.001$). Infective juvenile *S. glaseri* were more attracted to hosts colonized for 4 hr than 24 hr (attraction indices 9.1 ± 0.6 and 3.4 ± 0.4, respectively, $P < 0.01$).

DISCUSSION

Entomopathogenic nematodes, *Steinernema* spp., clearly distinguish between hosts that are unparasitized and those parasitized by conspecific or

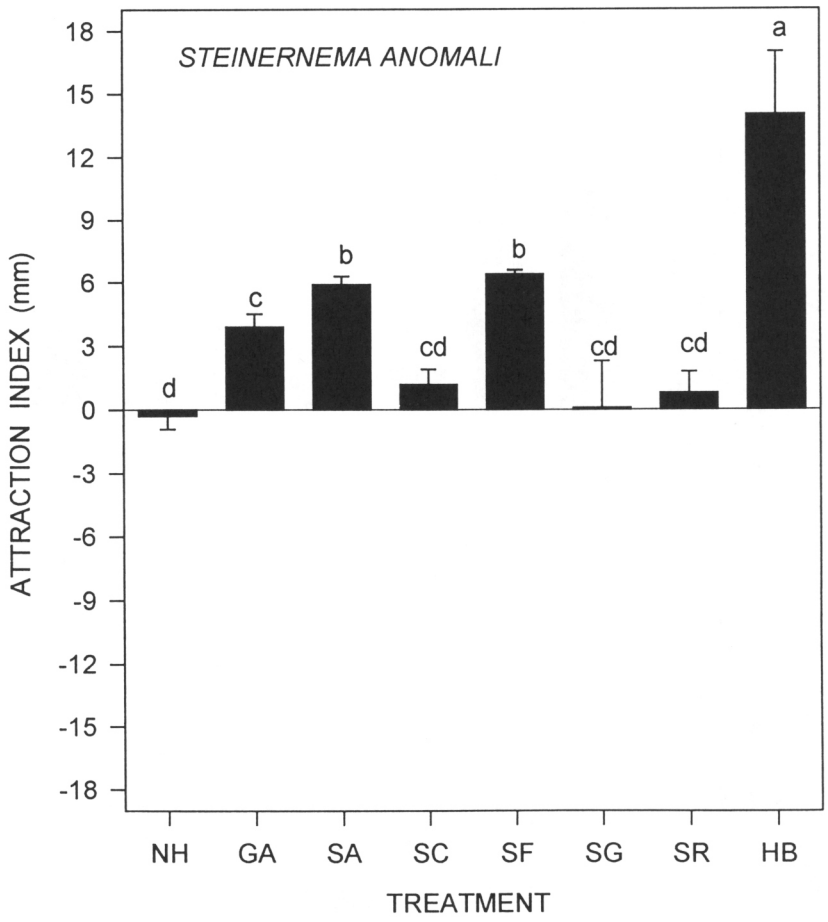

FIG 4. Attraction or repulsion of infective juvenile *Steinernema anomali* to uninfected or infected hosts. Treatments are: NH = no host; GA = uninfected host; SA = *S. anomali*; SC = *S. carpocapsae*; SF = *S. feltiae*; SG = *S. glaseri*; SR = *S. riobravis*; HB = *Heterorhabditis bacteriophora*. Bars with the same letters are not significantly different at $P > 0.05$.

heterospecific nematodes. Janssen et al. (1995) reported that *Leptopilina heterotoma* (Hymenoptera: Eucoilidae) an endoparasitoid that attacks *Drosophila* spp. larvae, avoids decaying stinkhorn mushroom patches containing parasitoids of another species (*L. clavipes*). Odor-mediated detection by *L. heterotoma* allows it to avoid superior competitors by smelling their presence from a distance. We propose that the odor-mediated responses of nematodes may reduce inter- and intraspecific competition among Steinernematidae and enhance their reproductive success.

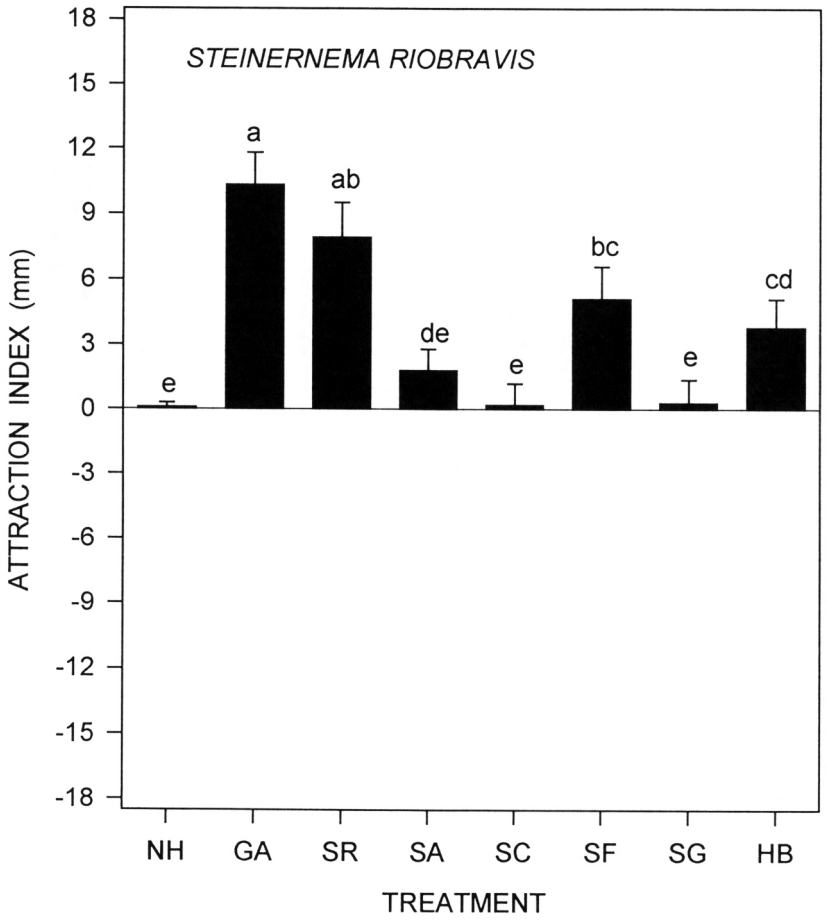

FIG. 5. Attraction or repulsion of infective juvenile *Steinernema riobravis* to uninfected or infected hosts. Treatments are: NH = no host; GA = uninfected host; SR = *S. riobravis*; SA = *S. anomali*; SC = *S. carpocapsae*; SF = *S. feltiae*; SG = *S. glaseri*; HB = *Heterorhabditis bacteriophora*. Bars with the same letters are not significantly different at $P > 0.05$.

Our data explain results of previous studies of entomopathogenic nematode community dynamics within hosts. Studies on intraspecific competition among *Steinernema* spp. reveal that infective juvenile production is greatest when an optimum number of infective juveniles initiate the infection (Zervos et al., 1991; Selvan et al., 1993). We found that early in an infection by *S. glaseri* and *S. carpocapsae*, attraction of conspecific infective juveniles was increased. How-

ever, as the infection progressed through time, this heightened level of attraction diminished. This scenario may favor the maintenance of an optimum infection level. Studies of interspecific competition indicate that some species of entomopathogenic nematode are more successful competitors than others. For example, *S. glaseri* always outcompeted *S. carpocapsae* in simultaneous infections due to their faster rate of development within the host (Koppenhoffer et al., 1995). Odor-mediated avoidance of heterospecific infections by *S. carpocapsae* reflects these competitive interactions.

Host colonization is initiated by infective juvenile nematodes releasing specific bacteria into the hemocoel. Specific association of each nematode species with a different *Xenorhabdus* sp. would suggest that the suitability of hosts may be due to the presence of appropriate bacteria. However, the responses of infective juveniles to hosts containing bacteria alone suggest that host recognition is not merely a food-selection process. For instance, infective juvenile *S. glaseri* were repelled from hosts colonized by *S. riobravis* but were attracted to those injected with symbiont bacteria from these nematodes. Thus the source of volatiles may be related to nematodes rather than to bacteria.

Enhanced attraction of *S. anomali*, *S. carpocapsae*, and *S. glaseri* to hosts colonized by conspecific nematodes bolsters the mate recruitment argument advanced by Grewal et al. (1993c). They demonstrated that male infective juveniles are dispersers and are more responsive to host cues than are females during host-finding and colonization. Colonization subdues live hosts and prepares them so that potential mates can more easily find them, enter, and reproduce (Grewal et al., 1993c). The absence of data that support recruitment in the other species included in this study may be due to different rates of the development of infection.

Differences in response of *Steinernema* spp. to uninfected hosts represent differences in their foraging strategies (Lewis et al., 1992, 1993, 1995; Grewal et al., 1994a). Cruise (widely ranging) searchers rely heavily on chemical cues to locate their remote prey, whereas the ambush (sit-and-wait) foragers rely on host mobility (Huey and Pianka, 1981; Bell, 1991). The weak response of *S. carpocapsae* infective juveniles to uninfected hosts is typical of their ambush foraging behavior, whereas the directional response of *S. anomali*, *S. glaseri*, *S. feltiae*, and *riobravis* represents a more mobile foraging strategy (Grewal et al., 1994a).

Mate acquisition is an adaptive process as important as avoidance of predators, the ability to find a refuge, or the acquisition of food (Paterson, 1985). The subset of adaptations that are involved in signaling between mating partners constitute a species-specific mate recognition system (Paterson, 1985). Our results indicate that together with the suitability of hosts for food, the availability of potential mates may also be assessed by the nonreproductive infective stages of *Steinernema* spp. Therefore, host-finding and recognition strategies of infec-

tive juvenile entomopathogenic nematodes may also reinforce reproductive isolation.

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INDUCED RESPONSES OF COMMON ANNUAL SUNFLOWER *Helianthus annuus* L. FROM GEOGRAPHICALLY DIVERSE POPULATIONS AND DETERRENCE TO FEEDING BY SUNFLOWER BEETLE

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Abstract—The coumarins ayapin and scopoletin, which are expressed in leaves and other tissues of *Helianthus annuus* L., have a variety of effects on fungi and insects. The relative importance of these coumarins in the defenses of *H. annuus* against the sunflower beetle was investigated. Unstimulated leaves of 107 populations of wild annual sunflower from various states of the United States were analyzed for coumarin expression. Contralateral leaves were stressed with cytotoxic $HgCl_2$ to provoke a typical wounding response (including the synthesis of defensive chemicals) and then were also analyzed. Some populations showed almost no increase in scopoletin content after stimulation, and others showed up to a 56-fold increase. The average increase of scopoletin content was about nine fold. Populations collected from the region where *H. annuus* ssp. *texanus* was prevalent had the highest concentration of scopoletin after stress. A subselection of those populations that had high coumarin content or that had been stimulated to high levels of coumarin was assayed for feeding deterrence against the sunflower beetle (*Zygogramma exclamationis* F.). A two-choice feeding bioassay was accomplished within a standard arena over 20 hr. When preferences for leaves of each sunflower population were tested against a commercial cultivar without leaf stress, only one of 11 populations was found to show significant deterrence when total leaf area eaten was analyzed. However, after leaf stress, eight of 11 populations became significantly deterrent, including the one that had been deterrent without stimulation. Differences between scopoletin content and between feeding deterrence before and after stimulation were displayed graphically to determine if a correlation could be made between coumarin content and feeding behavior.

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The plot showed that in six of 11 populations increased scopoletin content was indeed correlated with decreased feeding, but that in another two, these factors were not correlated; another two populations showed no difference in feeding from the control scores after stress and could not be used in this graphical correlation procedure. One population showed a decrease in both coumarins after stimulation along with decreased feeding, suggesting that additional unidentified deterrents were formed. From the analysis of coumarin distributions and the feeding results, we conclude that coumarins are widely occurring, inducible constituents in the wild common annual sunflower. Coumarins may be of general importance in *H. annuus* populations throughout their range either in deterring sunflower beetle or in additional effects on other organisms. Coumarins appear to be deployed typically in induced defenses and may not be important in constitutive defenses.

Key Words—Coumarin, scopoletin, induced responses, sunflower, *Helianthus annuus*, sunflower beetle, *Zygogramma exclamationis*, feeding deterrence, herbivory, defensive chemicals.

INTRODUCTION

Following damage to plants by insects or other organisms, subsequent feeding by insects may be reduced on adjacent plant parts or on even more distant ones (Harrison and Karban, 1986; Raupp and Sadof, 1989; Lin et al., 1990). Many of these induced changes in insect preference for a host plant may occur after a short or intermediate time interval following the damage and may be characterized as physical or chemical alterations to the plant (Green and Ryan, 1972; Schultz and Baldwin, 1983; Edwards and Wratten, 1983). These changes in the qualities of the host may affect the growth of the insect as well as the decision to feed (see examples in Coleman and Jones, 1991; Alborn et al., 1996).

Attributing changes in feeding preferences to specific induced constituents often has not been straightforward. Purported deterrence to squash beetles from rapidly induced squash cucurbitacins (Carroll and Hoffman, 1980) has been discounted by more appropriate experiments (Tallamy and McCloud, 1991). Deterrence of cabbage looper (*Trichoplusia ni* Hübner) due to inducible defensive responses of soybean has been attributed to a variety of constituents. The isoflavonoids daidzein and coumestrol may have provided deterrence (Hart et al., 1983; Neupane and Norris, 1990); other reports have suggested that the pterocarpan isoflavonoid glyceollin did likewise (Fischer et al., 1990; Neupane and Norris, 1991). Kogan and Fischer (1991) have appropriately referred to the deterrents active in soybean against herbivorous insects as "elusive."

One apparent defense of *Helianthus annuus* L., the common annual sunflower, is the induced synthesis of two coumarins, ayapin and scopoletin. These coumarins were formed in response to mechanical damage (Olson and Roseland, 1991), feeding by sunflower beetles or thrips (Olson and Roseland, 1991), infec-

tion by fungi (Tal and Robeson, 1986a,b), and stress induced by applied chemicals (Dieterman et al., 1964; Roseland et al., 1991). The biological responses to these coumarins have been identified as growth inhibition of nonpathogenic fungi on sunflower (Tal and Robeson, 1986a) and deterrence to feeding by the sunflower beetle (Olson and Roseland, 1991).

Simple coumarins related to scopoletin and ayapin occur in a variety of other plants (Murray et al., 1982) and have been correlated with deterrence to insect feeding. Gorz et al. (1972) reported that blister beetles of the genus *Epicauta* showed feeding preferences related to the content of coumarin and *cis*-O-hydroxycoumarin in different lines of sweet clover. Coumarin applied topically to radish cotyledon deterred feeding of flea beetles (Meisner and Mitchell, 1984) and dietary incorporation inhibited feeding of larval *Leptinotarsa decemlineata* (Muckensturm et al., 1981).

In contrast to the nontoxic, simple coumarins detected in sunflower (Olson, 1989) and other plants, certain of the more complex coumarins, such as the furanocoumarin xanthotoxin (from parsnip), have been characterized as photoactivated toxins (Berenbaum and Feeny, 1981; Zangerl and Berenbaum, 1993). The effects of toxic parsnip furanocoumarins on the feeding preference decisions of the parsnip webworm, a specialist insect, have not been fully described. Yajima et al. (1977) have shown that these and similar toxic furanocoumarins deter feeding when incorporated into an artificial diet of the polyphagous larval species, *Spodoptera litura*.

The sunflower beetle (Chrysomelidae: *Zygogramma exclamationis* F.) is primarily a specialist on a few species of *Helianthus* (Criddle, 1922), several of which produce the coumarins ayapin and scopoletin (Roseland, Grosz, and Seiler, unpublished). The concentration of these coumarins varies in different stages of growth or in different environmental conditions (Roseland, Grosz, and Seiler, unpublished). Olson and Roseland (1991) have shown that sunflower leaves treated topically with coumarin concentrations characteristic of stressed plants were less preferred by sunflower beetle than were untreated leaves. In seedling sunflower grown without applied stress in the greenhouse, scopoletin may be absent (Olson and Roseland, 1991) or present at a low constitutive concentration (Roseland and Grosz, unpublished). The second coumarin, ayapin, has usually been detected only in stressed plants (Olson and Roseland, 1991; Roseland et al., 1991). In this study, one objective was to confirm that endogenous coumarins cause deterrence comparable to that observed following topical application of coumarins to sunflower.

Although it seems counterintuitive that a specialist herbivore might be deterred by a constitutive chemical of its host, such chemicals have been shown to cause feeding deterrence of several other specializing insect species. When soybean cotyledons were exposed to high-intensity UV irradiation, synthesis of glyceollin was enhanced in plant tissues. The Fabaceae-specializing Mexican

bean beetle preferred controls to the treated host plants (Kogan and Fischer, 1991) as a consequence of elevated concentrations of the previously identified glyceollin deterrent (Fischer et al., 1990). The cherry oat aphid, a specialist of small grains and grasses (Poaceae), induced increased content of hydroxamic acids when feeding on wheat (Gianoli and Niemeyer, 1995). The same aphids were deterred from feeding on artificial diets or on wheat cultivars that contained increasing concentrations of the hydroxamic acid DIMBOA (Givovich and Niemeyer, 1996). Even the specialist insect *Manduca sexta*, the tobacco hornworm, showed reduced consumption of tobacco leaves that expressed an increased content of nicotine, despite an ability to resist alkaloid by means of an efficient excretion mechanism (Baldwin, 1991). Other deleterious effects of constitutive chemicals of the host to specialist insects are also known, besides induced deterrence. The isoflavonoid coumestrol, which is an inducible phytoalexin of legumes (Liu et al., 1992), has been shown to cause mortality in the Leguminaceae-specializing velvetbean caterpillar (Slansky and Wheeler, 1992).

Where insect preferences have been assayed with respect to a major deterrent, usually only one or several cultivars of a plant species were investigated, and preferences were not analyzed among natural plant populations. That is, a single deterring chemical has not been shown to cause feeding deterrence towards an herbivorous insect across diverse genotypes within a species. Given that coumarins are deterrent to sunflower beetles, the role of these chemicals in the defense of sunflower across several populations collected over a wide geographic area was sought.

In this series of observations and experiments, we first investigated the occurrence of coumarins in a large number of sunflower populations grown in a single environment. Next, we evaluated the role of both constitutive and induced coumarins on the palatability of sunflower to the beetles. We used a standard strategy for inducing the plant phytoalexins that are active against fungi: topical treatment with heavy metals (Darvill and Albersheim, 1984). Both cytotoxic stresses and mechanical wounds may activate synthesis of defensive chemicals. By analyzing the relationship between coumarin content and feeding deterrence across various populations, we expected to determine whether coumarins were principal chemicals that generally deter feeding of the sunflower beetle. Finally, we attempted to describe the known deterring effects of coumarins on herbivory by the sunflower beetle within the larger context of other chemical defenses of sunflower.

METHODS AND MATERIALS

Plants. In 1991, seeds of wild populations from 11 US states were obtained from the USDA Plant Introduction Station at Ames, Iowa. Seedlings were ger-

minated on moist paper in the cold for several weeks without scarification. Plants were grown in a greenhouse with supplemental 1000-W overhead sodium lamps on a 15-hr photophase. Eleven populations were selected in 1992 for characteristics that are described in the results. The USDA P.I. numbers assigned to these populations were abbreviated by letters and the correspondence between them is noted in Table 3 (below).

Seeds of the wild populations used in 1992 were surface-sterilized in a 2% sodium hypochlorite solution for 20 min and rinsed three times with sterile water. Seeds were scarified, put into a Petri plate with moist filter paper, and then held in the dark overnight. The next day the seed coat was removed and the embryos returned to a moist sterile Petri plate. Germination was completed in the dark at room temperature. Two days after germination, the seedlings were placed under fluorescent lights for one day and transplanted to soil (Sunshine Mix no. 1, Fison's Western Corp, Vancouver, British Columbia) in 10-cm pots the next day. Subsequently, seedlings were grown in a greenhouse supplemented with sodium lamps on a 15-hr photophase. Seeds of Interstate 3311 (IS3311; Interstate-Payco Seed Co.), the control population, were not pregerminated but were sown directly into soil of 10-cm pots in the greenhouse.

Stress Induction. One leaf of each plant was stressed with a solution of one mg/ml mercuric chloride (Sigma Chemical Co.) in 0.01% aqueous Tween 20 (Nutritional Biochemicals, Inc.), which was applied to the upper leaf surface with a brush. The opposite leaves were treated with Tween 20 only and served as control leaves. After 72 hr, leaf samples were harvested for coumarin analysis and sunflower beetle bioassay.

In a separate experiment, the qualitative effects of a variety of stresses were compared to those produced by a heavy metal. Plants were treated with UV, mercuric chloride, or acetyl salicylic acid, an analog of the known defensive coordinating chemical, salicylic acid (Raskin, 1992). For these experiments, the cultivated line IS3311 was used exclusively. Coumarin content was analyzed and feeding deterrence measured using techniques similar to those reported in Roseland et al. (1991). UV stress was administered by germicidal UV lamps (Sylvania mercury vapor germicidal lamps, #G30T8, 36-in. length), which were rotated in an arc about 20 cm above the 4-week-old plants. Two exposures of five min each were given five and two days before analysis. Identical, unexposed plants served as controls. Mercuric chloride (1 mg/ml) in aqueous Tween 20 solution was brushed on the upper leaf surfaces of the third or fourth leaves two days before the feeding experiment. The opposite leaves received Tween 20 treatment only. Acetyl salicylic acid (Sigma), 15 mM in Tween 20, was sprayed on the third and fourth pair of leaves eight days before analysis. The contralateral control leaf received only an aqueous Tween application.

Coumarin Analysis. Five 1-cm leaf discs were taken from each treated and control leaf and the fresh weights obtained. The leaf discs were homogenized

in 95% ethanol. A hexane wash removed lipophilic materials from the ethanol homogenate. The samples were hydrolyzed to free the sugar conjugates of scopoletin, and differential extraction into chloroform was accomplished. After drying and resuspension in methanol, samples were injected into a reverse-phase C_{18} HPLC column for separation and comparison with standards for ayapin (synthesized by M. Olson; see Olson and Roseland, 1991) and scopoletin (Sigma). Coumarins were quantitated with fluorescence detection at 330 nm (excitation) and 440 nm (emission). Coumarin recovery was 66% for scopoletin and 57% for ayapin. All coumarin values are reported without adjustment; for details, see Roseland et al. (1991).

Coumarin content was measured in treated and control leaves of five or six plants of each population. Samples were taken from the fourth or fifth leaf pairs, one leaf pair lower than that used in the feeding bioassay. Plants were 5 weeks old when young, fully expanded leaves developed at the fifth or sixth node. In the analysis that compared the effects of three inducing stressors, the third and fourth leaf pairs were used.

Insect Bioassays. The feeding preferences of beetles for the wild *H. annuus* populations and for the commercial hybrid IS3311 were compared in a dual choice feeding bioassay. All leaves were rinsed with water before the assay to remove mercuric chloride or acetyl salicylic acid. Two test leaves derived from the wild population and two leaves of the control were placed in a 25-cm aluminum dish whose surface was covered with a moist filter paper. Cut leaves were maintained in a turgid condition by placing the petioles in a cotton-plugged test tube filled with tap water. A plastic dome about 15 cm tall covered the arena. Leaves were equilibrated for 3 hr before the initial leaf area was measured.

Sunflower beetles used in the assay were collected from a field adjacent to the Carrington Research Center (Carrington, North Dakota). The insects had all been feeding on a commercial crop planted to a single variety of sunflower, and the plants were in the four leaf-pair stage. Beetles were later fed on IS3311 plants grown in the greenhouse until 24 hr before the assay and then were starved for one day prior to the bioassay. Four adult beetles were placed in each arena and allowed to feed for 20 hr in a rearing room with a 14L:10D regime at 22–26°C. Up to 26 leaf measurements were made of each unstimulated population tested, and up to 10 for each stimulated population. Unstimulated plants were assayed in June 1992, and both stimulated and unstimulated plants were assayed again two months later. Leaf areas were measured before and after feeding with a leaf area meter (LI-COR, Lincoln, Nebraska).

Statistical Analysis. The area of the leaves consumed in the test populations and the controls were compared using a t test at $P < 0.05$. The preference index of Kogan and Goeden (1970), $2T/(C + T)$ where C is the area of control leaf (cultivated hybrid) consumed and T is the area of the leaf consumed from the wild population, was also used to compute feeding scores. The scores range

from 0 to 2, in which 1 is no preference, 0 is maximum deterrence of the accession and 2 is maximum preference for the test accession.

Differences in coumarin content in successive years were analyzed by means of PROC GLM (SAS/STAT User's Guide version 2 GLM-VARCOMP, SAS Institute Inc., Cary, North Carolina, 1990). A linear regression analysis for coumarin content and feeding was performed on the data with the PROC REG procedure. Regional variation of coumarins was analyzed by t test, and by Waller-Duncan k-ratio t test (PROC TTEST) using both the geographic zones described by Rieseberg et al. (1987) and the geographic locations given for three of the four *H. annuus* subspecies.

Graphic Analysis Technique for Induced Changes in Feeding. Varying matrices of chemical constituents or differing arrays of physical characters in the sunflower populations may affect feeding independently of a single, specific chemical. A method was sought to limit the effects of these variables and thereby sort out the active role of coumarin in feeding nonpreference. Such a method would analyze insect feeding deterrence within each population and avoid making a conclusion based on the goodness of fit of all populations to a regression line.

The method selected is one that graphically displays differences between feeding scores (the x value) and differences between coumarin content (the y value) for stressed and unstressed plants of each population. These differences were assigned as coordinates on a scatterplot diagram. Thus, we subtracted feeding scores on leaves that were not stimulated from scores on leaves that were stimulated. Similarly, the change in scopoletin (or ayapin) concentration was represented using the formula for fold increase $(T - C)/C$, where C is the scopoletin concentration without stimulation, and T is the concentration after stimulation. By using both the difference and the unstimulated value, we took into account both the direction of the induced changes in concentration, as well as the magnitude of the coumarin concentration in the unstimulated plants. When T exceeded C, values were positive (the stimulated coumarin concentration exceeded the unstimulated concentration), and when T was smaller than C, values were negative. If there were no differences in coumarin concentration following stimulation, the y term had a value of 0. If the average concentration of ayapin was less than the minimum detectible amount of ayapin (0.06 ng/mg) in a single determination, we rounded the average concentration of ayapin down to 0, since accuracy at this concentration was likely to be low. This procedure will be referred to as the graphic analysis method.

RESULTS

Occurrence of Coumarin in Unstimulated Leaves. A frequency histogram of mean scopoletin concentrations was plotted for the leaves of 107 unstimulated

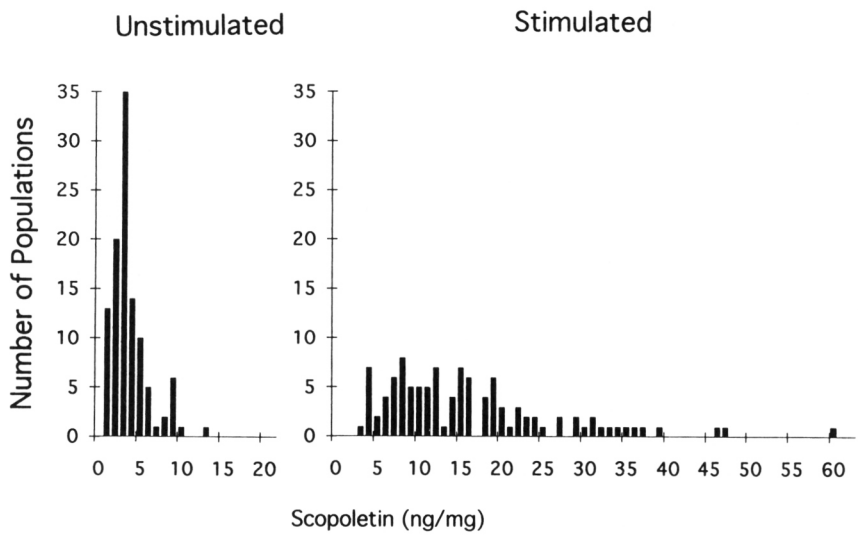

FIG. 1. Frequency histogram of scopoletin content in populations of *H. annuus*, stimulated and unstimulated.

sunflower populations that were anlayzed in 1991 (Figure 1). The mean content of scopoletin in all populations was 2.9 ng/mg. Relatively few populations had high concentrations of the coumarin. Thirty-seven populations had concentrations above the mean and 70 had concentrations below it. Only one population had less than 0.5 ng scopoletin/mg plant tissue.

A frequency histogram of ayapin concentrations in unstimulated leaves of the populations was also plotted (Figure 2). Only five populations contained detectable concentrations of ayapin, and of these usually only one sample from six leaves expressed ayapin. The mean content of ayapin among the five was 0.387 ng/mg. The highest quantity detected was 1.06 ng/mg.

Occurrence of Coumarin in Stimulated Leaves. The effects of stimulation by UV irradiation or by acetyl salicylic acid on coumarin content were compared to stimulation by topical treatment of mercuric chloride ($HgCl_2$). All three treatments induced elevated concentrations of scopoletin ($P < 0.05$) in IS3311 (Table 1). UV stimulation produced the highest concentration of coumarins.

After stimulation of 107 populations of *H. annuus* with $HgCl_2$, scopoletin concentrations (Figure 1) showed a more extended tailing of values not observed in the distribution of unstimulated leaves. The mean scopoletin content in the leaves of stimulated populations was 15.7 ng/mg. Sixty-six populations had a scopoletin content below the mean and 41 populations had a content above it. Scopoletin content increased an average of 8.6-fold between stimulated and

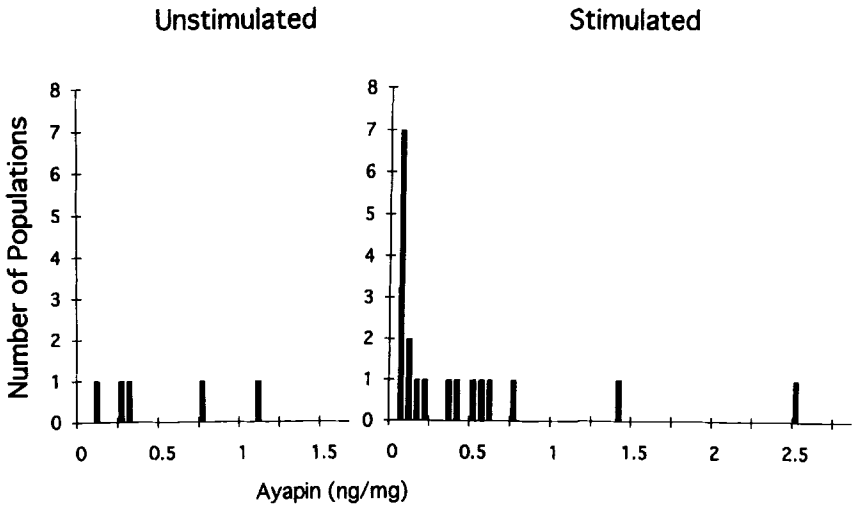

FIG. 2. Frequency histogram of ayapin content in populations of *H. annuus*, stimulated and unstimulated.

unstimulated leaves and varied from no increase to a 56-fold increase. Scopoletin contents for typical populations are given in Table 2 along with the stimulated increase for each.

About 18% of the populations (19) had detectable levels of ayapin in 1991 after stimulation (Figure 2). The mean content of ayapin of these populations was 0.40 ng/mg. The highest concentration was 2.45 ng/mg. Thirteen of the 19 populations producing ayapin had an ayapin concentration between 1% and

TABLE 1. SIMULATION OF COUMARIN RESPONSE BY $HgCl_2$, UV, AND ACETYL SALICYLIC ACID

	Scopoletin (ng/mg)		Ayapin (ng/mg)		Significant deterrence to feeding[a]
	Mean ± SE	*N*	Mean ± SE	*N*	
Mercuric chloride	13.1 ± 2.9	10	0.00 ± 0.00	10	$+(P < 0.06)$
Control	3.5 ± 1.3	10	0.00 ± 0.00	10	
UV exposure	25.8 ± 2.3	16	1.00 ± 0.25	16	$+(P < 0.0001)$
Control	0.83 ± 0.10	16	0.00 ± 0.00	16	
Acetyl salicylic acid	13.0 ± 1.5	10	0.00 ± 0.00	10	$-(P < 0.10)$
Control	1.3 ± 0.2	10	0.20 ± 0.16	10	

[a]Student's *t* test analysis.

TABLE 2. RANGE OF SCOPOLETIN CONCENTRATIONS IN STIMULATED WILD *H. annuus* LEAVES

Scopoletin (ng/mg)	Population (PI)[a]	Mean ± SE (ng/mg)	Stimulated increase over unstimulated
0–10	413137	2.6 ± 1.1	5.7
	413156	9.8 ± 3.3	5.2
10–20	413034	10.2 ± 7.2	17.4
	413027	19.9 ± 4.9	6.3
20–30	413105	20.9 ± 5.8	9.7
	413114	29.5 ± 19.0	8.4
30–50	413059	30.4 ± 9.2	4.2
	West Fargo-1[b]	47.5 ± 19.6	10.8
Above 50	413153	60.3 ± 14.3	26.2

[a]PI = Plant Introduction number.
[b]Personal collection of L. Charlet. All others accessions of USDA Plant Introduction Station.

18% by weight of the scopoletin content after stimulation and the remainder had less.

In 1992, eight of 11 populations had detectable ayapin concentrations (Table 3). K had the highest ayapin content with respect to scopoletin (43% by weight). The next highest content of ayapin was nearly 17% by weight of scopoletin (I). Those populations with higher concentration of scopoletin generally had somewhat higher concentrations of ayapin. Five of the 11 populations tested in 1992 had an ayapin content after stimulation that was at least 1% that of the scopoletin content.

Selection of Lines for Sunflower Beetle Bioassay. Some the wild populations assayed in 1991 were selected in 1992 for reanalysis of coumarin content and for insect bioassay. Four populations (C, G, I, and J) were selected because they showed a large increase in scopoletin content after stimulation ($\bar{X}$ = 32-fold, see Table 3). C also had the third highest concentration of ayapin among the 107 accessions. In 1992, however, C, G, I, and J had on average only a 2.3-fold increase in scopoletin after stimulation and attained only to the overall average content of all populations in that year. Two populations (A and K) were chosen for above average content of scopoletin in unstimulated plants (11 ng/mg) and an average capacity to be stimulated (1.9×). In 1992, however, these lines had below average content of scopoletin before stimulation (2 ng/mg) and an above average increase (4×) after stimulation. Three others that were chosen (B, D, and H) had an average scopoletin content and an average capacity to be stimulated. In 1992, these populations continued to have an average scopoletin content before stimulation and an average amount after stimulation. Two other

TABLE 3. LEAF CONCENTRATION OF SCOPOLETIN AND AYAPIN

		Scopoletin (ng/mg)				Ayapin (ng/mg)			
		Unstimulated		Stimulated		Unstimulated		Stimulated	
Population	Designated	1991	1992	1991	1992	1991	1992	1991	1992
413052	A	12.9	2.9	30.6	14.9	0.00	0.25	0.00	0.00
413130	E	8.7	4.6	13.5	5.5	0.00	0.24	0.07	0.34
413054	K	8.6	0.7	11.6	1.0	1.10	0.20	0.09	0.44
413138	H	4.2	5.5	28.3	9.0	0.00	0.00	0.28	0.17
413046	F	4.0	9.9	3.0	7.6	0.00	0.04	0.00	0.02
413162	B	3.5	12.9	46.2	30.7	0.00	0.08	0.00	0.17
413114	D	3.5	6.7	29.5	9.1	0.00	0.00	0.00	0.01
413153	C	2.3	2.7	60.3	8.3	0.00	0.54	0.43	0.95
413058	I	0.8	1.3	21.1	2.3	0.00	0.39	0.00	0.55
413049	G	0.7	1.5	18.3	4.1	0.00	0.03	0.00	0.00
413181	J	0.4	17.5	19.6	19.9	0.00	0.00	0.00	0.00

populations were chosen (E and F) because they contained above average concentrations of scopoletin (6 ng/mg) but stimulation caused only small increases in scopoletin content. E and F in 1992 had above average initial scopoletin content (7 ng/mg) along with a less than average increase in scopoletin content.

Comparisons between Years. The 11 selected populations had a mean scopoletin concentration after stimulation of 22.4 ± 6.7 ng/mg (mean ± SE) in 1991, but only 10.9 ± 2.8 ng/mg in 1992 (Table 3). The decline in scopoletin content was statistically significant (by PROC GLM: $P < 0.05$). Unstimulated leaves showed no differences between the two years.

The ayapin concentration in the stimulated plants (Table 3) was nearly threefold higher in 1992 (0.24 ± 0.07 ng/mg) than in the same 11 populations in 1991 (0.09 ± 0.04 ng/mg. Although the increase was significant between the two years ($P < 0.05$) for stimulated leaves, unstimulated leaves did not differ in ayapin content. If ayapin was strongly expressed in 1991, it was also strongly expressed when retested in 1992, except for population H, which declined slightly. Stimulation caused an increase in ayapin content in three of the populations in 1991 and in seven of the populations in 1992.

The coumarin content of sunflowers growing in the field was also measured. Progeny of previously cultivated plants growing as volunteers contained 1.8 ng/mg scopoletin at the four leaf-pair stage (Table 4). This value was only somewhat less than the average concentration of all the comparably staged greenhouse-grown wild plants (2.9 ng/mg). Older plants from the field (eight leaf pairs or more with a developing flower bud) had somewhat higher concentrations of scopoletin (22.6 ng/mg) than the average concentrations of the stimulated leaves of greenhouse grown plants in the vegetative, four leaf-pair stage (15.7 ng/mg).

Enhancement of Nonpreference by Mercuric Chloride Compared to Other Treatments. Plants treated with mercuric chloride or UV exposure significantly deterred ($P < 0.06$ and $P < 0.0001$, respectively) sunflower beetle feeding (Table 1). However, salicylic acid had no effect on beetle feeding ($P < 0.10$). Mechanical damage accomplished by pressure of sandpaper blocks against leaves

TABLE 4. COUMARIN CONTENT OF VOLUNTEER SUNFLOWER

	Scopoletin (ng/mg)		Ayapin (ng/mg)	
	Mean ± SE	*N*	Mean ± SE	*N*
4–5 leaf-pair stage	1.8 ± 0.6	10	0.0 ± 0.0	10
Flower bud stage	22.6 ± 8.4	10	91.0 ± 0.4	10

has also been shown to elicit feeding nonpreference by both adult beetles and larval budworms on sunflower leaves (Roseland and Grosz, unpublished).

Stimulation of Feeding Nonpreference in Selected Lines. Feeding preferences among the populations selected in 1992 were initially analyzed by comparing the mean leaf areas consumed (Table 5). In preference assays with leaves that were not stimulated, two of the populations (F and G), were significantly nonpreferred by beetles ($P < 0.05$) compared to the control population. Three other populations tested (B, I, and J) received twofold less feeding than controls, but these results were not significantly different by t test analysis.

The feeding data were then converted into feeding scores $[2T/(T + C)]$ and reanalyzed (Figure 3). A score between 0 and 0.5 has been selected by some authors to indicate nonpreference for test leaves (Fischer et al., 1990). The lower limit of acceptance was 0.5 in these assays, since essentially no plants showed overlap with those of higher feeding score by multiple range tests (Kogan and Goeden, 1970). In the present assays with unstimulated leaves, only one population (B) approached a score of 0.5 (Figure 3). It should be noted that two populations (F and G) were significantly less preferred compared to controls when analyzed by t test, but that the 0.5 threshold for feeding scores excluded both.

In feeding assays with stimulated leaves, a total of six populations (A, B,

TABLE 5. CONSUMED LEAF AREA OF UNSTIMULATED *H. annuus* AND IS3311 IN SUNFLOWER BEETLE FEEDING CHOICE ASSAYS

	Wild *H. annuus* population		IS3311[b]		
Population[a]	Mean ± SE	*N*	Mean ± SE	*N*	*P*
A	0.80 ± 0.21	26	1.29 ± 0.29	30	0.18
B	0.43 ± 0.10	22	0.82 ± 0.18	27	0.07
C	1.10 ± 0.31	24	0.96 ± 0.37	21	0.76
D	1.07 ± 0.26	24	1.06 ± 0.29	24	0.99
E	1.34 ± 0.32	27	2.26 ± 0.47	27	0.11
F	0.43 ± 0.11[c]	21	1.29 ± 0.32	21	0.02
G	0.90 ± 0.21[c]	24	2.08 ± 0.49	22	0.03
H	1.18 ± 0.42	14	1.97 ± 0.54	19	0.26
I	0.68 ± 0.25	23	1.41 ± 0.36	28	0.10
J	0.29 ± 0.09	26	0.89 ± 0.32	26	0.09
K	1.56 ± 0.31	13	2.11 ± 0.62	16	0.44

[a] Obtained from the Plant Introduction Station at Ames, Iowa; see Table 3 for PI numbers.
[b] Standard cultivar.
[c] Significantly different from IS3311 control, t test, $P < 0.05$.

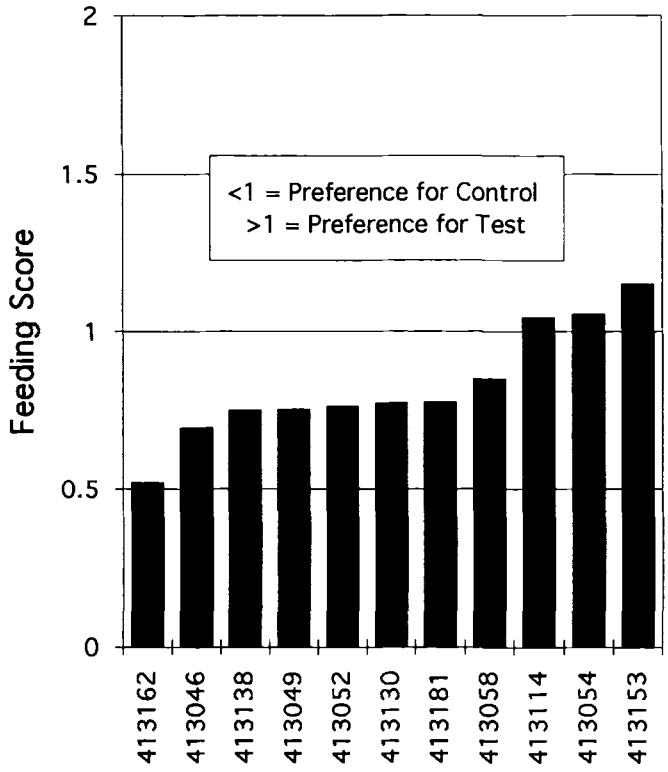

FIG. 3. Feeding scores from unstimulated populations of wild *H. annuus*.

D, F, G, and I) had significantly less mean leaf area consumed than controls ($P < 0.05$) based on pair-wise t test analysis (Table 6). Four of the other populations tested had mean leaf areas consumed that were substantially, although not statistically, less than that of controls. When the feeding scores were computed, six populations had scores below 0.5 (A, B, F, G, I, and K), indicating substantial feeding nonpreference (see Figure 4). These included all but one of the populations that were significant in the t test analysis and the addition of one more (K). A total of nine populations were on the nonpreferred side of the equal preference line, excluding only C and H.

Plant Stress and Coumarin Content in Relation to Changes in Feeding Scores. The concentrations of coumarins were regressed against the feeding scores. First, both scopoletin and ayapin were evaluated independently. The probability that scopoletin alone was correlated with feeding scores was $P < 0.42$ and for ayapin alone, $P < 0.16$. The probability that the concentration of

TABLE 6. CONSUMED LEAF AREA OF STIMULATED *H. annuus* AND IS3311 IN SUNFLOWER BEETLE FEEDING CHOICE ASSAYS

	Wild *H. annuus* population		IS3311[b]		
Population[a]	Mean ± SE	*N*	Mean ± SE	*N*	*P*
A	0.00 ± 0.00[c]	9	4.23 ± 1.53	8	0.02
B	0.19 ± 0.14[c]	10	2.31 ± 0.77	8	0.03
C	0.18 ± 0.12	9	0.24 ± 0.16	8	0.77
D	0.34 ± 0.22	10	2.16 ± 0.77	7	0.05
E	0.59 ± 0.40	10	1.04 ± 0.53	10	0.50
F	0.14 ± 0.16[c]	10	1.93 ± 0.54	10	0.01
G	0.06 ± 0.06[c]	10	2.12 ± 0.51	10	0.003
H	0.96 ± 0.89	9	0.52 ± 0.52	9	0.68
I	0.43 ± 0.27[c]	4	4.75 ± 2.30	10	0.01
J	0.04 ± 0.04	6	0.32 ± 0.32	9	0.43
K	1.14 ± 0.71	9	3.11 ± 0.84	9	0.09

[a] Obtained from the Plant Introduction Station at Ames, Iowa; See Table 3 for PI numbers.
[b] Standard cultivar.
[c] Significantly different from IS3311 control, *t* test, $P < 0.05$.

both coumarins simultaneously correlated with the feeding scores was evaluated, but no significance was found ($P < 0.19$).

Next, we used the graphic analysis method to correlate net changes in the coumarin content with net changes in feeding preference, between stimulated and unstimulated plants in each population. The graphic analysis for scopoletin produced three types of results (Figure 5). In quadrants I and II, 10 populations showed an increase in concentration of scopoletin following stimulation (Figure 5). In quadrant I, at least six of these populations (A, B, D, G, I, K) and possibly a seventh (J) had substantial decreases in feeding scores (increased nonpreference). Except for K ($P < 0.09$), the populations also showed significant decreases in feeding compared to controls by *t* tests analysis. K also had the fourth lowest stimulated increase of scopoletin compared to controls (43%). Another two populations with an increase in scopoletin were positioned in quadrant II (C and H), and these correlated with increased feeding scores (increased preference) following stimulation. However, feeding on leaves of these two populations after stimulation (C and H) was not significantly different from feeding on control leaves by *t* test ($P < 0.76$ and $P < 0.68$, respectively) or by feeding scores criteria: their assignment to quadrant II is therefore equivocal. Two populations (E and J) had feeding scores that indicated little or no change in preference after stimulation, but they were associated with a small increase

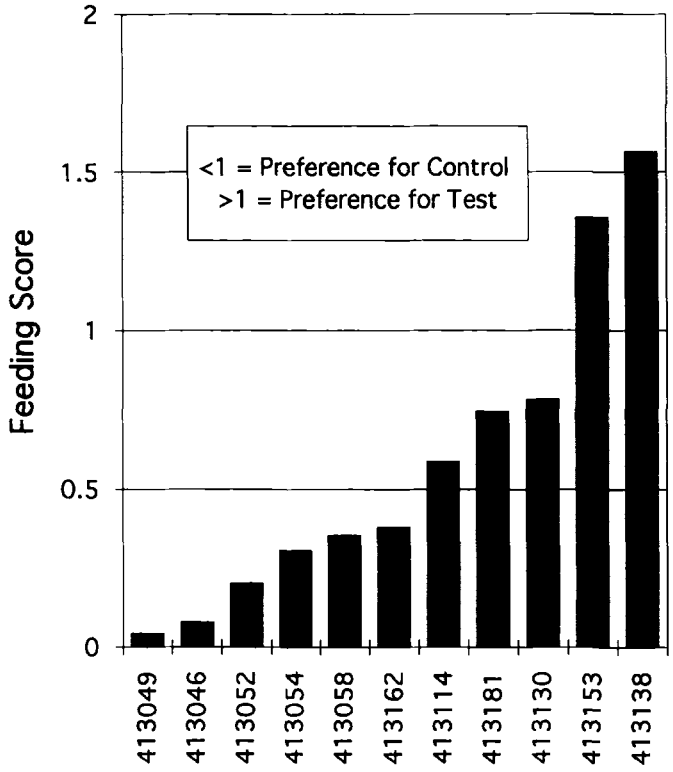

FIG. 4. Feeding scores from stimulated populations of wild *H. annuus*.

in scopoletin score. These two populations also had the lowest increase among the 11 in scopoletin content over unstimulated plants (20% and 14%, respectively). The single population (F) positioned in quadrant III indicated that a decline in feeding preference also correlated with a decline in scopoletin content after stimulation. In summary, when scopoletin content increased following stimulation, six of eight populations (and possibly a seventh) showed enhanced deterrence to feeding.

Graphic analysis for ayapin identified six of the 11 populations with an increased score (increased content) after stimulation (Figure 6). Of these, beetles showed a decreased feeding preference for leaves of three populations (B, I, K) that correlated with this increased ayapin content (quadrant I). Two populations with an increase in ayapin score (C, H) showed a net increase in feeding (quadrant II). As noted earlier, feeding by beetles on these two populations was not significantly increased over control leaves by *t* test or by feeding scores. One

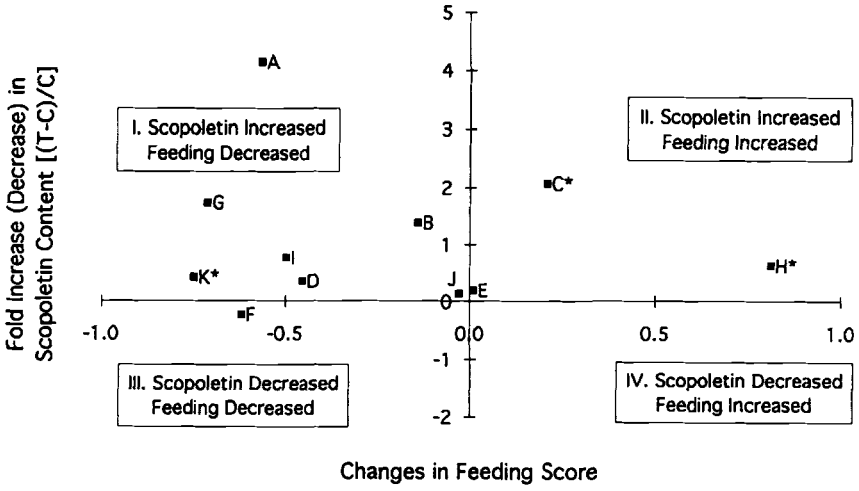

FIG. 5. Graphic analysis of changes in scopoletin concentrations and changes in feeding scores. *Feeding preferences not different from controls by *t* test analysis.

population (E) showed a modest increase in ayapin score but with a barely detectable increase in the feeding score (quadrant I/II). The single population (A) positioned in quadrant III indicates that a decline in ayapin score correlated with a decline in feeding preference. Three populations (F, G, D) showed no

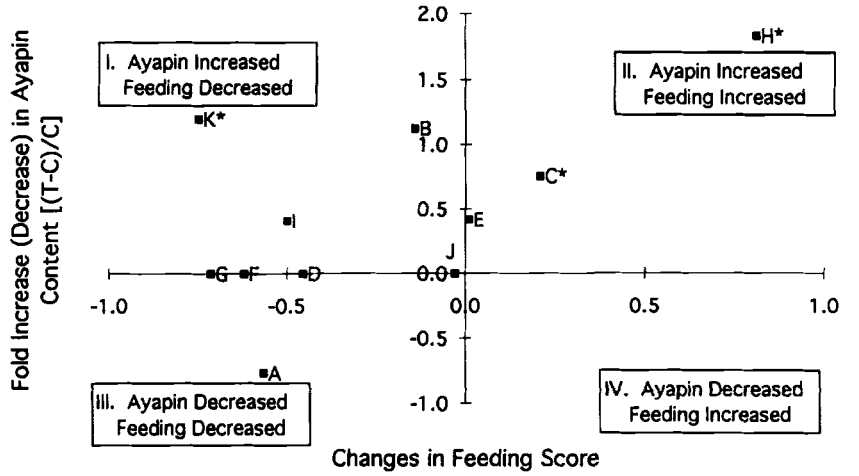

FIG. 6. Graphic analysis of changes in ayapin concentrations and changes in feeding scores. *Feeding preferences not different from controls by *t* test analysis.

changes in ayapin score but had a substantial decline in feeding preference (quadrant I/III border). Two of these populations had a score that placed them within quadrant I when scopoletin was analyzed (G, D). Finally, the population near the intersection of the two axes (J) did not show any significant changes in feeding or ayapin content. When ayapin increased in content, there was most commonly a decline in feeding by beetles (3/4).

Regional Differences in Coumarin Expression. Table 7 shows the mean scopoletin content and scopoletin inducibility in all populations when summarized by state and region. The populations that were collected in the region of subspecies *lenticularis* had lower concentrations of scopoletin when stimulated than did those that were collected in the region of subspecies *texanus*. Not unexpectedly, the scopoletin content of plants deriving from the intragrade area of these subspecies [region 7 of Rieseberg et al., (1987)] was not significantly different from that of either of the parental subspecies. The southwestern-most group of the *lenticularis* population (from California and Nevada, or region 2) had a higher stimulated ayapin concentration (unpublished) than did the southeastern-most part (region 5).

DISCUSSION

Induction of Coumarin Synthesis by Applied Stress. While mercuric chloride has been routinely used to generate chemical stress responses that mimic a response to a pathogenic invasion (Devlin and Gustine, 1992), it has not been routinely used to generate responses that mimic feeding damage by insect herbivores. Treatment with heavy metals such as mercuric chloride induces production of glutathione (Rennenberg and Burnold, 1994), synthesis of both P3-chitinases of bean (Margis-Pinheiro et al., 1993), and pathogenic response proteins (Jung et al., 1995). All these induced responses are characteristic of plants undergoing pathogenic invasion. In our study, UV stress and mercuric chloride both led to stimulated synthesis of coumarins as well as to nonpreference by sunflower beetles. Jung et al. (1995) working with sunflower showed that mercuric chloride could activate the expression of three of four protein classes that typify the response of pathogenic invasion; aspirin, UV irradiation, NAA, and organic acids could activate all four classes. Another cytotoxic metal salt, cupric chloride, when administered to detached sunflower leaves induced both coumarins (Gutierrez et al., 1995). Summarizing the previous work, it appears that heavy metal treatment of leaves adequately mimics various environmental stresses. In the present work, we have achieved a stress response with mercuric chloride in sunflower that appears to be comparable to that induced by more natural stressors (Tal and Robeson, 1986a,b; Olson and Roseland, 1991).

In order to reduce the variability involved in sampling plant chemicals, our

TABLE 7. DISTRIBUTION OF SCOPOLETIN VALUES IN STIMULATED WILD *H. annuus* LEAVES BY COLLECTION SITES

State	Stimulated mean (ng/mg ± SE)	*N*	Fold increase over unstimulated	Region[a]	Regional mean (ng/mg ± SE)	Subspecies[b]		
						H. lenticularis (1-5)	*H. texanus* (6)	Intergrade (7)
California	14.6 ± 5.2	60	6.8	2	14.6 ± 1.2	15.0 ± 1.1a		
North Dakota	24.6 ± 10.4	4	8.4	3	17.6 ± 4.8			
South Dakota	11.4 ± 4.2	3	5.6	3				
Wyoming	12.5 ± 4.9	4	15.3	4	15.7 ± 3.4			
Colorado	20.0 ± 11.2	3	6.5	4				
New Mexico	11.2 ± 3.9	7	9.5	5	15.1 ± 3.8			
Arizona	19.0 ± 5.0	7	17.9	5				
Texas	25.4 ± 6.4	5	10.0	6	25.4 ± 6.2		25.4 ± 6.2b	
Nebraska	16.8 ± 6.6	5	11.9	7	16.7 ± 2.8			16.7 ± 2.8ab
Kansas	16.7 ± 4.7	9	8.9	7				
Minnesota	7.7 ± 0	1	3.0	[c]				

[a] Geographic regions defined by Rieseberg et al. (1987).
[b] Waller-Duncan *k* ratio *t* test: means with the same letter are not different.
[c] Not reported in Rieseberg et al. (1987).

study analyzed opposite leaves from the same plant, one stimulated, the other not. Systemic effects caused by leaf stress to the contralateral leaf over three days were unlikely. Olson and Roseland (1991) measured scopoletin content in circumferential rings surrounding a damage site. One week after damage initiation, increased coumarin was localized to a ring on the leaf not more than 2.5–5.2 cm from an injury site. An increase in whole plant concentration of coumarins did not occur until at least 10 days after a stress was applied. This result suggests that a systemic stress response in sunflower would have occurred much later than at the time of leaf removal in the present investigation.

In this study, chemically induced stress caused increased production of at least one coumarin in nearly all populations (10 of 11) that were investigated. A few populations showed only a small increase. Perhaps these weakly induced populations possessed a high resistance to cytotoxicity and so did not activate a defensive response. Perhaps a more specific stress was required to induce these populations.

Relationship of Feeding Reduction to Coumarin Content. When the changes in scopoletin content and feeding were compared before and after stimulation, it was clear that in six populations feeding nonpreference correlated with increased concentration of scopoletin (quadrant I). In one population, increased nonpreference correlated with a decline in scopoletin content (quadrant III). This result suggests that scopoletin production declined and another deterrent chemical was formed instead. In two other populations, no changes were observed in feeding preferences even though scopoletin increased in stimulated plants. In the remaining populations (quadrant II), no significant differences were identified between leaf area consumed in control and stimulated plants. Strong correlations between ayapin content and feeding deterrence were also identified. A definite increase of ayapin concentration in four populations was correlated with increased feeding nonpreference in three. In one population, an increase of ayapin content was not correlated with a commensurate decline in feeding.

What is the relationship of the two coumarins to the induced nonpreference by beetles? In three populations (B, I, and K), nonpreference correlated with both increased scopoletin and ayapin concentration; in three other populations (A, D, G), nonpreference correlated mostly with increased scopoletin concentration, while ayapin concentration did not change after stimulation (except for A which declined). In one population (F, sector III) neither coumarin was correlated with the observed deterrence. We can conclude that the increase of scopoletin or scopoletin and ayapin together was important in causing a decline in feeding preference after stimulation for at least seven populations of eight that showed an increase in coumarin content.

Evidence has been presented that two coumarins are inducible to higher concentrations across many populations of sunflower. Ayapin is only infrequently found in unstimulated plants, but expressed more frequently after stress.

When deterrence could not be predicted from the plant coumarin content alone, some additional chemical (as yet unknown) is suggested. Other examples of this type of complex defense have been described, as for example in *Podocarpus gracilior*. This fern expresses at least four different types of chemical defenses, including phytoecdysones, norditerpene dilactones, podolides, and biflavones (Kubo, 1991). Multiple defenses arise even in related host plant species in the same environment when attacked by the same insect predator. Bruchid beetles that feed on palo verde seeds do not induce similar defenses in related sympatric host species (Siemens et al., 1992).

Effects of Constitutive Chemicals on Herbivores. Should scopoletin be considered a constitutive or an induced defense? It appears that scopoletin concentrations remain low at the stage sunflower beetle adults begin to feed and lay eggs on plants grown in the field or the greenhouse. From previous results (Olson and Roseland, 1991), it appears that an elevated threshold of ayapin and probably of scopoletin must be attained before a deterrent response is activated in the insect. Thus, although low constitutive concentrations of at least scopoletin may be continuously present in host plants, an insect may only perceive and respond to elevated coumarin concentrations found in stimulated plants.

Can constitutive chemicals such as coumarins become deleterious to specialist feeders? The frequent occurrence of these chemicals in the diet should lead to tolerance. However, the encounter of a sunflower beetle with a fully induced plant may not be a frequent occurrence. In the early summer, most plants would express only a low concentration of scopoletin. Those plants with a high concentration might actually be weakened plants that were responding to a pathogenic challenge or that had been previously attacked by insects. Consequently, there may be no selective advantage, and perhaps even a disadvantage, for insects to use such stressed plants for oviposition or feeding.

Another sunflower-specializing insect, the sunflower moth (*Homoeosoma electellum*), showed either a deterrent response or no response to sunflower chemicals, depending upon the age of the insect. Early-instars were deterred from feeding on certain parts of the florets of *Helianthus maximiliani* or from diet containing sesquiterpene lactones from the floret parts (Rossiter et al., 1986). Later instars did not avoid plant parts or diet containing sesquiterpene lactones.

Deleterious effects of some constitutive chemicals to specialist insects feeding on the host plant have been shown by several authors. In wild parsnips, applied stresses have induced elevated concentrations of furanocoumarin (Zangerl, 1990). When the parsnip constituent bergapten was incorporated into artificial diet, parsnip webworm (*Depressaria pastinacella*) showed decreased growth and digestibility on the diet (Berenbaum et al., 1989). Parsnip webworms may also discriminate between host plants of high and low furanocoumarin content, a result that was inferred a posteriori from chemical analysis of field plants that had been attacked (Zangerl and Berenbaum, 1993). Similarly, sun-

flower beetle adults are likely to discriminate against sunflower populations with high scopoletin content. If bioactive phytochemicals are induced to higher than constitutive concentrations, or such elevated concentrations are found in variant plants, deterrence to insects may be observed.

Ecological Role of Scopoletin in Sunflower Defenses. Scopoletin is expressed by nearly all populations of *H. annuus* and by several other species within the genus *Helianthus* (C. R. Roseland and T. J. Grosz, unpublished). We have shown that there are regionally specified differences in coumarin inducibility correlating with the occurrence of different subspecies of *Helianthus*. Direct exposure of sunflower beetles to sunflower leaves topically treated with coumarins revealed that these sunflower chemicals were indeed deterrent to adult feeding (Olson and Roseland, 1991). Why then are coumarins not deployed consistently in all populations, especially since their multiple roles in defense have been demonstrated? Perhaps a matrix of unique, inducible chemicals is found in each geographically separate population of sunflower. A species as cosmopolitan as *H. annuus* probably has developed extensive genetic and phenotypic diversity to respond to different challenges in the ecosystems in which it may be found.

Various insect species and pathogens may be relatively more important in some areas than in others. Although *Z. exclamationis* appears to be an herbivore nearly as extensive in range as the host sunflower, the red seed weevil is more important as a seed predator in the northern Great Plains, and the sunflower moth is a prominent infesting species in the central and southern Great Plains (Schulz, 1978). The carrot beetle is important in Texas and the central Great Plains (Rogers and Thompson, 1978). Systematic characterization of plant defenses by geographic location, occurrence of stressing organisms, and type of leaf or seed damage might reveal a relationship to specific predominant chemical defenses.

Regional variations in inducible coumarin content may be related to specific defenses against different herbivorous insect species. In a survey of 100 wild potato species and five herbivores or sucking insects, Flanders et al. (1992) found that the presence of leptine and tomatine correlated well with resistance to Colorado potato beetle, while the phenolic defenses associated with type A + B glandular trichomes correlated best with resistance to leafhopper and potato aphids. The variable appearance of herbivores may thus act on sunflower to select for defenses against one or more infesting species. Environmental factors including differences in regional climates are also relevant for selection favoring different plant defenses, because these also regulate insect populations.

The variation of induced coumarin expression that we detected may reflect differences in the role of scopoletin in different local populations. Cytotoxic metal salts, insect feeding, and mechanical damage all apparently provoke the wounding response and are correlated with different levels of coumarin synthesis

(Olson and Roseland, 1991; Gutierrez et al., 1995). Scopoletin content may be induced at one concentration as a response to pathogenic invasion. Induction at another level for a different duration may occur as a response to exploitation by an insect population. Finally, induced increases in coumarin content may regulate changes in plant quality and both may regulate host acceptance.

The breadth and variety of induced defensive chemicals that modulate herbivore and pathogen infestations have been but meagerly explored in most plants. Until the whole interrelationship of plant nutrition and the physical, chemical, and structural changes following wounding and other stresses are analyzed together, as advocated by Faeth (1991), the source of insect deterrence will be difficult to elucidate. In sunflower, we have begun to investigate the complex interactions between insects and the host by examining one response at a time to the stress of wounding or infestation.

The widespread occurrence of coumarins in different populations of sunflower and the inducibility of these constituents to higher levels in wild populations suggest that they are one component of a defensive strategy in sunflower. Regional variations in coumarin inducibility may respond to differences in defenses against different herbivorous species. A direct defensive role for coumarins appears likely, but it is one that also may involve significant interactions with other, unknown constituents of sunflower.

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ENVIRONMENTAL EFFECTS ON THE ACCUMULATION OF HYDROXAMIC ACIDS IN WHEAT SEEDLINGS: THE IMPORTANCE OF PLANT GROWTH RATE

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Abstract—The effects of temperature and photoperiod on accumulation of hydroxamic acids (Hx) in wheat (*Triticum aestivum* L.) seedlings were evaluated under laboratory conditions. Hx concentrations were significantly higher at higher temperatures. No such clear trend was found for the photoperiod effect. The significant effect of temperature and photoperiod on growth rate of seedlings and the significant positive correlation between growth rate prior to analysis and levels of Hx, suggested that environmental effects on Hx accumulation were at least partially mediated through their effect on plant growth rate. After uncoupling the effect of environmental conditions from the effect of plant growth rate by statistical means the effect of temperature on Hx was no longer significant. Therefore, temperature effect was fully mediated by plant growth rate. Implications of the patterns found are discussed in issues of plant-defense general theories.

Key Words—Temperature, photoperiod, hydroxamic acids, growth, defense, secondary metabolites, wheat, Gramineae.

INTRODUCTION

Plants growing under different environmental conditions may show changes in resource allocation to main biological functions such as growth, reproduction, and defense (Bazzaz et al., 1987; Chapin et al., 1987; Herms and Mattson, 1992). Concerning defense, numerous experimental studies have described the effect of environmental factors such as temperature, water, relative humidity,

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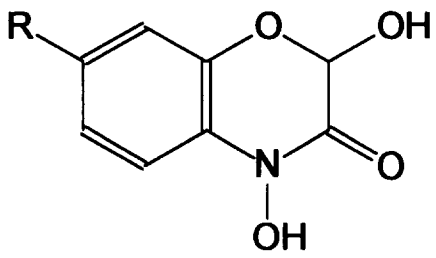
light, nitrogen, CO_2, etc. on plant resistance traits (reviewed in Smith, 1989 and Waterman and Mole, 1989). The effects of both temperature and photoperiod on plant resistance to insects, measured as effects on insect performance parameters (Khan et al., 1986; Salas and Corcuera, 1991), as well as on constitutive levels of plant secondary metabolites (Hanson et al., 1983; Anderson et al., 1991) have been described. However, these reports have often overlooked phenological differences between plants arising from being subjected to different environmental conditions. These phenological differences may account for the resistance pattern observed, in view of the known relationship between plant or plant tissue age and levels of secondary metabolites (Bazzaz et al. 1987). Hence, since environmental conditions affect growth rate of plants, this must be also considered when attempting to distinguish the effect of environmental variables on defenses in plants.

Hydroxamic acids (Hx) (4-hydroxy-1,4-benzoxazin-3-ones, Figure 1) are plant secondary metabolites present in both cultivated and wild Gramineae (Niemeyer, 1988) related to the resistance of cereals to insects, fungi, and bacteria (reviewed in Niemeyer and Pérez, 1995). Hx occur naturally as 2-β-O-D-glucopyranosides which are hydrolyzed by plant glucosidases released following tissue disruption (Hofman and Hofmanová, 1971). Hx are absent from the seed, increase upon germination, peak at the young seedling stage and decrease thereafter (Argandoña et al., 1981) yet retaining high levels of Hx in the youngest tissue of mature plants (Thackray et al., 1990).

We herein describe the effects of temperature and photoperiod on the accumulation of Hx on wheat (*Triticum aestivum* L.) seedlings under laboratory



R=H DIBOA

R=CH_3O DIMBOA

FIG. 1. Chemical structures of DIMBOA and DIBOA, the main hydroxamic acid aglucones in wheat extracts.

conditions and the involvement of seedling growth rate–which differed among treatments–on the patterns found. The experimental design used (seedlings analyzed were of the same phenological stage) allowed us to distinguish between environmental and plant phenology effects. Implications of our findings in issues of plant-defense general theories are discussed.

METHODS AND MATERIALS

Plants. Seeds of *T. aestivum* cv. Paleta were obtained from INIA, Chile, and germinated in a growth chamber in individual plastic pots (25 ml) filled with soil. Seedlings grew in a growth chamber under the nine different environmental conditions arising from a factorial array of three temperatures (15, 20, and 25°C) and three photoperiods (8, 12, and 16 light-hours). Light intensity, expressed as P.A.R., was 117 μmol photons $m^{-2} \cdot s^{-1}$ for all treatments. When seedlings attained growth stage 12 (first leaf completely unfolded, second leaf visible; Zadoks et al., 1974) the aerial biomass was analyzed for total Hx. Eight seedlings (height: 170 $\pm$ 5 mm) were used for each treatment. The utilization of seedlings of the same phenological stage–but different chronological age between treatments–allowed the distinction between environmental and plant phenology effects. Growth rate of all seedlings was determined as follows: height at growth stage 12 to the nearest mm/age at such stage, determined to the nearest hour.

Chemical Analysis. Plant material was macerated with 1 ml H_2O, using mortar and pestle. The aqueous extract was left at room temperature for 15 min and then adjusted to pH 3 with 0.1 N H_3PO_4. The extract was centrifuged at 13 000 g for 15 min and a 50 μl aliquot of the supernatant was directly injected into a high performance liquid chromatograph. An RP-100 Lichrospher-C18 column was used with a contant solvent flow of 1.5 ml/min and the following linear gradients between solvents A (MeOH) and B (0.5 ml H_3PO_4 in 11 H_2O): 0 to 7 min, 30% A; 7 to 9 min, 100% A; 7 to 13 min, 30% A. Compounds eluting from the column were detected at 263 nm.

Statistical Analysis. Hx concentrations (mmol/kg fresh weight) were analyzed with a two-way ANOVA with temperature and photoperiod as main effects and with a two-way ANCOVA with growth-rate as a covariate. Growth rate (mm/day) was determined for each individual seedling and correlated with its Hx level. All Hx concentrations were square-root transformed prior to analysis in order to normalize their distributions.

RESULTS

Accumulation of Hx was significantly affected by temperature ($P < 0.001$), higher temperatures leading to increased levels of Hx (Table 1). No clear trend

TABLE 1. LEVELS OF HYDROXAMIC ACIDS (Hx) (MMOL/KG FR. WT) IN WHEAT SEEDLINGS GROWN UNDER DIFFERENT TEMPERATURES AND PHOTOPERIODS. MEAN ± SE[a]

	Daylength		
Temperature	16	12	8
25	1.62 ± 0.24aA	1.31 ± 0.13aA	1.48 ± 0.14aA
20	1.35 ± 0.11aA	1.24 ± 0.09aAB	0.99 ± 0.07bB
15	0.84 ± 0.05bA	1.11 ± 0.08aA	0.95 ± 0.07bA

[a]Means sharing a lowercase letter within columns or an uppercase letter within rows are not significantly different ($P < 0.05$, LSD test).

was found for the effect of photoperiod ($P > 0.40$). In addition, the interaction of effects was marginally significant ($P < 0.05$) (Table 1). As expected, seedling growth rate prior to analysis was significantly affected by both temperature and photoperiod as well as by their interaction ($P < 0.001$ in all cases), higher temperatures and longer daylengths enabling plants to grow faster (Table 2). On the other hand, seedling growth rate (mm/day) prior to analysis for Hx was significantly positively correlated with Hx accumulation ($r = 0.44$, $P < 0.001$, $n = 72$) (Figure 2).

Since, as described above, both temperature and light regimes significantly affected seedling growth rate (Table 2), it may be questioned whether the described effect of temperature on Hx levels (Table 2) reflected merely the effect of growth rate on Hx. This situation was elucidated by performing the corresponding two-way ANOVA this time including "growth rate prior to analysis"

TABLE 2. GROWTH RATE (MM/DAY) PRIOR TO ANALYSIS FOR HYDROXAMIC ACIDS CONTENT OF WHEAT SEEDLINGS GROWN UNDER DIFFERENT TEMPERATURES AND PHOTOPERIODS. MEAN ± SE[a]

	Daylength		
Temperature	16	12	8
25	18.86 ± 0.59aA	16.84 ± 0.29aB	15.29 ± 0.35aC
20	14.14 ± 0.20bC	17.25 ± 0.31aA	15.69 ± 0.36aB
15	13.23 ± 0.48bA	11.80 ± 0.22bB	11.32 ± 0.25bB

[a]Means sharing a lowercase letter within columns or an uppercase letter within rows are not significantly different ($P < 0.05$, LSD test).

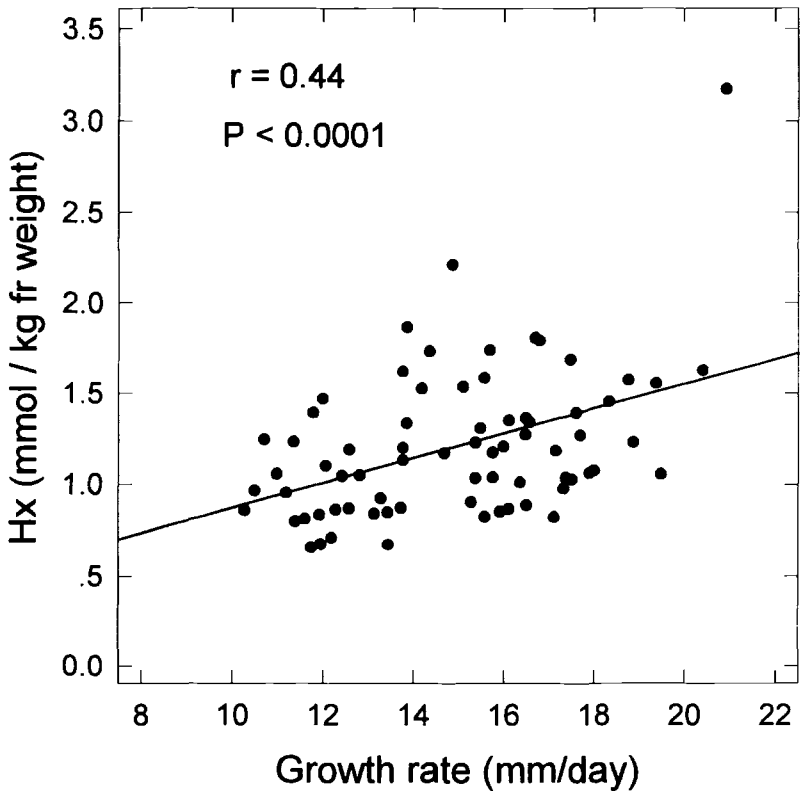


FIG. 2. The relationship between seedling growth rate prior to analysis and the accumulation of hydroxamic acids (n = 72).

as a covariate, i.e., removing its effect. In this new analysis the former strongly significant effect of temperature on accumulation of Hx disappeared upon the inclusion of the covariate ($P > 0.20$). No qualitative difference for the other sources of variance arose after the analysis (Table 3).

DISCUSSION

Accumulation of Hx in wheat seedlings under different temperatures showed a similar pattern to that found earlier (P. W. Wellings, pers. comm.). However, our findings are somehow in conflict with those of Epstein et al. (1986) regarding the effect of both temperature and photoperiod. These authors reported that shorter days and lower temperatures increased constitutive levels of 6-methoxy-

TABLE 3. ANALYSIS OF VARIANCE OF THE ACCUMULATION OF HYDROXAMIC ACIDS IN WHEAT SEEDLINGS GROWN UNDER DIFFERENT TEMPERATURE AND PHOTOPERIOD TREATMENTS. IN BRACKETS: VALUES OBTAINED INCLUDING "SEEDLING GROWTH RATE PRIOR TO ANALYSIS" AS A COVARIATE.

Source	*df*	Mean square	F-ratio	P-value
Temperature, T	2 (2)	0.292 (0.031)	14.808 (1.606)	0.000 (0.209)
Photoperiod, P	2 (2)	0.018 (0.002)		*n.s.*[a]
T × P	4 (4)	0.051 (0.061)	2.588 (3.138)	0.045 (0.020)
Error	63 (62)	0.0197 (0.019)		

[a] *n.s.* = not significant.

benzoxazolinone, a decomposition product of naturally occurring Hx (Niemeyer, 1988), in wheat plant extracts. However, these authors did not consider the fact that plants grown under low temperature and/or short photoperiod regimes were shorter and hence, contained a higher proportion of younger tissue, which in turn contains higher levels of Hx (Argandoña et al., 1981).

The results presented here show that temperature and photoperiod affect both primary and secondary metabolism of wheat seedlings, although their effects may be interwoven and, thereby, appear difficult to distinguish. It was clear that seedlings growing under higher temperatures and/or longer daylengths reached earlier the phenological stage considered for analysis. Consequently, a major evident effect on seedlings is variation of growth rate. Given that seedling growth rate prior to analysis was significantly positively correlated with accumulation of Hx it could be considered that the formerly stated temperature effect on accumulation of Hx was mediated by its effect on plant growth rate. An adequate statistical analysis showed that after removing the effect of plant growth rate the effect of temperature on accumulation of Hx was no longer significant. Therefore, the temperature effect was fully mediated through plant growth rate. This suggests the occurrence of an intrinsic effect of plant growth rate rather than a direct effect of the environment on metabolic processes along the Hx biosynthetic pathway.

Given that the effect of temperature (the factor most importantly affecting Hx accumulation) "vanished" after removing the effect of plant growth rate we will focus the discussion on the relationship of plant growth rate and the anti-herbivore defenses status of a given plant, population or species. General theories of plant defenses predict an inverse relationship between plant growth rate and defense investment (Zangerl and Bazzaz, 1992). This pattern is rationalized by the higher energetic cost of replacement of biomass loss by herbivory for

slow-growing plants in comparison with fast-growing ones. Although this was originally proposed and evaluated at interspecific level and dealing with an evolutionary rather than ecological time scale (growth-rate theory, Coley et al., 1985), some later reports support a broadening of its scope to comprise patterns at intraspecific level (e.g., Coley, 1986; Jing and Coley, 1990; Sagers and Coley, 1995).

The present work shows a direct relationship between plant growth rate and defense investment within a intraspecific context. Since this result cannot be explained within the former theoretical framework, it seems meaningful to seek proximal explanations for the pattern found e.g., a linkage between Hx and plant growth.

Although Hx have been thought to occur in the plant only as glucosides (Hofman and Hofmanová, 1971), the presence in wheat extracts of Hx aglucones and of the benzoxazolinones arising from the decomposition of the aglucones has been reported (Leszczynski and Dixon, 1990). Therefore, in order to discuss the physiological roles of Hx in the plant all three types of molecules should be considered.

A cytokinin activity (Petho and Dinya, 1992) and an inhibitory effect on auxin-induced coleoptile growth (Venis and Watson, 1978) have been described for benzoazolinones. In addition, both stimulant (Ray et al., 1977) and inhibitory (Venis, 1979) effects on the binding affinity of auxin analogues have been reported for benzoxazolinones and aglucones, respectively; hence, in view of such opposing evidence, this putative physiological role of Hx remains to be elucidated. On the other hand, a possible role of root-exuded Hx in iron uptake in grasses has been reported (Petho, 1993). This suggests at first glance a possible coupling of Hx accumulation with mineral nutrition and hence with growth. However, since the relative distribution of Hx in the shoot and in root exudates is unknown, this phenomenon cannot yet be considered to account for the pattern found (Figure 2). Further research is needed to determine how and to what extent Hx are related to the plant growth. The importance of the identification of the "primary" functions of a particular plant secondary metabolite has been stressed recently in the context of the expected physiological and ecological costs of chemically-based plant resistance to herbivores (Simms, 1992).

Further experiments broadening the range of environmental conditions evaluated or adding other growth-modifying variables and including plant performance measures should lead to a more definitive ascertainment of the pattern found as well as to a more clear notion of the ecological significance of the observed phenotypic plasticity in secondary metabolism coupled to the growth process. In addition, in view of the role of Hx in resistance against herbivores of crop plants, here it is suggested the usefulness of considering the effect of environmental conditions on such mechanism of plant defense against herbivores in order to assess accurately its efficiency as a tool in pest management programs.

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SPIROACETALS AND OTHER VENOM CONSTITUENTS AS POTENTIAL WASP ATTRACTANTS

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Abstract—The major volatile spiroacetals from the venom of both the common wasp, *Vespula vulgaris* and the German wasp *V. germanica*, viz. 7-methyl-1,6-dioxaspiro[4,5]decane and 7-ethyl-2-methyl-1,6-dioxaspiro-[4,5]decane, respectively, were synthesized by known methods. These acetals, along with *N*-isopentylacetamide (the major volatile amide from wasp venom), 2-heptanone (a honeybee pheromone), 2-methyl-3-buten-2-ol (a component of hornet venom), cuticle wax from *V. vulgaris*, and venom sacs from both wasp species were assayed by EAG and olfactory bioassay for attractancy to *V. vulgaris* workers. Antennal responses to all test chemicals were recorded. Acetal isomers (±)-**2** and (±)-**3**, *N*-isopentylacetamide, and 2-heptanone were attractive to *V. vulgaris* workers at levels of < 1 μmol. Greater quantities of the same compounds were repellent to *V. vulgaris* workers.

Key Words—Wasps, *Vespula vulgaris, Vespula germanica*, venom, attractants, spiroacetals, electroantennogram, olfactory bioassay.

INTRODUCTION

The German wasp, *Vespula germanica* (L), is thought to have arrived in New Zealand in 1945 (Thomas, 1960). This species has subsequently spread throughout most of the country (Clapperton et al., 1989b) and has reached high densities

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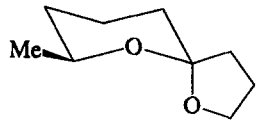
in some areas such as the beech forests of the South Island (Sandlant and Moller, 1989), where control measures have been initiated (Perrott, 1975). The common wasp, *V. vulgaris* (F), has been recorded sporadically in New Zealand since 1921 and became established in Dunedin in 1983 (Donovan, 1984). Since then, it has dispersed rapidly throughout much of the country (Clapperton et al., 1989b, 1994) and appears to have spread into all habitat types occupied by *V. germanica.*

These two wasp species have proliferated to the extent that they are now considered to be making a serious ecological and economic impact in areas of high infestation, particularly the beech forests of the northwestern region of the South Island and parts of Canterbury (Sandlant and Moller, 1989). In the middle of the summer, feeding wasps dramatically reduce the standing crop of honeydew, a sugary secretion of the scale insect *Ultracoelostoma* spp. (Moller et al., 1991). Ecologists (Moller and Tilley, 1989; Beggs and Wilson, 1991) have suggested that wasp foraging for protein and honeydew may be reducing the ability of the beech forests to support native fauna. The wasps are also an urban nuisance, a menace to tourists hiking in national parks, and a major pest to the apicultural (Clapperton et al., 1989a) and horticultural industries in New Zealand.

In recognition of the severity of the infestation of these wasps during the summer months, several scientific programs have been launched in recent years in New Zealand in an effort to control wasp populations.

Biological control, which involves the introduction, release, and establishment of a natural parasitoid, *Sphecophaga vesparum*, has been the principal research focus (Donovan and Read, 1987), but it suffers from the long periods of time required for evaluation of the extent of proliferation and of the overall effectiveness of the parasitoid. Furthermore, there is presently debate over whether or not biological agents such as *Sphecophaga* will ever significantly control vespid wasp colonies (Akre, 1991; Beggs et al., 1996).

Chemical control, on the other hand, appears to be a favorable strategy for dealing with localized infestations of wasps. Poison baits can be delivered to wasps via bait stations placed in the vicinity of the nests. To date, this approach has involved the use of sodium monofluoroacetate (1080) (Spurr, 1991a), hydramethylnon (Spurr, 1991b), and sulfluramid (Spurr, 1993) in sardine-based cat foods. The poisoned bait is taken by foraging wasps to their nests, resulting in the death or considerable reduction in populations of wasps. However, sardine cat-food bait is short-lived (1–2 days) and consequently baits have to be replaced frequently with fresh material. The success (cost-effectiveness) of this strategy might therefore be enhanced if a volatile chemical attractant, specific to wasps, could be found which, when combined with the poisoned bait, would increase its potency. The aim of the work described in this paper was to find such an attractant.



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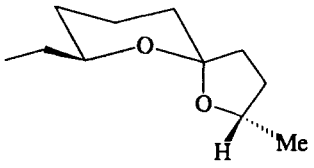

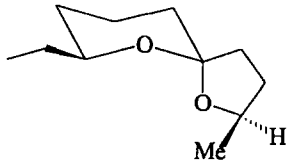

(2) (3)

FIG. 1. Structures of spiroacetal pheromones.

A small number of synthetic chemical products are known to attract wasps, but very few are sufficiently attractive in the field to have been developed commercially for this purpose (see Discussion). We therefore set out to synthesize and assay the attractiveness of some bicyclic spiroacetals (Figure 1, (±)-**1** and (±)-**2**), which are the major volatile components of the venom of the German and common wasps. These acetals were isolated in 1978 and characterized by Francke's group (Francke et al., 1978, 1979a), who demonstrated that application of a synthetic mixture of these acetals to dead wasps lacking any venom will attract wasps of the same species. The acetals therefore appeared to satisfy our requirements for an attractant, outlined above. As these acetals have been synthesized by several different routes and are well characterized, the description here of our synthetic work is therefore brief. The acetals, along with several other synthesized venom components, were assayed by electroantennogram (EAG) response and olfactory bioassay to determine whether they fulfil the promise suggested in Francke's work.

Electroantennogram recordings were used in this work to determine the relative response of *V. vulgaris* to the test chemicals, but these recordings provide no indication of whether or not the chemicals are attractive to the wasp. Such a determination was performed by olfactory choice experiments with captive wasps as a convenient laboratory bioassay.

METHODS AND MATERIALS

Organic Chemistry

Gas chromatography was carried out on a J & W Scientific fused silica bonded phase capillary column, 30 m × 0.2 mm, coated with DB-5. Helium was used as a carrier gas at a flow rate of 1 ml/min. The column was programmed from 100°C to 300°C at 10°C/min and the eluted substances were recorded by FID. ^{1}H and ^{13}C NMR spectra were recorded using $CDCl_3$ as solvent on a Bruker AC-300 MHz instrument.

Acetone-1,1-dimethylhydrazone. This was prepared by the method of Enders et al. (1990).

2-(2-Iodoethoxy)tetrahydro-2H-pyran. This was prepared from dihydropyran (726 mmol), 2-iodoethanol (145 mmol), and *p*-toluenesulfonic acid (1.45 mmol) in dichloromethane (500 ml) at room temperature. Flash chromatography on silica gel with hexane:ethyl acetate (95:5), followed by distillation afforded the product in 36% yield; bp 99°C (0.7 mm) (Paquette et al., 1992).

Pentane-1,3-diol. This was prepared by Reformatsky condensation of propanal with ethyl bromoacetate and reduction of the product with lithium aluminum hydride. The diol boiled at 86–90°C at 1 mm. Lit., bp 120–122°C at 16 mm (Hanschke, 1955).

2-Methyloxetane. This was prepared in two stages from butane-1,3-diol as described by Meltzer and King (1953) and Searles et al. (1957).

2-Ethyloxetane. This was prepared from pentane-1,3-diol according to the previous procedure (Clarke and Holbrook, 1977).

(1-Ethyl-3-iodopropoxy)trimethylsilane. 2-Ethyloxetane (0.23 mol) in $CHCl_3$ (120 ml) was stirred at room temperature under nitrogen while trimethylsilyliodide (0.26 mol) was added dropwise. The mixture was cooled in a water bath at room temperature and stirred under nitrogen for 1 hr. The $CHCl_3$ was removed on a rotary evaporator and the product was distilled through a Vigreux column, yield 55 g (83%); bp 60–65°C at 0.1 mm.(Hamilton et al., 1986).

(3-Iodo-1-methylpropoxy)trimethylsilane. This was prepared as above, from 2-methyloxetane; bp 46–50°C at 1.3 mm. (Hamilton et al., 1986).

7-Methyl-1,6-dioxaspiro[4,5]decane. This was prepared in 33% yield, according to the procedure depicted in Figure 2 and described by Fletcher et al. (1992). The product was purified by flash chromatography on silica gel (hexane–acetone, 98 : 2) and its mass spectrum was identical with that reported by Francke et al. (1979b).

7-Ethyl-2-methyl-1,6-dioxaspiro[4,5]decane. This was prepared in 25% yield according to the procedure depicted in Figure 3 (see preceding method).

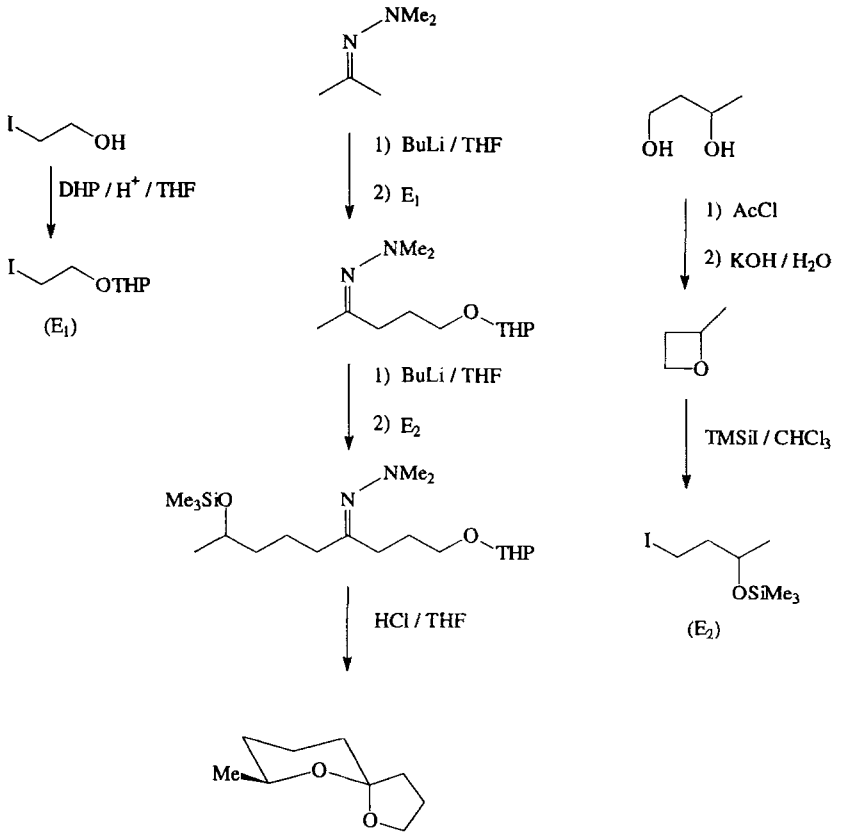

FIG. 2. Synthesis of 7-methyl-1,6-dioxaspiro[4,5]decane.

Electroantennogram Response

All chemicals tested were stored in a freezer prior to use. Dichloromethane was used as the solvent for the dilution series. Quantities of the materials assayed are shown in Table 1. Ten microlitres of a solution of each chemical to be tested was pipetted onto filter paper (2.5 × 10 mm), placed in a Pasteur pipet and stored in the freezer for use the next day. Venom was isolated from wasps by detaching the last gastral tergite and sternite from the remainder of the abdomen of recently emerged but active foragers and expressing the venom sac onto filter paper.

Comb from *V. vulgaris* nests was removed and kept in a rearing room at 30°C. Freshly emerged workers (<48 hr posteclosion) were collected and stored at 3°C (1–6 hr) prior to use. Antennae were cut at the end of the first segment

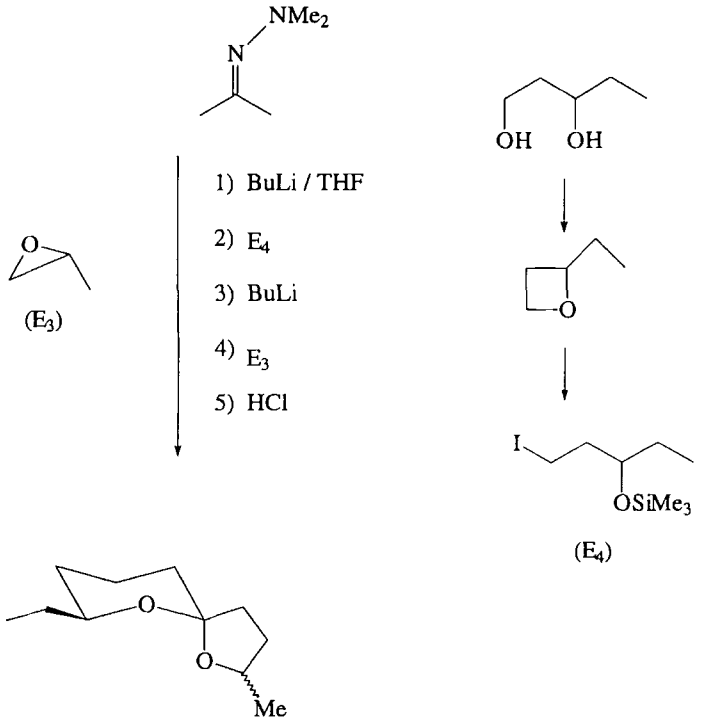

FIG. 3. Synthesis of 7-ethyl-2-methyl-1,6-dioxaspiro[4,5]decane.

and the severed sections placed between saline-filled wells of a Perspex antennae holder. The wells contained silver–silver chloride electrodes (Karg et al., 1994). Electrical contact was improved by dipping the underside (away from the airstream) in the saline solution. The holder was placed inside a glass chamber isolating it from ambient air. Before chemical stimulation, the antennae were exposed to the test conditions by placing them in a charcoal-filtered airflow generated by a suction pump. The holder was enclosed in a glass vessel mounted above the pump. The stimulus was added through a side valve located between the charcoal filter and the antennae. The Pasteur pipet containing the test chemical was mounted into the valve and a separate pump admitted the headspace of the test volatile into the airstream over the antennae.

Chemicals were assayed in three groups. For each group, chemicals were tested in random sequence with 2-heptanone (88 μmol) tested first in the middle and then at the end of the recording for each antenna. 2-Heptanone gave a large response and was used to measure any decay in antennal response through time. Each substance was admitted to the airstream five times for each antenna. EAG

TABLE 1. ELECTROANTENNOGRAM RESPONSES OF *Vespula vulgaris* ANTENNAE (N = 10) TO TEST COMPOUNDS NORMALIZED TO DICHLOROMETHANE[a]

Test	Chemical	Mass (μmol)	Response (mean ± 95% CI)
1	Acetal (±)-**1**	29.0	1.51 ± 0.25*
		2.9	1.11 ± 0.16
		0.3	1.13 ± 0.16
	Mixture of acetals (±)-**2** and (±)-**3**	42.0	1.25 ± 0.25
		4.2	1.38 ± 0.25*
		0.4	1.21 ± 0.22
	Dichloromethane		1.00
	Blank		0.85 ± 0.10*
2	Acetal (±)-**2** or (±)-**3**, isomer a	4.6	1.41 ± 0.12
	Acetal (±)-**2** or (±)-**3**, isomer b	5.1	1.37 ± 0.19*
	N-Isopentylacetamide	78.0	1.26 ± 0.12*
	N-Isopentylacetamide	7.8	1.25 ± 0.14*
	N-Isopentylacetamide	0.8	1.02 ± 0.08
	2-Heptanone	88.0	2.75 ± 0.45*
		8.8	1.28 ± 0.14*
		0.9	1.04 ± 0.10
	Dichloromethane		1.00
	Blank		0.80 ± 0.10*
3	Wasp cuticle wax		1.25 ± 0.14*
	V. vulgaris venom sac (5 sacs)		1.32 ± 0.18*
	V. vulgaris venom sac (1 sac)		1.12 ± 0.14
	V. germanica venom sac (1 sac)		1.16 ± 0.12*
	2-Methyl-3-buten-2-ol	116.0	1.19 ± 0.08*
	2-Methyl-3-buten-2-ol	11.6	1.09 ± 0.10
	2-Methyl-3-buten-2-ol	1.2	1.13 ± 0.08*
	Dichloromethane		1.00
	Blank		0.97 ± 0.08

[a]Each response was compared to that of the solvent (dichloromethane). Mean ± 95% confidence interval *$P < 0.05$.

signals were amplified and recorded using standard methods (Karg et al., 1994). For each sample, the average of the last four exposures was calculated, adjustment made for any decay in antennal response (response of treatment/average response of adjacent 2-heptanone samples), and normalized to the response of the dichloromethane to compare sample responses between test groups (adjusted response of treatment/adjusted response of solvent).

A blank tube was used to measure a combination of signal noise (random fluctuations in the antennal potential), additional mechanical stimulation from changes to the airflow created by the admission of the test substances, any

electrical interference caused by the second pump, and any contamination of glassware or tubing. Therefore responses above those to the blank were responses to the test sample. Paired *t* tests were used to compare the antennal responses to the test samples with those to the solvent.

Olfactory Bioassay

The responses of wasps to potential attractant compounds were tested in the following way. A piece of *V. vulgaris* nest comb with about 50 preemergent pupae was placed into each of two 1.5-liter plastic containers with a 50-ml disposable plastic test chamber attached to the side. The pupae were maintained at room temperature (approximately 20°C). Trials were then carried out with newly emerged workers. In each trial, a 50 × 15-mm piece of blank filter paper was placed inside the test chamber in one container and a similar-sized piece of filter paper with a measured amount of test chemical placed in the test chamber in the other container. The number of wasps per minute entering the test chambers was recorded for 10 min. The test chambers were then switched between containers and the procedure repeated to give 20 records for each test chemical. Three masses, over two orders of magnitude of acetal (±)-**1,** a mixture of (±)-**2** and (±)-**3,** *N*-isopentylacetamide, 2-heptanone, and 2-methyl-3-buten-2-ol, and one mass of the individual acetals (±)-**2** and (±)-**3** were tested (see Table 1). Clean test chambers were used for each chemical. The wasp responses to test chemicals were compared by analyses of variance and least significant difference tests (Wilkinson, 1990). Least significant differences were calculated from the formula given by Andrews et al. (1980).

RESULTS

Synthesis

Synthesis of the acetals (±)-**1,** (±)-**2,** and (±)-**3** (Figure 1) was achieved with the schemes outlined in Figures 2 and 3, the central reaction of which is described by Fletcher et al. (1992). The iodopyran (E_1, Figure 2) was employed for the synthesis of 7-methyl-1,6-dioxaspiro[4,5]decane, instead of ethylene oxide, which is both volatile and toxic. Initially it was hoped that the oxetanes could be used as electrophiles, but their addition to the hydrazones was unsuccessful. Consequently they were opened to the protected iodides E_2 and E_4 (Jung and Lyster, 1977).

Electroantennogram Response

The antennal response to 13 of the samples was significantly greater ($P < 0.05$) than to that of dichloromethane, which was the solvent for the test compounds (Table 1). 2-Methyl-3-buten-2-ol elicited a response at 1 μmol, equiv-

alent to that of one venom sac and the response did not significantly increase with any further increase in sample size. Isopentylacetamide induced a response at 0.8-μmol that was slightly less than that of one venom sac, but the response then increased 20% for a 10-fold increase in sample size, after which there was no further changes in response with change in sample size.

Acetals (±)-**2** and (±)-**3** or a mixture of these at levels 4–5 μmol elicited a significant response which was greater than that of five venom sacs. The response then decreased for a 10-fold increase in sample size, which suggested that the antennal receptors had become saturated. Acetal (±)-**1** gave a response at 0.3 and 2.9 μmol, equivalent to one venom sac, but this increased approximately 40% at 29 μmol to be greater than that for five venom sacs. Acetal (±)-**1** produced the largest EAG response of all the acetals.

2-Heptanone not only elicited an increasing response with increasing sample size, but also produced the greatest response of all the test materials, albeit at 88 μmol.

Olfactory Bioassay

The number of wasps per minute entering the chambers containing test chemicals varied significantly over all treatments ($F = 6.74$, $df = 13,506$, $P < 0.001$). On average, 0.6 wasps/min entered the test chambers containing blank filter paper (Figure 4). Small amounts (<1 μmol) of acetal (±)-**2**,

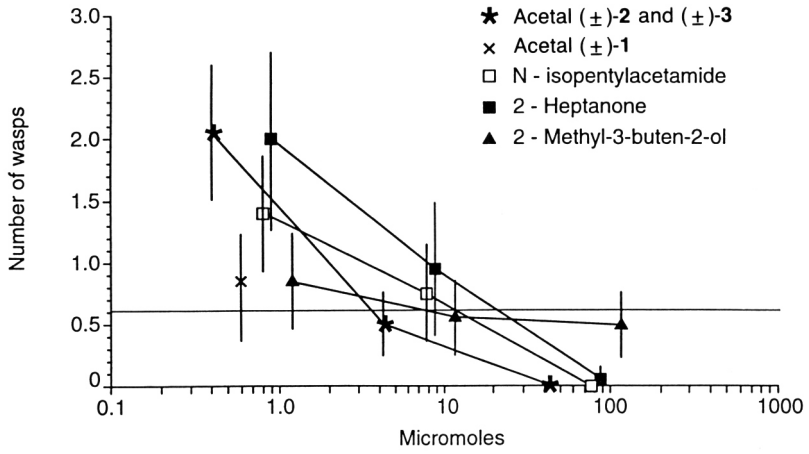

FIG. 4. Number of common wasps per minute attracted to test chemicals in a laboratory bioassay. The horizontal line represents the mean number of wasps per minute entering test chambers containing blank filter paper. Vertical lines represent least significant differences (LSDs). Means with LSDs overlapping the horizontal line are not significantly different from the blank mean ($P < 0.05$, least significant difference test).

N-isopentylacetamide and 2-heptanone attracted significantly more wasps than the blank, whereas larger amounts (40–90 μmol) of the same chemicals attracted significantly fewer wasps (Figure 4). Responses to acetal (±)-**1** and 2-methyl-3-buten-2-ol did not differ significantly from those to the blank.

DISCUSSION

Spiroacetals

Francke et al. (1978, 1979a) reported the occurrence of bicyclic spiroacetals in the venom of the common wasp, *V. vulgaris* and the German wasp, *V. germanica*. They suggested that these acetals might function as aggression inhibitors that could protect a wasp from attack by its kin. Application of a mixture of synthetic acetals to dead extracted wasps prevented these from being attacked by other wasps for a period of 8–10 min.

Bicyclic spiroacetals have been isolated from four orders of insects (Fletcher and Kitching, 1995); beetles (Colcoptera), flies (Diptera), bees and wasps (Hymenoptera) (Perron and Albizati, 1989), and bugs (Hemiptera) (Moore et al., 1994), and it is therefore unlikely that the acetals have a function specific to the wasps, but instead, have a more general function common to other insect families. So far this function appears to be aggregation, but it has not yet been satisfactorily verified for wasps.

The acetals that Francke et al. (1978, 1979a) found in the venom of *V. vulgaris* and *V. germanica* have been analyzed again more recently in order to define their absolute stereochemistry. Lübke (1990) found that two acetals are the major volatile components of the venom of both wasps; namely (5*S*, 7*S*)-7-methyl-1,6-dioxaspiro[4,5]decane **(1)** and (2*S*,5*S*,7*S*)-7-ethyl-2-methyl-1,6-dioxaspiro[4,5]decane **(2).** Both of these acetals are the thermodynamically most stable isomers (Deslongchamps et al., 1981). Several isomers of these acetals also occur in the venom of both wasp species in minor quantities, and it is possible that the profile of these acetals is characteristic of an individual insect species. Our synthesis of 7-methyl-1,6-dioxaspiro[4,5]decane (Figure 2) afforded a single product **(1),** together with its optical antipode. Synthesis of 7-ethyl-2-methyl-1,6-dioxaspiro[4,5]decane (Figure 3) provided two products that had identical mass spectra. These products were assigned the structures **2** and **3,** together with their respective antipodes, based on the work of Deslongchamps et al. (1981).

Of these six products, the enantiomers of **2** and **3** do not occur in the venom of *V. vulgaris* (Lübke, 1990), the antennae of which were used in this work for bioassays. The racemic mixtures described above were used for these bioassays, but nothing is known about the biological effect that one acetal will have on that of its optical antipode, in relation to insect behavior. The work of Bergström

et al. (1982) and of Tengö et al. (1990) has illustrated the importance of the absolute stereochemistry of spiroacetals in the behavior of *Andrena* bees and similar effects might be expected to be observed in the behavior of *Vespula* wasps towards these acetals.

Amides

The volatile components of the venom of the German wasp included *N*-isopentylacetamide. This amide, like the spiroacetals, has been isolated from a number of insect species. It was isolated by Heath and Landolt (1988) from the venom of the American southern yellowjacket, *Vespula squamosa*. These authors described the amide as a pheromone that elicits alarm and attack behavior in that species at a level of 1.0 μmol. At the time of their publication, only one other alarm pheromone had been identified in the venom of a social wasp, viz., 2-methyl-3-buten-2-ol from the hornet *Vespa crabro* (Veith et al., 1984). Bellas and Fletcher (1979) isolated *N*-isopentylacetamide and five closely related amides from the rectal glands of two species of male *Dacus* fruit flies. This was the first report of the isolation of simple amides from insects. Later work by Baker et al. (1982) yielded the same amides from melon flies *D. cucurbitae*. These amides appeared to be the only constituents of the rectal gland secretion, yet no pheromonal activity was ascribed to any of these compounds even though such activity was displayed by the whole secretion (Bellas and Fletcher, 1979). Although the simple acetamides are known to occur in cheese, tobacco, sherry, and wine, these authors suggested that the amides are synthesized by the flies rather than originating directly from a food source, since both laboratory-reared and wild flies afforded a secretion that had the same composition. They also suggested that the amine radicals of these amides arise from the amino acids leucine and isoleucine, respectively. Such a suggestion infers that protein is a source of these amides.

In the present case, the source of the amides in the wasps may well be the same as the source of the amides in the fruit flies. It is well known that wasps have a preference for either a carbohydrate or a protein food source at different periods of the summer (Wagner and Reierson, 1969). Consequently if the above hypothesis is true, the levels of amides in wasp venom may be high during a period of protein feeding and lower during a period of carbohydrate feeding. The levels will also be determined by the availability and proximity of a protein food source; e.g., wasps collected from an area in which protein had been placed might yield venom with high levels of amide.

The amides therefore appear to be products from protein metabolism and may not be true pheromones, a conclusion that agrees with the work of Bellas and Fletcher (1979) on fruit flies. Despite the work of Heath and Landolt (1988), a behavioral function for these amides in wasps has yet to be satisfactorily demonstrated in field studies.

Electroantennogram Studies

Newly emerged wasps were used in the EAG because research with honeybees suggested antennal sensitivity is enhanced by conditioning (De Jong and Pham-Delégue, 1991; Wadhams et al., 1994) and age (Allan et al., 1987), which would increase variability. In a colony, it is the older workers that guard the nest entrance and would be expected to show greater behavioral responses to venoms. Relatively large replication is needed due to variations in the response from one antenna to another. A gradual decline in the responsiveness of antennae was recorded over time. Since these wasps are generalist feeders, it is not surprising that their antennae respond to a wide range of chemicals, making interpretation of EAG responses in isolation difficult. The antennal response to acetals (±)-**2** and (±)-**3** was generally high at 4–5 μmol but much less at 42 μmol. This indicated that antennal receptor sites became saturated at the higher dose levels, after which no further response was recorded. This phenomenon also occurred in preliminary work with citral, where a 10% solution elicited a greater antennal response than the pure compound and a slower return of the antennae to resting levels. Our work has demonstrated that the acetals (±)-**2** or (±)-**3** at a dose of approx. 5 μmol and acetal (±)-**1** at a dose of approx 30 μmol elicit a stronger response from *V. vulgaris* antennae than one *V. germanica* venom sac and five *V. vulgaris* venom sacs.

One aspect of interest to emerge from this work is the response of *V. vulgaris* antennae to 2-heptanone. The antennae of *V. vulgaris* responded to approximately 10 μmol of 2-heptanone to the same degree as to 5 μmol of acetals (±)-**2** or (±)-**3,** but whereas the latter saturated the antennae at higher dose levels, 2-heptanone elicited increasing response at higher levels. A related substance, 2-undecanone, occurs at low levels in the venom of these two wasp species, and it is a potential precursor of the acetals **2** and **3.** 2-Heptanone was identified by Shearer and Boch (1965) as an alarm pheromone in the mandibular glands of the honeybee, but Simpson (1966) also demonstrated that the mandibular secretion is repellent to bees. This ketone has also been identified as an alarm pheromone in the ant *Iridomyrmex pruinosus* (Blum et al., 1963). 2-Heptanone and congeneric 2-alkanones may have a function in insects as general alarm substances, similar to that alluded to above for alkylacetamides, and Blum et al. (1966) have indeed shown that this is the case for *I. pruinosus.*

Development of a whole wasp mount would be advantageous to improve interpretation of antennal responses. Mauelhagen and Greggers (1993) identified a conditioned proboscis extension response in honeybees thought to indicate behavioral recognition of food substances. Wadhams et al. (1994) found the proboscis extension response to be absent in naive and present in conditioned honeybees despite both groups showing responses to the compounds. Indeed analysis of behavioral responses of loosely mounted individuals to known repel-

lent, attractive, and alarm-causing compounds may identify behavioral bioassays to link with EAG assays and improve interpretation and screening powers. Addition of a gas chromatographic link to the EAG–behavioral assays would improve isolation of compounds attractive to wasps, especially when dealing with blends of compounds (Wadham et al., 1994).

Olfactometry Bioassay

The apparent attractiveness of small amounts of acetal (±)-**2**, (±)-**3**, *N*-isopentylacetamide, and 2-heptanone was only weak. On average, only about 2 wasps/min (of about 50 wasps available) entered the test chambers containing the most attractive test chemicals. The lowest quantities of these chemicals tested may have been too great to show much attractancy in this bioassay. For example, acetal (±)-**1** was not attractive to *V. vulgaris* even at 0.6 μmol. The repellency of larger amounts of test chemicals was obvious. Wasps preparing to enter the test chambers containing larger amounts of test chemicals clearly turned away before entering.

CONCLUSION

Electroantennogram studies have demonstrated that the isomers of 7-ethyl-2-methyl-1,6-dioxaspiro[4,5]decane, **2** or **3** (or their antipodes), elicit responses in common wasp (*V. vulgaris*) antennae at levels of around 5 μmol, which is approximately equivalent to the same effect elicited by five venom sacs from *V. vulgaris* while acetal **1** (or its antipode) was active at 25–30 μmol. A laboratory bioassay demonstrated that a mixture of acetals **2** and **3** (and their antipodes) attracted *V. vulgaris* workers at a dose of < 1 μmol and repelled them at a dose of >40 μmol. *N*-Isopentylacetamide also attracted *V. vulgaris* workers at a dose of < 1 μmol. Additionally, 2-heptanone, which does not occur in the venom of German or common wasps, also attracted *V. vulgaris* at the same dose (approximately 1 μmol) as the acetal and amide above, which are the major volatile components of the venom of these wasps. Larger amounts of these materials were repellent to *V. vulgaris*. This work has indicated that the acetals (±)-**2** and/or (±)-**3** and 2-heptanone may be candidates for field trials to determine their efficacy as attractants for wide-area use with poisoned baits.

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VOLATILE COMPOUNDS FROM THE FOREHEAD REGION OF MALE WHITE-TAILED DEER (*Odocoileus virginianus*)

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Abstract—Secretions produced by sebaceous and apocrine glands of cervids may be important in identifying individuals, establishing dominance, and signaling sexual readiness. The secretions from these glands are transferred to the hair for both lubrication and scent communication via forehead rubbing. We collected hair samples from the forehead and back of 10 male white-tailed deer (*Odocoileus virginianus*) of various ages and analyzed them with gas chromatography–mass spectrometry to determine age-related differences. Fifty-seven compounds were identified, including alkanes, arenes, aldehydes, ketones, aliphatic alcohols, terpenes, terpene alcohols, and phenols. Although forehead apocrine glands of dominant deer become more active during the breeding season, we found that concentrations of eight compounds found on the forehead hair were higher in subordinate deer, while only one was higher in dominant deer. Subordinate deer may have higher concentrations of these compounds because they rub less frequently than dominant deer. Additionally, only five forehead hair volatiles differed in concentration from those taken from the back hair. This seems to indicate that an increase in forehead glandular activity may take place concurrently with an increase in general integumentary glandular activity. The variation in hair volatiles among individuals also may be indicative of an individual-specific odor that could aid in identification.

Key Words—Forehead hair, *Odocoileus virginianus*, pheromone, scent communication, semiochemical, volatiles, white-tailed deer.

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INTRODUCTION

Intraspecific communication in odocoileids commonly is accomplished by the production and detection of olfactory cues (Müller-Schwarze et al., 1977; Albone, 1984; Marchinton and Hirth, 1984). Antler rubs made by male deer immediately prior to and during the breeding season once were thought to aid in shedding velvet and strengthening neck muscles. However, several studies have indicated that these antler rubs also function as visual and olfactory signposts (Müller-Schwarze, 1972; Moore and Marchinton, 1974).

Both male and female black-tailed deer (*Odocoileus hemionus hemionus*) rub their foreheads on twigs and branches (Müller-Schwarze, 1971, 1972). Male black-tailed deer usually rub more often than females, but females often approach these rubs, presumably to obtain olfactory cues. Müller-Schwarze (1972) also determined that rubs could be either exclusive or shared. The fact that deer can differentiate between these two types of rubs provides evidence of the social function of rubbing behavior. Black-tailed deer also show interest in artificial branches rubbed on the head of a conspecific (Volkman et al., 1978). Johnson and Leask (1977) investigated the forehead of the roebuck (*Capreolus capreolus*) throughout the year and found an increase in glandular activity just prior to and during the breeding season. They also found sites of active testosterone metabolism in the skin during these time periods.

With the aid of trained dogs, Moore and Marchinton (1974) demonstrated that scent is left behind on the rubs made by white-tailed deer (*Odocoileus virginianus*). Furthermore, female white-tailed deer also seem to respond to rubs left by males. Sawyer et al. (1989) observed female deer licking, sniffing, and occasionally rubbing their foreheads on rubs made by bucks. They further noted that the dominant female was responsible for almost 50% of the observed behaviors. Forand and Marchinton (1989) reported that, during the breeding season, social grooming among male white-tailed deer tended to be concentrated in the forehead region. This evidence may point to either volatile or nonvolatile compounds produced by the forehead that aid in individual recognition. Atkeson and Marchinton (1982) examined the forehead skin of white-tailed deer and found that both sexes showed greater apocrine glandular activity in the fall than in the summer. They also reported that activity was higher in males than females and was highest in dominant males.

In a study of the effects of population density on antler rubbing, Miller et al. (1987) found that rub density was correlated with older (≥ 2.5 years old) buck density but not with overall buck density, suggesting that rubs are made predominantly by older males. Studies by Moore and Marchinton (1974), Kile and Marchinton (1977), and Ozoga and Verme (1985) also indicated that more antler rubs are made by dominant males than by subordinates. Recent studies

(Fudge et al., 1992) have shown that the effects of exogenous implants of testosterone were negligible on the rubbing behavior of subordinate male white-tailed deer but did seem to enhance the rubbing behavior of the dominant male. This suggests that rubbing behavior is influenced more by hierarchical position than by testosterone levels.

Changes in glandular activity of the forehead gland, the resulting volatiles on the forehead hair, and increased antler rubbing by older age males indicate that the forehead region may play an important role in scent communication whereby bucks communicate their presence and/or social status to conspecifics. We investigated hair from the forehead region of male white-tailed deer to identify the volatile compounds present and to determine if the concentration of these compounds varied markedly between dominant and subordinate animals. Concentrations of compounds taken from hair from the dorsal surface also were examined.

METHODS AND MATERIALS

Hair samples were collected from the forehead region of 10 male white-tailed deer (1.5–11.5 years old). Deer were housed at the Whitehall Deer Research Facility operated by the University of Georgia's Daniel B. Warnell School of Forest Resources. They were fed a commercial feed (Checkers Pellet, Purina Mills, Inc.) and alfalfa (*Medicago sativa*) ad libitum. These deer were divided into several groups and placed into 1-ha pens. Does and fawns also were placed in the pens to provide olfactory stimuli that would possibly induce the establishment of dominance.

Sampling of behavioral data began in October 1993 and ended December 1993. Observations were conducted within 2 hr after sunrise or 2 hr before sunset. The all-occurrences method described by Lehner (1979) was used due to pen visibility of $>95\%$ and frequency of behaviors. Matrices were then established per Lehner (1979) to determine the social ranking of each individual within his group. At the end of the sampling period, the hierarchies were stable, with all immature deer (1.5 years old) being subordinate and all mature deer (>3.5 years old) being dominant (Gassett, 1995).

In December 1993, deer were immobilized with a mixture of ketamine hydrochloride and xylazine hydrochloride (Mech et al., 1985). Hair from the forehead region of each deer was clipped with sterile scissors. Hair samples also were taken from the back to use as a control for area-specific compounds. Hair samples were frozen immediately in glass vials at $-60°C$. Anesthetized deer were revived with yohimbine hydrochloride.

Volatiles were removed from the hair and concentrated on a porous polymer (Tenax GC) with a modified headspace technique (Novotny et al., 1974;

McConnell et al., 1979). The hair containing the secretions was placed into a headspace-sampling apparatus and subjected to a stream of purified helium gas at a flow rate of 50 ml/min for 30 min at 75°C. Volatiles were passed through a cooled condenser to reduce water mist originating from the sample. Subsequently, the volatiles were trapped on a 4-mg Tenax GC precolumn. After the extraction, the precolumn was placed into a modified injection port of a Hewlett Packard model 5980A GC-MS. Volatiles were desorbed from the precolumn at 200°C and cryogenically retrapped for 15 min to compress the sample at the column inlet. The column used was a fused silica capillary column (Restek RTX 225, 30 m, 0.25 mm ID) provided with a cyanopropyl phase. Column temperature was programmed from 30°C to 190°C at a rate of 2°C/min. Volatile constituents were identified through their mass spectra with electron impact ionization at 70 eV. Peak areas were evaluated with a GC-MS data station.

Peak areas were $\log_{10}(x + 1)$ transformed because their variances were unequal. Because of the nonnormally distributed data, statistical comparisons of volatile concentrations between mature and immature bucks and between forehead and back hair were evaluated with a Mann-Whitney U test. Significance was accepted at $\alpha \leq 0.10$.

RESULTS

Fifty-seven different volatile compounds were identified from the forehead hair including alkanes, arenes, aldehydes, ketones, aliphatic alcohols, terpenes, terpene alcohols, and phenols (Table 1). Many of these also occurred on the samples of back hair. Only seven compounds occurred exclusively on the forehead and may have resulted from vegetative contamination. The GC-MS recording shown in Figure 1 is typical of the volatile profiles obtained from the forehead hair. Higher concentrations of borneol ($P \leq 0.10$) were found in the dominant group. Decane, undecane, *p*-cymene, carvone, tridecane, 4-methylnonane, an unidentified 13 carbon alkane (mol wt 184), and 1-octen-3-ol concentrations in subordinate deer were significantly higher than in dominant deer (Table 1).

Forehead hair volatiles are compared with those taken from the back in Table 1. Naphthalene, an alcohol (mol wt 154), and an unidentified compound (mol wt 152) were significantly higher ($P \leq 0.10$) in the forehead hair. Octanal and a styrene with (2)-carbon side chain were significantly higher in the back hair samples. Several terpene compounds, including allo-ocimene, myrcene, methyl *t*-butylphenol, an unidentified compound (mol wt 152), a monoterpene, carveol, and α-terpineol were present only in trace quantities on the back, but substantial quantities were found on the forehead hair. None of these differences were significant, however (Table 1).

TABLE 1. TRANSFORMED $Log_{10}(x + 1)$ MEAN PEAK AREA OF VOLATILE COMPOUNDS RECOVERED FROM FOREHEAD AND BACK HAIRS OF MATURE AND IMMATURE MALE WHITE-TAILED DEER[a]

Compound class	Peak number	Structure	Mean values of peak areas[b]							
			Mature	SEM	Immature	SEM	Forehead	SEM	Back	SEM
Alkane	2	4-methylnonane	3.75*	0.04	4.30*	0.09	4.02	0.14	4.83	0.84
	3	beta-ocimene	4.02	0.74	5.65	0.24	4.83	0.86	2.06	1.45
	4	decane	5.05*	0.10	5.76*	0.04	5.40	0.16	5.72	0.67
	7	beta-pinene	5.64	0.16	5.53	0.02	5.58	0.17	5.55	0.62
	8	12-carbon alkane (mol wt 170)	3.52	0.41	3.87	0.45	3.69	0.65	5.55	0.61
	9	allo-ocimene	2.19	0.60	2.15	0.59	2.17	0.89	0.00	0.00
	10	myrcene	1.16	0.52	2.58	0.50	1.87	0.80	0.00	0.00
	11	undecane	5.71*	0.10	6.45*	0.05	6.08	0.17	6.53	0.36
	13	limonene	4.75	0.58	5.77	0.12	5.26	0.64	2.91	2.06
	16	4-carene	4.88	0.11	5.52	0.07	5.20	0.17	5.75	0.37
	17	13-carbon alkane (mol wt 184)	5.29*	0.17	5.73*	0.03	5.51	0.19	5.88	0.40
	20	dodecane	5.77	0.08	5.09	0.57	5.43	0.62	6.56	0.21
	26	tridecane	5.74*	0.05	6.12*	0.04	5.93	0.09	6.11	0.12
	36	tetradecane	5.56	0.04	5.51	0.15	5.53	0.16	5.59	0.24
Arene	1	toluene	5.72	0.10	6.04	0.04	5.88	0.12	5.78	0.42
	12	styrene	4.02	0.45	3.98	0.45	4.00	0.67	5.55	0.58
	19	p-cymene	5.36*	0.21	5.65*	0.11	5.51	0.26	2.89	2.04
	23	a dimethylethylbenzene	3.75	0.43	5.40	0.09	4.58	0.54	5.42	0.65
	25	a dimethylethylbenzene	3.63	0.45	5.02	0.18	4.33	0.56	5.37	0.36
	27	4-carbon alkylbenzene	5.57	0.11	5.75	0.04	5.66^	0.13	5.68^	0.14
	32	a methyl butyl benzene	4.83	0.09	5.26	0.07	5.05	0.13	5.37	0.39
	50	naphthalene	3.07	0.56	5.40	0.17	4.24^	0.73	3.01^	2.13
	52	1,3-dimethylbenzene	5.59	0.26	4.63	0.53	5.11	0.64	2.36	1.67
	58	a methylnaphthalene	5.44	0.12	5.06	0.26	5.25	0.31	5.02	0.24

TABLE 1. Continued.

Compound class	Peak number	Structure	Mean values of peak areas[b]							
			Mature	SEM	Immature	SEM	Forehead	SEM	Back	SEM
Aldehyde	5	hexanal	6.34	0.08	6.16	0.04	6.25	0.10	5.83	0.21
	14	heptanal	5.73	0.03	5.75	0.05	5.74	0.06	5.44	0.03
	22	octanal	5.78	0.03	5.94	0.07	5.86^	0.09	5.98^	0.18
	29	benzaldehyde	6.03	0.12	5.86	0.06	5.95	0.14	5.78	0.15
	31	nonanal	6.44	0.03	6.20	0.12	6.32	0.14	6.13	0.08
	40	decanal	6.29	0.09	5.89	0.17	6.09	0.22	5.72	0.25
	51	myrtenal	4.96	0.57	5.29	0.23	5.13	0.65	2.23	1.58
Ketone	33	fenchone	4.95	0.12	5.24	0.03	5.10	0.14	5.40	0.35
	41	pinocamphone	3.47	0.64	4.37	0.50	3.92	0.87	2.55	1.80
	42	acetophenone	3.75	0.44	4.95	0.08	4.35	0.51	5.91	0.30
	55	carvone	4.81*	0.18	4.89*	0.11	4.85	0.23	4.69	0.32
	57	verbenone	5.70	0.20	5.53	0.20	5.62	0.23	5.19	0.00
Alcohol	24	1-octen-3-ol	3.98*	0.48	5.44*	0.06	4.71	0.57	5.36	0.14
	28	2-ethyl-2-hexanol	4.60	0.52	5.92	0.03	5.26	0.60	5.85	0.33
	30	linalool	1.85	0.51	4.65	0.53	3.25	0.90	2.47	1.74
	39	sabinol	6.11	0.19	5.56	0.20	5.84	0.30	4.97	0.25
	43	methyl, t-butylphenol (mw 164)	1.18	0.53	0.00	0.00	0.59	0.59	0.00	0.00
	44	carveol	6.33	0.13	5.92	0.21	6.13	0.27	0.00	0.00
	47	borneol	5.69*	0.18	5.59*	0.10	5.64	0.21	3.04	2.15
	48	alpha-terpineol	5.59	0.21	4.16	0.47	4.87	0.60	0.00	0.00
	49	mol wt 154 alcohol	3.51	0.64	4.43	0.50	3.97^	0.87	2.72^	1.92

Other (unidentified)	6	mol wt 134	5.47	0.17	5.50	0.12	5.49	0.22	5.25	0.65
	18	unknown	2.99	0.55	5.73	0.04	4.36	0.74	5.29	0.62
	21	mol wt 180	6.15	0.08	6.70	0.06	6.43	0.14	6.86	0.22
	34	a monoterpene	4.28	0.49	4.29	0.49	4.28	0.73	0.00	0.00
	35	mol wt 150	3.00	0.55	5.42	0.11	4.21	0.72	5.70	0.33
	37	mol wt 152	4.89	0.57	5.64	0.19	5.26	0.65	2.67	1.89
	38	a lactone	5.12	0.03	4.42	0.50	4.77	0.54	5.49	0.27
	45	mol wt 150	4.84	0.57	5.38	0.17	5.11	0.63	2.26	1.60
	46	mol wt 152	3.28	0.60	4.25	0.48	3.77^	0.83	2.65^	1.87
	53	mol wt 152	2.98	0.55	3.89	0.44	3.43	0.76	0.00	0.00
	54	a sesquiterpene	4.82	0.08	5.26	0.09	5.04	0.14	4.87	0.40
	56	mol wt 180	0.94	0.42	0.00	0.00	0.47	0.47	2.24	1.58

[a] Samples were collected at the University of Georgia's Whitehall Deer Research Facility in December 1993. SEM indicates standard error of the mean. *Indicates a significant difference ($P \leq 0.10$) between mature and immature animals. ^Indicates a significant difference ($P \leq 0.10$) between forehead and back hair.

[b] Mature is ≥ 3.5 years old ($N = 5$). Immature is 1.5 years old $N = 5$. "Forehead" indicates all forehead samples combined ($N = 10$). Back: $N = 2$.

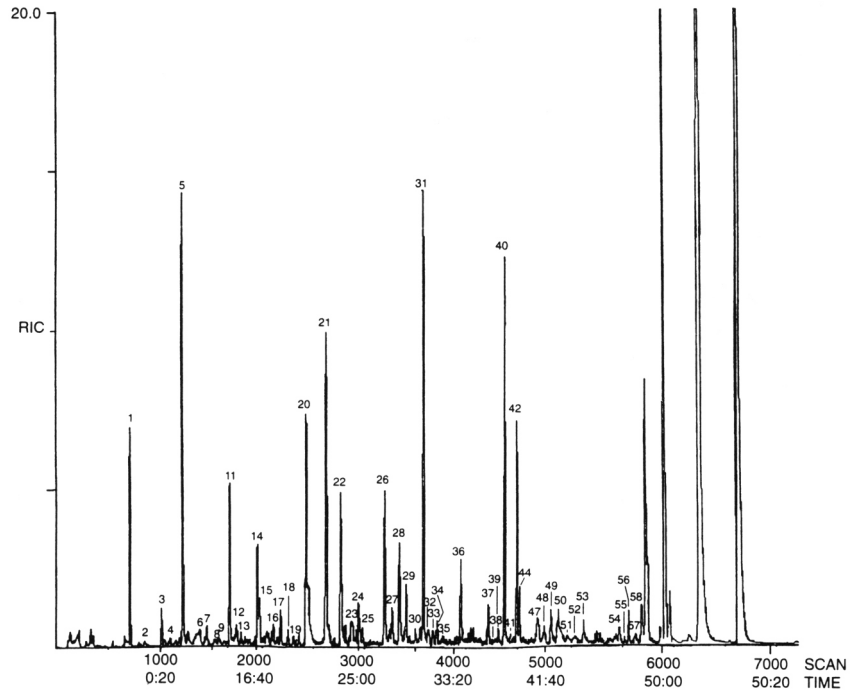

FIG. 1. Chromatogram of the volatile compounds recovered from hairs of the forehead region of male white-tailed deer. The number for each identified peak corresponds with peak numbers in Table 1. Some of these numbers were not placed on the chromatogram due to lack of space; however, the peaks are in sequential order.

DISCUSSION

There were few variations related to age or social rank among the forehead hair compounds. The deposition of chemicals following rubbing behavior may simply act as a calling card, indicating the presence of a deer to its conspecifics. The tremendous individual variation in compound concentrations, as seen by the large standard errors, may support the hypothesis of individual-specific odors. Furthermore, since we did not analyze for steroid metabolites, it is possible that testosterone is metabolized by the skin in this area and the metabolites subsequently are placed on a rub. This would provide a mechanism by which a dominant buck could be identified by his signpost. The compounds found to be significantly higher in subordinate bucks than in dominant bucks may be related to the lower serum testosterone levels, resulting in a variation of glandular activity, or to a difference in rubbing frequency by the subordinate males.

Of particular interest are the number of terpenes found in the forehead region. Terpenes are known to be associated with steroid metabolism, and some terpenes function as pheromones in other species as well. However, plants also produce terpenes as secondary compounds, and since male deer frequently rub their foreheads against vegetation, care must be taken in discriminating between terpenes produced by plants and those produced by animals. Most terpenes identified were probably produced by glands since they were also found on the back, which contacts vegetation less than the forehead. A few terpenes present on the forehead were present only in trace quantities on the back and may prove to be plant-derived.

The small sample sizes of back hair may have contributed to the lack of differences between the forehead and back. We expected the concentrations of compounds to be higher on the forehead than on the back due to increased glandular activity, but this was not so. Sebaceous and apocrine sudoriferous glands in the forehead region are similar to those found elsewhere on the integument, so the lack of variation between sites was not completely unexpected. In this case, increased glandular activity may not correlate with increased volatile production. Moreover, an increase in forehead glandular activity may take place concurrently with a corresponding increase in the glandular activity of the integument in general.

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ROLE OF ECOLOGICAL VARIABLES IN THE SEASONAL VARIATION OF FLAVONOID CONTENT OF *Cistus ladanifer* EXUDATE

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Abstract—The leaves and photosynthetic stems of *Cistus ladanifer*, a plant that colonizes arid lands, secrete an exudate that shows a large seasonal variation in its flavonoid content. The maximum secretion of flavonoids in the exudate is produced during summer, increasing approximately three-to fourfold with respect to the secretion measured in spring. Summer is the season in which the plant suffers the greatest stress from environmental physical variables such as UV irradiation, high temperatures, and hydric stress. Studies were conducted in plants from several locations, which were selected considering daily UV irradiation (open or shaded areas), annual precipitation, and annual average maximum and minimum temperatures. Additional studies to control UV irradiation, drought, and temperature separately were performed with *C. ladanifer* plants growing in a glasshouse and in a culture room. The UV irradiation was found to be the major inducer of the enhanced flavonoid secretion during summer, because no significant increase of flavonoid secretion during summer was observed when the *C. ladanifer* plants in the field were covered with a Plexiglas box (total UV absorption below 380 nm). These results support an ecophysiological role of the flavonoids in the exudate to protect the plant against the damaging effects of UV irradiation. The culture room experiments confirmed this point and also showed that the induction of flavonoid secretion by UV irradiation is synergistically augmented by drought. The glasshouse and culture room experiments showed drought and high temperatures (between 30° and 45°C) to correlate with the summer increase of the more methylated flavonoids (kaempferols and 7-methylated apigenins) in the exudate. Because these more methylated flavonoids have higher hy-

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dropathy than the less methylated, these results suggest that the secretion of more methylated flavonoids is part of the defense mechanism of the plant against the hydric stress of summer.

Key Words—*Cistus ladanifer*, flavonoids, exudate, seasonal variation, environmental stress, UV irradiation, hydric stress, hot climate.

INTRODUCTION

Cistus ladanifer is a plant that colonizes arid lands where peak temperatures reach 40–45°C during summer (Cabezas and Escudero 1989a). This plant is of potential value against erosion. Secondary metabolism is particularly active in most *Cistus* species, in which epithelial cells secrete an abundant exudate, especially *C. symphytifolius*, *C. monspeliensis*, *C. populifolius*, *C. laurifolius*, *C. ladanifer*, *C. palhinhae*, and *C. clusii*. The exudate represents between 8 and 15% of the dry mass of the leaves in these species (Vogt et al., 1987). Other *Cistus* species, such as *C. albidus* and *C. salvifolius*, secrete an exudate that only represents between 0.5 and 1.6% of the dry mass of their leaves. Flavonoids are one of the major components of the *Cistus* exudate (Vogt et al., 1987; Chaves et al., 1993). The amount of flavonoids in the *C. ladanifer* exudate shows a large seasonal variation (Chaves, 1991, 1994; Chaves et al., 1991; Chaves et al., 1993), with a peak secretion and enrichment of flavonols (kaempferols) over flavones (apigenins) during summer. It is therefore interesting to study the effect of ecological factors (drought, UV irradiation, temperature, etc.) that are responsible for the multifaceted stress of summer on the secretion of flavonoids by *C. ladanifer*.

Phenolic compounds, are widely distributed in plants (Harborne, 1977), and more than 400 different flavonoid structures have been reported (see for example Harborne et al., 1975). This structural diversity is due to a large genetic polymorphism and selective expression of the different genes encoding for enzymes leading to production of particular flavonoids in response to different environmental stimuli. The production of flavonoids varies widely, both qualitatively and quantitatively, between plant organs during plant senescence and is dependent on plant growth and on the seasons (Siegelman, 1964; Harborne, 1967; Graham, 1991; Chaves et al., 1993). Systematic studies with *Eucalyptus* (Curir et al., 1990) *Crataegus monogyna* (Lamaison and Carnart, 1991), and *Citrus aurantium* and *Cistus laurifolius* (Vogt et al., 1991; Castillo et al., 1992) have further extended and refined earlier experimental observations with application of modern methodology of analytical chemistry and biochemistry.

Seasonal variations in the production of phenolic compounds have been reported for a variety of plants, such as *Pteridium aquilinum*, *Robinsonia evenia*, *Menziesia ferruginea*, and *Calluna vulgaris* (Cooper-Driver et al., 1977; Pacheco et al., 1985; Bohm et al., 1984; Jalal et al., 1982). Among the recognized

inducers of the synthesis of specific flavonoids are attack by pathogens (bacteria and fungi), mechanical wounding, and physicochemical conditions that stress the plants (see Gottstein and Gross, 1992). Bell (1980) proposed that the synthesis of flavonoids should be regarded as a defense mechanism of the plant against stress. This hypothesis is supported by the observation that ultraviolet (UV) light is a very potent inducer of flavonoid production in plants (Chapell and Hahlbrock, 1984; Vogt et al., 1991; Ziska et al., 1993; Panagopoulos et al., 1992; Cen and Bornman, 1993).

Plants often suffer different types of stress simultaneously (multiple stress). For example, high levels of UV light irradiation are usually accompanied by other environmental stress factors such as drought and high temperatures. There could then be either an additive, synergistic, or antagonistic response to these stress factors. Synergistic and/or antagonistic responses are evidenced by the fact that the response of a plant species to multiple stress conditions does not fit the sum of responses to each stress imposed separately (Balakumar et al., 1993). For example, the phenolic content of *Vigna unguiculata* is increased by 8, 36, and 25% under conditions of water stress, UV-B irradiation, and both types of stress simultaneously, respectively. In addition, the response to a given stress has been shown to differ from one plant species to another. Studies carried out with rice plants in a glasshouse (Ziska and Teramura, 1992) have shown that while in the variety IR-36 the production of flavonoids increases in response to the stress produced by higher CO_2 levels but not to stress from UV-B irradiation; in the variety Furiyama 5 both types of stress (higher CO_2 and UV-B irradiation) increase the production of flavonoids when applied separately, and they are synergistic when applied simultaneously. It has been reported (Tevini et al., 1983) that UV-B irradiation under conditions of hydric stress produces a larger increase in the production of flavonoids by *Cucurbita peplo* than in *Brassica raphanus*. Since, in the absence of stress, the concentration of flavonoids in the *C. peplo* leaves was less than in the *B. raphanus* leaves, the authors suggested that production of flavonoids by the epidermal cells of the leaves might serve to prevent the damaging effects of excessive UV light reaching internal tissues, as flavonoids absorb UV light and act as UV filters.

The aims of the present work were to establish which environmental stress factors play a major role in the induction of flavonoid secretion by *C. ladanifer* and which influence seasonal variation in type of flavonoids present in the exudate from leaves and stems.

METHODS AND MATERIALS

Sampling of C. ladanifer

Samples were collected in the Sierra de los Conejeros (Alburquerque, Badajoz, Spain) over two years. The average rainfall in these mountains is 623 mm/

yr (Cabezas et al., 1983), and the annual average minimum and maximum temperatures are 10.7° and 19.8°C, respectively (Cabezas and Escudero, 1989b). In addition, seven other sampling sites were chosen (Figure 1) in the southwest of Spain to give the highest possible variation in average maximum and minimum temperatures and annual rainfall (ranging between highs of 16.8° and 23.8°C; lows of 3.5° and 10.7°C, and 455 and 933 mm of rainfall, respectively). At each site, samples were collected from both open and shaded areas. The shaded areas were in all cases in *Quercus* woods on the northern slopes of hills.

Sampling of C. ladanifer *Under Controlled Conditions.* UV irradiation and hydric stress were manipulated in three experimental situations: (1) field-grown *C. ladanifer* plants under plastic housing; (2) glasshouse studies; and (3) culture room studies.

Field-Grown C. ladanifer. Large (about 1–1.5 m per side) plastic boxes were constructed to cover selected groups of 15 plants located at Monesterio (Badajoz, Spain). Walls were built all around the groups of plants to protect the area against browsing. Two types of plastic were used for the boxes. Two boxes were made of Plexiglas (to cover a total of 10 plants), a plastic which filters out UV light below 380 nm (Vogt et al., 1991). One box was made of a greenhouse plastic with little ability to filter out UV light (N = 5 plants), and

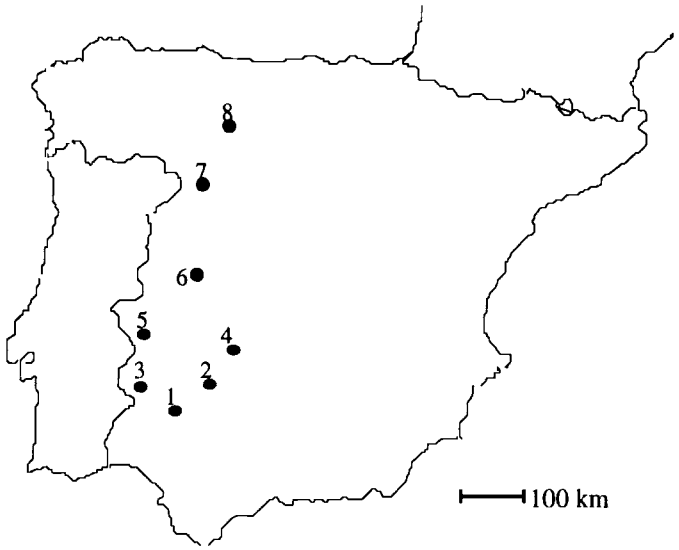


FIG. 1. Location of the field sites in Spain used for intensive sampling in this study: (1) Monesterio, (2) Llerena, (3) Jerez de los Caballeros, (4) Monterrubio, (5) Alburquerque, (6) Jaraiz de la Vera, (7) Zamora, and (8) Astorga.

10 plants were used as controls. The intensity of visible light inside and outside the Plexiglas box was measured with a Quantum LiCor-190SA photometer. To avoid excessive heating inside the boxes, several ventilation slits were made at the bottom and near the top of the plastic walls so that sunlight could not penetrate the box without passing through the plastic. The relative humidity and maximum and minimum temperatures inside and outside the boxes were measured weekly.

Glasshouse Studies. Small *C. ladanifer* plants (each treatment, $N = 10$) approximately 1 year old, 25 cm tall) were collected in November and replanted in a glasshouse and grown under temperature and humidity conditions close to those attained in summer (e.g., maximum and minimum temperatures of 37.5° and 15°C at midday and midnight, respectively, and 25% and 50% relative humidity during daylight and nighttime, respectively). Day/night cycles were simulated by illumination for 16 hr with the lamps indicated below, followed by 8 hr of darkness. Daylight was simulated by combining visible light Philips 36W/56 and UV light Philips TL/40W 09 and Mazoafluor TFWN 18 lamps. Hydric stress was simulated by controlled irrigation of the plants divided into two groups. The plants irrigated every day served as the control group without hydric stress, and the other group had hydric stress provoked by moderate irrigation every 15 days.

Culture Room Studies. In these experiments plants were grown in the absence of UV light and the effect of hydric stress was studied. Plants of *C. ladanifer* like those used for the glasshouse trials were placed in the culture room, separated into two groups ($N = 10$ plants per group): those irrigated daily (control unstressed group) and those irrigated every 15 days (hydric stress group). The culture room was kept at 30°C with a relative atmospheric humidity of 5%, and 16 hr irradiation with a daylight lamp (Sylvania Gro-lux F30W/Gro-T8), followed by an 8-hr dark period.

Characteristics and Treatment of Samples Used for Exudate and Flavonoid Extraction and Analysis

For field plants, the exudate was extracted from leaves produced during the spring (light green leaves) and from the photosynthetic stems of plants with similar morphology and age. From each plant 17 g of leaves and of photosynthetic stems were collected at different times of the year. The sampling period extended from 1990 to 1993. During 1990 and 1991, samples were collected each week from May to October and then every 15 days for the rest of the year. Subsequently, sampling was every 15 days. The samples were placed in plastic bags and maintained at 10°C for transport to the laboratory. They were treated the same day to extract the exudate as indicated below.

For plants covered by plastic boxes and controls (years 1993 and 1994),

additional samples were taken immediately before covering the plants with the plastic (mid-June) and then every 15 days until the end of December.

For glasshouse and culture room trials, plants were allowed to grow for two to three months in the glasshouse or culture room, and then samples of leaves (approximately 0.3 g, three to four leaves) grown during this period were collected as indicated above.

Exudate Extraction. The exudate was extracted from the samples by following the protocols indicated in Chaves et al. (1993) and Vogt and Gülz (1991). The two protocols were found to produce an exudate with identical flavonoid composition, although the yield was higher with the protocol of Vogt and Gülz (1991). With both methods, only aglycone flavonoids are detected in the extracted exudate (Vogt et al., 1987; Chaves et al., 1993; and the present work, see below). This indicated that neither of these methods produced any significant damage to the cells of the leaves, which would have resulted in the release of flavonoid glycosides (Vogt et al., 1987). To further confirm this point, the exudate was removed from selected leaves that were left on the plant. In less than a month these leaves had recovered the exudate layer and presented a normal morphological appearance, indistinguishable from that of control (untreated) leaves. Briefly, the protocol of Chaves et al. (1993) is as follows: 0.7 g of Whatman 118 paper soaked in absolute ethanol and held with pincers was gently passed across the surface of the samples several times and then placed in glass flasks containing 10 ml of ethanol. The flasks were sealed and kept at 4–6°C in a refrigerator until assay (see below). The protocol of Vogt and Gülz (1991) is basically a chloroform extraction of the exudate: the leaves were dipped several times into 2 ml of chloroform. Then the chloroform is evaporated and the exudate is redissolved in 2 ml of methanol, stored at 4–6°C, and analyzed as indicated below.

After extraction of the exudate, the leaves were weighed, oven-dried at 60°C for 12 hr, and weighed again to measure their dry biomass. From these measurements, we determined the water content of the leaves, which can be used as an index of the hydric stress suffered by the plant (Balakumar et al., 1993).

Analysis of Flavonoid Composition in the Exudate. The identification and quantification of the flavonoids in the exudate were done by HPLC-mass spectroscopy. Flavonoids were separated from waxes and terpenoids by chromatography through Sephadex LH-20, as indicated in Vogt and Gülz (1991). The operational protocol was as follows. The extract was dissolved in hot methanol, then cooled to −20°C, and after 12 hr the precipitate containing waxes and other hydrocarbons was removed. Flavonoids and terpenoids remain soluble in methanol and were separated by chromatography on a 25-cm × 1.5-cm Sephadex LH-20 column soaked in methanol for 24 hr, using methanol as eluent. Terpenoids elute first and are well separated from flavonoids, as shown in Vogt and Gülz (1991).

The HPLC separation of flavonoids was with a Nucleosil 5μ C-18 column (25 × 0.45 cm), and a water–methanol–acetonitrile–tetrahydrofuran (56:16:6:22) eluent at a 0.8 ml/min flow rate. Flavonoids were detected upon elution with a diode-array detector using a detection wavelength of 350 nm.

HPLC–mass spectroscopy was done using a Vestec interface (Universal Interface, Vestec, Houston, Texas), with vaporizer temperature of 78°C, nebulizer temperature of 116°C, membrane temperature of 48°C, and separation temperature of 115°C. Other conditions were: flows of helium of the carrier and of the scan, 35 and 55 ml/min, respectively. Mass spectra of the eluted flavonoids were made with a double-focus mass-spectrometer (MS30/VG-70) with an ionization energy of 70 eV and 100 μA of emission current.

The flavonoids identified in the exudate are the following: apigenin, $M^+ = 270$; apigenin-4′-(*O*)methyl, $M^+ = 284$; epigenin-7-(*O*)methyl, $M^+ = 284$; apigenin-7,4′-di(*O*)methyl, $M^+ = 298$; kaempferol-3-(*O*)methyl, $M^+ = 300$; kaempferol-3,4′-di(*O*)methyl, $M^+ = 314$; kaempferol-3,7-di(*O*)methyl, $M^+ = 314$ and kaempferol-3,7-4′-tri(*O*)methyl, $M^+ = 328$. These flavonoids were previously reported to be present in the exudate (Chaves, 1994).

Quantitative analysis of the flavonoids in all samples was done by HPLC, under the following conditions: 20 μl of the extract (eightfold dilution) was injected into Nucleosil 5μ C-18 (150 × 4 mm) column and eluted with water–methanol–acetonitrile–tetrahydrofuran (56:16:6:22) at a flow rate of 0.7 ml/min (Chaves et al., 1993).

Chemicals

Sephadex LH-20 was purchased from Fluka (Buchs, Switzerland). Nucleosil 5μ C-18 columns were supplied by Teknokroma (Barcelona, Spain). The solvents used in the HPLC–mass spectroscopy analysis were obtained from Merck (Darmstadt, Germany).

Statistical Analysis

Data were analyzed using the nonparametric Kruskal-Wallis (K-W) and Mann-Whitney U (M-W) test.

RESULTS

Production of Exudate by C. ladanifer

Upon evaporation of the solvent used for extraction, the total exudate extracted from different samples of *C. ladanifer* from spring to late autumn was weighed and found to range between 0.09 and 0.14 g/g dry mass of leaves ($N > 100$). Values in this range were found in leaves from different plants in the same sampling area and collected the same day, with no seasonal changes of

the total amount of exudate per gram of dry leaves noted. This allowed us to estimate that the production of exudate amounts to approximately 11.5 ± 2.5% of the annual biomass production of the plant, which is in good agreement with the results reported by Vogt et al. (1987). The flavonoids of the exudate varied from 5–6 (winter) to 22–25 (summer) mg flavonoids/g dry mass of leaves. Therefore, the production of flavonoids amounts to less than 2% of the annual biomass production of the plant.

Seasonal Variation of Flavonoids in Exudate

Recently, we have observed elsewhere that the amount of flavonoids in the exudate of *C. ladanifer* shows seasonal variation, increasing three- to fourfold during summer, with changes in the overall kaempferol/apigenin ratio (Chaves et al., 1993). However, the seasonal changes of the relative composition of kaempferols and apigenins in the exudate show different patterns for different kaempferols and apigenins (Table 1). For example, while the contribution of apigenin + apigenin-4′-(*O*)methyl to total apigenins decreases by approximately 48% from March to August (K-W: $P < 0.005$), the contribution of apigenin-7-(*O*)methyl and apigenin-7,4′-di(*O*)methyl increases in this period (K-W: $P < 0.01$). Similarly, the contribution of kaempferol-3,7-di(*O*)methyl to total kaempferols is increased (K-W: $P = 0.0012$), while the contribution of kaempferol-3-(*O*)methyl decreases by about 24% (K-W: $P = 0.0013$). These results show an increased methylation in position 7 of flavonoids in the summer exudate, in parallel with the increase of flavonoid secretion during this season. Studies carried out with *Vicia dumetorum* have shown differences in the composition of flavonoids extracted from leaves and from stems (Webb and Harborne, 1991). Therefore, we extracted and analyzed the composition of the exudate from *C. ladanifer* photosynthetic stems. The composition and seasonal variation of flavonoids in them is presented in Table 2. These results show that both the composition and the seasonal changes are similar in the exudates extracted from leaves and from photosynthetic stems (M-W: $P > 0.05$). Therefore, we conclude that the seasonal variation of the flavonoid composition of *C. ladanifer* exudate is a characteristic of the overall exudate of this plant.

Content of Flavonoids in Exudate is Altered by Environmental Changes

In order to study the environmental parameters that may affect the secretion of flavonoids, different locations were chosen as indicated in the Methods and Materials section. The relative amounts of flavonoids of plants growing in different locations are presented in Table 3. It can be seen that in all cases the secretion is enhanced during summer (K-W: $P = 0.0081$ for open areas; K-W: $P = 0.014$ for shaded areas). The samples collected in Zamora and in Astorga showed a delayed increase in flavonoid secretion, which is correlated with the

TABLE 1. SEASONAL VARIATION OF FLAVONOIDS OF EXUDATE EXTRACTED FROM LEAVES OF *C. ladanifer* PLANTS GROWING IN ALBURQUERQUE, EXPRESSED AS RATIOS OF DIFFERENT APIGENINS AND KAEMPFEROLS OVER TOTAL APIGENINS AND KAEMPFEROLS, RESPECTIVELY

	Ratios[a]							
	Ap/Apt	Ap4′/Apt	Ap7/Apt	Ap7,4′/Apt	K3/Kt	K3,4′/Kt	K3,7/Kt	K3,7,4′/Kt
March	0.43	0.32	0.2	0.03	0.84	0.04	0.07	0.02
May	0.24	0.3	0.35	0.1	0.76	0.08	0.13	0.02
August	0.18	0.19	0.47	0.14	0.6	0.05	0.29	0.05
October	0.14	0.2	0.49	0.15	0.5	0.06	0.3	0.07
December	0.19	0.23	0.49	0.08	0.61	0.05	0.26	0.03
P (K-W)[b]	0.0007	0.005	0.0027	0.013	0.0013	0.2832	0.0012	0.01

[a] Ap: apigenin; Ap4′: apigenin-4′-(*O*)methyl; Ap7: apigenin-7-(*O*)methyl; Ap7,4′: apigenin-7,4′-di(*O*)methyl; K3: kaempferol-3-(*O*)methyl; K3,4′: kaempferol-3,4′-di(*O*)methyl; K3,7: kaempferol-3,7-di(*O*)methyl; K3,7,4′: kaempferol-3,7,4′-tri(*O*)methyl; Apt: total apigenins; Kt: total kaempferols.

[b] Results of Kruskal-Wallis test for seasonal variation of each flavonoid.

TABLE 2. SEASONAL CHANGES OF FLAVONOIDS PRESENT IN EXUDATE OF *C. ladanifer* PLANTS GROWING IN ALBURQUERQUE, EXPRESSED AS PERCENT OF TOTAL FLAVONOIDS

	Percentage (%)[a]									
	March		May		August		October		December	
	L	S	L	S	L	S	L	S	L	S
Apigenin	14	12	6	8	4	3	3	6	4	4
Kaempferol-3-(*O*)methyl	57	48	57	54	47	44	43	50	48	44
Apigenin-4′-(*O*)methyl	11	20	8	1	4	5	4	7	5	4
Apigenin-7-(*O*)methyl	7	3	9	4	10	6	9	6	11	7
Kaempferol-3,4′-di(*O*)methyl	3	7	5	7	4	8	5	7	4	10
Kaempferol-3,7-di(*O*)methyl	4	7	10	10	22	26	26	18	21	22
Apigenin-7,4′-di(*O*)methyl	1	1	3	3	3	4	3	3	3	4
Kaempferol-3,7,4′-tri(*O*)methyl	2	1	2	1	3	2	2	2	2	2
Total apigenins	33	37	25	26	22	19	18	22	23	20
Total kaempferols	68	63	75	73	77	78	86	77	78	80
P^b	0.95		0.49		0.87		0.63		0.87	

[a]L: leaves; S: stems.
[b]Results of Mann-Whitney U test for composition of leaves and stems.

TABLE 3. SEASONAL VARIATION OF TOTAL FLAVONOIDS IN EXUDATE EXTRACTED FROM LEAVES OF *C. ladanifer* PLANTS GROWING IN DIFFERENT AREAS[a]

	Total flavonoids							
	Open areas				Shaded areas			
County	Mar	May	Aug	Nov	Mar	May	Aug	Nov
Monesterio	3.5	4.72	10.5	5.62	3.48	4.41	6.06	4.51
Llerena	4.87	5.42	8	4.55		4.87	7.21	4.46
Jerez de los Caballeros	4.19	3.43	3.98	3.79	3.37	4.32	3.1	3.09
Monterrubio	2.7	4.96	12.48	8.7		5.08	5.86	3.96
Alburquerque	3.5	5.5	12	5	2.69	3.69	3.29	3.13
Jaraiz	5.36	5.58	6.95	5.38	2.98	4.69	5.78	2.52
Zamora		1.52	6.74	4.05		2.9	6.35	5.04
Astorga		1.52	8	6.39		0.65	5.76	2.62
Average[b]	4.02 ± 0.9	4.08 ± 1.7	8.58 ± 2.8	5.44 ± 1.5	3.13 ± 0.3	3.83 ± 1.4	5.43 ± 1.4	3.67 ± 0.9
P (K-W)[c]		0.0081				0.014		

[a] In each case plants growing in open areas and in small *Quercus* woods (shaded areas) were selected. The data are expressed as areas of flavonoids obtained from HPLC chromatograms.
[b] Average values of all sampling areas with standard error.
[c] Results of Kruskal-Wallis test for seasonal variation of total flavonoids in open and shaded areas.

delayed phenology of the plant growth in these areas where cold weather lasts until April/May and as a result few new leaves can be observed in March. A delay of new leaf appearance is also observed in plants growing in shaded areas of Llerena y Monterrubio. In addition, plants growing in open areas had an enhanced flavonoid secretion over those growing in shaded areas, on average a 1.5-fold higher production as measured from May to August (M-W: $P < 0.05$).

Additional differences in the flavonoid content of the exudate of *C. ladanifer* located in the different sampling areas may be seen in the results presented in Table 4. A common feature observed in the different sampling locations is that the increase of the ratio apigenin-7-(*O*)methyl/total apigenin during the summer was systematically higher in open than in shaded areas. Other changes observed, such as the variation of the decrease of the apigenin-4′-(*O*)methyl/total apigenin ratio during summertime in different sampling areas, are likely to be a reflection of the variation of environmental parameters between these locations. Owing to the complex relationships between different environmental parameters that might selectively affect the production of the different flavonoids of the exudate, such as temperature, drought, and UV light, we carried out the following trials under more controlled conditions aiming to establish their relative influence on flavonoid secretion and composition.

Trials Under Controlled Environmental Conditions

These studies were done at environmental temperatures similar to those attained during summer. The UV irradiation and hydric stress were controlled separately.

The role of UV light in the increased secretion of flavonoids observed during summer was established in the trials done with the plants covered with boxes of Plexiglas in the field as indicated in the Methods and Materials section. The following environmental parameters were measured: maximum and minimum daily temperature, relative atmospheric humidity and water content of the leaves. These are listed in Table 5. The last two parameters were used as an indirect index of the hydric stress suffered by the plants. Figure 2 shows the flavonoid secretion from the control plants and covered by the non-UV-filtering plastic to be higher in summer than in spring (K-W: $P = 0.0002$ and $P = 0.04$, respectively). The UV-protected plants (Plexiglas), however, presented a nearly constant secretion over the study period (K-W: $P = 0.9023$). Since there were no significant differences in the other environmental parameters measured inside the Plexiglas boxes and the non-UV-filtering plastic boxes, and only temperature differed between the exterior (control group) and the two covered groups (Table 5), these results demonstrate the important role of UV light in the induction of flavonoid secretion. It is to be noted, however, that the relative composition of the different flavonoids in the exudate is not significantly different

TABLE 4. SEASONAL CHANGES OF APIGENIN DISTRIBUTION IN EXUDATE OF *C. ladanifer* LEAVES FROM MARCH TO AUGUST IN DIFFERENT SAMPLING AREAS, EXPRESSED AS RELATIVE RATIOS OF APIGENIN-4'-(*O*)-METHYL (AP4') AND APIGENIN-7-(*O*)-METHYL (AP7) CONTENT OVER TOTAL APIGENINS (APT)

	County area[a]									
	Ap4'/Apt Open area						Ap4'/Apt Shade area			
	M	LLE	JE	MO	AL	JA	M	JE	AL	JA
March	0.47	0.54	0.47	0.48	0.33	0.32	0.54	0.4	0.35	0.44
August	0.25	0.34	0.23	0.31	0.19	0.28	0.36	0.33	0.29	0.28
Decrease (%)	22	20	24	17	14	4	18	7	6	19
	Ap7/Apt Open area						Ap7/Apt Shade area			
March	0.12	0.12	0.13	0.11	0.2	0.18	0.1	0.15	0.22	0.13
August	0.38	0.32	0.4	0.32	0.47	0.32	0.27	0.28	0.34	0.35
Increase (%)	26	20	27	21	27	14	17	13	12	22

[a]Field sites: M: Monesterio; LLE: Llerena; JE: Jerez de los Caballeros; MO: Monterrubio; AL: Alburquerque; JA: Jaraiz.

TABLE 5. SEASONAL VARIATION OF PHYSICAL PARAMETERS MEASURED FROM JUNE TO DECEMBER INSIDE LARGE PLEXIGLAS AND PLASTIC BOXES COVERING *C. ladanifer* PLANTS AND IN THE VICINITY OUTSIDE THE BOXES (CONTROLS)

	Maximum daily temperature			Minimum daily temperature			% relative humidity			% water in leaves		
	Plexiglas box[a]	Plastic box[b]	Control	Plexiglas box	Plastic box	Control	Plexiglas box	Plastic box	Control	Plexiglas box	Plastic box	Control
June	34	34	34	15	15	15	37	37	37	57.1	56	59
July	47.2	44.3	37.2	17.5	15.6	17.4	34	44	28	51.7	53.6	53.5
August	47.8	45	37.3	15	13.6	14.8	33.6	40	26.5	41.8	42.3	43.3
September	43	41.2	28.2	11.8	11	12.8	47	43.3	40.3	48.7	44.6	49.3
October	31	27	18	15	14	15	71	83	92	60.5	59.3	62.8
December	24.5	31	15	1	0	1	75	85	95	64	65	63.2

[a] UV of sunlight filtered by Plexiglas.
[b] Negligible filtering of UV sunlight by these plastic boxes.

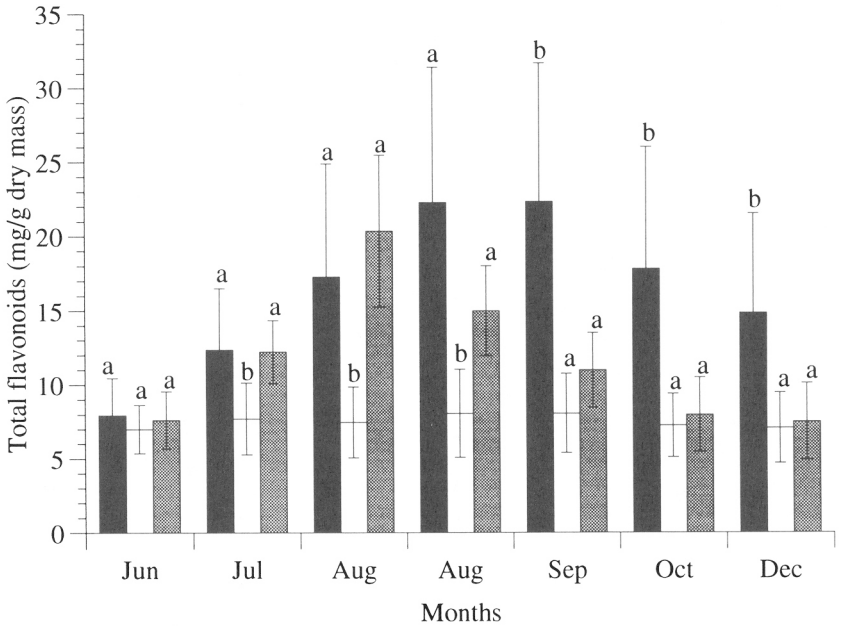


FIG. 2. Effect of UV light on the seasonal dependence of the flavonoid content of the exudate of *C. ladanifer*. Black bars, control plants; white bars, plants covered with a box of Plexiglas (which filters the UV radiation of sunlight); dashed bars, plants covered with a large plastic box (which does not filter the UV radiation of sunlight). Bars with the same letter above them are not significantly different (Mann-Whitney U test, $P > 0.05$). Vertical lines at the top of the bars show $\pm$ standard deviation.

between plants protected against UV irradiation and those exposed to UV irradiation in the field (M-W: $P > 0.05$) (Table 6). Thus, UV light does not appear to have a major role in the enrichment of the exudate in kaempferols and 7-methylated apigenins during summertime.

The higher hydropathy of flavonoids is associated with a higher degree of methylation. Therefore, we considered the possibility that the increase of kaempferols and 7-methylated apigenins in the exudate could be part of the plant's defense mechanism against the hydric stress of summer. To test this hypothesis, we carried out the glasshouse and culture room trials as indicated in the Methods and Materials section. As a result we observed that the secretion of flavonoids in the exudate was induced both by UV irradiation and by hydric stress (Figure 3). These two environmental factors operate synergistically, and therefore the highest content of flavonoids in the exudate is attained when UV irradiation and drought are imposed simultaneously on the plant (K-W: $P < 0.001$). The anal-

TABLE 6. EFFECT OF UV IRRADIATION ON SEASONAL VARIATION OF APIGENIN AND KAEMPFEROL CONTENT, EXPRESSED AS PERCENT OF TOTAL FLAVONOIDS IN EXUDATE OF *C. ladanifer* LEAVES OF PLANTS GROWING IN THE FIELD

	Total apigenins (%)			Total kaempferols (%)		
	Without UV irradiation	With UV irradiation	*P* (M-W)[a]	Without UV irradiation	With UV irradiation	*P* (M-W)[a]
June	34	34	0.26	65	63	0.26
July	26	21	0.39	74	78	0.39
August	26	19	0.095	74	80	0.095
October	27	24	0.22	72	75	0.22
December	33	32	0.84	67	70	0.84

[a]Results of Mann-Whitney U test for total apigenin and kaempferol composition in plants with and without UV irradiation.

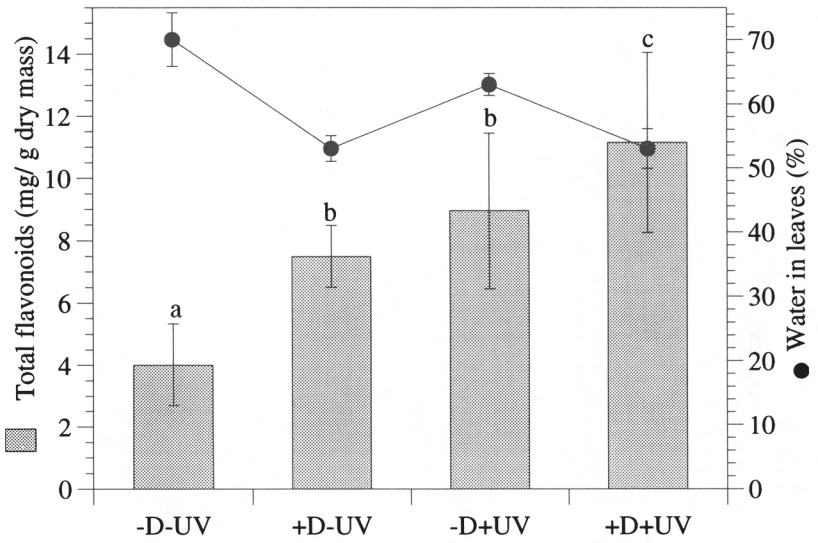


FIG. 3. Effect of UV light irradiation and drought stress (D) on total flavonoid content of the exudate (dashed bars) and water content of leaves (●): −D − UV, control plants without drought stress and without UV irradiation; +D − UV, plants with drought stress and without UV irradiation; −D + UV, plants without drought stress and subjected to UV irradiation; +D + UV, plants subjected to drought stress and UV irradiation. Bars with the same letter above them are not significantly different (Mann-Whitney U test, $P > 0.05$). Vertical lines at the top of the bars show ± standard deviation.

ysis of the flavonoid composition of the exudate secreted by plants suffering drought revealed that the apigenin/kaempferol ratio is significantly lower than in plants not subjected to hydric stress (Figure 4), both in the presence and in the absence of UV irradiation (K-W: $P < 0.001$). This difference is, however, less pronounced in the presence of UV light irradiation, probably because this highly energetic radiation also provokes some hydric stress in the leaves.

DISCUSSION

The exudate secreted by *C. landanifer* amounts to approximately 11.5 ± 2.5% of the total biomass production of this plant. While the total amount of exudate remains approximately constant during the year, the content of flavonoids in the exudate, which amounts to less than 2% of the total annual biomass production of the plant, shows a seasonal variation reaching a peak in midsummer at the time that the plant suffers the greatest stress by physical environmental variables such as drought, temperature, and UV irradiation.

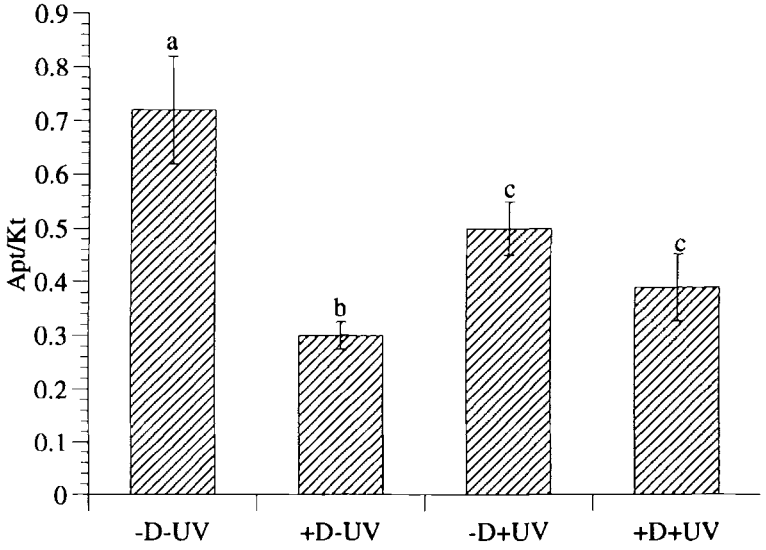


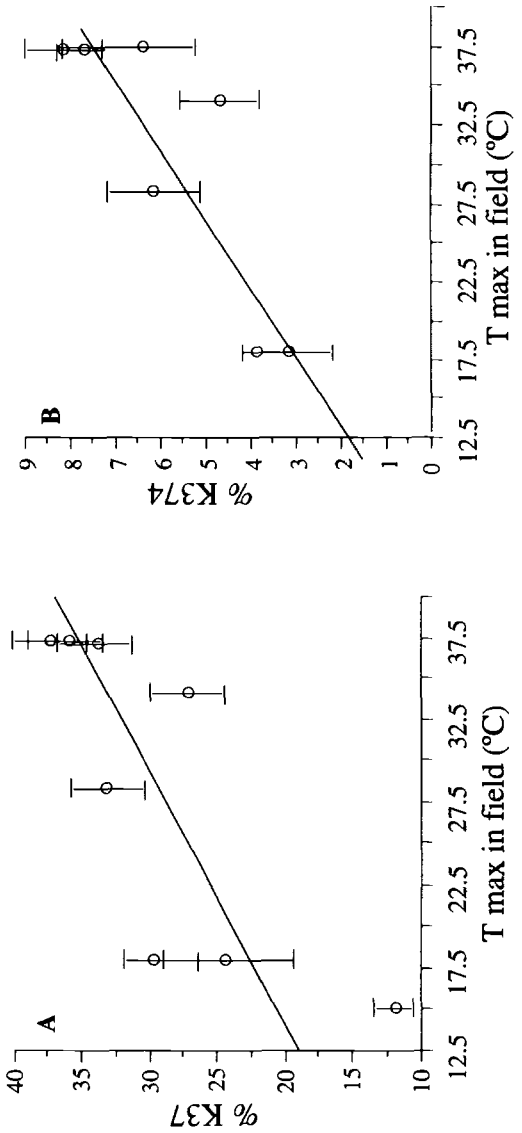
FIG. 4. Effect of UV light irradiation and of drought stress (D) on the apigenin/kaempferol ratio, Apt/Kt, of the exudate: −D − UV, control plants without drought stress and without UV irradiation; +D − UV, plants with drought stress and without UV irradiation; −D + UV, plants without drought stress and subjected to UV irradiation; +D + UV, plants subjected to drought stress and UV irradiation. Bars with the same letter above them are not significantly different (Mann-Whitney U test, $P > 0.05$). Vertical lines at the top of the bars show ± standard deviation.

Previous studies have led to the conclusion that flavonoids can be accumulated in plants in any of the following ways: (1) without apparent induction by environmental factors; (2) in response to an environmental signal; and (3) in response to cell damage or infection by a pathogen microorganism (McClure, 1986; Harborne, 1988). In earlier studies we have shown that aglycone flavonoids of the exudate of *C. ladanifer* undergo seasonal variation, reaching a peak secretion during summertime (Chaves et al., 1993). Green leaves and photosynthetic stems secrete the exudate, with similar composition and seasonal variation. The results obtained in the culture room and glasshouse under controlled conditions indicate that changes in environmental climatic factors (such as temperature, UV irradiation, and drought) play the major role in the large increase of the secretion of flavonoids observed during summer (four to fivefold over the secretion in spring).

The results obtained with samples of *C. ladanifer* from colonies located in open sites that differ in average annual precipitation (from 455 to 933 mm) and maximum and minimum temperatures (from 16.8° to 23.6° and from 3.5° to

10.7°C, respectively) show that there are no major differences in the seasonal variation of the exudate from different sampling sites. These results indicate that the environmental stress producing the induction of flavonoid secretion in the exudate during summer is not greatly different in these sites. However, plants protected against UV irradiation by a Plexiglas box (filtering UV) in the field lost the seasonal variation of flavonoids in the exudate from June to December, and only secreted the same amount of flavonoids as the control plants before the summer (Figure 2). It is noteworthy that the amount of flavonoids in the exudate is systematically lower for plants grown in shaded areas than for those grown in open areas located in close geographical proximity. Cooper-Driver et al. (1977) and McDougal and Parks (1986) have previously described a different concentration of flavonoids in plants (*Pteridium aquilinum*, *Quercus rubra*) grown in open or in shaded areas. These results demonstrate that UV irradiation plays a major role in the induction of flavonoid secretion by *C. ladanifer* during summer. The increase in flavonoid production caused by UV irradiation is now well documented in many plants (Chapell and Hahlbrock, 1984; Ziska et al., 1993; Panagopoulos et al., 1992; Cen and Bornman, 1993; Ziska and Teramura, 1992; Tevini and Termura, 1989; Balakumar et al., 1993; Arimoto and Homma, 1988). Vogt et al. (1991) demonstrated that UV irradiation of *C. laurifolius* induced the production of flavonoid glycosides. Our data showed that in *C. ladanifer* UV irradiation induced the secretion of flavonoid aglycones of the exudate. Since flavonoids absorb UV light and *C. ladanifer* colonializes lands with high light intensity (Matías, 1990), these results support the hypothesis of an ecophysiological role of flavonoids as UV filters to protect against the damaging effect of UV irradiation on photosynthetic pigments or on DNA, as proposed elsewhere (Les and Sheridan 1990; Chaves et al., 1993; Li et al., 1993). Attempts to study the effect of removal of the exudate on leaf growth, or on biochemical markers such as chlorophyll content, failed because the leaves regenerated the exudate layer in less than one month.

The studies in the culture room and glasshouse show that drought increased the content of flavonoids in the exudate of *C. ladanifer*, acting synergistically with UV irradiation (Figure 3). Moreover, in the culture room in the absence of UV irradiation and hydric stress, the amount of flavonoids measured in the exudate approached the value measured in the exudate from field plants in spring (4 mg/g leaf dry mass). The induction of flavonoid production by drought can also be considered as part of the defense mechanism of the plant, since flavonoids react with H_2O_2 and can serve as antioxidant agents by eliminating the H_2O_2 (Takahama, 1988), whose production is enhanced by hydric stress as a by-product of the glycolate pathway stimulated by closure of the stomas (Goyal, 1987). The maximum secretion of flavonoids obtained under controlled UV irradiation and drought in the culture room (11 mg/g leaf dry mass) is only approximately 40–50% of the maximum secretion attained in summer in plants


A
% K37
40
35
30
25
20
15
10
12.5
17.5
22.5
27.5
32.5
37.5
T max in field (°C)
B
% K374
9
8
7
6
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T max in field (°C)

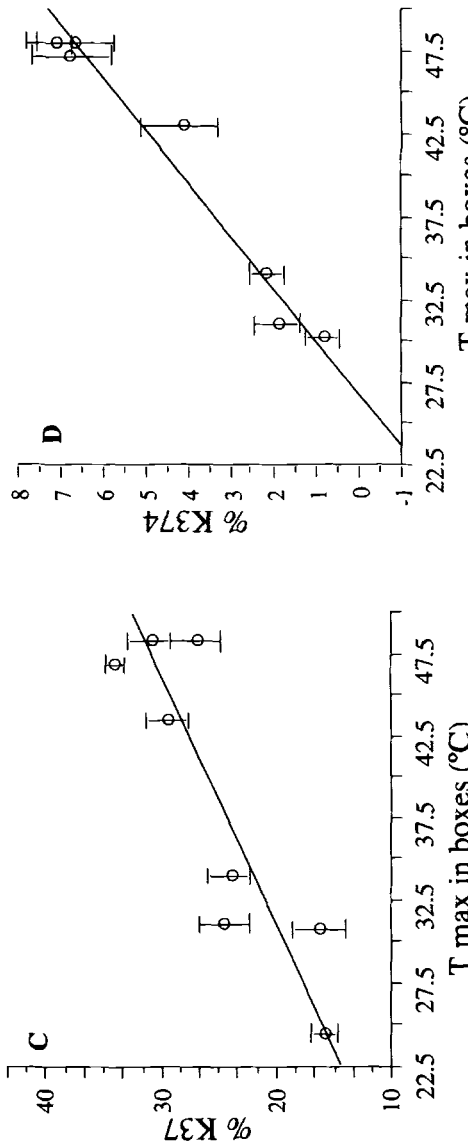

FIG. 5. Content of kaempferol −3,7-di(*O*)methyl (K37) (A and C) and of kaempferol −3,7,4′-tri(*O*)methyl (K374) (B and D) in the exudate of *C. ladanifer* plants in the field (A and B) and of plants covered with boxes of Plexiglas (C and D) as a function of the maximum daily temperature (T max) at each site during the sampling period. Correlation coefficient: $r = 0.77$ (A), 0.85 (B), 0.90 (C), and 0.97 (D). Error bars represent ± standard deviation.

in the field (22–25 mg/g leaf dry mass) and simply reflects the reduction of the total exudate secretion under these experimental conditions. A similar result was reported by Woodhead (1981) for phenolic compound production in plants and probably indicates that the stress applied in the culture room is less than that suffered by plants in the field. In particular, the drought suffered by *C. ladanifer* plants in the field during this study was certainly been more severe than that imposed in the culture room studies, because during the years 1991–1994 only occasional rainfalls took place from June to September at the sampling sites, and they were often separated by periods of more than one month. In addition, the temperature at midday in open places was a few degrees above the maximum temperatures listed, which were recorded in shaded areas. The variations in this latter variable can account for variations in the content of kaempferol-3,7-di(*O*)methyl and kaempferol-3,7,4′-tri(*O*)methyl in the exudate between plants in the Plexiglas box and control plants in the field, as there is a good correlation between the content of these flavonoids and environmental temperature (Figure 5).

During summer, the exudate was enriched in kaempferols and in apigenin-7-(*O*)methyl derivatives, while the relative content of apigenin-4′-(*O*)methyl was much smaller with respect to the spring exudate. Therefore, the exudate secreted by *C. ladanifer* during summer is enriched in methylated flavonoids, particularly in 7-methyl derivatives. The more methylated the flavonoid, the higher its hydropathy and because there is some correlation between the decrease of the less methylated apigenins and rainfall in the field (Figure 6A), the simple possibility that this could merely reflect the effect of differential washing of flavonoids by rain was considered. Thus, studies were carried out in the culture room to avoid any effect of rainfall while imposing the high temperatures and hydric stress on the plants. These studies showed that this change in the flavonoid composition of the exudate is seen when the plants are subjected to high temperatures and drought, with a good correlation between the decrease in apigenins and water content of the leaves (Figure 6B). These results suggest a role of the more methylated flavonoids of the exudate in the *C. ladanifer* response to hydric stress associated with high temperatures in contributing to decreasing the permeability to water of the exudate, since these flavonoids are more hydrophobic than the less methylated apigenins.

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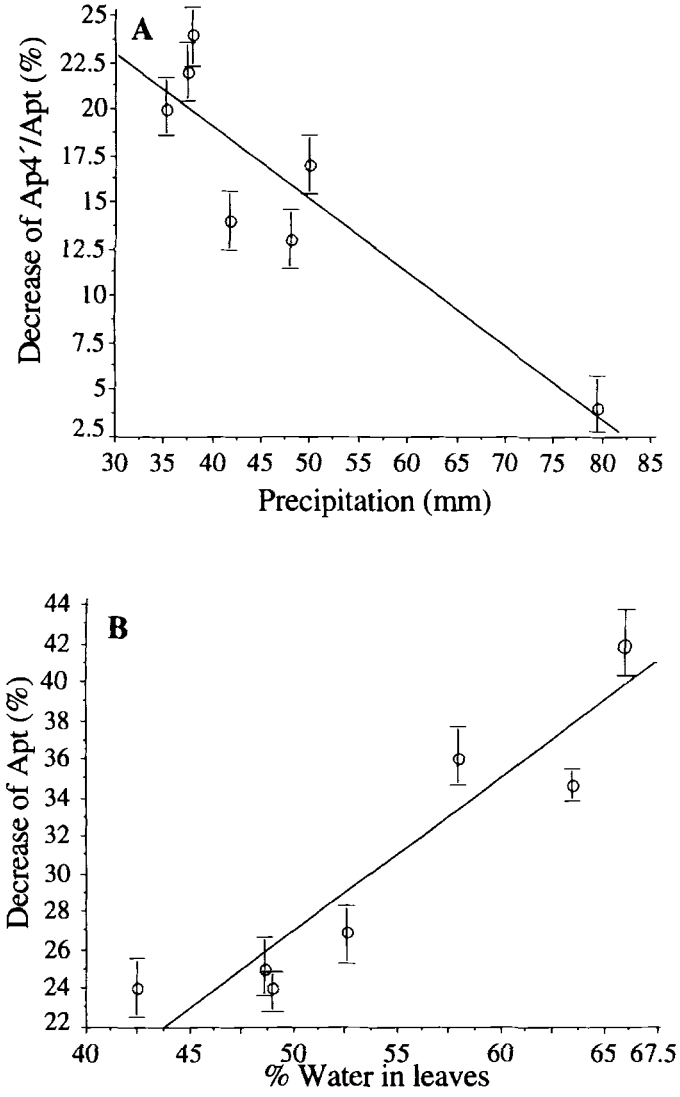

FIG. 6. A: Effect of rainfall from June to September on the seasonal decrease of the content of apigenin-4'-(*O*)methyl (Ap4') in the exudate, expressed as percent of total apigenins (Apt). Correlation coefficient $r = 0.90$. B: Seasonal decrease of the total apigenin content in the exudate as a function of the water content of the leaves. Correlation coefficient $r = 0.93$. Error bars represent $\pm$ standard deviation.

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A SERIES OF BICYCLIC INSECT JUVENILE HORMONE ANALOGS OF CZECH ORIGIN: TWENTY YEARS OF DEVELOPMENT[1]

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Abstract—Research results are summarized from a series of insect juvenile hormone analogs derived from 2-(4-hydroxybenzyl)-1-cycloalkanones, which have been investigated at the Institute of Organic Chemistry and Biochemistry in Prague during the past 20 years. At present, practical application of several prospective structures for insect control is under investigation. Biological activity values were determined to delineate the most important subseries of compounds and the most promising insect juvenile hormone analogs selected from the subseries. Carbamates, and in particular compound **47**, proved to be highly active against aphids, cockroaches, flies, and many other insect species.

Key Words—Insect juvenile hormone analogs, 2-(4-hydroxybenzyl)-1-cycloalkanones, *Tenebrio molitor*, Coleoptera, Tenebrionidae, *Dysdercus cingulatus*, *Pyrrhocoris apterus*, Heteroptera, Pyrrhocoridae, *Acyrthosiphon pisum*, *Phorodon humuli*, Homoptera, Aphididae, *Locusta migratoria migratortorioides*, Orthoptera, Acrididae, *Reticulitermes lucifugus*, *Prorhino-*

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termes simplex, Isoptera, Rhinotermitidae, *Blattella germanica*, Blattodea, Blattidae, *Musca domestica*, Diptera, Muscidae, *Daphnia magna* Cladocera, Daphnidae, *Trypanosoma cruzi*, Euglenozoa, Trypanosomatidae.

INTRODUCTION

The majority of insect species do not interfere with human life, and humans even have a long history of utilization of some insects, including culture for honey and silk production. Nevertheless, a considerable number of insect species are competing with man for food sources (e.g., stored product pests, aphids, locusts, or orchard pests) or are vectors of serious diseases (e.g., flies or mosquitoes). Due to a variety of influences, including the dramatic increase of the human population, increased need for food production, and environmental sensitivity, research efforts have been increasingly focused on environmentally safe ways of controlling insect pests.

Insects are dependent upon numerous hormones including neurohormones, molting hormones, and juvenile hormones, which play important roles in their development and reproduction. The concentration of juvenile hormones varies according to the development stage of individual insects, and addition of externally applied juvenile hormone to an insect during a stage in which the concentration of the natural juvenile hormone is low affects development and usually results in fatal morphological changes. Juvenile hormones are usually nontoxic to warm-blooded animals and fish. Some compounds with juvenile hormone activity have become important tools in insect pest control, since they fulfill the basic requirements for environmentally safe biorational pesticides and effectively control some pests (Henrick, 1995).

Since the discovery of juvenile hormones and related naturally occurring compounds showing the same type of biological activity, hundreds of compounds have been synthesized as insect juvenile hormone analogs. By 1975 several series of aliphatic compounds, some bearing one ring (preferably one aromatic moiety), were prepared at the Institute of Organic Chemistry and Biochemistry in Prague, and their biological effects were studied (for review see Sláma et al., 1974). Due to the low chemical stability of these compounds, a search for more stable but not long-term persistent structures with the same or more favorable biological activities was started. Attention has been focused on the derivatives of 2-(4-hydroxybenzyl)-1-cyclohexanone. This compound bears two rings, one of which is saturated, and has a ketone functional group, factors that enable a chemist to make variable modifications. This advantage was utilized to synthesize compounds in each particular series of insect juvenile hormone analogs that differ in the type of functionality, and, therefore, in physicochemical properties of each compound. The diversity in substituents led to detailed studies of the relationship between the chemical structure and the biological activity.

During the initial years of research in the juvenile hormone area (Sláma et al., 1974), many important discoveries were made, contributing to insights into structure–activity relationships. The most important results found during the development of the juvenile hormone analog series derived from 2-(4-hydroxybenzyl)-1-cycloalkanones were that the overall length of the molecule in a stretched conformation and substitution of the aromatic ring were most important with regard to biological activity. The overall length of the analog molecules, at about 17 Å, with some range of variation among analogs, mimics the overall length of the natural juvenile hormone in an extended conformation. However, little has been published on the conformation of juvenile hormone molecules when bound at a receptor. More exact knowledge of the structure at the receptor active site(s) must be gathered. With a majority of the juvenile hormone analogs studied, only compounds bearing a para-substituted aromatic ring in the molecule showed considerable biological activity. Other substitutions of the aromatic ring (i.e., very often ortho or meta) resulted in a decrease of the biological activity when compared with the para-substituted derivatives. We have included the present knowledge into the design of a general model of oligocyclic compounds. The model in Figure 1 was derived from the basic structural details of juvenile hormone-type compounds and was influenced independently and almost simultaneously by the work of Swiss and Czech scientists

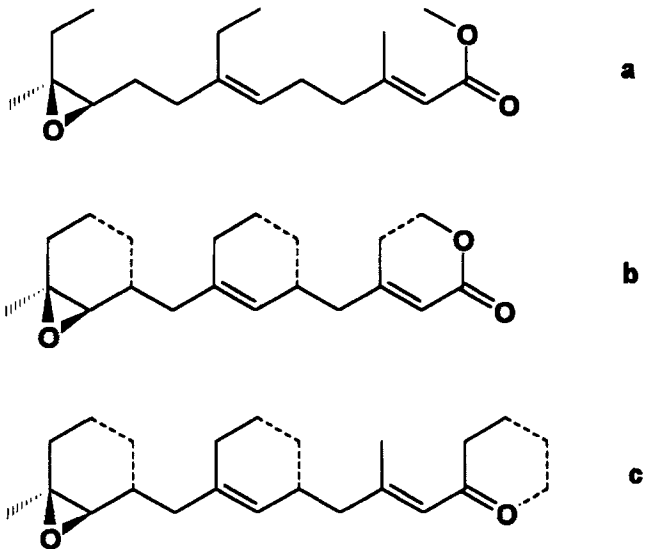


FIG. 1. Juvenile hormone I (a) and models (b and c) of oligocyclic juvenile hormone analogs.

(Karrer et al., 1974; Romañuk, 1976, but reported at a symposium held in 1974). The model illustrates several ways that cyclic compounds can be made by modifying the basic juvenile hormone skeleton. Figure 1 indicates representative compounds from a series in which either the ether-bearing aliphatic unit or the ester-bearing unit were incorporated into a series of compounds with two rings in the molecule. Studies with both series of compounds were initiated to investigate the role of the nature of the aliphatic subunit in the structure–activity relationship. Compounds with three ring cycles in the molecule were also included in this research. The presence of each ring in the product assists in fixing certain conformations in a selected part of the molecule and may increase the general knowledge on structural effects of selected subunits on overall biological activity. Compounds with five-membered and seven-membered saturated rings and those bearing an α-(4-alkoxybenzyl)-α'-methyl disubstituted six-membered C(1)-functionalized saturated ring were also subjects of our study.

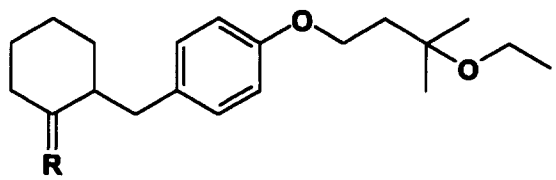
The absolute configuration at the chiral centers of the natural juvenile hormone may be the key for good binding of the juvenile hormone molecule to the receptor site. The chirality center of the juvenile hormone molecule could be substituted by a spacer, while the ester part of the molecule could give the desired physicochemical properties for good activity. However, some of the juvenile hormone analogs presented in this paper showed that even small modification of the structure can result in huge biological effects that are difficult to explain.

Biological activity data in this paper are given as the inhibitory dose (ID_{50}, in micrograms per individual) when the tested compound was applied topically (standard tests described by Sláma et al., 1974) or as the inhibitory concentration (IC_{50}, in either milligrams per milliliter or percent of active ingredient) when the compound was applied by surface treatment. The spray-residue method was used for testing juvenile hormone analogs on aphids (Hrdý, 1974; Hrdý and Kuldová, 1981; Kuldová et al., 1994). Juvenilizing effects on termites were scored according to numbers of soldiers and/or soldier–worker intercasts appearing in orphaline termite groups kept on a treated surface (Hrdý, 1985; Su and Scheffrahn, 1990).

The series of compounds consisting of about 100 biologically active compounds compiled in Tables 1–10 were synthesized by a small team of chemists and tested by entomologists.

ETHER FUNCTIONALITY IN ALIPHATIC SUBUNIT

The relatively unstable and toxic epoxide functionality that is present in the natural juvenile hormone may be successfully substituted by an alkoxy group (Sláma et al., 1974). We followed this finding in constructing the first series of



1, R = O
2, R = H, OH (*cis*)
3, R = H, OH (*trans*)
4, R = OCH_2CH_2O

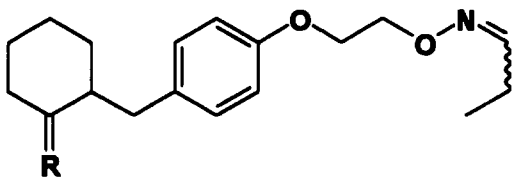
FIG. 2. Ether functionality in the aliphatic subunit: ω-alkoxy-ω,ω-dimethyl derivatives.

derivatives of 2-(4-hydroxybenzyl)-1-cyclohexanone (Wimmer and Romaňuk, 1981). A large series of compounds, the aliphatic subunit of which was derived from ω-alkoxy-ω,ω-dimethylalkanes, was synthesized. We found in this series that biological activity strongly depends on the overall length of the molecule. The only compounds (**1–4**) displaying biological activity are shown in Figure 2. All similar molecules in which the aliphatic subunit was shorter or longer in the carbon–carbon chain proved to be biologically inactive. Table 1 summarizes the biological activity values of **1–4** on the yellow mealworm (*Tenebrio molitor*), the Indian cotton stainer (*Dysdercus cingulatus*), and the pea aphid (*Acyrthosiphon pisum*). We found that compound **4** displayed high activity against aphids (Kuldová et al., 1990). Hop aphids (*Phorodon humuli*) are considered to be an insect pest of high importance due to damage caused to hop plants. Compound **4** was selected for further careful research as a potential aphid control agent. Later, other compounds proved to display more favorable physicochemical properties and biological activities for field applications.

TABLE 1. BIOLOGICAL ACTIVITY OF JUVENILE HORMONE ANALOGS **1–4** BEARING ETHER FUNCTIONALITIES IN ALIPHATIC UNIT

Compound	*Tenebrio molitor*[a]	*Dysdercus cingulatus*[a]	*Acyrthosiphon pisum*[b]
1	8.0×10^{-3}	10.0	
2	1.0×10^{-1}	1.0×10^{-1}	7.0×10^{-2}
3	4.0×10^{-2}	10.0	2.0×10^{-2}
4	3.0×10^{-4}	10.0	1.0×10^{-2}

[a] ID_{50} (μg per individual).
[b] IC_{50} (mg/ml).



5, R = O (*syn*)
6, R = O (*anti*)
7, R = H, OH (*cis*) (*syn*)
8, R = H, OH (*cis*) (*anti*)
9, R = H, OH (*trans*) (*syn*)
10, R = H, OH (*trans*) (*anti*)
11, R = OCH_2CH_2O (*syn*)
12, R = OCH_2CH_2O (*anti*)

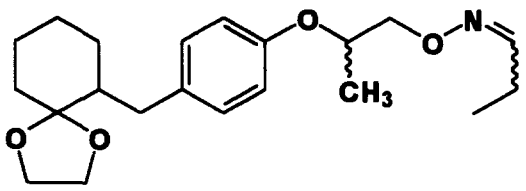
FIG. 3. Ether functionality in the aliphatic subunit: oxime ethers.

The ether part of the aliphatic subunit was modified later, resulting in synthesis of several series of oxime ether derivatives (Wimmer et al., 1988b) (Figure 3). We were successful in an easy and efficient separation of the respective *syn* and *anti* isomers, and isomers **2** and **3** were compared for biological activity with the preceding series of compounds **1–4.** Comparative biological activity studies of *syn* and *anti* isomers of **5–12** were done as well. The results are given in the Table 2, which shows that differences in biological activity

TABLE 2. BIOLOGICAL ACTIVITY OF OXIME ETHER JUVENILE HORMONE ANALOGS

Compound	*Tenebrio molitor*[a]	*Acyrthosiphon pisum*[b]
5	5.0	2.5×10^{-2}
6	5.0	1.0×10^{-1}
7	1.0×10^{-1}	1.0×10^{-1}
8	1.0×10^{-1}	1.0×10^{-1}
9	5.0	1.0×10^{-1}
10	5.0×10^{-3}	1.6×10^{-3}
11	20.0	6.2×10^{-3}
12	20.0	2.0×10^{-3}

[a] ID_{50} (μg per individual).
[b] IC_{50} (mg/ml).



13, racemate
14, (*R*)-enantiomer
15, (*S*)-enantiomer

FIG. 4. Ether functionality in the aliphatic subunit: chirality in oxime ethers.

values may be seen among all types of isomers presented. Compounds **10–12** displayed high biological activity against the pea aphid (*Acyrthosiphon pisum*), and these compounds were selected for more detailed studies for field treatments of hop aphid (*Phorodon humuli*).

The easy separation of *syn* and *anti* isomers of oxime ethers **5–12** focused our attention on introducing chirality into the aliphatic subunit (compounds **13–15,** Figure 4) (Wimmer et al., 1990). This effort resulted in several findings, including difficulty in separating of *syn* and *anti* isomers, and loss of most of the biological activity of compounds **13–15** as compared with **5–12.** Several compounds of this series, however, displayed surprisingly high selectivity against termites (Wimmer and Romaňuk, 1989) (Table 3). Even with optical purities of 85% ee of enantiomers **14** and **15** (the major components of the mixture), maximum biological effectiveness was observed with one enantiomer, no biological effectiveness with the other, and medium biological effectiveness for the racemic mixture. On the other hand, a high selectivity against the European subterranean termite, *Reticulitermes lucifugus* and a subterranean termite from Cuba, *Prorhinotermes simplex* was demonstrated. Superfluous soldier caste for-

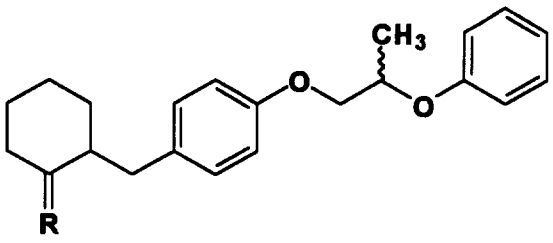
TABLE 3. BIOLOGICAL ACTIVITY OF JUVENILE HORMONE ANALOGS **13–15** ON TERMITE SPECIES

Compound	Conc. (%)	*Reticulitermes lucifugus*	*Prorhinotermes simplex*
13	0.12–0.50	medium–high	medium–high
14	0.12–0.50	none	none–medium
15	0.12–0.50	high	high

mation and disorders in the social structure were observed after administration of juvenile hormone analogs (Hrdý, 1985; Su and Scheffrahn, 1990).

An ether functionality present in the aliphatic subunit permits easy construction of a tricyclic juvenile hormone analog (Wimmer et al., 1994a) (Figure 5). Compounds **16–27** represent this series of analogs and form a series consisting of racemic compounds and all possible enantiomers. Generally, this series of compounds was not of great importance because biological activity values were low when compared with many other analogs. Nevertheless, differences were found in the biological activity values of racemic compounds and their enantiomers (Wimmer et al., 1994a) (Table 4). The presence of the third ring in the molecule and/or the overall structure of the appropriate subunit of the molecule did not meet the demands required for interaction with a receptor site. This result is important because it will be one of the targets for future investigations.

Results obtained with juvenile hormone analogs bearing an ether functionality in the aliphatic subunit indicated that the structure of this aliphatic subunit is crucial for biological activity.



16, R = O (racemate)
17, R = O (*R*)-enantiomer
18, R = O (*S*)-enantiomer
19, R = H, OH (*cis*, racemate)
20, R = H, OH (*cis*, (*R*)-enantiomer)
21, R = H, OH (*cis*, (*S*)-enantiomer)
22, R = H, OH (*trans*, racemate)
23, R = H, OH (*trans*, (*R*)-enantiomer)
24, R = H, OH (*trans*, (*S*)-enantiomer)
25, R = OCH_2CH_2O (racemate)
26, R = OCH_2CH_2O (*R*)-enantiomer)
27, R = OCH_2CH_2O (*S*)-enantiomer)

FIG. 5. Ether functionality in the aliphatic subunit: tricyclic derivatives.

TABLE 4. BIOLOGICAL ACTIVITY OF JUVENILE HORMONE ANALOGS BEARING THREE CYCLES IN MOLECULE

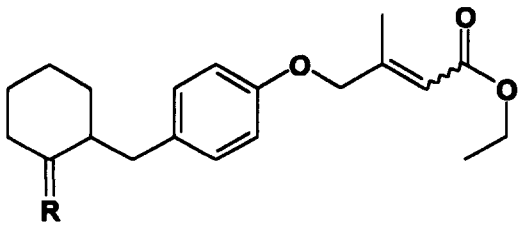
Compound	*Tenebrio molitor*[a]	*Dysdercus cingulatus*[a]	*Pyrrhocoris apterus*[a]
16	5.0×10^{-2}	5.0	inactive
17	1.0×10^{-1}	2.0	inactive
18	1.0×10^{-2}	5.0	inactive
19	5.0×10^{-2}	8.0×10^{-3}	8.0×10^{-2}
20	1.0×10^{-1}	5.0×10^{-2}	1.0×10^{-1}
21	1.0×10^{-3}	3.0×10^{-3}	5.0×10^{-2}
22	1.0×10^{-3}	< 1.0	< 1.0
23	5.0×10^{-4}	1.0	1.0×10^{-1}
24	5.0×10^{-3}	5.0×10^{-2}	inactive
25	5.0×10^{-3}	inactive	inactive
26	1.0×10^{-2}	3.0	inactive
27	1.0×10^{-3}	inactive	inactive

[a] ID_{50} (μg per individual).

ESTER FUNCTIONALITY IN ALIPHATIC SUBUNIT

A bicyclic skeleton of different shape than that described in the preceding section may be built on the basis of the general model shown in Figure 1. The aliphatic subunit, which bears the ester functionality present in the original juvnile hormone molecule, can be incorporated into the molecule. This approach enables formal comparison of the importance of the effect of respective parts of the natural juvenile hormone molecule on biological activity. In order to study this structural relationship, several subseries of insect juvenile hormone analogs with the ester functionality in the aliphatic subunit were synthesized.

The derivation of the aliphatic subunit was initiated from a precursor as similar as possible to the structure of the corresponding part of the juvenile hormone molecule, and generally an α,β-unsaturated ester (for example, ethyl 3-methyl-2-butenoate) met this requirement (Wimmer and Romaňuk, 1981). The resulting juvenile hormone analog subseries (**28–35**) is shown in Figure 6. The presence of a double bond in the aliphatic subunit resulted in formation of two series of geometric isomers, which were separated. The respective *E* and *Z* isomers were subjected to biological screening tests (Wimmer and Romaňuk, 1981), with selected results presented in Table 5. Compounds **31** and **35** gave identical biological activity values when tested on the pea aphid (*Acyrthosiphon*



28, R = O (*E*)-isomer
29, R = H, OH (*cis*) (*E*)-isomer
30, R = H, OH (*trans*) (*E*)-isomer
31, R = OCH_2CH_2O (*E*)-isomer
32, R = O (*Z*)-isomer
33, R = H, OH (*cis*) (*Z*)-isomer
34, R = H, OH (*trans*) (*Z*)-isomer
35, R = OCH_2CH_2O (*Z*)-isomer

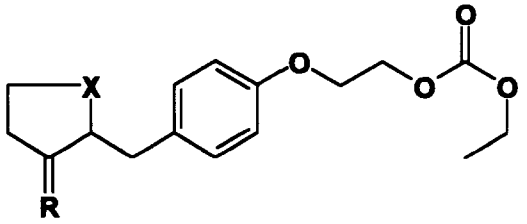
FIG. 6. Ester functionality in the aliphatic subunit: α,β-unsaturated esters.

pisum), indicating that separation of **31** and **35** is not necessary (Kuldová et al., 1990). These results moved the value of this subseries to the highest priority. Other subseries were shown to possess different advantages, which seem to be even more important in the selection of juvenile hormone analogs for potential application as insect pest control agents in the field.

TABLE 5. BIOLOGICAL ACTIVITY OF JUVENILE HORMONE ANALOGS BEARING α,β-UNSATURATED ESTER MOIETY IN ALIPHATIC UNIT

Compound	*Dysdercus cingulatus*[a]	*Acyrthosiphon pisum*[b]
28	1.0×10^{-1}	4.0×10^{-2}
29	1.0×10^{-3}	5.0×10^{-1}
30	1.0×10^{-2}	4.0×10^{-2}
31	5.0×10^{-2}	2.0×10^{-2}
32	100.0	2.0×10^{-1}
33	6.0×10^{-1}	inactive
34	7.0×10^{-1}	inactive
35	>1.0	2.0×10^{-2}

[a] ID_{50} (μg per individual).
[b] IC_{50} (mg/ml).



36, R = O, X = CH_2CH_2
37, R = H, OH (*cis*), X = CH_2CH_2
38, R = H, OH (*trans*), X = CH_2CH_2
39, R = OCH_2CH_2O, X = CH_2CH_2
40, R = O, X = CH_2
41, R = H, OH (*cis*), X = CH_2
42, R = H, OH (*trans*), X = CH_2
43, R = OCH_2CH_2O, X = CH_2

FIG. 7. Ester functionality in the aliphatic subunit: carbonates.

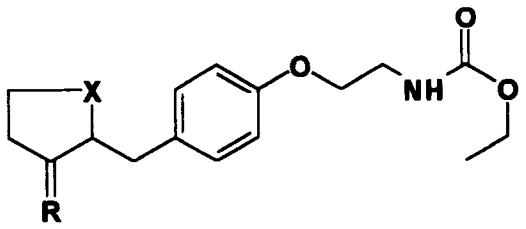
A series of carbonate insect juvenile hormone analogs was generated by further modification of the aliphatic subunit (Rejzek et al., 1991, 1994a) (Figure 7). Those compounds (**36–43**), however, displayed lower biological activity in general (Table 6).

The development of the carbamate juvenile hormone bioanalogs started in the 1980s. The original synthetic carbamate subseries (**44–47**, Figure 8) (Wim-

TABLE 6. BIOLOGICAL ACTIVITY OF CARBONATE JUVENILE HORMONE ANALOGS

Compound	*Tenebrio molitor*[a]	*Dysdercus cingulatus*[a]	*Pyrrhocoris apterus*[a]
36	1.6×10^{-3}	10.0	inactive
37	3.4×10^{-2}	1.0×10^{-1}	1.0×10^{-2}
38	3.7×10^{-2}	5.0	5.0
39	4.8×10^{-3}	10.0	10.0
40	8.1×10^{-2}	10.0	5.0
41	6.9×10^{-3}	1.0×10^{-1}	5.0×10^{-1}
42	1.1×10^{-2}	1.0	1.0
43	4.7×10^{-3}	inactive	inactive

[a] ID_{50} (μg per individual).



44, R = O, X = CH_2CH_2
45, R = H, OH (*cis*), X = CH_2CH_2
46, R = H, OH (*trans*), X = CH_2CH_2
47, R = OCH_2CH_2O, X = CH_2CH_2
48, R = O, X = CH_2
49, R = H, OH (*cis*), X = CH_2
50, R = H, OH (*trans*), X = CH_2
51, R = OCH_2CH_2O, X = CH_2
52, R = O, X = $CH_2CH_2CH_2$
53, R = H, OH (*cis*), X = $CH_2CH_2CH_2$
54, R = H, OH (*trans*), X = $CH_2CH_2CH_2$
55, R = OCH_2CH_2O, X = $CH_2CH_2CH_2$

FIG. 8. Ester functionality in the aliphatic subunit: carbamates I.

mer et al., 1985) displayed very promising biological activity on a variety of insect species (e.g., Wimmer et al., 1991a; Novák et al., 1991; Kuldová et al., 1991, 1994; Kodrík, 1991; Kontev et al., 1991). A relatively easy synthetic procedure, which later modifications made even easier, attracted general attention (Rejzek et al., 1992; Wimmer et al., 1994b). Biological screening included a wider variety of insect species and increased the importance of the carbamate juvenile hormone analogs for potential practical application.

The carbamate juvenile hormone analogs were investigated very carefully to prove that their mode of action does not differ from the mode of action of the natural juvenile hormones. Toxicological tests (Table 7) indicated very low toxicity of this type of insect juvenile hormone analog for warm-blooded animals, fish, and for nontarget arthropods living in water (e.g., water flea, *Daphnia magna*).

On the basis of the preliminary results obtained with **44–47** (Figure 8) (Wimmer et al., 1985), more than 30 new carbamate juvenile hormone analogs were tested (**48–83**, Figure 9) (Wimmer et al., 1994b; Rejzek et al., 1994a). The carbamic acid unit may give rise to two different series of carbamate deriv-

TABLE 7. TOXICOLOGICAL DATA OF CARBAMATE JUVENILE HORMONE ANALOG **47**

Type of test	LD_{50}
Rat (feeding test)	6 g/kg
Rabbit (eye test)	0.1 g (no persistent effect)
Daphnia magna	>6000 ppm

atives when different substituents are introduced at the *N*- or *O*-terminus, and both series of carbamates were synthesized. Moreover, the size of the saturated ring was modified as well (Figures 8 and 9), and a methyl substituent was introduced at the C(α') position to the carbon atom bearing either of the oxygen-containing functionalities [while the opposite C(α) carbon atom bears the para-substituted benzyl group, as is usual in this series). Table 8 lists all carbamate juvenile hormone analogs (**44–83**) prepared so far.

The difference in the biological activity values of a variety of isomers involved in this research (*cis* and *trans* isomers of alcohols, *syn* and *anti* isomers of oxime ethers, and *E* and *Z* isomers of α,β-unsaturated esters) focused our attention on the chiral juvenile hormone analogs during the investigation of the carbamates. Compounds **45** and **46,** racemic carbamates, displayed satisfactory biological activity on a variety of insect species and were chosen as suitable models for such a study. Using chemoenzymic procedures, we synthesized the chiral juvenile hormone analogs **84–87** shown in Figure 10 (Rejzek et al., 1994b). The biological activity of chiral analogs **84–87** was determined as well as the biological activity of their parent racemic structures **45** and **46** in Table 9. These data and those mentioned above prove that a chiral receptor site is involved in the complex recognition/transport/action process. Further research in this field has been focused on the synthesis of a variety of chiral juvenile hormone analogs bearing C(1), C(α), and C(α') substituents with defined absolute configurations.

The aliphatic subunit with an α,β-unsaturated ester moiety plays an important role in the biological activity of **28–35** in most cases, but its role does not seem to be crucial (cf., the result obtained from testing **31** and **35** on aphids or the relatively high biological activity of **33** and **34** against *Dysdercus cingulatus* species). Conversely, all carbamate analogs (**44–83**) indicate the importance of the terminal unit bearing a 1,2-disubstituted saturated ring for biological activity. This conclusion was strongly supported by results found with chiral carbamates **84–87.** Contrary to this finding, carbonate derivatives **36–43** do not seem to follow the hypothesis. The importance of the results obtained with this juvenile hormone analog series is not clear at the moment, and without more detailed

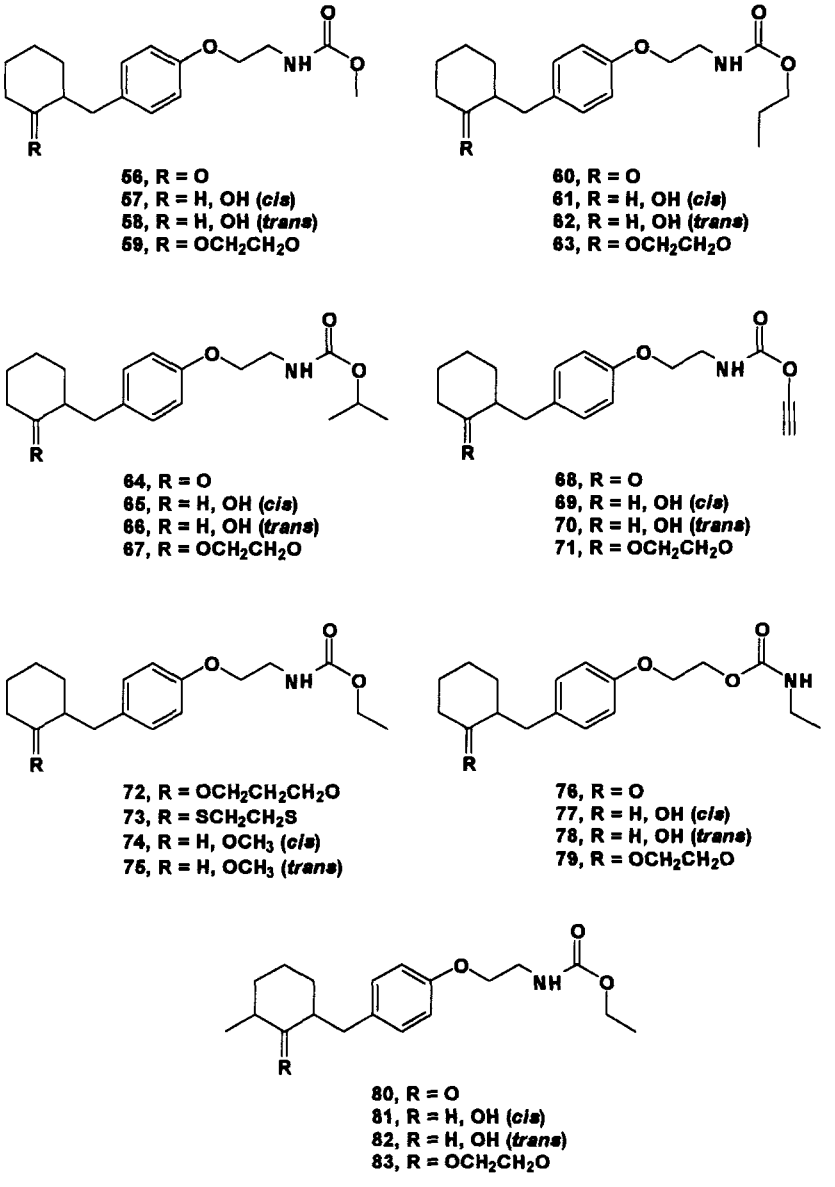
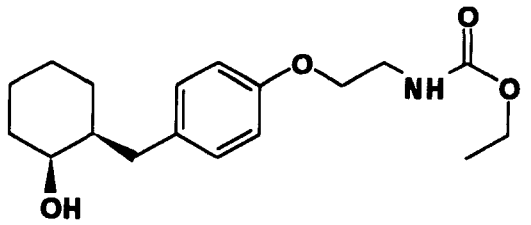


FIG. 9. Ester functionality in the aliphatic subunit: carbamates II.

TABLE 8. BIOLOGICAL ACTIVITY OF CARBAMATE JUVENILE HORMONE ANALOGS

Compound	*Tenebrio molitor*[a]	*Pyrrhocoris apterus*[a]	*Dysdercus cingulatus*[a]	*Galleria mellonella*[a]	*Locusta migratoria*[a]
44	1.2×10^{-6}		1.0×10^{-1}	1.0×10^{-2}	
45	5.3×10^{-5}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-1}	
46	1.2×10^{-4}	1.0	5.0×10^{-2}	1.0×10^{-2}	
47	2.7×10^{-6}	1.0×10^{-1}	1.0×10^{-2}	1.0×10^{-2}	5.0×10^{-1}
48	5.0×10^{-5}	2.0×10^{-1}	5.0×10^{-3}		
49	1.7×10^{-4}	1.0×10^{-3}	1.0×10^{-4}	1.0×10^{-2}	
50	8.3×10^{-5}	5.0×10^{-2}	5.0×10^{-3}		
51	1.1×10^{-5}	2.0	2.0	1.0×10^{-2}	
52	1.4×10^{-5}	1.0×10^{-2}	1.0×10^{-1}	1.0×10^{-2}	
53	6.5×10^{-6}	5.0×10^{-3}	5.0×10^{-4}	1.0×10^{-3}	1.0×10^{-3}
54	2.9×10^{-5}	5.0×10^{-3}	3.0×10^{-4}	1.0×10^{-2}	1.0×10^{-2}
55	2.8×10^{-5}	3.0×10^{-1}	5.0×10^{-1}	1.0×10^{-1}	
56	5.0×10^{-4}	1.0	5.0×10^{-2}	1.0×10^{-1}	
57	5.0×10^{-4}	1.0×10^{-2}	5.0×10^{-4}	1.0	
58	5.0×10^{-3}	5.0×10^{-1}	5.0×10^{-2}	1.0×10^{-2}	
59	1.0×10^{-5}			1.0×10^{-1}	
60	1.0×10^{-2}	1.0	1.0×10^{-3}	1.0	
61	1.0×10^{-1}	1.0×10^{-1}	1.0×10^{-3}	10.0	
62	1.0×10^{-2}	1.0×10^{-1}	1.0×10^{-3}	1.0	
63	1.0×10^{-3}			1.0	
64	1.0×10^{-3}	1.0	5.0×10^{-1}	1.0	
65	5.0×10^{-4}	1.0×10^{-2}	5.0×10^{-3}	10.0	
66	5.0×10^{-2}	inactive	1.0	1.0	
67	5.0×10^{-4}			5.0×10^{-1}	
68	3.0×10^{-4}	5.0×10^{-1}	5.0×10^{-1}	1.0	
69	3.0×10^{-2}	1.0×10^{-3}	5.0×10^{-4}	10.0	
70	5.0×10^{-1}	1.0×10^{-2}	5.0×10^{-4}	10.0	
71	1.0×10^{-4}			1.0	
72	2.0×10^{-5}	1.0	1.0		
73	1.3×10^{-4}	5.0×10^{-5}	5.0×10^{-5}		
74	4.2×10^{-6}	5.0×10^{-2}	1.0×10^{-4}		
75	3.7×10^{-6}	1.0×10^{-1}	1.0×10^{-1}		
76	1.1×10^{-5}	5.0×10^{-3}	5.0×10^{-5}	1.0×10^{-3}	1.0×10^{-2}
77	9.8×10^{-5}	1.0×10^{-3}	5.0×10^{-5}	1.0×10^{-4}	1.0
78	3.4×10^{-5}	5.0×10^{-1}	1.0×10^{-5}	1.0×10^{-4}	
79	2.1×10^{-6}	1.0	1.0×10^{-5}	1.0×10^{-6}	
80	4.3×10^{-5}	2.0	5.0×10^{-1}		
81	2.0×10^{-5}	5.0×10^{-3}	1.0×10^{-3}		
82	2.9×10^{-6}	1.0×10^{-2}	5.0×10^{-3}		
83	1.6×10^{-5}	inactive	inactive		

[a] ID_{50} (μg per individual).



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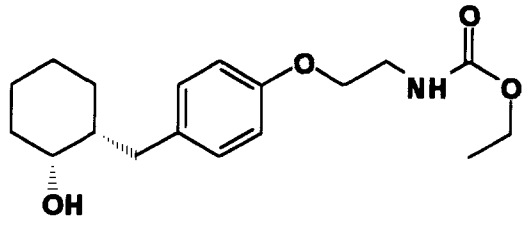

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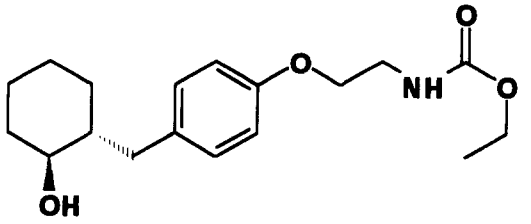

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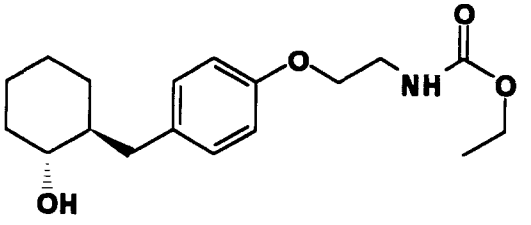

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FIG. 10. Ester functionality in the aliphatic subunit: chirality in carbamate derivatives.

knowledge on the mode of action of insect juvenile hormone analogs it is difficult to explain all the experimental data satisfactorily.

Additional juvenile hormone analogs were synthesized during this 20 years of research dealing with 2-(4-hydroxybenzyl)-1-cycloalkanone derivatives, but

TABLE 9. BIOLOGICAL ACTIVITY OF CHIRAL CARBAMATE JUVENILE HORMONE ANALOGS

Compound	*Tenebrio molitor*[a]	Compound	*Tenebrio molitor*[a]
45	5.3×10^{-5}	**46**	1.2×10^{-4}
84	3.1×10^{-6}	**86**	5.2×10^{-6}
85	6.8×10^{-5}	**87**	7.2×10^{-4}

[a] ID_{50} (μg per pupa)

their biological activity values were not high enough for insect pest control, and they are not, therefore, included in this review (cf., Wimmer and Romaňuk, 1981; Rejzek et al., 1994a).

JUVENOGENS

Juvenogens were discovered by Sláma and Romaňuk (1976). They are complex chemical substances, usually displaying low or no biological activity toward insect species, but capable of liberating the biologically active ingredient under the effect of abiotic or biotic conditions. The basic idea of the juvenogen concept arose from the understanding of the first steps of biodegradation of the juvenile hormone molecule. Natural juvenile hormones are metabolized by enzymes, in which the ester is hydrolyzed with liberation of the corresponding acid and a small alcohol (Figure 11). The hydrolysis products are biologically

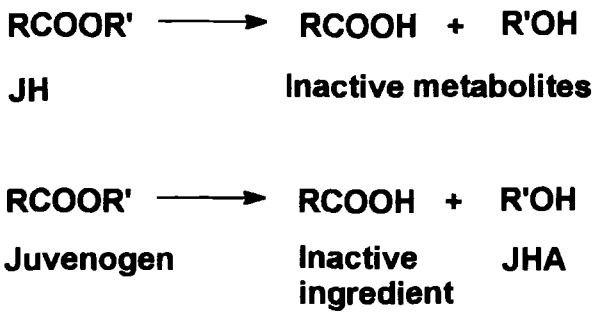

FIG. 11. Degradation of juvenile hormones and juvenogens.

inactive. Sláma and Romaňuk (1976) prepared a fatty acid ester with the alcoholic part derived from an alcoholic juvenile hormone analog. This complex substance was introduced into insects, and it was found that enzymes present within the test insects were able to metabolize the compound into a biologically active juvenile hormone analog (Figure 11) that caused morphological and physiological changes in the insect development. This discovery suggested that a large variety of juvenogens might be synthesized and that insects might convert them into compounds with juvenile hormone activity. We synthesized glycosidic derivatives of the alcoholic juvenile hormone analogs (**88–91**) that proved to be useful as systemic insecticides (Sláma et al., 1977; Wimmer et al., 1988a), esters of dicarboxylic organic acids (e.g., those of succinic acid, **92–95**), and analogs of insect glycerides (**96** and **97**) (Wimmer and Romaňuk, 1982). These juvenogen compounds are presented in Figure 12.

The systemic application of the isomeric glycosidic juvenogens **90** and **91** was investigated using pea plants grown hydroponically, on which sucking insects (fire bug, *Pyrrhocoris apterus*) were studied. The insects received only dry food and were forced to ingest water from the stems and/or leaves of the treated plants. The effects produced in the insects proved absorption of a juvenile hormone analog from the hydroponic medium through the plant root system into its stem and leaves. Sequentially, stems and leaves of the plants were collected and extracted, and analysis of this extract showed the presence of alcohols **2** and **3** (Wimmer et al., 1988a, 1991a).

The biological effect of juvenogens can be explained in more detail now. Most of the substances synthesized up to now were found to be inactive when applied topically (Sláma and Romaňuk, 1976; Sláma et al., 1977; Wimmer et al., 1991a). On the other hand, if a juvenogen is applied orally, it directly enters the insect digestive system in which enzymes attack the molecule and liberate a biologically active juvenile hormone analog that may affect insect development. Table 10 summarizes additional results obtained with juvenogens (Sláma et al., 1977; Wimmer et al., 1991a). The data in Table 10 demonstrate that the biological activity recorded with juvenogen **88** in tests on the penultimate instar of the Indian cotton stainer (*Dysdercus cingulatus*) may be even higher than the corresponding values recorded for the parent juvenile hormone analog **29** (Sláma et al., 1977). This phenomenon is explained by the slow liberation of a biologically active juvenile hormone analog from the juvenogen molecule that supplies a biologically active analog to target cells in smaller quantities during a longer period. Such an application of a biologically active compound may result in higher practical "usage" of such a juvenogen compound in comparison with topical application of a juvenile hormone analog that reaches the target receptor site(s) in smaller quantities. This area of research is now to be one of our high-priority targets.

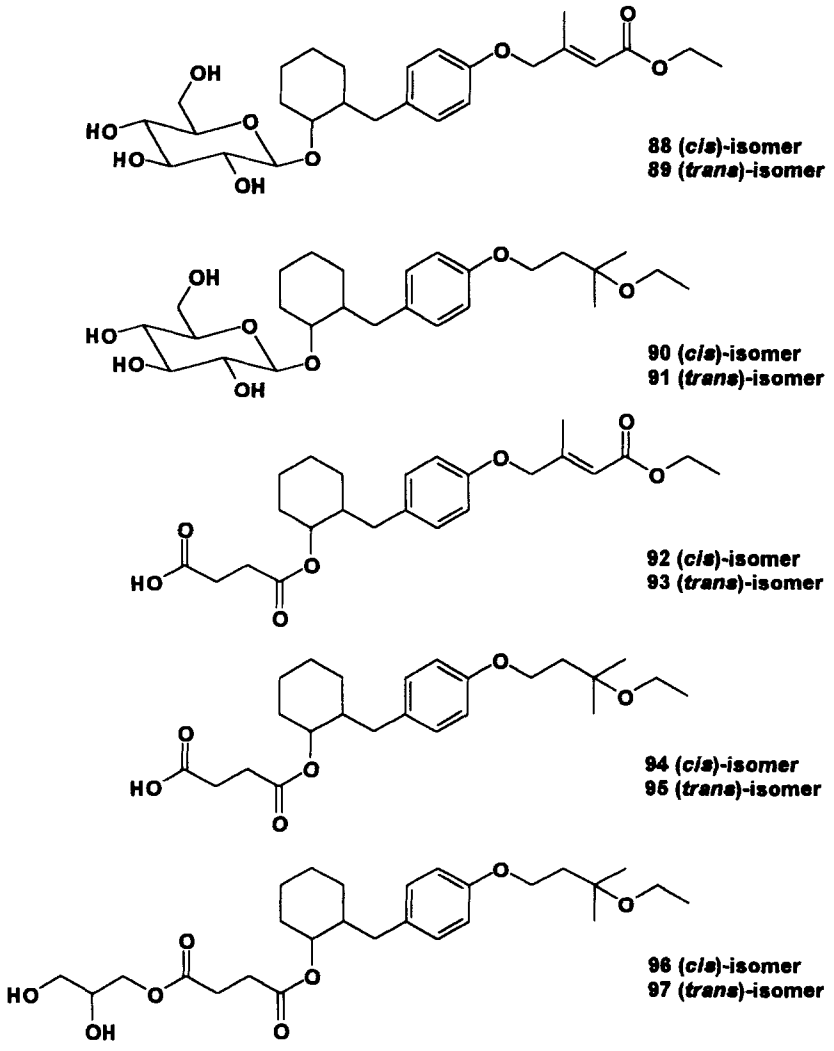

FIG. 12. Juvenogens.

COMPARATIVE SCREENING TESTS

A comparison of juvenile hormones I and II with several commercially available compounds is presented to show briefly the advantages and disadvantages of the Czech compounds. The commercially available compounds were

TABLE 10. BIOLOGICAL DATA OF JUVENOGENS **88–91** AND THEIR PARENT JUVENILE HORMONE ANALOGS **2**, **3**, **29**, and **30**

Compound	*Dysdercus cingulatus*[a]	E/P index[b]
Treated in the last instar		
29	1.0×10^{-2}	0.1
30	4.0×10^{-1}	0.25
2	1.0×10^{-1}	1.0
3	4.2	2.38
88	7.0×10^{-2}	>7143[c]
89	4.0×10^{-1}	>1250[c]
90	1.2	>416[c]
91	>500	
Treated in penultimate and last instar		
29	8.0×10^{-3}	
88	5.0×10^{-3}	

[a] ID_{50} (μg per larva).
[b] Sláma *et al.* (1977).
[c] Calculated for 500 μg of the topical ID_{50}.

obtained as gifts from the producers and were tested using the same screening methods by the same team of entomologists as with the Czech compounds. The results, summarized in Table 11, are fully comparable with those shown in Tables 1–10.

The compounds tested were as follows: Juvenile hormones I and II (pre-

TABLE 11. BIOLOGICAL ACTIVITY OF NATURAL JUVENILE HORMONES AND SELECTED COMMERCIALLY AVAILABLE JUVENILE HORMONE ANALOGS

Compound	*Tenebrio molitor*[a]	*Pyrrhocoris apterus*[a]	*Dysdercus cingulatus*[a]	*Galleria mellonella*[a]	*Locusta migratoria*[a]
Juvenile hormone I	4.4×10^{-5}				
Juvenile hormone II	2.0×10^{-3}	3.0×10^{-1}	1.0×10^{-1}		
Methoprene	3.2×10^{-6}	1.0×10^{-2}	1.0×10^{-4}		
Fenoxycarb	3.2×10^{-6}	1.0×10^{-1}	5.0×10^{-3}	5.0×10^{-3}	5.0
Pyriproxyfen	7.9×10^{-6}	1.0×10^{-1}	1.0×10^{-1}		5.0×10^{-1}

[a] ID_{50} (μg per individual).

pared synthetically by the Czech chemists; Henrick et al., 1972; Anderson et al., 1972; Rejzek et al., 1994a), isopropyl (2*E*,6*E*)-11-methoxy-3,7,11-trimethyl-2,6-dodecadienoate (methoprene, Sandoz, USA), ethyl *N*-(4-phenoxyphenoxyethyl)carbamate (fenoxycarb, Ciba-Geigy, Switzerland), and 1-(4-phenoxyphenoxy)-2-(1-pyridyloxy)propane (pyriproxyfen, Sumitomo, Japan). Based on toxicological data obtained with compound **47,** methoprene is less toxic but fenoxycarb displays higher toxicity against *Daphnia magna.* The data for pyriproxyfen are not available in this comparison.

Table 11 shows that juvenile hormones I and II are less active than the majority of insect juvenile hormone analogs described in this paper. Biological activities comparable with those obtained for the juvenile hormone analogs of Czech origin were obtained with methoprene, fenoxycarb, and pyriproxyfen. Nevertheless, at least several carbamate juvenile hormone analogs display higher activity on tested insect species than compounds tested for comparison. Another comparison of fenoxycarb with **2, 3, 4, 29, 30, 31, 35, 44,** and **47** was made in screening tests against aphids, *Acyrthosiphon pisum* (Kuldová et al., 1994). Fenoxycarb was inactive at concentrations up to 1 mg/ml, while **47** displayed the highest biological activity against aphids among the juvenile hormone bioanalogs compared. The compounds of Czech origin formed an interesting series of juvenile hormone bioanalogs, and further investigation may result in important results.

No computer-aided structure design was used during this long-term research, but structure designing was based on experimental experience of a team of scientists. To formulate quantitative structure–activity relationship conclusions based on the compounds presented in this paper is one of our future tasks.

CONCLUSION

The carbamate **47** was tested under field conditions on the hop aphid (*Phorodon humuli*) (Kuldová et al., 1994), against the German cockroach (*Blattella germanica*) in a housing estate in a small town near Prague (Zeman et al., 1992), against the house fly (*Musca domestica*) in calf houses (Wimmer et al., 1991b), and on some other insect pest species (e.g., Novák et al., 1991; Kuldová et al. 1991, 1994; Kodrík, 1991; Kontev et al., 1991; Vinuela et al., 1994). The compound controlled cockroaches very well, but control of the hop aphid in commercial orchards on rapidly growing high-trellis hops was not satisfactory. Only aphids on the middle and bottom parts of plants were suppressed due to the residual action of **47,** while those on the young untreated shoots of the upper parts were unaffected (carbamate **47** does not translocate in plant tissues). These results indicate that the strategy of using the insect juvenile hormone analogs as one part of an integrated pest management strategy may be useful in low-trellis or dwarf hop plantations (Kuldová et al., 1997).

Recent results obtained in collaboration with the University of Buenos Aires (Fichera et al., 1995) demonstrated the advantage of carbamate structure **47,** which displays high lytic activity on the development of *Trypanosoma cruzi,* a triatomine Chagas disease vector. Compound **47** is the first of the series of insect juvenile hormone analogs of Czech origin to be tested for this type of activity. The results point to another possible advantage of the carbamate series of juvenile hormone analogs as promising candidates for *T. cruzi* elimination from blood, a role already demonstrated from compound **47.**

Research is under way to trace metabolites of several radioisotopically labeled carbamates (Elbert et al., 1993, 1994, 1996). Knowledge of the fate of the compound within an insect has been determined to contribute to a better understanding the general mode of action of natural juvenile hormones and their synthetic analogs (Tykva et al., 1997; Pener et al., 1997). Attention has been paid also to chiral compounds to determine the role of chiral receptor sites (Rejzek et al., 1994b) in the juvenile hormone/juvenile hormone analog field.

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LEAF SURFACE COMPOUNDS AND OVIPOSITION PREFERENCE OF TURNIP ROOT FLY *Delia floralis*: THE ROLE OF GLUCOSINOLATE AND NONGLUCOSINOLATE COMPOUNDS

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Abstract—The role of leaf surface compounds influencing the oviposition of the turnip root fly, *Delia floralis*, was investigated using bioassays and fractionation of leaf surface extracts from four *Brassica* genotypes. Polar leaf surface extracts contained between 65 and 175 nM/g leaf equivalent of glucosinolates. However, following fractionation it was found that nonglucosinolates were the major stimuli for *D. floralis* oviposition. Electrophysiological studies of leaf surface extracts and their fractions were performed by using *D. radicum*, the cabbage root fly, as an analytical tool. The most behaviorally active fractions contained stimulatory compound(s) that had an activity profile identical to that previously described for recently discovered nonglucosinolate compounds. The role of leaf surface chemicals in influencing antixenotic resistance to *D. floralis* is discussed.

Key Words—*Delia floralis*, turnip root fly, oviposition stimuli, chemoreception, leaf surface chemistry, *Brassica*, host plant resistance, glucosinolates.

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INTRODUCTION

Antixenotic resistance (Kogan and Ortman, 1978) to oviposition may be influenced by plant secondary compounds. Glucosinolates, typically found in members of the Brassicaceae, are linked with the oviposition specificity or feeding stimulation of more than 20 insect species associated with host plants of this family (reviewed Städler, 1992). The physical and chemical stimuli that influence the oviposition behavior of one of these 20 species, the cabbage root fly (*Delia radicum*), have been extensively investigated. *D. radicum* oviposition site selection is influenced by the volatile hydrolysis products of glucosinolates (Wallbank and Wheatley, 1979; Ellis et al., 1980; Nottingham and Coaker, 1985; Tuttle et al., 1988), nonvolatile chemicals on the leaf surface (Roessingh et al., 1992a, b), and leaf color (Prokopy et al., 1983a, b). Furthermore, physical characteristics, including possession of a waxy surface, a stem, and vertical folds, increased *D. radicum* oviposition on surrogate plants (Roessingh and Städler, 1990). Purified glucosinolates will stimulate *D. radicum* to oviposit (Roessingh et al., 1992b). However, following fractionation, the most stimulatory part of a cauliflower leaf extract contained no glucosinolates, and Roessingh et al. (1992b) concluded that other compounds were of greater importance.

Antixenosis can be a major component of resistance to the turnip root fly *Delia floralis* under field and field cage conditions (Birch, 1985, 1989). For *D. floralis*, the leaf surface is critical in the selection of an oviposition site and the host selection sequence is interrupted on plant genotypes exhibiting antixenotic resistance (Hopkins et al., 1992; Hopkins, 1994). Alborn et al. (1985) concluded that chemical components in *Brassica* leaf surface washings influenced the host selection of *D. floralis* and that tarsal contact with leaf surface chemicals stimulated oviposition. They noted that differences in leaf surface glucosinolate patterns did not appear to relate to differences in *D. floralis* oviposition preferences. However, glucosinolates have been shown to stimulate oviposition by *D. floralis* when tested individually on surrogate plants (Simmonds et al., 1994). This stimulation varies with the structure of individual molecules and can be related to both the length of side chains and the presence of different functional groups (Simmonds et al., 1994).

This paper addresses the following questions about *D. floralis* host selection. Do the glucosinolates in the leaf surface explain the oviposition-stimulating activity of extracts? Is there evidence for additional oviposition stimulating activity due to nonglucosinolate compounds on the leaf surface?

METHODS AND MATERIALS

Biological Material. Four *Brassica* genotypes were selected that had exhibited a range of susceptibility to oviposition by *Delia floralis* (Hopkins et al.,

1992). Ranked by decreasing resistance to oviposition (antixenosis), they were the kales (*Brassica oleracea* var. *fimbriata*), cv. Fribor and cv. Dwarf Green Curled, and swedes (*B. napus* ssp. *rapifera*), GRL aga (a Scottish Crop Research Institute breeding line) and cv. Doon Major (Albom et al., 1985; Ruuth, 1988; Birch, 1989). Plants were glasshouse grown in 5-cm-diameter pots containing a 3:1 mixture of Levington® Universal compost and sand. Test plants were grown under a 16:8-hr light–dark regimen and at a temperature ranging from 16 to 21°C. *D. floralis* came from a laboratory culture established in 1985 at the Scottish Crop Research Institute from a local Scottish population and replenished frequently from local populations.

Bioassay for Oviposition Preference. D. floralis (30–40 gravid females and 10 males) aged 7–18 days were introduced into a rotating turntable chamber at 0900 hr with normal culture foods placed off the turntable. The turntable was 60 cm in radius and gave equal access to all oviposition sites, combating the effect of preferred positions within an arena (Ellis and Hardman, 1975). For each replicate, one undamaged plant of each genotype was introduced onto the turntable at 1300 hr and exposed to the gravid females for 24 hr. Plants were at the 4–5 true-leaf stage and in a pot containing compost covered with moist sand (grain size 1.0–1.4 mm diameter).

Leaf surface extracts and their fractions were sprayed onto surrogate plants (total surface area 130 cm^2) manufactured by Stiftung Behindertenbetriebe im Kanton Schwyz, Switzerland, from green cardboard and covered with paraffin wax, based on a larger model (Roessingh and Städler, 1990). Each surrogate plant was embedded in wax in a 4-cm-diameter pot and the wax covered with a 1-cm layer of sand (grain size 1.0–1.4 mm). Surrogate plants were sprayed using an airbrush (Aerograph Sprite, Devilbiss) in a fume hood with 1.25 g leaf equivalents (gle) of the extract or fraction under test, between 0900 and 0930 hr on the day of the bioassay. Solvents were allowed to evaporate in the fume hood before surrogate plants were introduced to the flies at 1300 hr. After oviposition preference bioassays, the eggs were recovered by flotation in water, sieved (0.2-mm mesh), and counted.

The number of eggs laid on each plant or surrogate plant was then expressed as a percentage of the total number of eggs laid on all (surrogate) plants during the 24 hr of the replicate. When less than 200 eggs were laid during any 24-hr test period, the results of that replicate were disregarded. Replication varied and *N* values are given, where appropriate, in the Results section. The data from the choice tests were analyzed by a Kruskal-Wallis test on the percentage of eggs laid and Mann-Whitney U test on paired groups (Statview 4.0, Abacus Concepts Inc., Berkeley, California).

Leaf Surface Extraction and Fractionation. Leaf surface extracts were prepared from leaves taken from plants at the 5–8 true-leaf stage using methods based on those of Roessingh et al. (1992b). The leaves were successively dipped

once into dichloromethane and then once into methanol for a period of 5 sec in each solvent, to produce nonpolar and polar extracts, respectively. Solvent was allowed to drip back into the solution for a further 10 sec after each dipping. These were then filtered (Whatman GF/D, glass-fiber filters) and evaporated to near dryness under vacuum at 35–40°C. A single bulk extract was made of each plant genotype to avoid variation from different batches and individual plants. Extracts were stored at −20°C prior to use. To produce fractions, 10 gle of a methanol leaf surface extract were made up to 2 ml with distilled water and loaded onto a minicolumn containing 100 mg A-25 DEAE Sephadex, preequilibrated with 0.02 M pyridine acetate and thoroughly rinsed with distilled water. These columns were then treated with different sequences of solutions, the eluents were bioassayed, using the oviposition preference test, and analyzed to investigate the content. Fractions are referred to by a name based on their subsequently identified principal components.

Glucosinolate Analysis. Desulfoglucosinolates from the methanol extracts were separated and quantified by high-performance liquid chromatography (HPLC) by the method of Spinks et al. (1984). The identity of glucosinolates was confirmed by coelution with authenticated standards (supplied by the Food Research Institute, Norwich, U.K.) or were tentatively identified on the basis of their relative retention times as compared with those published by Sang et al. (1984) with similar chromatographic conditions. Response factors for the conversion of peak areas to molar concentrations were determined for the identified glucosinolates (McGregor, 1985).

Electrophysiology. Specific fractions were investigated for contact chemosensory activity on tarsal chemoreceptors of *D. radicum*, a species closely related to *D. floralis*. This species was used in the present study as a model insect because its tarsal chemoreceptors and their sensitivity to glucosinolates and to nonglucosinolate oviposition stimulants, in particular to a compound at present called CIF (cabbage identification factor), are well investigated (Städler, 1978; Roessingh et al., 1992a, b; Baur et al., 1996). This biological test for CIF is required because at this time no chemical analysis technique has been developed for CIF. Electrophysiological recordings were obtained from chemoreceptors on the ventral side of tarsi on the prothoracic leg of females (age <3 days) with methods and equipment described by Roessingh et al. (1992b). Standard tip recording techniques (Städler, 1984) were used to stimulate receptors in the ventrolateral D sensilla on the second (D2), third (D3), and fourth tarsomere (D4) as well as in the ventromedial C sensillum on the fifth tarsomere (C5). These sensilla are known to possess the following sensitivities: D2 sensilla are not sensitive to glucosinolates or CIF (control response); D3 and D4 sensilla respond to glucosinolates, but are not sensitive to CIF; C5 sensilla respond to both glucosinolates and CIF (Roessingh, 1992a, b; R. Baur and P. Roessingh, unpublished observations).

Fractions were tested at concentrations of 0.1 gle/ml and 1.0 gle/ml dissolved in 10 mM potassium chloride. KCl at 10 mM evoked less than 10 impulses/sec on its own. Stimulus pipets were freshly filled immediately before each series of stimulations (one recording per sensillum), and an interval of at least 2 min was maintained between consecutive stimulations of the same sensillum. The recordings were digitized and impulses within the period 50–1050 msec were counted on a Macintosh II computer with the software STA (P. Roessingh, R. Baur, and A. Fritschy, unpublished results).

RESULTS

Oviposition Preference Bioassay of Whole Plants and Surface Extracts. The oviposition preference bioassay of whole plants showed that the percentage of total eggs laid by *D. floralis* on each *Brassica* genotype differed ($P < 0.001$, $N = 10$) between plant genotypes. The decreasing order of preference for oviposition was Doon Major, GRL aga, Dwarf Green Curled, and Fribor (Figure 1). The methanol leaf surface extracts of the four plant genotypes tested on surrogate plants had higher proportions ($P < 0.001$, $N = 13$) of eggs laid upon

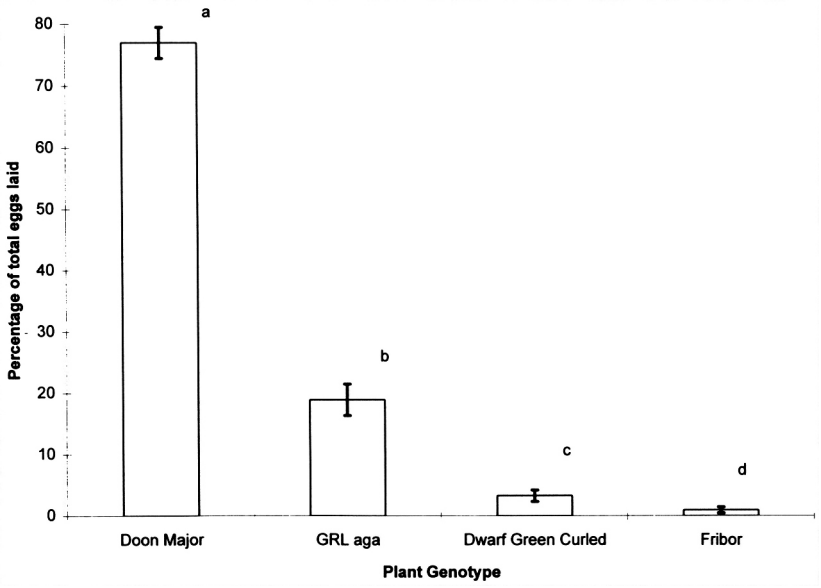

FIG. 1. Oviposition by *D. floralis* on real plants of four *Brassica* genotypes under "choice" conditions. Error bars show SD, columns bearing different letters are significantly different, $P < 0.05$, Mann-Whitney U test on paired groups.

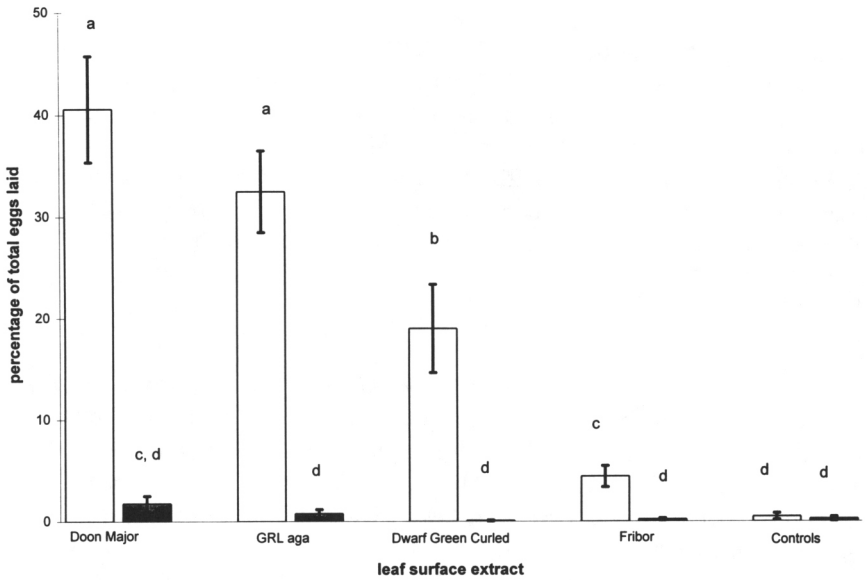

FIG. 2. Oviposition by *D. floralis* on surrogate plants sprayed with methanol (polar) and dichloromethane (nonpolar) extracts of four *Brassica* genotypes under "choice" conditions. Error bars show SD, columns bearing different leaves are significantly different, $P < 0.05$, Mann-Whitney U test on paired groups. Unshaded bars = methanol extract; shaded bars = dichloromethane extract.

them than the dichloromethane extracts from the same plants (Figure 2). Solvent controls of methanol and dichloromethane both received a negligible proportion of the eggs laid. The percentage of eggs laid on the methanol leaf surface extracts of the four *Brassica* genotypes differed ($P < 0.05$) between extracts (Figure 2). The ranking for oviposition on both methanol and dichloromethane extracts applied to surrogate plants was the same as had been found using real plants (Figure 1).

Glucosinolate Content of Methanol Extracts. Analysis of the glucosinolate content of the methanol leaf surface extracts demonstrated that detectable levels of glucosinolates were present (Table 1). Thirteen individual glucosinolates, six of which were common to the two kale and two swede genotypes, were detected. All four genotypes had relatively high levels of 3-indole methyl glucosinolate (glucobrassicin), and no statistically significant difference was found between the mean values for kales and swedes ($P > 0.05$). In contrast, 3-methyl thiopropyl glucosinolate (glucoibervirin) was detected at consistently higher ($P < 0.05$) levels in swedes than in kales, where only trace quantities were detected. The total glucosinolate content of the methanol leaf surface extracts ranged from 66.4 nM/gle (Dwarf Green Curled) to 173.6 nM/gle (Doon Major) (Table 1).

TABLE 1. LEVELS OF GLUCOSINOLATES (nM/gle) FOUND IN THE METHANOL EXTRACT OF FOUR *Brassica* GENOTYPES

	Crop type and genotype[a]			
	Swede		Kale	
Glucosinolate	Doon Major	GRL aga	Dwarf Green Curled	Fribor
Aliphatic				
Prop-2-enyl	n/d	n/d	2.1	7.3
3 methylsulphinyl propyl[b]	n/d	n/d	15.2	32.4
3-methyl thiopropyl[b]	62.7	42.7	tr	tr
But-3-enyl	tr	1.1	n/d	n/d
2-hydroxy but-3-enyl	22.5	5.4	tr	tr
4-methyl sulphinyl butyl[b]	1.2	1.4	3.4	5.2
5-methyl sulphinyl pentyl/ 2-hydroxy pent-4-enyl[c]	27.4	5.1	n/d	n/d
Pent-4-enyl	7.0	n/d	n/d	n/d
Unknown	4.1	2.2	n/d	n/d
Aromatic				
3-Indolemethyl	30.5	29.5	40.2	19.5
1-methoxy-3-indolemethyl	1.5	7.5	0.8	tr
4-methoxy-3-indolemethyl	16.7	12.3	3.5	3.1
Total	173.6	107.2	65.2	67.5

[a]n/d: not detected; tr: trace (less than 0.5 mM/gel).
[b]Indentification based on retention time.
[c]Peak contains two compounds.

Fractionation of Methanol Leaf Surface Extracts. Each methanol leaf surface extract was loaded on to a DEAE Sephadex preequilibrated column. The unretained portion of the extract (i.e., that which passed straight through the column) contained no detectable glucosinolates. A range of different solutions was tested to investigate the efficiency for eluting glucosinolates from the column. Eluting a column with 0.5 M pyridine acetate removed all glucosinolates intact from the column, producing a fraction termed the glucosinolate+ fraction (+ indicating that the fraction contained, in addition to glucosinolates, other unidentified compounds). It was found that potassium sulfate, over a range of molarities, consistently eluted all detectable aliphatic glucosinolates from the column and left behind all detectable aromatic glucosinolates. A fraction removed with 0.5 M potassium sulfate was termed the aliphatic+ fraction. Application of first potassium sulfate, then collecting separately the eluent produced by pyridine acetate produced a fraction that contained only aromatic glucosinolates, and this was termed the aromatic+ fraction. The effect of these different frac-

tions derived from the crude extract of cv. Doon Major was used to investigate the role of aliphatic and aromatic glucosinolates in the oviposition of *D. floralis*.

Role of Different Fractions of Methanol Surface Extract in Stimulating Oviposition. Flies were offered a six-way choice of surrogate plants, one each treated with either the crude Doon Major extract, the aliphatic+ fraction, the aromatic+ fraction, the glucosinolate+ fraction, the unretained fraction, or methanol (control). Significantly different ($P < 0.001$, $N = 6$) proportions of total eggs were laid on the differently treated surrogate plants (Figure 3). The oviposition stimulating activity of the aliphatic+ fraction was approximately 60% of the activity of the crude methanol leaf surface extract from which it had been derived. Lower proportions of eggs were laid on the glucosinolate+ fraction, the aromatic+ fraction, and the unretained fraction (15%, 1%, and 4% of the activity of the crude methanol leaf surface extract, respectively).

The above result was not entirely conclusive; the very low activity of the glucosinolate+ fraction and the aromatic+ fraction might be due to adverse effects of pyridine acetate on *D. floralis*. However, the aliphatic+ fraction did stimulate relatively high levels of oviposition. The aliphatic+ fraction was therefore tested in a five-way choice, comprising the aliphatic + fraction from all

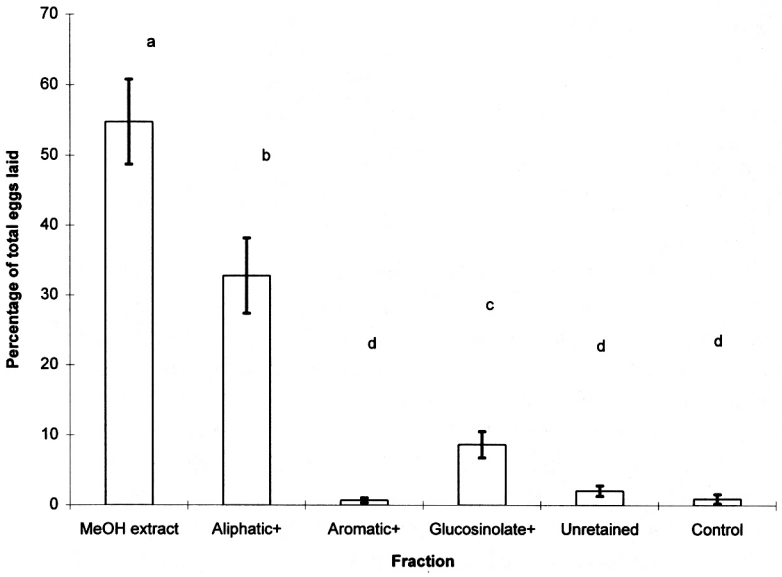

FIG. 3. Oviposition by *D. floralis* on surrogate plants treated with different fractions of the methanol (polar) surface extract of the swede genotype GRL aga under "choice" conditions. Error bars show SD, columns bearing different letters are significantly different, $P < 0.05$, Mann-Whitney U test on paired groups.

the *Brassica* genotypes tested and a methanol (solvent) control. There were significant differences ($P < 0.001$, $N = 8$) in the proportions of eggs laid on the aliphatic+ fraction of the different *Brassica* genotypes (Figure 4). The ranking of the aliphatic+ fractions derived from GRL aga and the two kale genotypes for oviposition stimulating activity was consistent with those of the whole plant and methanolic extract bioassays. However, the aliphatic+ fraction from Doon Major received fewer ($P < 0.05$) eggs than that from GRL aga, which differed from the ranking of oviposition preference using real plants.

The extent to which desulfoglucosinolates stimulate *D. floralis* oviposition when applied to surrogate leaves was also investigated. A set of desulfoglucosinolates was produced from the four leaf surface extracts by eluting a sulfatase treated A-25 DEAE Sephadex column with water. The four desulfoglucosinolate extracts were then offered to gravid *D. floralis* females in a nine-way choice with the four crude methanol extracts and a solvent control. The oviposition on the crude methanol extracts was consistent with the results of previous tests of the extracts, but desulfoglucosinolates were not stimulatory. Only the desulfoglucosinolates derived from the GRL aga methanol surface extract received any eggs at all (mean = 0.8% of total eggs laid, $N = 4$), and this was less than on the control surrogate. This result facilitated investigations of whether the activity in aliphatic+ fraction was due to the glucosinolates or other compound(s).

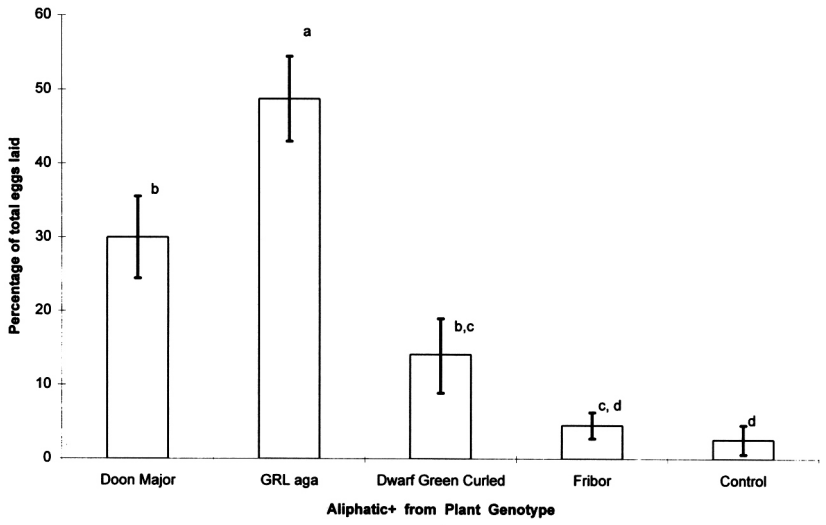

FIG. 4. Oviposition by *D. floralis* on surrogate plants sprayed with aliphatic+ of the methanol (polar) leaf surface extracts of four *Brassica* genotypes under "choice" conditions. Error bars show SD, columns bearing different letters are significantly different, $P < 0.05$, Mann-Whitney U test on paired groups.

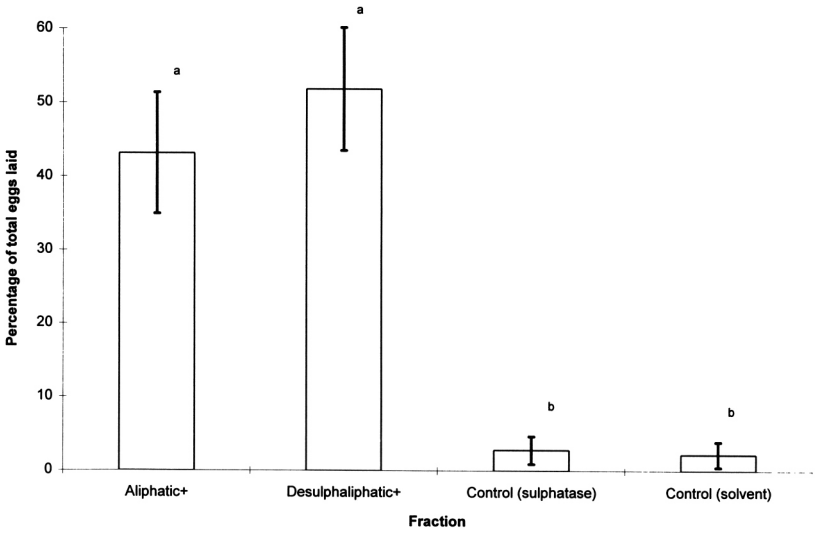

FIG. 5. Oviposition by *D. floralis* on surrogate plants sprayed with either aliphatic+ or desulfaliphatic+ 5 of the methanol (polar) leaf surface extracts of four *Brassica* genotypes under "choice" conditions. Error bars show SD, columns bearing different letters are significantly different, $P < 0.001$, Mann-Whitney U test on paired groups.

A desulfated aliphatic+ (desulfaliphatic+) fraction of GRL aga methanol leaf surface extract was compared with a normally treated aliphatic+ fraction (Figure 5). There was no significant difference ($P > 0.05$, $N = 10$) between oviposition on the aliphatic+ fraction and oviposition on the desulfated aliphatic+ fraction, the latter actually receiving a marginally higher proportion of the eggs. Two controls, a control from a blank column treated with sulfatase and a solvent (water) control both received a significantly ($P < 0.001$, $N = 10$) lower proportion of the eggs than the two fractions under test.

Electrophysiological Activities of Two Fractions on Tarsal Chemoreceptors of Cabbage Root Fly. The study investigated the stimulation of *D. radicum* sensilla by the aliphatic+ fraction of GRL aga and its desulfated derivative. Figure 6 shows the mean number of spikes per second produced by the two fractions on the three sensilla. The number of impulses produced with a level of 0.1 gle/ml was reflected when tests were performed at 1.0 gle/ml. The response of the D2 sensilla (control) was very low for both fractions. The D3/D4 sensilla were highly stimulated by the aliphatic+ fraction (glucosinolates intact) and stimulated very little by its desulfated derivative (glucosinolates inactivated). The C5 sensilla was highly stimulated by both the aliphatic+ fraction and its desulfated derivative at both concentrations tested. These results suggest

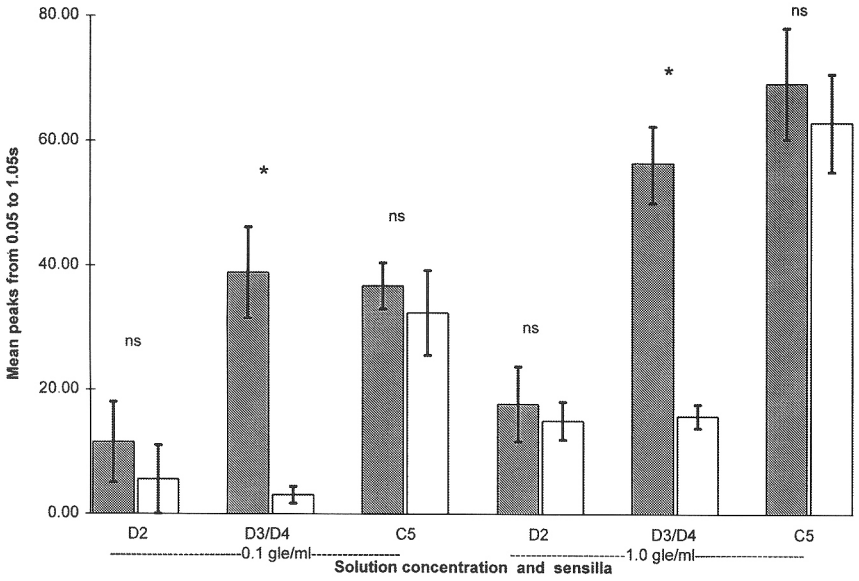

FIG. 6. The responses of different tarsal sensillae of *D. radicum* to fractions derived from methanol (polar) leaf surface extracts of GRL aga. Error bars show SD, pairs of columns marked with an asterisk show significantly different levels of stimulation from each other, $P < 0.05$, Mann-Whitney U test on paired groups. Shaded bars = aliphatic+ fraction, unshaded bars = desulfated derivative.

that the high level of stimulatory activity in both fractions is due, not to glucosinolates, but to other compound(s). The indications from the electrophysiological experiment are that the aliphatic+ fraction contains compound(s) that have activity similar to the compound referred to as CIF, which was previously found to stimulate *D. radicum* C5 sensilla and also oviposition when sprayed onto surrogate plants (Roessingh et al., 1992a; Baur et al., 1996).

DISCUSSION

The initial landing phase of *Delia floralis*, the period between landing on the leaf and the first movement across the leaf, is known to be an important part of the host plant exploration prior to oviposition site selection (Hopkins et al., 1992; Hopkins, 1994). The stimulation of *D. floralis* to oviposit by methanol leaf surface extracts reflects previously published work (Havukkala and Virtanen, 1985) and the results found with leaf extracts tested on *D. radicum* (Städler and Schöni, 1990; Roessingh et al., 1992a, b). This study has clearly

demonstrated the importance of nonglucosinolates in the oviposition site selection behavior of *D. floralis*. Chemical analyses of the methanol fraction demonstrated that the levels of glucosinolates in the leaf surface extracts were of the same order of magnitude as those that stimulate *D. floralis* (Simmonds et al., 1994) and *D. radicum* (Städler, 1978; Roessingh et al., 1992b) when tested individually on surrogate plants. The total glucosinolate content of the leaf surface extracts analyzed in this study was of the same order of magnitude as that found by Roessingh et al. (1992b). Individual glucosinolates differ widely in their stimulation of *D. floralis* and two of the most stimulatory, but-3-enyl and pent-4-enyl, are aliphatic (Simmonds et al., 1994). Both but-3-enyl and pent-4-enyl glucosinolate were present in the aliphatic+ fraction tested here, but their removal (desulfation) did not reduce the activity of the fraction.

The very low oviposition-stimulating activity of the aromatic+ and glucosinolate+ fractions may be due to an adverse effect of pyridine acetate. However, increasing the effort to remove pyridine acetate from fractions did not increase their activity in oviposition bioassays. The low activity of the aromatic+ and glucosinolate+ fractions may simply be due to the presence of naturally occurring oviposition deterrent(s). The effect of naturally occurring oviposition deterrents in extracts was not within the scope of this study. The absence of oviposition deterrents from the aliphatic+ fraction could be an explanation for the change in the ranking of extracts seen between the total and the aliphatic+ fractions (Figure 2 and Figure 4, respectively). Alternatively, this may be explained by the complex additive or synergistic interactions of the individual glucosinolates in the fractions. Little is known about the influence of combinations of leaf surface glucosinolates on *D. floralis*, but it is likely that interactions modifying behavior will occur (Simmonds et al., 1994). The aliphatic+ fraction had approximately 60% of the activity of the crude methanol leaf surface extract from which it had been derived. Although the components of the desulfated aliphatic+ fraction currently remain unidentified, characteristics have been defined by the extraction and fractionation procedures. The compounds remaining are all polar, stable, not volatile, and not degradable with the enzyme sulphatase.

Studies have shown that for the four *Brassica* genotypes tested the ranking of oviposition by *D. floralis* on real *Brassica* spp. and on the methanol extracts matches that for *D. radicum* with the same plants (Hopkins, 1994) or extracts (Baur et al., 1996). In both fly species, a leaf surface fraction that contained the compound CIF, but did not contain glucosinolates, brought about similar patterns of oviposition preference to those found here for the methanol extract (Baur et al., 1996). CIF, an as yet unidentified nonglucosinolate molecule, strongly stimulates *D. radicum* to oviposit (Roessingh et al., 1992a, b). Further work is in progress to elucidate if CIF is responsible for the oviposition stim-

ulation in the desulfated derivative of the aliphatic+ fraction. Figure 6 clearly demonstrates that this fraction strongly stimulates neurons in C5 sensilla, a response that is consistent with the presence of CIF. Both the oviposition bioassay and electrophysiological results of this study indicate a secondary role for glucosinolates, as oviposition stimuli on the leaf surface of *Brassica* spp.

Many correlative studies associate glucosinolates with insect damage or host selection (reviewed by Städler, 1992), and it is accepted that such data are suggestive but not definitive (Louda and Mole, 1991). More direct evidence for the involvement of glucosinolates in the host selection of crucifer-feeding insects stems from testing of compounds or fractionated extracts on surrogate plants or using electrophysiological techniques. However, the analysis of the most active part of a series of fractions does not necessarily lead to the most stimulatory molecule nor to the molecule that has the most influence on the host selection procedure. The most active fraction may be so as a result of the combination of molecules it contains, with the overall activity mediated by synergistic and antagonistic interactions. The presence of unidentified active molecules in highly stimulatory fractions of leaf extracts, as was found here in the aliphatic+ fraction, does question the overall role of glucosinolates as the principal mediators of root fly behavior on *Brassica* spp. *D. floralis* behavior is known to be influenced by glucosinolates (Simmonds et al., 1994), yet the role of glucosinolates compared to nonglucosinolate molecules is not fully appreciated. More work is clearly needed to identify the structure and distribution of nonglucosinolate molecules on the leaf surface of *Brassica* spp. and to search for other as yet undetected molecules that may be influencing the behavioral patterns of *Brassica*-feeding root flies.

This study shows that other compound(s) are more important as mediators of *D. floralis* oviposition than glucosinolates. However, the leaf surface has been shown to contain a range of compounds at varying levels, and their effect is unlikely to be merely additive. The fractionation of leaf surface extracts and the testing of single pure compounds approach the problems of host selection chemistry from opposite directions. Currently these two approaches remain some distance apart, but both indicate that host selection is a function of combinations of compounds rather than of single compounds.

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BEHAVIORAL AND CHEMICAL DEFENSES OF MARINE PROSOBRANCH GASTROPOD *Calliostoma canaliculatum* IN RESPONSE TO SYMPATRIC SEASTARS

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Abstract—The gastropod *Calliostoma canaliculatum* displays a series of aggressive escape behaviors upon contact with tube feet of the predatory seastars *Pycnopodia helianthoides* and *Pisaster giganteus*. Escape behaviors are predator specific. *Calliostoma canaliculatum* moves away from contact with *P. giganteus* more frequently than *P. helianthoides*, clamping down with the foot or retracting the head and foot into the shell when exposed to *P. helianthoides*. If escape from the grasp of either seastar fails, *C. canaliculatum* releases a yellow-colored exudate from the hypobranchial gland and subsequently retracts both the head and foot fully into the shell. This exudate contains noxious compound(s) as evidenced by retraction of tube feet and arms away from the exudate in both seastars. Tube-foot retraction responses to dilutions of the exudate indicates that both species of seastars are able to detect the exudate at a concentration of 3.2×10^{-1} mg exudate/ml seawater. *Pisaster giganteus* is more responsive to the exudate than *Pycnopodia helianthoides*, moving away from the source as well as retracting the tube feet and arm. Snails spread the exudate over their shells with their foot, perhaps to ensure defense from predators for some time period after exudate release. The exudate was collected and extracted in chloroform–ethyl acetate (1 : 1), then fractionated using flash chromatography. The most bioactive fraction, as evidenced by tube-foot retraction, was soluble in ethyl acetate and appeared to contain two major compounds.

Key Words—Gastropod, predation, chemical secretion.

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INTRODUCTION

Many studies describe the escape responses of marine gastropods to live predators (Gore, 1966; Dayton et al., 1977; Phillips, 1977; Hoffman et al., 1978; Harvey et al., 1987; Miller, 1986) and predator odors (Mackie et al., 1968; Mackie, 1970; Harvey et al., 1987; Duval et al., 1994). Most marine gastropods display similar suites of avoidance and escape behaviors. Some species possess the ability to detect predator odors at a distance (Dayton et al., 1977; Harvey et al., 1987). These gastropods often alter their direction of locomotion and begin to move quickly away from the odor source (Feder, 1963, 1967; Mackie, 1970; Phillips, 1977). Escape behaviors are displayed upon contact with a potential predator. Many gastropods display a "mushrooming" behavior and/or violently rotate their shells back and forth (Weldon and Hoffman, 1979; McClintock, 1985). *Calliostoma ligatum* has been observed to turn and "bite" seastar predators before fleeing (Harrold, 1982).

Another adaptation common in sessile or sluggish marine invertebrates to reduce predation is the use of chemical defense mechanisms (reviewed by Bakus et al., 1986; Paul, 1992; Pawlik, 1993). Secondary metabolites that make tissues unpalatable to predatory fish and invertebrates have been identified in marine sponges (Walker et al., 1985; McClintock, 1987; McClintock et al., 1994), ascidians (Paul et al., 1990; Lindquist and Fenical, 1991), soft corals (Wylie and Paul, 1989; Fenical and Pawlik, 1991; VanAlstyne and Paul, 1992; Slattery and McClintock, 1995), and opisthobranch and prosobranch gastropods (reviewed by Faulkner, 1992). The majority of these marine invertebrates rely on sequestering defensive chemicals in their tissues to deter potential predators (Pennings, 1994). Chemical antipredator defenses among the Gastropoda have focused on the opisthobranchs (Faulkner, 1992). As many opisthobranchs lack a protective shell, they are particularly vulnerable to predation and must rely on defensive chemistry and crypsis for their protection.

Prosobranch gastropods possess a calcified shell, which is assumed to be an effective barrier against predation (Norton, 1988). Nonetheless, many prosobranch gastropods are significant prey for fish (Norton, 1988), other gastropods (Menge, 1974; Ansell and Morton, 1987), and echinoderms (Harrold, 1982; Paine, 1969, 1974). Avoidance behaviors of prosobranch gastropods to predatory seastars have been investigated (Feder, 1963, 1967; Philips, 1976, 1977). The ability of gastropods to flee from seastars or withdraw into the shell are believed to be the predominant methods of defense against predation (Feder, 1963, 1967).

When foraging on the benthos, juvenile and adult *Calliostoma canaliculatum* are vulnerable to predation by the large, highly mobile, seastar *Pycnopodia helianthoides*. While gastropods make up approximately 80% of the diet of *P. helianthoides*, *C. canaliculatum* represents only 1.5% of this total (Herr-

linger, 1980). *Calliostoma canaliculatum* has been observed to release a brightly colored yellow exudate from its mantle following contact with *P. helianthoides* (Watanabe, personal communication), suggesting that this released exudate could be a defensive response to predation. The objectives of the present study were to: (1) determine if *C. canaliculatum* can detect waterborne odors from predatory seastars, (2) describe the behavioral escape response of *C. canaliculatum* to the predatory seastars *P. helianthoides* and *P. giganteus*, (3) determine if *C. canaliculatum* differentiates between predatory seastars, (4) record the reaction of predatory seastars to the exudate released by *C. canaliculatum*, and (5) chemically fractionate the exudate utilizing flash chromatography to isolate the active compound(s).

METHODS AND MATERIALS

Collections. Gastropods and seastars were collected in August 1995 at 10 m depth from *Macrocystis pyrifera* beds in Stillwater Cove, Monterey, California. Gastropods and seastars were placed in ambient seawater and shipped separately to the University of Alabama at Birmingham. Immediately upon arrival they were placed in separate holding tanks equipped with recirculating filtration and containing unfiltered artificial seawater (35 ppt) held at 10–12°C. Blades of *M. pyrifera* were placed in tanks with gastropods as a food source. The seastars *Pycnopodia helianthoides* and *Pisaster giganteus* were fed the gastropod *Tegula funebralis*.

Gastropod Responses to Seastar Odor. Individual gastropods were placed in one of 60 sterile plastic containers (10 cm long × 10 cm wide × 30 cm tall) with 200 ml of artificial 0.45-μm-filtered seawater (ASW). Fresh ASW was used for preparation of all bath water and in all assays. Water used in an assay was not used again in any part of the study. After allowing individuals to adjust to the container for a period of 1 min, 200 ml of ASW (control) or 200 ml of seastar bath water was added to the containers such that 30 control and 30 experimental treatments were conducted for each species of seastar odor. Bath water was prepared by submerging whole seastars of each species in ASW at a ratio of 200 g of seastar per liter of seawater for a period of three hours. After a period of 2 min, the height each gastropod had climbed up the side of the container was measured to the nearest mm. Bath water of both species of seastar was prepared and tested in the same fashion.

Contact with Seastar Tube Feet. Five *Pisaster giganteus* and five *Pycnopodia helianthoides* were maintained in separate seawater aquaria. Tube feet from randomly selected individuals were removed with tweezers from the aboral surface of the arm and immediately employed in an avoidance trial. Thirty *Calliostoma canaliculatum* were held in individual glass crystallizing dishes (15

cm diameter × 7.5 cm deep) containing 200 ml of ASW. Gastropods were allowed to adjust to the dishes for a period of at least 15 min prior to each avoidance trial. Each trial consisted of placing a tube foot held in a pair of forceps in contact with the mantle tissue of a gastropod. Responses were classified into a hierarchy where level 0 = no reaction; level 1 = movement away from the tube foot; level 2 = movement away and shell twisting; level 3 = movement, twisting and exudation of exudate; level 4 = movement and exudate release; level 5 = withdrawal into the shell and clamping down; level 6 = withdrawal and exudate release. Contact trials were performed on 30 individual snails, with each snail exposed to both tube feet from *P. helianthoides* and *P. giganteus*. Controls consisted of contacting gastropods with the forceps alone and with a cotton swab alone. A Wilcoxon signed-rank test was performed to compare levels of responses between seastars. The numbers of gastropods releasing exudates in response to each seastar species were compared with a Student's *t* test ($\alpha = 0.05$).

Seastar Responses to Gastropod Exudate. Gastropods were induced to release exudate by contacting them with seastar tube feet. The exudate was collected in a Pasteur pipet as it was expelled from the hypobranchial gland. The seawater/exudate mixture was transferred to a 20-ml glass vial and held at 2°C. The seawater/exudate mixture was then desalted by passing it through an Amberlite XAD-2 column (Quinn et al., 1980). Collected material was dried and then weighed to approximate the concentration of exudate in seawater as it is expelled (the exudate becomes increasingly dilute as it diffuses away from the gastropod; baseline concentrations were determined to calculate appropriate concentrations for bioassay). The exudate was then resolubilized in ASW to the original concentration and tested in a behavioral assay at concentrations of 3.2, 0.32, 0.032, 0.0032, and 0.00032 mg/ml seawater. Behavioral assays consisted of placing individual seastars, either *Pycnopodia helianthoides* or *Pisaster giganteus* ($N = 5$ of each) in plastic bowls (30.5 cm diameter × 15.2 cm deep) containing 2 liters/ASW. Each seastar was tested five times, with a different arm tip being utilized in each trial. Seventy-five microliters of seawater/exudate mixture was released approximately 1 cm from the tip of the arm. The level of response was separated into one of four hierarchical categories: level 0 = no response; level 1 = tube-foot withdrawal (TFW); level 2 = TFW and closing of the ambulacral groove; level 3 = TFW, tightening, and coiling of the arm; and level 4 = TFW, tightening, coiling, and movement away from the exudate. In addition, overall time of sustained tube-foot withdrawal and arm coiling in response to the exudate or a seawater control was recorded to the nearest second. Levels of responses to exudate and seawater controls were compared using a Wilcoxon signed rank test. Times of sustained tube-foot withdrawal and arm coiling in response to exudates or seawater controls were analyzed with a Student's *t* test ($\alpha = 0.05$).

Fractionation of Exudate. Exudate was collected from gastropods by inducing the release through contact with a tube foot of either *Pisaster giganteus* or *Pycnopodia helianthoides*. Forty milliliters of seawater/exudate was partitioned twice in a separatory funnel with a 3× volume of chloroform; then the mixture was partitioned twice with ethyl acetate (3× volume). The ethyl acetate and chloroform extracts were combined to yield a lipophilic exudate extract and dried under rotary evaporation at 35°C. The dried extract (95.6 mg) was resolubilized in ethyl acetate and loaded onto a flash column of silica 60 A, 300–400 mesh. Sequential solvent systems ranging from very nonpolar to polar (100% hexane; 8:2 Hex–ethyl acetate; 4:6 Hex–EtOAc; 2:8 Hex–EtOAc; 9:1 EtOAc–methanol; 6:4 EtOAc–MeOH; 3:7 EtOAc–MeOH; 100% MeOH) were employed and each fraction collected. The collected fractions were spotted onto silica TLC plates (Whatman Silica Gel 60 A; 250 μm thickness) and run with various solvent systems to optimize separation. Plates were visualized by spraying the plate surface with 50% sulfuric acid and heating. Fractions that contained compounds/mixtures with similar R_f values were combined and the resultant five fractions were reassayed using the same techniques as described for the original exudate. Four concentrations (3.2, 0.32, 0.032, and 0.0032 mg/ml seawater) were tested for each fraction.

RESULTS

The climbing response of gastropods exposed to the waterborne odors of *Pisaster giganteus* and *Pycnopodia helianthoides* did not differ significantly from the seawater controls ($P = 0.356$, Wilcoxon). Forty-nine percent of the gastropods ($N = 30$) exposed to the control filtered seawater climbed the sides of containers a mean distance of 9.2 cm. Fifty-four and 40% of the gastropods climbed the containers when exposed to the waterborne odors of *P. helianthoides* and *P. giganteus*, respectively. The mean distance climbed by gastropods exposed to waterborne odors of *P. helianthoides* and *P. giganteus* were 8.5 and 9.1 cm, respectively.

Behavioral responses of *C. canaliculatum* to contact with the tube feet of the two species of seastars were significantly different ($P < 0.001$, Wilcoxon). Gastropod responses to contact with the tube feet of *P. giganteus* were primarily level 3 and 4 responses. A very low percentage of snails (7%) displayed a level 6 response to *P. giganteus*, whereas 50% displayed a level 6 response to *P. helianthoides* (Figure 1). Additionally, 90% of snails contacted by *P. helianthoides* tube feet released exudate, whereas 75% released exudate in response to *P. giganteus* tube feet.

Exudate tested at a concentration of 3.2 mg/ml seawater caused a mean

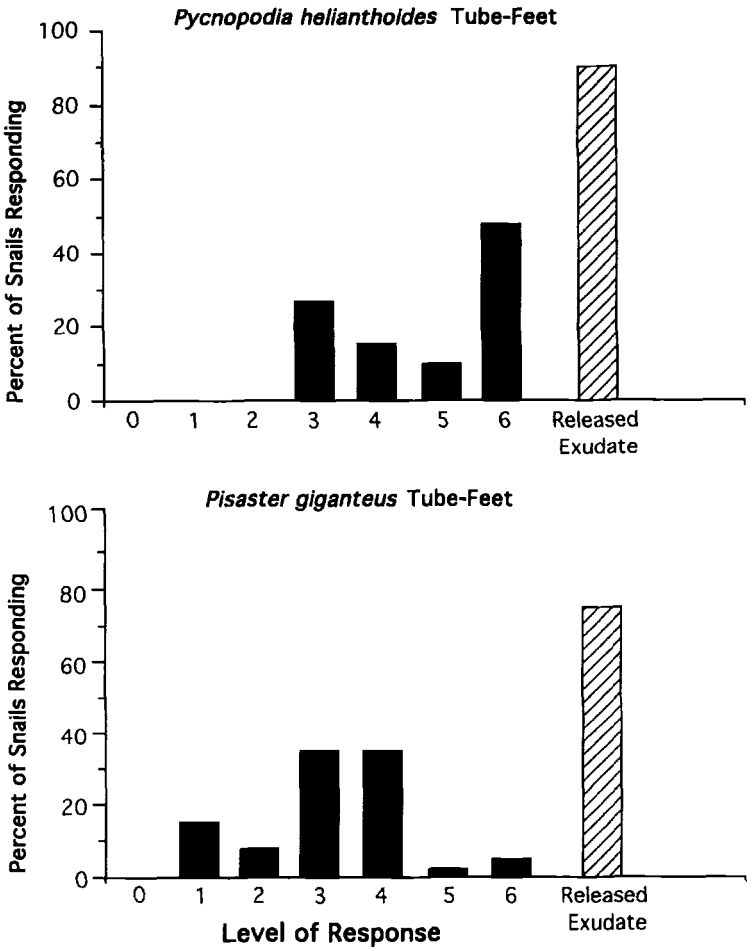

FIG. 1. *Calliostoma canaliculatum* response to seastar tube-foot contact. Levels of response are indicated by numbered ranks: 0 = no reaction, 1 = movement away from tubefoot; 2 = movement away and shell twisting; 3 = movement away, shell twisting, and exudation; 4 = movement away and exudation; 5 = withdrawal into shell; 6 = withdrawal and exudation. No response was observed for contact with forceps or cotton swabs (mechanical controls). Bars represent responses of 40 individual snails assayed.

tube-foot retraction time of 51.7 and 101.2 sec for *P. helianthoides* and *P. giganteus*, respectively (Figure 2). Arm withdrawal times were longer than tube-foot retraction, averaging 76.2 and 122.1 sec for *P. helianthoides* and *P. giganteus*, respectively, at 3.2 mg exudate/ml seawater. Both species of seastar were insensitive to exudate at a concentration of 3.2×10^{-4} mg/ml seawater. The

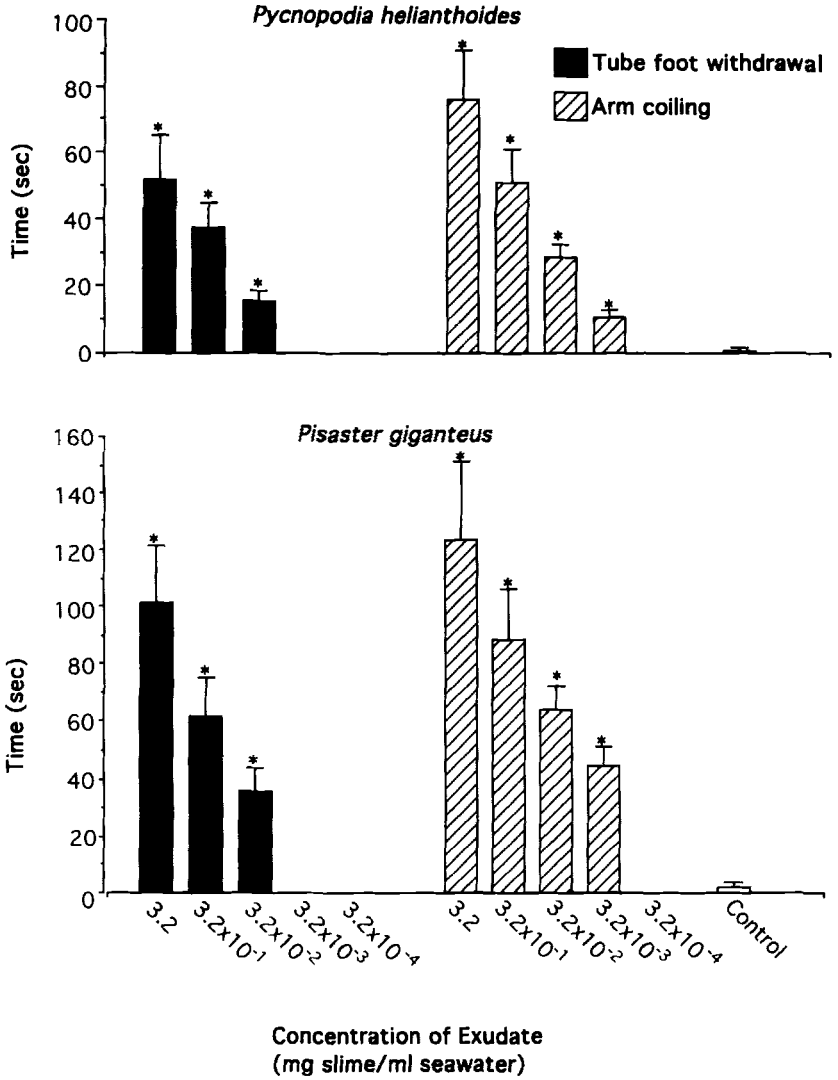

FIG. 2. Tube-foot and arm withdrawal responses of *Pycnopodia helianthoides* and *Pisaster giganteus* to exudate of *Calliostoma canaliculatum*. The arm withdrawal response of both species of seastar lasted longer and was initiated at lower concentrations than the tube-foot withdrawal response. Asterisks indicate a significant ($P < 0.05$, Student's t test) difference in the mean retraction time in comparison to the control, for each concentration. Data plotted are mean $\pm$ standard deviation of 30 replicates.

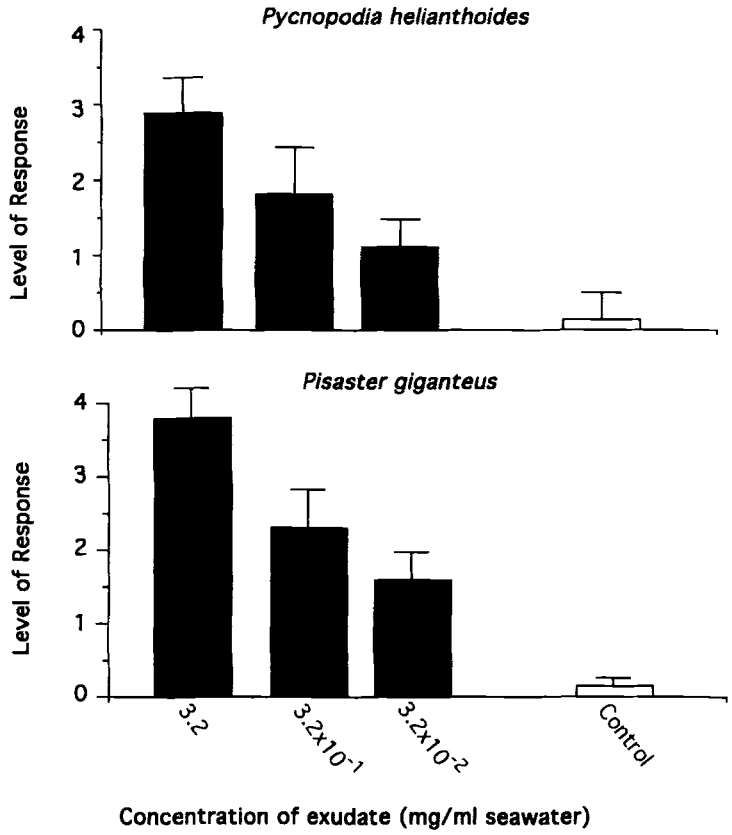

FIG. 3. The level of response to *Calliostoma canaliculatum* exudate for *Pycnopodia helianthoides* and *Pisaster giganteus*. The level of response (*y* axis) were scored by numerical ranks: 0 = no response; 1 = tube foot (TF) withdrawal and tightening of arm tip around ambulacral groove; 2 = TF withdrawal and tightening of arm tip around ambulacral groove; 3 = TF withdrawal, tightening, and arm coiling; 4 = TF withdrawal, tightening, arm coil, and movement away from source of mucous. Data plotted are mean ± standard deviation of 30 replicates.

mean level of response of *P. giganteus* (3.8 of 4) at 3.2 mg/ml seawater was higher than that of *P. helianthoides* (2.8 of 4) (Figure 3). The level 4 response is the most extreme, indicating tube-foot retraction, arm withdrawal, tightening of the ambulacral groove, and directional movement away from the exudate. The mean response of both species of seastar to the control (seawater) was a level of 0.2 response, indicating slight tube-foot retraction.

The fractionation of the exudate and reassaying of the fractions indicated that the active component(s) were present in fraction 3 (Figure 4). These com-

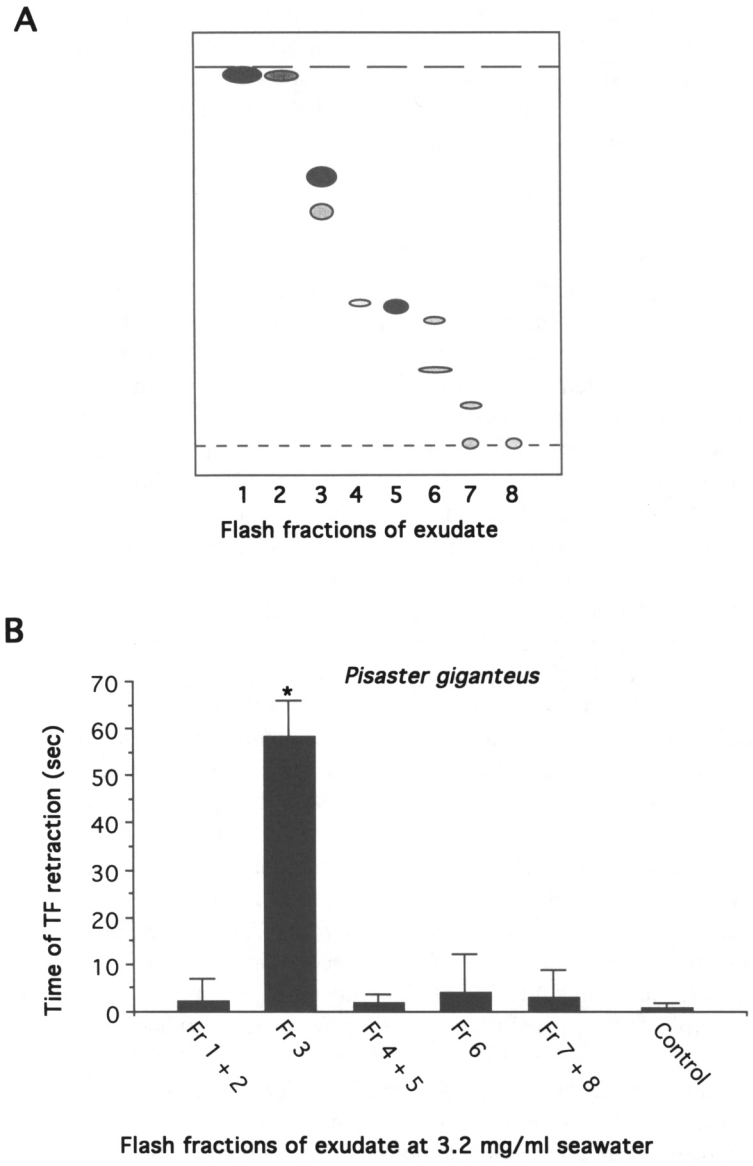

FIG. 4. A: Thin layer chromatography visualization of flash chromatography separated fractions of *Calliostoma canaliculatum* exudate. The solvent system was 3:2 ethylacetate–hexane. B: Response of the seastar *Pisaster giganteus* to fractions of *C. canaliculatum* exudate. Asterisks indicate a significant ($P < 0.05$, Student's t test) difference in the mean retraction time in comparison to the control. Data plotted are mean ± standard deviation of 10 replicates.

pounds were eluted with a 40% hexane–60% ethyl acetate solvent system. The higher eluting of the two spots in fraction 3 appeared yellow in color. Further purification and reassaying of compounds from the active fraction have revealed that two compounds (A and B) cause mean tube-foot retraction times of 31 and 28 sec, respectively, in *P. giganteus*.

DISCUSSION

The lack of escape behaviors in *Calliostoma canaliculatum* in response to odors from predatory seastars indicates escape is initiated only upon contact with the tube feet. Escape behavior typically is initiated by movement away from the area contacted by the tube feet, followed almost immediately by shell twisting. Occasionally gastropods will also retract into the shell. The release of the yellow exudate appears to be a final tactic to escape from the grasp of a predatory seastar. *C. canaliculatum* may release exudate while fleeing or while retracting their head and foot into their shells. The initial escape behaviors exhibited by *C. canaliculatum* are classic gastropod escape responses described in many previous studies (Feder, 1963; Phillips, 1977). However, the release of a defensive substance from the hypobranchial gland is rare among mollusks. The release of ink as a camouflaging defense and potential chemical irritant is known in sea hares (DiMatteo, 1981, 1982; Nolen et al., 1995) and cephalopods (Barnes, 1987). Ink release differs somewhat from the behavior observed in *C. canaliculatum*, since the exudate from *C. canaliculatum* is strictly chemodefensive in nature. *Aplysia californica* ink acts primarily to visually confuse potential predators (DiMatteo, 1982; Nolen et al., 1995) but may also be distasteful (DiMatteo, 1981). In squid, release of ink forms a dummy squid to divert a predator's attention. However, alkaloids in the ink may also be repellent to predators and/or anesthetize their chemoreceptors (Barnes, 1987). Several gastropods including *Cirostrema* sp., *Nucella lapillus*, *Ocenebra erinacea*, *Clathrus* sp., and *Ianthina janthia* release a purple secretion from their hypobranchial gland (Fretter and Graham, 1962). This purple dye contains dibromoindigo, which is produced once colorless chromogens in the exudate are exposed to oxygen and light (Fretter and Graham, 1962). The release of dye in these gastropods could serve a defensive function; however, the ecological significance of these compounds has yet to be investigated.

Calliostoma canaliculatum attempted to flee following contact with *Pisaster giganteus* more frequently than in response to contact with *Pycnopodia helianthoides*. In contrast, gastropods retracted into their shells more often when contacted with tube feet of *P. helianthoides* than *P. giganteus*, and higher percentages of gastropods released exudate when provoked by *P. helianthoides*. These striking differences in the responses of *C. canaliculatum* to contact with

tube feet of these two seastars indicates that individuals may be able to employ contact chemoreception to detect differences in seastar metabolites (Phillips, 1978). Investigations of the secondary metabolite chemistry of *P. helianthoides* and *P. giganteus* indicate both species produce a unique variety of water-soluble saponins and polyhydroxy steroids (Bruno et al., 1989; Zollo et al., 1990). Several studies have determined that gastropods can differentiate between odors of different predatory seastars (Phillips, 1976), crab predators (Alexander and Covich, 1991; Duval et al., 1994), and detect the dietary condition of a predator (Feder, 1963; Duval et al., 1994). A similar type of chemical recognition could be involved in *C. canaliculatum*. One possible explanation for the different responses shown by *C. canaliculatum* to contact with both seastars is related to the relative speeds at which *P. helianthoides* and *P. giganteus* move. *Pycnopodia helianthoides* moves at a much faster rate than *P. giganteus* (P. Bryan, personal observation). It may be possible for *C. canaliculatum* to flee effectively from the sluggish *P. giganteus*, whereas individuals would be easily captured by the more rapid *P. helianthoides*.

Both species of seastars showed avoidance behaviors to the yellow exudate released by *C. canaliculatum*. The exudate was active at retracting seastar tube feet at a concentration as low as 3.2×10^{-2} mg/ml seawater. It is likely that the exudate itself or active compounds in the exudate entail some energetic cost for the snail to produce. It would not be advantageous to release energetically valuable defensive material without just cause. The cost of chemical defense has been extensively studied in terrestrial plants (Rhoades and Cates, 1976; Coley et al., 1985). Recent studies suggest that similar investments and trade-offs are made in marine invertebrates as in plants (Hay and Fenical, 1988).

Based on analysis by thin-layer chromatography, there are at least two major compounds present in the bioactive fraction of the exudate. These non-polar compounds are soluble in ethyl acetate and relatively insoluble in seawater. The use of water-insoluble exudate to contain these compounds seems to be an effective adaptation to ensure retention of compound efficacy as long as possible. Moreover, *C. canaliculatum* was observed to spread the exudate over its shell with its foot. This behavior would place the defensive compounds on the surface of the shell where they would be detected upon contact by a predatory seastar. Water-insoluble compounds are also advantageous in that they would diffuse slowly into the seawater, ensuring that the potency of the exudate will remain intact for some period. Future analyses will attempt to reveal the chemical structure of these compounds. Estimates of energetic cost can then be determined once metabolic pathways have been identified.

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TASTE PREFERENCES FOR FIVE FOOD-ASSOCIATED SUGARS IN THE SQUIRREL MONKEY *(Saimiri sciureus)*

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Abstract—The taste preferences of six adult squirrel monkeys for sucrose, fructose, glucose, lactose, and maltose were assessed in two-solution choice tests of brief duration (5 min). In experiment 1 the monkeys were given the choice between all binary combinations of the saccharides presented in equimolar concentrations of 50, 100, 200, and 400 mM, respectively. Preferences for individual sugars were stable across the concentrations tested and indicate an order of relative effectiveness (sucrose > fructose > glucose ≥ maltose ≥ lactose), which is identical to the order of sensitivity found in this species and similar to findings on relative sweetness in man. In experiment 2 the squirrel monkeys were given the choice between a standard sucrose solution of 50 mM and ascending series of concentrations of the other saccharides in order to determine concentrations that were consumed in equal amounts compared to the standard. Solutions of 100 mM fructose, 300 mM glucose, and 200 mM lactose and maltose were found to be equally effective to the 50 mM sucrose, whereas other concentrations led to marked preferences for one of the alternatives. The finding of concentrations of equal effectiveness are consistent with findings on concentration-dependent loss of discrimination ability in man and support the hypothesis that for squirrel monkeys sucrose may indeed be indiscriminable from fructose, glucose, lactose and maltose when concentrations are suitably adjusted. The results suggest that squirrel monkeys and man share important features of sweet-taste perception.

Key Words—Taste preferences, sugars, squirrel monkey, *Saimiri sciureus*, relative sweetness.

INTRODUCTION

Species differences in both taste sensitivity and taste preferences are well established (Beidler et al., 1955; Carpenter, 1956; Feigin et al. 1987), and there is

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general agreement that these differences in gustatory performance and behavior are likely to reflect evolutionary adaptations to dietary specialization and physiological needs of a species (Kare, 1971; Kare and Brand, 1986; Scott, 1990; Simmen and Hladik, 1993). This is most obvious for substances that taste sweet to humans. Frugivorous mammalian species that rely on soluble carbohydrates as a primary source of metabolic energy (Portman, 1970) usually display marked preferences for sugars (Pfaffman, 1977), whereas carnivorous species such as the cat show little or no preference for sweet-tasting substances (Beauchamp et al., 1977). However, species have also been shown to differ in their preferences for individual sugars. While rats or gerbils, for example, prefer both sucrose and maltose over water, other species such as the dog or the armadillo only prefer the former but are indifferent or even averse to the latter (Ferrell, 1984; Harriman, 1970; Maller and Kare, 1967; Sclafani and Mann, 1987). One possible explanation for such differences in taste preferences for individual sugars is that the substances may have different taste qualities which, for whatever reason, evoke different behavioral responses. Alternatively, the substances may have the same taste quality, but may differ in their stimulating efficiency and concomitantly in attractiveness.

In humans, the existence of both phenomena has been documented. Several studies using multidimensional scaling or taste profiling procedures (Kuznicki and Ashbaugh, 1979; Schiffman et al., 1979) have shown qualitative differences between some of the numerous substances that taste predominantly sweet to man. However, there is considerable debate as to whether common sugars actually differ in taste quality. Recent psychophysical studies strongly suggest that sucrose, fructose, glucose, and maltose are indeed indiscriminable for humans when their relative concentrations are suitably adjusted (Breslin et al., 1994, 1996). The molecular explanation for this monogeusia is that these saccharides probably bind reversibly to a single class of membrane receptor in taste cells. Other studies using either magnitude estimation (Moskowitz, 1971) or paired comparison methods (Cameron, 1947; Pangborn, 1963; Pfaffman and Bartoshuk, 1971) have shown that sweet-tasting substances vary widely in their stimulating efficiency, and when presented with equimolar solutions of different sugars, human subjects assign the following order of relative sweetness to common sugars: sucrose > fructose > maltose > glucose > lactose.

In nonhuman species it is difficult to decide whether differences in taste quality or stimulating efficiency are responsible for specific preferences for a particular sweetener. However, conditioned taste avoidance procedures (Ramirez, 1994) and electrophysiological studies (Frank, 1977) suggest that both mechanisms may also be functional in animals. Nonhuman primates have repeatedly been shown to be a useful animal model of human taste perception. Whereas detection or preference thresholds for sweeteners have been determined in a variety of monkey species (for a review see Glaser, 1986) comparatively

few studies have so far investigated sweet-taste preferences (Glaser, 1979; Maller, 1973; Pfaffman, 1969; Sunderland and Sclafani, 1988). Almost all of these studies have measured sugar preferences indirectly, i.e., using either single-bottle tests or sugar solution vs. water test, and inferred preferences for individual sugars by comparing relative amounts of consumption. Furthermore, most studies on sweet-taste preferences in nonhuman primates have been based on long-term exposure (1–24 hr) to sugar solutions, and thus results from these tests are likely to be influenced by postingestive factors.

The squirrel monkey (*Saimiri sciureus*) has been the subject of considerable research on taste perception (Clark and Harriman, 1968; Dua-Sharma and Smutz, 1977; Ganchrow and Fisher, 1968; Laska, 1994; Pfaffman, 1969, 1970, 1974, 1977; Pfaffman et al., 1976; Snell, 1965; Sunderland and Sclafani, 1988; Wagner et al., 1965) and taste thresholds for common sugars have been established by the author (Laska, 1996). In order to determine the squirrel monkey's preference for different sugars, it was the purpose of the present study to systematically compare the intake of sucrose, fructose, glucose, maltose, and lactose—all important constituents of the natural diet in *Saimiri sciureus*—using two-solution choice tests of brief duration (5 min). This methodology makes it possible to directly measure preferences and largely rules out the influence of postingestive factors on the animal's ingestive behavior.

The assessment of such preferences is considered interesting in three respects: First, as all primate species investigated so far prefer the sweeter of two alternative sapid solutions so long as physiological concentrations (<0.5 M) are used (Pfaffman, 1977), the data obtained can be regarded as a first approximation of the relative sweetness of the saccharides under investigation. Second, comparing the ranking of sugars according to their relative effectiveness in the squirrel monkey to those of other species may help clarify whether this measure represents a general pattern in the order of primates or rather reflects an evolutionary adaptation to the dietary specialization of this frugivorous New World species. Third, by assessing whether concentrations of equal effectiveness exist among the sugars tested, the data obtained can give at least an indication of similarities or differences in sweet-taste quality perception between humans and nonhuman primates.

METHODS AND MATERIALS

Animals. Testing was carried out using three male and three female adult squirrel monkeys (*Saimiri sciureus*) weighing 0.8–1.2 kg and ranging from 4 to 8 years of age. This social group was housed in a 10-m^3 enclosure, which could be subdivided by sliding doors to allow temporary separation of animals for individual testing, and was maintained on a 12 : 12-hr light–dark cycle at 20–

24°C. Animals were fed marmoset pellets (Ssniff) and fresh fruit and vegetables ad libitum, but were deprived of water overnight before testing on the following morning.

Procedure. Taste preferences for sucrose, fructose, glucose, lactose, and maltose (reagent grade, Merck) were determined using a two-bottle solution vs. solution choice test. Twice each day, approximately 3 and 1 hr before feeding, the animals were separated and allowed 5 min to drink from a pair of simultaneously presented graduated 100-ml cylinders with metal drinking tubes. All animals had served in previous studies using the same methodology (Laska, 1994, 1996) and were completely accustomed to the procedure. To establish preference values, the monkeys were given the choice between defined concentrations of sugar solutions prepared with tap water. Each pair of stimuli was presented 10 times, and the position of the stimuli was randomized in order to counterbalance possible position preferences.

Statistical Analysis. For each animal, the amount of liquid consumed from each bottle was recorded, summed for the 10 test trials with a given stimulus combination, and 66.7% (i.e., two thirds of the total amount of liquid consumed) was taken as criterion of preference. This rather conservative criterion was chosen for reasons of comparability of data, as the same criterion has been used in previous studies using the same methodology and individuals (Laska, 1994, 1996); and in order to avoid misinterpretation of data due to a too liberal criterion. With the data obtained in the present study, for example, paired-sampled *t* tests would have allowed a preference of 56% to be regarded as statistically significant. However, it is questionable whether this really reflects a biologically significant, i.e., taste-based preference, particularly as water vs. water tests performed with the test animals and summed over 10 test trials sometimes also yield preferences of up to 56% for a given bottle.

Preliminary analysis of the data indicated that there were no reliable differences in preferences between the male and female subjects and between the first and the second presentation of the day. Intraindividual variability of the amount of liquid consumed across the 10 test trials with a given stimulus combination was low and averaged less than 20%. Thus a theoretically possible bias in the overall preference score due to excessive drinking in aberrant trials did not occur. Therefore, the data for the males and females obtained in the 10 test trials were combined and reported as group means and standard deviations.

Comparisons across tasks were made using the Friedman two-way analysis of variance. When ANOVA detected differences between tasks, this was then followed by separate pairwise Wilcoxon signed-rank tests for related samples to evaluate which tasks were responsible.

Experiment 1. This experiment was designed to provide information about the squirrel monkey's preference for five food-associated sugars in short-term two-solution choice tests and thus to establish an order of relative effectiveness

for these saccharides. Under the assumption that the sugars under investigation do not differ in taste quality—a hypothesis that experiment 2 was designed to clarify—the results from this experiment can be regarded as a first approximation of the relative sweetness of these saccharides in the squirrel monkey.

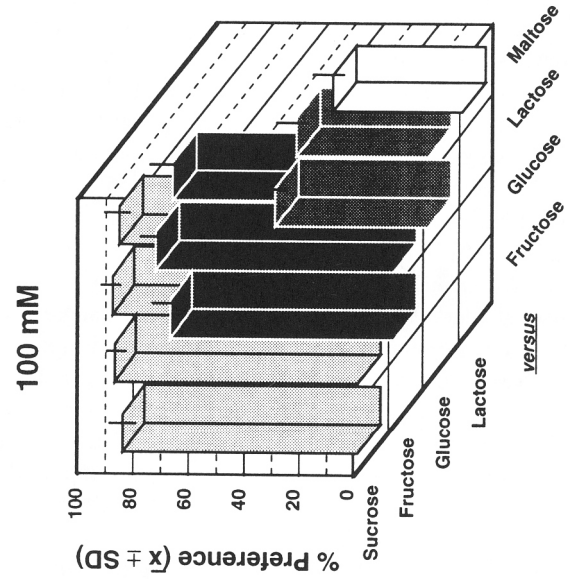
The six squirrel monkeys were given the choice between all binary combinations of sucrose, fructose, glucose, lactose, and maltose presented in equimolar concentrations. In order to investigate whether preferences are stable at different concentration levels, four test series were performed: at 50, 100, 200, and 400 mM, respectively.

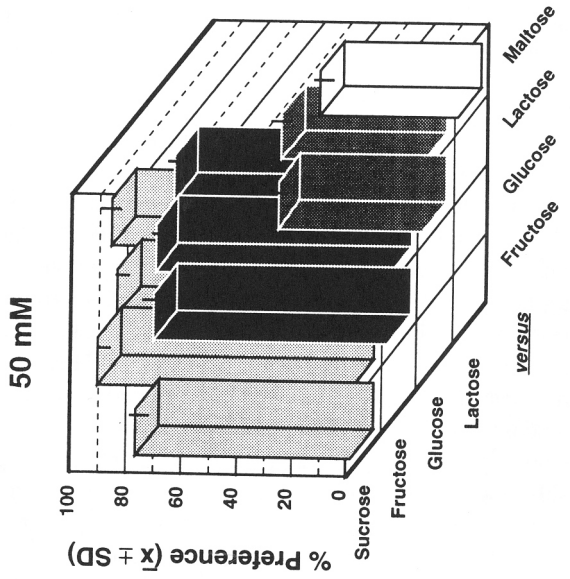
Experiment 2. The results from experiment 1 showed that squirrel monkeys display marked preferences for individual sugars when given a direct choice between two sapid alternatives. However, it is unclear whether these preferences are based on differences in taste quality or stimulating efficiency. Thus, experiment 2 was designed to provide information about equally effective concentrations of the sugars under investigation. If such concentrations at which neither alternative is preferred exist, this would support the hypothesis that the saccharides are indeed indiscriminable and results can be regarded as a first approximation of equal sweetness. If, on the other hand, such concentrations of equal effectiveness cannot be found, this would favor the hypothesis that the sugars tested may differ in taste quality.

The six squirrel monkeys were given the choice between a standard sucrose solution of 50 mM and an ascending series of concentrations (50, 100, 200, 400, and 800 mM, plus an intermediate concentration, if necessary) of fructose, glucose, lactose, and maltose in order to determine the concentrations which were equally effective, i.e. consumed at equal amounts.

RESULTS

Figure 1 shows the mean group preference ± SD for the six squirrel monkeys given a choice between two saccharides presented at equimolar concentrations of 50, 100, 200, and 400 mM, respectively. At all four concentrations tested the monkeys significantly preferred sucrose over all other sugars, and fructose over glucose, lactose, and maltose. Choice tests between the latter three sugars did not yield significant preferences. However, at the highest concentration tested (400 mM) the animals showed a nonsignificant tendency to prefer glucose over lactose and maltose, and at 200 and 400 mM there was a significant tendency to prefer maltose over lactose. Interindividual variability was remarkably low as can be inferred from the small SDs, and with only few exceptions all six animals either reached the criterion of preference in a given task or failed to do so. Preferences for a given stimulus combination were rather stable across the range of concentrations tested. Accordingly, ANOVA only detected signif-


100 mM
% Preference (x̄ ± SD)
100
80
60
40
20
0
Sucrose
Fructose
Glucose
Lactose
versus
Fructose
Glucose
Lactose
Maltose


50 mM
% Preference (x̄ ± SD)
100
80
60
40
20
0
Sucrose
Fructose
Glucose
Lactose
versus
Fructose
Glucose
Lactose
Maltose

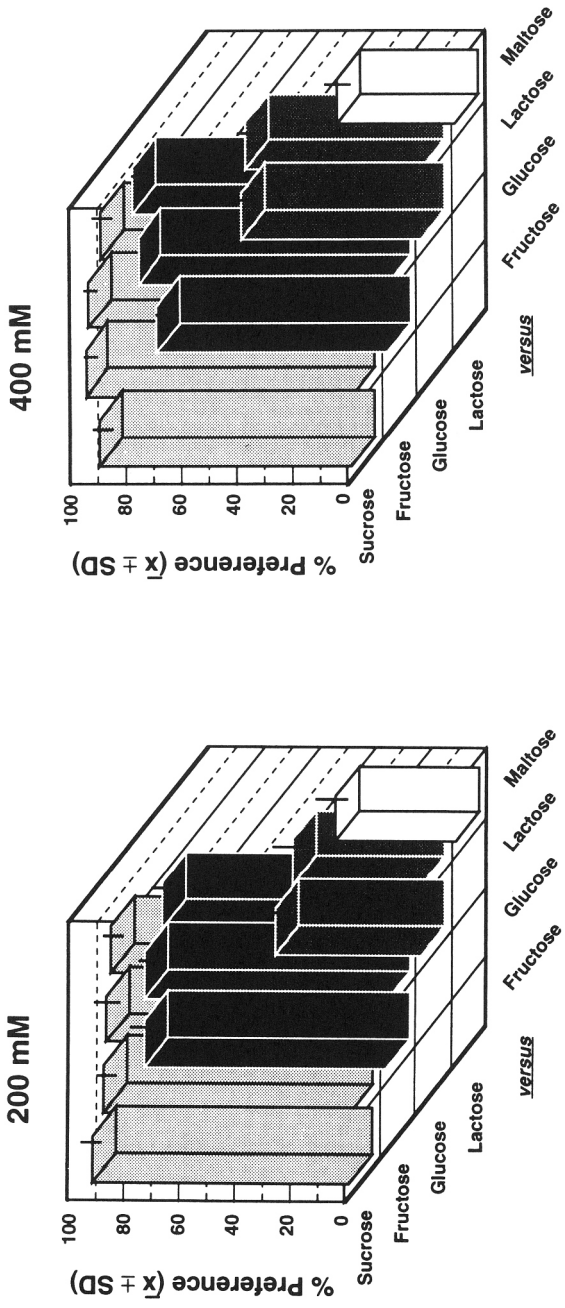


FIG. 1. Mean preference ± SD for six squirrel monkeys given a choice between two saccharides presented at equimolar concentrations of 50, 100, 200, and 400 mM, respectively.

icant changes in preferences across concentrations for glucose vs. maltose, glucose vs. lactose, and lactose vs. maltose.

Figure 2 summarizes the mean group preference ± SD for the six squirrel monkeys when given a choice between a 50 mM sucrose standard and variable concentrations of fructose, glucose, lactose, and maltose. With all four test sugars a concentration was found that was equally preferred to the 50 mM sucrose standard. Solutions of 100 mM fructose, 300 mM glucose, and 200 mM lactose and maltose, respectively, were consumed in equal amounts compared to the 50 mM sucrose. This was true for all six individuals with the exception of the 100 mM fructose at which one of six monkeys still preferred the sucrose. Lower concentrations of the test sugars yielded significant preferences for the sucrose standard whereas higher concentrations led to marked preferences for the test sugars. However, with the highest concentration of fructose tested (800 mM), the monkeys failed to show a significant preference for either alternative, and a similar tendency towards a U-shaped function of preference could be observed with maltose.

DISCUSSION

The results of experiment 1 indicate the following order of relative effectiveness in the squirrel monkey: sucrose > fructose > glucose ≥ maltose ≥ lactose. This is similar to findings in man on relative sweetness (Cameron, 1947; Pfaffman and Bartoshuk, 1971) and corresponds nicely to the order of sensitivity for these sugars established in a previous study using short-term solution vs. water tests: Three of the six animals used in the present study were found to significantly prefer solutions of 10 mM sucrose, 40 mM fructose, 90 mM glucose, and 100 mM maltose and lactose over tap water (Laska, 1996). As all preference tests in which both alternatives were presented at subthreshold concentrations (e.g., all combinations of glucose, lactose and maltose at 50 mM) yielded a lack of preferences, the results clearly suggest that fluid intake in these tests was very likely based on taste sensations only. Further, the results confirm the reliability of the threshold values.

The results of experiment 2 confirm the conclusion drawn from experiment 1 that sucrose has a higher stimulating efficiency for squirrel monkeys than fructose, glucose, maltose, and lactose and that fructose in turn is a behaviorally more potent stimulus than the remaining three saccharides. However, the concentrations needed to evoke equal consumption of test solution and sucrose standard suggest an order of relative effectiveness (sucrose > fructose > maltose = lactose > glucose) that differs slightly from the one found in experiment 1.

A similar discrepancy was found in human psychophysical studies using different methodologies. Paired comparison tests using equimolar solutions—a

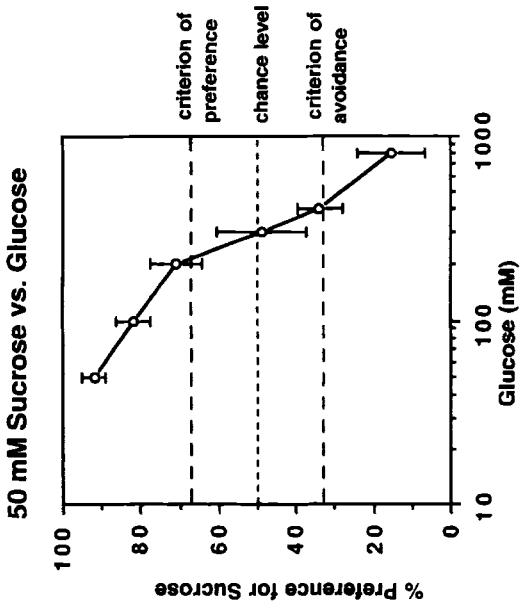
method similar to the one used in experiment 1—yielded the order of relative sweetness: sucrose > fructose > maltose > glucose > lactose (Cameron, 1947; Pfaffman and Bartoshuk, 1971), whereas magnitude estimations using ratings of saccharide sweetness against a glucose standard–a method similar to the one used in experiment 2–resulted in an order of relative sweetness of sucrose > fructose > lactose > glucose > maltose (Moskowitz, 1971). Thus, the difference between the results of experiments 1 and 2 more likely reflects effects of methodology rather than a contradiction. Furthermore, these differences were restricted to the least effective substances whereas the order of relative effectiveness of sucrose > fructose > other sugars emerged in both experiments.

The finding that concentrations of equal effectiveness were found in all cases suggests that, similar to humans, sucrose may indeed be indiscriminable from fructose, glucose, lactose, and maltose when concentrations are carefully matched. Thus, the results support the hypothesis of monogeusia for these saccharides in *Saimiri sciureus.*

The present study assessed the squirrel monkey's preference for five common sugars. Three findings emerged from the two-solution choice tests employed. First, *Saimiri sciureus* displayed an order of relative effectiveness (sucrose > fructose > glucose ≥ maltose ≥ lactose) that is identical to the order of sensitivity found in this species (Laska, 1996) and similar to findings in man tested for relative sweetness of these saccharides (Cameron, 1947; Pfaffman and Bartoshuk, 1971). Second, this pattern is stable for concentrations ranging from 50 to 400 mM. Third, concentrations of equal effectiveness were found in all cases tested, which again is in accordance with findings in humans tested for their ability to discriminate between these saccharides (Breslin et al., 1994, 1996).

The present findings confirm and extend the results obtained in previous studies on sweet-taste preception in the squirrel monkey using methods different than the one employed here. In two-bottle solution vs. water tests *Saimiri sciureus* preferred sucrose to a higher degree compared to fructose and glucose (Clark and Harriman, 1968; Pfaffman, 1969; Sunderland and Sclafani, 1988). With an operant conditioning procedure, the squirrel monkey showed the following sequence in the frequency of bar press responses in order to get a drop of sugar solution as a reward: sucrose > fructose > glucose = lactose = maltose (Ganchow and Fisher, 1968; Pfaffman, 1970). In the only other study so far that employed two-solution choice tests, one single squirrel moneky was found to equally prefer a 100 mM sucrose standard to a 140 mM fructose and a 400 mM glucose solution (Pfaffman, 1977). Thus, all behavioral measures, including those obtained in the present study, are in agreement that among the sugars tested sucrose is the most effective stimulus, followed by fructose, which in turn is more effective than glucose, lactose, and maltose.

This pattern differs strikingly from the one found in rats, which have repeat-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criterion of preference
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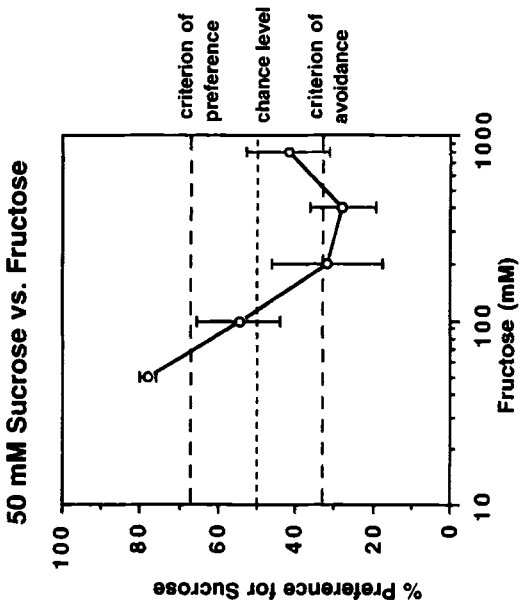
Glucose (mM)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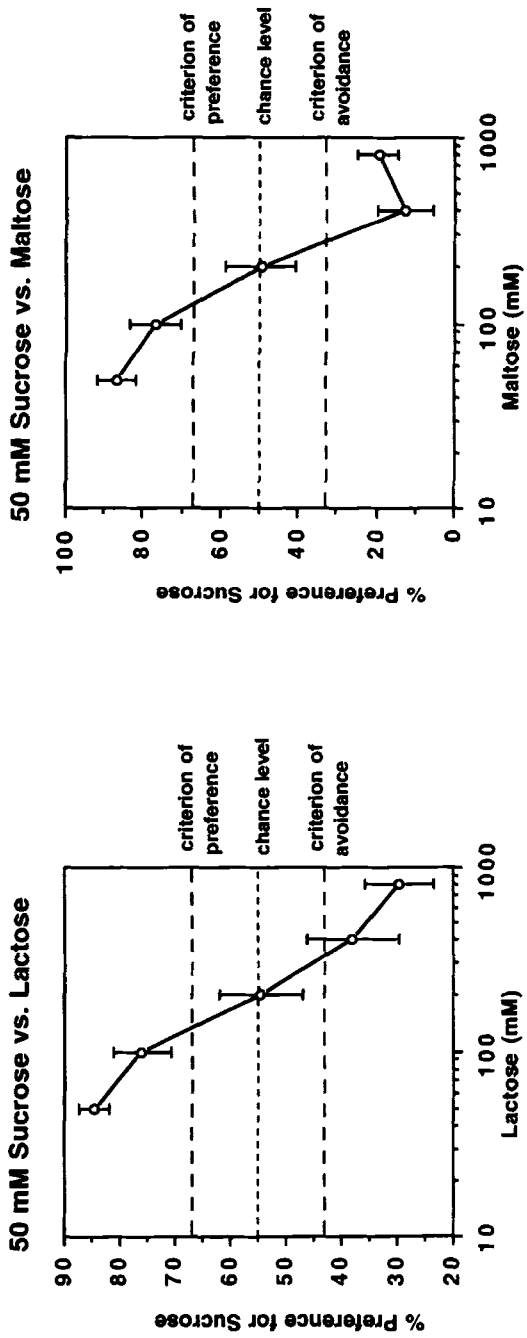

FIG. 2. Mean preference ± SD for six squirrel monkeys given a choice between a 50 mM sucrose standard and variable concentrations of fructose, glucose, lactose, and maltose.

edly been shown to prefer maltose to a higher degree compared to sucrose (Feigin et al., 1987; Richter and Campbell, 1940; Sclafani and Mann, 1987). This finding was confirmed in other rodent species (Feigin et al., 1987) and emerged in both solution vs. water and solution vs. solution tests. The explanation proposed to underlie this marked difference in relative effectiveness of sugars between primates and rodents is that the latter are presumed to have additional taste receptors for starch-derived saccharides (which are presumably responsive to polycose, maltose, and glucose but not to sucrose) that the former lack (Sclafani and Mann, 1987). This hypothesis is supported by findings from human psychophysical studies reporting starch-derived polysaccharides as bland-testing (Feigin et al., 1987).

However, as bonnet macaques have been reported to prefer maltose and polycose as strongly as sucrose in solution vs. water tests (Sunderland and Sclafani, 1988), it seems more likely that the species differences found in the preferences for individual sugars reflect evolutionary adaptations to dietary specialization rather than a generalizable pattern that can be assigned to an order of mammals. Macaque species, for example, include starchy plants in their diet and possess cheek pouches that are used for salivary predigestion of starch (MacKinnon and MacKinnon, 1980), both features that *Saimiri sciureus* lacks. Squirrel monkeys, on the other hand, rely heavily on fruits that usually contain sucrose, together with its monosaccharide components fructose and glucose, as quantitatively predominant saccharides (Kinghorn and Soejarto, 1986; Nagy and Shaw, 1980).

The methodology used in the present study does not allow a conclusive decision as to whether the observed preferences for individual sugars are based on differences in taste quality or stimulating efficiency. However, the finding of concentrations of equal effectiveness in experiment 2 suggests that sucrose may indeed have the same taste quality as fructose, glucose, lactose, and maltose in *Samiri sciureus*. Whereas recent human psychophysical studies provide evidence in favor of monogeneusia for the sugars tested (Breslin et al., 1994, 1996), rats have been reported to perceive the tastes of sucrose and fructose as qualitatively different (Ramirez, 1994). Other studies have shown that rats are able to discriminate between sugars and even between different concentrations of the same sugar on a basis other than taste, presumably olfactory cues (Rhinehart-Doty et al., 1994), and it still seems an unsettled question as to whether common sugars really have different tastes to rats.

In summary, the results of the present study suggest that squirrel monkeys and man share important features of sweet-taste perception, such as conformity in relative effectiveness of saccharides (sucrose > fructose > other sugars), and presumptive monogeusia for common sugars. Whether these similarities in sweet-taste perception between the two species extend to other aspects of gustatory performance–which would make the squirrel monkey a useful nonhuman primate model of human taste function–merits further investigation.

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FACTORS AFFECTING SCENT-MARKING BEHAVIOR IN EURASIAN BEAVER (*Castor fiber*)

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Abstract—We tested the hypothesis that a main function of territory marking in Eurasian beaver (*Castor fiber*) is defense of the territory. The results showed that: (1) beaver colonies with close neighbors scent-mark more often than isolated ones; (2) the number of scent markings increased significantly with the number of neighboring territories and individuals, the mean distance to all other territories, duration of territory occupancy and length of wooded banks within the territory; (3) the number of scent markings by a colony was not related to the number of animals in the colony, the distance to nearest neighbors' main resting-site, or age of beaver; (4) the number of scent mounds or scent markings in territories with breeding and nonbreeding beaver did not differ; (5) there was no significant difference in the number of scent markings during the season (16 April–31 August); and (6) some of the scent mounds were concentrated at feeding sites, resting sites, and near trails, but most had no apparent relationship to any of these sites. Therefore we concluded that scent marking apparently plays an important role in territory defense of the Eurasian beaver.

Key Words—Beaver, *Castor fiber*, scent communication, scent mound, scent marking, radiotelemetry, territorial behavior, Netherlands.

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INTRODUCTION

By marking their territories, animals might deter or intimidate potential intruders (Geist, 1964; Hediger, 1949; Mykytowycz, 1965). A scent mark may provide an intraspecific warning signal ("no trespassing") or even a "psychological fence," both decreasing the probability of an agonistic encounter with its risk of injury or even death (Welsh and Müller-Schwarze, 1989). The individuals resident in a territory have more to gain from retaining the territory than intruders do from taking it over, and will, therefore, be more likely to escalate an encounter than will an intruder. This is because residents will have invested a great deal of energy and time into getting to know their areas and resources, may have modified the habitat, and may have dependent young (Gorman, 1990). In essence, the scent marks in a territory act as a cue to potential fighting ability and willingness to fight in an asymmetric contest between resident and intruder (Maynard Smith and Parker, 1976). Gosling (1982) presented an alternative hypothesis, suggesting that the function of territory marking is to provide an olfactory association between the resident and the defended area that allows intruders to identify the resident when they meet and thus reduce the frequency of escalated agonistic encounters. Other possibilities are discussed by Gosling (1990) and Richardson (1991).

All age classes of beaver (*Castor* spp.) and both sexes participate in marking the territory at scent mounds close to the water's edge (Aleksiuk, 1968; Wilsson 1971; Butler and Butler, 1979; Svendsen, 1980a). Beaver mounds are marked with urine and castoreum from the castor sacs, and possibly with anal gland secretion (Bollinger, 1980; Schulte et al. 1994; see also Müller-Schwarze and Houlihan, 1991). Scent communication is important in beaver; they lack long-distance acoustic communication, and as a primarily nocturnal species, they cannot rely on visual communication (Tang et al., 1993). While marking may serve multiple purposes, recent studies have supported a territorial function as primary (Houlihan, 1989; Welsh and Müller-Schwarze, 1989). Hay (1958) and Aleksiuk (1968) also suggested that scent marking is related to territoriality in *C. canadensis*. Wilsson (1971) suggested a similar function for scent marking in *C. fiber*. Müller-Schwarze and Heckman (1980) demonstrated for the first time that experimentally used odor cues affect colonization by free-ranging beaver (*C. canadensis*). The experimentally scented sites were colonized less often than the unscented. However, aggressive encounters are not rare. For instance, in a dense population of *C. fiber* along the Elbe, bite wounds from conspecifics are the most important cause of death in adult beaver, occurring mostly in May at the time new territories are established (Piechocki, 1977).

Beaver should scent-mark before the main arrival period of intruders if the maximum preventive effect is to be obtained. Therefore, a marking peak in

April should be expected, because at that time young beaver usually disperse from their natal site in order to establish their own territory (Beer, 1955; Bergerud and Miller, 1977; Svendsen, 1980b). Most transients are dispersing subadults (2 years of age, in their third summer of life), but they may also include adult males temporarily leaving their family lodge or adult females without young (Townsend, 1953).

We hypothesize that the Eurasian beaver scent-marks as a means of territory defense. The following predictions were deduced and subsequently tested: (1) the number of scent markings in resident beaver increases with increasing number of neighboring territories or individuals; (2) residents scent-mark more frequently with increased duration of territory occupancy and territory quality; (3) the number of scent mounds present and the number of scent markings does not differ between breeding and nonbreeding beaver territories; (4) scent marking is more frequent at the presumed period of dispersal; and (5) most of the scent mounds are not located at specific resources (feeding sites, resting sites, trails).

METHODS AND MATERIALS

Study Area. The study was conducted in the Biesbosch region (about 100 km^2) in the freshwater estuary of the rivers Rhine and Meuse in the Netherlands (51°45′N, 4°50′E). The central part is a nature reserve (about 50 km^2) with a tidal amplitude of 30 cm, but the water levels are influenced much more by wind and river discharge than by the tide. The nature reserve consists of willow coppices and reedbeds intersected by creeks and no longer exploited by man. It is surrounded by agricultural fields with creeks with stable water levels and more or less intact banks. The dominant vegetation on the banks of waterways >5 m wide (615 km in total) was classified either as wooded (184 km), with herbs (126 km), with reeds (217 km), or barren (88 km). The banks were digitized from a 1 : 10,000 map into a geographical information system (ARC/INFO).

Study Animals. In October–November 1988–1991, a total of 42 Eurasian beaver originating from the river Elbe, Germany, were sequentially released as part of a reintroduction program (Nolet, 1995). The founder group contained 17 adults, 7 subadults, 10 yearlings, and 8 kits. Prior to release, the animals were marked with colored plastic and aluminum eartags for visual identification. We attempted to catch beaver born in the study area. However, the tidal fluctuation in water levels negatively affected trapping success with Hancock livetraps and we caught only four unmarked beaver (total trapping success 5%).

Delineation of Territories. From April 1 to August 31, 1993, we mapped all 13 territories present either by locating scent mounds supplemented with sight observations of mostly marked individuals (eight territories) or by radio-

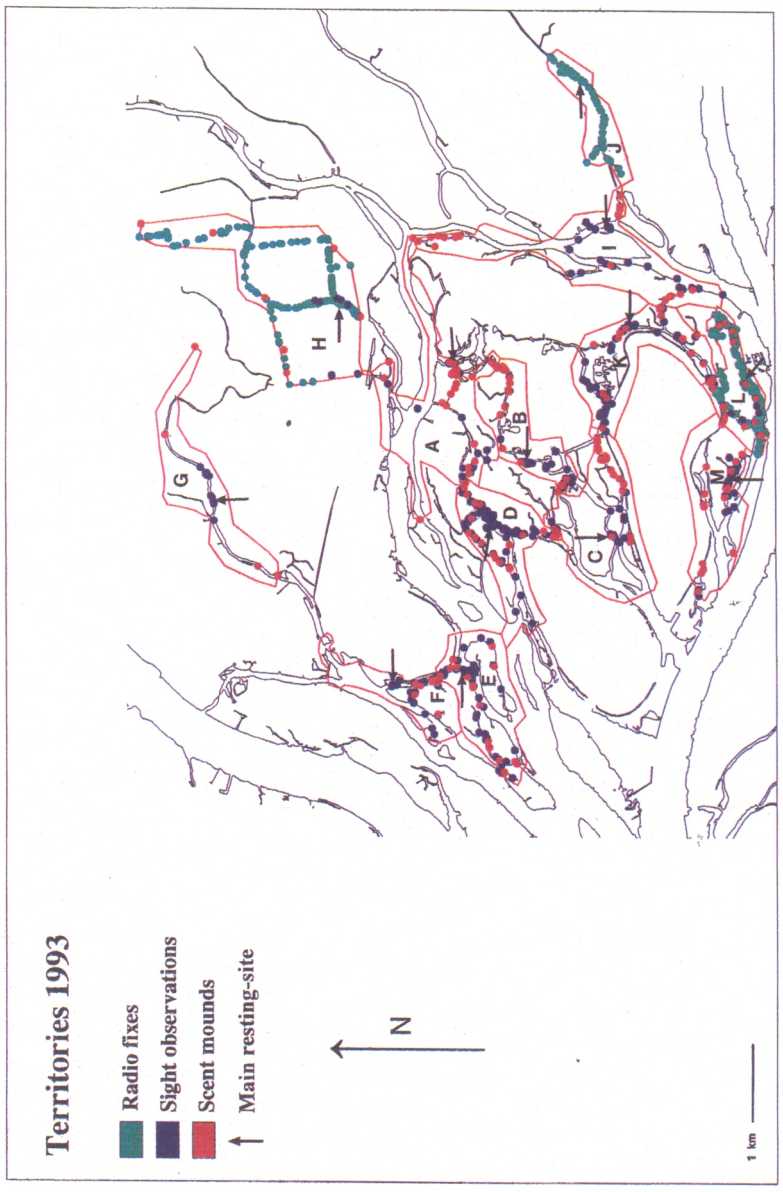

FIG. 1. Map of the Biesbosch with the location of radio fixes, sight observations, and scent mounds in the summer of 1993 and the delineation of beaver territories based on these data plus fresh feeding sites and sight observations by others.

telemetry (three territories) (Figure 1). We used the locations (concentrations) of scent mounds, sight observations, and radiotelemetry data to define the territorial boundaries (see also below). In three territories (A, C, and F, Figure 1), this did not provide enough information, and the locations of fresh feeding sites and sight observations by others were used in addition. The scent mounds, sight observations, and radio fixes were digitized from a 1 : 10,000 scale map into a GIS system (ARC/INFO) (Figure 1). We considered territories to be one- rather than two-dimensional, i.e., territory sizes were expressed as total length of banks [see Nolet and Rosell (1994) for further details].

Scent Mounds. This phase of the study was conducted between April 16 and August 31, 1993. The type, smell (strong or weak), and locations of scent mounds (see definition in Table 1) were recorded once a week in the whole study area, except in one territory (G, Figure 1, see also Table 2) located outside the nature reserve, which was visited only once a month. Scent mounds were assigned to a beaver colony according to the origin of an ear-tagged beaver sighted nearest to the scent mound. A scent mound that had recently been used was called a scent marking (see definition in Table 1). Freshly built scent mounds without odor were also designated as scent markings because they can contain chemical substances from the castor sacs (castoreum) and/or the anal glands that cannot be smelled by the human nose (see Bollinger, 1980; Schulte, 1993). The approximate distance to the nearest neighboring territory (see definition in Table 1) was measured as a straight line between the main resting sites of neighboring territories (den used most during the study period). To classify peripheral and central territories we measured the straight-line distance to all other territories (main resting site) and classified mean distances of <2.0 km as central and those distances ≥ 2.0 km as peripheral. To test the validity of the use of scent

TABLE 1. EXPLANATION OF TERMINOLOGY USED IN TEXT

Term	Definition
Scent mound	A location (for instance, a pile of mud, vegetation, twigs, leaves or grass) where scent marking was recorded at least once. The minimum distance between separate scent mounds was 10 cm.
Scent marking	Freshly used scent mound, i.e., the characteristic odor was smelled or new vegetation had been deposited on the mound since last check.
Scent-mound site	A site with one or more scent mounds. Sites are considered separate if more than 5 m apart.
Neighboring territories	Territories that share a common water border, i.e., land borders were not included in the definition (see also Figure 1).

TABLE 2. NUMBER OF NEIGHBORING TERRITORIES, SCENT MOUNDS, SCENT-MOUND SITES, SCENT MARKINGS, NEAREST-NEIGHBOR DISTANCE (KILOMETERS BETWEEN MAIN RESTING SITES), COLONY SIZE, AND AGE (OF OLDEST PAIR MEMBER) AT 13 OCCUPIED BEAVER TERRITORIES IN BIESBOSCH (1993)

Territory	Neighboring territories (*N*)	Scent mounds (*N*)	Scent-mound sites (*N*)	Scent markings (*N*)	Nearest neighbor distance (km)	Colony size	Age in 1993[a]
A	4	38	25	136	1.2	2	7
B	2	25	21	103	0.8	6[c]	4
C	3	26	19	91	1.0	4[c]	12.5
D	3	30	24	81	0.8	8[c]	12.5
E	2	27	22	88	0.7	2	12.5
F[b]	1	23	16	80	0.7	2	12.5
G[b]	0	4	4	4	2.3	2	12.5
H	1	7	7	7	1.4	2	12.5
I	3	23	18	58	1.1	2	5
J[b]	1	0	0	0	1.5	3[c]	3
K	3	33	19	119	1.1	2	12.5
L	2	34	23	117	1.0	2	4
M	1	16	16	49	1.0	6[c]	12.5
Total		286	214	933			

[a] Beavers released as adults were assumed to be 7.5 years old at the time of release, which was the average age of adults in the Elbe population (Heidecke, 1991).
[b] Peripheral territories (see Methods and Materials).
[c] Breeding beaver territories.

mounds in delineation of the territories, we arbitrarily distinguished three zones of 0–299 m, 300–600 m, and >600 m from the drawn territorial borders (measured along the waterway) and counted the number of scent markings in each zone (Figure 2). All three zones were searched with equal intensity. Eleven (of 13) of the main resting sites were found in the latter zone, and two were located in the middle zone. Although the zone >600 m from the border contained more length of banks, the zone nearest to the border had the highest mean number of scent markings (Figure 3). These data indicated that the scent mounts were not randomly distributed over the territory but were lumped instead. This gave us confidence that the scent mound data could be used to delineate the territories. However, beaver in colonies located at the periphery of the study area marked much less frequently than those in the central part (see results), but fortunately we had radio-tracking data for two of the three territories outside the nature reserve (see Figure 1).

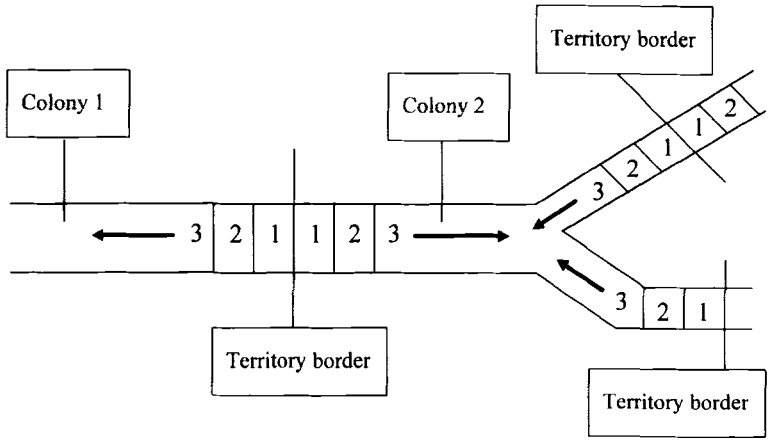

FIG. 2. A graphical illustration of the location of zones 1, 2, and 3 with respect to territorial borders. Zone 1 was located 0–299 m from the border, zone 2 at 300–600 m, and zone 3 at >600 m.

Breeding Status of Territories. Breeding territories were defined as those containing young of the year.

Statistical Methods. Statistical analyses were performed with SPSS (version 6.0) (Norusis, 1990). We used nonparametric statistics in accordance with Siegel and Castellan (1988). Nonparametric tests were corrected for ties. Probability

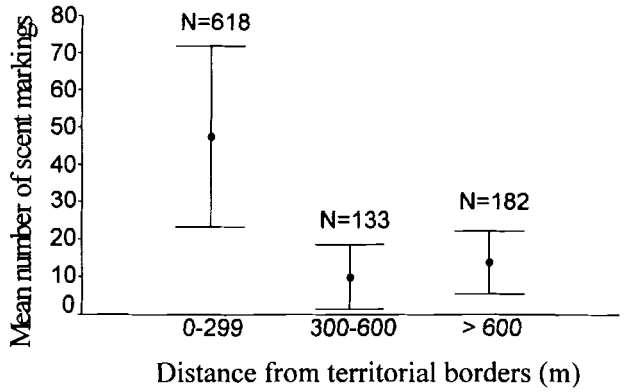

FIG. 3. Pooled data from 13 beaver territories in the Biesbosch showing the mean number of scent markings in three zones at arbitrarily chosen distances from the territorial border. Vertical bars show 95% confidence intervals; N = total number of scent markings in each zone (Kruskal-Wallis one-way ANOVA, $X^2 = 7.23$, $df = 2$, $P = 0.027$).

values are two-tailed and 5% was used as the level of significance. Mean values are shown with standard deviations.

RESULTS

Number of Scent Markings. The 286 scent mounds were located at 214 sites (see definition in Table 1) within the 13 occupied territories (Table 2). Scent mounds were checked a total of 3326 times, of which 933 (28.1%) were determined to be freshly used (scent marked) (Table 2). Of the 933 freshly used mounds, 352 (37.7%) had a strong smell, 175 (18.8%) a weak smell, and 406 (43.5%) no discernible smell. Of the 214 scent mound sites, 46 had more than one scent mound (range 2–8). Beaver did not maintain all mounds following initial use, and 36.4% of the scent mounds were marked only once, whereas others were marked repeatedly. Beaver in the three colonies located at the periphery (F, G, and J, Figure 1, Table 2) scent-marked 28.0 ± 45.1 times on average during the study period ($N = 3$), which was significantly less often than in the central colonies ($\bar{x} = 84.9 \pm 38.4$, $N = 10$) (Mann-Whitney U-test, $z = -2.02$, $P = 0.043$). The number of scent markings increased significantly with the number of neighboring territories (Figure 4). The same pattern was also shown for the number of neighboring individuals (Figure 5). The number of scent markings decreased with the mean distance to all other territories (Figure 6). A significant correlation was also found between number of scent markings and duration of territory occupancy (of the pair member that established first) (Figure 7) and length of wooded banks within the territory (Figure 8). No

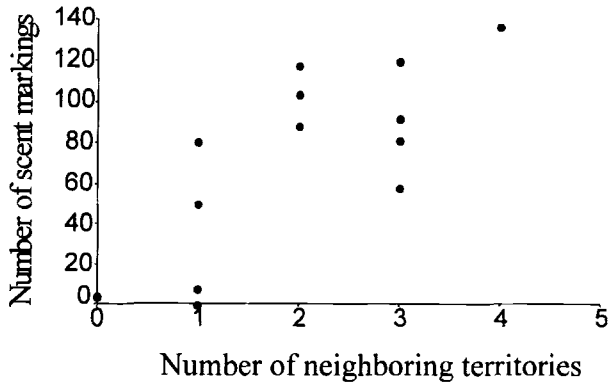

FIG. 4. The relationship between the number of neighboring territories and the number of scent markings for the period April 16 to August 31, 1993, for all 13 beaver territories ($r_s = 0.753$, $N = 13$, $P = 0.003$).

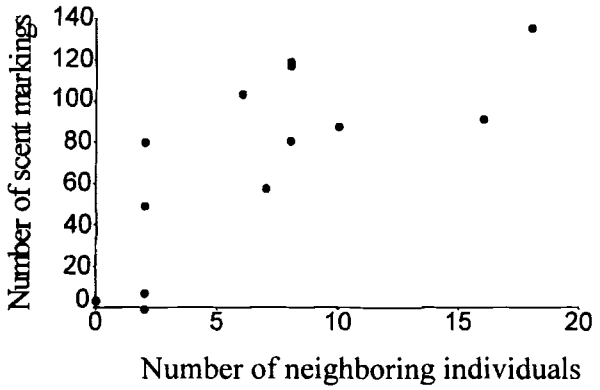

FIG. 5. The relationship between the number of neighboring individuals and the number of scent markings for the period April 16 to August 31, 1993, for all 13 beaver territories ($r_s = 0.807$, $N = 13$, $P = 0.001$).

significant correlation was found between number of scent markings by a colony and distance to the nearest neighbor's main resting-site (Table 2, $r_s = -0.197$, $N = 13$, $P = 0.520$), number of beaver per territory ($r_s = -0.100$, $N = 13$, $P = 0.744$), or age of beaver (of the pair member that was oldest) ($r_s = -0.088$, $N = 13$, $P = 0.775$). Colonies of breeding beaver ($N = 5$) (Table 2) made an average of 19.4 $\pm$ 12.0 scent mounds and scent-marked on the average 64.8 $\pm$ 41.4 times, which was not significantly different from nonbreeding beaver colonies ($N = 8$) ($\bar{x} = 26.6 \pm 12.4$ and 76.1 ± 50.1, respectively, Mann-

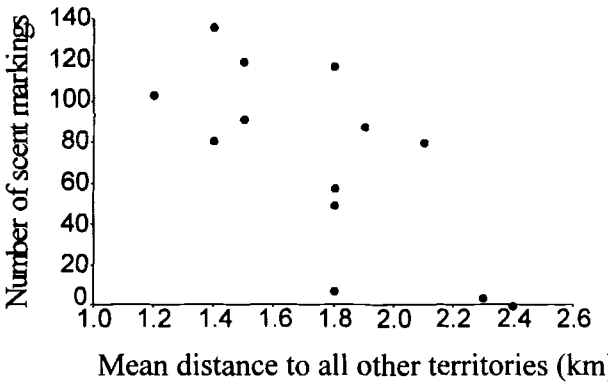

FIG. 6. The relationship between the mean distance to all other territories and the number of scent markings for the period April 16 to August 31, 1993, for all 13 beaver territories ($r_s = -0.710$, $N = 13$, $P = 0.007$).

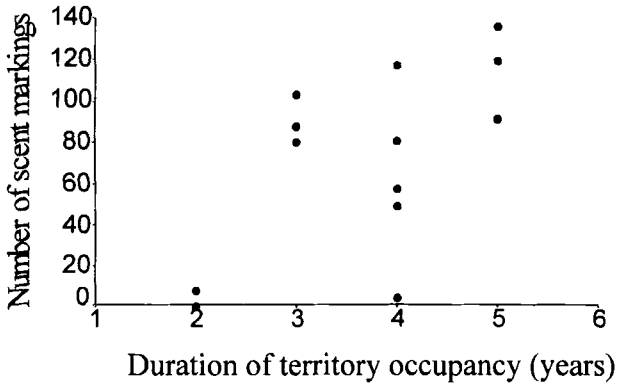

FIG. 7. The relationship between the duration of territory occupancy in years, for the pair member that established first, and the number of scent markings for the period April 16 to August 31, 1993, for all 13 territories ($r_s = 0.601$, $N = 13$, $P = 0.030$).

Whitney U-test, $z = -0.73$, $P = 0.464$ for number of scent mounds and $z = -0.59$, $P = 0.558$ for number of scent markings).

Variation with Season. Table 3 shows how the significant trends in Figures 4–8 hold up over the course of the season. The results in Table 3 indicate a significant relationship between the number of scent markings and both the number of neighboring territories and the number of neighboring individuals for most bimonthly periods. There also were significant correlations between the number of scent markings and all five parameters for the period August 1–15.

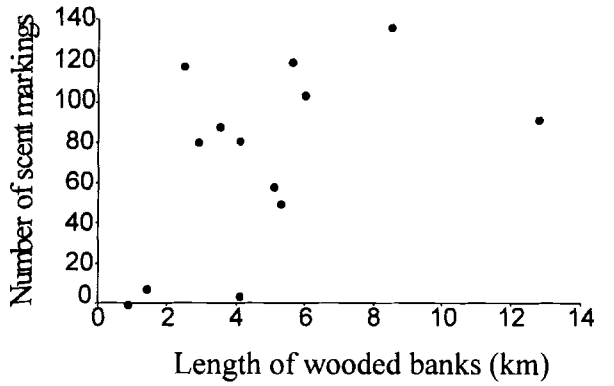

FIG. 8. The relationship between the length of wooded banks within the territory and the number of scent markings for the period April 16 to August 31, 1993, for all 13 territories ($r_s = 0.575$, $N = 13$, $P = 0.040$).

TABLE 3. SPEARMAN RANK CORRELATION COEFFICIENTS FOR CORRELATION BETWEEN NUMBER OF SCENT MARKINGS (y AXIS IN FIGURES 4–8; 13 TERRITORIES) PER TERRITORY AND 5 PARAMETERS LISTED BELOW (x AXIS IN FIGURES 4–8) FOR BIMONTHLY PERIODS FROM APRIL 16 TO AUGUST 31[a]

Date	Correlation coefficients				
	Number of neighboring territories	Number of neighboring individuals	Mean distance to all other territories	Duration of territory occupancy	Length of wooded banks
April 16–30	0.53	0.57*	−0.40	0.19	0.19
May 1–15	0.57*	0.57*	−0.53	0.35	0.19
May 16–31	0.62*	0.75**	−0.64*	0.62*	0.39
June 1–15	0.75**	0.65*	−0.69**	0.56*	0.54
June 16–30	0.67*	0.55	−0.43	0.57*	0.76**
July 1–15	0.55	0.59*	−0.28	0.27	0.37
July 16–31	0.53	0.74**	−0.55	0.47	0.35
August 1–15	0.86***	0.83***	−0.74**	0.78**	0.70**
August 16–31	0.59*	0.50	−0.37	0.42	0.60*

[a] *$0.01 < P < 0.05$; **$0.001 < P < 0.01$ and ***$P < 0.001$.

There was no significant difference in the number of scent markings from April 16 to August 31 (Figure 9). The scent mound construction was highest during the last week in April and the first week in May (Table 4).

Location of Scent Mounds. Of the total number of scent mounds ($N = 286$) observed during the entire period April 16–August 31, 34.6% were located at feeding sites, 6.6% near (<5 m) a resting site (den, hole, lair), and 5.3% near a trail; 53.5% had no apparent relationship to any of these sites. Of the 286 scent mounds, 97.2% were self-constructed, while the rest (2.8%) were located on rocks, tussocks, or directly on the ground. The week-to-week variation in scent mound numbers and locations during the peak period of marking/dispersal by beaver is showed in Table 4.

DISCUSSION

Beaver sites with more neighbors, and therefore a greater number of potential intruders, were expected to be scent marked more often. In this study beaver colonies in the central part scent-marked significantly more than did colonies at the periphery. The number of scent markings increased significantly with the number of neighboring territories and individuals. This pattern held for most of the bimonthly periods from April 16 to August 31, further indicating the impor-

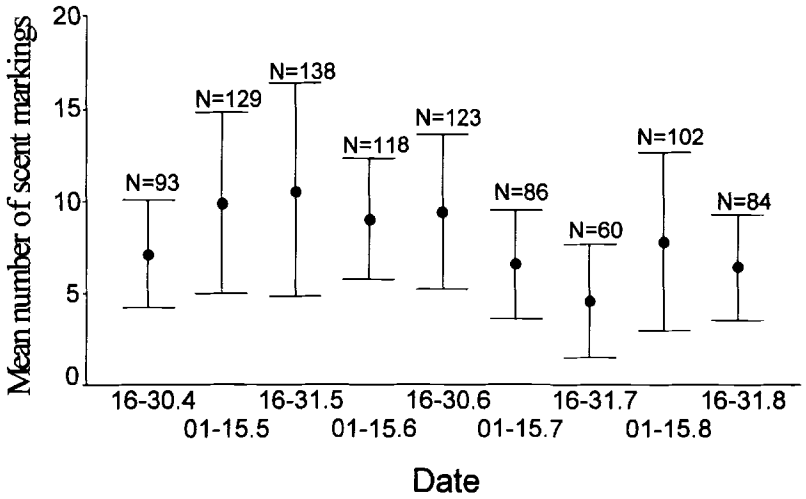

FIG. 9. Bimonthly changes in the mean number of scent markings for all 13 territories combined for the period April 16 to August 31, 1993 (Kruskal-Wallis one-way ANOVA, $X^2 = 7.38$, $df = 8$, $P = 0.497$). Vertical bars show 95% confidence limits; N = total number of scent markings. The date notation 16-30.4 indicates April 16 to April 30.

tance of scent marking as a defense against neighbors. We also found that the number of scent markings decreased with increasing mean distance to all other territories. This may be regarded as a measure of how central a territory was situated. Thus, these findings are in accordance with our first prediction. We found no significant correlation, however, between the number of scent markings and distance to the nearest neighbor's main resting site, as found by other authors

TABLE 4. WEEK-TO-WEEK VARIATION IN 13 TERRITORIES IN SCENT MOUND NUMBERS AND LOCATIONS DURING PEAK PERIOD OF MARKING AND DISPERSAL BY BEAVER

Date	Total number of scent mounds	Number of scent mounds at			
		Feeding sites	Resting site	Trails	Other sites
April 16–22	21	5	2	0	14
April 23–30	46	7	6	4	29
May 1–8	44	12	2	3	27
May 9–16	21	7	4	1	9
May 17–24	12	7	0	0	5
May 25–31	19	10	0	0	9

(Butler and Butler, 1979; Müller-Schwarze and Heckman, 1980; Svendsen, 1980a; Houlihan, 1989).

That the number of scent markings is density-dependent has previously been shown for both *C. canadensis* (Butler and Butler, 1979; Müller-Schwarze and Heckman, 1980; Houlihan, 1989) and *C. fiber* (Andersson and Westerling, 1984). Thus, when beaver have many close neighbors, they apparently need to scent-mark more often to be unambiguously recognized. Gosling (1982) predicted that the owner should remove or replace marks in the territory that do not match its own odor. We recorded two observations that support this prediction. In both cases a nonresident adult male beaver scent-marked and the resident adult male beaver scent-marked the same mound minutes later.

Nolet et al. (1995) found that, in contrast to other food studies on beaver, in the Biesbosch they nearly exclusively ate woody plants all year round. Wooded banks within the territory were therefore clearly an important resource. Beaver released in unoccupied habitat spent considerable time exploring their surroundings, especially during the first two years of the reintroduction (Nolet and Rosell, 1994). Thus, once established, these (large) territories were presumably well worth defending. Theoretically, the greater potential value of the territory for residents, in contrast to intruders, makes it worth fighting harder for. Thus intruders should retreat (Maynard Smith, 1976). Nolet and Rosell (1994) found that the earliest arrivals claimed larger territories and territories of better quality than later arrivals. We found a significant positive correlation between both the number of scent markings and the duration of territory occupancy and length of wooded banks, as did Hodgdon (1978), which is in accordance with our second prediction. It appears that residents invest more in scent marking in good-quality territories, and when a territory has been occupied for a long time, as a means of defending it better.

The number of scent mounds and scent markings in breeding and nonbreeding beaver territories did not differ, which is in accordance with our third prediction. This indicates that scent marking is not associated with having young. It also suggests that constructing scent mounds is relatively cheap and that breeding beaver are not forced to save energy by scent marking less.

There was no significant difference in the number of scent markings during the season. However, we observed a small peak in the number of scent markings in May. We also observed a peak in scent-mound construction in the last week in April and the first week in May, which is in accordance with our fourth prediction. These peaks could be explained by the greater need for scent marking early in the season (spring) to inform transient beaver that the area is occupied (intercolony communication) (Butler and Butler, 1979). Intrusion by neighboring beaver, especially during spring dispersal of 2-years-olds, has been hypothesized to be the cause of the increase in the construction of scent mounds observed by

some authors (Aleksiuk, 1968; Houlihan, 1989). Molini et al. (1980) predicted that scent marking would be greatest when interactions between dispersing and resident beaver peaked. As individuals begin to find available sites, the number of "floaters" also decreases, as should scent marking. Their model predicted a peak in spring, which nearly coincided with an observed peak in scent marking for *C. canadensis*. Nitsche (1985) found a peak in April and May for *C. fiber* (see also Klenner-Fringes, 1992), whereas for *C. canadensis* a peak from March through July has been found (Fabel, 1977; Hodgdon, 1978; Butler and Butler, 1979; Bollinger, 1980; Svendsen, 1980a; Walro, 1980). Svendsen (1980a) suggested that both residents and transients respond to each others scent mounds and that the high numbers of scent markings in spring reflect the combined behaviors of both residents and transients. The model advanced is one where the presence of one fresh scent mound induces another. The significant increase in number of scent markings in the beginning of August may coincide with the period for the emergence of kits from the dens. Buech (1995) also recorded a peak in scent-mound construction (for males) during the beginning of August in North American beaver. However, this peak in scent markings could also be associated with dispersal of yearlings. Hartman's (1994) results, from a low density population, showed that six of nine beavers dispersed as yearlings, three of these in the early fall.

Müller-Schwarze and Heckman (1980) found that scent mounds were located at trails, lodges, and dams. Hay (1958) reported that scent mounds occurred in concentrated patterns around inhabited lodges. The examination by Butler and Butler (1979) of scent-mound placement indicated that 84% of the scent mounds constructed by beaver colonies occurred in areas of high colony activity (e.g., feeding areas, trails leading away from the pond, grooming areas). The scent mounds in this study were located near feeding sites, resting sites, and trails (near areas with high activity), but more than half of the scent mounds had no apparent relationship to any of these sites, which is in accordance with our fifth prediction. These scent mounds at "other sites" also seem to play a role in territory defense, as indicated by the high frequency of scent-mound construction here at the end of April and first week in May.

The number of scent markings by a colony of beaver in this study was not related to the number of animals in the colony or age of beaver. Other authors also found no correlation between the number of scent markings and the number of animals in the colony (Butler and Butler, 1979; Svendsen, 1980a; Houlihan, 1989). It seems that the beaver in the Biesbosch manage to maintain, independent of group size and age, the scent mounds intact so that they can serve their function in territory maintenance. Apparently, a pair can defend the territory as well as a large family.

The scent-marking communication system therefore appears to play a major

role in maintaining territorial borders. An important aspect of the system is that transient beaver and members of neighboring colonies appear to voluntarily avoid areas harboring scent mounds (Müller-Schwarze and Heckman, 1980; Welsh and Müller-Schwarze, 1989).

The scent marking communication system of maintaining territories suggested here is not necessarily the only functional mechanism, as one function need not necessarily exclude others.

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SYMBIONT REGULATION AND REDUCING ERGOT ALKALOID CONCENTRATION BY BREEDING ENDOPHYTE-INFECTED TALL FESCUE

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Abstract—Ergot alkaloids in endophyte-infected (E+) tall fescue (*Festuca arundinacea*) are responsible for livestock toxicosis. The host plant is capable of modifying the extent to which endophyte produces ergot alkaloids, possibly via endophyte nutrition by the host. Endophytes vary in alkaloid production. Maintaining the E+ tall fescue is essential for plant survival. Therefore, crop scientists are interested in reducing alkaloid concentration of E+ tall fescue. The objectives of this study were to determine maternal and paternal effects as an estimate of plant and endophyte genotype interactions for ergot alkaloid phenotype and the heritability of the ergot alkaloid trait in divergently selected E+ tall fescue populations. Diallel crosses were conducted among four tall fescue genotypes that varied in alkaloid concentration. Five of six crosses had progeny means less than the parental means when genotypes with lowest alkaloid were the female parents. Four of six crosses had progeny means similar to the parental means of crosses when high alkaloid genotypes were the female parents. Large ranges in progeny suggest an interaction between endophyte and plant affects alkaloid phenotype. In a second experiment, a tall fescue population was screened for alkaloid concentration. Low- and high-alkaloid plants were selected and poly-crossed within each class. Mean alkaloid concentrations of the high- and low-alkaloid progeny were higher or lower than the parental population. The trait was highly heritable and alkaloid concentration was reduced by 86% in the low-alkaloid population after two generations of selection.

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Key Words—*Neotyphodium*, *Acremonium*, ergot alkaloids, diallel cross, heritability, fescue toxicosis, endophyte, tall fescue, mutualism, symbiosis.

INTRODUCTION

Ergot alkaloids present in endophyte-infected (E+) tall fescue are responsible for fescue toxicosis (Hill et al., 1994). The endophyte [*Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon, and Hanlin comb. nov.] is in a mutualistic association with the plant and provides its host with enhanced vigor (Hill et al., 1991a), resulting in resistance to insect pests (White and Cole, 1985; West et al., 1988; Siegel et al., 1990), nematodes (West et al., 1988); diseases (White and Cole, 1985; Siegel and Latch, 1991), drought tolerance (Archevelata et al., 1989; Elmi and West, 1989); and tolerance to mammalian herbivores (Siegel et al., 1987; Stuedemann and Hoveland, 1988). Because of these characteristics, *N. coenophialum* has been shown to be critical for persistence of tall fescue where biotic and abiotic stresses are present (Bouton et al., 1993).

Three classes of antiherbivore alkaloids are associated with endophytic infection of tall fescue: the pyrrolizidine alkaloids including the loline alkaloids (Yates and Tookey, 1965), the pyrrolopyrazine alkaloids including peramine (Rowan et al., 1990), and the ergot alkaloids comprised of the ergopeptine and clavine alkaloids (Yates et al., 1985; Lyons et al., 1986). Loline alkaloids are believed to be plant derived as a response to the endophyte, but peramine and the ergot alkaloids are products of the fungus (Bush et al., 1979; Siegel et al., 1990). Both the loline and peramine alkaloids are associated with insect resistance (Siegel et al., 1990; Rowan and Gaynor, 1986), but there is no evidence suggesting that loline or peramine alkaloids are toxic to mammalian herbivores.

Studies have shown that ergot alkaloid concentration varies depending upon the genotype of the E+ tall fescue plant (Agee and Hill, 1994; Roylance et al., 1994), implying plant-mediated chemical or nutritional control of endophyte production of ergot alkaloids. Although the endophyte produces the alkaloids, the genotype of the plant may alter expression (Hill et al., 1991b). Plant mediation may be associated with calcium or tryptophan nutrition to the endophyte by its host (Roylance et al., 1994). Calcium is a coenzyme for dimethylallyl tryptophan synthase (DMATase) the enzyme that catalyzes the first pathway-dependent reaction necessary for the production of ergot alkaloids (Lee et al., 1976; Cress et al., 1991). Variation exists for calcium concentration in tall fescue, and the trait is highly heritable (Sleper, 1979). Calcium nutrition to the plant increases ergot alkaloid concentration in E+ tall fescue independent of a soil pH effect (Hill, 1995). Tryptophan is also necessary for the DMATase reaction (Lee et al., 1976; Cress et al., 1991), but there is no evidence that

variation exists for tryptophan concentration in tall fescue. Despite a lack of knowledge of variables controlling ergot alkaloid concentration, there appears to be little effect on the agronomic properties of the plant. Hill et al. (1991b) reported that the ergot alkaloid content in E+ tall fescue does not appear to be associated with competitiveness or persistence of the plant.

Peramine and ergot alkaloid concentrations in E+ tall fescue are independently regulated (Roylance et al., 1994). Development of E+ tall fescue populations that are low in ergot alkaloid concentration may improve animal weight gain and reproduction since peramine has no known effect on mammalian species. The beneficial effects of the endophyte to the plant may be maintained in low-alkaloid populations of E+ tall fescue, thereby creating a sustainable alternative to current E+ and endophyte-free cultivars. The objectives of this study were to determine: (1) maternal and paternal effects as estimates of plant and endophyte genotype interactions affecting ergot alkaloid phenotype and (2) the heritability of selecting for low- and high-ergot alkaloid concentration in E+ tall fescue.

METHODS AND MATERIALS

Experiment 1. Maternal and Paternal Effects on Ergot Alkaloid Phenotype. Four replicate plots of four endophyte-infected tall fescue genotypes (DN2, DN11, DN12, and DN15) were vegetatively propagated in a crossing block in the fall of 1992 at the University of Georgia Plant Sciences Farm near Watkinsville, Georgia. These genotypes were selected because they are known to vary in ergot alkaloid concentration (Roylance et al., 1994). The soil was a Pacolet sandy clay loam (Typic Kanhapludult, clayey, kaolinitic, thermic). Field-grown plants were fertilized in the spring of 1993 with approximately 100, 42, and 84 kg/ha of N, P, and K, respectively.

Diallel crosses were made among all plant genotypes in May 1993 by pairing individual inflorescences of each genotype with inflorescences of the other genotypes. The inflorescences were covered with glassine bags (Brown Paper Goods Co., Libertyville, Illinois) prior to anthesis. Seedheads were harvested at physiological maturity during the first week of June, and seed from each cross was harvested and stored separately. Individual seedheads were also placed into glassine bags to check for selfing, but no selfing occurred because tall fescue is primarily self-infertile (Asay et al., 1979).

On July 7, 1993, harvested seeds from the 12 diallel crosses were planted in a greenhouse in flats containing a growth medium of 1:1:1:1 Cecil sandy clay loam (Typic Hapludult, clayey, kaolinitic, thermic)–shredded peat moss–perlite–fritted clay. Three weeks after emergence, subsets of 25 progeny

were randomly selected from each cross and planted in 0.5-liter pots containing a 1:1:1:1 mix of Cecil sandy clay loam–shredded peat moss–perlite–fritted clay. Pots were randomly placed on the greenhouse bench. The plants were fertilized weekly with 0.3, 0.13, and 0.25 g/pot of N, P, and K, respectively.

In October 1993, 20 of the most vigorous plants of each cross were selected and divided into two halves. Each half of the plant was planted into 3.0-liter pots containing the soil mix with fritted clay and randomly assigned to two blocks, each serving as a replication. The parent plants were divided and planted in similar fashion and included in each replication. After 90-days of growth, herbage was clipped to a height of 7.5 cm above the soil surface and discarded. The plants were grown for an additional 90 days and then 60 days, and the herbage was clipped to 2.5 cm above the soil surface after each period. Harvested herbage from each of the last two clipping dates was lyophilized, ground in a cyclone-type mill to pass through a 1-mm mesh screen, and stored at −70°C.

Experiment 2. Heritability When Selecting for Low and High Ergot Alkaloid Concentration in E+ Tall Fescue. Seeds from GA-Jesup Improved-EI (endophyte-infected) tall fescue were planted into cell packs measuring 2.5 × 2.5 × 5.0 cm (width, length, and depth, respectively) containing a commercial potting mix in the summer of 1993. Seedling plants were grown for eight weeks in the greenhouse under 29/24°C day/night temperatures. Seedlings were fertilized weekly with approximately 0.3, 0.13, and 0.25 g/cell of N, P, and K, respectively.

Six hundred thirty randomly selected seedlings were transferred to the University of Georgia Plant Sciences Farm located near Watkinsville, Georgia, in October 1993. The field location had been previously planted to soybean, *Glycine max* (L.) Merr., and was fertilized with 72, 13, and 59 kg/ha of N, P, and K, respectively. The plants were established in the field on 0.67-m centers. The field site was a Cecil sandy clay loam (Typic Hapludult, clayey, kaolinitic, thermic). The plot area was sprayed with 2,4-dichlorophenoxyacetic acid in early March 1994 for broadleaf weed control. No additional fertilizer was applied to the plot.

Beginning April 7 and continuing through April 23, 1994, plants were monitored for development. Upon full emergence of the flag leaf from the most advanced tiller, tillers from half of the crown area were hand-clipped to a height of 7.5 cm above the soil surface. Harvested tillers were oven-dried at 60°C for 48 hr, ground to pass a 1-mm screen in a Cyclone-type mill, and immunochemically screened for ergot alkaloid concentration. Forty-five plants were selected each for low and high alkaloid concentration based upon their ELISA absorbance values.

Twelve preanthesis reproductive tillers from each of the 45 plants testing

lowest and from 45 plants testing highest for ergot alkaloid concentration were transferred to the greenhouse and planted into 2.5- × 2.5- × 5-cm (width, length, and depth, respectively) cell-packs (6 cells/pack) containing a commercial potting mix. After rooting and emergence of seedheads, plants were reassigned to a polycross with six replications. Low- and high-alkaloid-containing plants were physically separated to avoid cross-pollination between groups. Seeds were harvested at physiological maturity in June 1993, and seeds from each female genotype were bulked. The seeds were stored at 4°C and 40% relative humidity.

In August 1994, 12 seeds from each of the 45 low- and 45 high-alkaloid-containing parent plants were planted into two 2.5- × 2.5- × 5-cm (width, length, and depth, respectively) cell-packs (6 cells/pack) containing a commercial potting mix. One cell pack of each was randomly assigned to two replications. Control populations of two cell-packs per rep of GA-Jesup Improved-EF (endophyte-free) tall fescue, and three cell-packs per replication of GA-Jesup Improved-EI tall fescue were planted to compare alkaloids in the selected populations to the original GA-Jesup Improved-EI population and an endophyte-free population. All plants were grown in the greenhouse.

After 60 days of growth, plants were sampled by hand-clipping to a height of 3 cm. Harvested tissue was transported to the laboratory and stored at −70°C. Samples were lyophilized, ground in a Cyclone-type mill to pass through a 1-mm mesh screen, and stored at −70°C until analyzed for ergot alkaloid content. All plants were transported and planted in the field to vernalize.

The procedure was repeated in 1995 to determine the effects of making a second cycle of selection on reducing ergot alkaloid concentration. Selection pressure was modified slightly from the first cycle in that 48 of the plants testing low in alkaloid concentration and 48 of the plants testing high in alkaloid concentration were selected from 540 plants within each population. Crossing and plant cultivation procedures were identical to those in 1994, except that eight cell packs (48 plants) of the E+ and E− base populations were tested per replication for controls.

Ergot Alkaloid Analysis. Plant tissue was immunochemically analyzed for ergot alkaloids using the competitive ELISA method using the monoclonal antibody described by Hill and Agee (1994). Ninety-six-well Immulon 4 microtiter plates (Dynatech Co., Chantilly, Virginia) were coated with 188 pg of human serum albumin–glutaryl–lysergol (HSA-LYS) conjugate diluted in 50 μl borate saline solution (6.19 g boric acid, 9.5 g $Na_2B_2O_7H_2O$, 4.9 g NaCl, 1 l water, pH 8.5) by incubating overnight at 4°C. The plates were rinsed three times with ELISA wash (1.21 g Tris, 500 μl Tween 20, 0.2 g NaN_3, 1 liter distilled water, pH 8.0) by using a Denley Wellwash 5000 (Denley Instruments, Ltd.) to remove excess conjugate. The microtiter plate wells were blocked with 125 μl bovine

serum albumin blocking solution (10 g bovine serum albumin, 1.17 g Na_2HPO_4, 0.244 g NaH_2PO_4, 8.2 g NaCl, 1 liter distilled water) for 30 min at room temperature and washed three times with ELISA wash.

Ergot alkaloids were extracted from freeze-dried tall fescue plants by vortexing 0.10 g plant tissue in 8 ml phosphate buffer–Tween solution (EPBST) (1.17 g Na_2HPO_4, 0.243 g NaH_2PO_4, 8.175 g NaCl, 500 μl Tween 20, 1 liter distilled water, pH 7.35–7.45), incubating for 15 min at room temperature, and vortexing a second time prior to sampling the liquid fraction. Fifty microliters of liquid extract from each sample were added to a microtiter well. Monoclonal antibody 15F3.E5 hybridoma supernatant (Hill and Agee, 1994) was diluted 1:100 in borate saline solution, and 50 μl of the diluted antibody was added to each well containing liquid extracts. The antibody was permitted to competitively bind with extracted ergot alkaloids and the lysergic moiety of HSA-LYS for 2 hr at 25°C. Plates were washed three times with ELISA wash. Bound antibody was measured with 50 μl rabbit anti-mouse antibody alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Missouri; Cat #A1902) diluted 1:500 in ELISA diluent (1.17 g Na_2HPO_4, 0.243 g NaH_2PO_4, 8.175 g NaCl, 500 μl Tween 20, 0.2 g NaN_3, 10 g bovine serum albumin, 1 liter distilled water). After incubating at room temperature for 2 hr, the plates were washed three times with ELISA wash. Fifty microliters of chromogen solution containing 0.83 mg *p*-nitrophenyl phosphate (Sigma) per milliliter substrate buffer (0.10 g $MgCl_2$, 96 ml diethanolamine, 1 liter distilled water, pH 9.8) was added to each well. Plates were incubated at 25°C and color development was stopped after 30 min by adding 50 μl of 3 M NaOH. The optical density of each well was measured at 405 nm with a Bio-Tek Instruments (Winooski, Vermont) EL311 microplate reader.

Alkaloids were quantified in the progeny of the selected populations by regressing ELISA values to those of a standard dilution of lysergic acid. Pure D-lysergic acid (9,10-didehydro-6-methyl-ergoline-8β-carboxylic acid) (Sigma) was diluted to 1.02×10^{-10}, 7.65×10^{-11}, 5.10×10^{-11}, 2.55×10^{-11}, 1.27×10^{-11}, and 6.35×10^{-12} M solutions in EPBST. Absorbance values of the standards were fit to a cubic regression equation and used to quantitateergot alkaloid content in each sample. A typical fit of standards to the regression line had a R^2 of 0.95 or greater, with coefficients of variation for each standard of 0.28–5.0%. Three repeated measures of each sample were analyzed and average alkaloid values were calculated for each treatment. All selected plants were histologically stained to verify the presence of the endophyte by the method of Clark et al. (1983).

Statistical Analysis of Experiment 1. To determine paternal and maternal effects, parents and progeny from the diallel cross were analyzed as a split plot design with progeny as the main plot and harvest time as the subplot. Means and variances were calculated for parents of the diallel cross and for the progeny

from each cross using the PROC GLM procedure of PCSAS (SAS Institute, Cary, North Carolina). Variances were tested using Bartlett's test for homogeneity (Little and Hills, 1978). The variances were determined to be unequal and were proportional to the mean. Data were subjected to a $\log_{10}$ transformation, and the analysis of variance was recalculated after confirming homogeneous variances among the parents and progeny.

All two-way, three-way, and four-way interactions among paternal, maternal, harvest date, and replication effects were tested (Table 1). There were no harvest date interactions, and data were combined over harvest dates to determine male, female, and male × female effects. To test for the paternal effect, the mean squares of the male × replication and male × female × replication interactions were summed and used as the error mean square. To test for the maternal effect, the mean squares of the female × replication and male × female × replication interactions were summed and used as the error mean square. To test for the male × female interaction, the mean square of the male × female × replication was used as the error mean square. Means were separated by using a Fisher's protected LSD. The parental mean of each cross was calculated,

TABLE 1. ANALYSIS OF VARIANCE FOR LOG_{10}-TRANSFORMED ALKALOID CONCENTRATION OF TWO HARVESTS AND FOUR TALL FESCUE GENOTYPES USED AS MALE AND FEMALE PARENTS IN A DIALLEL MATING DESIGN

Source	*df*	Mean squares	*F* statistic	Error term
Rep (R)	1	0.195		
Harvest (H)	1	5.769		1
(1) R * H	1	0.610		
Male (M)	3	0.421	70.2[a]	2 + 4
Female (F)	3	2.921	97.4[a]	3 + 4
M * F	5	0.105	21.0[a]	4
(2) M * R	3	0.001		
(3) F * R	3	0.025		
(4) M * F * R	5	0.005		
M * H	3	0.097	2.69 NS[b]	5 + 7
F * H	3	0.087	1.26 NS	6 + 7
M * F * M	5	0.013	0.89 NS	7
(5) M * R * H	3	0.017		
(6) F * R * H	3	0.050		
(7) M * F * R * H	5	0.019		
Residual	853			

[a]Significant at the 0.05 level of probability.
[b]NS: not significant at the 0.05 level of probability.

and the mean of the progeny tested for similarity to the parental mean using the LSD value calculated from the ANOVA for transformed data.

Statistical Analysis of Experiment 2. Progeny means were calculated for families from each maternal parent within each replication. To determine if plant selection affected alkaloid concentration, data were analyzed using the PROC GLM procedure of PCSAS (SAS Institute). To do so, family means within the low- and the high-alkaloid-containing populations and randomly selected plants from the GA-Jesup-Improved-EI base population were assigned to a randomized complete block design. Variances were tested by using Bartlett's test for homogeneity among the populations (Little and Hills, 1978). The variances were determined to be heterogeneous and were proportional to the mean. A $\log_{10}$ transformation of the data was performed, and the variances of the transformed data were tested and determined to be homogeneous. Transformed means of the three populations were separated using a Fisher's protected LSD.

Realized heritabilities were calculated for both the high and the low ergot alkaloid-containing populations for each generation of selection. Realized heritabilities were determined as the actual gain from selection divided by the expected gain from selection according to equation 1.

$$h^2 = [x_{S2}/x_{BP2} - 1]/[x_{S1}/x_{BP1} - 1] \quad (1)$$

where x_{S2} is the mean progeny alkaloid concentration of the selected high or low population, x_{BP2} is the mean base population alkaloid concentration at the time of progeny evaluation, x_{S1} is the mean ELISA value of the high or low selected plant population from the previous generation of selection or the base population, whichever is appropriate, and x_{BP1} is the mean ELISA absorbance value of the Jesup Improved population from the previous generation of selection or the base population, which ever is appropriate.

RESULTS AND DISCUSSION

Experiment 1. Maternal and Paternal Effects on Ergot Alkaloid Phenotype. Results from the analysis of variance procedure suggest that there were large male, female, and male by female interaction effects (Table 1). The statistical interaction between male and female suggests that both endophyte and plant genotypes affected alkaloid concentration. Because the endophyte is maternally transmitted, large male effects suggest the plant's genotype has a major impact on ergot alkaloid concentration in tall fescue. If plant genotype had no effect on ergot alkaloid concentration, each of the progeny would have the same concentration as the female parent.

Among the parents, DN11 and DN15 have the highest numeric concentra-

TABLE 2. ERGOT ALKALOID CONCENTRATION FOR FOUR PARENTS USED IN DIALLEL MATING DESIGN

Parent	Ergot alkaloids	
	Transformed [$\log_{10}$ (μg/kg)]	Raw data (μg/kg)
DN11	3.279	1933
DN15	3.220	1706
DN2	3.103	1345
DN12	2.879	774
LSD (0.05)[a]	0.157	

[a]Least significant difference at the 0.05 level of probability.

tions of ergot alkaloids, but they were not different from one another (Table 2). DN12 had the lowest concentration of ergot alkaloids, and DN2 was intermediate to the extremes and not different from DN15.

Because the endophyte is asexually transmitted through the maternal parent, progeny from each cross contain the same endophyte as the female parent. One potential source of variation for alkaloid concentration, therefore, is the endophyte associated with the female parent. The endophyte is not transmitted through the pollen; consequently, when the alkaloid concentration of progeny differs from that of the female parent, the difference may be attributed in part to genetic recombination of genes from the maternal and paternal parents, which is a second source of variation in alkaloids. Interactions between plant and endophyte genotypes may also affect ergot alkaloid phenotype.

The ergot alkaloid concentration in progeny from each cross ranged from 0.07 to 2.4 times the progeny mean (Table 3). When plant genotypes DN15 and DN11 were the female parent, four of the six crosses resulted in progeny means similar to the mean of the parents of each cross. These crosses are among female parents whose endophytes appear to have the genetic capacity to produce ergot alkaloids and, therefore, the plant genotype limits the expression of the ergot alkaloid phenotype. When plant genotypes DN2 and DN12 were the female parent, five of six crosses had progeny means less than the parental mean for each cross. This suggests that the endophyte associated with these maternal parents was less capable of producing ergot alkaloids, and the effect of plant genotype may have been masked by the endophyte effect.

High alkaloid expression is likely only when the endophyte and the plant genotype have the genetic capacity to complement each other for alkaloid production. Thus, alkaloid concentration could potentially be limited by plant

TABLE 3. MEAN PROGENY ERGOT ALKALOID CONTENT FROM DIALLEL MATING DESIGN AMONG HIGH- AND LOW-ALKALOID-CONTAINING TALL FESCUE PLANTS

Parents		Transformed mean	Raw data		
Male	Female	[$\log_{10}(\mu g/kg)$]	Mean ($\mu g/kg$)	Range ($\mu g/kg$)	Variance $(\mu g/kg)^2$
DN15	DN2	3.063	1300	386-3131	407,861
DN15	DN12	3.022	1201	165-3015	373,847
DN15	DN11	3.084	1515	107-3920	764,785
DN11	DN2	3.041	1228	215-3691	343,994
DN11	DN12	3.037	1248	233-4500	470,404
DN11	DN15	3.147	1653	523-6400	1,127,440
DN2	DN12	2.891	935	70-2665	274,398
DN2	DN11	2.995	1136	331-4157	459,426
DN2	DN15	2.996	1126	355-4320	407,325
DN12	DN2	2.823	751	178-1759	139,584
DN12	DN11	2.903	938	191-3485	325,425
DN12	DN15	2.799	745	119-2083	192,054
LSD (0.05)[a]		0.104			

[a] Least significant difference at the 0.05 level of probability.

genotype, endophyte genotype, or both. To the plant breeder, it is important to know if alkaloid concentration is limited by plant or endophyte to effectively select plants that reduce the alkaloid production by the endophyte. To identify whether plant alkaloid concentration is due to endophyte or plant genotype, plants testing low in alkaloid concentration may be crossed as females to a known high-alkaloid paternal parent, and the progeny screened for alkaloid concentration.

Experiment 2. Heritability When Selecting for Low and High Ergot Alkaloid Concentration in E+ Tall Fescue. Means of the selected populations in generation 1 differed by as much as 30% of the base population (Table 4). Populations that were divergently selected for ergot alkaloid concentration were different from the base population and from one another after one generation. A second generation of selection further reduced ergot alkaloids in the low-alkaloid population. Alkaloid concentration increased in the high-alkaloid population following the second generation of selection. Therefore, progress can be made when selecting for high or low ergot alkaloid containing plant populations.

When the nontransformed data were incorporated into equation 1, the realized heritability estimates were 0.56 and 0.49 for the low- and the high-alkaloid

TABLE 4. MEAN ERGOT ALKALOID CONCENTRATIONS OF POPULATION AND FAMILIES (12 PROGENY FOR EACH FAMILY) WITHIN LOW AND HIGH ERGOT ALKALOID POPULATIONS OF JESUP IMPROVED TALL FESCUE

	Ergot alkaloid					
	Generation 1			Generation 2		
Population	N^a	Raw data (μg/kg)	Transformed [$\log_{10}$ (μg/kg)]	N^a	Raw data (μg/kg)	Transformed [$\log_{10}$ (μg/kg)]
Low	37	1231	2.92	48	237	2.13
Base	25	1722	3.08	48	1223	2.92
High	44	2304	3.19	48	1662	3.14
LSD (0.05)[b]	low vs. base	1092	0.12	LSD (0.05)	896	0.10
	high vs. base	1061	0.11			
	low vs. high	754	0.08			

[a] Number of families used to characterize mean ergot alkaloid concentrations within the low and high population; number of individuals used to characterize alkaloid concentration for the base population of Jesup Improved.
[b] Least significant difference at the 0.05 level of probability.

containing populations from the first generation of selection, respectively. The mean of the parents for the second generation of selection were 104 μg/kg in the low population, and 4113 μg/kg in the high population. Realized heritabilities for the second generation of selection were 0.91 and 0.45 for the low- and high-alkaloid containing populations, respectively.

In a similar study, Agee and Hill (1994) determined that ergot alkaloid concentration was higher in greenhouse-grown plants than those grown in the field, especially during summer, fall, and winter. Relative rankings for the high- or low-alkaloid trait were stable among plant genotypes over three locations in that study. Therefore, growing plants in the greenhouse is more likely to permit E+ tall fescue genotypes to reach their potential for alkaloid production than field locations. Hence, screening progeny of plants that had been selected for low or high alkaloids was limited to the greenhouse in this study.

Based on realized heritability estimates, progress was made in selecting and breeding tall fescue populations that are low or high in ergot alkaloid concentration. After two generations of selection, the low-alkaloid population had an 86% decrease and the high-alkaloid population had a 36% increase over the base population. Tall fescue is an autoallohexaploid plant, which makes gene action difficult to assess. In a previous study, the alkaloid trait was found to be normally distributed among progeny from a cross between high- and low-alkaloid parents (Agee and Hill, 1994). However, heritability improved and gain increased in the second generation of selection in the low-alkaloid population. Therefore, it is likely that the low-alkaloid trait is controlled by multiple genes that are either additive, recessive, or both.

Crawford et al. (1989) conducted studies to determine the relationship between the level of endophyte infection and weight gain in cattle grazing tall fescue. One hundred eighty yearling steers and heifers were grazed on a series of 0.81-ha tall fescue pastures over a 3-year period. Pasture endophyte infection ranged from 3 to 83%. The relationship between endophyte infection frequency (EIF) and average daily gain (ADG) was described by the following equation:

$$\text{ADG} = 0.662 - 0.0045 \times \text{EIF} \quad (2)$$

where ADG is kilograms per day and EIF is expressed as a percentage. Therefore, ADG was decreased by 0.045 kg/day for each 10% increase in EIF. Assuming that a reduction in alkaloid concentration had a similar effect on ADG, an 86% reduction in ergot alkaloid levels from a 100% infected stand of GA-Jesup Improved-EI tall fescue would improve ADG in cattle from 0.21 kg/day in the base population to 0.60 kg/day in the population grazing the 86% reduced ergot alkaloid containing fescue. Therefore, breeding tall fescue for reduced levels of ergot alkaloid concentration should improve the profitability of the livestock producer if all other inputs remained equal.

It remains to be determined what effects reduced ergot alkaloid levels have

on tall fescue persistence, insect resistance, and tolerance to environmental stresses. A better understanding of the ecological significance of the ergot alkaloids and the interactive effects of other endophytic alkaloids is imperative to developing E+ tall fescue populations that are persistent and provide improved animal performance. In this study, it was easier to make progress towards lower than higher ergot alkaloid levels. This suggests that populations of tall fescue have been selected for the high-alkaloid condition in agricultural ecosystems and that ergot alkaloids may be an important component to the persistence of the population. In addition, little is understood as to what effect breeding might have on the plant/endophyte relationship. Selection against fungal secondary products, e.g., ergot alkaloids, may result in a concomittant selection for plants that are antagonistic to the endophyte (Hill, 1993). If so, the selected population may not retain the beneficial agronomic qualities of the original E+ population, and this may result in a population with reduced persistence. Hence, the ecological consequences of reducing ergot alkaloids within E+ tall fescue need to be ascertained prior to distribution of such populations to the marketplace.

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EXPLANATION OF BITTER TASTE OF VENOM OF PONERINE ANT, *Pachycondyla apicalis*

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Abstract—The venom gland of workers of *Pachycondyla* (= *Neoponera*) *apicalis* (Hymenoptera: Formicidae) contains the bitter-tasting cyclic dipeptide of leucine and phenylalanine [cyclo-leu-phe or 3-benzyl-6-(2-methylpropyl)-2,5-piperazinedione]. The venom also contains proteins of undetermined activity. It is suggested that the function of the venom may be both defensive and offensive. The mandibular glands of *N. apicalis* contains δ-decalactone and benzaldehyde.

Key Words—Ponerine ant, venom, bitter dipeptide, diketopiperazine, mandibular gland secretion, Dufour gland secretion.

INTRODUCTION

Schmidt (1986) has pointed out that the venoms of social bees and wasps have essentially one function, i.e., defense in its broad sense, and the venom of solitary Hymenoptera have the function of prey capture, with possibly a minor function of defense, but the ant venoms have a great variety of functions. We tend to think of venoms as injectable, stinging defensive agents, but Schmidt points out that for ants they can also be topically applied defensive agents; trail, alarm, sex, queen recognition, aggregation, attractant–recruitment and recognition pheromones; as well as repellents and toxic agents for prey capture (Schmidt, 1986). In further expansion of this theme, he pointed out that some venoms are gustatory repellents. He listed, among others, several ponerine spe-

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cies that have venoms with a bitter, burning taste (to humans); among these he mentioned *Pachycondyla* (= *Neoponera*)A *apicalis* (Schmidt, 1986).

We have examined the venom of *Pachycondyla apicalis* and identified a known bitter compound that is recorded here in a venom and in an ant for the first time. We have also examined the mandibular glands and postpharyngeal glands of this species.

METHODS AND MATERIALS

Maintenance of Colony. Workers of *Pachycondyla apicalis* were collected in Tapachula, Chiapas, Mexico. The ants were reared in an artificial nest made from a small plastic box partially filled with moistened plaster of Paris. The ants were kept in the laboratory on a diet of sugar solution and dead flies.

Preparation of Glands for Gas Chromatographic Analysis. The samples for injection were prepared by cooling individual ants in a refrigerator, then dissecting out the venom reservoir in distilled water under a binocular microscope. The upper part of the head capsule was used for postpharyngeal glands. Whole heads of workers were used to examine the mandibular glands. Excess water was removed by touching the gland with a fragment of filter paper and the tissue was then attached to a fragment of glass and placed individually in short glass capillaries sealed at one end; the other end was then sealed with a flame (Morgan, 1990).

Gas Chromatography–Mass Spectrometry. Chromatography was carried out with a Hewlett-Packard 5890 gas chromatograph directly coupled to a 5970B mass selective detector. The system was controlled by a Hewlett-Packard Series 300 computer with HP 59970C Chemstation.

Chromatography was performed on an immobilized polydimethylsiloxane phase (equivalent to OV-1) in a fused silica column (12 × 0.2 mm) (SGE, Milton Keynes, UK). Helium was used as carrier gas at 1 ml/min. The sample was heated in the injector to 200°C for 4 min before crushing the capillary, as described by Morgan and Wadhams (1972). The oven was programmed from 30°C (2 min) at 8°C/min to 280°C for venom and mandibular gland secretion. The identification of the compounds was confirmed by comparison of their mass spectra and retention times with those of synthesized material.

Microozonolysis, and hydrogenation of Dufour gland substances were carried out as described by Attygalle and Morgan (1983). Analysis of cuticular hydrocarbons was as described by Bagnères and Morgan (1990).

Synthesis of Cyclic Peptide. The cyclic dipeptide was first synthesized following the method of Johnstone and Povall (1975) by heating methyl phenylalanyl-leucinate (50 mg) previously prepared from phenylalanyl-leucine with diazomethane, in refluxing dimethyl formamide (3 ml) for 12 hr. The product

was obtained by evaporation of the solvent in vacuo. It was also prepared by heating phenylalanyl-leucine itself (10 mg) in dimethyl formamide (4 ml) at 100–110°C for 10 hr. The product was purified by sublimation at 160–170°C under water pump vacuum. Its purity was checked by gas chromatography and NMR spectroscopy. The reaction was carried out with both (L)-phenylalanyl-(L)-leucine and (D, L)-phenyalanyl-(D, L)-leucine (both Sigma, Gillingham, Dorset).

Cyclo-(L)-leucyl-(L)-phenylalanyl was produced by heating (L)-leucyl-(L)-phenylalanine in dimethylformamide and purifying the product for sublimation, which gave essentially one enantiomer. ^{1}H NMR spectrum (270 MHz) in CF_3CO_2D: δ 0.184 (1H ddd, J approx. 14 Hz, 10.74, 3.42 Hz), δ 0.805 (3H, d, J 6.34 Hz), δ 0.847 (3H, d, J 6.34 Hz), δ 1.200 (1H, ddd, J 13.7, 10.74, 3.42 Hz) δ 1.47 (1H, m), δ 3.206 (1H, dd, J 14.16, 4.88 Hz), δ 3.432 (1H, dd, J 14.16, 4.39 Hz) δ 4.165 (1H, dd, J 11.23, 3.42 Hz) δ 4.804 (1H, dd, J, 4.88, 4.39 Hz), δ 7.20 (2H, m), δ 7.40 (3H m). The ^{13}C NMR spectrum (67.5 MHz, in CF_3CO_2D) had absorptions at 20.7 and 23.3 ppm (q, CH_3); 25.3 ppm (t, CH_2); 40.7 (d, CH); 44.6 (t, PhCH_2); 54.8, 57.9 (d, CHCO); 130, 131, 131.8, 135 (phenyl); 172.1, 174 (s, CO).

The mass spectra were very similar for the isomers: cyclo-L-phenylalanyl-L-leucyl (and its enantiomer) M^+, 260 (30), 204 (71), 175 (4), 169 (28), 141 (54), 120 (16), 113 (59), 103 (9), 91 (100), 86 (12), 65 (7), 57 (16), 41 (22). The mixture of cyclo-D-phenylalanyl-L-leucyl and cyclo-L-phenylalanyl-D-leucyl gave M^+, 260 (30); 204 (60), 175 (5), 169 (30), 141 (47), 120 (12), 113 (52), 103 (8), 91 (100), 86 (9), 65 (14), 57 (15), 41 (18).

Electrophoresis of Venom Proteins. The venom of *P. apicalis* was collected in a Pasteur pipet, drawn out to a fine capillary, by piercing the venom sac of a dissected gland. The venom was then added to aqueous sodium dodecyl sulphate (SDS, 19 μl) and the solution heated to boiling for 10 min to denature the proteins.

The solution was placed in a channel of polyacrylamide gel, and solutions of bee venom, venom of *Dinoponera australis*, phospholipase A_2 from bee venom (Sigma, Gillingham), and molecular mass standards were placed in parallel channels and electrophoresis carried out. The protein bands were stained first with Coomassie blue and then with silver to obtain a more sensitively stained gel.

RESULTS

When venom glands of workers of *P. apicalis* were examined by gas chromatography–mass spectrometry for volatile compounds, essentially only two peaks, eluting close together, were seen, and these gave almost identical mass

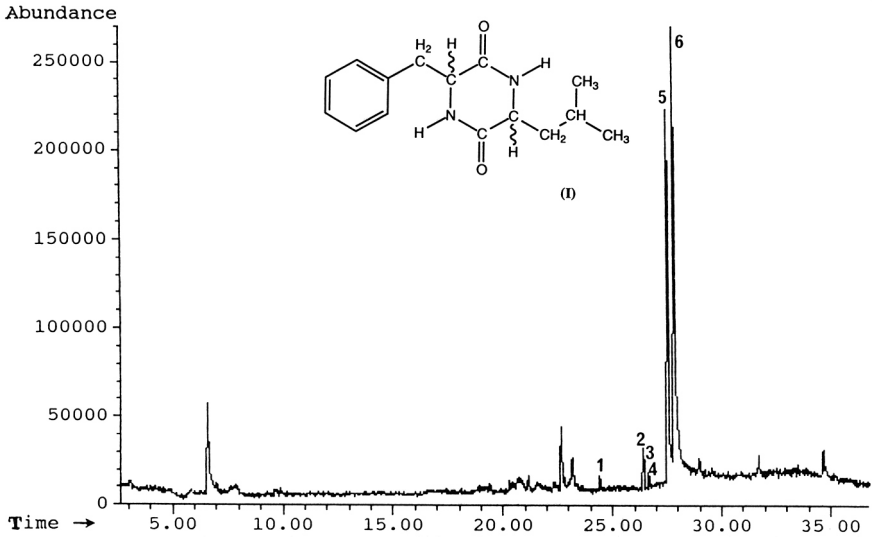

FIG. 1. Gas chromatogram of the volatile portion of the venom of *P. apicalis*. Peaks 1, 2, 3, and 4: hydrocarbons from cuticle (see Figure 2); 5: cyclo-L-leucyl-D-phenylalanyl; and 6: cyclo-L-leucyl-L-phenylalanyl, both represented by structure I.

spectra, showing they were closely similar isomers (Figure 1). Some very small amounts of linear C_{23} hydrocarbons were also evident, but these were also seen in the analysis of heads and cuticle. The mass spectra of the two major peaks showed prominent losses of benzyl and butyl groups and the presence of an ion at *m/z* 113, typical of a piperazinedione. This led us to suspect an amino-acid origin and to identification of the two substances as the diastereoisomeric forms of the cyclic dipeptide cyclo-leucyl-phenylalanyl or 3-benzyl-6-isobutylpiperazine-2,5-dione (**I**) (Figure 1). The identification was confirmed by synthesis of **I** from DL-leucyl-DL-phenylalanine, which gave two gas chromatographic peaks of equal areas and having retention times and mass spectra identical to those of the natural compounds. The mass spectrum of cyclo-leu-phe has been recorded and the fragmentation studied in detail (Szafranek et al., 1976). The spectrum recorded earlier by Heyns and Grützmacher (1966) looks unlike ours or that of Szafranek et al. (1976).

When L-leucyl-L-phenylalanine was subjected to the same cyclization process, the same compounds were formed, except that the second peak was much larger than the first, indicating that some slight racemization had occurred during cyclization, that the first-eluting peak contained cyclo-L-leu-D-phe and the second peak cyclo-L-leu-L-phe, and that the venom probably contained all four possible enantiomers. The ratio of the areas of the two peaks from the venom

(Figure 1) varied with sample but was approximately 1 : 1. When chromatography was carried out with a lower preheating and injection temperature to see if isomerization was occurring, no change was observed. There were no other volatile substances detected in the venom.

Quantification of the amount of dipeptide from three venom glands of workers was estimated by comparison with known quantities of synthetic dipeptide. Values of 2.7, 5.9, and 17.0 μg were obtained.

The ^{1}H and ^{13}C NMR spectra of the synthetic material were recorded, since these had not been available before.

The venom of *N. apicalis* was also examined by polyacrylamide gel electrophoresis (PAGE) to see if it also contained venom proteins. It was carried out in parallel with the venom of the ponerine ant *Dinoponera australis*, bee venom, and phospholipase A from bee venom. The venoms of *P. apicalis* and *D. australis* seemed identical and quite different from bee venom. The venom of *P. apicalis* gave essentially five protein bands, one of low molecular mass belonging to an unknown protein, one at ~15,000 Da, close to the phospholipase A_2 of bee venom; one at ~21,500 Da, not found in bee venom, but parallel to the soybean trypsin of the protein standards; one at ~35,000 Da, parallel to the hyaluronidase of bee venom; and one at 45,000 Da, close to the acid phosphomonoesterase of bee venom. Since the proteins of ant and bee venoms have quite different chemical structures (Leluk et al., 1989), we cannot conclude that these comigrating protein bands have corresponding enzymatic activity.

The mandibular glands contained a mixture of acetamide, benzaldehyde, and δ-decalactone in variable proportions (Figure 2). These were identified by their mass spectra and confirmed for the first two by coinjection of authentic materials. The postpharyngeal glands in the heads contained a mixture of four substances (Figure 2). The first and last eluting were readily identified as heneicosane ($C_{21}H_{44}$, M^+ 296) and tricosane ($C_{23}H_{48}$, M^+ 324) respectively. The other two (M^+ 322 and M^+ 320) eluted just before tricosane and are presumed to be a methyldocosene and a methyldocosadiene, respectively. They are not the linear C_{23} alkene and diene, because the order of elution on the column used is normally diene, then alkene. These four substances were the only ones detected when we examined a sample of cuticle using our micromethod (Bagnères and Morgan, 1990). They also appeared as contaminants in the chromatograms of the venom gland (Figure 1).

DISCUSSION

The bitter taste of some cyclic dipeptides has been known for many years, and was mentioned by Emil Fischer in 1906, but the bitter taste was studied

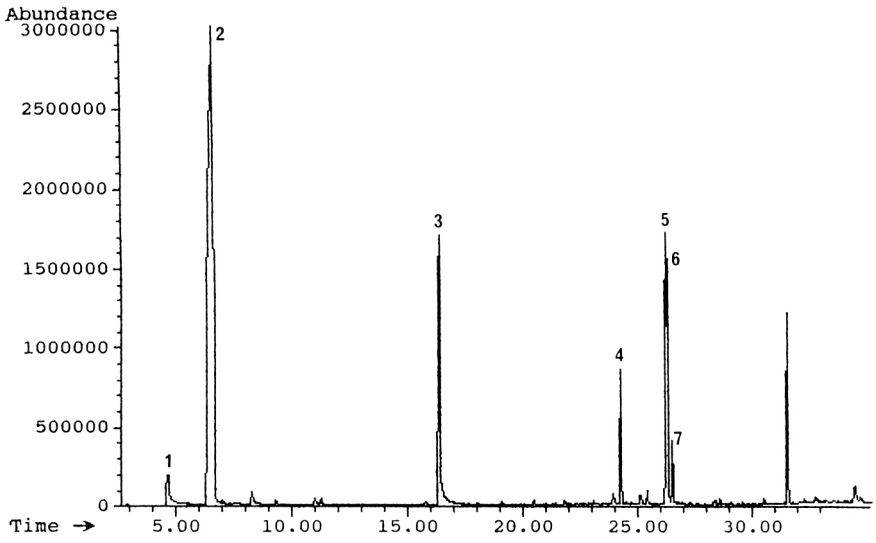

FIG. 2. Gas chromatogram of the volatile compounds in the head of a single worker of *P. apicalis*. Compounds are: 1, acetamide, 2, benzaldehyde, 3, δ-decalactone (all from the mandibular glands), 4, heneicosane, 5, unknown alkene (M^+ 222); 6, unknown alkadiene (M^+ 220); and 7, tricosane (all from the postpharyngeal gland).

systematically only much later (Matoba and Hata, 1972). It was found that there is a good correlation between bitterness and hydrophobicity of the side chains (Shiba et al., 1981) and that cyclo-leucyl-phenylalanyl is one of the most bitter compounds (Pickenhagen et al., 1975; Gardner, 1980; Ney, 1986). Moreover, the receptors for bitterness do not recognize chirality or require a strict conformation for the substrate (Shiba et al., 1981). While this information applies to human experimental subjects, generally what is bitter to us is judged to be unpleasant to other mammals by their avoidance of such materials. The presence of this cyclic dipeptide explains the bitter taste of *P. apicalis* venom reported by Schmidt (1986).

The volume of a worker venom reservoir, treated as a prolate spheroid was approximately 350 nl, and it contained on average 8.5 μg of dipeptide—a concentration of about 2.5%. We examined the venom by gel electrophoresis to see what, if any, proteins were also present. Five protein bands were detected, but we were unable to correlate them with enzymic activity.

The bitter taste suggests that the venom is a defensive agent, but Schmidt (1986) quotes this venom as having a moderate effect in causing pain. Schmidt (personal communication) also reports seeing *Pachycondyla* (= *Neoponera*) *villosa* in Costa Rica stinging wasp prey, which were then immobilized within

seconds, and the prey promptly carried off. It may well be that *Pachycondyla* venom is both offensive and, through its bitterness, defensive.

The cyclic dipeptide has been identified in insects and in venom for the first time. It is recorded as produced by the microorganism *Streptomyces noursei* (Kelley and Brown, 1966) and has been found in the skin of some amphibians (Erspamer et al., 1986), although the link with bitter taste was not apparently recognized there.

The mandibular gland secretion is noteworthy because it did not contain the alkylpyrazines that are commonly found in ponerine glands. Lactones have been identified in the secretion of cockroaches, beetles, phasmids, and bees, as well as in ants (Blum, 1981; Attygalle and Morgan, 1984). δ-Decalactone itself has been identified in the mandibular glands of stingless bees of the *Trigona carbonaria* group (Blum 1981, p. 243, quoted as Wheeler et al. 1975 but untraceable).

Blum et al. (1969) first reported benzaldehyde in the mandibular glands of a harvester ant, *Messor* (= *Veromessor*) *pergandeyi* (Myrmicinae). It was described as a defensive secretion, and a minor component of the gland was said to release alarm. Blum and Wheeler (unpublished, quoted in Blum, 1981) also found benzaldehyde in the mandibular glands of *Azteca* species (Dolichoderinae). We now have examples of benzaldehyde in the mandibular glands of ants from three subfamilies. There are several reports of benzaldehyde in the mandibular secretion of stingless bees. Blum (1981) referred to unpublished work, identifying it in *Trigona postica*, *T. xanthotricha*, and *T. tubiba*. He found benzaldehyde very attractive to workers of *T. tubiba*. Luby et al. (1973) reported it in *T. mexicana* and *T. pectoralis* but did not find any distinct behavioral response in these species, although it did enhance the alarm response of ketones also present.

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REINVESTIGATION OF FEMALE SEX PHEROMONE OF PROCESSIONARY MOTH (*Thaumetopoea pityocampa*): NO EVIDENCE FOR MINOR COMPONENTS

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Abstract—The female sex pheromone of the processionary moth *Thaumetopoea pityocampa* has been reinvestigated to look for possible minor components. Examination by GC-MS and GC-EAD of the contents of virgin female glands, after stimulation with PBAN (pheromone-biosynthesis-activating neuropeptide), showed that the major component, (Z)-13-hexadecen-11-ynyl acetate (**1**), appears to be the only pheromone compound present in the gland. Comparison of female attractivity with that of the natural extract and synthetic (Z)-13-hexadecen-11-ynyl acetate showed that this chemical is able to elicit a similar activity to that displayed by virgin females in a wind tunnel. In single cell recording experiments, two specialist receptor cell types were found in the trichoid sensilla. One cell type was tuned to enyne acetate **1**. The other one was tuned to (Z,Z)-11,13- hexadecadienal and (Z)-13-hexadecen-11-ynal, the major components of the pheromone blend of other *Thaumetopoea* spp., and constitutes a further example of interspecific inhibitor receptor cells. Our results show that the processionary moth may not need minor components for successful mate recognition.

Key Words—Sex pheromone, processionary moth, *Thaumetopoea pityocampa*, Lepidoptera, Thaumetopoeidae, minor component, single cell recording, behavior.

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INTRODUCTION

The pine processionary moth *Thaumetopoea pityocampa* (Lepidoptera, Thaumetopoeidae) is one of the most devastating pine pests in southern Europe, North Africa, and occasionally in Central Europe. Almost all species of pines can be defoliated by the larvae, but other conifers, such as cedars, can also be attacked (Montoya and Hernández, 1991). Moreover, the pest poses a serious threat in suburban areas since last-instar larvae release microscopic urticating hairs into the air, causing severe irritations on the skin and mucous membrane in humans.

Only one compound has been found so far in the female sex pheromone gland and characterized as (*Z*)-13-hexadecen-11-ynyl acetate (**1**) (Guerrero et al., 1981). The chemical has been synthesized by several routes (Camps et al., 1981a,b, 1983; Cardillo et al., 1982; Michelot et al., 1982; Stille and Simpson, 1987) and has displayed a remarkable attractant activity of males in the laboratory and in the field (Cuevas et al., 1983; Einhorn et al., 1983). This compound represents the first sex pheromone of a moth containing an enyne group (Arn et al., 1986) as an essential functionality for biological activity (Camps et al., 1988). This functional group has also been found in *Thaumetopoea wilkinsoni* Tams., which has been considered an ecotype of *T. pityocampa* by Frérot and Demolin (1993), and in *T. jordana* Powell (Frérot et al., 1990; Frérot and Demolin, 1993). We have occasionally noticed unsatisfactory results in field tests with acetate **1** and, consequently, suspected that some minor components might be missing in our formulations. Therefore, we have set out a series of experiments in the search for minor components of the sex pheromone, including a reexamination of the contents of virgin female glands by gas chromatography–mass spectrometry (GC-MS) and gas chromatography–electroantennographic detector (GC-EAD), after stimulation with PBAN (pheromone-biosynthesis-activating neuropeptide) to increase pheromone titer, comparison of the attractivity of females to natural extract and synthetic material in a wind tunnel, as well as single cell recording (SCR) experiments. We describe the results of these investigations, which suggest that the insect may not need additional minor components for successful mate recognition.

METHODS AND MATERIALS

Chemicals. (*Z*)-13-Hexadecen-11-ynyl acetate (**1**) was prepared by stereospecific coupling of (*Z*)-1-bromo-1-butene with 2-(11-dodecynyloxy)-tetrahydropyran catalyzed by tetrakis(triphenylphosphine)palladium(0) followed by hydrolysis and acetylation, as previously described (Michelot et al., 1982). The stereochemical purity was *Z*:*E* 98:2 on a SE-54 50-m × 0.32-μm-ID fused

silica capillary column. 11-Hexadecynyl acetate was prepared by alkylation of the corresponding acetylide with *n*-butyl bromide. (*Z*,*Z*)-11,13-Hexadecadienal, (*Z*)-13-hexadecen-11-ynal, and (*Z*)-13-hexadecen-11-ynoic acid were prepared by oxidation of the corresponding alcohol with pyridinium dichromate in methylene chloride for the aldehydes and dimethylformamide for the acid, repectively (Corey and Schmidt, 1979). The synthetic compounds were purified by column chromatography eluting with hexane–ether or hexane–ethyl acetate mixtures and were homogeneous by TLC, NMR, and GC analyses. Grace's medium was obtained from Sigma (St. Louis, Missouri) and Bom-PBAN I was purchased from Peninsula Lab. (Belmont, California).

Insects. Thaumetopoea pityocampa pupae were collected in an infested pine forest in Mora de Rubielos (Teruel) by personnel of the Servicio de Protección de Vegetales (Teruel) in the spring of 1990–1995, sexed, covered with a 3- to 6-cm layer of sawdust, and sent to our department. The pupae were kept in wooden boxes in a 16:8 light–dark reversed photoperiod at 23 ± 1°C and 55–65% humidity until emergence. Several toothpicks were placed standing on the sawdust to encourage perching of males after emergence. Due to the short lifetime of the insects and because young males responded better than the old ones in the wind tunnel, only individuals 8–20 hr old were used in the bioassays. Newly emerged moths were separated from the remaining pupae every day, and placed into 31- × 12- × 21-cm plastic boxes containing a 2-cm layer of sawdust.

Gland Incubation and Extraction. Tissue for gland incubations was prepared as described previously (Fabriàs et al., 1995). Briefly, the ovipositor was removed from the abdomen and placed on a drop of 0.9% NaCl aq. solution. The ovipositor valves and muscle were removed, an incision was made along the dorsal midline, and the gland was then dissected and cleaned of internal tissues. The tissue thus prepared was gently blotted on a strip of filter paper and transferred to a 10-μl drop of Grace's medium, containing 5 pmol of Bom-PBAN I. After 3 hr of incubation, glands were extracted with hexane (10 μl/gland), and aliquots of the pheromone extracts corresponding to one to three glands were mixed with 10 ng/gland of dodecyl or tridecyl acetate as internal standard for quantification.

Analytical Methods. Pheromone titer was determined by GC on a Carlo Erba 4130 gas chromatograph equipped with a flame ionization detector. Analyses were run on a Supelco SPB-5 fused silica capillary column (30 m × 0.25 mm ID, Supelco, Bellefonte, Pennsylvania), using a temperature program from 60° to 280°C at 10°C/min. Samples were injected in the splitless mode, the split valve being opened 35 sec after injection. GC-MS analyses under electron impact conditions were carried out on a Fisons gas chromatograph (8000 series) coupled to a MD-800 mass selective detector. The system was equipped with a Hewlett-Packard HP-1 fused silica capillary column (30 m × 0.20 mm ID), which was programmed from 80°C (2 min) to 225°C at 5°C/min and then to

300°C at 12°C/min. Extracts corresponding to 12 glands were concentrated to 2–3 μl and injected under the SCAN mode.

Behavioral Tests. The tests were carried out in a wind tunnel, whose features were previously described (Quero et al., 1995). Illumination of the tunnel was 3–5 lux, and the airspeed, measured at the center of the flight compartment, was 45 cm/sec. The temperature of the experiment room was 22 ± 1°C and the relative humidity 60 ± 5%. Before testing, the insects were allowed to acclimate to the tunnel conditions for 1 hr. The toothpicks supporting one to two males were carefully fixed on a polyurethane block, and placed in the tunnel on a stainless steel jack at 150 cm downwind from the source. The fan and blower were turned on simultaneously and the males allowed to respond for 5 min. Moths were used only once and then discarded. The synthetic pheromone and the natural extract were dissolved in nanograde hexane, and the volume required to achieve the test doses applied to a 1.5- × 1-cm cotton wick. The solvent was allowed to evaporate and the lure suspended at 18 cm from the top and 40 cm from the far end of the tunnel. When virgin females were used as the pheromone source, five individuals were placed in a 6.5- × 4 × 3-cm stainless steel cages of 2- × 2-mm mesh and hung on the same holders as the wicks. Males were scored according to the following behaviors: wing fanning and taking flight (TF), arrival to the middle of the tunnel (65 cm, HW), close approach to the lure (ca. 10 cm, CA), and contact with the source (SC). Only those males arresting at the source for a minimum period of 5 sec were recorded as SC. The behavioral effect was calculated as the percentage of males showing a particular behavior relative to the total number of insects released. Generally, 10–15 insects were assayed per day, while a total of 25–37 males was considered for each treatment, except in the choice experiment between the synthetic pheromone and virgin females in which 12 males were tested. The combined data corresponding to the same treatment were subjected to statistical analysis using a chi-square 2 × 2 test of independence (Sokal and Rohlf, 1969). Bars bearing the same letter are not significantly different at $P < 0.05$.

In the behavioral test with PBAN-treated females, the following protocol was applied. To the seventh abdominal segment of five virgin females 10–18 hr old was applied 20 pmol PBAN in 20 μl of Meyers and Miller's saline (Meyers and Miller, 1969) 1 hr before the onset of the scotophase. After 2 hr of incubation, the insects were introduced in the tunnel room, acclimated for one additional hour, and were then ready for the assay. Only females that had adopted the calling position were considered for the test.

GC-EAD. A Carlo Erba Vega 6000 gas chromatograph, equipped with a BP-20 25-m × 0.22-mm-ID (SGE, Austin Texas) fused silica capillary column and a flame ionization dectector (FID), was linked to an EAG set up (Guerrero et al., 1986) through a deactivated fused silica column (0.32 mm ID). The oven temperature was kept at 80°C for 1 min and then programmed at 10°C/min to

240°C and held at this temperature for 10 min. The effluent from the column was split between the FID and the antennal preparation, using a variable outlet splitter (SGE) with a built-in makeup (II) gas tee. The ratio between the EAD and the FID effluents was 5.4 : 1 and the flow of the makeup II was 30 ml/min. The transfer line to the antenna was heated to 230°C and directed to a purified airstream of 300 ml/min. In the EAG preparation mounted on a live insect, the indifferent electrode was inserted onto the base of the head while the recording electrode housed the antennal tip, which was bathed in Ringer solution. The input electrode was connected to a high impedance amplifier (100×) and the amplified signal sent to a storage oscilloscope (Guerrero et al., 1986).

Electrophysiology. Single sensillum recordings (SSRs) were performed on whole insect preparations according to the standard tip recording technique (Kaissling and Thorson, 1980). Glass electrodes were filled with sensillar saline for the recording electrode and with hemolymph saline for the reference electrode, which covered the tip of the antenna after excising the more distal segments. The tips of several sensilla were cut off using sharpened forceps, and the recording electrode was slipped over the cut end of one hair. The SSR responses were filtered (150–5000 Hz) and amplified (1000×). The recordings were stored on a PC-AT microcomputer via a DASH 16 (Metrabyte) analog-to-digital conversion board. Acquisition and analysis of the recordings were performed by programs home-developed in Asyst (McMillan Software Co.). Records were plotted with Awave, a software developed in Visual C++ under MS-Windows (Marion-Poll, 1995).

RESULTS

Initial analyses of gland extracts showed that females produce minute amounts of pheromone in the laboratory (< 1 ng/individual). Therefore, we decided to stimulate pheromone production by incubating pheromone glands with PBAN. Analyses of PBAN-treated female glands presented, as expected, a notable enhancement of the pheromone contents of up to 40 ng/specimen (Figure 1). This afforded us a more reliable way to search for minor components in the gland. In GC-MS (SIM mode) diagnostic ions at *m/z* 79 and 94 for the conjugated enyne group appeared only in two compounds with retention times of 28.75 and 32.62 min. The two compounds were identified as (*Z*)-13-hexadecen-11-ynonic acid and (*Z*)-13-hexadecen-11-ynyl acetate (the expected pheromone **1**), respectively, by comparison of their mass spectra (Figure 2) and chromatographic behavior with authentic samples. Careful analysis of every peak present in the mass chromatogram did not show any evidence for any compound expected from the biosynthetic pathway or having a structure similar to that of known pheromone components.

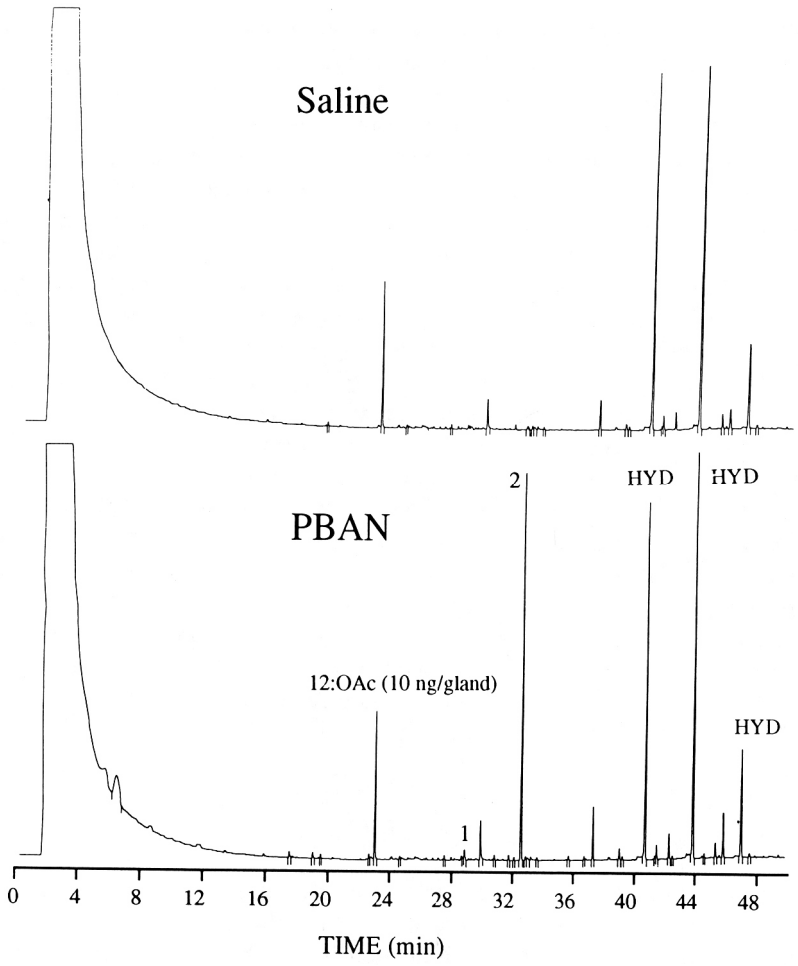

FIG. 1. GC-FID trace of in vitro PBAN-stimulated gland extract and saline control. Dodecyl acetate (10 ng/gland) was added as internal standard. See Methods and Materials for details. Peak **1**: (*Z*)-13-hexadecen-11-ynoic acid; peak **2**: (*Z*)-13-hexadecen-11-ynyl acetate; HYD: hydrocarbons.

These results were confirmed when GC-EAD analyses of the natural extract were carried out. Thus, the male antenna elicited a significant depolarization response (0.5 mV) to the natural pheromone, while a lower depolarization was also detected at the internal standard (tridecyl acetate) retention time (Figure 3).

In the wind tunnel, virgin females, PBAN-treated females, and a natural extract containing 600 ng of acetate **1** elicited similar responses on males dis-

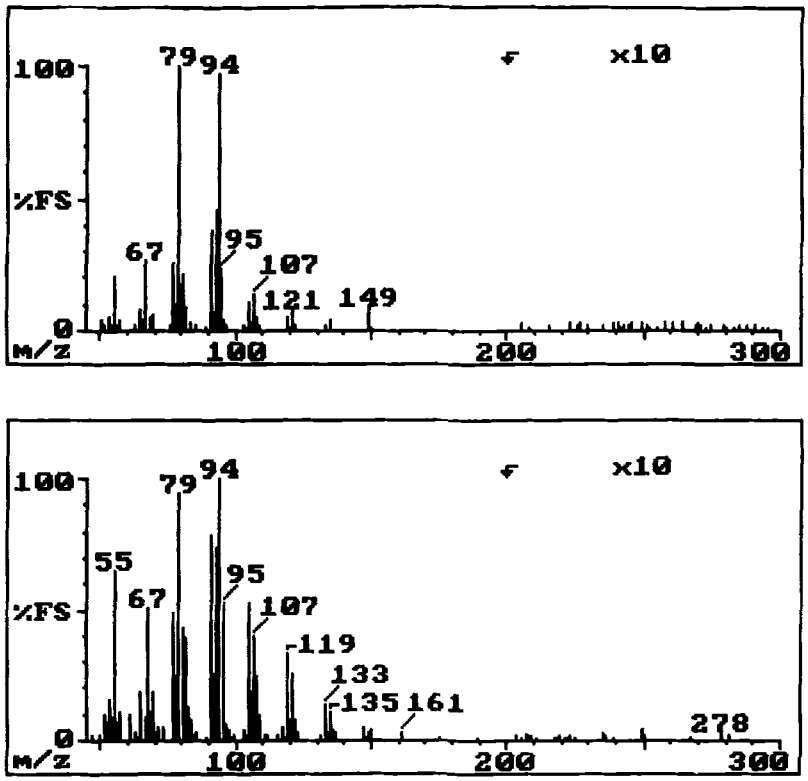

FIG. 2. Mass spectra of (Z)-13-hexadecen-11-ynoic acid (top) and (Z)-13-hexadecen-11-ynyl acetate (bottom) obtained in in vitro PBAN-stimulated glands extract.

playing every type of behavior (TF, HW, CA, and SC) (Figure 4). The number of males taking flight was 92–100%, of which 56–65% successfully attempted copulation with the source. In a separate experiment, virgin female attractivity was also compared with that of 1 μg of the synthetic compound **1**. In both cases, a large number of males successfully moved close to the lure (97% responding to the synthetic pheromone and 86% to the caged females), but only 65% attempted copulation with the females, a value significantly lower ($P < 0.05$) than the number of males contacting the pheromone lure (90%) (Figure 5). In the presence of 0.5 and 0.1 μg of compound **1**, the number of contacts decreased to 70% and 20%, respectively (results not shown). When males were simultaneously attracted to females and synthetic pheromone (1 μg), the number of individuals eliciting every type of behavior was significantly lower ($P < 0.05$) than in the presence of females or the chemical alone. The number of source

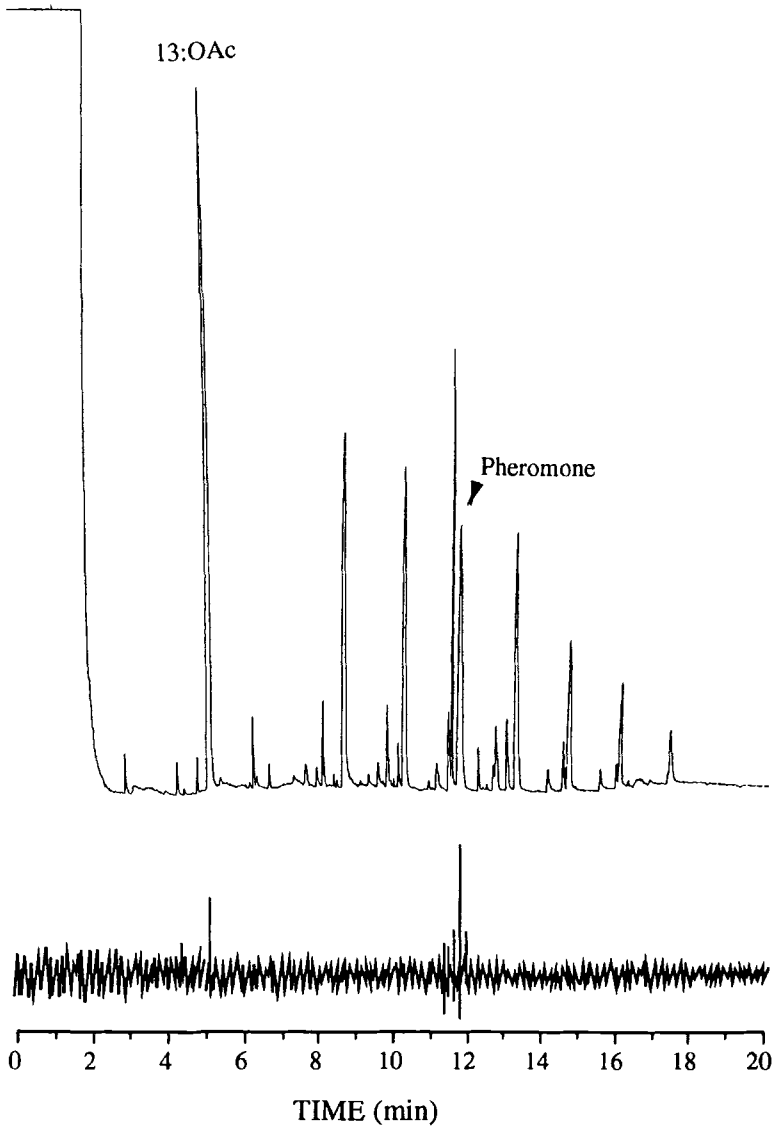

FIG. 3. GC-FID (top) and GC-EAD (bottom) traces of in vitro PBAN-stimulated glands extract showing depolarization responses to the natural pheromone and, to a lesser extent, to tridecyl acetate, which was added to the preparation as an internal standard. See Methods and Materials for details.

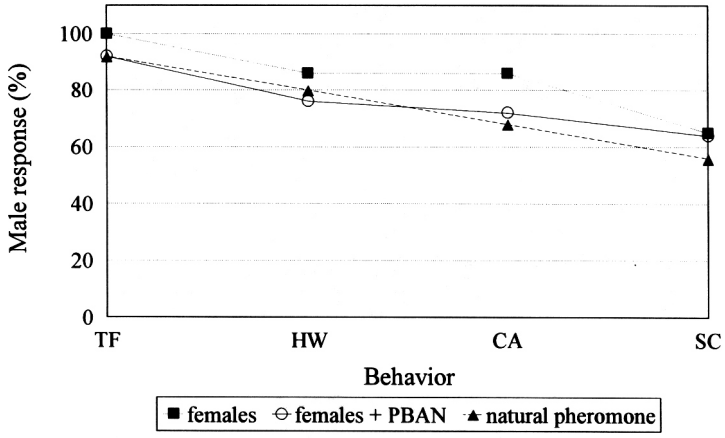

FIG. 4. Percentage of males responding to virgin females, PBAN-treated virgin females and natural extract containing 600 ng of pheromone in a wind tunnel. TF: taking flight, HW: upwind flight and arrival to the middle of the tunnel, CA: close approach to the lure (ca. 10 cm), SC: source contact.

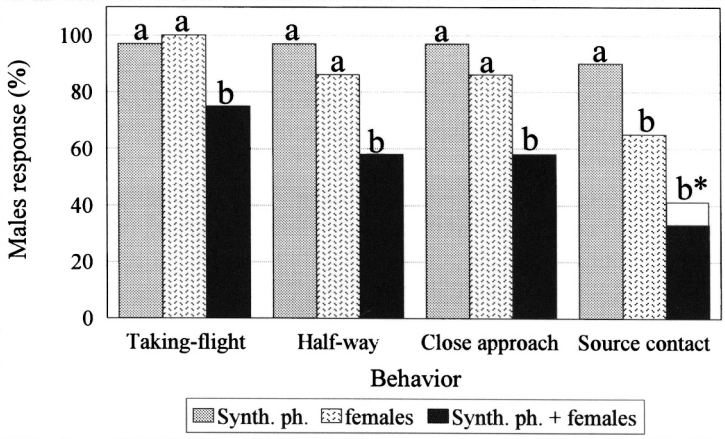

FIG. 5. Percentage of males responding to synthetic pheromone (1 μg) in comparison to virgin females in a wind tunnel. Histograms with different letters are significantly different using a chi-square 2 × 2 test of independence ($P < 0.05$). *In SC behavior: ■, mean percentage of males contacting the lure with synthetic pheromone; whereas □, mean percentage of males contacting with females cage.

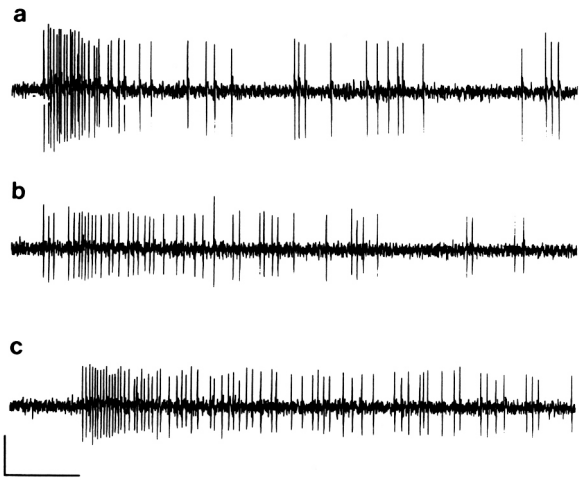

FIG. 6. Pheromone receptor cell responses from a trichoid sensilla on the male antenna after stimulation with 0.5 μg of (a): (*Z*)-13-hexadecen-11-ynyl acetate; (b) (*Z*)-13-hexadecen-11-ynal; (c): (*Z*,*Z*)-11,13-hexadecadienal. Vertical scale: 1 mV; horizontal scale: 200 msec.

contacts was 40%, of which 32% were attracted to the wick and only 8% to the females cage.

Two types of receptor cells were found in the long trichoid hairs located on the branches of the male antennae. The firing activities of these two types could be discriminated on the basis of the amplitude of the spikes they fired. A first cell type fired large spikes in response to 0.5 μg of the enyne acetate **1** (Figure 6a). The second cell type fired spikes of smaller amplitude in response to two aldehydes, i.e., (*Z*)-13-hexadecen-11-ynal (Figure 6b) and (*Z*,*Z*)-11,13-hexadecadienal (Figure 6c). No specific receptor cell for (*Z*)-13-hexadecen-11-ynol was found in the sampled olfactory hairs. The acetylenic analog, 11-hexadecynyl acetate, was found to activate the large spike cells when presented at doses over 5 μg.

DISCUSSION

Insect sex pheromones are generally composed of blends of chemicals (Roelofs, 1977; Arn et al., 1986), and only in a very few cases has a single component been substantiated as the only pheromone compound of a moth (McDonough et al., 1995). In the case of the processionary moth, the unique enyne structure orginally identified (Guerrero et al., 1981) raised the question

of whether any other minor components had possibly been missed in our investigation.

The search for new minor components in the sex pheromone of a moth relies on the extraction of large amounts of pheromone, particularly in the processionary moth, which produces, under laboratory conditions, less than 1 ng of the major compound per specimen. Furthermore, a pheromone collection from a large number of individuals is thwarted by the fact that adults are only available for three to four weeks per year. Artificial stimulation of pheromone production was achieved by incubation of isolated glands with PBAN (Fabriàs et al., 1995). However, a reexamination of virgin female glands, which had been treated or not with the neuropeptide, failed to disclose any clues that would point to the presence of a new component in the extract. Only traces of (*Z*)-13-hexadec-11-ynoic acid were also detected as well as the expected compound **1** (see below).

In wind-tunnel bioassays, extracts of female pheromone glands elicited in males very similar responses to those brought about by calling females, previously activated in vivo or not with the neuropeptide. In the same type of tests, the synthetic compound **1** induced in males the complete mate-finding sequence, in a similar manner as did virgin females. Furthermore, the number of males displaying SC behavior was clearly higher when the lure contained 1 μg of the synthetic pheromone **1**. Lower amounts of **1** (0.5 μg) elicited lower numbers of contacts with the source (70%), a similar value obtained in the presence of virgin females, while 0.1 μg of **1** only induced arresting at the source on 20% of males. In a competition experiment using caged females and the synthetic chemical (1 μg), males preferentially made contact with the cotton wick containing the chemical. Although the dose of **1** was apparently far above the amount of pheromone found in female glands, the intrinsic difference of the two emission sources should be pointed out. Thus, the female gland shows a dynamic release vs. a more continuous, permanent emission of pheromone from the wick. In addition, the higher volume of the cotton wick than that of the female gland may cause a greater adsorption of the attractant onto the surface.

In previous biosynthetic studies (Fabriàs et al., 1989; Arsequell et al., 1990), we showed that the main pheromone component is biosynthesized from palmitic acid through the combined action of Δ-11 and Δ-13 desaturases. The acyl-CoA intermediates were identified as their corresponding methyl esters, i.e., (*Z*)-11-hexadecenoate, 11-hexadecynoate, as well as (*Z*,*Z*)-11,13-hexadecadienoate and other minor fatty acid esters. By gland incubation of appropriately labeled precursors we proved that the acetylenic acid, and not the dienoic acid, was the precursor of the pheromone enyne system (Barrot et al., 1994). Accordingly, the corresponding 11-hexadecynyl acetate was not found in female gland extracts, activated or not with PBAN. The acetylenic acetate, however, behaved as a pheromone mimic, being able to induce the complete mate-finding

sequence in the wind tunnel (Quero et al., 1995) and to attract a good number of males in the field (Camps et al., 1988). Its activity was, nevertheless, lower than that of the synthetic pheromone. In SSR the acetylenic acetate activated the receptor cell type tuned to compound **1**, but only at high concentrations.

In the same way, no traces of (*Z*,*Z*)-11,13-hexadecadienyl derivatives (alcohol, acetate, or aldehyde) were found in the gland, although the methyl ester was detected after methanolysis of the gland extract in our biosynthetic assays. Interestingly, in SSR experiments, a receptor cell type of the male antenna responded to (*Z*,*Z*)-11,13-hexadecadienal, the major component of *T. pinivora* and *T. bonjeani* sex pheromones (Frérot and Demolin, 1993). On the other hand, (*Z*)-13-hexadec-11-ynoic acid, found in minute quantities in PBAN-treated females, did not exhibit any synergistic effect when mixed with the synthetic pheromone in field tests. The presence of this acid in the hexane extract might indicate that PBAN acts by stimulating the release of the enynoyl precursor from the lipidic pools for further reduction and acetylation. (*Z*)-13-Hexadecen-11-ynal, the main component of the sex pheromone of *T. jordana* (Frérot and Demolin, 1993), activated a second receptor cell and was found to be a very potent inhibitor of the upwind flight response of males when mixed with the synthetic pheromone even at a 1:99 ratio (Quero et al., 1995).

In summary, and within the limits of our study, the processionary moth apparently uses (*Z*)-13-hexadecen-11-ynyl acetate as the only pheromone compound, although the male antenna contains receptor cells responding to other unsaturated aldehydes, which are major components of the pheromone blend of other *Thaumetopoea* spp. Thus, the receptor-cell type tuned to these aldehydes constitutes a further example of interspecific inhibitor receptor cells, which have been described in several moths (Lucas and Renou, 1989; Zagatti et al., 1991). The presence of an enyne functionality confers an unique structure to the pheromone of some members of the Thaumetopoeidae family, in comparison with those of other lepidopteran forest pests, such as *Rhyacionia buoliana*, *Choristoneura fumiferana*, or *Eucosma sonomana* (Borden, 1990), among others. This suggests that in the processionary moth the presence of minor components may not be necessary to ensure specificity of the pheromone communication system of the insect and that the unsatisfactory field results eventually observed must be caused by other factors.

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MILD CONDITIONED FOOD AVERSIONS DEVELOPED BY SHEEP TOWARDS FLAVORS ASSOCIATED WITH PLANT SECONDARY COMPOUNDS

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Abstract—The objectives of the experiment were to test whether sheep develop conditioned flavor aversions (CFAs) towards a food flavor associated with the administration of a plant secondary compound, and providing that such CFAs develop, to determine how long they persist. For experimental purposes, two natural secondary compounds were used—quebracho (mainly condensed tannins) and oxalic acid—at doses previously known to produce negative post-ingestive consequences in sheep. The experiment consisted of four conditioning periods (each eight days long) during which the novel flavored foods (flavored hay) were offered concurrently with oral administration by gavage of the secondary compounds. In the conditioning period four groups of sheep (N = 6 each) were arranged such that each group received different flavored feeds (orange and aniseed) for two-day sequential periods. One flavor was paired with one of the model secondary compounds. Between the two sets of two days and after the second two-day period there was a period of two "rest" days, to avoid residual physiological effects of secondary compound administration. The intakes of flavored hay were recorded during each conditioning day and the effects of the secondary compounds were quantified in rumen (for volatile fatty acids and ammonia concentrations) and blood samples (for plasma calcium levels). At the end of each conditioning period, the preference for the two flavors was measured by a two-choice short-term preference test (20 min each); the persistence of the CFAs was similarly measured at 0, 10, 30

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and 60 days after the completion of the conditioning with two-choice long-term preference tests (3 hr each). Results of the experiment indicated that quebracho administration did not lead to development of CFAs at the level administered. This does not necessarily reflect the sheep's inability to develop CFAs towards tannins but may reflect possible trade-offs between the ingestion of nutrients and secondary compound concentration that might have been imposed on the sheep in this experiment. In other words, it might reflect the increased familiarization with the hay (i.e., increase in its intake and hence of nutrients), which overcame the negative effects of quebracho administration. However, oxalic acid caused CFA in sheep: hay intake tended to be reduced during the days that the secondary compound was administered, and importantly the secondary compound associated flavored hay was partially avoided during the two-choice preference tests. The latter avoidance was not affected by time (consistent across conditioning periods). This CFA persisted for up to 60 days after the completion of conditioning in the absence of intervening oxalic acid exposure. Thus, sheep were able to form CFAs towards a flavor associated with the administration of a naturally occurring organic plant secondary compound.

Key Words—Conditioned flavor aversions, condensed tannins, intake, oxalic acid, plant secondary compounds, sheep, toxins.

INTRODUCTION

Over the few years there has been some strong evidence (e.g., Launchbaugh et al., 1993; Launchbaugh and Provenza, 1994; Provenza et al., 1994a,b) that ruminants develop aversions towards food flavors that are associated with the administration of lithium chloride (LiCl). These conditioned food (flavor) aversions (CFAs) result from the strong negative postingestive consequences (activation of the emetic system) (Provenza et al., 1994) brought about by the administration of LiCl. The results of these experiments using LiCl have been put forward as a possible general explanation of the mechanism by which ruminants learn to avoid the consumption of harmful (toxic) foods, and it has been suggested that the activation of the emetic system is the predominant mechanism that ruminants use to learn to avoid such foods (Provenza et al., 1994a,b; De Rosa et al., 1994; Provenza, 1995a).

The effect of LiCl, however, is probably an extreme one and may not account for how ruminants might learn to avoid foods (plants) that contain plant secondary compounds that result in less extreme physiological consequences, such as digestion inhibition or mild toxicity. The experiment reported here was designed to give some further consideration to how herbivores might learn which plant species to eat and which to avoid while grazing pastures that contain a variety of potential foods (plant species) that vary in both nutritive value and toxicity. For these purposes we have used two plant secondary compounds that occur naturally in plants and have the potential of inducing mild negative post-

ingestive consequences: one primarily at a digestive level (condensed tannins) and the other at a systemic level (oxalic acid).

Condensed tannins are a major group of secondary compounds and are found in a wide range of plants commonly consumed by ruminants. They have been shown to affect both the nutritive value of plant forage (Zucker, 1983) and to influence diet selection (Provenza and Malechek, 1984). They do not appear to be absorbed from the digestive tract of ruminants and act by forming complexes with dietary protein and digestive enzymes (Terrill et al., 1994). Oxalic acid, also found in many plants consumed by ruminants, forms calcium oxalate crystals in blood capillaries, causing direct cellular damage as well as reducing the systemic availability of calcium (Vonburg, 1994). Although rumen microbes are known to degrade oxalic acid (Allison et al., 1977), nonadapted animals may absorb significant amounts of oxalic acid with potentially toxic consequences (James and Butcher, 1972). The primary question addressed in the experiment was whether sheep learn to develop conditioned flavor aversions when the flavor of the food is associated with the administration of one of the above example secondary compounds. The doses of the two secondary compounds used have been shown to result in negative postingestive consequences in sheep (quebracho: Robbins et al., 1991; oxalic acid: Libert and Franceschi, 1987). The experiment also addressed the question of the persistence of conditioned flavor aversion to secondary plant compounds in the absence of continual reinforcement.

METHODS AND MATERIALS

Animals and Housing

Twenty-four female sheep of the Texel × Greyface breed, born indoors in spring 1995, were used in this experiment. They had been reared indoors since weaning on a moderate quality pelleted basal food with a crude protein (CP) content of 120 g/kg and metabolizable energy (ME) content 9.4 MJ/kg fresh food. At the start of the experiment (August 1995), sheep were 5–6 months old and had a mean live weight of 45.8 (SD, 6.5) kg. At that point they were penned individually and were given a liveweight-based allowance of the basal food daily (3% of their liveweight) (Kyriazakis and Oldham, 1993); half of the food was offered in the morning (0800 hr) and half in the afternoon (1600 hr).

Experimental Procedure

Experimental Plant Secondary Compounds. Two secondary plant compounds were used. Each compound had a different site and mode of action, one acted primarily at the level of the digestive system (quebracho) by limiting

nutrient absorption (Robbins et al., 1991), and the other at a systemic level (oxalic acid) by perturbing the animal's metabolism (James and Butcher, 1972). Quebracho (Roy Wilson Dickson Ltd., Chester, UK) is a crude extract of the heartwood of *Sinopsis* spp. sold commercially for use in the leather industry but commonly used in herbivore feeding studies as a model condensed tannin (e.g., McArthur and Sanson, 1993). Quebracho consists predominantly of condensed tannins with small amounts of simple phenolics. The preparation of quebracho used in this experiment was the purest available. Although specific information on the physiological effects of quebracho are not known, it has been shown (Robbins et al., 1991) that its inclusion in diets at the 3–6% level leads to significantly reduced digestibility of dry matter, protein, and carbohydrates. Oxalic acid (Aldrich, Poole, Dorset, UK) is found at high concentrations in many plants including fodder beet (*Beta vulgaris* L.) and *Rumex* species (Libert and Franceschi, 1987).

Flavors and Experimental Food. The flavors to be associated with the administration of the secondary compounds were chosen in a series of two-choice preference tests (10 min) in which the hedonic acceptability of three flavors (aniseed, apple, and orange; inclusion 0.3% w/w within the basal pelleted food) was assessed. The order of the flavor pair offered was randomized within sheep, but all sheep received all three possible flavor pairs as a choice. These flavors and level of inclusion had been suggested as equally preferred and suitable for sheep (International Additives Ltd, Wallasey, UK). First, the flavors were mixed with maize starch in a bakery mixer (dilution 1 : 10) and subsequently each flavor mix was mixed with the pellets (basal food) in the mixer. Since aniseed and orange were the two flavors equally preferred (see Results) by the experimental animals, they were chosen as test flavors for the main part of the experiment (hedonic acceptability of novel food).

A high-quality chopped molassed alfalfa hay [crude protein (CP): 143 g/kg and metabolizable energy (ME): 9.9 MJ/kg fresh food; dry matter (DM): 860 g/kg] was the novel test food for the sheep. To prepare sheep to accept the novel food (hay), they were offered 500 g/day for three days from 0800 to 1200 hr; at 1300 hr they were given the pelleted basal food at an allowance of 2.5% of their liveweight. The feeding motivation of the sheep was thus increased by slightly decreasing their basal food allowance from 3 to 2.5%. On the third day of this familiarization period, sheep had on average consumed 319.8 $\pm$ 31.4 g/day of the novel food; this amount was considered a sufficient intake for familiarization purposes.

Subsequently the hay was mixed with either aniseed or orange flavors in a powder form (diluted as above, 1 : 10 in maize starch). Hay was perceived by us to have a strong natural smell and at the 0.3% level of inclusion we were unable to detect any strong aniseed or orange aroma. Two more levels of inclusion were therefore prepared (0.5 and 0.7% w/w) in an attempt to overcome

the natural flavor of the hay, and they were also tested for their hedonic acceptability. It was decided that the inclusion level of 0.7% fresh feed was satisfactory because the hay with this inclusion level clearly had the characteristic flavor of aniseed and orange. Hedonic acceptability tests at these three flavor inclusion levels were performed for 20 min and a preference ratio was recorded. The time of the test was increased to take account for the slower consumption of the hay. A similar preference for both flavors at all levels of inclusion was found; hay intake in 20 min was also unaffected by level of inclusion (see Results). Therefore, the 0.7% level of inclusion was used throughout the main experiment.

Conditioning. Sheep were randomly assigned to one of the two secondary compound source treatments (12 sheep per treatment). Within each treatment (secondary compound: quebracho or oxalic acid) sheep were randomly assigned to four groups ($N = 3$) and each group received a unique combination of the hay mixed with either aniseed flavor or orange flavor that was associated with the administration of one of the secondary compounds or not associated (control) (Table 1). Each conditioning period lasted for eight days, during which flavors and secondary compound were associated as follows. For the first two days one of the flavored foods was offered while dosing with the chosen substance. There followed two days (days 3 and 4) of rest, during which the background (basal) pelleted food was offered as above. These rest days were considered necessary to avoid any carryover of physiological effects into the subsequent conditioning

TABLE 1. CONDITIONING AND PREFERENCE SCHEDULE OF ONE CONDITIONING PERIOD OFFERING A UNIQUE COMBINATION OF FLAVOR AND SECONDARY COMPOUND (TOXIN) TO FOUR GROUPS OF SHEEP ($N = 3$ PER SECONDARY PLANT COMPOUND SOURCE) (SPC) (QUEBRACHO[a] AND OXALIC ACID)[b]

	Sheep groups on each of the two treatments			
Day	1	2	3	4
1	Aniseed/SPC+[c]	Orange/SPC−[d]	Aniseed/SPC−	Orange/SPC+
2	Aniseed/SPC+	Orange/SPC−	Aniseed/SPC−	Orange/SPC+
3		Rest		
4		Rest		
5	Orange/SPC−	Aniseed/SPC+	Orange/SPC+	Aniseed/SPC−
6	Orange/SPC−	Aniseed/SPC+	Orange/SPC+	Aniseed/SPC−
7		Rest		
8		20 min preference test—rest		

[a] Quebracho consists predominantly of condensed tannins (McArthur and Sanson, 1993).
[b] This schedule was repeated on four consecutive occasions.
[c] Gelatin capsules with the respective secondary compound (quebracho or oxalic acid) were given.
[d] Empty gelatin capsules were given.

event. For the subsequent two days (days 5 and 6) of the conditioning period, the opposite flavored food was offered and the animals dosed with empty capsules. The last two days of the conditioning period (days 7 and 8) were also rest days. In half the groups the order of association was reversed with sheep receiving a flavored food with empty capsules followed by the opposite flavored food together with the chosen secondary compound. The same protocol was followed for each conditioning period and was repeated four times (i.e., each animal followed the same flavor/toxin association throughout the experiment).

The flavored hay was offered to the sheep from 0800 to 1200 hr on days 1, 2, 5, and 6 of the conditioning days. At the mid-point of this period (1000 hr), sheep were orally dosed with gelatin capsules that either contained the secondary compound or were empty. The level of administration of the two test compounds were based on the likely daily food intake of sheep together with typical concentrations present in food plants. The choice of an appropriate dose rate for tannins is complicated by the wide variation in natural tannin concentrations, together with the well-known difficulties in standardizing tannin concentrations across studies because of the lack of correlation between absolute concentrations and structure of tannins and biological potency. A typical concentration of condensed tannins in browse species is 50 g/kg DM (Singleton, 1981) and inclusion rates of quebracho in previous nutritional studies have been of a similar order. Dose rates for quebracho were therefore calculated as 5% of typical hay intakes on a DM basis over the 4 hr of the test (600 g of hay for a 50-kg sheep). The dose rate of quebracho used in this experiment has previously been shown to have significant effects on nutrient absorption from the digestive tract of sheep (Robbins et al., 1991). Similarly, *Rumex* spp. typically contain 10 g/kg DM soluble oxalate (Libert and Franceschi, 1987) and the dose rate of oxalic acid was therefore set at 1% of the hay intake on a DM basis. The two compounds were thus used at dose rates that represent typical concentrations found in browse plants and, more importantly, at dose rates that have been shown to have negative postingestive consequences on sheep. Compounds were weighed into gelatin capsules, and the level of administration was adjusted (for kilograms of liveweight) to account for variation in liveweight (LW) of experimental animals (quebracho: 0.6 g/kg LW; oxalic acid: 0.12 g/kg LW). The amount of the flavored hay consumed by sheep during this period (4 hr) was measured over each of the days on which it was offered during the conditioning period. Sheep were given the basal food (pelleted ration) corrected for the hay consumption in the afternoon as usual.

Measurements Taken During Conditioning. During each conditioning period the physiological effects of the secondary compounds were quantified by blood samples and by collecting rumen fluid via a stomach tube connected to a pump. Approximately 60 ml of rumen fluid and 10 ml of blood were collected per sample. Samples were collected at 1600 hr on days 2 and 6 of the conditioning

periods. The timing of sample collection (6 hr after administration of the test compounds) was chosen to allow the development of physiological effects (Sanz and Reig, 1992; Salawu et al., 1976).

Rumen fluid was filtered through muslin and divided into two aliquots immediately following collection. Microbial activity was arrested in the aliquot of rumen fluid for ammonia analysis by adding saturated mercuric chloride (250 μl). Ammonia concentrations were determined with a continuous flow spectrophotometric method (Chapman et al., 1967). The aliquot for volatile fatty acid (VFA) analysis was acidified by adding three drops of concentrated sulfuric acid and was then stored at $-20°C$ pending analysis. At the time of analysis, rumen fluid was again filtered through muslin and centrifuged at 12,000 rpm for 15 min. To 1 ml of supernatant was added 0.25 ml internal standard (20 mM ethylbutyric acid in 20% H_3PO_4). Following transfer to a chromatography vial, 0.2 μl was injected onto the GC column. Chromatography was carried out on a Pye Unicam 4550 capillary gas chromatograph with a Supelco SBP-20 bonded-phase capillary column (0.53 μm $\times$ 30 m) with direct injection. Column oven temperature was raised from 90°C to 122°C at 3°C/min. Peak detection was by a flame ionization detector and peak areas were quantified with a Spectra Physics computing integrator. Concentrations were calculated by relating unknown peak areas to that of the internal standard. Response factors were calculated following injection of a mixture of VFAs (C_2–C_5) in approximately the same proportions and concentrations found in the rumen samples.

Blood samples were collected from the jugular vein into evacuated heparinized glass tubes (Vacutainer, Becton Dickinson, Oxford, UK) and centrifuged at 2500 rpm for 20 min before removing plasma. Plasma aliquots were stored at $-20°C$ pending analysis for calcium concentrations (Voth, 1981).

Preference Tests. During rest days sheep were fed the basal pelleted ration at their usual allowance. At the end of each conditioning period all animals were given a choice between hay with aniseed and orange flavors (Table 1). These tests (short-term preference tests) lasted for 20 min and were conducted in the morning (0800 hr) of day 8 (second day of the rest periods between conditioning.) To investigate the persistence of conditioned food aversions in the absence of continuous reinforcement (secondary compound administration), four longer preference tests, lasting 3 hr each, were performed at the end of the last conditioning period (0 day in terms of the long-term preference test; this test was conducted concurrently with the short-term test of the fourth conditioning period), and then 10, 30, and 60 days later.

Sheep were offered 500 g of aniseed- and 500 g of orange-flavored hay in separate food boxes in the short-term preference tests; 1000 g of each was offered in the long-term preference tests. The position of the flavored hays was completely randomized within each preference test. Preference for a flavored hay was calculated as the relative intake of the respective flavored hay in the choice

tests. Preference could thus be affected by hay flavor, flavor order, and secondary compound administration, and their respective interactions. Avoidance ratios for the flavored hay associated with a secondary compound were calculated for each of the four flavor/secondary compound combinations using the following formula. Avoidance ratio = flavored hay (aniseed or orange) intake associated with the particular toxic substance/total intake of flavored hay (aniseed + orange).

Statistical Analyses

Data were analyzed separately for the quebracho and oxalic acid parts of the experiment. Since the experiment consisted of either four identical (conditioning) periods, or four similar short- and long-term preference tests, it was analyzed as a split-plot design in time (Horgan and Sword, 1995) with the food (secondary compound or no secondary compound associated), flavor (aniseed or orange), and flavor presented first (flavor order) as the main effects, with individual sheep ($N = 12$) nested within treatments. This was the case for the analyses for hay intake, preference, and avoidance ratio during the short- and long-term preference tests. For hay intake during the conditioning phase and the physiological variables in which more than one measurement (sample) was taken into conditioning, the nesting structures used were conditioning phase within individual sheep and sample within individual sheep.

RESULTS

Hedonic Acceptability of Flavored Pellets and Hay

Sheep showed an equal preference ($P > 0.05$) for the pelleted food flavored (at the 0.3% inclusion level) either with aniseed (49%) or with orange (51%). Similarly, sheep manifested a similar preference for the hay flavored with aniseed or orange, for all three levels of inclusion tested (see Methods and Materials—0.3%: 43.1 vs. 56.9; at 0.5%: 51.6 vs. 48.4; and 0.7%: 53.4 vs. 46.6%, respectively; in all cases $P > 0.05$).

Intake of Flavored Hay during Conditioning

As the conditioning progressed, the consumption of the flavored hay over the 4 hr during which it was offered increased for animals both on the quebracho ($P < 0.001$) and oxalic acid ($P < 0.05$) schedules (Table 2). Hay intake in the fourth trial for sheep on quebracho rose from 383 (1st conditioning) to 634 g (4th conditioning; SE 50.0). For sheep on the oxalic acid regime hay intake rose from the first to the third conditioning (from 387 to 602 g/4 hr; SE 64.4); hay intake, however, was similar between the third and fourth conditioning offerings. No differences in intakes between hays paired with quebracho or the

TABLE 2. INTAKE OF FLAVORED HAY (g/4 hr) BY SHEEP RECEIVING SECONDARY PLANT COMPOUND (QUEBRACHO[a] OR OXALIC ACID) DURING EACH OF FOUR CONDITIONING PERIODS

Conditioning	Secondary compound administered	Toxin	
		Quebracho	Oxalic acid
1st	+[b]	394	374
	−[c]	371	400
	Mean	383	387
2nd	+	442	508
	−	479	540
	Mean	460	524
3rd	+	579	625
	−	629	580
	Mean	604	602
4th	+	630	480
	−	639	609
	Mean	634	545
	s.e.d.	56.2	72.1
Means	+	511	497
	−	529	532
Significance of:			
Toxin association		NS	†[d]
Conditioning		***	*
Interaction		NS	NS

[a]Quebracho consists predominantly of condensed tannins (McArthur and Sanson, 1993).
[b]Gelatin capsules with the respective secondary compound (quebracho or oxalic acid) were given only.
[c]Gelatin empty capsules were given.
[d]†, $P \leq 0.1$; *, $P < 0.05$; ***, $P \leq 0.001$.

control (no toxic substance) were detected (toxic substance paired: 511 vs control: 529 g/4 hr; SE 20.0, NS; Table 2). However, sheep tended to consume less from the flavored hay paired with oxalic acid compared to control hay (497 vs 532 g/4 hr, respectively, SE 16.6, $0.1 > P > 0.05$). There was no effect of flavor or flavor order on hay consumed during the conditioning periods.

Avoidance of Flavored Hay Associated with Secondary Compounds

Tests End of Each Conditioning Period (Short-Term Preference Tests). The intake of flavored hays when offered as a choice and the avoidance ratios for the flavored hay associated with the administration of the secondary compounds—as means across conditioning periods—are shown in Table 3. There was no effect of conditioning period, flavor, or flavor order (and no effect of

TABLE 3. AVERAGE INTAKE OF FLAVORED HAY BY SHEEP DURING PREFERENCE TESTS AT END OF EACH CONDITIONING (MEAN OF FOUR TESTS ± SE)[a]

Flavor choice	Secondary compound association	Quebracho		Oxalic acid	
		Intake (g)	Avoidance ratio for toxin	Intake (g)	Avoidance ratio for toxin
Aniseed	+	117.1 ± 13.9	0.56	85.7 ± 11.5	0.43
Orange	−	98.5 ± 13.3		104.5 ± 14.5	
Aniseed	−	79.8 ± 10.6		108.2 ± 14.5	
Orange	+	80.0 ± 13.8	0.49	42.1 ± 7.3	0.33
SE			0.112		0.095
Means	+	98.6 ± 10.0	0.52	63.9 ± 7.4	0.38
	−	89.1 ± 8.5		106.4 ± 10.1	
Significance of					
Flavor			NS		NS
Toxin association			NS		[b]
Interaction			NS		NS

[a] Sheep were given a choice between aniseed- and orange-flavored hays associated with either a secondary plant compound (quebracho or oxalic acid; secondary compound +) or nothing (secondary compound −). Quebracho consists predominantly of condensed tannins (McArthur and Sanson, 1993).

[b] $P \leq 0.05$.

their respective interactions) on either hay intake, the preference of a flavored hay, or the avoidance ratio. Sheep consumed significantly less of the secondary compound associated flavored hay than the control ($P < 0.05$) in the choice situation only when oxalic acid was the test substance. As a result the preference for a flavored hay was significantly affected by oxalic acid association, and this preference was consistent across conditioning periods (Table 3; Figure 1, in which the avoidance ratios for the four conditioning periods are shown). The weakest response appeared to be during the first short-term preference test.

Tests after Completion of Conditioning (Long-Term Preference Tests). There was no effect of time of test, flavor, or flavor order on the intake of flavored hays, on the preference for a flavored hay, and on the avoidance ratios for the secondary compound associated hay. Sheep consumed less of the secondary compound associated hay than the nonassociated one in the choice situation when oxalic acid was the test substance: 166 ± 18.3 vs 278 ± 24.4 g/test (P

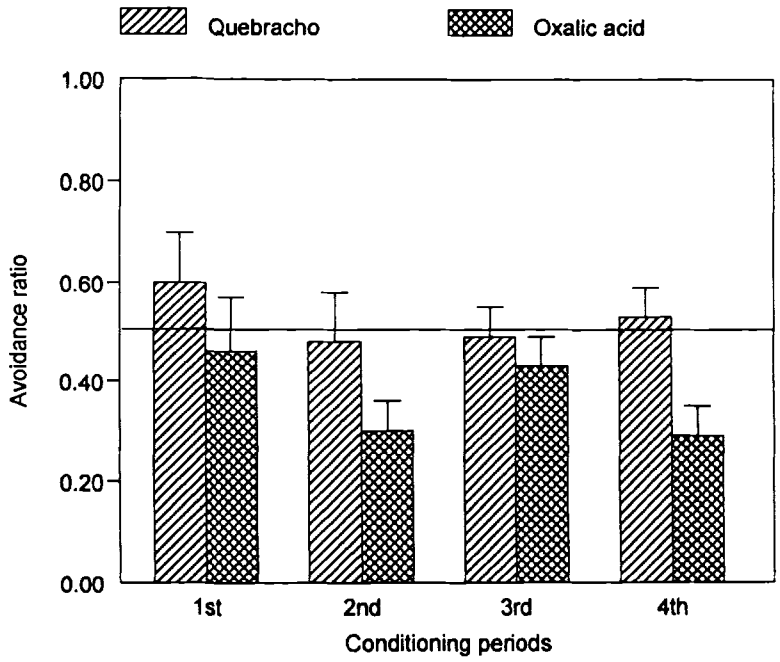

FIG. 1. Avoidance ratio of flavored hay previously associated with a secondary plant compound (quebracho or oxalic acid) by sheep during the short-term preference tests at the end of each conditioning period. The solid line represents the 0.50 avoidance ratio (i.e., equal preference/avoidance for the hay associated with the secondary compound and the control hay).

< 0.05), respectively. The consumption of both (secondary compound and nonassociated) hays in the choice situation was similar when quebracho was the test substance: 205 $\pm$ 20.6 vs 197 $\pm$ 16.5 g/test, respectively. As a result, the preference for a flavored hay was affected ($P < 0.01$) by oxalic acid association (avoidance ratio for toxin associated hay: 0.38 $\pm$ 0.033) but not by quebracho association (avoidance ratio: 0.49 $\pm$ 0.029) in all four long-term preference tests (Figure 2). The strongest effect appeared to be on day 0 and the weakest 60 days later (last test) with avoidance ratios for secondary compound associated hay of 0.31 $\pm$ 0.058 and 0.43 $\pm$ 0.072, respectively.

Effects on Physiological Variables Measured During Conditioning

Conditioning period significantly affected the concentration of all variables measured for both the secondary compounds used and their respective controls. In general, the concentrations of rumen VFAs, rumen NH_3-N, and Ca plasma

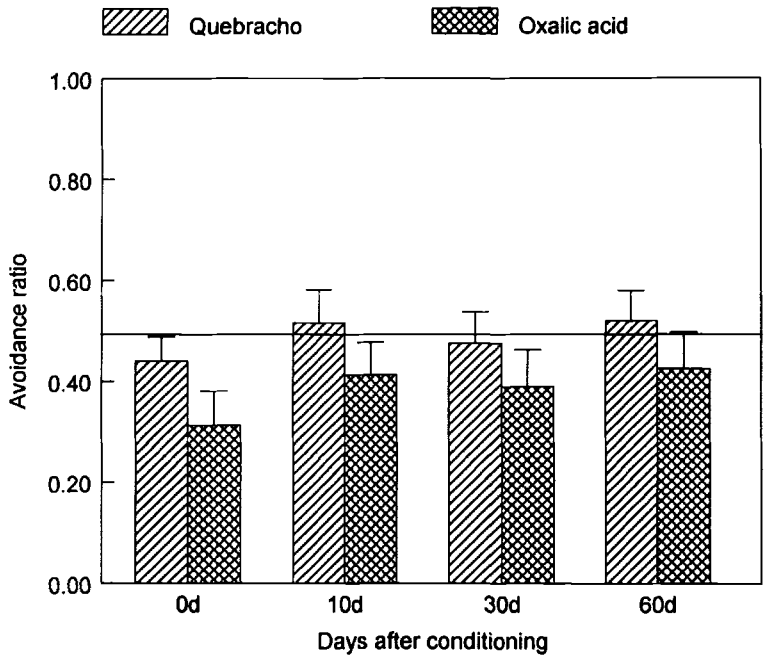

FIG. 2. Avoidance ratio of secondary plant compound associated flavored hay performed (quebracho or oxalic acid) by sheep, during the long-term preference tests performed at 0, 10, 30, and 60 days after the completion of conditioning periods. The solid line represents the 0.50 avoidance ratio (i.e., equal preference/avoidance for the hay associated with the secondary compound and the control hay).

increased as the conditioning progressed. For example, the Ca plasma concentrations for the quebracho schedule were: 105, 112, 117, 110 (SE 1.9; $P < 0.001$) and for the oxalic acid: 101, 107, 110, 109 mg/liter (SE 2.1; $P < 0.01$) for the four conditioning periods respectively. Similarly rumen NH_3-N concentrations were 68, 107, 108, 133 mg/l (SE 12.6; $P < 0.001$) for quebracho, and 79, 97, 123, 114 mg/l (SE 17.5; $0.1 < P < 0.05$) for oxalic acid.

The effects of the secondary compound administration on the physiological variables measured on the samples taken 6 hr after their administration are shown on Table 4. Oxalic acid administration significantly affected only the plasma Ca concentration, whereas quebracho administration significantly affected only the VFA concentrations of the rumen liquor (in particular the concentration of acetate, propionate, butyrate, and valerate). Rumen NH_3-N concentrations were unaffected by either of the two secondary compounds administered.

DISCUSSION

The objective of the experiment was to test the hypothesis that sheep are able to form conditioned flavor aversions toward a flood flavor associated with the administration of secondary compounds that occur naturally in food plants. These secondary compounds were chosen such that their negative postingestive consequences had a different site and mode of action. Postingestive consequences were quantified by monitoring physiological parameters relevant to the secondary compound administered to allow meaningful interpretation of the conditioned flavor aversions observed.

Physiological Consequences of Administration of Plant Secondary Compounds

We used two natural plant secondary compounds: one that was expected to act at the digestive tract level (quebracho, predominantly condensed tannins) and the other to act at a more systemic level (oxalic acid). The two model secondary compounds selected for study in this experiment represent the two broad categories of plant defense proposed by Feeny (1976). Condensed tannins fall into the quantitative defense category in that they act by limiting nutrient assimilation in a dose-dependent manner. Oxalic acid, while not a true secondary compound, shares characteristics with the so-called qualitative defenses in that its effects are acutely toxic above a certain threshold. Evolutionary advantages can be envisaged for the development of avoidance behavior for both types of secondary compounds. In the case of quantitative defenses, however, avoidance behavior is likely to be more sensitive to the balance between the nutritive composition of a particular food item and its secondary compound content.

The administration of the condensed tannins (CTs, quebracho) at the dose used was expected to lead to an inhibition of protein and carbohydrate digestion

TABLE 4. MEAN EFFECTS OF SECONDARY PLANT COMPOUND ADMINISTRATION (QUEBRACHO OR OXALIC ACID; SECONDARY COMPOUND +) OR NOTHING (SECONDARY COMPOUND −) ON CONCENTRATIONS OF RUMEN VOLATILE FATTY ACIDS (VFA) AND AMMONIA (NH_3-N), AND PLASMA CALCIUM[a]

Variable	Secondary compound							
	Quebracho				Oxalic acid			
	+	−	SE		+	−	SE	
VFA concentration (mmol/l)								
Acetate	31.3	28.1	0.64	***[b]	27.8	29.0	1.00	NS
Propionate	11.4	9.7	0.31	***	10.4	9.9	0.55	NS
Isobutyrate	0.31	0.38	0.040	NS	0.41	0.37	0.028	NS
Butyrate	3.49	2.88	0.261	*	2.72	2.89	0.144	NS
Isovalerate	0.28	0.31	0.045	NS	0.38	0.33	0.039	NS
Valerate	0.92	0.64	0.050	***	0.77	0.66	0.061	NS
NH_3-N concentration (mg/l)	111.2	96.7	14.65	NS	109.1	97.3	14.16	NS
Ca plasma concentration (mg/l)	110.8	111.3	2.11	NS	105.0	108.8	1.53	*

[a]The rumen liquor and blood samples were taken 6 hr after the oral administration of the compounds.
[b]NS, non-significant; *, $P \leq 0.05$; ***, $P \leq 0.001$.

in the rumen (Barry, 1985; Mangan, 1988; Robbins et al., 1991) and, therefore, we were expecting effects on both the NH_3-N and VFA concentrations of the rumen liquor. We expected that its administration and the dose used would mainly lead to digestion inhibition. We were, however, unable to detect any differences in rumen NH_3-N concentrations between CT-treated and control animals, and most of the changes in VFA concentrations were in the direction opposite to that expected (i.e., higher in the sheep CTs administered than in the control animals). It may be that the absence of an effect on NH_3-N concentrations was an outcome of rumen sampling time (i.e., too late to observe an effect 6 hr after quebracho administration), rather than a result of a low CT dose used. The CT dose in our experiment was based on previous experience (Singleton, 1981; Robbins et al., 1991), in which effects on carbohydrate and protein digestion had been seen. The above suggestion would also partly account for the higher VFA concentrations observed in the animals receiving CTs, since they could be seen as the outcome of a short-term decrease (and hence a delay) in the rumen fermentation rates of sheep administered CTs.

The administration of oxalic acid (OA) was expected to lead to a reduction in the systemic availability of Ca, and hence in plasma Ca concentrations (James and Butcher, 1972). This effect was mainly expected to be the direct result of acute toxicity created by OA administration. On the other hand, chronic OA toxicity may lead to uremia due to the damage to kidney blood capillaries, but this was not expected to be seen in this experiment. The experimental results are in line with the above expectation, since OA administration led to a mild hypocalcemia, which was transient and reversible within the two to four days of OA withdrawal (i.e., when the next blood sample was taken). This mild hypocalcemia was not accompanied by any other signs of distress. One of our experimental objectives was to mimic levels of intake and consequences of OA similar to those occurring under natural grazing conditions. It is, however, possible that OA administration and the resulting hypocalcemia could also have consequences for plasma phosphorus concentration, pH, and acid–base balance changes in the blood of the sheep (Kyriazakis et al., 1994). The latter were not quantified.

All three physiological variables measured (NH_3-N, VFAs, and Ca) were affected by the conditioning period, since they generally increased as conditioning progressed. This was probably a consequence of the increased intake of hay over the 4-hr periods as conditioning progressed. This outcome could have had an effect on the development of conditioned flavor aversions and towards the quebracho-associated food; this hypothesis is elaborated below.

Development of Conditioned Flavor Aversions

The process of development of conditioned flavor aversions can be seen in the reduction of the intake of flavored hay associated with the secondary com-

pound when this food is offered on its own, and in the degree of its avoidance when it is offered as a choice versus flavored hay that is not associated with the secondary compound (short-term preference tests). The former reduction in intake, however, should be distinguished and be independent from any potential direct effect of the secondary compound on the voluntary food intake of the animals. Such a process was seen in the sheep on the OA schedule: they tended, overall, to consume less of the hay accompanied by OA administration within the conditioning periods and showed mild aversions towards it when they were offered it as a choice (average aversion ratio <0.40). The weakest aversion was seen during the first and the strongest during the fourth short-term preference tests, respectively. OA administration did not seem to have a direct effect on the feeding motivation of the animals, since they all consumed their corrected pelleted food allowance offered 6–7 hr after OA administration.

Sheep on the quebracho schedule (at a dosage level of 0.6 g/kg LW) did not show any evidence of development of conditioned aversions towards the food flavor associated with quebracho administration, and this was further confirmed by the absence of such an aversion during the long-term preference tests. This finding contradicts existing evidence that ruminants develop conditioned flavor aversions to CTs either as a result of their postingestive consequences in the rumen (Cooper and Owen-Smith, 1985) or internal malaise (Provenza et al., 1990). The contradiction between previous results and the results of this experiment might simply lie in the structure and concentrations of the tannins used in the various studies. An explanation that we favor, however, given that it was previously shown that quebracho at the dose used inhibits digestion (Robbins et al., 1991), might lie in the fact that as conditioning progressed intake of both quebracho-associated and control hays was increased by a factor of almost 1.7 (from the first to the fourth conditioning; Table 2). This increase is taken to denote familiarization of the sheep with the relatively novel food: hay (Thorhallsdottir et al., 1987; Provenza et al., 1994a,b). The justification for using the flavored chopped hay was that we wanted a truly novel food that differed from the basal food in a number of characteristics, including form and texture as well as nutritional composition. Consequently, the increase in hay intake and its positive postingestive consequences, which could be seen as an increase in the provision of nutrients to the animal over the 4-hr period, may have overwhelmed any possible negative postingestive consequences that could have resulted from the administration of quebracho (CTs). In other words, quebracho ingested relative to hay consumed was reduced over the course of the conditioning and, as a consequence, examination of the physiological parameters relevant to quebracho administration (VFAs, rumen NH_3-N) indicated that effects on digestion were relatively slight. Development of CFAs towards digestion-inhibiting secondary compounds may depend on trade-offs between nutritive value and secondary compound concentrations. Implicit to this suggestion is the

assumption that negative postingestive consequences resulting from the administration of quebracho can only be seen as a decrease or inhibition of protein and/or carbohydrate digestion.

The questions of whether CTs are able to induce conditioned flavor aversions and, most importantly, the mechanism by which this might be achieved could not be elucidated by our experiment. Future experiments that address the issue should take into account some of the trade-off issues raised above and consider the use of different doses of CTs and perhaps introduce a fixed feeding schedule for the (novel) test food (i.e., a definite amount of food offered), as well as the separation of the effects of food flavor from those associated with the postingestive consequences of CTs (Provenza et al., 1990).

Persistence and Degree of Developed Conditioned Flavor Aversions

The second objective of the experiment was to test for the persistence of developed conditioned flavor aversions in the absence of continuous reinforcement. Given the outcomes of the conditioning phase of the experiment (see above), this question becomes relevant only for the animals on the OA schedule. These sheep continued to demonstrate aversions towards the flavor previously associated with the administration of OA up to 60 days after the last conditioning. The degree of these aversions did not change significantly with time and continued to be mild with an aversion ratio of 0.30–0.40.

The first question that arises from these results is why sheep developed only mild aversions, i.e., why they consumed any of the food flavor associated with OA when they were given a choice? The answer to this may lie in the degree of magnitude of the physiological changes observed. This was in line with our original objective of mimicking the physiological impact of naturally consumed food plants. There is now sufficient evidence in the literature to suggest that the degree of aversion is dependent on the strength of the negative consequences (for a review see Provenza, 1995a). In addition, the maintenance of sampling behavior by grazing herbivores created by the continuous small-scale consumption of the toxin-associated food could be beneficial (Cooper et al., 1995), since the concentrations of toxins in the foodstuffs (plants) change with time as a result of plant growth processes and previous herbivory, and the physiological consequences for animals may also alter as herbivores adapt to the secondary compounds in their diets (Allison et al., 1977; Provenza et al., 1990). The issue of trade-offs between the positive increase in provision of nutrients and the negative consequences of OA administration might also be of some relevance here, and most relevant to why animals needed more than one conditioning before they started exhibiting the development of conditioned aversions. It is clear, however, that the consequences of the OA administration were the overwhelming factor in the conditioning.

The second issue that arises is the reason for retention of these mild aversions over a relatively long period and the potential advantages of such a behavior to the animal. Similar behavior has previously been observed in ruminants with evidence in the literature that young ruminants can remember food that produced aversive consequences for at least one to three years without intervening exposure (e.g., Green et al., 1984). This finding, however, is somewhat at odds with the idea that the maintenance of sampling behavior is important for optimum flexibility in the animal's dietary habits. Currently there is no theoretical framework that could encompass such apparent contradictions, although the issue is attracting attention (Provenza, 1995b).

CONCLUSIONS AND IMPLICATIONS

The experiment demonstrates that sheep are able to form conditioned flavor aversions towards a flavor that is associated with the administration of a potentially toxic substance (oxalic acid) that occurs naturally in plants. Once such conditioned flavor aversions are formed, they can persist for a relatively long period (in the case of the experiment up to 60 days) without intervening exposure to the secondary compound and its consequences. Conditioned flavor aversions may therefore be a wider phenomenon and mechanism than previously thought and could perhaps be invoked more widely to account for how herbivores learn to select a diet that minimizes the ingestion of toxins. The fact that sheep failed to develop conditioned flavor aversions towards a flavor that was associated with the administration of a physiologically realistic dose of a condensed tannin (quebracho) suggests that the issue of trade-offs between the ingestion of nutrients and avoidance of secondary compounds might have to be investigated for a better understanding of the mechanism and implications of conditioned food aversions.

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CONTACT SEX SIGNALS IN TWO SYMPATRIC SPIDER SPECIES, *Tegenaria domestica* and *Tegenaria pagana*

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Abstract—Bioassays in two sympatric spider species, *Tegenaria domestica* and *T. pagana*, demonstrate that the total female chemical extract, and in particular the polar fraction, are involved in the sexual behavior of males and reproductive isolation mechanisms. In *T. domestica* female attractiveness was correlated with changes in 18 chemical compounds. In *T. pagana* little difference was observed between 12 compounds in extracts of attractive and unattractive females. 12- + 10-Methyloctacosane was not detected in attractive females of either species. Reproductive isolation was linked not only to quantitative changes in 18 chemical compounds detected in both species, but also to nine compounds present in one or the other species.

Key Words—Bioassay, sexual behavior, chemical signals, communication, spiders, *Tegenaria domestica*, *Tegenaria pagana*.

INTRODUCTION

In most spider species females lead sedentary and solitary web-bound lives. It is usually the male that leaves its web in search of a suitable mate. Many studies have shown that searching males are guided by silk threads left around female

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webs (Dijkstra, 1976; Tietjen, 1977; Tietjen and Rovner, 1980; Roland and Rovner, 1983; Roland, 1984). These threads are thought to bear chemical signals that play a role in maintaining the interspecific barrier of sympatric species. When placed in the presence of conspecific and heterospecific female threads, most males are attracted to conspecific threads.

The existence of sex pheromones in spiders has been confirmed by numerous authors. Ayyagari and Tietjen (1986) reported that low-molecular-weight lipids in the silk of *Schizocosa ocreata* (Lycosidae) act as a male inhibitory pheromone. Schulz and Toft (1993) identified sexual pheromones on the silk of unmated female *Linyphia triangularis* (Clerck). These sex pheromones, (*R*)-3-hydroxybutyric acid, and (*R*)-3-[(*R*)-hydroxybutyryloxy]butyric acid were found to elicit web reduction behavior in males.

In addition to locating the nest, completion of male courship behavior in many spider species requires close-range recognition by the mating partners (Kaston, 1936; Suter and Renkes, 1982; Suter and Hirscheimer, 1986). This probably involves surface contact signals. The chemical composition, structure, and role of cuticular compounds in insect behavior has been extensively investigated (Lockey, 1988; de Renobales et al., 1991; Howard, 1993; and see Stanley-Samuelson and Nelson, 1993). However, few studies have been done on semiochemicals, particularly cuticular lipids in arachnids, (Toolson and Hadley, 1977; Hadley and Hall, 1980; Hadley and Quilan, 1989). All previous studies have focused on cuticular permeability rather than behavior. In a recent analysis of cuticular products in *Tegenaria atrica* (Agelenidae), Trabalon et al. (1996) observed a gregarious period, and chemical identification of sexual contact pheromones was attempted.

The purpose of this paper was to study contact sex recognition signals influencing sexual behavior and reproductive isolation in two sympatric spider species: *Tegenaria domestica* and *Tegenaria pagana.* These closely related species present few morphological differences.

METHODS AND MATERIALS

Tegenaria domestica (Clerck) and *T. pagana* (C. L. Koch) were reared under laboratory conditions for three generations at 20 ± 1°C with a 12L:12D photoperiod. They were regularly supplied with lab crickets, *Gryllus bimaculatus*, and water. Both females and males were tested 30 days after the final molt.

Ethological Tests. Observations were conducted in a open circular testing arena with a 9.5-cm radius. Experimental females were placed in the arena first. Males were introduced and observed for 15 min. Only contact time between males and females was recorded. In order to evaluate the effects of chemical extracts on sexual behavior and reproductive isolation, males were exposed and allowed to choose between intact and extracted females.

In *Tegenaria* sp., male courtship behavior is manifested by drumming of pedipalps on the substrate and leg-waving (Roland, 1984). In our bioassays anesthetized females were considered as attractive (A) if males exhibited courtship behavior after contact was made and as unattractive (U) if males did not exhibit courtship behavior. The number and duration of male contacts with attractive and unattractive females were also noted.

Three hundred intact females were cold anesthetized for 15 min at 4°C, then a first round of tests were carried out. After classification as attractive or unattractive, females were deodorized in two successive 15-min baths consisting of pentane followed by methanol and retested.

In the second round of ethological testing, deodorized females (30 per group) were treated with 1 ml of different cuticular extracts and retested with males. Tests were carried out using conspecific extracts, heterospecific extracts, male total extracts, and organic solvents, i.e., pentane as control for apolar extracts and methanol for polar extracts.

Chemical Identification. Each female tested was soaked in 1 ml pentane to extract cuticular chemicals. Samples were dried under nitrogen and redissolved in 50 μl of pentane with 375 ng of hexadecane as an internal standard. Ten quantifications were performed for each female group (attractive and unattractive). After gas chromatography (GC), total extracts obtained from attractive females were eluted from a Sep-pak silica cartridge (Waters-Millipore) using pentane or methanol as eluant. Each extract was reanalyzed by GC on a Delsi 200 apparatus equipped with a CP-Sil-5CB WCOT apolar capillary column (25 m, 0.2 mm ID; Chrompack) or a CP-Sil-8CB WCOT polar capillary column (25 m, 0.2 mm ID; Chrompack), and with a flame ionization detector (FID). Oven temperature was programmed from 100°C to 150°C at 5°C/min and from 150°C to 320°C at 3°C/min. Peak areas for each GC sample were obtained by integration. Results were expressed as percentages and in nanograms per total body weight.

Analysis by gas chromatography–mass spectroscopy (GC-MS) of the total extract, polar fraction, and apolar fraction was performed with pooled samples of 80 attractive and unattractive *T. domestica* and *T. pagana* females. The apparatus was a Hewlett Packard GC-MS system comprising an HP 5890 GC Series II equipped with the same CP-Sil-5CB column as for GC, an HP 5989A MS Engine and a HPUX Chemsystem control unit. The carrier gas was helium (2 bars). The source was set at a temperature of 240°C, the quadrupole at 100°C, and the interface at 300°C. Masses were scanned between m/z 35 and 600 at 0.95 scans/sec. The temperature program for the GC oven was from 30°C to 200°C at 8°C/min and from 200°C to 320°C at 3°C/min, and then isothermal for 5 min.

Statistical Analysis. Behavioral data were analyzed by ANOVA. Percentages for each peak were divided by 100 and converted to $\sqrt{\text{arcsine}}$ to normalize data before ANOVA.

RESULTS

Ethological Tests. In the first round of tests about two thirds of the anesthetized *T. domestica* females were classified as attractive (A). The duration of contact between males and attractive females was about two times longer than between males and unattractive females in the remaining third of the test population (Table 1). Males made only fleeting contacts with deodorized females and never manifested courtship behavior. The same results were observed in *T. pagana.*

When *T. domestica* males were confronted with deodorized females treated with conspecific extracts, behavior was variable. There was no significant difference in the number of contacts between females treated with total extract, polar fraction, or apolar fraction obtained from attractive females used in the first round of testing. However, the duration of contact with deodorized females treated with the apolar fraction was significantly shorter than with females treated with the other two extracts. The duration of contact with washed females treated with total extract and apolar fraction was comparable to the duration observed in the first round of testing with anesthetized attractive females. The number and duration of contacts with unattractive females or males was significantly shorter than corresponding observations using deodorized females treated with total extracts.

T. pagana males contacted deodorized females treated with total extract, polar fraction, and apolar fraction obtained from attractive females significantly less often than they contacted anesthetized attractive females in the first round of testing, and the duration of contact with treated females was significantly longer. The number of contacts with females treated with total extract obtained from unattractive females or males was significantly lower than the number of contacts observed in the first round of testing with attractive anesthetized females, but was comparable to the number of contacts observed with females treated with total extracts from attractive females. The duration of contact with deodorized females treated with total extracts from attractive or unattractive females was significantly shorter.

In both species, contacts were significantly lower in number and shorter in duration when males were exposed to females treated with heterospecific female extract than with conspecific female extract. Males never attempted to complete courtship behavior with females treated with heterospecific female extract.

Chemical Identification. GC and GC-MS showed that total extracts (Table 2) from *T. domestica* and *T. pagana* contained acids, methylesters, saturated hydrocarbons, cholesterol, and unknown compounds. There was no significant difference between *T. domestica* and *T. pagana* with regard to the total weight of these chemical compounds (2321 and 1824 ng/female, respectively). Qualitatively there were significantly more methylesters and unknown compounds and less dimethylakanes in *T. domestica* females than from *T. pagana* females.

TABLE 1. CONTACT INVOLVING COURTSHIP BEHAVIOR BY MALE *T. domestica* AND *T. pagana*[a]

	Contacts (N/min)	Duration of contact (sec)
Contacts between male *Tegenaria domestica* with		
Anesthetized		
Attractive female	13.41 ± 1.72	245.78 ± 37.90
Unattractive female	5.95 ± 0.91***	141.30 ± 53.72**
Deodorized female	3.91 ± 0.72***	67.94 ± 38.35***
Total extract from		
Attractive female	8.50 ± 2.75	314.67 ± 53.76
Unattractive female	3.10 ± 0.50**	25.15 ± 7.36***
Male	1.55 ± 0.48***	31.70 ± 13.59***
T. pagana female	5.85 ± 0.64**	13.70 ± 3.82***
Polar fraction from		
Attractive female	8.60 ± 1.20	295.25 ± 40.83
Methanol female	4.47 ± 0.84***	85.27 ± 18.93**
Apolar fraction from		
Attractive female	8.20 ± 1.36	197.70 ± 53.80a
Pentane	5.67 ± 1.15***	18.57 ± 5.23**
Contacts between male *Tegenaria pagana* with		
Anesthetized		
Attractive female	14.36 ± 1.43	89.05 ± 14.80
Unattractive female	6.35 ± 1.46***	27.40 ± 15.52***
Deodorized female	2.38 ± 0.29***	7.26 ± 2.89***
Total extract from		
Attractive female	7.85 ± 2.07a	195.60 ± 36.26***
Unattractive female	6.27 ± 0.50a	61.30 ± 28.90**
Male	4.75 ± 0.83*	13.40 ± 4.14***
T. domestica female	2.95 ± 0.61***	21.25 ± 10.38***
Polar fraction from		
Attractive female	5.74 ± 1.20*	230.26 ± 40.83***
Methanol	1.55 ± 0.84***	55.27 ± 8.98*
Apolar fraction from		
Attractive female	5.84 ± 0.81*	144.35 ± 29.29b**
Pentane	1.95 ± 0.97**	63.15 ± 2.83*

[a]Values are ± SE (N = 30 per group). Analysis of variance: *** significantly different from attractive female, and from total extract of attractive females at $P \leq 0.0001$; ** significantly different from attractive female, and from total extract of attractive females at $P \leq 0.001$; a: significantly different from total extract and polar fraction of attractive females at $P \leq 0.01$; b: significantly different from polar fraction of attractive females at $P \leq 0.05$.

TABLE 2. PERCENTAGE COMPOSITION ± SE OF TOTAL EXTRACT FROM *Tegenaria domestica* AND *Tegenaria pagana* FEMALES

Chemical composition	*Tegenaria domestica*	*Tegenaria pagana*
Acids	9.31 ± 1.28	10.64 ± 0.88
Methyl esters	2.40 ± 0.48	0.38 ± 0.06[a]
Hydrocarbons	82.95 ± 9.21	85.00 ± 8.44
n-Alkanes	38.45 ± 4.19	34.15 ± 2.49
Monomethylalkanes	35.41 ± 3.81	32.38 ± 3.68
Dimethylalkanes	9.09 ± 1.21	18.47 ± 2.27[a]
Cholesterol	0.52 ± 0.01	0.80 ± 0.05
Unknowns	2.07 ± 0.20	0.79 ± 0.06[a]

[a] Analysis of variance: $F = 4.93$ to 5.55, significantly different from *Tegenaria domestica* at $P \leq 0.05$.

Table 3 lists the compounds found in attractive (A) and unattractive (U) *T. domestica* and *P. pagana* females. In both species the polar fraction was composed of fatty acids C_{14}, C_{16}, and two C_{18}s), and methylesters (three in *T. domestica* and one in *T. pagana*). Both species also contained three *n*-alkanes (*n*-C_{21}, *n*-C_{22}, and *n*-C_{24}). Attractive *T. domestica* females displayed significantly more acid content and less methylester content than their unattractive counterparts (247 vs. 171 ng/female and 50 vs. 94 ng/female, respectively).

T. domestica attractive and unattractive females differed in that attractive females had more myristic acid (peak 1), methyl palmitate (peak 2), palmitic acid (peak 3), *n*-heneicosane (peak 6), and *n*-docosane (peak 9) and less methyl stearate (peak 4) and methyl oleate (peak 5) than unattractive females. *T. pagana* attractive females had more *n*-heneicosane (peak 6) and *n*-docosane (peak 9) and less octadecadienoic acid (peak 7) than unattractive females.

Regardless of attractiveness, *T. domestica* and *T. pagana* females presented different amounts of methyl palmitate (peak 2), palmitic acid (peak 3), octadecadienoic acid (peak 7), and octadecanoic acid (peak 8). Methyl stearate (peak 4) and methyl oleate (peak 5) were not detected in *T. pagana*.

There were 40 saturated hydrocarbons in *T. domestica* and 48 in *T. pagana*. In both species the *n*-alkanes presented chain lengths of 21–32 carbon atoms. Methylalkanes contained 25–33 carbon atoms in *T. domestica* and 25–41 in *T. pagana*.

Attractive *T. domestica* females exhibited significantly more dimethylalkanes than unattractive females (305 vs. 280 ng/female). Other hydrocarbons, especially *n*-alkanes, were lower in attractive than unattractive females (717 vs. 1033 ng/female, respectively).

TABLE 3. IDENTIFICATION AND PERCENTAGE (±SD) OF COMPOUNDS OBTAINED FROM ATTRACTIVE (A) AND UNATTRACTIVE (U) FEMALES OF *Tegenaria domestica* AND *Tegenaria pagana*

Peak no.	Chemical compounds	*Tegenaria domestica*		*Tegenaria pagana*		ANOVA[a]
		A	U	A	U	
1	myristic acid	6.12 (0.16)	4.63 (0.06)	6.71 (0.05)	6.28 (0.14)	a
2	methyl palmitate	6.18 (0.15)	3.48 (0.02)	3.01 (0.06)	3.69 (0.09)	a, c
3	palmitic acid	44.73 (0.79)	32.24 (1.42)	68.51 (0.65)	68.96 (0.89)	a, c
4	methyl stearate	7.50 (0.22)	15.48 (0.31)			a
5	methyl oleate	1.61 (0.11)	14.86 (0.04)			a
6	*n*-heneicosane	4.45 (0.08)	2.43 (0.09)	3.17 (0.35)	1.47 (0.04)	a, b
7	octadecadienoic acid	23.79 (0.30)	22.76 (1.30)	10.82 (0.15)	13.60 (0.45)	b, c
8	octadecanoic acid (stearic acid)	1.22 (0.03)	2.24 (0.26)	3.06 (0.03)	3.62 (0.23)	c
9	*n*-docosane	4.38 (0.07)	1.87 (0.09)	4.73 (0.05)	2.39 (0.37)	a, b
10	*n*-tricosane	0.56 (0.03)	0.36 (0.06)	0.52 (0.09)	0.28 (0.04)	b
13	*n*-tetracosane	5.76 (0.34)	4.26 (0.38)	2.58 (0.02)	2.80 (0.31)	c
15	*n*-pentacosane	8.44 (0.56)	4.14 (1.04)	6.84 (0.47)	7.56 (0.57)	a
16	13- + 11- + 9-methylpentacosane	3.31 (0.56)	4.59 (0.39)	1.06 (0.15)	1.23 (0.10)	c
18	3-methylpentacosane	3.81 (0.08)	4.05 (0.20)	0.76 (0.10)	0.84 (0.06)	c
19	*n*-hexacosane	3.77 (0.66)	5.95 (0.31)	1.79 (0.15)	2.45 (0.13)	a, c
20	3,11-dimethylpentacosane	4.50 (0.05)	3.98 (0.19)	0.78 (0.09)	0.65 (0.04)	c
21	12- + 10- + 8-methylhexacosane	2.71 (0.20)	1.82 (0.16)	0.74 (0.02)	0.90 (0.11)	a, c
22	4/2-methylhexacosane	1.56 (0.11)	0.74 (0.06)	0.66 (0.03)	0.36 (0.05)	b, c
23	3-methylexacosane	2.65 (0.16)	2.59 (0.14)	3.11 (0.07)	2.72 (0.19)	
24	*n*-heptacosane	12.54 (1.06)	21.89 (1.32)	13.20 (1.02)	17.55 (0.79)	a, b
25	13- + 11- + 9-methylheptacosane	4.28 (0.84)	3.34 (0.22)	1.80 (0.09)	2.00 (0.67)	c
26	5-methylheptacosane	0.20 (0.01)	0.20 (0.02)	0.19 (0.01)	0.31 (0.04)	c
27	9,13- + 9,15- + 9,17-dimethylheptacosane	0.30 (0.04)	0.27 (0.02)	0.11 (0.03)	0.14 (0.02)	c
28	2-methylheptacosane	1.79 (0.08)	2.30 (0.12)	1.62 (0.15)	2.12 (0.11)	a

TABLE 3. Continued

Peak no.	Chemical compounds	*Tegenaria domestica* A	*Tegenaria domestica* U	*Tegenaria pagana* A	*Tegenaria pagana* U	ANOVA[a]
29	3-methylheptacosane	1.71 (0.11)	1.97 (0.08)	1.62 (0.08)	1.94 (0.10)	
30	5,17-dimethylheptacosane	0.72 (0.32)	0.62 (0.02)	0.24 (0.03)	0.31 (0.02)	c
31	*n*-octacosane	2.07 (0.18)	2.47 (0.22)	3.74 (0.15)	4.36 (0.14)	c
32	3,9- + 3,11-dimethylheptacosane	2.47 (0.41)	1.49 (0.23)	0.30 (0.01)	0.42 (0.07)	c
33	12- + 10-methyloctacosane		0.13 (0.05)		0.27 (0.05)	
34	6-methyloctacosane	0.84 (0.10)	0.65 (0.05)			
35	2-methyloctacosane	0.59 (0.08)	0.39 (0.06)	2.81 (0.09)	2.11 (0.13)	c
36	3-methyloctacosane	1.42 (0.23)	1.70 (0.11)	2.08 (0.44)	1.62 (0.11)	c
37	*n*-nonacosane	1.58 (0.04)	2.62 (0.18)	5.29 (0.61)	5.77 (0.21)	a, c
38	15- + 13- + 11- + 9-methylnonacosane	3.15 (0.21)	2.25 (0.16)	3.14 (0.20)	2.30 (0.16)	b
39	5-methylnonacosane	0.60 (0.02)	0.59 (0.03)	0.95 (0.10)	0.87 (0.04)	c
40	9,13- + 7,17-dimethylnonacosane	0.88 (0.14)	0.65 (0.04)	1.09 (0.08)	0.94 (0.05)	c
41	5,15-dimethylnonacosane	0.47 (0.05)	0.33 (0.02)	0.39 (0.02)	0.36 (0.02)	
42	*n*-triacontane	2.05 (0.35)	3.00 (0.25)	0.56 (0.04)	0.63 (0.06)	a, c
43	14- + 12- + 10-methyltriacontane	3.63 (0.26)	4.08 (0.57)	4.44 (0.22)	2.83 (0.58)	
45	2-methyltriacontane	0.54 (0.03)		1.45 (0.31)	0.89 (0.08)	b, c
46	*n*-hentriacontane	2.05 (0.11)	2.26 (0.21)	0.68 (0.06)	0.94 (0.14)	c
47	15- + 13- + 11-methylhentriacontane	5.26 (0.37)	4.33 (0.30)	4.95 (0.82)	3.93 (0.20)	
48	7-methylhentriacontane	1.46 (0.07)	0.84 (0.15)	1.71 (0.09)	1.80 (0.11)	
49	13,17-dimethylhentriacontane + 11,15-dimethylhentriacontane	0.48 (0.03)	0.48 (0.04)	0.41 (0.05)	0.33 (0.06)	
50	9,21- + 7,19-dimethylhentriacontane			0.43 (0.02)	0.64 (0.09)	
51	*n*-docotriacontane	2.85 (0.38)	3.73 (0.36)	0.53 (0.02)	0.71 (0.07)	c
52	14- + 12- + 10-methyldocotriacontane	3.70 (0.40)	1.65 (0.53)	1.37 (0.05)	1.94 (0.13)	a, c

[a]a: significantly different from attractive and unattractive females of *T. domestica* at $P \leq 0.05$; b: significantly different from attractive and unattractive females of *T. pagana* at $P \leq 0.05$; c: significantly different from *T. domestica* and *T. pagana* at $P \leq 0.005$.

Attractive (A) *T. domestica* females exhibited significantly higher amounts of *n*-pentacosane (peak 15), 12- + 10- + 8-methylhexacosanes (peak 21), 14- + 12- + 10-methyldocotriacontanes (peak 52), and 13,19- + 11,17-dimethyltritriacontanes (peak 55) than unattractive (U) females, and significantly less *n*-hexacosane (peak 19), *n*-heptacosane (peak 24), 2-methylheptacosane (peak 28), *n*-nonacosane (peak 37), and *n*-triacontane (peak 42). 12- + 10-Methyloctacosanes (peak 33) were detected in attractive females, and 2-methyltriacontane (peak 45) was not detected in unattractive females.

Unattractive *T. pagana* females had significantly more hydrocarbons than their attractive counterparts (1793 vs. 1265 ng/female). Attractive females had significantly more *n*-tricosane (peak 10), 4/2-methylhexacosane (peak 22), 15- + 13- + 11- + 9-methylnonacosanes (peak 38), 2-methyltriacontane (peak 45), 13,21- + 13,19-dimethylpentatriacontanes (peak 59), and 13,23- + 13,19-dimethylhentetracontanes (peak 63) and significantly less *n*-heptacosane (peak 24) and 17- + 15- + 13-methyltetratriacontanes (peak 56) than unattractive females. No 12- + 10-methyloctacosanes (peak 33) were detected in attractive females.

Compound-by-compound comparison of the apolar fraction in both species revealed several differences (Table 3). No 6-methyloctacosane (peak 34) was detected in *T. pagana*. No mono- and dimethylalkanes were detected after peak 55 in *T. domestica*. A significant qualitative difference between *T. domestica* and *T. pagana* females was noted with respect to seven *n*-alkanes and 14 methylalkanes.

DISCUSSION

Bioassays indicated that *T. domestica* and *T. pagana* males discriminate solvent extracts obtained from attractive females and solvent extracts obtained from unattractive females or males. In both species duration of contact with females treated with apolar extract (hydrocarbons) was significantly shorter than with females treated with the polar extract (acids and methylesters) or total extract. The signals contained in total extracts appeared to be fairly conspecific since males showed only weak reactions to heterospecific extracts. These findings indicate that reproductive isolation of these two sympatric species may depend on a cross-checking process involving silk, pheromones, and behavior of both males and females.

The qualitative composition of the total extract obtained from *T. domestica* and *T. pagana* was similar to that reported in another spider species (Trabalon et al., 1996), various insects (Hadley and Jackson, 1977; Blomquist and Jackson, 1979), and scorpions (Lockey, 1980). Saturated hydrocarbons and fatty acids, particularly palmitic acid, octadecadienoic acid, *n*-pentacosane, and

n-heptacosane, were the most abundant compounds in both spider species studied here. Like *Tegenaria atrica* (Trabalon et al., 1996), neither species exhibited unsaturated hydrocarbons.

The predominance of *n*-alkanes in cuticular lipids is in agreement with previous findings in insects (Jackson and Blomquist, 1976; Lockey, 1980, 1988; Trabalon et al., 1990; Bagnères et al., 1991) and at least one other species of spider *T. atrica* (Trabalon et al., 1996). The *n*-alkane fraction accounted for 42% and 38% of total hydrocarbons in *T. domestica* and *T. pagana*, respectively. In the scorpion *Paruroctonus mesaensis*, normal alkanes accounted for 54–63% of the total hydrocarbons (Hadley and Jackson, 1977).

Hydrocarbon components of cuticular lipids, particularly methyl-branched alkanes, contribute to cuticular waterproofing in the desert beetle *Eleodes armata* (Hadley, 1977) and the scorpion *Pauroctonus mesaensis* (Hadley and Jackson, 1977). Monomethylalkanes branched at positions 15, 13, 11, 9, and 3 were the most abundant components in both species. No 6-methyloctacosane was detected in *T. pagana* and no 4-monomethylakanes in *T. domestica*. Only 12- + 10-methyloctacosanes were detected in unattractive *T. domestica* and *T. pagana* females. In Diptera dimethylalkanes produced by some females have been observed to induce male tsetse flies to copulate on contact (Carlson et al., 1984). Low levels of dimethylalkanes were found in the two *Tegenaria* species (9 and 18% of total extract, respectively), but 13,21- + 13,19-dimethylpentatriacontanes and 13,23- + 13,19-dimethylhentetracontanes were involved in female attractivity. Our results suggest that in *T. domestica* and *T. pagana* hydrocarbons could also play a role in chemical communication, since males of both species are able to discriminate the apolar fractions of attractive females versus unattractive ones.

Nevertheless ethological tests indicate that female attractiveness in *T. domestica* seems more related to the polar fraction, but careful chemical analysis linked attractiveness in *T. domestica* to variations in both polar and apolar compounds.

Our results did not clearly delineate the compounds involved in female attractiveness in *T. pagana*. In ethological tests, female attractiveness seemed to be related to both the total extract and polar fraction. We observed minor chemical differences between attractive and unattractive females with respect to three compounds in the polar fraction and nine compounds in the apolar fraction. Although chemical signals certainly facilitate recognition of suitable females by *T. pagana* males, initiation of male sexual behavior was not dependent on chemical contact stimulant alone. Thus in *T. pagana* we speculate that chemical information from the female must be validated by a vibratory response to the male.

Reproductive isolation in both species was correlated not only to quantitative changes in shared compounds, but also to qualitative differencies.

The results of this study show that bioassays are highly useful to assess the relationship between female attractiveness and levels of cuticular products. Further work is needed to test the effect of synthetic compounds alone or in combination on male behavior.

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TALL LARKSPUR INGESTION: CAN CATTLE REGULATE INTAKE BELOW TOXIC LEVELS?[1]

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Abstract—Tall larkspur (*Delphinium barbeyi*) is a toxic forb often consumed by cattle on mountain rangelands, with annual fatalities averaging about 5%. This study examined the relationship between food ingestion and toxicity in cattle. Two grazing studies suggested that larkspur consumption above 25–30% of cattle diets for one or two days led to reduced larkspur consumption on subsequent days. We subsequently hypothesized that cattle can generally limit intake of larkspur to sublethal levels. This hypothesis was tested by feeding a 27% larkspur pellet in experiment 1. Cattle given a 27% larkspur pellet ad libitum showed distinct cyclic patterns of intake, where increased larkspur consumption on one or two days was followed by reduced ($P < 0.025$) consumption on the following day. The amount of larkspur (mean 2007 g/day; 17.8 mg toxic alkaloid/kg body wt) consumed was just below a level that would produce overt signs of toxicity. Experiment 2 was conducted to examine cattle response to a toxin dose that varied with food intake. Lithium chloride (LiCl) paired with corn ingestion was used as a model toxin, and we hypothesized that if increased (decreased) consumption was followed by a stronger (weaker) dose of LiCl, cattle would show a transient reduction (increase) in corn intake. There was no difference ($P > 0.05$) between controls and treatment animals at the 20 or 40 mg LiCl/kg dose in the percentage of

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[1]All experimental procedures with animals followed guidelines approved by the Utah State University Animal Care and Use Committee and were conducted under veterinary supervision.

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corn consumed, but the 80 mg LiCl/kg dose induced a cyclic response (mean 46%) compared to intake by controls (mean 96%) ($P < 0.001$). At the 80 mg/kg dose, LiCl induced an aversion to corn; when corn intake decreased on subsequent days and LiCl dose also decreased, cattle responded by increasing corn intake and apparently extinguishing the transient food aversion. Experiment 3 was similar to the LiCl trial, except that tall larkspur was the toxin. Cattle responded to oral gavage of ground larkspur with distinct cycles; days of higher corn consumption were followed by one to three days of reduced consumption. Corn intake for controls was higher ($P < 0.01$) than for larkspur-treated animals (means 84 and 52%, respectively; day × treatment interaction $P < 0.01$). The threshold for toxic effects on corn intake was 14 mg toxic alkaloid/kg body weight. In conclusion, cattle apparently limit ingestion of some toxins so that periods of high consumption are followed by periods of reduced consumption to allow for detoxification. Cyclic consumption generally enables cattle to regulate tall larkspur consumption below a toxic threshold and allows cattle the opportunity to safely use an otherwise nutritious, but toxic, plant.

Key Words—Larkspur, alkaloids, intake regulation, toxicity, cattle.

INTRODUCTION

Generalist herbivores may regulate ingestion of food and minimize aversive feedback from toxins. For instance, aversive postingestive feedback caused cattle to reduce intake of tall larkspur (*Delphinium barbeyi*), a forb that contains toxic alkaloids (Pfister et al., 1990). The extent of larkspur ingestion appeared to be limited by the concentration of toxic alkaloids. Olsen and Ralphs (1986) were also able to cause strong food aversions to a familiar food in cattle by using large doses of alkaloid extracts from tall larkspur. Nausea may be one basis for aversive postingestive feedback in livestock (Provenza et al., 1992). In the case of larkspur, nausea may be the result of larkspur alkaloids inhibiting the function of cholinergic parasympathetic nerves of the gastrointestinal tract, similar to nicotine-induced nausea (Taylor, 1990).

Larkspur contains numerous diterpenoid alkaloids, but the principal toxic alkaloids are methyllycaconitine (MLA) and 14-deacetylnudicauline (DAN) (Manners et al., 1992). These two alkaloids are similar in toxicity ($LD_{50} \approx 4.5$ mg/kg in mice; Manners et al., 1995), and account for most of the toxicity of tall larkspurs (Panter and Manners, unpublished data). The larkspur alkaloids block acetylcholine (ACh) receptors in the central and peripheral nervous system (Benn and Jacyno, 1983; Dobelis et al., 1993), eventually leading to muscular weakness, paralysis, and death from respiratory failure. Of course, the toxin dosage while foraging depends on the amount and toxicity of the plant ingested.

In this paper, we first provide a retrospective analysis of individual animal records for the pattern of larkspur ingestion by cattle foraging on rangelands. On the basis of that analysis, we hypothesized that cattle can limit intake of

some toxic foods to sublethal levels. We tested this hypothesis experimentally in the following three experiments.

Experiment 1: Cyclic Consumption of Larkspur Pellets by Cattle. The objective of this experiment was to determine if cattle consumption of larkspur pellets followed a cyclic pattern, indicating subclinical intoxication and detoxification. We hypothesized that since larkspur is very nutritious (Pfister et al., 1989), animals may immediately experience positive postingestive consequences, followed by delayed adverse gastrointestinal illness due to alkaloids or other secondary compounds (Provenza et al., 1992). This delayed illness may reduce food consumption until toxicity passes, then animals may show an increasing propensity to consume larkspur, presumably due to positive nutritional feedback.

Experiment 2: Food Ingestion as Affected by LiCl. Conditioned food aversion studies have shown that cattle can be trained to avoid tall larkspur if illness (i.e., nausea induced by LiCl) is paired with larkspur ingestion (Lane et al., 1990; Ralphs, 1992). If cattle sample the plant, however, the aversion is eventually extinguished (Ralphs and Olsen, 1990; Lane et al., 1990). Sheep also continue to sample harmful foods after experiencing illness from ingesting the food (Burritt and Provenza, 1989). Launchbaugh et al. (1993) found that lambs could detect levels of LiCl mixed with barley and would adjust feed intake to a level slightly below the amount necessary to induce a food aversion. This study was done to examine cattle response to a toxin dose that varied with food intake. We used LiCl because this compound produces rapid emesis without other undesirable side effects. The objective of this experiment was to determine if cattle would adjust intake when food consumption was paired with gastrointestinal distress produced by LiCl, and no change in flavor cues were present to predict toxicity. We hypothesized that if increased consumption was followed by a stronger dose of LiCl, animals would show a transient reduction in intake of the food.

Experiment 3: Food Ingestion as Affected by Tall Larkspur. This experiment was conducted in a similar manner to experiment 2, except that tall larkspur replaced LiCl as the toxic agent. The objective of this experiment was to determine if cattle would adjust intake when novel food consumption was paired with subclinical toxicosis produced by tall larkspur. We hypothesized that if increased consumption of the novel corn was followed by a higher dose of larkspur alkaloids, animals would show a transient reduction in corn intake.

METHODS AND MATERIALS

Retrospective Study of Larkspur Ingestion by Individual Grazing Animals. Grazing studies were conducted during 1986 and 1987 with Hereford cows to determine when cattle consumed tall larkspur in relation to plant alkaloid levels

(Pfister et al., 1988a,b). These studies provided mean daily larkspur consumption by groups of animals. We retrospectively examined records of individual animals from those studies to determine if any larkspur consumption patterns were apparent. Detailed methods for the field grazing trials are given in Pfister et al. (1988a,b). Both grazing trials were conducted on the Manti-LaSal National Forest at about 3000 m elevation. Plant communities were dominated by patches of tall larkspur. During both years, all animals were focally sampled in a predetermined random order during all active grazing periods. The same animals were used each year. Bite counts were used to determine diet composition (i.e., percent of bites); diets were categorized as grasses, forbs, shrubs, and larkspur. Tall larkspur was in the flower and pod stages of growth during these two studies.

Experiment 1: Cyclic Consumption of Larkspur Pellets by Cattle. Tall larkspur was collected from high elevation (>3000 m) rangeland in central Utah during the summer of 1989. Plants were air-dried, ground through a 2-mm screen, and mixed with alfalfa hay to form a 27% larkspur pellet. We chose to make 27% pellets because cattle often consumed 25–30% of their diets as larkspur (Pfister et al., 1988a,b). Toxic alkaloid content of the larkspur pellet was determined using HPLC (Manners and Pfister, 1993).

Ten 32-month old Hereford × Angus heifers weighing 412 ± 56 kg were used in the study during the fall of 1989. Feed intake was monitored using locking head stanchions with individual feeders. Animals were habituated to being locked into the stanchions each morning at 0800 hr, and were allowed to eat until 1100 hr. Alfalfa pellets were fed ad libitum for 10 days to all animals to establish a baseline; consumption was stable on days 8–10. Feed intake was measured as the difference between feed offered and that remaining after 3 hr.

Cattle were randomly divided into two groups ($N = 5$); the control group was fed only alfalfa pellets ad libitum for nine days. Subclinical larkspur intoxication produces no measurable biochemical lesions or quantifiable diagnostic signs, thus reductions in feed intake were used as an index of intoxication for the larkspur group. Consumption was compared to the last three days of baseline for alfalfa, when intakes had stabilized, by calculating daily consumption as a percentage of baseline (100%). Percentage values were analyzed using ANOVA with repeated measures over days.

Experiment 2: Food Ingestion as Affected by LiCl. Twelve 2-year-old Charolais × Angus × Hereford cows (431 × 35 kg) that were ruminally cannulated were given 35 days to become familiar with alfalfa pellets and were allowed ad libitum access to alfalfa pellets from 1300 to 1500 hr each day. Whole shelled corn, a highly palatable but novel food, was used as the target food.

Cattle were randomly divided into controls and a treatment group ($N = 6$) and remained in the same group throughout the study. We began with a low

dose of LiCl (20 mg/kg body weight) for eight days, then increased the dose (40 mg/kg body weight) for 11 days, and ended with a high dose (80 mg/kg) for eight days. A dose of 100 mg/kg body weight induces mild aversion to a novel food (Ralphs and Cheney 1993). We extended the 40 mg LiCl dose for an extra three days because food intake of two animals began to fluctuate near the end of the eight-day period.

Corn (1000 g) was offered for 10 min at 0800 hr, and LiCl was administered intraruminally in 100 ml of water in proportion to the amount of corn consumed and the body mass of the animal. For example, during the 20 mg/kg body weight period, if a 400-kg animal consumed 500 g of corn (50% of corn offered), it was immediately dosed intraruminally with 4 g of LiCl (50% dose). Provenza et al. (1993) found that LiCl levels peaked rapidly (i.e., within 15 min) when given intraruminally to sheep. Cattle could easily consume 1000 g of corn in a 10-min period. Each period was analyzed separately using a split-plot ANOVA model, with repeated measures over days.

Experiment 3: Food Ingestion as Affected by Tall Larkspur. Ten 3- to 4-year-old Hereford × Angus cows (598 × 42 kg) were randomly assigned to two treatment groups ($N = 5$). These cows had all grazed on summer range where they had ingested some tall larkspur.

After an initial seven-day period with ad libitum access to alfalfa pellets, a familiar food, ad libitum pellet intake was measured for each animal for 10 days, and mean intake over the last five days constituted the baseline value. Thereafter, alfalfa pellets were offered at 90% of baseline to each animal individually from 1300 to 1500 hr each day.

Tall larkspur (*D. barbeyi*) was collected from the Wasatch Plateau at 3200 m elevation in late July, 1994, air-dried at 20°C, ground through a 2-mm screen, and stored at 16°C in sealed plastic bags. Larkspur was analyzed by an HPLC method to determine toxic alkaloid concentration (Manners and Pfister, 1993).

Whole, shelled corn (1000 g) was offered to each animal individually in a food box for 5 min/day at 0700 hr. Cattle had to eat avidly to consume all the corn within 5 min. Immediately thereafter, cattle were dosed with either ground larkspur or ground alfalfa via oral gavage in direct proportion to the amount of corn consumed. The amount of larkspur that was dosed varied as a function of the amount of corn eaten. A 100% larkspur dose was equivalent to 16 mg toxic alkaloid/kg body weight; alfalfa was dosed to controls at the equivalent amount of 2.22 g/kg body weight. This dose of tall larkspur was estimated to be just below an amount (>22 mg/kg) that would cause muscular weakness and possible temporary collapse (Pfister et al., 1994b). The onset of overt toxicosis from tall larkspur is generally delayed by 7–10 hr from time of ingestion (Pfister et al., 1994c), so cattle would presumably have experienced illness from tall

larkspur during midafternoon. Livestock can acquire taste aversions if the delay between food ingestion and postingestive consequences does not exceed 12 hr (Burritt and Provenza, 1989).

The experiment was analyzed as a repeated-measures ANOVA with treatment, animals nested with treatment, with a day effect, and the day × treatment interaction. When a significant F test was found ($P < 0.05$), the PDIFF procedure of SAS (1987) was used to separate means.

RESULTS

Retrospective Study of Larkspur Ingestion by Individual Grazing Animals. Larkspur consumption by individual animals in the 1986 and 1987 studies varied in a cyclic pattern, generally over a two- to four-day period (Figure 1). In both studies, there was a significant day effect ($P < 0.01$). During the 1986 study, cattle ate substantial amounts of larkspur early in the grazing period, whereas larkspur consumption was initially low during 1987 before increasing over time. Overall, the pattern of consumption suggests that when larkspur was ingested at levels above 25–30% of the diet during a specific day, cattle decreased consumption over the next day or two.

Experiment 1: Cyclic Consumption of Larkspur Pellets by Cattle. Intake of alfalfa pellets was low during the first few days of the trial, until daily consumption leveled to about 10 kg/head by day 8–10 (Figure 2). Toxic alkaloid concentration of the 27% larkspur pellet was 1.06 mg toxic alkaloid/g (3.92 mg/g in plant). Previous toxicity tests have shown that no overt signs of intoxication (i.e., muscular tremors, periodic collapse) are apparent unless cattle are given a single pulse dose equivalent to about 2300 g larkspur (>22 mg toxic alkaloid/kg body weight) (Pfister et al., 1994b). In this trial, no overt signs of intoxification were noted, presumably because cattle ate the pellets over several hours. Mean larkspur intake was 2007 g/day.

Cattle given only the 27% larkspur pellet showed distinct cycles of increasing consumption, a period of rejection, and increased intake again (Figure 2; day × treatment interaction, $P < 0.025$). Intake by controls ranged from 93 to 115% of baseline, whereas intake by larkspur animals ranged from 53 to 97% of baseline. Controls averaged 105% of baseline; the larkspur group averaged 79% ($P < 0.008$).

Experiment 2: Food Ingestion as Affected by LiCl. All animals ate 100% of the basal ration except for one treatment animal that refused about 1.5 kg of alfalfa pellets on days 6 and 7 during the 40-mg dose and on day 4 during the 80-mg dose. No differences in corn consumption ($P > 0.1$) between treatment and controls were found at the 20 or 40 mg/kg dose of LiCl (Figure 3). There was a day effect and day × treatment interaction for corn intake ($P < 0.01$)

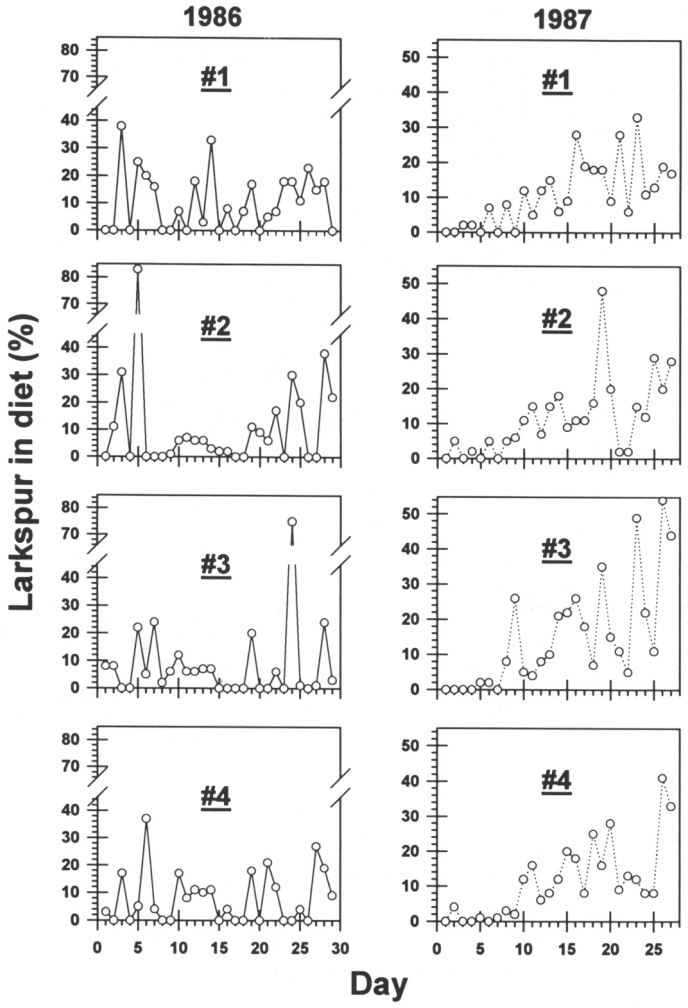

FIG. 1. Consumption of tall larkspur (% of diets) by individual heifers, numbered 1 to 4, grazing in Utah during summer, 1986 and 1987. The same animals were used both years. Both trials began when larkspur was in the flower stage and ended when larkspur was in the pod stage.

for the 20 mg/kg dose, due mainly to initial variability in acceptance of a novel food. Corn intake for the control and treatment groups differed at the 80 mg/kg dose ($P < 0.001$, Figure 3). There was a day effect ($P < 0.001$) and a day × treatment interaction at this dose ($P < 0.03$). At the 80-mg level, the controls averaged over 96% consumption of the corn, while the treatment group averaged

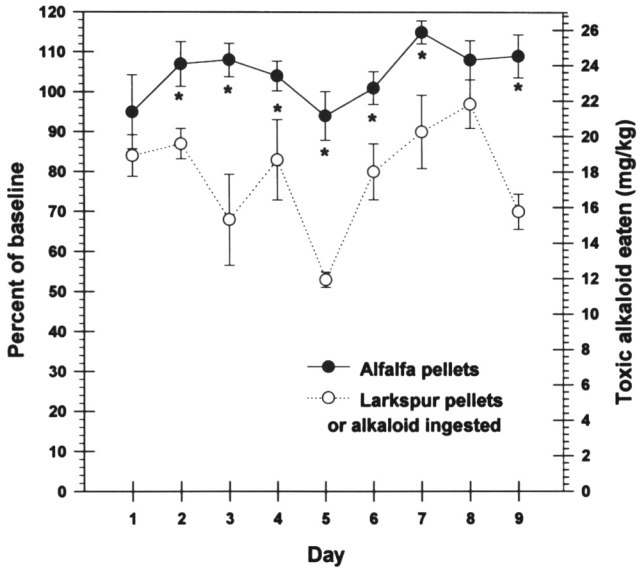

FIG. 2. Experiment 1: pellet consumption (% of baseline on left *y* axis) by cattle receiving either larkspur pellets (treatment group), or alfalfa pellets (controls) during a nine-day pen trial. The right-hand *y* axis is mean daily amount of toxic alkaloid ingested per kilogram of body weight and applies only to animals receiving larkspur pellets. Bars are ±SE for pellet consumption. An asterisk indicates a difference ($P < 0.05$) between controls and treatment groups for pellet consumption.

only 46% corn consumption. The LiCl group decreased corn intake to below 20% by day 3, then intake increased to 40–50% of the offered corn from days 5 to 8.

Cattle on the 80 mg/kg dose received an average daily dose of 37 mg LiCl/kg. Cattle generally did not refuse any alfalfa pellets in the afternoon after being dosed with the 80-mg dose of LiCl in the morning, indicating that only corn intake was affected by LiCl. Most animals who received the 80-mg dose decreased corn intake on days 2 and 3, then increased corn intake in a fluctuating manner. An actual dose of about 60–70 mg LiCl/kg body weight on one or two days would result in decreased corn consumption on subsequent days. Because all animals on the 80-mg dose were not in sync with respect to daily fluctuations after day 3, two patterns representative of individual animal variability are shown in Figure 4. One animal (Figure 4A) was apparently more susceptible in LiCl than the other animals, and corn consumption by that animal fluctuated greatly at the 40-mg dose. The 40-mg dose apparently induced a transient aversion, whereas the 80-mg dose also induced an aversion interspersed with several days

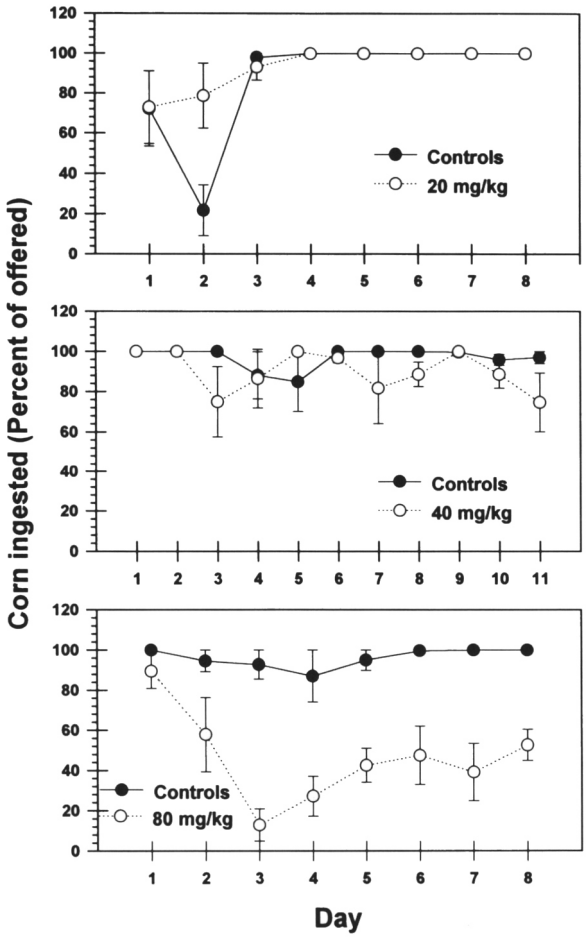

FIG. 3. Experiment 2: consumption of corn (% of 1000 g offered) during a 10-min trial when corn consumption was followed by an intraruminal dose of 20, 40, or 80 mg LiCl/kg body weight or no treatment was given (controls). At each dose level, LiCl was dosed in proportion to the amount of corn consumed during each period. At the 80-mg dose, the treatment and control groups differed ($P < 0.05$) each day after day 1. There was a treatment × day interaction ($P < 0.05$) at the 20- and 80-mg doses and no interaction at the 40 mg dose. Bars are ±SE for corn intake.

of acceptance of corn (Figure 4B). Conversely, the other animals were generally not affected by the 40-mg dose (Figure 4C), but showed distinct cyclic patterns when receiving the 80-mg dose (Figure 4D).

Experiment 3: Food Ingestion as Affected by Tall Larkspur. There was a

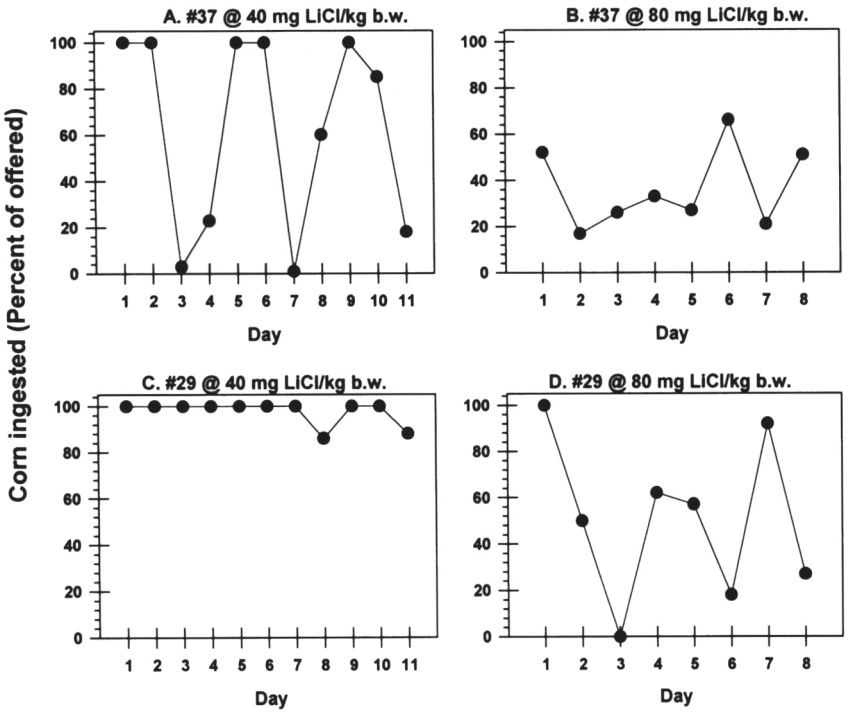

FIG. 4. Experiment 2: daily corn consumption (% of 1000 g offered) by animals #37 (A and B) and #29 (C and D) during a 10-min trial for eight days using either 40 or 80 mg LiCl/kg body weight. During each period, LiCl was dosed in proportion to the amount of corn consumed.

treatment effect ($P < 0.01$) and a day × treatment interaction ($P < 0.01$) for corn consumption (Figure 5). Overall, corn intake was lower ($P < 0.01$) for the larkspur group compared to controls (52 vs. 84%, respectively). Daily corn intakes for each individual animal are shown in Figure 6. Corn intakes were generally low initially because of its novelty, but some animals were not initially neophobic to corn. Larkspur animals showed various cyclic patterns of corn intake; in general one to three days of higher corn consumption (and higher larkspur doses) were followed by one to three days of reduced corn consumption. For most treatment animals the threshold for larkspur effects on food intake was about 14 mg/kg body weight. One animal (#6) did not respond to the 16 mg/kg dose of larkspur, so her dose was increased to 20 mg/kg on day 8, after which she showed a cyclic pattern of intake. The tall larkspur in this collection had a toxic alkaloid concentration of 7.2 mg/g. No overt signs of toxicosis were noted during the experiment. One control animal (#49) was injured by another cow on day 13 and was removed from the study.

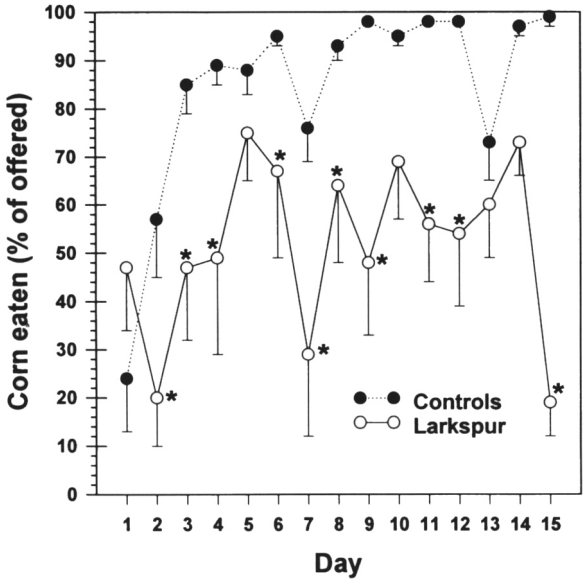

FIG. 5. Experiment 3: mean consumption of corn (% of 1000 g offered) during a 5-min trial when corn consumption was followed by an oral dose of tall larkspur (larkspur) or alfalfa (controls). Larkspur and alfalfa were dosed in proportion to the amount of corn consumed. An asterisk indicates that the larkspur group differs ($P < 0.05$) from controls. There was a treatment × day interaction ($P < 0.01$). Bars are −SE.

There was no difference in alfalfa pellet consumption ($P > 0.1$) between the two groups, nor was there a day × treatment interaction ($P > 0.4$). Mean alfalfa pellet intake was 95% of that offered, which again shows that the aversion was specific to the corn.

DISCUSSION

Retrospective Study of Larkspur Ingestion by Individual Grazing Animals. Individual animal consumption of toxic plants is often overlooked and is of paramount importance in understanding how animals react to toxins (Provenza et al., 1992). Diets of grazing ruminants vary within and between days; this grazing strategy is thought to provide sufficient nutrients (Kyriazakis and Oldham, 1993; Provenza, 1995), avoid food-specific satiety or toxicity (Provenza, 1995), and alleviate dietary monotony (Newman et al., 1992; Early and Provenza, unpublished data, 1996). A major mechanism for dietary variety may be acquired aversions for both nutritious and toxic foods, which diminish preferences for selected foods (Provenza, 1996). The cyclic pattern noted in Figure

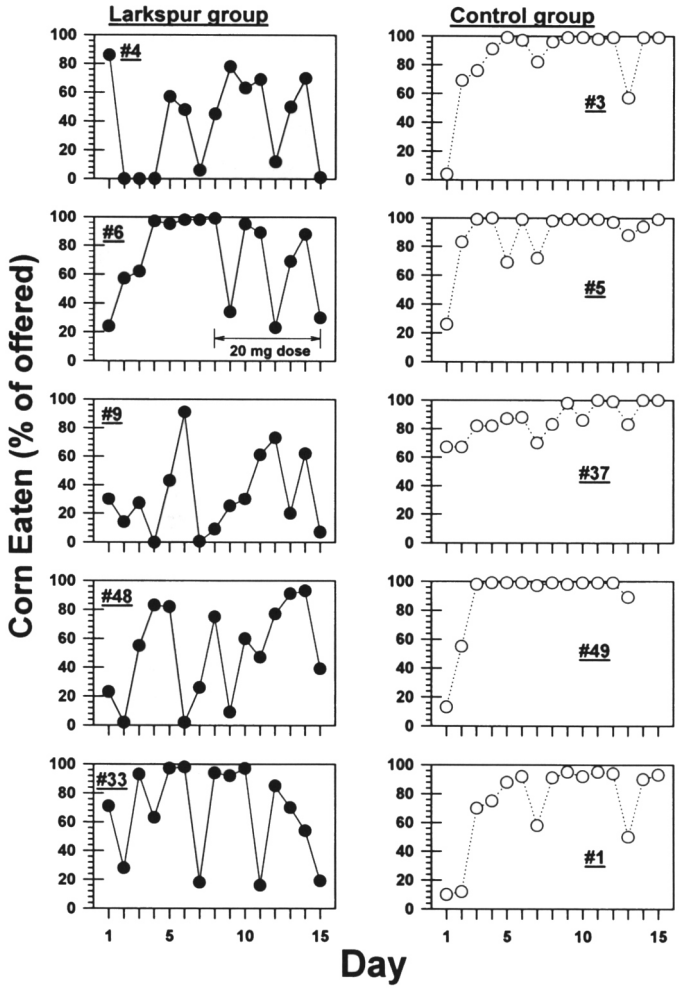

FIG. 6. Experiment 3: daily corn consumption (% of 1000 g offered) by individual animals in the larkspur-treated group and controls. The tall larkspur dose was 16 mg toxic alkaloids/kg body weight for all animals except where noted for animal #6; the corresponding alfalfa dose for controls was 2.2 g/kg body weight. Control animal #49 was injured by another animal and removed from the study after day 13.

1 appears to be a specific response to tall larkspur. The data suggest that cattle ingest larkspur up to some critical threshold, then reduce larkspur intake, which presumably allows a detoxification period. We have examined daily grazing records of cattle consumption of other major palatable forbs (e.g., tall bluebell, *Mertensia arizonica*; cow parsnip, *Heracleum lanatum*). Variability is evident

as one would expect, but cyclic changes are not apparent as with tall larkspur (Pfister, unpublished data). These observations provided a rationale for three pen studies to examine cattle consumption patterns of either tall larkspur or food ingestion paired with toxic larkspur or LiCl.

Experiment 1: Cyclic Consumption of Larkspur Pellets by Cattle. Cattle in the larkspur group ingested pellets in amounts (mean 7628 mg toxic alkaloid; 17.8 mg toxic alkaloid/kg body weight) near the threshold for overt toxic symptoms (9400 mg toxic alkaloid, >22 mg/kg) (Pfister et al., 1994a; Pfister and Cheney, 1997). The results suggest that under these conditions cattle can regulate consumption of larkspur and therefore generally avoid overingestion and overt intoxication. One to several days of high consumption were followed by a day of reduced consumption. These periods of reduced consumption apparently allow animals to recover from subclinical larkspur toxicity. Because MLA and DAN reversibly bind to ACh receptors (Dobelis et al., 1993), the degree of intoxication will depend on receptor site saturation, ACh and toxin concentration and subsequent competition in the synaptic cleft, and alkaloid binding affinity to ACh receptors. The rate of detoxification will depend primarily on toxin concentration, metabolism, and excretion at the active site (Lefkowitz et al., 1990). We have previously found that cattle recover within 24–48 hr after being dosed with sufficient larkspur alkaloids to produce muscular tremors and recumbency (Pfister et al., 1994b).

Experiment 2: Food Ingestion as Affected by LiCl. Within each experimental period (i.e., 20-, 40-, or 80-mg dose), the amount of LiCl actually dosed to each animal depended upon how much corn was consumed. Thus, on days when corn consumption was very low or very high, the amount of LiCl was varied accordingly. This scenario resembles grazing conditions, where the amount of toxic alkaloids ingested depends primarily on the amount of larkspur consumed, since alkaloid levels change only gradually over the growing season (Pfister et al., 1988b, 1994a; Pfister and Manners, 1991).

The results suggest that cattle can generally regulate intake of LiCl below an individual threshold of about 60–80 mg/kg. Four of six animals required ≥70 mg/kg for two consecutive days before intakes decreased, whereas a fifth animal reduced intake after one 67 mg/kg dose, and the sixth animal reduced intake after an initial dose of 41 mg/kg. Once the individual threshold was reached, animals reduced corn intake for one to three days, but began consuming some corn again when LiCl dosage decreased. The aversion to corn paired with LiCl was fragile, probably because 80 mg (the highest dose used) is not a particularly strong dose (Ralphs and Cheney, 1993) and perhaps due to positive postingestive feedback from corn. Corn is high in digestible energy and provides rapid postingestive reinforcement (Villalba and Provenza, 1997).

Experiment 3: Food Ingestion as Affected by Tall Larkspur. Tall larkspur doses paired with corn consumption resulted in transient aversions at higher

amounts. Cattle apparently sense tall larkspur toxicosis and make an association between the larkspur dose and the corn flavor. This aversion is fragile, however, as subsequent sampling of corn generally leads to rapid extinction and heightened corn intake during later tests. We suspect that the aversion was not strong or permanent because the nausea was relatively mild (e.g., animals did not reduce basal feed intake), and because the positive feedback from corn encouraged renewed consumption. These results with corn and larkspur are similar to the patterns noted in grazing cattle (Figure 1). Cattle apparently have the ability to moderate tall larkspur intake based on gastrointestinal feedback from larkspur, even though ingestion and toxicosis may not be closely linked in time. This experiment also indicates that overall food intake is not adversely affected by subclinical larkspur toxicosis. We have noted in other studies (Pfister et al., 1994c; Pfister and Cheney, 1997) that food intake (i.e., long-stem hay) is reduced for two or three days after cattle show overt signs of intoxication, including muscular tremors and periodic collapse.

General Discussion. Cattle regulated consumption of larkspur following intoxication. Grazing animals showed marked fluctuations in amounts of larkspur consumed; one or two days of high consumption were generally followed by a day or two of reduced consumption. During experiment 1, cattle followed days of high consumption of larkspur pellets with a day of reduced consumption; average daily consumption (17.8 mg toxic alkaloid/kg) was just below a toxic threshold, above which cattle show overt signs of intoxication. In experiments 2 and 3, cattle displayed similar cyclic patterns when LiCl and tall larkspur were used as the toxic agents. When larkspur was given as a single dose mixed with water, the lowest threshold for effects was about 14 mg/kg. We have noted significant reductions in operant responding when cattle were dosed with 14–18 mg toxic alkaloid, but no clinical signs were noted (Pfister and Cheney, 1997). Periods of reduced consumption after ingesting the larkspur toxin probably allow animals time to recover from food-specific illness and detoxify after the toxic insult.

Other studies have shown variable or cyclic food intake patterns in response to ingestion of toxic foods. Dietary diversity may result in part because herbivores limit consumption of specific toxic plants (Freeland and Janzen, 1974). Mice attempted to minimize toxic effects by varying dietary intake of toxic foods (Freeland and Saladin, 1989). Kyriazakis and Emmans (1992) reported that pigs showed a cyclic intake pattern when given rapeseed-based food. Nutritional deficiencies such as imbalances of zinc can reliably provoke cyclic food intake patterns similar to toxicoses from food (Wallwork et al., 1981).

We did not determine how cattle become adverse to tall larkspur and not other plants during peak larkspur ingestion, but two variables may be important: (1) the amount of larkspur eaten during a meal compared to the amount of other foods eaten (e.g., goats acquire an aversion to the food eaten in the greatest

amount when toxicosis follows a meal of novel foods) (Provenza et al., 1994); and (2) prior illness associated with larkspur ingestion (e.g., lambs avoid the food that made them ill in the past when toxicosis follows a meal of several foods) (Burritt and Provenza, 1991, 1996). Thus, the proportion of tall larkspur eaten and prior illness associated with larkspur may cause cattle to limit intake of larkspur and not other plants.

Why do animals continue to consume a food that has been aversive in the recent past? Many toxic compounds cause food aversions (Riley and Tuck, 1985), but sampling can quickly extinguish the aversion (Provenza et al., 1992; Ralphs and Olsen, 1990). In the case of larkspur, it appears that cattle begin sampling the food because ingestion of low to moderate amounts causes few or no adverse effects while simultaneously providing energy. Increased consumption probably results in heightened adverse effects because of the dose–response nature of diterpenoid alkaloids (Manners et al., 1992, 1993). We speculate that above the 14–18 mg/kg threshold, larkspur alkaloids interact with cholinergic receptors in the gastrointestinal tract and produce nausea. However, highly nutritious larkspur also provides sufficient positive postingestive feedback for animals to quickly extinguish a food aversion and resume consumption once the gastrointestinal distress passes. Learned aversions to larkspur formed in a pen situation are quickly extinguished under field conditions when cattle begin sampling the plant (Lane et al., 1990; Ralphs and Olsen, 1990). Although the results of experiments 2 and 3 may have changed slightly had animals been given the basal food ad libitum, we believe that the basic pattern would not have changed since the degree of dietary restriction was not great. This conclusion is supported by the results from experiment 1 where animals were given food ad libitum.

duToit et al. (1991) found a similar fluctuating pattern of food ingestion in sheep given LiCl-treated feed. They noted that LiCl intake fluctuated around 39 mg/kg, a level just below a dose necessary to form a mild aversion. We found that at the highest LiCl dose, cattle averaged 37 mg/kg of LiCl per day. This suggests that mechanisms for food aversion learning are about as sensitive in cattle as in sheep or goats (duToit et al., 1991).

Why then are about 5% of animals killed by overingestion of larkspur each summer on western US mountain ranges (Ralphs et al., 1988)? The reasons why some animals fail to learn to avoid lethal doses of toxic plants are complex (Provenza et al., 1992). Our observations indicate that many cattle deaths occur during brief (i.e., 20–30 min) episodes of frenetic overingestion, when environmental circumstances apparently alter plant acceptability (Pfister et al., 1988a). At other times, cattle overingest tall larkspur for several hours when the plant is apparently very palatable, and animals that are more susceptible are sometimes fatally poisoned (Pfister, unpublished observations). Other extenuating circumstances that may play a role in cattle deaths are bloat and handling stress, but these have not been studied.

Based on the results of these trials and previous dosing experiments with tall larkspur, there appear to be at least three distinct thresholds involved in tall larkspur toxicosis (Figure 7): first, a subclinical toxicosis that results in reduced tall larkspur intake for one to three days, but no overt signs nor overall reductions in intake of safe foods (14–21 mg/kg); second, a short-acting toxicosis with overt clinical signs that results in reduced food intake for several days, but no long-term effects (near 22 mg/kg); and third, a potentially fatal toxicosis with severe clinical signs that can result in death ($\geq$40 mg/kg). Obviously, there is a dose–response gradient with respect to severity of clinical signs as the dose increases above 22 mg/kg. We conclude that cyclic consumption enables cattle to generally regulate larkspur consumption below the second threshold. This

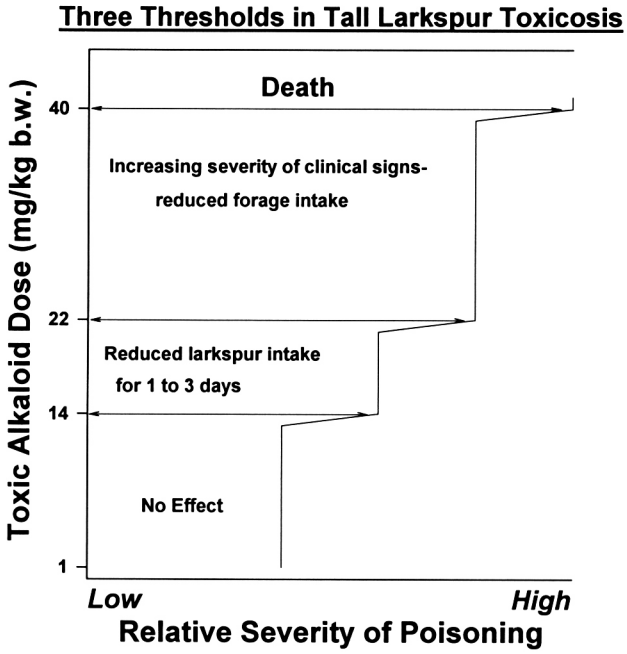

FIG. 7. Representation of three thresholds in tall larkspur toxicosis. The *y* axis is amount of toxic alkaloid ingested, and the *x* axis represents increasing severity of intoxication. The thresholds are: first, a subclinical toxicosis that results in reduced tall larkspur intake for one to three days, but no overt signs or overall reductions in intake of safe foods (14–21 mg/kg); second, a short-acting toxicosis with overt clinical signs that results in reduced food intake for several days, but no long-term effects (near 22 mg/kg); and third, a potentially fatal toxicosis with severe clinical signs that can result in death at $\geq$40 mg/kg. Severity of clinical signs increases in a dose-dependent manner above the 22 mg/kg threshold, until animals die from respiratory failure.

allows most cattle the opportunity to use an otherwise nutritious, but potentially toxic, plant. Additional research is needed to better define when this process is upset and animals are lethally poisoned.

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MELLEIN, A TRAIL PHEROMONE COMPONENT OF THE ANT *Lasius fuliginosus*[1]

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Abstract—3,4-Dihydro-8-hydroxy-3-methylisocoumarin (mellein) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one are among the volatile constituents identified from the hindgut of the formicine ant *Lasius* (*Dendrolasius*) *fuliginosus* Mellein induces trail-following behavior in worker ants of this species and evokes electrophysiological responses from their antennae. The trail-following activity released by (*R*)-(−)-mellein is significantly higher than that elicited by its (*S*)-(+) antipode, or the racemic mixture. The above-mentioned pyranone is found also in the honeydew of aphids, the customary diet of this ant species. The pyranone also evokes some trail-following behavior, but its activity is far less pronounced than that of mellein. Apparently, extra pheromone components are present in the hindgut, since the activity of these two constituents, either individually or as a mixture, does not completely account for the total activity released by a hindgut extract containing similar amounts of these two compounds.

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Key Words—Ant, Formicinae, *Lasius fuliginosus*, hindgut, trail pheromone, 3,4-dihydro-8-hydroxyisocoumarin, mellein, 2,3-dihydro-3,5-dihydroxy-6-methyl-4-*H*-pyran-4-one.

INTRODUCTION

A wide variety of ant species are known to utilize chemicals from exocrine glands for laying trails during foraging and nest movement (Hölldobler and Wilson, 1990). The trail communication of the formicine ant *Lasius fuliginosus* Latr. was one of the earliest systems to be studied in detail (Carthy, 1950; Hangartner and Bernstein, 1964; Hangartner, 1967). A major part of the diet of *L. fuliginosus* consists of honeydew obtained from aphids. Honeydew serves also as a nutrient for the symbiotic fungus, *Cladosporium myrmecophilum*, which provides mechanical stability to the nests of this ant (Maschwitz and Hölldobler, 1970). From a three-year field study on the trail communication system of a single colony of *L. fuliginosus*, Quinet and Pasteels (1991) reported the existence of a permanent network of trails that leads the ants to their aphid sites. Apparently the trails and aphid sites remain unchanged for several years.

First attempts to identify the trail pheromone of *L. fuliginosus* were reported by Huwyler et al. (1973, 1975). They attributed the activity to a mixture of fatty acids. However, in preliminary investigations, we failed to elicit any trail-following behavior in *L. fuliginosis* by laying artificial trails made from solutions containing hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids, the reported trail pheromone mixture. Independent research conducted by Quinet and Pasteels (1995) also confirms that these fatty acids are not effective as trail pheromone components when tested at physiological concentrations. These observations and the discovery of 3,4-dihydro-8-hydroxyisocoumarins as trail pheromones of several other formicine species (Bestmann et al., 1992) led us to reinvestigate the chemical composition of this pheromone. Our results, which contradict those of Huwyler et al. (1973, 1975), are reported in this publication.

METHODS AND MATERIALS

Chemicals. A racemic mixture of (±)-3,4-dihydro-8-hydroxy-3-methyl-isocoumarin (mellein, **1**) was provided by Prof. K. Mori (Tokyo University, Japan), and (*R*)-(−)- and (*S*)-(+)- enantiomers of mellein (enantiomeric purity >99%) were from Prof. T. Ueno (Kyoto University, Japan) (Figure 1). (±)-3,4-Dihydro-8-hydroxy-3,5-dimethylisocoumarin **5** (purity 98%) and (±)-3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin **6** (purity 98%) were synthesized by Dr. M. C. Witschel (University of Erlangen-Nürnberg, Germany) (Figure

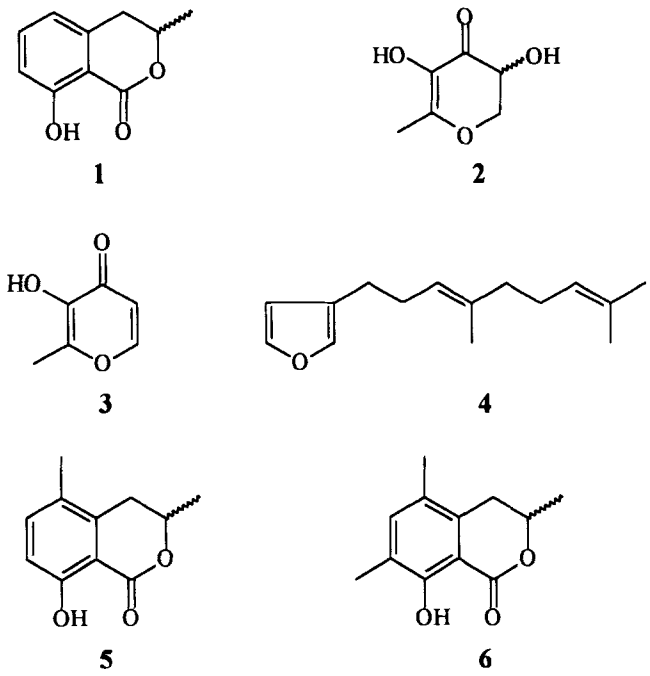

FIG. 1. Structures of 3,4-dihydro-8-hydroxy-3-methylisocoumarin (**1**, mellein), 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (**2**), 3-hydroxy-2-methyl-4*H*-pyran-4-one (**3**, maltol), 3-(4,8-dimethyl-3,7-nonadienyl)furan (**4**, dendrolasin), 3,4-dihydro-8-hydroxy-3,5-dimethylisocoumarin (**5**), and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (**6**).

1). (±)-2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one **2** (pyranone, purity 92%) was a gift of Dr. M. Glomb (Institut für Lebensmittelchemie, Stuttgart, Germany) (Figure 1). Hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids as well as hexadecane, hexadecanol, and isopropyl hexadecanoate were purchased from Aldrich Chemical Co. (Steinheim, Germany).

Ants. Ants were collected from several sites in Erlangen and Frankfurt. For behavioral bioassays, colony fragments consisting of several hundred workers without brood were kept in plastic boxes and provided with water, aqueous honey, and occasionally with dead insects.

Preparation of Biological Material. The ants were anesthetized with carbon dioxide and kept in a freezer at −20°C for 20 min before their hindguts were excised in water and extracted with diethyl ether. Ether extracts made from about 10 hindguts were used for behavioral tests and microreactions. Similarly,

ether extracts of hindguts, Dufour glands, and 35 individual heads were also prepared for GC-MS analyses. For the analysis of volatiles in the midgut by GC-MS, midguts of 105 ants were extracted with diethyl ether and the extract was condensed to 1 μl. For GC-MS analysis by a solid-sampling injection technique (Morgan and Wadhams, 1972), 55 hindguts and 40 midguts were sealed in glass tubes and crushed in the GC injection port (Attygalle et al., 1989). Ether extracts of aphids (Homoptera: Aphidina), which provided honeydew to the ant colonies of this study, and the honeydew were also analyzed by GC-MS.

Gas Chromatography. Gas chromatographic analyses were carried out using a Hewlett-Packard 5890 instrument equipped with a splitless injector, a flame ionization detector, and a solid sampler. Analyses were performed using a 25-m × 0.25-mm fused-silica capillary column coated with SE-30 stationary phase, using nitrogen as carrier gas (2 ml/min). The oven temperature was held at 60°C for 4 min and increased 6°C/min to 260°C. Enantiomeric purity of (*R*)-(−)- and (*S*)-(+)-mellein was determined using a Cyclodex-B chiral column (30 m × 0.25 mm; J&W Scientific) and helium as carrier gas (90°C for 4 min and increased 1°C/min to 180°C).

Different fractions of the GC effluent were collected by micropreparative gas chromatography. Material trapped in glass tubes cooled in methanol–Dry Ice was washed out with hexane, and the extracts were tested by a trail-following bioassay.

Gas Chromatography–Mass Spectrometry. GC-MS was performed using a Finnigan MAT90 (EI, 70 eV) instrument linked to a Varian 3000 GC. Analyses were conducted using a fused-silica column coated with SE-52 (25 m × 0.25 mm; 60°C for 4 min, 60–260°C at 6°C/min; splitless injection; carrier gas He, 2 ml/min). For quantification, standard solutions of (±)-mellein (100 μg/ml hexane) and (±)-2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (pyranone, **2**) (100 μg/ml hexane) were used as external standards.

Thin-Layer Chromatography. The contents of 10 hindguts of *L. fuliginosus* workers were squeezed directly onto a silica gel plate (Merck 60 F_{254}, 50 × 100 × 0.25 mm). The debris of hindguts was removed, and the plate was developed using diethyl ether–hexane (5 : 1). Five segments between R_f values 0–0.36, 0.36–0.41, 0.41–0.72, 0.72–0.81, and 0.81–1.00 were scraped from the silica gel plate, extracted with ether, and the extracts were tested by bioassay (Attygalle and Morgan, 1984). Under identical conditions, synthetic samples of mellein and pyranone were also chromatographed to determine their R_f values.

Microreactions. An aqueous extract of about 10 hindguts (200 μl) was basified with 1 M NaOH; the solution was sealed in a glass tube and heated for 20 min at 100°C. The reaction mixture was allowed to cool to room temperature and divided into two fractions. One fraction was extracted with 100 μl of diethyl ether while the other half was acidified with HCl before extraction. The trail-

following activity elicited by the two extracts was tested by the bioassay procedure described below.

Ten microliters of a diethyl ether extract of six hindguts were silylated with 5 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) for 10 min at room temperature. The reaction mixture was separated by TLC, and the relevant silica gel layers were scraped and extracted with diethyl ether. Biological activity of each fraction was tested by trail-following experiments.

Trail-Following Studies. Artificial trails were drawn with a pencil on a index card (14.8 × 21 mm), a part of which had been cut off to form a peduncle of 2.5 cm length. Starting from the peduncle, a pencil line was drawn, which divided after 2.5 cm into two intersecting S-shaped lines (each 20 cm) for test and control trails. The common section and the test trail were streaked with each test solution (100 μl) using a 50-μl disposable micropipet. Similarly, the common section and the control trail were treated with 100 μl of hexane. The card was held just above the ant colony until an ant climbed onto the peduncle. The card was then lifted slightly to prevent other ants climbing to it. The ant was observed until it left the trail or reached the 15-cm mark of the trail. The test ant was then removed to a separate box, and the procedure was repeated with nine more worker ants (the duration of each experiment was about 3–7 min for 10 ants). In this way, a trail-following activity value between 0 and 10 was obtained for each test. A new index card was used for each test and the position of test and control trails (left or right) was interchanged randomly.

Hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids were tested individually at trail concentrations of 0.003, 0.3, and 30 ng/cm against a trail made of 0.15 hindgut equivalents/cm or pure hexane as control. Each test was repeated five times. Similarly, each fatty acid at 0.003, 0.03, 0.3, 3.0, and 30 ng/cm trail concentrations and a mixture consisting of equal amounts of each acid at 0.3 ng/cm were tested against a trail drawn with 0.15 hindgut equivalents/cm or hexane.

The trail-following activity evoked by synthetic mellein and pyranone **2** was tested using a laboratory "colony" consisting of 500–750 workers that had been starved for several days before each test session. Stock solutions of mellein **1** (100 μg/ml in hexane) and pyranone **2** (100 μg/ml in ether) were diluted with hexane. For each bioassay, 100 μl of test solution was applied to the 20-cm pencil trail. In the first test series, each of the two compounds was tested over a relatively wide range of concentrations covering five degrees of magnitude (mellein, 0.25 pg/cm to 25 ng/cm; pyranone, 2.5 pg/cm to 250 ng/cm). In another series of tests, the two compounds were tested over a narrower range of concentration. Mixtures of the two substances (mellein–pyranone = 1 : 10 and 1 : 100) were also tested for possible synergistic effects. During each bioassay session each substance was tested randomly either individually or as mixtures for each given concentration. For comparison, the trail-following response to a

L. fuliginosus hindgut extract (0.017 gland equivalent/cm) was also tested during each test session.

Furthermore, choice tests were conducted with mellein and pyranone **2** at concentrations of 2.5 and 25 pg/cm, respectively. Similarly, samples of (*R*)-(−)-mellein and (*S*)-(+)-mellein, (±)-mellein, (±)-3,4-dihydro-8-hydroxy-3,5-dimethylisocoumarin **5** and (±)-3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin **6** were also tested at a concentration of 5 pg/cm.

Electroantennography (EAG). Electrophysiological responses of *L. fuliginosus* workers, evoked by synthetic (±)-mellein **1,** pyranone **2,** (±)-3,4-dihydro-8-hydroxy-3,5-dimethylisocoumarin **5** and (±)-3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin **6** were recorded by the electroantennogram technique previously described by Kern and Bestmann (1993). The head of an ant was excised, and a glass capillary electrode filled with insect Ringer solution was connected to the laceration at the base of the head. Another microcapillary electrode was inserted to the distal end of the antenna. Compounds to be tested (0.01–100 μg) were applied to filter paper strips (7 × 20 mm) as hexane solutions and placed in small glass cartridges. Control cartridges were loaded with hexane. The antenna was stimulated by passing an airflow of 0.5 liters/min for 1 sec over the filter paper strips. The response amplitudes for each test series were normalized by dividing each value by the maximum value obtained for that series (EAG/EAG_{max}). These normalized values were used to compute the mean and standard error values for each test series.

RESULTS

GC-MS analysis of volatiles present in the hindguts of *L. fuliginosus* showed the presence of many components (Figure 2). The main components were identified as hexadecane (peak D), 1-hexadecanol (E), hexadecanoic acid (F), and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one **2** (pyranone, A) by comparison of their mass spectra and retention times with those of authentic standards. Similarly, a few of the minor components were identified as 3,4-dihydro-8-hydroxy-3-methylisocoumarin **1** (mellein, B), 3-(4,8-dimethyl-3,7-nonadienyl)furan **4** (dendrolasin, C, Figure 1), and isopropyl hexadecanoate (G) by comparing their mass spectra with those in the literature. In addition, mass chromatographic analysis revealed the presence of trace quantities of maltol **3** (3-hydroxy-2-methyl-4*H*-pyran-4-one, Figure 1); this substance elutes before the pyranone, and is therefore not shown within the retention time range presented in Figure 2.

Of all the fractions collected by micropreparative gas chromatography of hindgut volatiles, only two released trail-following activity. Thick double-headed arrows in Figure 2 indicate the retention time ranges of these trail-active frac-

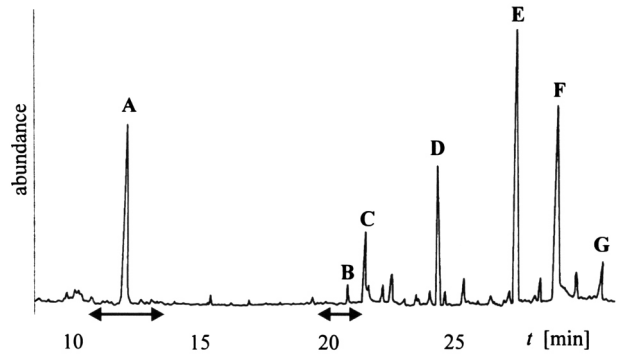

FIG. 2. A partial reconstructed gas chromatogram obtained by solid-sampling GC-MS analysis of volatiles from 55 hindguts of *L. fuliginosus* workers: 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (A), 3,4-dihydro-8-hydroxy-3-methylisocoumarin (B, mellein), 3-(4,8-dimethyl-3,7-nonadienyl)furan (C, dendrolasin), hexadecane (D), hexadecanol (E), hexadecanoic acid (F), and isopropyl hexadecanoate (G). Thick double-headed arrows indicate the regions corresponding to the GC effluent fractions that elicited trail-following activity.

tions. The two active fractions corresponded to the sections of the chromatogram where pyranone **2** (peak A, Figure 2) and mellein **1** (peak B) appear. Quantification of these two components, using authentic samples as external standards revealed that the amount of mellein **1** and pyranone **2** in an individual hindgut is approximately 50–100 pg and 0.6–1.1 ng, respectively.

Dendrolasin **4** has been identified from the mandibular glands of *L. fuliginosus* (Bernadi et al., 1967), and from plant sources (Belardini, 1983). In order to show that mellein and pyranone **2** are not contaminants from some other glandular secretions, ether extracts of Dufour glands and individual heads were analyzed by GC-MS, which showed that volatile components of the Dufour gland consist of hydrocarbons and a series of 2-alkanones, a mixture very similar to that described previously by Ali et al. (1988). In the extracts of individual heads, we identified a number of terpenes, including perillen, citronellal, citronellol, neral, geranial, farnesal, and dendrolasin, by comparison of their mass spectra with those in the literature. However, in none of the extracts obtained from mandibular or Dufour glands were mellein or pyranone **2** detected even in trace amounts. Therefore, these two compounds found in hindgut contents are not contaminants from mandibular or Dufour glands.

The volatile compounds present in the *L. fuliginosus* midgut were also investigated by analyzing solvent extracts and samples sealed in glass capillaries. Among the components identified were undecane, 2-pentadecanone, tetradecanoic acid, pyranone **2** (approx. 600–900 pg/midgut) and large quantities of

dendrolasin **4.** Traces of mellein **1** (approx. 25–70 pg/midgut) were also detected in the concentrated diethyl ether extract of 105 midguts.

The trail-following activity evoked by hindgut extracts disappeared when the extracts were treated with NaOH. The activity could be regenerated upon acidification. Similarly, silylation of the ether extract resulted in a significant loss of trail-following activity in comparison to that of the untreated extract.

We failed to elicit any appreciable trail-following behavior from synthetic samples of hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acid when tested at concentrations of 0.003, 0.3, and 30 ng/cm individually against a trail made of a hindgut extract or hexane as control. A mixture consisting of equal amounts of each of these fatty acids (0.3 ng/cm) was equally inactive. The test ants always selected and followed only the trail drawn with hindgut extract.

The R_f values obtained for synthetic pyranone **2** and mellein **1** by thin-layer chromatography (TLC) were 0.39 and 0.77, respectively (diethyl ether–hexane 5:1). When contents of 10 hindguts were separated by TLC and five sections between R_f values of 0–0.36, 0.36–0.41, 0.41–0.72, 0.72–0.81, and 0.81–1.00 were scraped, extracted with ether, and the extracts tested by bioassay, fraction 4 (R_f 0.72–0.81) elicited the highest trail-following activity. All 20 ants tested followed the trail made of this fraction to the end, while none selected the control trail made of hexane. Similarly, fraction 2 (R_f 0.36–0.41) also showed significant activity; 18 of 20 ants completed the trail. The activity of fraction 3 (R_f 0.41–0.72) was very weak (only 2 ants of 20 followed the trail) while fraction 1 (R_f 0–0.36) and fraction 5 (R_f 0.81–1.00) were completely inactive.

Since mellein **1** was identified by GC-MS as a component of hindgut volatiles, and the R_f value of mellein (0.77) is within the R_f range of the most active TLC fraction 4 (R_f 0.72–0.81), mellein was considered as one of the trail pheromone components. Similarly, fraction 2 (R_f 0.36–0.41), which corresponds to the R_f value of pyranone **2** (0.39), showed some activity indicating that pyranone **2** may also contribute to the overall trail-following response.

The results from trail-following biotests conducted with synthetic substances are presented in Figure 3. Both, mellein **1** and pyranone **2** elicited trail-following at all concentrations tested; however, the responses were weaker than those from a hindgut extract containing approximately the same amount of mellein and pyranone (Figure 3). As expected, not a single ant of the 550 test ants followed the control trail made of hexane. Furthermore, the trail-following responses released from this hindgut extract showed far less variability than those observed for both synthetic substances.

When tested at equal concentrations, mellein **1** produced slightly higher trail-following activity than pyranone **2** (Figure 3). Mellein elicited the highest trail-following activity at about 25 pg/cm, and concentrations above or below this value showed less activity. For pyranone the results were even more variable

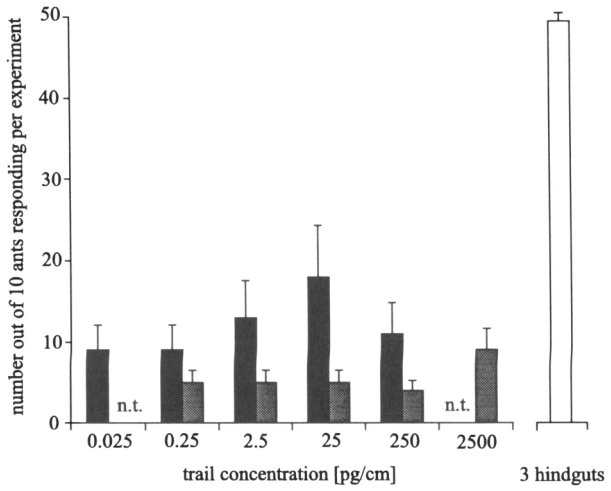

FIG. 3. Trail-following responses of *L. fuliginosus* workers to artificial 20-cm trails of synthetic mellein (black bars) and pyranone (hatched bars) at different concentrations. Response to a natural extract of three hindguts containing approximately 7.5 pg/cm of mellein **1** and 90 pg/cm of pyranone **2** is shown separately (open bar). Each column represents the average trail-following activity of 10 ants ($N = 5$) and vertical lines represent the standard errors; n.t., concentrations not tested.

than those for mellein. Trail-following activity released by mixtures of both components was compared to that of hindgut extracts. From the results, it was apparent that combinations of mellein and pyranone (1 : 10 and 1 : 100, mellein–pyranone) are not more effective than mellein alone. In this way, the existence of any synergistic effect could be ruled out.

Furthermore, in a choice experiment with mellein and pyranone at trail concentrations of 2.5 and 25 pg/cm, respectively, 26 of 30 ants followed the trail made of mellein, while only three selected the pyranone trail and one ant did not respond at all. Interestingly, when both enantiomers of synthetic mellein were offered in a choice test, the ants preferred to follow the trail of (*R*)-(−)-enantiomer. Of 30 ants tested, 21 ants followed the trail drawn with (*R*)-(−)-mellein, while four ants followed that of (*S*)-(+)-mellein, and the rest did not respond at all [enantiomeric purity of both (*R*)-(−) and (*S*)-(+) isomers were >99% as determined by gas chromatographic analysis on a chiral column (Cyclodex-B)]. Similarly, when ants were offered (*R*)-(−)-mellein against the racemate (5 pg/cm), they clearly showed their preference for the (*R*)-(−) isomer (19 vs. 8, of 30 test ants). On the other hand, the (*S*)-(+) enantiomer showed almost no differences in activity compared to that of (±)-mellein (13 vs 14). The structural analogs of mellein, (±)-3,4-dihydro-8-hydroxy-3,5-dimethyl-

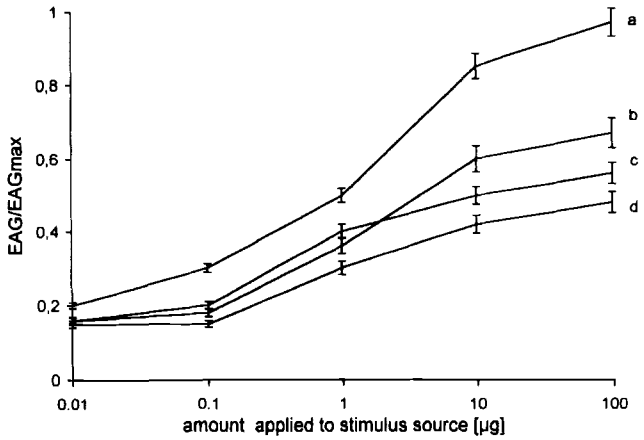

FIG. 4. Dose–response curves constructed from EAGs recorded from *L. fuliginosus* worker antennae. Each point represents the mean of 10 replicates. Vertical bars represent standard errors: 3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein) (a), 3,4-dihydro-8-hydroxy-3,5-dimethylisocoumarin (b), 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (c), and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (d).

isocoumarin **5** and (±)-3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin **6,** applied at 2.5 and 25 pg/cm, failed to evoke any trail-following activity.

Electroantennography (EAG). The electrophysiological responses recorded from antennae of *L. fuliginosus* workers obtained by puffing air over different doses of synthetic mellein **1,** 3,4-dihydro-8-hydroxy-3,5-dimethylisocoumarin **5,** pyranone **2,** and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin **6** are shown in Figure 4. The results showed that mellein (profile a) evokes higher electrophysiological responses compared to those from similar amounts of pyranone **2** (profile c) and the other two mellein analogs **5** (profile b) and **6** (profile d).

DISCUSSION

The hindgut has long been known as the source of trail pheromones in formicine ants (Blum and Wilson, 1964). Behavioral aspects of *L. fuliginosus* trail pheromone were first investigated by Carthy (1950, 1951). In 1964, Hangartner and Bernstein demonstrated that *L. fuliginosus* workers follow traces made of hindgut extracts, whereas extracts from other glands elicit only weak responses or do not lead to any significant trail-following behavior. Later, Hangartner (1967) showed that the trail-following activity evoked by a *L. fuliginosus* hindgut extract disappears upon basification and reappears upon acidification.

Based essentially on this observation, Huwyler et al. (1973, 1975) concluded that the fatty acids they identified from the hindgut were the components of the trail pheromone. Although they mentioned in a single sentence that commercial samples of the six acids elicited trail-following behavior, details of experimental procedures or results were not published (Huwyler et al., 1975). We failed to elicit any appreciable trail-following behavior from synthetic samples of these fatty acids when tested individually, or as a mixture consisting of equal amounts of all the fatty acids, against a hexane control or hindgut extract.

Subsequent to Huwyler's studies, Hayashi and Komae (1977) also reported a mixture of fatty acids as the trail pheromone of the myrmicine ant *Pristomyrmex pungens*. Although the trail-following activity was found in one fraction of a whole body extract of worker ants in which nine fatty acids were also identified, experimental procedures and detailed results of trail-following experiments were not presented (Hayashi and Komae, 1977). We have reinvestigated the trail pheromone of *P. pungens*, and our results, which contradict those of Hayashi and Komae (1977), will be published elsewhere.

On the other hand, the chemical properties of the pheromone components here identified, mellein and pyranone **2**, agree well with the observations of Hangartner (1967), who conducted microreactions with glandular extracts. Since mellein is a lactone, the addition of alkali is expected to open the lactone ring and thereby destroy the activity. A subsequent acidification of this mixture would cause relactonization and regeneration of the activity. In fact, we made similar observations during our trail-following tests with *L. fuliginosus* when synthetic mellein was subjected to changes of pH. Similar experiments with the mellein analog **6**, the trail pheromone of *Lasius niger*, also gave comparable results (Bestmann et al., 1992).

Considering the fact that the pyranone **2** is found also in aphids and their honeydew and that it is far less active as a trail releaser than mellein (which was not detected in aphids or honeydew), we are inclined to believe that the presence of pyranone is not vital for orientation communication. However, the pyranone may act as a semiochemical that may indicate the quality and quantity of an available sugary food source.

A trail pheromone system based on hindgut secretions that appears to consist of substances of different volatilities has been described for another formicine ant, *Formica polyctena* (Horstmann, 1982). When the colony was deprived of sugar for a longer period of time, *F. polyctena* workers were able to follow not only their own hindgut trails, but also trails laid by *L. fuliginosus* workers to their aphid sites (Horstmann, 1982).

In a more recent study of trail networks in *L. fuliginosus*, Quinet and Pasteels (1991) observed differences among temporary honeydew collecting trails, temporary hunting trails, and ephemeral recruitment trails. It is reasonable to expect qualitative and quantitative differences in the chemical compositions

of these trails. Although both, mellein **1** and pyranone **2** occur in the hindgut contents used for marking trails, the qualitative composition of the trails could change according to the physiological conditions of the trail-laying ant. We observed a considerable enlargement of the hindgut sac in our laboratory ants after a large intake of aqueous sugar or honey. In the field, the honeydew from a rich food source would similarly increase the hindgut volume. Although we have no evidence at present, it will not be surprising if future experiments reveal that relatively more pyranone is applied to trails laid during the return journey of a well-fed ant to the nest.

From electrophysiological investigations, it was evident that among the 3,4-dihydroisocoumarins tested, mellein elicited the highest electrophysiological responses. Structural analogs of mellein **1,** 3,4-dihydro-8-hydroxy-3,5-dimethylisocoumarin **5** and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin **6,** although eliciting some electrophysiological responses, failed to release trail-following behavior in our bioassays. Therefore it is reasonable to assume that *L. fuliginosus* workers are able to differentiate between the presence or absence of any extra methyl groups on the aromatic ring.

Unfortunately, a natural mellein sample from *L. fuliginosus* was not available when the chiral column for the separation of enantiomers was accessible during the last stages of our experiments. However, the significantly better trail-following activity released by (*R*)-(−)-mellein as opposed to its (*S*)-(+) isomer or a racemic mixture indicates that the natural pheromone is either the (*R*)-(−) enantiomer or a mixture enriched with that isomer.

We showed previously that 3,4-dihydro-8-hydroxyisocoumarins play an important role as trail pheromones in formicine ants. We identified a number of these substances as a new class of trail pheromones in the hindgut of *Lasius niger*, *Formica rufa* (Bestmann et al., 1992), and recently also in *Camponotus silvicola* and *C. rufipes* (Übler et al., 1996). In *L. niger* and *C. silvicola*, 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin **6** was established as the major trail pheromone component, whereas in *C. rufipes* 3,4-dihydro-8-hydroxy-3,7-dimethylisocoumarin is the active substance. On the other hand, *F. rufa* (Bestmann et al., 1992) and *L. fuliginosus* follow artificial trails made of 3,4-dihydro-8-hydroxy-3-methylisocoumarin **3** (mellein). Since mellein is common to both species as a trail-active substance, we are now in a position to explain Hangartner's (1967) previous observation that workers of *L. fuliginosus* in some cases, also followed trails of *F. rufa*.

Pyranone **2** and the minor component maltol (**3**), are typical products that could originate from mixtures of monosaccharides and amino acids (Maillard reaction) when subjected to elevated temperatures (Kramholler et al., 1993). The high temperature of the GC injection port could have produced these compounds as analytical artifacts of the GC procedure that was used to analyze hindgut samples. However, this possibility was excluded since extracts from

silica gel fractions corresponding to the R_f value of pyranone **2**, obtained from a TLC separation of a hindgut extract, also elicited trail activity. This observation suggested that the pyranone **2** is a genuine hindgut component.

Moreover, we found mellein **1** and pyranone **2** are not only present in the hindgut but also in the midgut of *L. fuliginosus*. Interestingly, Hangartner (1964) had showed previously that an extract of midguts also elicited some trail-following activity. Both components, mellein **1** and pyranone **2**, occurring in sections of the digestive tract could originate from the diet. However, we detected only the pyranone in the food; therefore it appears that only the pyranone is related to food intake. On the other hand, the origin of mellein in formicine ants, since it was not detected in the ants' diet, remains unknown.

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CAN ACORN TANNIN PREDICT SCRUB-JAY CACHING BEHAVIOR?

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Abstract—We tested the hypothesis that animals caching acorns for future food use may preferentially cache acorns high in tannin content. We used a population of Florida scrub-jays (*Aphelocoma coerulescens*) that cache acorns from four oak species. Tannin activity in acorns was measured by protein precipitating ability (PPA). Logistic regression models accurately predicted frequency of acorn caching among the oak species, using frequency of usable acorns and PPA loss during burial as predictor variables. Among the four oak species, frequency of caching increased with both increasing frequency of undamaged acorns at peak harvest time, and increasing loss of PPA during burial. Other regression models accurately predicted frequency of undamaged acorns for each species, using green acorn PPA and buried acorn PPA as predictor variables. Among the four oak species, frequency of usable acorns tended to increase with increasing green acorn PPA, and decreased with buried acorn PPA. We suggest that a deterrent effect of tannins on insect larvae is responsible for the differences in frequency of acorns usable to jays among oak species, and that jays select acorns for burial by assessing insect damage. Species differences in PPA change during burial probably reinforce this pattern of scrub-jay caching behavior.

Key Words—Acorns, *Aphelocoma*, caching, oaks, *Quercus*, scrub-jay, tannins.

INTRODUCTION

Many birds and mammals cache seeds (reviewed by Turcek and Kelso, 1968; Smith and Reichman, 1984; Sherry, 1985; Vander Wall, 1990), and many of

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these animals are thought to make caching decisions on the basis of seed physical or chemical attributes (Smith and Follmer, 1972; Bossema, 1979; Lewis, 1980; Smallwood and Peters, 1986). Based on an experimental study using gray squirrels (*Sciurus carolinensis*), Smallwood and Peters (1986) hypothesized that some animals may selectively cache acorns, the seeds of oaks, according to their tannin attributes; acorns with greater tannin activity will be cached in preference to acorns with low tannin activity. Acorns with greater tannin activity are hypothesized to be more resistant to insect predation and microbial decay (Koenig and Mumme, 1987). These acorns also may contain such high concentrations of tannin at the time of storage as to be deleterious to the herbivore prior to storage.

Tannins are a diverse class of phenolic compounds that give plants a characteristic bitter taste and astringent quality (Bate-Smith, 1972). These compounds are known to deter feeding by many herbivores, although the deterrence mechanisms are in dispute (Bernays et al., 1989, and references therein). Tannins may negatively affect digestibility (Short, 1976; Featherston et al., 1978; Butler et al., 1986; Robbins et al., 1987; Elkin et al., 1990) because they can precipitate proteins, which may make the protein indigestible by herbivores (Hagerman and Klucher, 1986; Lindroth et al., 1986). Tannins also can precipitate digestive enzymes, greatly reducing enzyme activity (Feeny, 1969; Petersen and Hill, 1991), and attack gut tissue (Singleton and Kratzer, 1973). Tannins also may reduce microbial damage to plant tissues (Swain, 1978; Scalbert, 1991) and reduce insect growth rates (Mole et al., 1993). In our study, we emphasized the protein precipitating activity (PPA) of tannins because it is the feature of tannin compounds thought to be most responsible for deterrence of vertebrate herbivores (Swain, 1979; Martin and Martin, 1982).

We sought to test Smallwood and Peters' (1986) hypothesis that food-caching animals would preferentially store high-tannin acorns in a field setting. We also wanted to determine how acorn tannin content affected the perishability of stored acorns and to determine how storage affected acorn tannin activity. To do this, we searched for selective caching of acorns by Florida scrub-jays (*Aphelocoma coerulescens*), which annually harvest thousands of acorns from several species of oaks (DeGange et al., 1989), and examined acorns from different oak species for variation in tannin activity and insect damage.

METHODS AND MATERIALS

Study Site. Our study was conducted at Archbold Biological Station, near the southern terminus of the Lake Wales Ridge in Highlands County, Florida. Archbold Biological Station encompasses about 2000 ha of oak scrub (mostly Scrubby Flatwoods, sensu Abrahamson et al., 1984), which consists of low-

growing, xerophytic shrubs, especially oaks (*Quercus*), palmettos (*Serenoa repens* and *Sabal etonia*), and widely scattered pines (*Pinus elliottii* and *P. clausa*) growing on the well-drained sandy soils of paleodunes. The highest and driest dune tops are vegetated, in order of decreasing abundance, by four oak species: *Quercus inopina*, *Q. geminata*, *Q. chapmanii*, and *Q. myrtifolia*. *Q. inopina* and *Q. myrtifolia* are members of the black or red oak subgenus *Erythrobalanus*; *Q. geminata* and *Q. chapmanii* are members of the white oak subgenus *Quercus*. Acorns of all these species are relatively small, between 1.0 and 1.5 g mean weight (Fleck, unpublished data). Jay territories have been mapped carefully each year since 1970 (Woolfenden and Fitzpatrick, 1984). For this project we observed jays in 13 territories. *Quercus inopina*, *Q. geminata*, and *Q. chapmanii* were present in all 13 territories, while *Q. myrtifolia* was present in only three.

Florida scrub-jays feed on acorns during most of the year. From late summer through early winter jays eat mostly acorns picked from oaks. In fall each jay caches about 6500–8000 acorns, scattered about, but almost entirely within its territory (DeGange et al., 1989). During late winter and spring jays eat the acorns they cached during fall. Few acorns are eaten in early summer.

Acorn Tannin Activity. Protein precipitating ability data for green and mature brown acorns picked from trees of each oak species were taken from Fleck and Layne (1990). We obtained data for buried acorns by assaying acorns picked and buried by us. These acorns were buried in December and January and retrieved, without replacement, at monthly intervals two to four months after burial (February through June). We buried acorns in sites similar to those in which jays cached acorns, about 2 cm deep in well-drained sandy soil. Buried acorns were placed in small hardware-cloth exclosures. Retrieved acorns were examined for damage by insects and microorganisms, and assayed for tannin activity.

We used Hagerman's (1987) assay for tannin protein precipitating ability to measure tannin in acorns. Results of this assay are reported as quantity of protein precipitated per milligram of plant material. Because measurements are taken of the area of a Petri dish in which protein precipitation has occurred, the quantity of protein precipitated is given per square millimeter. Hagerman (1987) and Fleck and Layne (1990) described the procedures in detail.

Acorn Damage. Insect damage (mostly from *Curculio* spp. larvae) and fungal decay were estimated by dissecting acorns. For each acorn, we recorded the number of larvae and estimated the percent of cotyledon damaged. Because the jays regularly fed only on acorns with minor or no damage (Fleck, unpublished data), we considered acorns with less than 25% damage usable by jays, and acorns with more than 25% damage unusable. Only a single instance of a jay consuming the weevil larvae found in an acorn was seen, out of more than 450 observations of jays using acorns (contra Johnson et al., 1993).

Scrub-Jay Observations. Florida scrub-jays are cooperatively breeding birds, forming family groups that reside in permanent, year-round territories. All the jays watched were individually color-marked and part of a population that had been under intensive study for 18 years (Woolfenden and Fitzpatrick, 1990). Many of the jays are extremely tame and allow close approach without altering their behavior. This permitted us to observe closely acorn handling by the jays. From July 1986 to January 1987 we made quantitative observations on 45 individuals, chosen to represent males and females, young and adults, breeders and nonbreeders, and a range of family sizes.

For acorns handled by jays, we recorded oak species, stage of maturity (green vs. brown), and the use of the acorn (eaten or cached). We omitted observations made during the first 60 min after sunrise, because when the jays first became active they appeared hungry and less selective, and tests of Smallwood and Peters' hypothesis would be invalid.

Analyses of acorn caching for a given oak species were restricted to the several weeks when acorns of that particular oak species were most available to jays. To assess availability, we estimated acorn abundance, acorn size, and the proportion of usable acorns monthly from August through December 1986 at 39 stations, three from each territory (for details, see Fleck, 1988).

Statistical Methods. G tests were used to analyze behavioral data. Williams' correction was used for all G tests (Sokal and Rohlf, 1981). Because the PPA data did not meet requirements for parametric tests, a nonparametric multiple range test (Dunn, 1964) was used to test for significant interspecific differences in acorn PPA. To keep the overall significance at 0.05 for these comparisons, the significance level per comparison was set at 0.0083 (0.05/number of comparisons), where the number of comparisons among means was 6 (Dunn, 1964). This conservative technique reduces the possibility of type I statistical error. When comparing two means, we used Mann-Whitney U tests (Sokal and Rohlf, 1981).

We used logistic regression (SAS version 6.04) to predict acorn caching as a function of acorn PPA, proportion of usable acorns, and change in PPA during burial. As it was not possible to obtain data on the individual acorns cached or eaten by jays, we constructed regression models using the mean values of green acorn PPA, buried acorn PPA, and proportion of usable green acorns for each species. By subtracting species buried acorn PPA from species green acorn PPA, we obtained a measure of loss of PPA activity during burial.

To determine how well these models' predictions fit observed data, we compared predicted acorn caching frequencies for regression models using both the proportion of usable acorns and PPA loss and using acorn damage only as predictor variables. We also compared predicted usable acorn frequencies for regression models with both green acorn PPA and buried acorn PPA as predictor variables.

RESULTS

Acorn Tannin Activity. Dunn's (1964) multiple range test showed that green *Q. chapmanii* acorns had significantly greater PPA than green *Q. myrtifolia* or green *Q. inopina* (Figure 1). No other comparisons among green or brown acorns were significant at the $P < 0.0083$ level. During burial, PPA decreased significantly in three species (*Q. chapmanii*, $U = 60$, $P < 0.001$, $N = 16$; *Q. myrtifolia*, $U = 32$, $P < 0.01$, $N = 12$); *Q. inopina*, $U = 40$, $P < 0.005$, $N = 13$), but not in *Q. geminata* ($U = 24$, $P < 0.15$, $N = 12$). During burial, relative PPA among the species changed (Figure 1). At the end of a four-month period, *Q. geminata* had the greatest PPA, significantly higher than *Q. chapmanii* at the $P < 0.0083$ level (Figure 1).

Acorn Damage. The proportion of usable green acorns varied with oak species (Figure 2) ($G = 79.990$, $P < 0.001$, $df = 3$, $N = 262$). *Q. chapmanii* had the greatest frequency of usable green acorns, *Q. myrtifolia* and *Q. inopina* acorns had intermediate frequencies, and *Q. geminata* had the lowest frequency of usable green acorns. The proportion of usable buried acorns also varied with oak species (Figure 2) ($G = 11.208$, $P < 0.025$, $df = 3$, $N = 400$). Again, *Q. chapmanii* had the greatest frequency of usable acorns, followed by *Q. myrtifolia*. Fewer than 15% of *Q. inopina* and *Q. geminata* acorns remained usable to jays after only 2 months of burial.

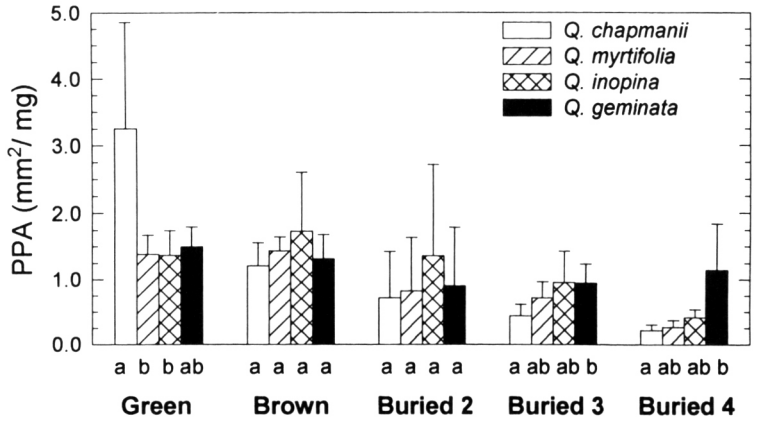

FIG. 1. Change in acorn PPA: mean PPA (protein precipitating ability, with SD) of acorns from four oak species (*Quercus*) at five stages (green on shrub, brown on shrub, buried 2, 3, and 4 months). Letters below each bar indicate results of multiple range tests; within a stage, bars sharing a letter are not significantly different. Data for green and brown acorns from Fleck and Layne (1990).

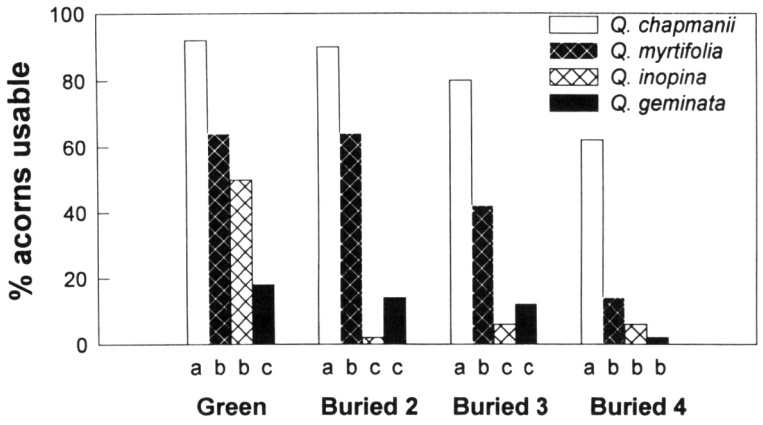

FIG. 2. Frequency of usable acorns: Percentage of acorns usable by jays at four stages (green on shrub, buried 2, 3, and 4 months). Letters below each bar indicate results of pairwise G tests; within a stage, bars sharing a letter are not significantly different.

Use of Acorns by Scrub-Jays. Jays in our study harvested more green than brown acorns in most species, both for caching and eating (Table 1). Scrub-jay caching of green acorns varied with oak species ($G = 17.121$, $P < 0.001$, $df = 3$, $N = 208$), but caching of brown acorns did not ($G = 1.140$, $P > 0.50$, $df = 3$, $N = 91$) (Table 1). Caching rates for each of the four species were compared using multiple G tests on each pair of species (Sokal and Rohlf, 1981). These indicated that green *Q. geminata* acorns were cached significantly less often than acorns of the other three species ($G > 6.95$ in each comparison; P

TABLE 1. USE OF GREEN AND BROWN ACORNS

		Quercus species				
Stage	Use	*chapmanii* ($N = 43$)	*myrtifolia* ($N = 19$)	*inopina* ($N = 174$)	*geminata* ($N = 63$)	Total ($N \leq 299$)
Green	cached	30 (88%)[a]	14 (82%)	85 (69%)	15 (44%)	144
	eaten	4 (12%)	3 (18%)	38 (31%)	19 (56%)	64
Total		34 (100%)	17 (100%)	123 (100%)	34 (100%)	208
Brown	cached	8 (89%)	1 (50%)	42 (82%)	24 (83%)	75
	eaten	1 (11%)	1 (50%)	9 (18%)	5 (17%)	16
Total		9 (100%)	2 (100%)	51 (100%)	29 (100%)	91

[a]No. of observations (percent of total for stage and species).

< 0.05, $df = 1$ in each comparison; $N = 51$ to 157). Overall, 73% of acorns harvested were cached. Of green acorns used by jays, 88% of *Q. chapmanii* were cached ($N = 34$), 82% of *Q. myrtifolia* ($N = 17$), 69% of *Q. inopina* ($N = 123$), and 44% of *Q. geminata* ($N = 34$). These results, based on data collected for each species during the time that species' acorns were most abundant, parallel those based on data collected over the entire season ($G = 13.767$, $P > 0.01$, $df = 3$, $N = 450$; *Q. chapmanii*, 84% cached, $N = 56$; *Q. geminata*, 63% cached, $N = 115$).

Species Comparisons. Three regression models were found that did not differ significantly from observed patterns in their prediction of acorn caching and usable acorn frequencies (likelihood ratios for these models >0.6). The proportion of green acorns of a species that remain usable was positively correlated with the probability of that species' acorn being cached (Table 2, A); *Q. chapmanii*, with the greatest frequency of usable green acorns, was most likely to be cached. *Q. geminata*, with the fewest usable green acorns, was least likely to be cached. Scrub-jay acorn caching patterns could be accurately predicted by a logistic regression equation containing frequency of usable green acorns as the only predictor variable (Table 2, B).

Neither green acorn PPA nor buried acorn PPA could predict caching patterns. PPA loss, the change in mean PPA between green and buried acorns of a species, correlated well with acorn caching patterns; *Q. chapmanii* experienced the greatest loss of PPA (3.038 mm^2/mg) and was cached most frequently, while *Q. geminata* lost very little PPA (0.351 mm^2/mg) and was cached

TABLE 2. LOGISTIC REGRESSION: FREQUENCY OF USABLE ACORNS AS PREDICTOR OF ACORN CACHING

A. Dependent variable: Acorn use
Predictor variable: Frequency of usable acorns

Source	Estimate	Error	*df*	χ^2	Prob.
Intercept	−0.781	0.4247	1	3.38	0.0659
Usable	0.318	0.00828	1	14.76	<0.001
Likelihood ratio			2	0.28	0.869

B. Proportion of acorns cached:

Species	Observed	Predicted
Q. chapmanii	0.88	0.90
Q. myrtifolia	0.82	0.78
Q. inopina	0.69	0.69
Q. geminata	0.44	0.45

TABLE 3. LOGISTIC REGRESSION: FREQUENCY OF USABLE ACORNS AND PPA LOSS AS PREDICTORS OF ACORN CACHING

A. Dependent variable: Acorn use
Predictor variables: Frequency of usable acorns, PPA loss of buried acorns

Source	Estimate	Error	*df*	χ^2	Prob.
Intercept	−0.8897	0.5139	1	3.00	0.0834
Usable	0.0388	0.0203	1	3.64	0.0565
PPA	−0.2213	0.5850	1	0.14	0.7052
Likelihood ratio			1	0.14	0.7102

B. Proportion of acorns cached:

Species	Observed	Predicted
Q. chapmanii	0.88	0.88
Q. myrtifolia	0.82	0.79
Q. inopina	0.69	0.70
Q. geminata	0.44	0.43

least often. However, PPA loss alone was not a good predictor of the frequency of caching. A model containing frequency of usable green acorns and PPA loss during burial as predictor variables also fit the observed pattern, but not as well as a model containing frequency of usable green acorns alone (Table 3, A and B).

A positive correlation existed between frequency of usable green acorns and green acorn PPA (Table 4, A). A negative correlation existed between frequency of usable green acorns and buried acorn PPA (Table 4, A). Neither green acorn PPA nor buried acorn PPA alone was an accurate predictor of the proportion of usable green acorns. In combination, these variables proved to be accurate predictors of acorn usability. The frequency of usable green acorns could be accurately predicted by a logistic regression equation containing green acorn PPA and buried acorn PPA as predictor variables (Table 4, B). In all cases, observed data frequencies closely matched frequencies predicted by the various logistic regression models (Tables 2–4).

DISCUSSION

Smallwood and Peters (1986) hypothesized that the low-tannin, early-germinating white oak acorns are more valuable as immediate food, and the high-tannin, late-germinating black oak acorns are more valuable as a stored food. Black oak acorns are less "perishable" and preferred for caching despite, or

TABLE 4. LOGISTIC REGRESSION: ACORN PPA AS PREDICTOR OF FREQUENCY OF USABLE ACORNS

A. Dependent variable: Frequency of usable acorns
Predictor variables: Green acorn PPA, Buried acorn PPA

Source	Estimate	Error	*df*	χ^2	Prob.
Intercept	−0.2744	0.5863	1	0.22	0.6398
Green acorn PPA	0.9952	0.3020	1	10.86	<0.001
Buried acorn PPA	−2.4069	0.3955	1	37.03	<0.001
Likelihood ratio			1	0.25	0.6165

B. Proportion of undamaged acorns:

Species	Observed	Predicted
Q. chapmanii	0.92	0.92
Q. myrtifolia	0.64	0.62
Q. inopina	0.50	0.52
Q. geminata	0.18	0.18

perhaps because of, their high tannin activity. Smallwood and Peters' hypothesis is not directly supported by our data; acorn PPA alone is not a good predictor of scrub-jay use of acorns. However, acorn PPA may not be irrelevant to jay choice, because of its relationship to the proportion of acorns remaining usable by jays.

The acorn characteristic that best predicted caching patterns was usability, i.e., lack of damage. This characteristic is also one that jays can, and do, assess with relative ease (Ligon and Martin, 1974; Vander Wall and Balda, 1977; Johnson et al., 1987). Like many corvids, jays often, if not always, manipulate acorns in their beaks immediately after picking or recovering them. Many are quickly discarded. Discarded acorns often show only a tiny hole in the husk (left by ovipositing adult weevils), but have considerable internal damage (Fleck, unpublished data). The cues the jays use to distinguish undamaged from damaged acorns are unknown. An acorn with no detectable internal damage would, on average, make a good storage food; acorns with a small amount of damage, while largely edible, would make a poor storage food because weevil larvae inside would consume the greater proportion of the acorn tissue. An acorn with considerable internal damage may be discarded, with or without a larva inside, because it may not be worth the time and energy required to open it.

Unlike acorn usability and damage, which follows a consistent trend in all species after burial, changes in acorn PPA after burial differ by species. In all species, acorn PPA drops; in *Q. chapmanii* and *Q. myrtifolia*, it drops so far

as to be nearly undetectable after three months. The proportion of usable green acorns for a given species was roughly inversely correlated with loss of PPA during burial. This association may explain the ability of regression models containing both of these factors as predictor variables to predict acorn use; loss of acorn PPA is merely closely associated with the proportion of usable green acorns, not an important factor on its own. On the other hand, to the extent that acorn tannins are deleterious to scrub-jays (Koenig and Heck, 1988; Fleck and Tomback, 1996), a preference for caching acorns that will lose most of their PPA (e.g., *Q. chapmanii*) and an avoidance of acorns that will retain most of theirs (e.g., *Q. geminata*) would be understandable.

Tannins are an extremely diverse group of compounds, often with very specific biological actions (e.g., Clausen et al., 1990). In addition, plants such as oaks may produce a wide range of tannin compounds (Haslam, 1989). This makes generalization about the action and fate of tannins very difficult. The reason for the species differences in rates of loss of PPA in buried acorns found in this study is unknown, but could have to do with differences in the tannins produced by each species. Hydrolyzable tannins are susceptible to hydrolysis under a wide range of pH environments. Such hydrolysis forms simple phenolic acids that are not able to precipitate proteins. Condensed tannins are chemically very stable and are probably unlikely to degrade rapidly during storage (Hagerman and Butler, 1991).

Q. geminata showed little reduction in PPA between green, brown, and buried acorns. If *Q. geminata* acorns contain greater proportions of condensed tannins than *Q. chapmanii*, *Q. myrtifolia*, and *Q. inopina*, then the observed pattern of rapid loss of PPA to low levels in these three species, and slow loss of PPA in *Q. geminata* would be expected.

The probability of usable green acorns was accurately modeled by the combination of green acorn PPA and buried acorn PPA, but not by either variable alone. Lack of damage was positively associated with green acorn PPA. This is as expected if tannin activity were partially deterrent to weevils, as has been noted in other studies (Koenig and Mumme, 1987; Steele et al., 1993). The rough association between green acorn PPA and usability in buried acorns is largely a function of the high PPA of *Q. chapmanii* acorns; the other oak species, although differing in the frequencies of usable acorns, had very similar acorn PPA values. This finding gives tentative support to one of the predictions of Smallwood and Peters' hypothesis—that high tannin activity in acorns permits long-term caching and that food-hoarding animals could use high tannin as a cue of suitability for caching. However, because *Q. chapmanii* is the only high PPA species in this study, it is difficult to generalize this result.

Weevil eggs are oviposited in green acorns while still on the oak, and the larvae hatching from eggs damage increasing amounts of acorn tissue over time as they feed. Few additional sources of damage are introduced underground.

High-tannin acorns, which have little initial damage, will accumulate damage more slowly than acorns with greater initial damage. Lack of damage was negatively associated with buried acorn PPA. The retention of PPA in buried acorns may result from higher levels of acorn damage inducing the production of tannins within acorn tissues, possibly after separation from the parent oak (Clausen et al., 1989; Hunter and Schultz, 1993; Fleck, unpublished data); or buried acorn PPA may be the measured manifestation of some unknown factor that influences acorn damage. Changes in tannin activity underground may have less influence on any damage that occurs underground. The relatively rapid loss of PPA in buried *Q. chapmanii* acorns did not lead to a rapid increase in damage; neither did the slower reductions in PPA in *Q. myrtifolia* and *Q. inopina* acorns prevent the loss of most acorns.

Johnson and Adkisson (1985) noted that ". . . green [beech]nuts form the bulk of what is transported and stored by jays," and hypothesized that jays would treat acorns similarly. Jays in our study harvested more green than brown acorns of most species, both for caching and eating. Jays appeared to differentiate between green acorns of different species in terms of caching decisions; no such differentiation was apparent between brown acorns (Table 1). We have no damage measures for brown acorns. The frequency of usable brown acorns of different species may follow the pattern shown by green and buried acorns (Figure 2). However, brown acorns may be less usable by jays, because they have experienced a longer period of potential weevil attack while cached acorns are safely underground.

If few brown acorns were usable, why do caching rates remain high? Possibly jays may be able to make more reliable caching decisions regarding brown acorns; if an acorn contains a weevil larva, by the time the acorn has browned, the larva will have grown large enough to be easily detectable. Jays will be able to cache the smaller pool of usable brown acorns more confidently. By then, acorns containing the mature larva will be neither cache-worthy nor usable as immediate food.

Based on our study, we suggest that the roles of acorn tannins in mediating scrub-jay caching behavior in the wild are more complex than Smallwood and Peters' hypothesis would imply. We suggest that the protein-precipitating activity of tannins impinges upon jay caching in two ways. Most importantly, acorn tannin activity may help mediate weevil infestation of green acorns, which is the storage characteristic both most easily assayed by jays and most indicative of acorn "survival" while buried. Acorn PPA thus indirectly affects caching behavior through its effects on acorn insect infestation and acorn susceptibility to damage while buried. Secondarily, jays may indeed respond to the change in levels of PPA in acorns and selectively cache acorns of species likely to lose greater levels of PPA after burial, because of the deleterious effects of these acorn tannins on the jays (Koenig, 1991). These decisions on the part of jays

are not absolute; no cutoff point exists above which all acorns are cached or below which all acorns are eaten. Higher (or lower) levels of tannin activity may produce a tendency or preference for caching (or eating), which will be followed or not based on immediate circumstances at the time of caching.

DeGange et al. (1989) showed that acorn production was variable and inconsistent among species and years, and that 1986 was an above average year for *Quercus inopina* and *Q. geminata*, and below average for *Q. chapmanii*. However, even in 1986 *Q. chapmanii* and *Q. myrtifolia* produced many acorns per shrub. From August through November 1986, many acorns of the three most abundant oaks were readily available to jays in all families in our study.

In field tests such as ours it is often difficult to separate out important covariates. In the present study, *Q. chapmanii* would seem to produce a near-perfect acorn for storage for several reasons: high in tannin initially, relatively undamaged both before and after storage, and very low in tannin activity when recovered. Similarly, *Q. geminata* would seem to be a poor acorn for storage: extensive damage initially, practically no intact acorns after a few months of burial, and still retaining tannins after retrieval. It is not possible with our present data to untangle the many factors leading to jay caching decisions, but our study does show that the effects of acorn tannins on the herbivores that eat and cache them are more varied and complex than they first seem.

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PHEROMONE RELEASE BY INDIVIDUAL FEMALES OF CODLING MOTH, *Cydia pomonella*

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Abstract—The gland titer and release of sex pheromone was studied in individual *Cydia pomonella* females. An increase of the main compound (*E*,*E*)-8,10-dodecadien-1-ol (codlemone) from 5.5 to 9 ng/gland, during the first 2 hr of calling, matched an increase in the release rate from 5 to 7 ng/hr/female. The average proportion of the minor compounds dodecan-1-ol, (*E*)-9-dodecen-1-ol, and tetradecan-1-ol in the effluvium decreased within 2 hr from 59% to 33%. Their proportion in the gland peaked 1 hr before the onset of the scotophase at 78% and decreased to 16% at 2 hr after onset of calling. Addition of these minor components to codlemone did not increase male attraction to field traps.

Key Words—Sex pheromone, codlemone, gland volatiles, female effluvia, release, emission, blend ratio, synergism, field attraction. *Cydia pomonella*.

INTRODUCTION

The sex pheromone of the codling moth, *Cydia pomonella* L., has been reported to consist of (*E*,*E*)-8,10-dodecadien-1-ol (*E*8,*E*10–12:OH; codlemone), dodecan-1-ol (12:OH), and tetradecan-1-ol (14:OH) (Roelofs et al., 1971; Einhorn et al., 1984, 1986; Arn et al., 1985; Bartell et al., 1988). A blend of these compounds is currently used to control codling moth by mating disruption (Barnes

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et al., 1992; Howell et al., 1992; Pfeiffer et al., 1993), although the behavioral role of the saturated alcohols is still controversial.

Wind-tunnel tests by Arn et al. (1985) and Einhorn et al. (1986) have shown that a 1- to 20-fold addition of 12:OH widens the dose range at which males are attracted to codlemone. However, at the proportions released by calling females (Arn et al., 1985), the saturated alcohols enhanced only male wing-fanning close to the source, but did not increase attraction in the field (Bartell et al., 1988) or in the wind tunnel (McDonough et al., 1993, 1995).

The pheromone emission of codling moth has previously been collected from a batch of females confined in a static atmosphere during the entire calling period (Arn et al., 1985). We have now measured the release by individual females under a steady airflow during 30-min sampling intervals. Release rates and ratios were compared to gland extracts. The effect of the most abundant minor components, 12:OH, *E*9–12:OH, and 14:OH, on male attraction to the main compound codlemone was tested in the field.

METHODS AND MATERIALS

Insects. Codling moths were field-collected in Skåne (Sweden) and reared for less than 10 generations on a semiartificial diet (Mani et al., 1978). They were held at 23°C under a 18L:6D hr photoperiod. After emergence, the females were kept individually in plastic jars (200 ml) and supplied with sucrose solution.

Gas Chromatography (GC). GC analysis was done on a Hewlett-Packard 5890 instrument with flame ionization detection (FID) on a DB-Wax column (splitless injection, 30 m × 0.25 mm; J&W Scientific, Folsom, California), programmed from 80°C (hold 2 min) at 10°/min to 230°C.

Gland Extracts. Glands of 2-day-old females were extracted 2 hr and 1 hr before, immediately at the onset of the scotophase, and 5 min, 1 hr, and 2 hr after onset of calling during the scotophase ($N = 10$). Glands were extracted during 1 min in 5 μl of redistilled heptane, and the extract was immediately analyzed by GC. The recovery of codlemone was 63%, as determined by a second wash with 5 μl heptane ($N = 5$); data were corrected accordingly.

Collection of Volatiles. Collection of effluvia from calling females was done according to Witzgall and Frérot (1989). Thirty minutes before lights-off, an unmated female was placed into a glass bulb. The female was resting freely on a support in the center of the bulb. A capillary glass tube (1 mm ID, 120 mm long) was connected to its outlet for collection of pheromone and was exchanged without disturbing the female. The glassware was kept for 8 hr at 400°C before use. A constant flow (200 ml/min) of purified air (activated charcoal) passed over the female and into the capillary tube. Pheromone was collected from the onset of calling (on average 45 min after lights-off) during four consecutive

intervals of 30 min. The capillary was immediately rinsed with 7 μl of redestilled heptane, and the extract was analyzed by GC. Only data from females calling continuously during 2 hr, with the gland close to the outlet of the bulb, was taken into account ($N = 10$). The glands of these females were extracted immediately after the last sampling interval (see above).

The release from 10-day-old red rubber septa (Thomas Scientific), loaded with 100 μg of *E*8,*E*10-12:OH ($N = 5$), was measured in the same apparatus.

Estimation of Collection Efficiency. To determine recovery rates from glass capillaries, 10 ng of synthetic *E*8,*E*10-12:OH, *E*9-12:OH, 12:OH, and 14:OH in pentane (5 μl) was injected into the capillaries. They were rinsed with 7 μl of redestilled heptane after 0.25, 0.5, 1, 2, and 4 hr ($N = 3$), and the extracts were analyzed by GC. In contrast to a constant recovery of 12:OH, *E*9-12:OH, and 14:OH, the recovery of synthetic *E*8,*E*10-12:OH from glass capillaries decreased with time (Table 1). After 18 hr, *E*8,*E*10-12:OH was no longer detected. The amount of pheromone released by calling females (30-min sampling interval) was corrected according to the recovery of synthetic compounds from glass capillaries after 15 min. Data were also corrected for 10% loss due to breakthrough, as determined by a second capillary.

Field Trapping. Tetra traps (Arn et al., 1979) were baited with various blends of *E*8,*E*10-12:OH, *E*9-12:OH (>98.7% isomeric purity by GC), 12:OH, and 14:OH on red rubber septa. Traps were placed along tree rows in an 8-ha apple orchard in Bjärred, Sweden, between June 18 and July 13, 1995. Trap distance was 5 m within a replicate ($N = 10$), and one additional buffer trap with *E*8,*E*10-12:OH was placed at each end. Traps were checked and rotated four times.

Statistical Analysis. An analysis of variance, followed by a Tukey test ($\alpha = 0.05$), was calculated from the amounts and proportions of each compound

TABLE 1. RECOVERY OF SYNTHETIC *E*8,*E*10-12:OH AND 12:OH FROM GLASS CAPILLARY TUBES WITH HEPTANE, 0.25–4 HR AFTER APPLICATION ($N = 3$)

	Recovery (%)	
Time (hr)	*E*8,*E*10-12:OH	12:OH
0.25	70 ± 6	85 ± 8
0.5	62 ± 6	85 ± 4
1	55 ± 4	92 ± 3
2	35 ± 3	93 ± 5
4	17 ± 1	87 ± 7

in effluvia and glands and from the number of trapped males, after transformation to log $(x + 1)$ or $\sqrt{x}$.

RESULTS

Female Gland Titer. Glands dissected 2 hr before onset of the scotophase contained 2.3 ± 1.6 ng *E*8,*E*10-12:OH; the codlemone titer increased significantly after the onset of female calling and peaked at 8.7 ± 2.6 ng after 2 hr of calling (Figure 1).

The amount of 12:OH in the gland, relative to codlemone, was highest 1 hr before onset of the scotophase (56.0 ± 52.6%) and decreased significantly to 10.7 ± 4.9% after 2 hr of calling (Figure 1). The gland proportions of *E*9-12:OH (mean titer during calling period: 5.6 ± 4.9% relative to *E*8,*E*10-12:OH) and 14:OH (7.1 ± 11.0%) did not change significantly over time, but the diel pattern was the same as for 12:OH (Figure 1).

Female Release. The females ($N = 10$) started to call within 30–75 min after onset of the scotophase. The mean release rate of *E*8,*E*10-12:OH increased significantly after the first half hour of calling and was then rather constant; the highest release was measured betwen 1 and 1.5 hr of calling (6.5 ± 3.8 ng/hr) (Figure 1).

The relative release rate of the most abundant minor component, 12:OH, was highest during the first half hour (47.6 ± 29.6%) and decreased significantly to 26.3 ± 13.4% between 1.5 and 2 hr of calling (Figure 1). All females emitted detectable amounts of *E*9-12:OH during at least one sampling interval, while 14:OH was detected in only four of 10 females. The proportions of *E*9-12:OH and 14:OH were highest during the first sampling interval (8.4 ± 7.2% and 7.9 ± 2.4%, respectively), the differences between sampling intervals were not significant (Figure 1).

The amounts of *E*8, *E*10-12:OH released, as well as the *E*8,*E*10-12:OH/12:OH blend ratio showed a strong interindividual, but a low individual, variation.

Release from Rubber Septa. Red rubber septa, loaded with 100 μg *E*8, *E*10-12:OH and aged in the field for 10 days, released 60.6 ± 15.7 ng/hr ($N = 5$), almost 10 times more than a calling female (see above). By comparison, a mating disruption dispenser aged for 14 days in the field released approximately 3000 times more *E*8,*E*10-12:OH (Brown et al., 1992) than a female; codling moth males are attracted to such dispensers in the field (Kehat et al., 1994; Witzgall et al., 1996).

Field Trapping. Addition of various amounts of 12:OH, *E*9-12:OH, and 14:OH to *E*8,*E*10-12:OH had no significant effect on male attraction to traps. Fewest males were caught at a 100%-addition of *E*9-12:OH (Table 2).

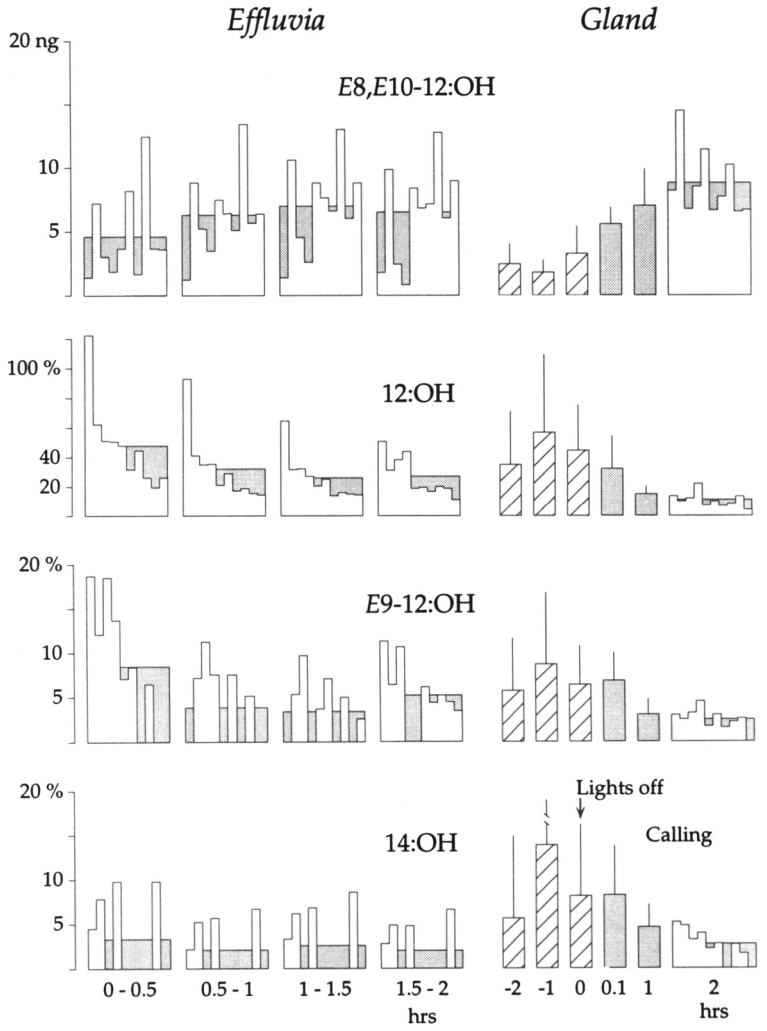

FIG. 1. Amounts of *E*8,*E*10–12:OH and relative amounts of 12:OH, *E*9–12:OH, and 14:OH in effluvia (ng/hr) and gland extracts (ng/gland; SD) of individual *C. pomonella* females. Collection of effluvia was done during four consecutive 30-min intervals after onset of female calling ($N = 10$; empty bars, mean release shaded). Glands were extracted at 2, 1, and 0 hr before onset of scotophase (hatched bars), and after 0.1, 1, and 2 hr of calling ($N = 10$; empty bars: females used for collection of effluvia). Females started to call on average 45 min after lights-off.

TABLE 2. FIELD ATTRACTION OF *C. pomonella* MALES TO 10 μg OF *E*8,*E*10-12:OH BLENDED WITH FEMALE GLAND COMPONENTS[a]

Compound	Amount (μg/trap)						
*E*8,*E*10-12:OH	10	10	10	10	10	10	10
12:OH		1	10				10
*E*9-12:OH				0.2	0.5	10	0.5
14:OH							1
Males caught	56 a[b]	74 a	68 a	65 a	49 a	48 a	70 a

[a]Bjärred (1995); $N = 10$.
[b]Tukey test, $P = 0.05$.

DISCUSSION

The amount of *E*8,*E*10-12:OH in pheromone glands of *C. pomonella* females increased rapidly at the onset of the scotophase. An increase of the female gland titer during the early phase of calling has been reported in various other Lepidoptera (e.g., Raina et al., 1986; Dunkelblum et al., 1987; Kou, 1992; Kamimura and Tatsuki, 1993).

The comparison of codlemone gland titer and release (Figure 1) suggests a continuous production during calling, at a turnover rate of approximately 1 hr. The release rate of codlemone is higher than previously measured in a static atmosphere during longer sampling intervals (Arn et al., 1985). This difference may in part be due to the decrease in the recovery rate of synthetic *E*8,*E*10-12:OH from glass (Table 1). Similarly, the recovery of *E*8,*E*10-12:OH from C-18 bonded silica decreased within 1 hr of air sampling from 71 to 54% (see Pop et al., 1993).

The gland titer of 12:OH, relative to *E*8,*E*10-12:OH, decreased significantly during the late photophase and the early scotophase; *E*9-12:OH and 14:OH followed the same pattern. The minor components stem from biosynthetic precursors of codlemone (Löfstedt and Bengtsson, 1988), which probably accumulate in the gland before the onset of calling. The decrease in the release ratios of 12:OH and *E*9-12:OH may also be due to their higher vapor pressures, compared to codlemone (Butler and McDonough, 1981; Liu and Haynes, 1994).

Individual females of several lepidopterous species have been reported to release behaviorally active pheromone components in rather constant ratios, such as *Heliothis virescens* (Pope et al., 1982), *Pectinophora gossypiella* (Haynes et al., 1984), *Yponomeuta padellus* and *Y. rorellus* (Du et al., 1987), and *Cacoecimorpha pronubana* (Witzgall and Frérot, 1989). In *C. pomonella*, the indi-

vidual variation and the proportional decrease of 12:OH, *E*9-12:OH, and 14:OH during calling argues against a behavioral activity of these compounds; this is also supported by the field trapping test (Table 2).

Saturated analogs of the main pheromone compound are commonly found in tortricid pheromone glands and effluvia (Arn et al., 1992, 1995), but have only rarely been shown to synergize male behaviors (Arn et al., 1986; Löfstedt et al., 1986). Addition of 100% or more 12:OH to *E*8,*E*10-12:OH has shown a synergistic effect on male attraction in the wind tunnel (Arn et al., 1985; Einhorn et al., 1986). One of the females tested here released 12:OH at 122% relative to codlemone during the first half hour of calling, but the much lower average release ratio (Figure 1) raises doubts about a behavioural effect of female-produced 12:OH.

On the other hand, green-leaf volatiles have been reported to augment attraction of codling moth males to pheromone (Light et al., 1993). Apples release a wide range of alcohols, including 12:OH (Drawert et al., 1968). This may be an indication that the behavioral effect of large amounts of 12:OH observed in clean wind tunnel air is not an artifact, but may rather reflect a synergism between host volatile compounds and pheromone.

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EFFECTS OF ROOT EXUDATES OF CUCUMBER (*Cucumis sativus*) AND ALLELOCHEMICALS ON ION UPTAKE BY CUCUMBER SEEDLINGS

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Abstract—The effects of root exudates of cucumber, aromatic carboxylic acids in root exudates, and their analogs upon the uptake of NO_3^-, $H_2PO_4^-$, SO_4^{2-}, K^+, Ca^{2+}, Mg^{2+}, and Fe^{2+} by intact cucumber seedlings were examined. Root exudates inhibited the uptake of all the ions analyzed except for $H_2PO_4^-$. Inhibition of ion uptake by cinnamic acid, a main component of root exudates, was both concentration- and pH-dependent. With decreasing pH, the inhibitory effect on the ion uptake increased. With benzoic and cinnamic acids, the substitution of hydrophilic group(s) on the benzene ring alleviated the inhibition of ion uptake. Aromatic acids enhanced ion leakage. The potency was in proportion, but not equal, to the extent of uptake inhibition. The lipophilicity was a valuable index for evaluating the allelopathic potential of aromatic acids.

Key Words—Allelochemicals, allelopathy, aromatic carboxylic acids, autotoxicity, *Cucumis sativus* L., ion leakage, ion uptake, phytotoxicity, root exudates.

INTRODUCTION

Some plant species have allelopathic potential by releasing allelochemicals into the rhizosphere (Rice, 1984; Yu and Matsui, 1994). Allelochemicals affect plant metabolism such as photosynthesis, respiration, and ion uptake (Balke, 1985; Einhellig, 1986). The effect of allelochemicals on ion uptake is particularly

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important, since the root is the first organ to come into contact with allelochemicals in the rhizosphere. Our previous studies found that cucumber plant possess allelopathic potential by exuding allelochemicals such as benzoic and cinnamic acids (Yu and Matsui, 1993, 1994). The objective of this study was to determine the effects of root exudates of cucumber, excuded allelochemicals, and their analogs on uptake and leakage of inorganic ions such as NO_3^-, $H_2PO_4^-$, SO_4^{2-}, K^+, Ca^{2+}, and Mg^{2+} by intact seedlings of cucumber. The effects of true root excudates on the ion uptake by plants have scarcely been examined (Balke, 1985; Newman and Miller, 1977). On the other hand, several studies have reported on the effects of aromatic acids on ion uptake by plant roots (Balke, 1985; Booker et al., 1992; Glass, 1973, 1974; Harper and Balke, 1981; Lyu and Blum, 1990; Vaughan and Ord, 1991). Those studies were carried out mainly with monocotyledonous plants using specific allelochemicals.

METHODS AND MATERIALS

Materials. All the reagents and solvents used were commercially available and used without further purification. Cucumber seeds (*Cucumis sativus* L., cv. Tokiwa; Sakata Seed Co., Japan) and lettuce seeds (*Lactuca sativa* L., cv. Shisuko; Takii Seed Co., Japan) were also commercially available.

Collection of Root Exudates of Cucumber Plants. Root exudates of cucumber were collected as described in a previous paper (Yu and Matsui, 1994). The collection was carried out with three cucumber plants and lasted for 80 days. Root exudates were adsorbed onto Amberlite XAD-4 resin, eluted with methanol, and evaporated to dryness at reduced pressure. One hundred twenty milligrams of root exudates were obtained.

Preparation of Seedlings. Cucumber seeds were germinated on filter papers moistened with 0.5 mmol/dm^3 $CaSO_4$ solution and kept at 27°C for six days in the dark. Germinated seeds were exposed to light for 10 hr and then transferred to a solution containing KNO_3, 0.5; $Ca(NO_3)_2$, 0.3; KH_2PO_4, 0.1; $MgSO_4$, 0.15 (in mmol/dm^3) and Fe (EDTA-Fe), 0.50; Mn, 0.14 and B, 0.05 (in mg/dm^3). The pH was adjusted to 5.5. The seedlings were allowed to grow for 8–10 days under natural light and continuous aeration in a greenhouse maintained at 28°C by day and 20°C by night. Seedlings thus obtained were used for the following experiments.

Ion Uptake Experiment. The 14- to 16-day-old seedlings were transferred to 0.5 mmol/dm^3 $CaSO_4$ solution and allowed to stand for 24 hr. After rinsing the seedlings with distilled water, pairs of seedlings were then incubated in 25 cm^3 nutrient solution containing exudates or various aromatic acids. The composition of the nutrient solution was the same as that used for the preparation of seedlings. Aromatic acids and root exudates were dissolved in ethanol and

added to the nutrient solution. The final concentration of ethanol in each solution was 0.2% (v/v), at which concentration ethanol has a negligible effect on ion uptake. The pH of the test solution was adjusted to 5.5 with 0.1 mmol/dm^3 HCl or 0.1 mmol/dm^3 NaOH except for the pH experiments, for which pHs were adjusted to 4.5, 6.0, and 7.5 respectively. The seedlings were incubated in a growth chamber maintained at 7500 Lx and 25°C. After 5 hr, the seedlings were taken out of the solution and the roots were weighted. The residual nutrient solution was adjusted to 25 cm^3 with distilled water for chemical analysis. Each treatment was done in triplicate.

Ion Leakage Experiment. After rinsing the 14-day-old seedlings with distilled water, pairs of seedlings were incubated with 0.5 mmol/dm^3 $CaSO_4$ solutions containing 0.25 mmol/dm^3 aromatic acids for 4 hr. The concentration of ions in solution was determined as described below.

Chemical Analyses. The nitrate N was determined with a UV spectrophotometer (Shimadzu UV-2100) after the solution was treated with activated charcoal to remove interfering organic compounds (Kato et al., 1992). Other elements such as P, S, K, Ca, Mg, Fe, Mn, and B were determined with an inductively coupled plasma atomic emission spectrometer (Shimadzu ICPS-2000) (Lee et al., 1991). The rate (k) of ion uptake or the rate (k') of ion leakage per unit of fresh weight of root was calculated from changes in the amounts of the ions in the solutions before and after incubation. The index (R) for ion uptake inhibition by allelochemicals was calculated by:

$$R = 100(k_0 - k)/k_0.$$

where k_0 is k for the control.

Phytotoxicity Bioassay. The bioassay was carried out according to the methods of Tang and Young (1982), and lettuce was used as plant material. The details have been described in a previous paper (Yu and Matsui, 1994). The phytotoxicity index (PI) was calculated in a manner similar to that for R.

RESULTS AND DISCUSSION

Effect of Root Exudates on Ion Uptake. The root exudates of cucumber were collected by adsorbent Amberlite XAD-4, extracted by organic solvent, and evaporated to dryness. The residue thus obtained is widely believed to be true root exudates (Tang and Young, 1982). The effects of root exudates on ion uptake by intact cucumber seedlings were examined with a nutrient solution containing 80 mg/dm^3 root exudate. The addition of the exudates significantly inhibited the uptake of all ions examined except for $H_2PO_4^-$ (Table 1). There were no significant differences in the inhibition indexes (57–73%) for NO_3^-, K^+, Ca^{2+}, Mg^{2+}, and Fe^{2+}, whereas the indexes for $H_2PO_4^-$ (29%) and Mn^{2+}

TABLE 1. EFFECTS OF ROOT EXUDATES ON ION UPTAKE (k) BY CUCUMBER SEEDLINGS

	k		
	Control	Exudates[a]	R[b]
NO_3^- (μmol/g/hr)	7.3	2.0***	73
$H_2PO_4^-$	0.41	0.29	29
K^+	1.4	0.43***	68
Ca^{2+}	0.72	0.25**	65
Mg^{2+}	0.44	0.19*	57
Fe^{2+} (μg/g/hr)	1.0	0.46*	58
Mn^{2+}	0.16	0.12*	25
BO_3^{3-}	4.7	1.6*	66

[a]Concentration of root exudates in the solution was 80 mg/dm^3. *, **, and *** refer to significant differences compared with the control at 0.05, 0.01, and 0.001 levels by Student's t test, respectively.
[b]Inhibition index for ion uptake by root exudates.

(25%) were lower. These results indicate that the root exudates of cucumber contain allelochemicals that inhibit ion uptake by intact cucumber seedlings and that ions such as $H_2PO_4^-$ and Mn^{2+} were less sensitive to the exudates than the other ions such as NO_3^-, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, and BO_3^-. The lower sensitivity of $H_2PO_4^-$ to aromatic acids than K^+ has been reported in other studies (Glass, 1973, 1974; Lyu and Blum, 1990), although the reason is not clear.

Effect of Cinnamic Acid on Ion Uptake. Cinnamic acid is one of the main constitutes of cucumber root exudates (Yu and Matsui, 1994). Table 2 shows the effect of cinnamic acid at various concentrations on the uptakes of NO_3^-, $H_2PO_4^-$, SO_4^{2-}, K^+, Ca^{2+}, and Mg^{2+}. The uptakes of SO_4^{2-} and Mg^{2+} were significantly inhibited even at 0.01 mmol/dm^3 cinnamic acid. The uptakes of NO_3^-, K^+, and Ca^{2+} were significantly inhibited at 0.1 mmol/dm^3 cinnamic acid, a high concentration. On the other hand, this concentration had no significant effect on the uptake of $H_2PO_4^-$. At the acid concentrations of 0.5 mmol/dm^3 or more, the k values were negative, indicating that the rates of ion efflux were higher than those of influx, resulting in net leakage of ions from roots. The concentrations of cinnamic acid for 50% inhibition were estimated to be 0.16, 0.11, 0.06, 0.04, 0.07 and 0.02 mmol/dm^3 for NO_3^-, $H_2PO_4^-$, SO_4^{2-}, K^+, Ca^{2+}, and Mg^{2+} respectively. The inhibitory effects of vanillic acid (4-hydroxy-3-methoxybenzoic acid), p-hydroxycinnamic acid, and ferulic acid (4-hydroxy-3-methoxycinnamic acid) on the uptake of $H_2PO_4^-$ by cucumber seedling have been examined by Lyu et al. (1990). However, the activities of these acids were not as strong as that of cinnamic acid observed in this study, indicating that the

TABLE 2. EFFECTS OF CINNAMIC ACID AT DIFFERENT CONCENTRATIONS ON ION UPTAKE BY CUCUMBER SEEDLINGS[a]

Concentration (mmol/dm³)	Ion uptake (μmol/g/hr)					
	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}
0	4.2a	0.45a	0.28a	1.7a	0.71a	0.53a
0.005	4.0a	0.48a	0.21ab	1.3ab	0.63a	0.35ab
0.010	3.2ab	0.44a	0.15b	1.2ab	0.63a	0.28b
0.100	2.4b	0.34a	0.16b	0.79bc	0.35b	0.21b
0.250	2.0b	0.09b	0.09b	0.12c	0.12b	0.02bc
0.500	−0.42c	−0.51c	−0.24c	−1.8d	−0.38c	−0.21c
1.000	−5.4d	−1.4c	−0.41c	−5.9e	−0.51c	−2.4d

[a]Numbers with different letters within a column refer to significant difference at the 0.05 level according to Duncan's multiple-range test.

activity of a phenolic acid was partly modified by substitutions such as hydroxyl and methoxyl groups. This will be discussed later in this paper.

The ion uptake was significantly influenced by the pH of the nutrient solution. The inhibition index increased with decreasing pH (Table 3). For example, the index for K^+ uptake ranged from 45 at pH 4.5 to 17 at pH 7.5. The other

TABLE 3. EFFECTS OF PH ON ION UPTAKE RATE (K) AND INHIBITION INDEX (R) FOR ION UPTAKE BY CINNAMIC ACID[a]

pH	Cinnamic acid (mmol/dm³)	k (μmol/g/hr)					
		NO_3^-	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}
4.5	0	5.6b	0.18a	0.28b	1.7b	0.57b	0.33b
	0.1	4.3a	0.15a	0.17a	0.94a	0.30a	0.17a
6.0	0	6.6bc	0.19a	0.31b	2.1bc	0.73c	0.38b
	0.1	5.7b	0.18a	0.25ab	1.6b	0.45b	0.18a
7.5	0	6.8c	0.44b	0.33b	3.2c	1.0c	0.48b
	0.1	6.5bc	0.33ab	0.41b	2.7bc	1.0c	0.52b
		R					
4.5	0.1	23	17	39	45	47	48
6.0	0.1	14	5	19	24	38	53
7.5	0.1	6	25	−24	17	0	−8

[a]Numbers with different letters with a column refer to significant differences at the 0.05 level according to Duncan's multiple-range test.

ions showed similar patterns. Since the pK_a of cinnamic acid is 4.4, the ratio of undissociated to dissociated species increases with the decreasing pH of the solution. It has been shown with oats that the uptake of the allelochemical *o*-hydroxybenzoic acid increased and the ATP production decreased with a decrease in pH (Balke, 1985; Harper and Balke, 1981). It is the undissociated acid species that is concerned with the inhibition (Balke, 1985). The dependence of phytotoxicity of phenolic acids on rhizosphere pH was also observed by Blum et al. (1985).

Effect of Various Carboxylic Acids on Ion Uptake. The inhibitory effect on the ion uptake was examined with three groups of carboxylic acid: phenyl-substituted aliphated acids (G1), substituted benzoic acids (G2), and substituted cinnamic acids (G3) (Table 4). The concentrations of the acids were adjusted to 0.25 mmol/dm^3, where cinnamic acid exhibited significant effects on all the ions examined. As the number of the methylene group between the phenyl and carboxyl moities of G1 carboxylic acids increased, inhibition indexes for $H_2PO_4^-$, K^+, and SO_4^{2-} increased. The indexes decreased with an increase in the number of hydroxyl groups on the benzene ring for G2 and G3 carboxylic acids. The substitition of the methoxyl group for the hydroxyl group (vanilic and ferulic acids) also increased the indexes for these ions. These results suggest that the hydrophobicity (or lipophilicity) of the carboxylic acids plays an important role in the inhibitory activity. However, this tendency was not clearly observed for NO_3^-, Ca^{2+}, and Mg^{2+}. Among the compounds tested, *o*-hydroxybenzoic acid showed the strongest activity. Elucidation of the role of the methylene, and hydroxyl and methoxyl groups in the inhibitory activity on ion uptake will help in evaluating the activity of other phenolic analogs ubiquitous in nature.

Effect of Allelochemicals on Ion Leakage and Their Phytotoxicity. Ion leakage (k_0') was not significant in the absence of aromatic acid. K^+ was the main ion leaked (Table 5). However, ion leakages (k') were increased up to five times by the addition of aromatic acids. Generally, strong inhibitors against ion uptake, such as phenylbutyric, *o*-hydroxybenzoic, and cinnamic acids were also strong inducers of ion leakage.

The K^+ ion was chosen to elucidate the relationships among K^+ leakage, inhibited amount of K^+ uptake ($k_0 - k$), the octanol-water partition coefficient (log P) of aromatic acid, and its phytotoxicity. Figure 1a shows a plot of K^+ leakage induced by aromatic acid ($k' - k_0'$) against the inhibited amount of K^+ uptake ($k_0 - k$). The plot demonstrates a strong positive correlation between the two variables. Moreover, the values of the former were only about 40% of those of the latter, indicating that ion leakage induced by acid was not the only process responsible for the inhibition of the ion uptake. In other words, allelochemicals affect not only ion leakage but also the active processes associated with ion uptake. The potential difference, activity of ATPase, and ATP pro-

TABLE 4. INHIBITION INDEX (R) FOR ION UPTAKE BY VARIOUS AROMATIC ACIDS[a]

Group	Acid	R					
		NO_3^-	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}
G1	Benzoic	57	55	67	61	60	44
	Phenylacetic	55	45	87	79	115	44
	Phenylpropionic	65	82	104	99	116	45
	Phenylbutyric	32	128	124	126	107	33
G2	*o*-Hydroxybenzoic	65	180	122	140	151	119
	p-Hydroxybenzoic	46	−4	91	44	104	46
	3,4-Dihydroxybenzoic	5	−12	18	3	31	−51
	Vanillic	68	42	82	76	92	43
	3,4,5-Trihydroxybenzoic	8	−21	61	30	38	−3
G3	Cinnamic	74	49	87	99	114	80
	o-Hydroxycinnamic	74	31	99	72	119	66
	p-Hydroxycinnamic	71	15	63	38	87	42
	3,4-Dihydroxycinnamic	62	7	41	23	119	42
	Ferulic	74	40	85	85	92	38
	Sinapic	62	22	68	51	107	46

[a]Each value is the mean of the results of three replicates and the concentration of each acid was 0.25 mmol/dm^3.

TABLE 5. EFFECTS OF AROMATIC ACID ON ION LEAKAGE (k') AND PHYTOTOXOCITY INDEX (PI)[a]

Acid	k' (μmol/g/hr)						PI
	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}	
Control	0.10a	0.04a	0.02a	0.41a	0.05a	0.06a	0a
Benzoic	0.14a	0.28b	0.05b	0.98b	0.24bc	0.21b	58b
Phenylbutyric	0.48c	0.37c	0.11b	2.24c	0.18b	0.25b	57b
o-Hydroxybenzoic	0.38b	0.36c	0.10b	2.01c	0.25bc	0.34bc	64b
p-Hydroxybenzoic	0.23b	0.09a	0.05b	1.09b	0.17b	0.19a	36b
3,4-Dihydroxybenzoic	0.28b	0.05a	0.03a	0.80a	0.18b	0.17a	3a
Cinnamic	0.27b	0.25b	0.08b	1.97c	0.18b	0.22b	51b
p-Hydroxycinnamic	0.31b	0.09a	0.04b	1.37b	0.16b	0.19b	30b
3,4-Dihydroxycinnamic	0.45b	0.03a	0.03a	1.04b	0.12ab	0.14ab	30b

[a]Numbers with different letters within a column refer to significant difference at 0.05 level according to Duncan's multiple-range test and the concentration of each acid was 0.25 mmol/dm^3.

duction appear to be the most likely processes affected by allelochemicals (Einhellig, 1986; Levitan and Barker, 1972).

The plot of phytotoxicity vs. inhibition index (R) for K^+ uptake with regard to tested acids showed that a correlation existed between these variables (Figure 1b). The slope (0.5) of the correlation line was considerably less than unity, indicating that these acids had less effects on phytotoxicity than on ion uptake. These results indicate that the inhibition of ion uptake was partly responsible for the growth inhibition by allelochemicals.

The octanol–water partition coefficient (log P) has been widely adopted as an index of lipophilicity (Glass, 1973, 1974; Leo, et al., 1971). When the logarithms of ion leakage and ion uptake inhibition induced by aromatic acid were plotted against log P, strong correlations were observed (Figures 2a and b). It seems likely that the acids under examination are distributed between the lipid components of the cell membrane and the aqueous nutrient solution according to their respective partition coefficients between the two phases. The acid within the membrane may induce a change in the permeability of the membrane, resulting in an increase in ion leakage and a decrease in ion uptake. This study also showed that lipophilicity of allelochemicals is a valuable index in determining the phytotoxic activity of allelochemicals. The dependence of the ion uptake inhibition by aromatic acids on pH (Table 3) may also be explained in this way.

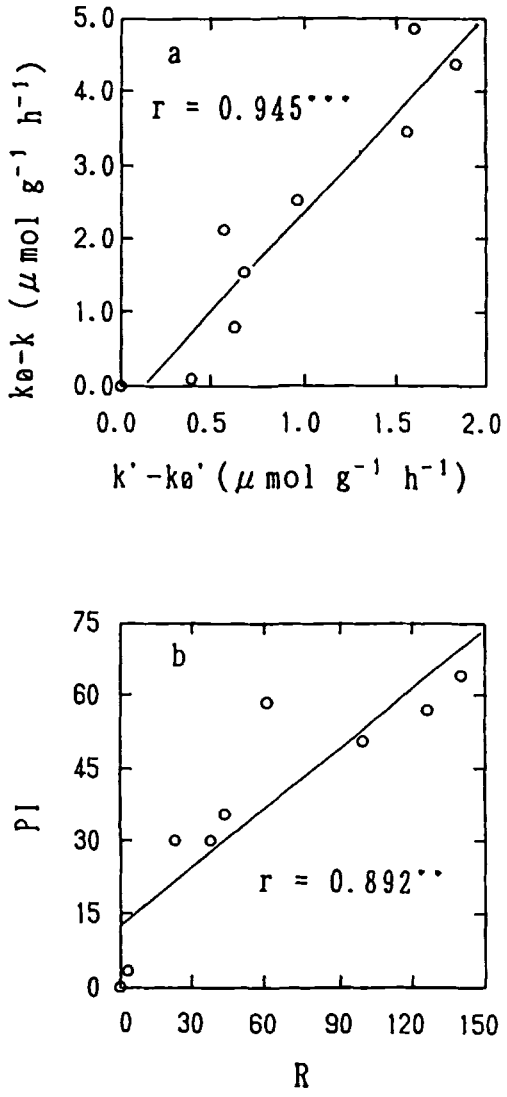

FIG. 1. (a) Relationship between the potency of aromatic acid in inducing K^+ leakage ($k' - k_0'$) and that in inhibiting K^+ uptake ($k_0 - k$). (b) Relationship between the inhibition index (R) for K^+ uptake by aromatic acid and its phytotoxicity index (PI).

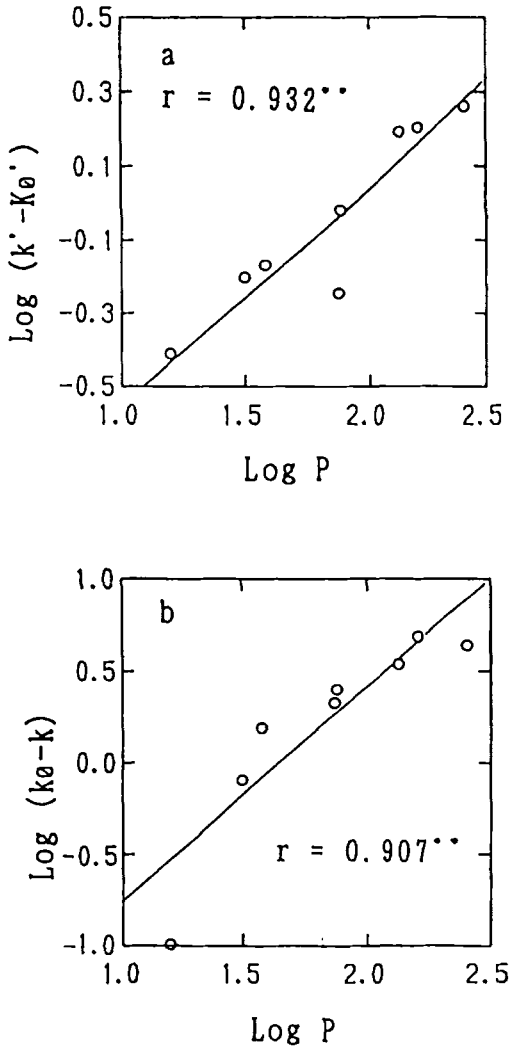

FIG. 2. Relationships between octanol–water partition coefficient (log P) and the potency of aromatic acid in inducing ion leakage (a) and that in inhibiting ion uptake (b).

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RESPONSE TO RODENT SALIVA BY TWO SPECIES OF RODENTIOPHAGOUS SNAKES

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Abstract—Brown tree snakes (*Boiga irregularis*) and prairie rattlesnakes (*Crotalus viridis*) responded with higher rates of tongue flicking to rodent saliva than to water. Both materials were presented on cotton-tipped applicators touched gently to the snakes' lips. Rattlesnakes also struck more frequently at applicators bearing saliva than at control applicators. Since rodents frequently lick themselves during bouts of grooming behavior, saliva is certainly a component of the chemicals associated with rodent integuments. It is concluded that this association has given rise in rodentiophagous predators to a sensitivity to saliva or to salivary components.

Key Words—Chemoreception, squamata, rodentiophagous snakes, saliva, predation, tongue flicking.

INTRODUCTION

Grooming behavior, a frequent activity of rodents and other mammals, involves licking the hair as well as various body parts; therefore, saliva is spread about the integument. Mammalogists have long speculated about the functions of self-anointing behavior (Heck, 1912; Herter, 1938, 1965). Recent work reveals one function to be individual recognition, and it is likely that other functions will be confirmed (Baldwin and Meese, 1977; Smith and Block, 1989; for reviews see Adams, 1980; Brown and MacDonald, 1985; see also Halpin, 1974). Since

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saliva is unquestionably a social cue among mammals, and since saliva is characteristically present on their body surfaces, salivary molecules may become associated with resting sites, burrows, or other refugia occupied by mammals, either through passive contact between the animal and its surroundings or as the result of active processes (Balasingh et al., 1995; Brockie, 1976). Reliable correlation between the presence of salivary molecules and recent mammalian occupation of an area suggests that predators might use salivary cues during foraging activities, as garter snakes (*Thamnophis sirtalis*) use chemical cues arising from earthworms that are associated with fresh soil castings deposited near burrow openings (Gillingham et al., 1991). Similar hypotheses involving mammalian urine, blood products, and other chemical cues have been tested with generally positive results (Cushing, 1984; Thiessen and Cocke, 1986), but we are aware of no direct test involving mammalian saliva and any species of predator.

Adult brown tree snakes (*Boiga irregularis*) are opportunistic predators of rodents and other endothermic vertebrates on Guam and in the native range, and the snakes also take lizards and their eggs (Fritts and McCoid, 1991; Greene, 1989; Rodda, 1992; Savidge, 1988). These predators responded strongly in laboratory tests to rodent and human blood (Chiszar et al., 1992, 1993a). Subsequent work revealed similar results with prairie rattlesnakes (*Crotalus viridus*), indicating that effects of mammalian blood were not unique to *B. irregularis*, but probably are fairly general among rodent-feeding snakes (Chiszar et al., 1991, 1992, 1993a,b). Field tests on Guam obtained more captures of free-ranging *B. irregularis* in traps baited with porcine blood than in control traps that were unbaited or that were baited with pungent but biologically irrelevant substances such as floral perfume. Nevertheless, traps baited with live mice caught far more snakes than did any trap baited with blood or other mammal-derived chemical cues (Rodda and Chiszar, 1993). Hence, either additional chemical cues must be added to blood to produce a more effective bait, or cues appropriate for other sensory modalities (vision, vibration) of a foraging snake must be added to the trap. These two possibilities are, of course, not mutually exclusive, and we are purusing both of them.

Among the mammal-derived chemicals that snakes might use to detect the presence of rodents is saliva, but we could find no test of this hypothesis in the ophidian chemical ecology literature (Burghardt, 1970; Halpern, 1992). Several studies quantified the response of rat snakes to aqueous suspensions of rodent integumentary materials, with Burghardt and Abeshaheen (1971) finding significantly greater response to these suspensions than to water. Morris and Loop (1969) obtained comparable but less clear-cut results. While rodent saliva must have been a component of the suspensions used in these studies, many other components were also present. Consequently it is not possible to draw conclusions about response to saliva by rat snakes or any other species (see also Brock

and Myers, 1979). The present experiments were designed to test rodent saliva in isolation from other chemical cues that likely entered the eluates created by Burghardt and others.

METHODS AND MATERIALS

Experiment I. Ten *B. irregularis* were randomly selected from our laboratory population of 18 animals from Guam. The 10 subjects ranged in weight from 212 to 980 g. All had been in captivity for at least three years and had been feeding on rodents (*Mus musculus* and *Rattus norvegicus*, one prey/14 days, fresh killed and thawed in about equal numbers). 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 cycle, photophase 0700–1900 hr); relative humidity was elevated at 40% by vaporizers; and temperature was kept at 26°C by a solid-state unit controlling a hot-water heating system. Snakes had last eaten 10–12 days prior to this study.

Each snake received two trials separated by no less than four days. Trials were slightly modified versions of the cotton applicator technique developed by Wilde (1938) and used extensively by Arnold (1981), Burghardt (1970), Cooper (1994, 1995), and others. Their tests, usually conducted with garter snakes or other small, nonvenomous reptiles, typically involve bringing an applicator tip within 2 cm of the snake's lips and keeping it there for 30–60 sec, recording tongue flicks and strike latency. The snakes we used are large, aggressive, and venomous, such that holding an applicator in front of them for 30–60 sec risks either a bite to the observer or an escape. Therefore, we simply touch the snake gently on its lips with the applicator, then withdraw from the cage, lock it, and record tongue flicks for the next 10 min. Control tests involved applicators soaked with tap water at 26°C. Experimental tests involved applicators that were gently rolled in the buccal cavity of a fresh killed rat, provided that no blood appeared on the swab. Rats were killed by cervical dislocation. Five snakes received control trials followed by experimental trials, while the remaining snakes received the reverse order.

One week after this study, another random sample of 10 *B. irregularis* was drawn from the colony and the entire study was repeated.

Experiment II. To extend the generality of our findings regarding snake response to rodent saliva, we conducted essentially the same experiment with prairie rattlesnakes (*Crotalus viridis*), as subjects. The choice of *C. viridis* was, of course, based upon their availability in this laboratory, but also upon the fact that adult *C. viridis* are rodent specialists (Klauber, 1956).

Eleven adult *C. viridis* were observed, all well-acclimated, long-term cap-

tives (at least five years) that had been routinely accepting rodent prey (*M. musculus* and *R. norvegicus*, fresh killed and thawed) at the rate of one per 14 days. The snakes ranged in weight from 373 to 647 g. Laboratory temperature, photoperiod, and cage dimensions were the same as those described in experiment I. Rattlesnakes were housed in a separate room from the *B. irregularis* but the two rooms were identical in all respects except that vaporizers were unnecessary for the rattlesnakes.

Procedures were the same as those used with *B. irregularis*. However, during pilot observations with other rattlesnakes we noticed that predatory strikes were sometimes aimed at applicators as they were touched to the snakes' lips. This did not occur with *B. irregularis*. Because the strikes might provide useful information, they were systematically recorded in experiment II. Rattlesnakes had last eaten 10–12 days prior to this study.

RESULTS

Experiment I. Mean rates of tongue flicking are shown in Table 1, and in both replications the rates were significantly higher after snakes' lips were touched with saliva-bearing applicators than after control presentations. Student's t tests for paired comparisons were 3.80 and 2.79 for the respective replications, $df = 9$; $P < 0.05$ for both tests. Six snakes in replication two had also served in replication one, and four snakes had not. Partitioning the data into these two categories generated the last two rows of Table 1 (replication 2a consists of the subjects that served in both replications and 2b consists of those that served only in the second replication). Mixed analysis of variance was

TABLE 1. MEAN RATE OF TONGUE FLICKING BY BROWN TREE SNAKES (*Boiga irregularis*) IN TWO REPLICATIONS OF EXPERIMENT I[a]

Replication	N	Tongue Flicks (per min ± SE)	
		Water control	Rat saliva
1	10	10.5 ± 2.0	25.8 ± 4.0
2	10	7.0 ± 2.2	16.3 ± 2.9
2a	6	4.8 ± 2.3	14.5 ± 3.9
2b	4	10.3 ± 3.9	19.2 ± 4.2

[a] See text for an explanation of rows 2a and 2b.

applied to this set of scores, treating groups 2a and 2b as a between-subjects factor and trials (control vs. experimental) as a repeated-measures factor. The main effect of trials was reliable ($F = 7.07$, $df = 1, 8$; $P < 0.05$), agreeing with the paired t tests reported above. The main effect of groups was not significant ($F = 1.80$, $df = 1, 8$; $P > 0.05$); and, most importantly, the group × trials interaction was negligible ($F < 1.0$), suggesting, within the limits of our statistical power (0.78), that the two groups responded with comparable elevation in tongue flicking to the presentation of rodent saliva. In other words, snakes tested twice with control and saliva responded as strongly during their second tests ($t = 4.13$, $df = 5$; $P < 0.05$) as did all snakes during their first tests with each applicator ($t = 3.82$, $df = 13$; $P < 0.05$).

Experiment II. Mean rates of tongue flicking after control and saliva presentations are shown in Table 2. The difference was significant by paired t test ($t = 2.41$, $df = 10$; $P < 0.05$). Likewise, proportions of control and saliva trials on which strikes occurred also differed significantly by a two-tailed sign test ($P < 0.05$).

Composite scores that combine tongue flicks and attack latencies have long been used by ophidian chemical ecologists in evaluating the effects of prey extracts (see Cooper & Burghardt, 1990, for a detailed discussion). Because both components differed significantly between control and saliva presentations in experiment II, it is not surprising that composite scores did so as well. Since the latter analysis does not add substantially to those already described, we will not discuss this matter further.

Another fact emerged during statistical analyses; namely, that snakes fell into two categories after presentation with saliva. Five snakes exhibited high rates of tongue flicking ($\bar{X} = 21.3$/min, SE = 4.7), approaching those seen in the context of strike-induced chemosensory searching (Chiszar et al., 1993b). The remaining six snakes had low rates of tongue flicking ($\bar{X} = 4.1$/min, SE = 1.1), comparable to those seen in the control condition. Several interpretations

TABLE 2. MEAN RATE OF TONGUE FLICKING BY PRAIRIE RATTLESNAKES (*Crotalus viridis*, $N = 11$) AND PROPORTION OF TRIALS ON WHICH STRIKES WERE DELIVERED TO APPLICATORS

Measure	Water control	Rat saliva
Rate of tongue flicking (per min ± SE)	3.5 ± 0.5	11.9 ± 3.4
Strike (proportion ± SE)	0.09 ± 0.09	0.63 ± 0.15

of this binary response to saliva are possible (see Chiszar et al., 1993b). For example, the applicator presentations may not have delivered sufficient quantities of chemical cues to snake chemoreceptors in the case of those animals with low rates of tongue flicking. Present data do not permit a test of this hypothesis or others discussed by Chiszar et al. (1993b). Instead of speculating further about the causation of this response variability, we want to point out that although rodent saliva is a potent cue for prairie rattlesnakes, it is not sufficient to guarantee strikes or strong chemosensory searching in all individuals.

DISCUSSION

Brown tree snakes and prairie rattlesnakes responded significantly more to rat saliva than to water when both materials were delivered to their lips by cotton-tipped applicators. Although this response suggests that saliva represents a food scent, additional studies are needed to rule out alternative interpretations involving novelty or pungency effects of saliva. We will conduct such studies, but these effects are unlikely to account for the present results because previous use of novelty and pungency controls (e.g., Chiszar et al., 1992) have never produced responses as large as those seen with saliva in the present study.

Another point requiring additional research is to ascertain whether the present response is specific to saliva or if the response occurs to other rodent-derived chemicals, in which case the active molecules may be relatively common constituents of rodent fluids or tissues. Because these issues are not yet resolved, we conclude that saliva probably triggered predatory responses in our snakes, but the effect might not be specific to saliva.

Whether rodent saliva or its components will be useful as baits for brown tree snakes on Guam remains to be seen in appropriate field studies. The work of Rodda and Chiszar (1993) cautions that robust laboratory findings sometimes transfer only modestly to field applications. Therefore, it is unwise to make unduly optimistic statements about the bait value of saliva until confirmatory tests are conducted on Guam.

Saliva and other materials from various mammals including rodents have long been known to have allergenic effects in humans (Olfert, 1986), stimulating considerable research aimed at identification of the specific chemical components mediating production of immunoglobulin E antibodies (e.g., Ohman, 1979; Ohman et al., 1975; Siraganian and Sandberg, 1979; Schumacher, 1980; Taylor et al., 1977). With a growing list of specific proteins of relatively low molecular weight all verified as having potent biological activity, perhaps it is not surprising that whole saliva is detectable by snakes dependent upon rodent prey. Furthermore, we wonder if the cues recognized by snakes are related to those recognized by human lymphocytes and homologous cells.

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FIELD RESPONSE OF SOUTHERN PINE BEETLE PARASITOIDS TO SOME NATURAL ATTRACTANTS

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Abstract—Studies were performed to isolate and identify semiochemicals that mediate location of host-infested trees by parasitoids of the southern pine beetle (SPB), *Dendroctonus frontalis*. Bark or bolts removed from pines infested with SPB broods attracted significant numbers of the hymenopterous parasitoids *Spathius pallidus* and *Roptrocerus xylophagorum* to sticky traps placed in an active SPB infestation. Traps baited with the water distillate of SPB brood-infested bark also attracted both species of parasitoids. In contrast, a synthetic bait composed of 18 compounds identified from the headspace volatiles of attractive bark failed to trap parasitoids. The oxygenated and hydrocarbon components of the bark distillate were partitioned by silica gel liquid chromatography, and the resulting two fractions were tested in the field. Parasitoid attraction was greatest when both fractions were released from traps simultaneously. The hydrocarbon fraction, which failed to attract parasitoids, enhanced the weak attractiveness of the oxygenated fraction. Hence, it appears that no single compound is responsible for mediating SPB parasitoid host-tree location and that both oxygenated and hydrocarbon semiochemicals are involved in this process.

Key Words—Parasitoid, bark beetle, *Dendroctonus frontalis*, *Spathius pallidus*, *Roptrocerus xylophagorum*, host location, semiochemical, water distillation, terpenes, fractionation.

INTRODUCTION

Numerous species of hymenopterous parasitoids are associated with the southern pine beetle (SPB), *Dendroctonus frontalis* Zimmermann (Coleoptera: Scoly-

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tidae), the most destructive forest pest in the southeastern United States (Franklin, 1969; Berisford, 1980). Evidence indicates that these parasitoids may use olfactory cues to locate hosts and host habitats. Several SPB parasitoids have been reported to respond to traps baited with beetle pheromones, pine resin terpenes, or a combination of these (Camors and Payne, 1972; Dixon and Payne, 1980), and these odors may serve as cues in the location of host habitats, namely, infested stands (Payne, 1989). However, bark beetle parasitoids arrive on infested pines in the greatest numbers when their preferred host life stages, late-instar larvae and pupae, are most abundant (Camors and Payne, 1973; Dixon and Payne, 1979; Berisford et al., 1995). This period of maximum parasitoid arrival on host-infested trees generally follows the period of maximum beetle pheromone production and host-tree resin exudation (Sullivan et al., unpublished data), and it has been suggested that these compounds are likely not used as cues by parasitoids during selection of host-infested trees (Berisford and Franklin, 1969; Payne 1989). Susceptible brood stages are normally concealed within the bark, and, although heat perception (Richerson and Borden, 1972a, b) and tactile recognition (Ryan and Rudinsky, 1962) have been proposed as the host location mechanism for at least one species of bark beetle larval parasitoid, other evidence suggests that the cues used by bark beetle parasitoids in selecting infested trees and locating hosts are olfactory (Kudon and Berisford, 1980, 1981; Mills et al., 1991). Possible sources of parasitoid host-tree location cues include the pheromones of late-arriving SPB associates, odors produced by SPB larvae or their activities (Payne, 1989; Camors and Payne, 1973), as well as metabolites of yeasts and fungi introduced by the attacking beetles (Leufvén and Birgersson, 1987; Birgersson and Leufvén, 1988). Electroantennogram tests of the SPB parasitoids *Coeloides pissodis* (Ashmead) and *Dinotiscus dendroctoni* (Ashmead) have shown that both species possess olfactory receptors for a variety of beetle pheromones as well as host-tree odors associated with SPB larval development stages (Salom et al., 1991, 1992).

The identities of compounds used by SPB parasitoids to locate individual trees infested with susceptible host stages are currently unknown. Previous efforts in our laboratory to produce a synthetic attractant as effective at attracting SPB parasitoids as naturally infested materials have been unsuccessful (Dalusky et al., unpublished data). Synthetic SPB parasitoid attractants are potentially important tools for furthering current knowledge of bark beetle–parasitoid interactions. Practically, these attractants might be employed to monitor parasitoid populations levels, to evaluate individual parasitoid species as potential biocontrol agents (Mills and Krüger, 1988), or, possibly, to enhance levels of parasitism within active infestations (Lewis and Martin, 1990). The purpose of our study was to isolate and identify those compounds arising from infested trees that likely serve as host-tree location cues to foraging parasitoids. Three sets of experiments were conducted in pursuit of this goal. In the first, we identified

beetle-infested materials in the field that were likely to contain quantities of parasitoid-attractive volatile compounds. In the second, we used a water distillation procedure to produce a parasitoid-attractive crude extract from such beetle-infested materials. In the third, the chemical composition of the crude extract was analyzed using GC-MS, and tests were performed to determine whether the biologically active components of the extract were primarily oxygenated or hydrocarbon compounds.

METHODS AND MATERIALS

Sticky traps baited with SPB-infested materials or their extracts were used to assay parasitoid attraction in the field. Insects were trapped on Stikem Special-coated hardware cloth cylinders (31 cm tall, 18 cm diam.) arranged concentrically around either infested bolts or trap bodies containing infested bark or extracts. Trap bodies consisted of cylinders of heavy-gauge, 1-cm-mesh black plastic netting (27 cm tall, 13 cm diam.) lined on their interior surfaces with four layers of cotton cheesecloth held in place by duct tape. The rationale behind this design was to provide a bole-like visual stimulus to parasitoids in the form of a dark, vertical shape and produce a more even diffusion of bait volatiles from the surface of the traps. Waxed, corrugated cardboard disks (13 cm diam.) were inserted into both ends of the trap bodies flush with the top and bottom of each cylinder. These disks served to maintain the trap shape, contain the enclosed bait, and attach the trap body to a pipe standard, which was inserted through holes cut into the center of each disk. Bolts or baited trap bodies were placed on 1.8-m-tall pipe standards within areas of an SPB infestation that contained beetle activity. Treatments were assigned at random to trap positions within blocks in a randomized complete block design. At any one time, two to four blocks were set up within a given SPB infestation, and traps were spaced a minimum of 5 m apart both within and between blocks. The total trapping time for a single replicate was 4 hr, and during that time traps were rotated within each block regularly so that each treatment spent an equal length of time at every position within the block. Trapping was normally performed during daylight hours on days with fair weather. Following trapping, sticky screens were removed from the trap standards, separated by sheets of wax paper, and returned to the laboratory. Parasitoids were removed from the screens by hand using a dissecting probe and cleaned in hexane before being stored in 70% ethanol for later identification and sexing.

Experiment 1. Trapping assays were conducted in an active SPB infestation located in a mature, mixed stand of loblolly and shortleaf pine at Ft. Benning, Georgia, on June 7–15, 1995. Four candidate attractive sources were assayed in 12 complete blocks: (1) "late brood SPB bark" consisted of a trap body

filled with pieces of bark (total approximate surface area was 0.2 m^2) taken from trees with both fourth instar SPB larvae mining in the corky bark and numerous parasitoids on the bark surface, presumably searching for oviposition sites; (2) "early brood SPB bark" was a trap body filled with bark taken from trees that contained predominantly early larval instar or egg stage brood SPB and had few or no parasitoids searching the bark surface; (3) "infested bolt" consisted of a ~25-cm-long, ~12-cm-diam. bolt cut from the mid- or lower bole of a pine that met the same criteria as the trees used in treatment 1; and (4) "blank" was an empty trap body presented as a check.

"Bark" as used in treatments 1 and 2 and in the remainder of the experiments consisted of the outer corky bark with the phloem, cambium, and cork cambium tissues still attached. Bark was collected at a height of 1–3 m on the bole of each tree after loose outer bark flakes had been shaved off with a machete. Pieces of bark were removed by hand with a knife and placed into a trap body within 1 hour. Care was taken to prevent parasitoid adults from entering the trap bodies. The age of the beetle brood larvae was gauged in the field by the appearance of the larval galleries in the bark phloem: larvae were considered "early" only if they had not yet begun producing visible larval chambers in the phloem as described by Bridges et al. (1984). Bark used in each trap was collected from a different infested tree, and bolt baits used on consecutive trapping days were cut from a single tree.

Experiment 2. Trapping was carried out on July 14–21, 1995, in an active SPB infestation in a mature, mixed loblolly and shortleaf pine stand located in the Oconee National Forest, Georgia. Four trap treatments were compared in eight complete blocks: (1) "bark extract" consisted of a trap body baited with the water distillate of late brood SPB-infested bark; (2) "synthetic bait" was a trap body baited with a synthetic parasitoid bait formulated using commercially available compounds; (3) "infested bark" consisted of a trap body filled with pieces of infested bark as distilled in treatment 1 above; and (4) "blank" was an unbaited trap body presented as a check.

The bark extract used in treatment 1 above was obtained from pieces of loblolly pine bark as described in experiment 1, treatment 1, that were collected in the field, placed into resealable freezer bags, and kept frozen (−80°C) until extracted. Extractions were carried out using an apparatus designed for the distillation of small amounts of essential oils having a lower density than water (Guenther, 1948, pp. 316–319). In our distillations, a 4-liter Erlenmeyer flask heated on an electric hot plate/stirrer was used for boiling the extractable material, and an Allihn condenser was used to recondense volatilized oils and steam (Figure 1). Frozen bark (500 g) was ground for 30 sec at low speed in a Waring blender with deionized water (2–2.5 liters) and placed into the Erlenmeyer flask. The contents of the flask were heated to a steady boil, and the distillation was carried out for 3 hr after extract first began to accumulate in the oil trap. A

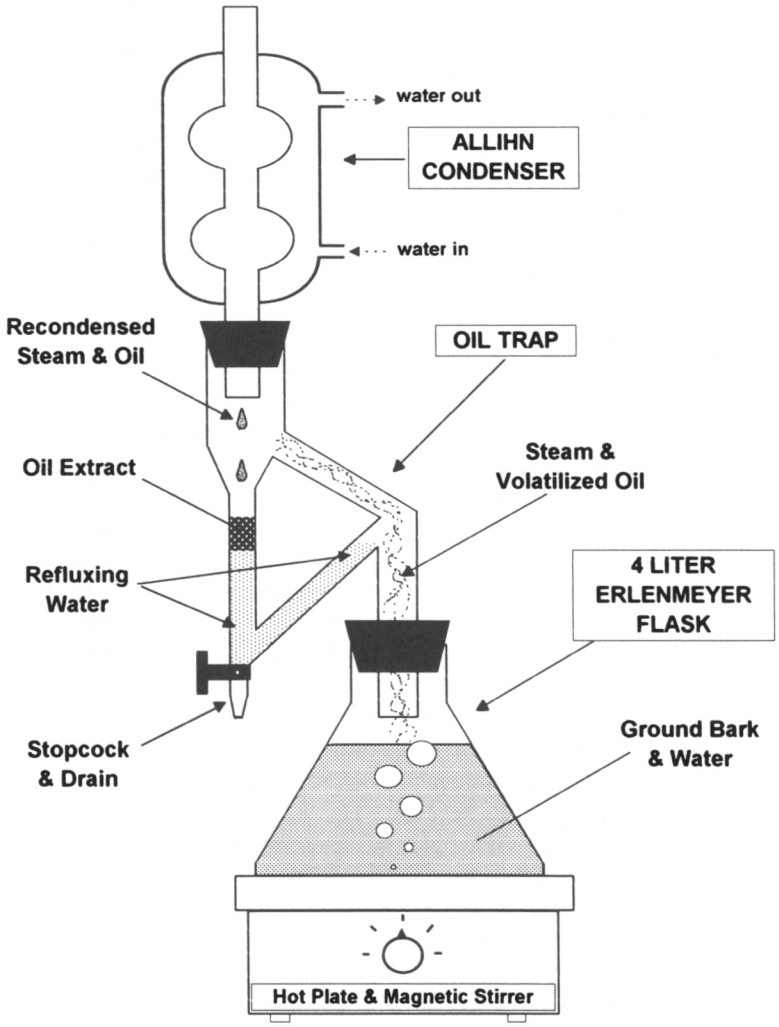


FIG. 1. Diagram of water distillation apparatus used to extract SPB-infested bark (after Guenther, 1948). Steam and oil volatilized from the flask of ground bark and boiling water are trapped in the water-cooled Allihn condenser, and the resulting condensate drips into the oil trap. The oil trap separates recondensed essential oils (they form a distinct, floating layer in the left-hand side of the oil trap) from recondensed steam while permitting the latter to reflux back into the boiling flask. The stopcock and drain allow accumulated oil extract to be removed from the still while it is in operation.

magnetic stirring bar was used to circulate the heated material in the flask. The extracted oil was collected into screw-top vials and stored in a freezer at −30°C.

The synthetic bait consisted of 18 compounds identified by means of GC-MS analysis of headspace volatiles of the parasitoid-attractive pine bark used in experiment 1, treatment 1, and the relative proportions of compounds employed in the blend were based on those found in the headspace samples. For these analyses, bark was collected in the field, placed into 1-qt heavy-gauge, reseal-able freezer bags, and transported and stored at −80°C until use. Prior to analysis the bags were allowed to equilibrate at 32°C, and then a 1-ml sample of air from within the bags was injected with a gas-tight syringe directly onto the GC-MS. Chirality of the identified compounds was not determined; however, racemic blends of compounds were incorporated in the bait whenever practical. The percentage composition of the synthetic bait by weight was as follows (all compounds were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, unless otherwise noted): 56% (±)-α-pinene, 1.8% (±)-camphene, 9.2% (−)-β-pinene, 1.5% myrcene, 4.6% (±)-limonene, 0.93% *p*-cymene, 1.8% terpinolene, 0.83% (−)-fenchone (source: Fluka Chemical Corp., Ronkonkoma, New York), 2.3% camphor (source: Fluka), 0.19% pinocamphone (source: see text), 0.32% isopinocamphone (source: see text), 5.5% terpinen-4-ol, 6.2% 4-allylanisole, 3.3% α-terpineol, 1.2% (−)-verbenone (source: Bedoukian Research Inc., Danbury, Connecticut), 0.14% (−)-borneol (source: Fluka), 1.3% ethanol (source: Aaper Alcohol, Shelbyville, Kentucky), and 1.3% acetone (source: J. T. Baker Inc., Phillipsburg, New Jersey). The purity of all compounds as measured by GC analysis was between 95% and 99%, with the exception of myrcene and camphene, which were 85% and 81% pure, respectively. Pino/isopinocamphone was obtained by the oxidation of isopinocampheol (source: Aldrich) with CrO_3/H_2SO_4 (Jones reagent), and was added to the mixture in a 1:3 decane solution.

For treatments 1 and 2, liquid bait (3 ml) was soaked into an exposed cellulose sponge (8.5 × 5.0 × 0.2 cm), which was suspended in the interior of a trap body. Sponge baits were prepared in the field immediately before the trapping was begun. The release rate of bait from the sponges was determined gravimetrically to be approximately 0.5 g/hr. Trap bodies not containing bark were lined with three paper towels moistened with deionized water to approximate the moisture released by freshly peeled bark.

Experiment 3. The bark distillate of experiment 2 was fractionated using the procedures of Chamblee et al. (1991) as a general guide. Open column chromatographic separations were performed on a 18-cm × 1.6-cm-ID column dry packed with silica gel (30.9 g, Merck, grade 10180, 70–230 mesh, Aldrich). The column was preconditioned by rinsing sequentially with ethyl ether (100 ml), methylene chloride (100 ml), and pentane (100 ml). Following conditioning, bark extract (3 ml) collected as described above for experiment 2, treatment

1, was absorbed onto the column. The hydrocarbon fraction was eluted from the column with redistilled pentane (120 ml), and the oxygenated components were then eluted with redistilled methanol (130 ml). Additional methanol (30 ml) passed through the column as a check showed no traces of compound. The two fractions were concentrated to a convenient volume (10 ml) by using a Snyder distillation column. GC-MS analysis of the collected distillate revealed that less than 0.1% of either the hydrocarbon or oxygenated fractions were lost during the process of concentration. Hence 10 ml of each concentrated fraction was assumed to contain the same quantity of bark oil components as 3 ml of undiluted bark extract. Like concentrated fractions were combined to produce two stock extract fractions for use in all replicates of experiment 3. The composition of the two fractions as well as the unfractionated bark extract were determined by GC-MS analysis (see below).

Trapping was carried out August 19–September 9, 1995, in the same SPB infestation utilized in experiment 2. For each treatment, baits were applied to cellulose sponges (10 × 10 × 0.4 cm) and suspended using wire inside a single trap body. Parasitoid responses to five trap treatments were compared within 10 complete blocks: (1) "bark extract" consisted of the water distillate (3 ml) of late brood SPB-infested bark produced and released in the same manner used above for experiment 2, treatment 1; (2) "hydrocarbon fraction" was the concentrated pentane eluted fraction (10 ml) of the bark distillate and redistilled methanol (10 ml) released from separate sponges; (3) "oxygenated fraction" was the concentrated methanol eluted fraction (10 ml) of the bark distillate and redistilled pentane (10 ml) released from separate sponges; (4) "both fractions" consisted of the hydrocarbon fraction (10 ml) and the oxygenated fraction (10 ml) released from separate sponges; and (5) "blank" consisted of redistilled pentane (10 ml) and methanol (10 ml) released from separate sponges.

Statistical Analysis. Trap catch data for each species and sex as well as for species and sexes combined were normalized using the formula $\log_{10}(X + 1)$ and subjected to a two-way analysis of variance using block and treatment as variables and the Student-Newman-Keuls (SNK) procedure for multiple pairwise comparisons (SigmaStat, Jandel Scientific, 1992–1994). All tests were performed with $\alpha = 0.05$.

Chemical Analysis. Bait compositions were analyzed on a Hewlett-Packard GCD G1800A GC-MS system equipped with a HP-FFAP fused-silica capillary column (Hewlett-Packard Corp., Palo Alto, California) (50 m × 0.2 mm ID; 0.33-mm film thickness). Bark extracts and concentrated fractions were diluted 1/50 in redistilled pentane prior to injection (1 μl). The temperature program was 32°C for 1 min, then 15°C/min to 75°C, then 6°C/min to 220°C for 12 min (use of two temperature ramps improved peak spacing and resolution); the flow rate of the helium carrier gas was 0.7 ml/min. Compounds were identified by their mass spectra and by retention time matches with known standards.

Percentage composition was calculated using peak integration areas of the total ion chromatograms; raw peak area percentages were corrected using response factors calculated by injecting known quantities of standards. For compounds with unknown response factors (ones for which no standard was available), response factors were assigned based on the compound's structural similarity to compounds with known response factors.

RESULTS AND DISCUSSION

Ten species of recognized SPB parasitoids (Berisford, 1980) were caught on test traps during the course of the three experiments (Table 1). At both trapping sites the composition of species responding to traps baited with infested materials or their extracts was, overall, quite similar, and more than 80% of the parasitoids caught were *Roptrocerus xylophagorum* (Ratzeburg) and *Spathius pallidus* (Ashmead). However, these two species represented only 39% of the total trap catch on blank traps, and, in this regard, species composition of parasitoids recovered from blank traps differed significantly from that recovered from the infested material/extract baited ones (χ^2 test, $P < 0.0001$). These data

TABLE 1. SPB PARASITOIDS CAUGHT IN FIELD-TRAPPING EXPERIMENTS

Parasitoid species	Experiment 1			
	Total trapped	% of catch	M/F	Catch on blank
Braconidae				
Atanycolus sp.	1	0.1	all F	0
Cenocoelius sp.	3	0.3	all F	0
Coeloides pissodis (Ashmead)	29	2.7	1:6.3	6
Dendrosoter sulcatus Muesbeck	36	3.3	all F	4
Spathius pallidus (Ashmead)	459	42.7	1:458	9
Eupelmidae				
Eupelmus sp.	20	1.9	all F	3
Eurytomidae				
Eurytoma spp.	7	0.7	all F	1
Pteromalidae				
Dinotiscus dendroctoni (Ashmead)	12	1.1	all F	2
Heydenia unica Cook & Davis	42	3.9	1:41	6
Roptrocerus xylophagorum (Ratzeburg)	466	43.3	1:76.7	7
Total	1075	100.0	1:88.6	38

suggest that the superabundance of *S. pallidus* and *R. xylophagorum* relative to the other species caught on baited traps resulted from an overall stronger response to baits by these two species rather than a disproportion of their numbers in the local populations or an inherent selectivity in the basic trap design.

Very few males of any parasitoid species were caught except for *Heydenia unica* Cook and Davis, and, in general, the sex ratio was strongly female-biased. Male parasitoid responses to treatment traps were not significantly different in any of the three experiments either when the parasitoid species were considered singly or grouped together. In previous studies, very few male parasitoids arrived on SPB-attacked trees when susceptible hosts were present (Camors and Payne, 1973; Dixon and Payne, 1980). Salom et al. (1991) suggested that male parasitoids of bark beetles remain on the trees from which they emerge in order to mate with emerging females and do not generally search for other infested trees. Adult males do not parasitize hosts and therefore probably do not respond to odor cues associated with host insects in the same manner as females.

Experiment 1. Infested Materials. Total SPB parasitoid catch was greater for traps baited with any of the host-infested materials than for the blank, and bark infested with either early or late SPB brood stages attracted significantly

Experiment 2				Experiment 3				Experiments 1–3 combined			
Total tapped	% of catch	M/F	Catch on blank	Total trapped	% of catch	M/F	Catch on blank	Total trapped	% of catch	M/F	Catch on blank
0	0.0	N/A	0	1	0.4	F	1	2	0.1	all F	1
0	0.0	N/A	0	0	0.0	N/A	N/A	3	0.2	all F	0
5	1.5	all F	0	5	1.8	1:4	1	39	2.3	1:7.8	7
7	2.0	all F	0	8	2.9	1:3	1	51	3.0	1:25.5	5
191	55.7	1:30.8	6	68	24.3	1:67	1	718	42.3	1:89.8	16
8	2.3	all F	1	6	2.1	all F	1	34	2.0	all F	5
5	1.5	1:4	0	0	0.0	N/A	0	12	0.7	1:12	1
10	2.9	all F	0	3	1.1	all F	0	25	1.5	all F	2
38	11.1	1:1.53	9	46	16.4	1:1.09	4	126	7.4	1:3.32	19
79	23.0	all F	1	143	51.1	1:142	2	688	40.5	1:98.3	10
343	100.0	1:15.6	17	280	100.0	1:10.4	11	1698	100.0	1:27.8	66

more parasitoids than the infested bolt. The higher trap catch for bark-baited traps compared to bolt-baited traps was likely the result of the greater exposure of attractant-releasing surface area in the former. Total parasitoid catch was somewhat higher for traps baited with late brood SPB-infested bark than traps baited with earlier stages, but this difference was not significant. When the data were broken down by species and sex, only female *R. xylophagorum* and female *S. pallidus* showed a statistically significant response to traps baited with any of the SPB-infested materials (Figure 2). Females of these two species alone comprised 87% of the total trap catch on all baited (i.e., not blank) traps used in experiment 1.

Although female *S. pallidus* were attracted in significant numbers to both bark treatments, they showed a significant preference for bark containing late brood SPB as compared to bark infested with predominantly earlier developmental stages (Figure 2). Peak abundance of most parasitoids including *S. pallidus* has been shown to occur on SPB-infested trees when late-instar SPB larvae, the parasitoids' most frequently utilized host stage (Berisford, 1980), are present in large numbers (Camors and Payne, 1973; Dixon and Payne, 1980). However, previous studies have not established whether the cues that mediate this aggregation on suitable trees are arrestants or long- or short-range attractants. Our data suggest that *S. pallidus* females are able to select materials infested with appropriate SPB host stages while in flight in that they showed a distinctly greater attraction to bark infested with the host stages that they are known to

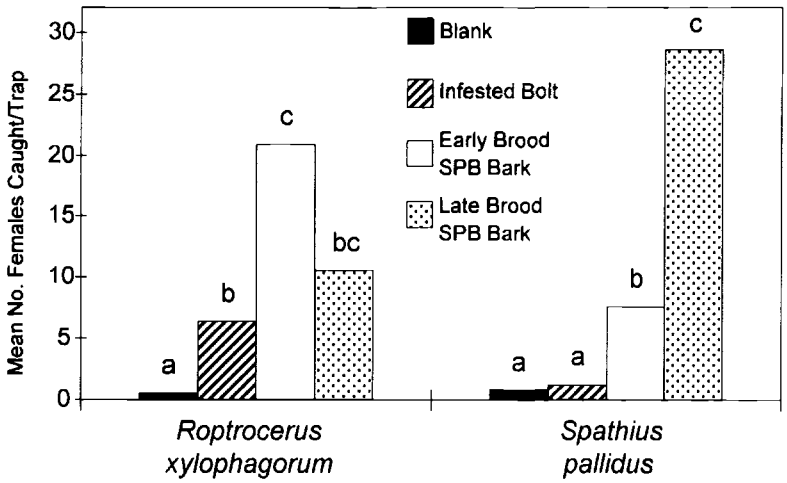


FIG. 2. Mean numbers of female parasitoids caught in traps baited with SPB brood-infested materials. Within each species, means with the same letter are not significantly different at the $P < 0.05$ level, SNK test.

parasitize most frequently. This finding supports the hypothesis that *S. pallidus* aggregation on trees infested with susceptible host stages is mediated by an attractant that can be detected by these parasitoids at some distance (at least a few centimeters) away from its source.

Female *R. xylophagorum* also responded in significant numbers to both bark baits; however, this species appeared to differ from *S. pallidus* in its treatment preferences (Figure 2). The proportions of *S. pallidus* and *R. xylophagorum* responding to the two bark treatments differed significantly (χ^2 test; $P < 0.0001$), suggesting that each species was responding maximally to cues that differed quantitatively or qualitatively. Unlike *S. pallidus*, *R. xylophagorum* did not show a preference for bark containing late-instar SPB larvae, (Figure 2). This was a somewhat surprising result since both of these species are reported to parasitize the same host stages (Franklin, 1969; Moore, 1972), and one study has reported that peak abundance of these two species on infested trees occurs approximately at the same time, that is, when the predominant SPB life stages in infested trees are late larvae and pupae (Dixon and Payne, 1980). However, Camors and Payne (1973) reported that arrival of *R. xylophagorum* on SPB-infested trees preceded that of *S. pallidus* by approximately one week, and one study that examined the two sexes of *R. xylophagorum* separately indicated that peak arrival of females occurs when early-instar larvae are the predominant SPB life stage (Dix and Franklin, 1981). Although evidence suggests *R. xylophagorum* normally parasitizes the same brood stages as *S. pallidus*, we hypothesize that an earlier arrival on SPB-infested trees might offer some benefits to *R. xylophagorum* females. This species enters into bark beetle galleries and oviposits onto hosts while they are still in the phloem tissue (Berisford et al., 1970); in contrast, all other species of SPB parasitoids oviposit through the outer bark, which has been shown to act as a physical barrier to parasitization of beetle larvae that are deep within the bark tissues (Goyer and Finger, 1980; Gargiullo and Berisford, 1981) or that have not yet tunneled into the outer bark layers to pupate (Payne, 1980). Hence, *R. xylophagorum* have physical access to SPB hosts at an effectively earlier time than the other parasitoid species, and response to host-associated semiochemicals or other cues that are specific to this earlier time period might allow this parasitoid to take greater advantage of its unique access to hosts.

Based on these results, pine bark infested with larval SPB brood appeared to be a good candidate material for extraction of parasitoid-attractive volatiles since: (1) it was attractive when separated from the rest of the tree bole to at least two species of parasitoids, implying that volatile attractants were present in the infested bark tissues, and (2) bark could be readily collected, transported, and manipulated in the laboratory.

Experiment 2: Bark Extract and Synthetic Bait. Only traps baited with either the bark extract or the infested bark trapped significantly more parasitoids than

the blank, and there was no significant difference in the total number of parasitoids caught by these two attractive trap baits. As with experiment 1, only female *R. xylophagorum* and female *S. pallidus* showed a statistically significant response to any of the baits when the trap catch data were broken down by parasitoid species and sex (Figure 3). The responses of these two species to the different treatments were essentially identical, and together they represented the majority (79%) of parasitoids caught on baited traps during the experiment.

The data for experiment 2 demonstrate that a water-distilled extract of SPB-infested bark is an effective SPB parasitoid attractant and is capable of luring the same composition of parasitoid species as the unextracted, infested bark. These findings support the hypothesis that the attractiveness of trees and bark infested with susceptible host stages is an olfactory-based phenomenon and suggest that the water distillation process can isolate, at least to some degree, semiochemicals mediating parasitoid host-tree location. It is difficult to estimate whether there was any change in activity of the extracted mixture caused by the extraction process itself, since the bark-baited traps and the extract-baited traps were likely releasing volatiles at very different rates. It should be noted, however, that the water distillation process used to produce the bark extract does not accumulate water-soluble compounds, and the absence of such compounds may have influenced the extract's biological activity. In addition, water distil-

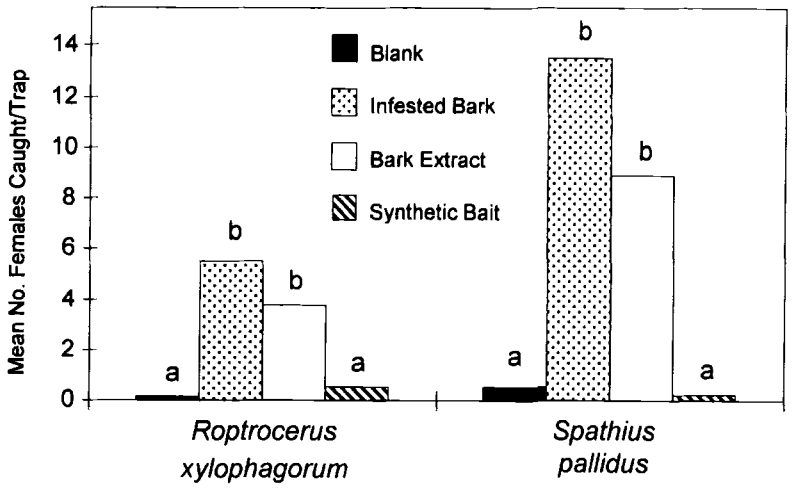


FIG. 3. Mean numbers of female parasitoids caught in traps baited with a water-distilled extract of SPB brood-infested bark, a synthetic bait, and controls. Within each species, means with the same letter are not significantly different at the $P < 0.05$ level, SNK test.

lation is known to cause some chemical changes in extracted essential oils (Guenther, 1948).

In contrast to the bark extract, our synthetic bait was not attractive to parasitoids either when species and sexes were considered individually or grouped together (Figure 3). This result was somewhat surprising since most of the major chemical constituents present in attractive bark extract were included in the synthetic mixture (Table 2), the relative proportions of these compounds were roughly similar in both baits, and both were released at the same rate. These observations suggest that no individual compound in the synthetic bait is responsible for the observed attraction of parasitoids to the crude extract of infested bark and that possibly: (1) critical constituents present in the bark extract were absent from the synthetic blend, (2) the relative proportions of components in the synthetic blend were not those required to trigger parasitoid attraction, or (3) the particular enantiomers of chiral compounds presented in the synthetic bait were not the biologically active ones.

Experiment 3: Fractionated Bark Extract. Analysis of the products of the silica gel chromatography by GC-MS revealed nearly complete separation of the bark extract into hydrocarbon and oxygenated fractions. Some 4-allylanisole (9.3% of the total) eluted with the pentane fraction, but otherwise all identifiable compounds in the pentane fraction were hydrocarbons, while all those in the methanol fraction were oxygenated (Figure 4; Table 2).

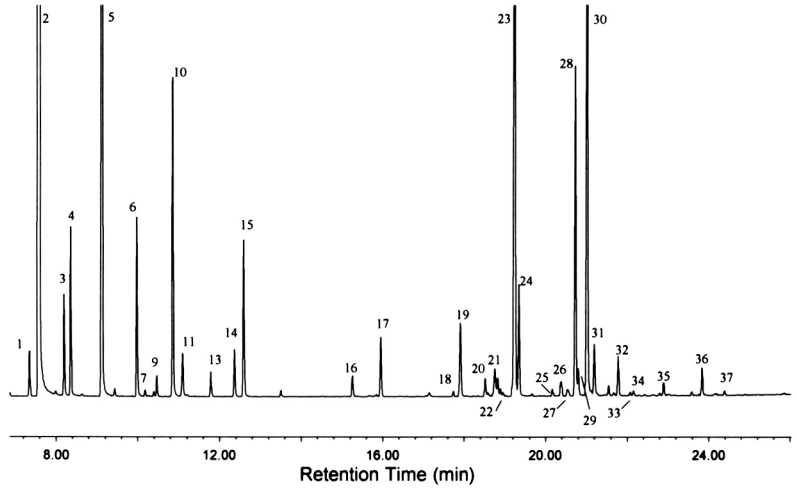
Among the five treatments tested, total parasitoid catch was greatest for traps baited with either the unfractionated bark extract or both the hydrocarbon and the oxygenated bark extract fractions simultaneously, and these treatments caught significantly greater numbers of parasitoids than traps baited with either the hydrocarbon or the oxygenated fractions alone. Traps baited with the oxygenated fraction alone were somewhat attractive, while the hydrocarbon fraction alone appeared to be unattractive to parasitoids. The fractionation process itself did not appear to substantially alter the attractiveness of the extracted components of the bark, since traps baited with both fractions caught a similar total number of parasitoids as traps baited with the original, unfractionated bark extract.

When the trap catch data for experiment 3 were categorized by species and sex, only female *R. xylophagorum* and female *S. pallidus* showed a significant response to any of the baited treatments (Figure 5). Responses of female *R. xylophagorum* and female *S. pallidus* to the treatment baits were very similar, except that female *S. pallidus* were attracted in somewhat greater numbers to traps baited with both extract fractions than those baited with the unfractionated extract, while female *R. xylophagorum* found both treatments nearly equally attractive. This difference may be the result of disparities in the relative release rates of components from the whole extract baits when compared to the fractionated baits.

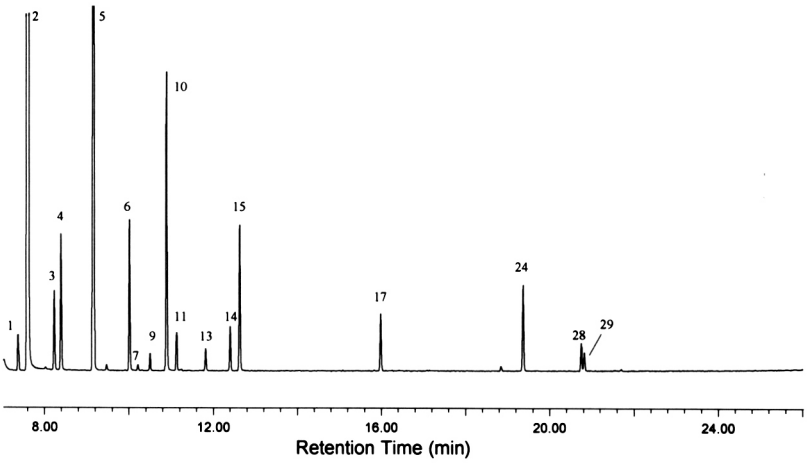
TABLE 2. CHEMICAL COMPOSITION OF BARK EXTRACT USED IN EXPERIMENT 3

Peak	Compound name	ID[a]	Type of compound[b]	Wt % in whole bark extract	Present in synthetic bait[c]
1	Tricyclene	MS, RT	HM	0.46	C
2	α-Pinene	MS, RT	HM	54.73	Y
3	α-Fenchene	MS, RT	HM	1.00	C
4	Camphene	MS, RT	HM	1.65	Y
5	β-Pinene	MS, RT	HM	9.77	Y
6	Myrcene	MS, RT	HM	1.96	Y
7	α-Phellandrene	MS, RT	HM	0.06	N
8	1,4-Cineol	MS, RT	OM	0.04	N
9	α-Terpinene	MS, RT	HM	0.18	N
10	Limonene	MS, RT	HM	3.18	Y
11	β-Phellandrene	MS	HM	0.44	N
12	Eucalyptol	MS, RT	OM	0.05	N
13	γ-Terpinene	MS, RT	HM	0.23	N
14	*p*-Cymene	MS, RT	HM	0.47	Y
15	Terpinolene	MS, RT	HM	1.73	Y
16	Fenchone	MS, RT	OM	0.25	Y
17	*p*,α-Dimethylstyrene	MS	HM	0.61	N
18	Linalool	MS, RT	OM	0.05	N
19	Camphor	MS, RT	OM	0.96	Y
20	Isopinocamphone	MS, RT	OM	0.23	Y
21	Fenchyl Alcohol	MS, RT	OM	0.37	N
22	Bornyl Acetate	MS, RT	OM	0.07	N
23	Terpinen-4-ol	MS, RT	OM	6.99	Y
24	Caryophyllene	MS, RT	HS	1.26	N
25	Myrtenal	MS, RT	OM	0.10	N
26	*trans*-Pinocarveol	MS, RT	OM	0.23	N
27	Isoborneol	MS, RT	OM	0.11	N
28	4-Allylanisole	MS, RT	OM	3.31	Y
29	α-Humulene	MS, RT	HS	0.32	N
30	α-Terpineol	MS, RT	OM	5.69	Y
31	Borneol	MS, RT	OM	0.63	Y
32	Verbenone	MS, RT	OM&P	0.56	Y
33	Piperitone	MS, RT	OM	0.05	N
34	Carvone	MS, RT	OM	0.07	N
35	Myrtenol	MS, RT	OM&P	0.17	N
36	*p*-Cymen-8-ol	MS, RT	OM	0.32	N
37	*cis*-Myrtanol	MS, RT	OM	0.04	N
	Unidentified			1.67	
	Total			100.00	

[a] MS = identification by mass spectrometry; RT = retention time matching that of known standards.
[b] HM = hydrocarbon monoterpene; HS = hydrocarbon sesquiterpene; OM = oxygenated monoterpene; P = SPB pheromone.
[c] Y = included in formulation of synthetic bait; N = not present in synthetic bait; C = present in synthetic bait as a contaminant.

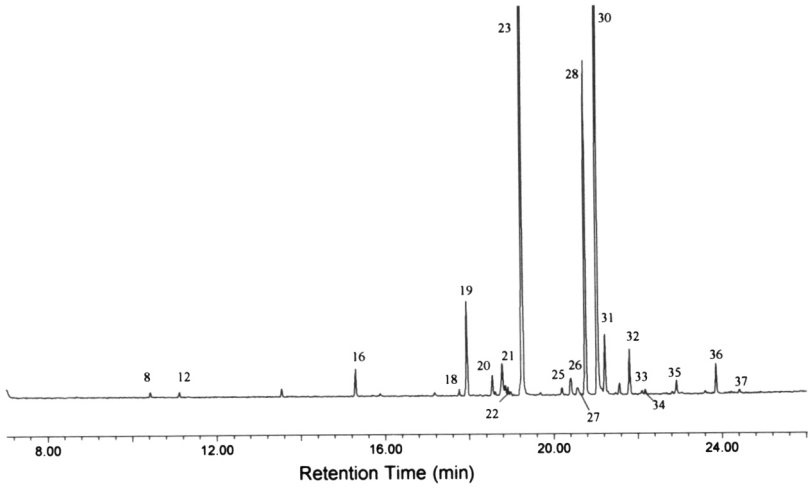


(a)



(b)

FIG. 4. Total ion chromatogram (TIC) of water-distilled extract of SPB brood-infested loblolly pine bark (a) and TICs of fractions of this extract produced on a silica gel column: the pentane-eluted (hydrocarbon) fraction (b) and the methanol-eluted (oxygenated) fraction (c). The labeled compounds are listed in Table 2.



(c)

FIG. 4. Continued.

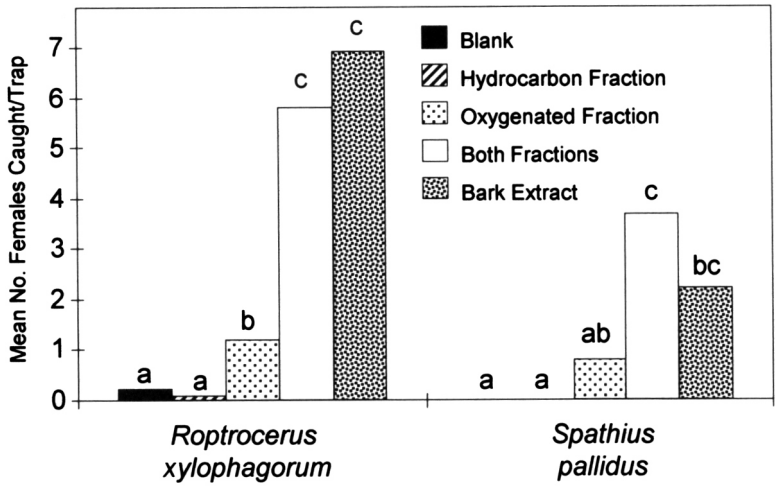


FIG. 5. Mean numbers of female parasitoids caught in traps baited with components of a water-distilled bark extract fractionated using liquid chromatography. Within each species, means with the same letter are not significantly different at the $P < 0.05$ level, SNK test.

It appears that no single compound was responsible for the observed parasitoid attraction to bark extract-baited traps and that compounds from both the hydrocarbon and oxygenated chemical classes were being used simultaneously during orientation by the two responding species of parasitoids. Electroantennogram studies on two other species of SPB parasitoids found that they possess receptors for a large number of host insect and tree-produced compounds—including both hydrocarbon and oxygenated monoterpenes (Salom et al., 1991, 1992). In foraging for hosts, the SPB parasitoids may be responding to a complex blend of odors associated with trees infested with susceptible stages of beetle brood, and presentation of the total natural blend of odors may be necessary to elicit maximal parasitoid attraction.

The hydrocarbon fraction, which comprised 78% of the original bark extract, consisted largely of hydrocarbon terpenes normally found in fresh loblolly pine resin (Paine et al., 1987; Coyne and Keith, 1972). Although the hydrocarbon fraction failed to attract parasitoids on its own, when released with the oxygenated fraction it produced a fourfold increase in the number of parasitoids attracted by its counterpart; hence the hydrocarbon components appear to be acting as synergists in the whole bark extract. Seventy percent of the hydrocarbon fraction was the monoterpene α-pinene, which is a known synergist of the attractants of numerous conifer-infesting scolytids and some of their natural enemies (Borden, 1982; Chénier and Philogeène, 1989; Schroeder and Lindelöw, 1989). In addition, α-pinene was found to be attractive to the SPB parasitoid *Heydenia unica* Cook and Davis when presented in the field using a sleeve olfactometer (Camors and Payne, 1972).

The oxygenated fraction, which was significantly attractive to parasitoids on its own, represented merely 22% of the components in the unfractionated bark extract and consisted almost entirely of oxygenated monoterpenes (Table 2). It has been found that the overall levels of oxygenated monoterpenes arising from the boles of some bark-beetle-attacked conifers increases as an attack progresses (Leufven and Birgersson, 1987; Birgersson and Bergstrom, 1989), and the presence of large numbers of searching parasitoids on SPB-infested trees has been found to coincide with the occurrence of such elevated levels of oxygenated monoterpenes (Birgersson et al., 1992). Increased concentrations of airborne oxygenated monoterpenes may signal the presence of susceptible hosts to foraging parasitoids, and the observed parasitoid responsiveness to the oxygenated fraction is in agreement with this hypothesis. Two known SPB pheromones, myrtenol and verbenone (Payne, 1980), were present in this fraction in relatively small amounts (Table 2) and may have played a role in the observed attraction.

Further experiments will be necessary to identify the precise blend of chemical constituents in the crude bark extract that are responsible for mediating SPB parasitoid host-tree location. The bark used to produce the attractive extract

contained biomass from bark beetle brood, associated arthropods, symbiotic microbes, as well as the host-tree; hence the attractive compounds may have their origin in any one or several of these biological sources. Another important goal for future research will be to define the biological and chemical events that trigger the release of critical olfactory cues from bark-beetle-infested trees and are responsible for the characteristic timing of parasitoid arrival.

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ORIENTATION OF SUGARCANE ROOTSTALK BORER WEEVIL, *Diaprepes abbreviatus*, TO WEEVIL, FRASS, AND FOOD ODORS[1]

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Abstract—Adults of the sugarcane rootstalk borer weevil, *Diaprepes abbreviatus*, form aggregations on citrus trees, where they feed on new foliage. The relative roles of male and female weevils, frass, food, and combinations of these odor sources in aggregation formation were studied using a y-tube olfactometer. Female and male *D. abbreviatus* were attracted by food, males, females, and female or male frass. Females were most often attracted by damaged food (broken green beans), whereas males were similarly attracted to damaged food and either female frass, male frass, or heterosexual pairs. No enhancement of attraction by either sex was found when males and male frass were combined with damaged food.

Key Words—Olfactometer, pheromone, volatiles, aggregation, behavior, attractant.

INTRODUCTION

The sugarcane rootstalk borer weevil, *Diaprepes abbreviatus* (L.) is a major pest of sugarcane and vegetable crops in the West Indies and is a serious pest of citrus in Florida, responsible for the death of many trees in infested areas

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(Schroeder, 1981; Beavers et al., 1982; Cruz and Segrarra, 1992; Sirjusingh et al., 1992). The larvae destroy root systems of seedlings and mature citrus trees, while adult weevils feeding on new foliage may significantly reduce growth and productivity of trees (Schroeder and Beavers, 1977). Methods are needed for monitoring the distribution of the pest in infested areas and for control.

Adult weevils form aggregations of equal sex ratio on or near new growth on citrus trees and may remain on the same trees for weeks (Beavers et al., 1982; Jones and Schroeder, 1984). Evidence for chemical mediation of the formation of aggregations is somewhat contradictory. Schroeder (1981) suggested a male-produced pheromone attracts females and a female-produced pheromone attracts males to aggregation sites. Beavers et al. (1982) indicated that odor of young citrus foliage is the main cue effecting aggregation and that subsequently produced frass at an aggregation site may serve as a secondary attractant for the opposite sex. Jones and Schroeder (1984) implied that a male-produced pheromone released from frass is attractive to both sexes of *D. abbreviatus*. Utilization of the chemical cues that attract *D. abbreviatus* to aggregation sites could provide attractants to lure one or both sexes to traps and hence serve as a useful lure for monitoring or trapping out weevils.

The goal of this study was clarification of the chemical-stimuli that are of most significance in attracting *D. abbreviatus* weevils to aggregation sites. This knowledge is needed to pursue the isolation and identification of pheromones and kairomones to use as lures in traps.

METHODS AND MATERIALS

Weevils. Adult *D. abbreviatus* were collected by hand picking from a citrus grove in Lake County, Florida, during April–October 1995. Beetles were maintained in climatic chambers in the USDA-ARS laboratory in Gainesville, Florida, at 25°C, 40–60% relative humidity, and 16L:8D photoperiod. Freshly cut citrus foliage and fresh green beans were supplied as food sources for the first five days and then beans thereafter. Beetles were separated by sex using differences in the last abdominal sternite shape: triangular sternite for females and rounded sternite for males. Beetles of each gender were kept in Plexiglas frame and screened cages (30 × 30 × 30 cm) with a Plexiglas plate at the bottom. Up to 100 beetles were held in a cage. All beetles were used in these studies, either as attractant sources or subjects tested for attraction to a volatile source, were held at least seven days prior to testing to adjust to laboratory environmental conditions. Age of collected weevils was variable, and females probably were mated before collection, as indicated by the fact that all egg clutches laid on the first seven days following collection hatched ($N > 200$).

Bioassay. All experiments were conducted using two y-shaped glass tube olfactometer sets as described by Harari et al. (1994), in which each of the two olfactometer arms led to a spherical glass trap, followed by a glass bulb containing a volatile source. Air (350 ml/min) was pushed into the system, directed from the source of volatiles to the starting point of the tested beetle. Beetles were released individually at the starting point and walked against the air current toward the control and treatment airstreams. A positive response was recorded when a beetle crawled into the spherical glass trap, while a negative or no response was recorded when a beetle did not reach the trap within 20 min of release. After testing five weevils, the olfactometer was turned 180° in order to avoid bias from uncontrolled directional factors. Since mating is apparently a diurnal behavior that starts before first light and lasts all day (Schroeder and Beavers, 1985), experiments were begun in the scotophase (red light) 60 min before lights-on and were conducted until 150 min after the onset of the photophase of the light cycle. Only one type of treatment comparison was made in any of the olfactometers in a given day, and 25–55 weevils were tested to that comparison during the 3.5-hr test period of a given day. Each treatment comparison was repeated over a period of five to eight days to comprise an experiment. Olfactometers were rinsed daily with hot water and ethyl alcohol.

Treatment Comparisons. Bioassays were conducted to determine whether *D. abbreviatus* are attracted to food, frass of either sex, individual weevils of either sex, interacting same-sex or heterosexual pairs of weevils, or to combinations of either sex with their frass or food.

Green beans (*Phaseolus vulgaris* L.) (2), either intact or broken by hand, were tested as a food attractant. Fecal material of males or females (0.5 ± 0.1 g), deposited over the two days before the experiment, was collected 20 min before the experiment was commenced. Adult weevils when held together either as a unisex group or as a mixed sex group often interacted, including mounting and copulating attempts. Therefore, multiple adult weevils were used as attractant sources either as an interacting group or held separately. A group was composed of five or 10 weevils held together in the olfactometer glass bulb, while weevils held separately were each put into a small plastic screen cage (6 cm high $\times$ 1.5 cm diam) to prevent physical interaction. Five or 10 cages of individual weevils were placed within the olfactometer glass bulb as an attractant source. When weevils of either sex were used as the attractant source in one arm of the olfactometer, empty small screen cages were added to the other arm together with the second treatment as a control.

Insect Experiments. The olfactometer was first assessed as a means to bioassay the weevils' attraction by conducting an experiment with no volatile sources in both arms of the olfactometer. After movement of the weevils along the tubes to both arms was confirmed, three experiments were performed with

TABLE 1. COMPARISON SETS OF TREATMENTS FOR ALL EXPERIMENTS CONDUCTED USING TWO-CHOICE OLFACTOMETER

Test category	Exp.	Source 1	Source 2	No. of replicates (no. of weevils) Female response	Male response
System control	1	blank	2 intact beans	6 (183)	6 (195)
	2	blank	5 males	6 (178)	6 (189)
	3	blank	5 females	6 (175)	6 (170)
Food	4	2 intact beans	2 broken beans	8 (273)	5 (223)
	5	5 females	2 broken beans	5 (194)	6 (209)
	6	5 males	2 broken beans	5 (203)	5 (178)
	7	female frass	2 broken beans	5 (175)	5 (198)
	8	male frass	2 broken beans	5 (199)	5 (159)
	9	5 males + 5 females held together	2 broken beans	5 (195)	6 (184)
	10	male frass + 5 males + broken beans	2 broken beans	6 (196)	6 (193)
Frass	11	male frass	5 males	8 (258)	6 (189)
	12	female frass	5 females	5 (161)	5 (117)
	13	female frass	male frass	7 (245)	6 (218)
Weevils	14	5 females (separated)[a]	5 males (separated)	7 (287)	5 (187)
	15	5 females (separated) + 5 males (separated)	5 males + 5 females held together	6 (214)	5 (172)
	16	10 males (separated)	10 males held together	7 (268)	5 (195)
	17	10 males held together	5 males + 5 females held together	6 (168)	6 (177)

[a]Five males or five females kept individually separated in small screen cages.

no source of potentially attractive volatiles in one arm of the olfactometer (Table 1) and either intact green beans (experiment 1), separated males (experiment 2), or separated females in the other arm (experiment 3).

The relative role of damaged food as a weevil attractant was assessed in a second series of experiments (Table 1, experiments 4–10). These experiments involved the following comparisons: (1) intact green beans vs. broken green beans (experiment 4), (2) a group of female weevils held together vs. broken green beans (experiment 5), (3) a group of males held together vs. broken green beans (experiment 6), (4) female frass vs. broken green beans (experiment 7), (5) male frass vs. broken green beans (experiment 8), (6) a group of males and females held together vs. broken green beans (experiment 9) and (7) male frass,

a group of males held together, and broken green beans vs. broken green beans (experiment 10).

The relative role of weevil frass in male or female attraction was assessed in a third series of experiments (Table 1, experiments 11–13). These experiments involved the following comparisons: (1) male frass vs. males held separately (experiment 11), (2) female frass vs. females held separately (experiment 12) and (3) female frass vs. male frass (experiment 13).

The attractiveness of female and male weevils to other weevils and effects of interacting weevils on female and male weevil attraction was assessed in a fourth series of olfactometer experiments (Table 1, experiments 15–17). These experiments involved the following comparisons: (1) female weevils held separately vs. male weevils held separately (experiment 14), (2) female weevils held separately and male weevils held separately vs. a mixed group of male and female weevils held together (experiment 15), (3) male weevils held separately vs. male weevils held together (experiment 16), and (4) male weevils held together vs. a mixed group of male and female weevils held together (experiment 17).

Statistical Analysis. The results were analyzed as two-choice data. The choice of one arm was compared to the choice of the other after nonresponsive individuals (less then 10% in any experiment) were omitted from the calculation. G statistics (Sokal and Rohlf, 1969) were used for replicated goodness-of-fit tests. The heterogeneity of the replicates for each experiment was tested first and when it was found to be significant ($P < 0.01$) more tests were conducted. A second goodness-of-fit test was performed for the pooled data. Both tests were used to compute the significance of deviation from expectation (1 : 1) for each experiment ($\alpha = 0.05\%$).

RESULTS

The heterogeneity of the replicates was significantly high ($P < 0.05$) in only one experiment (Table 2, experiment 11), meaning that the replicates of that experiment differed significantly among different days.

Interactions of males, females, or males and females were frequently observed when weevils were held together as a group in one arm of the olfactometer. These interactions included female–female mounting, male–male mounting with copulatory attempts, and male and female mounting and copulating.

Both male and female weevils chose the treatment arms more often ($P < 0.05$) in all experiments comparing a potential odor source with a system control. This indicated that adult *D. abbreviatus* were capable of moving toward a source

TABLE 2. RESPONSE OF FEMALE *D. abbreviatus* TO DIFFERENT ATTRACTANT SOURCES IN *Y*-TUBE OLFACTOMETER[a]

Exp.	Total	Source 1	Attracted (%)	Source 2	Attracted (%)	G_h	G_p	G_t	df
1	183	blank	35	green beans	65	7.45	16.70	**	6
2	178	blank	37	males	63	10.15	13.05	**	6
3	175	blank	37	females	63	8.22	1.72	**	6
4	273	intact beans	38	broken beans	62	16.85	15.54	**	8
5	194	females	28	broken beans	72	0.22	37.53	**	5
6	203	males	36	broken beans	64	2.64	17.35	**	5
7	175	female frass	30	broken beans	70	1.33	21.54	**	5
8	199	male frass	37	broken beans	63	4.81	19.53	**	5
9	195	males + females held together	37	broken beans	63	0.86	13.05	**	5
10	196	male frass + males + broken beans	48	broken beans	52	9.44	0.27	ns	6
11	258	male frass	47	males	53	15.48	1.64	ns	8
12	161	female frass	68	females	32	0.62	20.46	**	5
13	245	female frass	53	male frass	47	0.85	1.71	ns	7
14	287	females (separated)[b]	38	males (separated)	62	4.04	15.07	**	7
15	214	female (separated) + male (separated)	48	males + females held together	52	9.50	0.20	ns	6
16	268	males (separated)	44	males held together	56	9.31	4.25	ns	7
17	168	males held together	53	males + females held together	47	4.81	0.55	ns	6

[a]Total: number of tested weevils; G_h: G test for heterogeneity; G_p: G test pooled data; G_t: G test total (**$P < 0.05$).

[b]Five males or five females kept individually separated in small screen cages.

of volatile attractant in the olfactometer and were attracted by intact green beans, males, or females (Tables 2 and 3; experiments 1, 2, and 3, respectively).

Female Response. Significantly more females (Table 2) were attracted to broken green beans than to intact green beans ($P < 0.05$) (experiment 4), and more females were attracted to broken green beans than to female frass (experiment 7), male frass (experiment 8), females (experiments 5), males (experiment 6) or males and females that were held together (experiment 9) ($P < 0.05$). The combination of broken green beans, males, and male frass was not more

TABLE 3. RESPONSE OF MALE *D. abbreviatus* TO DIFFERENT ATTRACTANT SOURCES IN *Y*-TUBE OLFACTOMETER[a]

Exp.	Total	Source 1	Attracted (%)	Source 2	Attracted (%)	G_h	G_D	G_t	df
1	195	blank	37	green beans	63	6.41	13.44	**	6
2	189	blank	37	males	63	2.53	12.79	**	6
3	170	blank	34	females	66	1.62	18.70	**	6
4	223	intact beans	37	broken beans	63	6.74	14.62	**	5
5	209	females	38	broken beans	62	10.61	12.91	**	6
6	178	males	37	broken beans	63	5.55	13.00	**	5
7	198	female frass	40	broken beans	60	1.20	6.86	ns	5
8	159	male frass	47	broken beans	53	2.51	0.71	ns	5
9	184	males + females held together	46	broken beans	54	8.06	3.63	ns	6
10	193	male frass + males + broken beans	48	broken beans	52	6.07	0.20	ns	6
11	189	male frass	63	males	37	3.63	12.80	**	6
12	117	female frass	31	females	69	2.34	14.02	**	5
13	218	female frass	54	male frass	46	2.07	1.77	ns	6
14	187	female (separated)*	47	males (separated)	53	2.11	0.61	ns	5
15	172	females (separated) + males (separated)	35	males + females held together	65	2.02	14.70	**	5
16	195	males (separated)	37	males held together	63	1.64	13.43	**	5
17	177	males held together	43	males + females held together	57	5.22	3.49	ns	6

[a]Total: number of tested weevils; G_h: G test for heterogeneity; G_D: G test pooled data; G_t: G test total (**$P < 0.05$).

[b]Five males or five females kept individually separated in small screen cages.

attractive to females than broken green beans alone (experiment 10) ($P > 0.05$). Females did not choose male frass more often than female frass (experiment 13). Females did not choose male frass over males (experiment 11) ($P > 0.05$), but significantly more females were attracted to female frass than to female weevils (experiment 12) ($P > 0.05$). More females were attracted to males than females held separately (experiment 14) ($P < 0.05$), but females were not attracted more to males and females held together than males and females held separately (experiment 15) ($P > 0.05$). Females also were not attracted more

to males held together compared to males held separately (experiment 16) or males held together compared to males and females held together (experiment 17) ($P > 0.05$) (Table 2).

Male Response. Significantly more males (Table 3) were attracted to broken green beans than to intact green beans (experiment 4), and more males were attracted to broken green beans than to females (experiment 5), or to males (experiment 6) ($P < 0.05$). Males, however, were not attracted more to broken beans than to female frass (experiment 7), male frass (experiment 8) or males and females held together (experiment 9) ($P > 0.05$). Broken green beans with males and male frass were not more attractive to males than broken green beans alone (experiment 10) ($P > 0.05$). Males were not significantly more attracted to male frass than to female frass (experiment 13) ($P > 0.05$), but males were significantly more attracted to male frass than to male weevils (experiment 11) and were attracted more to female frass compared to female weevils (experiment 12) ($P < 0.05$). Males were not attracted more to females held separately compared to males held separately (experiment 14) ($P > 0.05$). However, males were significantly more attracted to males and females held together than to males and females held separately (experiment 15) and to males held together compared to males held separately (experiment 16) ($P < 0.05$). Males were attracted similarly to males held together compared to males and females held together (experiment 17) ($P > 0.05$) (Table 3).

DISCUSSION

Plant volatiles that act as insect attractants are known to occur widely (Finch, 1980; Visser, 1986). Among the curculionids, the boll weevil, *Anthonomus grandis* Boheman, orients to volatile chemicals from its host plant, cotton, (Hardee et al., 1966) and responds to crushed cotton squares (Dickens, 1989). Male and female plum curculio, *Conotrachelus nenuphar* (Herbst.), are similarly attracted to host odor (Prokopy et al., 1995), and both males and females of the banana weevil, *Cosmopolites sordidus* (Germar), are attracted to chopped banana (Budenberg et al., 1993).

Beavers et al. (1982), using a bouquet of citrus foliage, reported that both male and female *D. abbreviatus* weevils were attracted to volatiles emitted by citrus foliage, whereas Jones and Schroeder (1984) did not find a significant difference between numbers of weevils attracted to traps containing a methanol extract of citrus leaves or unbaited traps. Both males and females in our study were attracted to food odors and were more attracted to damaged food compared to intact food. A heightened response to damaged plant tissue may explain aggregations of large number of weevils observed on a single tree (Beavers et al., 1982; Jones and Schroeder, 1984). Aggregation sites first may be occupied

by individual weevils that initiate feeding and, by damaging foliage, may attract more weevils to that tree, leaving nearby trees undisturbed.

In this study, male and female *D. abbreviatus* also were attracted to females and to males. While females were more attracted to males than to females, males were similarly attracted to males and to females. A similar situation may occur in the pepper weevil, *Anthonomus eugenii* Cano, where females are more attracted to males than to females but males cannot distinguish between females or males (Coudriet and Kishaba, 1988). Adults of the plum curculio also may respond positively to odor of the same or opposite sex (Prokopy and Cooley, unpublished data, cited in Prokopy et al., 1995). In olfactometer experiments by Beavers et al. (1982), *D. abbreviatus* weevils were attracted to but did not choose one sex over the other. This contradiction with our data may be explained by: (1) in our assay individual males or females may have been contaminated by frass or (2) the experiment of Beavers et al. (1982) was conducted in the scotophase while *D. abbreviatus* mating behavior occurs during the photophase (Schroeder, 1981; Schroeder and Beavers, 1985). Thus, the time of day of the bioassays by Beavers et al. (1982) may have led to a different pattern of attraction responses.

Male and female *D. abbreviatus* were more attracted to frass than to either males or females, and both were similarly attracted by male frass and female frass. These results are in agreement with those of Jones and Schroeder (1984), who reported that males and females were similarly attracted to traps baited with either male or female frass, and with those of Schroeder (1981) and Beavers et al. (1982), who assumed that an attractant in weevil frass attracts the opposite sex as well as the same sex. A pheromone released from frass is known for the boll weevil, (Tumlinson et al., 1969). Sex pheromones are released by both sexes in the pecan weevil, *Curculio caryae* (Horn), (Hedin et al., 1979), the cabbage looper, *Trichoplusia ni* (Hübner) (Lenczewski and Landolt, 1991), and the salt marsh caterpillar, *Estigmene acrea* (Drury), (Willis and Birch, 1982), among others.

Male attraction to female-released pheromone is common in insect species (Mayer and McLaughlin, 1990) and easily explained in the context of sexual selection theory, where males are expected to maximize their mating potential and females are expected to perform the less costly role in pair formation (Greenfield, 1980; Thornhill and Alcock, 1983). Female attraction to a male-produced pheromone is far less common (Mayer and McLaughlin, 1990). Two explanations (among others) for male signaling for females provided by Thornhill and Alcock (1983) are: (1) the female may benefit from better mate choice opportunities among males in aggregations, and (2) males offer something of value to females, such as advertising a food location. The latter may be accomplished by augmenting the attractiveness of the food odor by a volatile male sex pheromone released from the frass. Male *D. abbreviatus* may signal their location

as a potential mate and the location of food to attract females. Other males, as well as females, may be attracted to the combined plant volatiles and pheromones, resulting in aggregations. The observed female attraction to female frass may have evolved as a means of food location as well.

Male, but not female, *D. abbreviatus* weevils were more attracted to males held together as a group than to males held separately. Males, but not females, also were more attracted to males and females held together as a group than to females and males that were held separately. Homosexual male mounting was frequently observed among males that were held together as a group in the olfactometer experiments. This behavior often was observed in the laboratory among weevils held in cages and was twice seen on citrus foliage in the field. Homosexual male mounting in the laboratory took place while the mounted male continued copulating with a female or in the vicinity of a female or a mating pair ($N > 100$). The mounting male actually penetrated the lower male's genitalia with its aedeagus ($N > 100$). Thus, the lower male may have exhibited "female mimicry" behavior. According to Trivers (1985) and Alcock and Gwynne (1991) such a male may convey the message to a conspecific male that he is a receptive female, in order to promote his own mating opportunities. A male may carry such a message by emitting an odor that resembles the female pheromone or by acting like a female (Alcock and Gwynne, 1991). Male *D. abbreviatus* courting or copulating with a female may try to deceive a rival male by emitting a "female odor" and may allow a homosexual mounting in order to avoid mating competition. In our experiments the possibility of contamination of those males with a female-produced sex pheromone was not excluded. Male-courtship pheromones that resemble components of female-produced pheromone are known for the velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner) (Heath et al., 1988), and the Oriental fruit moth, *Grapholitha molesta* (Busck) (Baker, 1983). In the case of the velvetbean caterpillar, male responses to male pheromone are interpreted as a secondary effect that may reduce the mating success of the courting male (Heath et al., 1988). *D. abbreviatus* "female mimicry" may be intended to draw the rival male away from the female in order to avoid fighting over mates. Release of a pheromone by males that mimics female sex pheromone as a mean of avoiding combat with other males is known in the rove beetle *Aleochara curtula* (Goeze) (Peschke, 1987), whereas a female mimicry behavior (pseudofemale behavior) with copulatory attempts is a widespread occurrence in cockroaches of the genera *Blaberus, Archimandrita,* and *Byrsotrias* (Wendelken and Barth, 1985).

The observed attractiveness of males to females, of females to males, and of each sex to their own sex, to their frass, and to food may be a result of responses to a bouquet of aromas comprised of a blend of pheromone and plant chemicals. The next step in understanding the role of each attractant in conjunction with the others as it occurs under field conditions may be to use distinct

components as attractants in traps in field cage experiments and then expand such tests to open field conditions.

The attractiveness of damaged food to males and even more so to females overshadowed the other attractant components in our olfactometer tests. Such results with female weevils is encouraging: developing lures for females may be more useful for reducing pest populations. Thus, it may be worthwhile to bait traps with food lures for *D. abbreviatus*, as has been implemented for monitoring and controlling populations of the scarabaeids *Maladera matrida* Argaman (Ben-Yakir et al., 1995), the Japanese beetle, *Popillia japonica* Newman (Fleming, 1969), and others (Reed et al., 1991).

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AGGREGATION PHEROMONES AND HOST KAIROMONES OF WEST INDIAN SUGARCANE WEEVIL, *Metamasius hemipterus sericeus*

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Abstract—Coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses and coupled GC-mass spectrometry (MS) of volatiles produced by male and female West Indian sugarcane weevils (WISW), *Metamasius hemipterus sericeus* (Oliv.), revealed eight male specific, EAD-active compounds: 3-pentanol (**1**), 2-methyl-4-heptanol (**2**), 2-methyl-4-octanol (**3**),

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4-methyl-5-nonanol (**4**), and the corresponding ketones. In field experiments in Florida, alcohols **1–4** in combination with sugarcane were most attractive, whereas addition of the ketones or replacement of alcohols with ketones significantly reduced attraction. In Costa Rica field experiments testing alcohols **1–4** singly and in all binary, ternary, and quaternary combinations revealed **4** in combination with **2** was the major aggregation pheromone, equally attracting male and female WISW. Stereoisomeric **4** and (4*S*,5*S*)-**4**, the only isomer produced by WISW, were equally attractive. Addition of 4*S*-, 4*R*- or (±)-**2** to (4*S*,5*S*)-**4** significantly enhanced attraction. Sugarcane stalks in combination with **2** plus **4** (ratio of 1 : 8) were highly synergistic, whereas EAD-active sugarcane volatiles ethyl acetate, ethyl propionate, or ethyl butyrate only moderately increased attractiveness of the pheromone lure.

Key Words—Coleoptera, Curculionidae, *Metamasius hemipterus sericeus*, aggregation pheromones, pheromone chirality, (4*S*, 5*S*)-4-methyl-5-nonanol, 2-methyl-4-heptanol, sugarcane, ethyl acetate, ethyl propionate, ethyl butyrate.

INTRODUCTION

The West Indian sugarcane weevil (WISW), *Metamasius hemipterus sericeus* (Oliv.) (Coleoptera: Curculionidae), is a pest of banana, pineapple, palms, and sugarcane in Central and South America, the Caribbean and Africa (Vaurie, 1966). In the mid-1980s the WISW was introduced into Florida, where it has become a significant pest of ornamental palms and sugarcane (Giblin-Davis et al., 1994a). The WISW has recently been implicated in Colombia as a possible vector of the nematode, *Bursaphelenchus cocophilus*, responsible for red ring disease in oil palm (Calvache et al., 1995).

Attraction of *Metamasius* spp. to sugarcane (Teran, 1968; Raigosa, 1974; Woodruff and Baranowski, 1985) is used in Central America to infect field populations with entomopathogens (Carballo and Arias de Lopez, 1994). Volatiles emitted by several host plants and by male WISW attract male and female weevils (Giblin-Davis et al., 1994a). We report the identification of and field attraction to male-produced aggregation pheromones and to host volatiles.

METHODS AND MATERIALS

Volatile Collection

Adult weevils of mixed age, sex, and mating status were collected in a banana plantation 7 km west of Homestead, Dade County, Florida. Thirty-eight male and 38 female weevils were aerated separately for four days (12L:12D photoperiod) in modified Nalgene (5311) desiccators (32.9 cm high × 25.1 cm ID) containing water-moistened Kimwipe papers. A mechanical pump was used

to draw charcoal-filtered air (2 liters/min) through the chamber and insect-produced volatiles were trapped on Porapak Q (10 g) (Oehlschlager et al., 1988). Using another desiccator, freshly harvested sugarcane stalk (1 kg, cut in 5 cm sections) was aerated for 48 hr. Volatiles from all aerations were eluted separately from Porapak Q with pentane (~175 ml) and concentrated to ~5 ml by slow distillation of the solvent through a Dufton column.

Instrumental Methods

Porapak Q-trapped volatiles were analyzed by gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975) employing a Hewlett-Packard (HP) 5890 gas chromatograph equipped with a fused silica column coated with SP-1000 (30 m × 0.25 mm ID; Supelco, Inc., Bellafonte, Pennsylvania). Antennal responses were amplified using a custom-built amplifier with a passive low-pass filter and a cutoff frequency of 10 kHz. A HP 5985B coupled GC-mass spectrometer (GC-MS) fitted with the same column, a DB-5-coated (30 m × 0.25 mm ID), or a Cyclodex-B-coated fused silica column (30 m × 0.25 mm ID; both from J & W Scientific, Folsom, California) were used for GC-MS analyses of extracts employing both electron impact (EI, 70 eV) and chemical ionization (CI) using isobutane as the proton source in full-scan modes. Diastereoisomeric derivatives of 4-methyl-5-nonanol (**4**) were analyzed on a 5890 Hewlett-Packard gas chromatograph using a DB-1 coated fused silica column (15 m × 0.25 mm ID; J & W Scientific).

Chemicals and Syntheses

Racemic 4-methyl-5-nonanol (**4**) was prepared (80% yield, 95% purity, bp 90°C at 10 mm Hg) as in previous work (Perez et al., 1996). Stereoisomers of 4-methyl-5-nonanol [(4*S*,5*S*)-**4**, 98% ee; (4*R*,5*R*)-**4**, 97% ee] were available in this laboratory from previous work (Perez et al., 1996). Racemic 2-methyl-4-heptanol (**2**) was prepared by reaction of propyl magnesium bromide with isovaleraldehyde in ether solution at room temperature. Standard work-up yielded **2**, 89% yield, 96% purity, bp 70°C at 15 mm Hg. Racemic 2-methyl-4-octanol (**3**) was synthesized in a fashion similar to that used for the preparation of **2** but using butyl magnesium bromide (87% yield, 95% purity, bp 73°C for 13 mm Hg). 3-Pentanol (**4**) and 3-pentanone (**5**) were purchased (Aldrich Chemical Company, Milwaukee, Wisconsin). 2-Methyl-4-heptanone (**6**, 87% yield, 95% purity; bp 60°C at 15 mm Hg) and 2-methyl-4-octanone (**7**, 86% yield, 95% purity; bp 67°C at 13 mm Hg) were obtained by oxidation of the corresponding alcohols with Jones' reagent (Fieser and Fieser, 1967). 4-Methyl-5-nonanone (**8**, 85% yield, 95% purity, bp 80°C at 11 mm Hg) was prepared as in previous work (Perez et al., 1996).

(E)-6-Methyl-2-hepten-4-ol (9). This was prepared as previously described by reaction of isobutyl magnesium bromide and crotonaldehyde in diethyl ether (Oehlschlager et al., 1992). Purification by fractional distillation at reduced pressure (61°C at 12 mm Hg) yielded 80% of (±)-**9.**

(R,E)-6-Methyl-2-hepten-4-ol [(RE)-9] (Figure 1). Titanium(IV) isopropoxide (2.28 ml, 7.4 mmol) in dry CH_2Cl_2 (80 ml) was mixed under argon with 0.5 g of 3A powdered, activated molecular sieves. After cooling to −78°C, diethyl (2*R*,3*R*)-tartrate [L-(+)-DET, 1.56 ml, 9.12 mmol] was added via syringe followed by addition of (±)-**9** (1.84 g, 14.4 mmol) and 15 mmol of decane as internal standard. The mixture was stirred 15 min prior to dropwise addition of precooled 6.6 M anhyd. *tert*-butyl hydroperoxide in CH_2Cl_2 (4.25 ml, 28 mmol). After the reaction warmed to −20°C, it was stirred at this temperature for 0.5–1 hr. The reaction was monitored by GC and was stopped at ~50% conversion. Optical purity of (*RE*)-**9** was determined by gas chromatography of the corresponding acetyllactate derivatives (Slessor et al., 1985) on a DB-1 column. Aqueous work-up followed by column chromatography (2:8 Et_2O–pentane) gave (*RE*)-**9** (0.75 g, 81% yield based on 50% conversion, 97% ee) as a colorless liquid. ^1H NMR ($CDCl_3$, ppm): 0.89 (3H, d, J = 8.1 Hz), 0.91 (3H, d, J = 8.1 Hz), 1.36 (1H, m), 1.44 (2H, m), 1.70 (3H, d, J = 8.1 Hz), 1.90 (1H, brs), 4.08 (1H, q), 5.44 (1H, dd, J = 8.1, 16.2 Hz), 5.65 (1H, dq, J = 8.1, 16.2 Hz); ^{13}C NMR ($CDCl_3$, ppm): 137.76, 126.44, 71.33, 46.94, 28.23, 24.57, 22.92, 22.50, 22.25, 17.60. This process has been reported previously in lower enantiopreference (Oehlschlager et al., 1992).

(S,E)-6-Methyl-2-hepten-4-ol [(SE)-9] (Figure 1). This enantiomer was prepared (0.86 g, 93% yield based on 50% conversion, 95% ee) following the above procedure using diethyl (2*S*,3*S*)-tartrate [D-(−)-DET] as the epoxidation catalyst. ^1H and ^{13}C NMR spectra were identical to those given for (*RE*)-**9.**

(S)-2-Methyl-4-heptanol [(S)-2] (Figure 1). A modification of Brown's method (Fieser and Fieser, 1967) was used to reduce (*RE*)-**9.** A 50-ml filtration

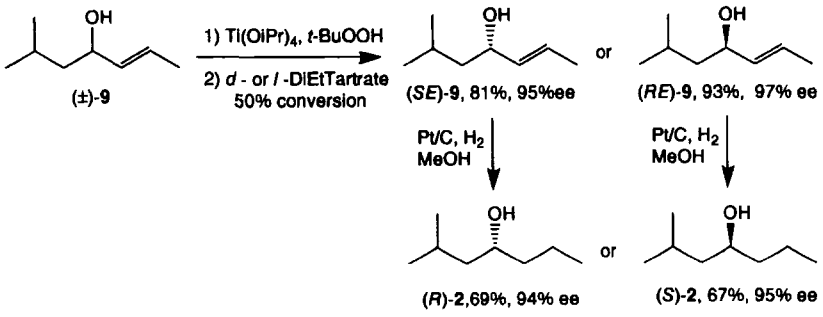

FIG. 1. Scheme for synthesis of (*S*)- and (*R*)-2-methyl-4-heptanol [(*S*)- and (*R*)-**2**].

flask was charged with a solution of (*RE*)-**3** (0.73 g, 5.7 mmol) in dry methanol (20 ml) and 0.05 eq. of 5% Pt/C. The flask was capped with a septum and a rubber bulb was secured with wire to the side arm. After flushing with nitrogen gas, H_2 was introduced until the rubber bulb inflated. The suspension was stirred at room temperature until deflation of the balloon ceased. GC analysis (DB-1) of aliquots withdrawn periodically indicated completion of the reaction after 3 hr. The system was purged with N_2 and the mixture filtered, diluted with water, and extracted (3 × 10 ml) with Et_2O. The extract was dried over anhyd. $MgSO_4$ and the solvent removed in vacuo to afford a light yellow oil. The residue was distilled at reduced pressure (70°C at 15 mm Hg) to give (*S*)-**2** as a colorless liquid (0.51 g, 67% yield, 95% ee). Optical purity was determined by gas chromatographic analysis of the acetyllactates and acetates on a Cyclodex B column. $[\alpha]_D^{20} = +14.3°$ (*c* = 2.230, Et_2O); ^1H NMR ($CDCl_3$, ppm): 0.89 (3H, d, *J* = 7.5 Hz), 0.92 (3H, d, *J* = 7.5 Hz), 0.94 (3H, t, *J* = 7.5 Hz), 1.24 (1H, m), 1.38 (6H, m), 1.76 (1H, m), 3.77 (1H, m); ^{13}C NMR ($CDCl_3$, ppm): 69.69, 46.86, 40.26, 24.62, 23.28, 22.03, 18.68, 13.97; El-MS *m/z* (relative intensity): 112 (M^+-18, 17), 87 (M^+-C_3H_7, 42). Anal. calcd. for $C_8H_{18}O$: C, 73.78; H, 13.93. Found: C, 73.68; H, 13.85.

(R)-2-Methyl-4-heptanol [(R)-2]. (Figure 1). A procedure as described above for the preparation of (*S*)-**2** was used to prepare (*R*)-**2**: $[\alpha]_D^{20} = -10.20°$ (*c* = 2.280, Et_2O); 0.60 g, 69% yield, 94% ee. Anal. calcd. for $C_8H_{18}O$: C, 73.78; H, 13.93. Found: C, 73.63; H, 13.81.

Field Experiments

Field tests of candidate semiochemicals were conducted in a 2-year-old banana plantation near Homestead, Florida, and in commercial oil palm plantations near Coto, Costa Rica. Experiments with 7–10 replicates each employed pitfall traps (Giblin-Davis et al., 1994b) or Dipel traps (Oehlschlager et al., 1993) set up in complete randomized blocks with treatments and blocks 20 m apart. Pitfall traps used in pheromone experiments contained 2–3 cm of soapy (3% by weight of Alkonox) or, equally effective, insecticide-laced (3 g/liter of Sevin 80) water to retain captured weevils. Traps were buried in the shade with their openings 2–3 cm above the soil surface. Dipel traps used in kairomone experiments were hung at chest height from palms. Attractants were suspended from the lid, and insecticide-treated food or water covered the trap bottom. Racemic candidate pheromones **1–8** were dispensed from membrane release devices [ChemTica Internacional (CTI), San José, Costa Rica] emitting 3 mg/24 hr of each component at 25°C under laboratory conditions (Hallett et al., 1993). In experiment 7 (Figure 11, below) mixtures of **2** and **4** were released at a total rate of 3 mg/24 hr from membrane release devices (CTI). Chiral isomers were released from glass capillaries (1 mm ID) cut 1 cm above the

liquid meniscus, and placed in 300-μl capped plastic centrifuge tubes with two 4-mm-diameter holes near the top. Each capillary tube released approximately 0.3 mg/24 hr of **2** or **4** at 25°C. Release of stereoisomeric 4-methyl-5-nonanol at 1.2 mg/24 hr was achieved by use of 4 capillary tubes. Host volatiles were released at 20 mg/24 hr (at 25°C under laboratory conditions) from 10 ml plastic vials. Freshly cut (20 cm pieces) sugarcane stalk (250 g per trap) was prepared immediately before placement.

The first trapping experiment (experiment 1) tested attraction of WISW to traps containing sugarcane alone and in combination with either alcohols **1–4,** ketones **5–8,** or both (**1–4** plus **6–9**). Experiment 2 tested attraction of male and female WISW to sugarcane, alcohols **1–4,** or both combined. Experiment 3 tested attraction of WISW to sugarcane and alcohols **1–4** quaternary or all possible ternary combinations. Experiment 4 tested attraction of WISW to sugarcane and alcohols **1–4** in quaternary or all possible binary combinations. Experiment 5 tested attraction of WISW to alcohols **1–4** in quaternary and all possible binary combinations. Experiment 6 tested attraction of male and female WISW to sugarcane and alcohols **1–4** singly or in quaternary combination. Experiment 7 tested attraction of WISW to **2** or **4** alone and in combination at three different ratios. Experiment 8 tested attraction of WISW to (±)-**4** or (4*S*,5*S*)-**4** alone or combine with (±)-**2,** (4*R*)-**2** or (4*S*)-**2.** Experiments 9, 10, and 11 tested attraction of WISW to **2:4** (1:8) alone or in combination with sugarcane or with ethyl acetate, ethyl propionate, or ethyl butyrate singly (experiment 9) and in all binary (experiment 10) and ternary combinations (experiment 11).

Statistical Analysis

Assumptions of normality and homogeneity of variance were tested on all data by graphical assessment of log variance vs. log mean and Bartlett's test, respectively (SAS, 1985; Systat Inc., 1992). Data that did not exhibit a normal distribution were transformed by $(x + 0.5)^{0.5}$ or log $(x + 1)$ and subjected to analysis of variance (ANOVA) [PROC GLM (SAS, 1985) or MGLH Fully Factorial (Systat Inc., 1992)] with means compared by Bonferonni's or the Waller-Duncan test at $P = 0.05$ (SAS, 1985; Systat Inc., 1992). Means presented are untransformed.

RESULTS

Laboratory Analyses

Gas chromatographic (GC) analysis of volatiles with flame ionization detector (FID) and electroantennographic detector (EAD) revealed eight male-specific compounds (Figure 2). Hydrogenation and reanalysis of weevil volatiles by GC-

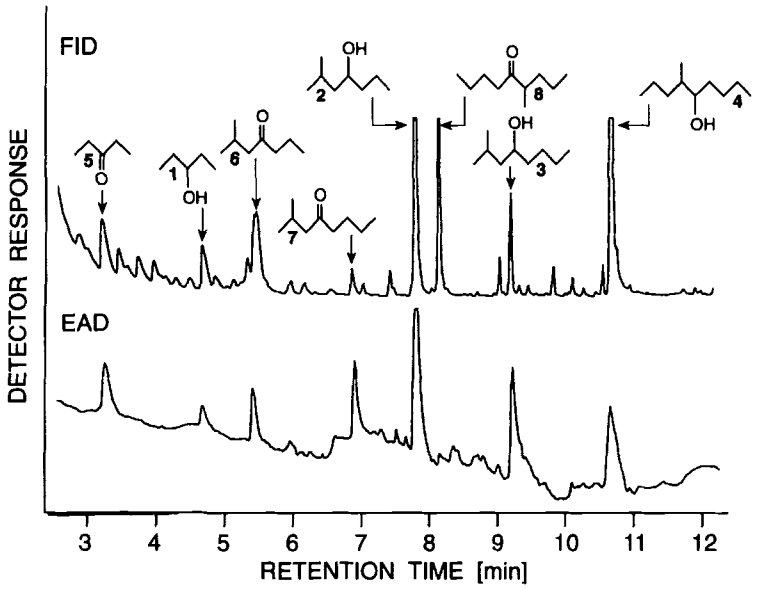

FIG. 2. Flame ionization (FID) and electroantennographic detector (EAD: female WISW antenna) responses to volatiles obtained from unfed male WISW. Chromatography: SP-1000-coated fused silica column; temperature program: 1 min at 50°C, 10°/min to 180°C. Carrier gas, He; linear flow 35 cm/sec; injector temperature 250°C; detector temperature 275°C. **1** = 3-pentanol; **2** = 2-methyl-4-heptanol; **3** = 2-methyl-4-octanol; **4** = 4-methyl-5-nonanol; **5** = 3-pentanone; **6** = 2-methyl-4-heptanone; **7** = 2-methyl-4-octanone; **8** = 4-methyl-5-nonanone.

EAD revealed antennal responses at the same retention times, indicating all EAD-active compounds contained no C–C double or triple bonds. Coupled GC-MS analysis of EAD-active volatiles in both EI and CI modes and calculation of their retention indices indicated that the EAD-active compounds were methyl-branched ketones and secondary alcohols with molecular weights of 86, 88, 128, 130, 142, 144, 156, and 158. Structures of **1** and **4** (Figure 2) were hypothesized to be 3-pentanol (**1**) and 4-methyl-5-nonanol (**4**) based on similarities of their mass spectra with those previously reported (Heller and Milne, 1978; Hallett et al., 1993). Retention indices (RI) of **2** (RI-1310) and **3** (RI-1410) on a SP-1000-coated fused silica column suggested they were a saturated analog and homolog, respectively, of (*E*)-6-methyl-2-hepten-4-ol (**9**), the aggregation pheromone of *Rhynchophorus palmarum*, the American palm weevil (APW) (Rochat et al., 1991; Oehlschlager et al., 1992) available in this laboratory. Hydrogenation of synthetic **9** afforded **2** whose retention time coincided with that of WISW-produced **2** on two GC columns (SP-1000 and DB-5). Thus,

it was hypothesized that **2** and **3** were 2-methyl-4-heptanol and 2-methyl-4-octanol, respectively. Identical retention times on two GC columns (SP-1000 and DB-5) and identical mass spectrometric characteristics of authentic alcohols (**1–4**) and ketones (**5–8**) with male-produced compounds confirmed structural assignments of all candidate pheromones. Analysis of male-produced **4** on a Cyclodex B fused silica column (Figure 3) revealed that only the 4*S*,5*S*-isomer was present. Since no separation of the enantiomers of **2** was possible on this column, formation of diastereoisomeric derivatives was necessary. GC analysis of the acetyllactate derivatives (Slessor et al., 1985) of male-produced **2** on a DB-1 column revealed that both enantiomers of **2** were present in the weevil extract in a ratio of 4:6 (*R*:*S*). This finding was confirmed by EI-SIM-MS analyses of the derivatized weevil extract and synthetic (*R*)- or (*S*)-**2**.

Semiquantitative analysis of **1–8** in male-produced volatiles revealed the following quantities produced per weevil-hour: **1**, 2 ng; **2**, 10 ng; **3**, 2 ng; **4**,

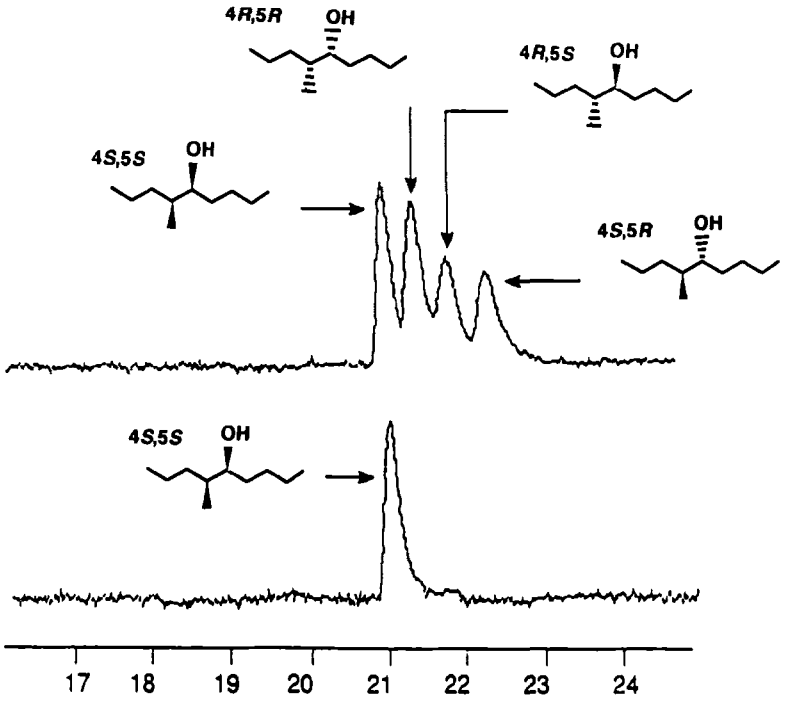

FIG. 3. Selected ion (*m/z* = 69, 87, and 101) chromatogram (Hewlett-Packard 5985B GC-MS) of (a) synthetic stereoisomeric and (b) weevil-produced 4-methyl-5-nonanol. Chromatography, Cyclodex-B column, isothermal 100°C; linear flow velocity of carrier gas 35 cm/sec, split injection and injector temperature 220°C.

40 ng; **5**, 2.2 ng; **6**, 2.1 ng; **7**, 0.7 ng; and **8**, 4.8 ng. Male WISW produce both enantiomers of **2** but only one enantiomer of **4**. Having assumed that male WISW produced one enantiomer of **2** and **4**, we employed a 1:8 mixture of racemic **2** and isomeric **4** in our experiments to determine kairomonal synergism. We found no significant difference in the response of WISW to widely varying ratios of **2** and **4** (Figure 11, below). The natural ratio is 1:16 of **2:4**.

GC-EAD analyses of sugarcane volatiles revealed four to seven compounds with antennal responses (Figure 4). Through GC-MS analyses and comparison with authentic standards, the three compounds eliciting the strongest EAD activity were identified as ethyl acetate, ethyl propionate, and ethyl butyrate. Compounds with moderate EAD activity below 2 min and ~4.5 min (Figure 4) could not be identified.

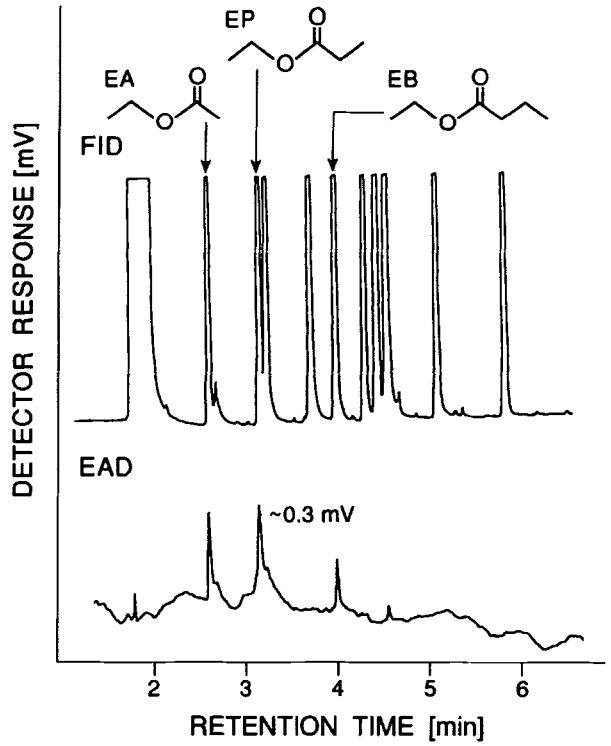

FIG. 4. Flame ionization (FID) and electroanntennographic detector (EAD: female WISW antenna) responses to volatiles obtained from sugarcane. Column and chromatographic conditions as in Figure 2. EA = ethyl acetate; EP = ethyl propionate; EB = ethyl butyrate.

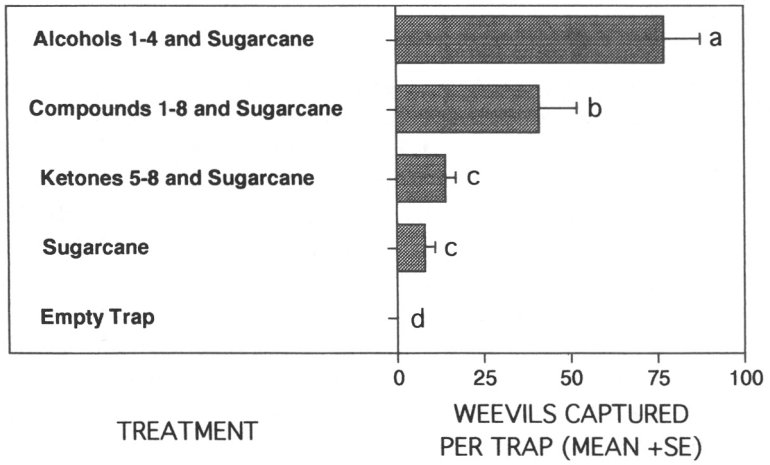

FIG. 5. Mean (+standard error) captures of WISW (experiment 1) in pitfall traps baited with sugarcane (250 g) alone and in combination with alcohols **1–4,** ketones **5–8,** and alcohols plus ketones **1–8** vs an empty trap. Alcohols **1–4** and ketones **5–8** were released from devices that emitted 3 mg/24 hr at 25°C of each component. The experiment (N = 10) was conducted in a banana plantation in Dade County, Florida, March 5–10, 1993. Data [transformed by $(X + 0.5)^{0.5}$ to approximate homogeneity] are presented untransformed. Means followed by the same letter are not significantly different according to a Waller-Duncan k-ratio t test (k = 100, $P < 0.05$).

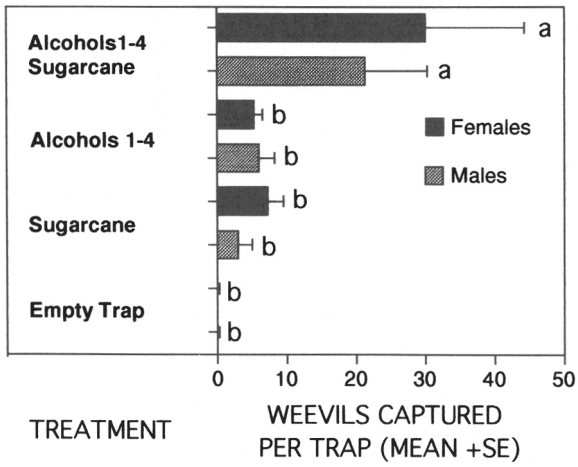

FIG. 6. Mean (+standard error) captures of WISW (experiment 2) in pitfall traps baited with sugarcane or alcohols **1–4** alone and in combination. Alcohols **1–4** were released from devices that emitted 3 mg/24 hr at 25°C of each component. The experiment (N = 10) was conducted in a banana plantation in Dade County, Florida, March 31–April 5, 1993. Data [transformed by $(X + 0.5)^{0.5}$ to approximate homogeneity] are presented untransformed. Means followed by the same letter are not significantly different according to a Waller-Duncan k-ratio t test (k = 100, $P < 0.05$).

Field Experiments

In field trapping experiments, alcohols **1–4,** but not ketones **5–8,** enhanced attraction of WISW to sugarcane (experiment 1, Figure 5). The presence of ketones **5–8** reduced attraction of WISW to alcohols **1–4** and sugarcane (Figure 5). Alcohols **1–4,** but not ketones **5–8,** enhanced attractiveness of sugarcane (experiment 1, Figure 5). Sugarcane and alcohols **1–4** were similarly effective

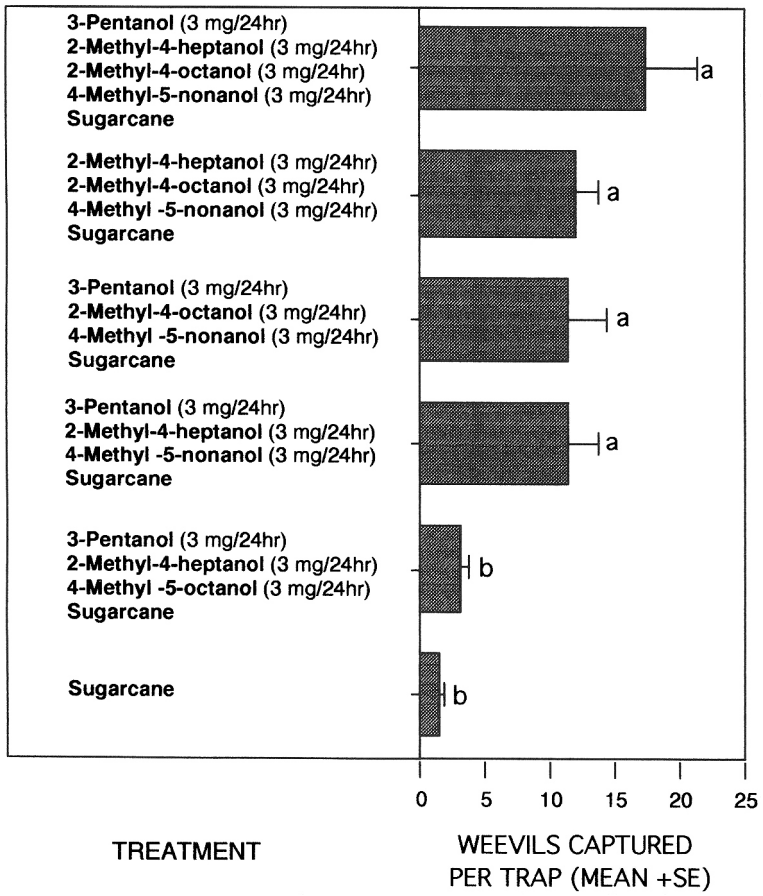

FIG. 7. Mean (+standard error) captures of WISW (experiment 3) in Dipel traps baited with sugarcane alone and in combination with alcohols **1–4** in quaternary and all possible ternary combinations. The experiment ($N = 8$) was conducted in an oil palm plantation surrounding Coto, Costa Rica, September 11–13, 1993. Data [transformed by $(X + 0.5)^{0.5}$ to approximate homogeneity] are presented untransformed. ANOVA, $F = 12.38$; $df = 5, 42$; $P < 0.0001$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

(experiment 2, Figure 6) and equally attracted male and female WISW. In combination with sugarcane, alcohols **1–4** or ternary blends thereof containing **4** were equally effective (experiment 3, Figure 7). Likewise, sugarcane plus alcohols **1–4** or plus binary blends including **4** were equally attractive (experi-

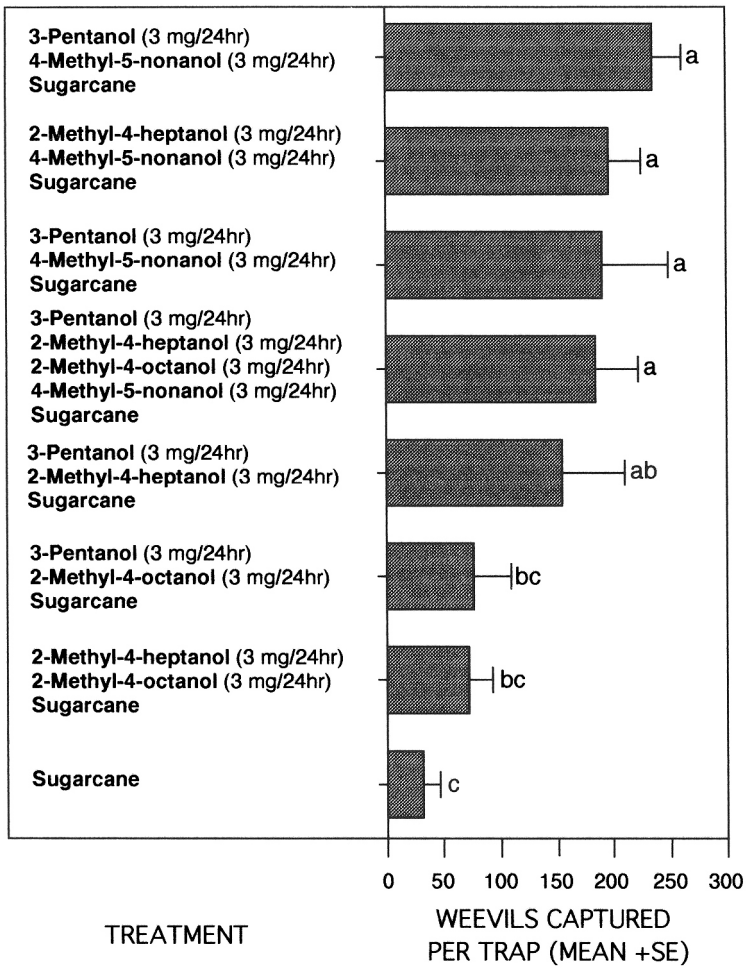

FIG. 8. Mean (+standard error) captures of WISW (expriment 4) in Dipel traps baited with sugarcane alone and in combination with alcohols **1–4** in quaternary and all possible binary combinations. The experiment ($N = 6$) was conducted in an oil palm plantation surrounding Coto, Costa Rica, January 5–8, 1994. Data [transformed by $(X + 0.5)^{0.5}$ to approximate homogeneity] are presented untransformed. ANOVA, $F = 4.98$; $df = 7$, 40; $P < 0.004$. Means followed by the same letter are not significantly different (Bonferronni's t test, $P < 0.05$).

ment 4, Figure 8). Without sugarcane, attractiveness of **2** plus **4** exceeded that of any other binary alcohol blend (experiment 5, Figure 9). Attractiveness of **1**, **2**, or **3** was significantly lower than of **4** or the quaternary blend of **1–4** (experiment 6, Figure 10). Alcohols **2** plus **4** at each of three ratios attracted more WISW than either component alone (experiment 7, Figure 11). In each treatment of experiment 7, statistically equivalent numbers of male and female WISW were captured. Experiments 3–7 were repeated a minimum of three times. Invariably, compound **4** was essential for attraction and capture of WISW males and females. Stereoisomeric **4** and (4*S*,5*S*)-**4** were equally attractive and attractiveness of (4*S*,5*S*)-**4** was enhanced by addition of *R*-, *S*- or (±)-**2** (experiment 8, Figure 12).

Because alcohols **2** plus **4** in experiments 5, 7, and 8 were most attractive, this alcohol blend was chosen to examine possible synergism with EAD-active

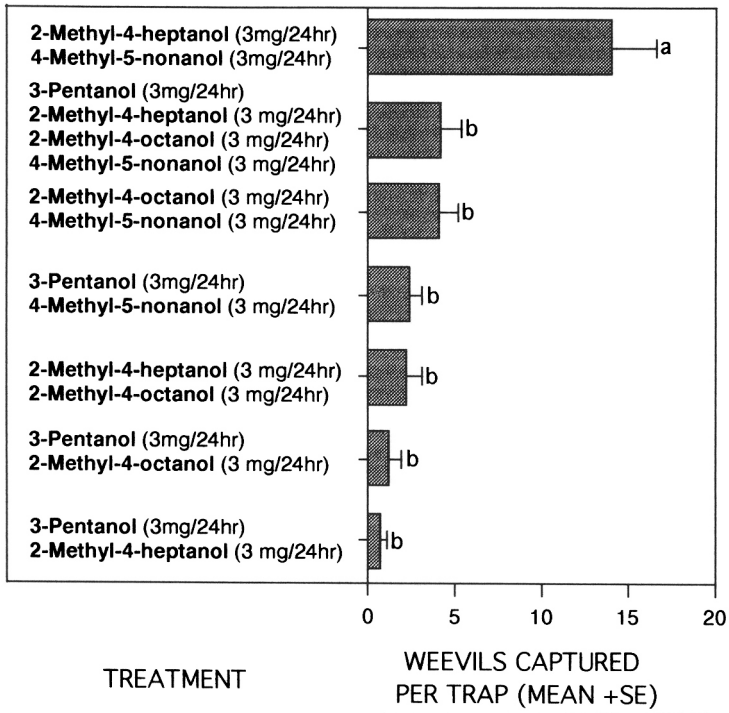

FIG. 9. Mean (+standard error) captures of WISW (experiment 5) in pitfall traps baited with alcohols **1–4** in quaternary and all possible binary combinations. The experiment ($N = 7$) was conducted in an oil palm plantation surrounding Coto, Costa Rica, November 8–11, 1993. Data [transformed by $(X + 0.5)^{0.5}$ to approximate homogeneity] are presented untransformed. ANOVA, $F = 10.04$; $df = 6, 36$; $P < 0.0001$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

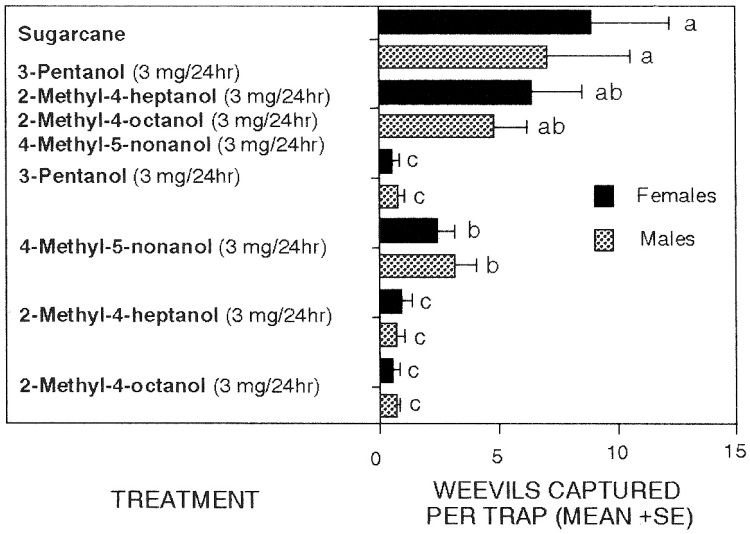

FIG. 10. Mean (+standard error) captures of WISW (experiment 6) in pitfall traps baited with alcohols **1–4** singly and in a quaternary blend. The experiment ($N = 8$) was conducted in an oil palm plantation near Río Claro, Costa Rica, January 12–15, 1994. Data [transformed by $(X + 0.5)^{0.5}$ to approximate homogeneity] are presented untransformed. Females: ANOVA, $F = 10.52$; $df = 5, 35$; $P < 0.0001$; males: ANOVA, $F = 11.31$; $df = 5, 35$; $P < 0.0001$. Means followed by the same letter are not significantly different (Bonferonni test, $P < 0.05$).

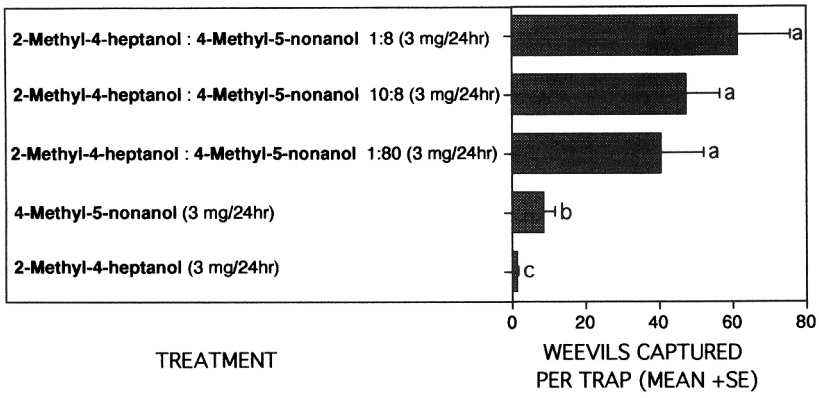

FIG. 11. Mean (+standard error) captures of WISW (experiment 7) in pitfall traps baited with 2-methyl-4-heptanol and 4-methyl-5-nonanol individually and in three ratios. The experiment ($N = 9$) was conducted in an oil palm plantation surrounding Coto, Costa Rica, November 20–28, 1994. Data [transformed by log $(X + 0.5)$ to approximate homogeneity] are presented untransformed. ANOVA, $F = 22.46$; $df = 4, 40$; $P < 0.0001$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

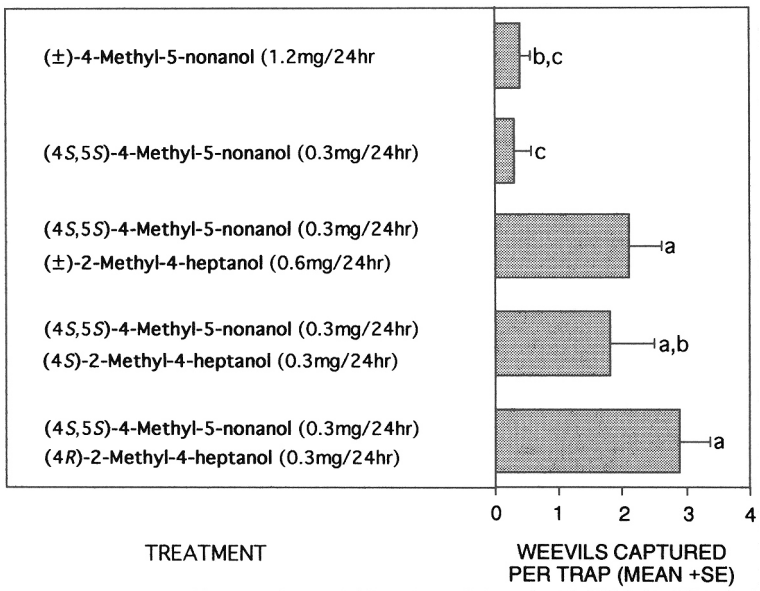

FIG. 12. Mean (+standard error) captures of WISW (experiment 8) in pitfall traps baited with stereoisomeric 4-methyl-5-nonanol (**4**) and (4*S*,5*S*)-**4** alone and combined with racemic 2-methyl-4-heptanol (**2**), *S*-**2** or *R*-**2**. The experiment ($N = 10$) was conducted in an oil palm plantation surrounding Coto, Costa Rica, August 11–24, 1994. Data [transformed by log $(X + 0.5)$ to approximate homogeneity] are presented untransformed. ANOVA, $F = 7.67$; $df = 4, 45$; $P < 0.001$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

sugarcane volatiles. Attractiveness of alcohols **2** plus **4** at a ratio of 1 : 8 increased upon addition of ethyl acetate, ethyl propionate or ethyl butyrate singly or in binary or ternary combinations (experiments 9, 10, and 11; Figure 13). Synergistic activity of fresh sugarcane exceeded that of the above esters 10–100 times.

DISCUSSION

The production of four EAD-active alcohols and corresponding ketones suggested a complex chemical communication system in WISW. While initial field tests revealed that attraction of WISW depended on the presence of EAD-active alcohols and not the corresponding ketones, further tests identified **4** as the major pheromonal component (Perez et al., 1995). Although **2** was not attractive by itself, it enhanced attractiveness of **4** and is obviously a second WISW pheromonal component. Superior EAD-activity of **2** rather than **4** is

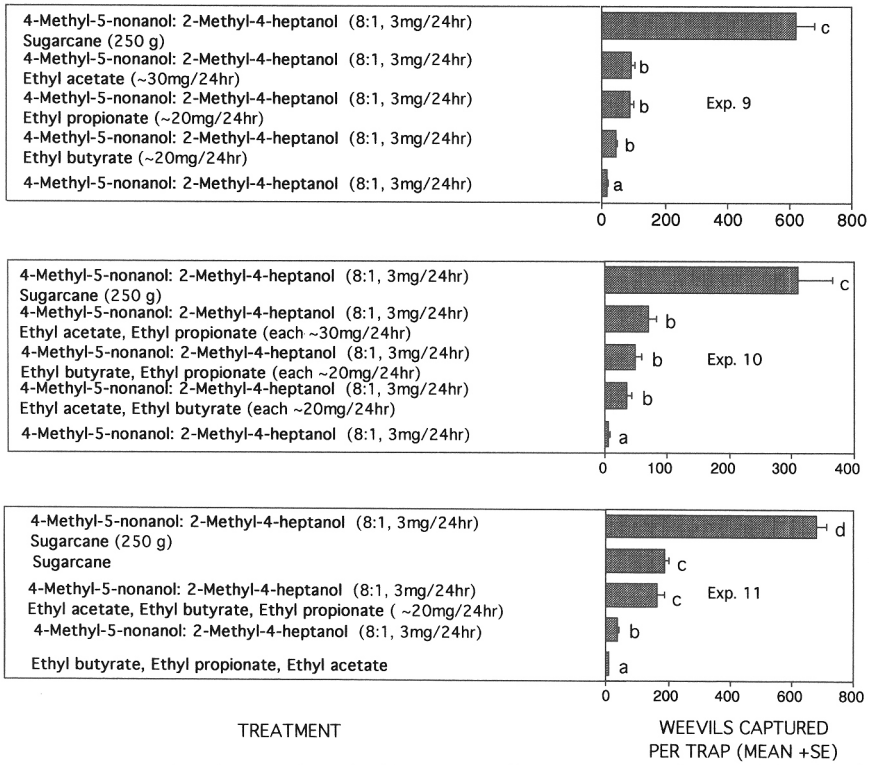

FIG. 13. Mean (+standard error) captures of WISW (experiments 9–11) in Dipel traps baited with a 1:8 mixture of 2-methyl-4-heptanol (**2**) and 4-methyl-5-nonanol (**4**) alone and combined with sugarcane (presoaked in Sevin 80) or with ethyl acetate, ethyl propionate, or ethyl butyrate singly (experiment 9) or in binary combination (experiment 10). Experiment 11 tested the blend of ethyl acetate, ethyl propionate, and ethyl butyrate alone and combined with **2** plus **4** vs. sugarcane alone and combined with **2** plus **4**. Treatments without sugarcane used detergent-laced water as the killing agent. All experiments were conducted in an oil palm plantation surrounding Coto, Costa Rica. Data of all experiments were transformed to approximate homogeneity but are presented untransformed. Experiment 9: $N = 9$; October 11–21, 1994; data transformed $(X + 1)^{0.5}$; ANOVA, $F = 13.56$; $df = 4, 38$; $P < 0.0001$. Experiment 10: $N = 9$; October 21–28, 1994; data transformed by log $(X + 1)$; ANOVA, $F = 49.66$; $df = 4, 39$; $P < 0.0001$. Experiment 11: $N = 9$; October 29–November 6, 1994; data transformed by $(X + 1)^{0.5}$; ANOVA, $F = 176.01$; $df = 4, 40$; $P < 0.0001$. In each of experiments 9–11 means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

surprising but may be attributed to the fact that **2** constitutes both a second WISW pheromonal component and a component of sympatric *Paramasius distortus* (Gemminger & Harold) [=*Metamasius inaequalis* (Gyllenhal); Perez et al., unpublished)]. Because blends of **2** plus **4** attracted both male and female WISW, they are an aggregation rather than a sex pheromone. A recent report suggests that a 1 : 10 ratio of **3** to **4** is as attractive as a similar ratio of **2** and **4** when presented with sugarcane; an interesting aspect of that report is that it is claimed that the sex ratio captured depends on the ratio of **3:4** or **2:4** used (Ramirez-Lucas et al., 1996b). Although male WISW may produce additional components that could add to the attractiveness of **2** plus **4** or **3** plus **4** and sugarcane, the current combination of **2** plus **4** and sugarcane is sufficiently attractive to warrant investigation for monitoring and population reduction through mass trapping. In a separate work we have determined that addition of ethyl acetate to the ternary blend of **2, 4,** and sugarcane significantly increases capture rates (Giblin-Davis et al., 1996).

The major aggregation component of the WISW, **4,** has been reported as an aggregation pheromone component in several species of *Rhynchophorus* weevils (Hallett et al., 1993; Oehlschlager et al., 1995; Perez et al., 1996). The presence and EAD activity of **4** in the American palm weevil, *R. palmarum* (Oehlschlager et al., unpublished) and in *Dynamis borassi* (Fabr.) (Giblin-Davis et al., unpublished) indicate that this compound occurs in at least three genera of tropical curculionids. All tropical Curculionidae investigated to date that use **4** as an aggregation pheronome component employ the 4*S*,5*S* isomer to attract conspecifics (Hallett et al., 1993; Oehlschlager et al., 1995; Perez et al., 1996). Prior to our initial report of (4*S*,5*S*)-**4** as a pheromonal component of the WISW (Perez et al., 1995), reports by Rochat et al. (1993) revealed **4** as a "pheromone-related structure" in WISW without establishment of behavioral activity, and Mori et al. (1993) determined the absolute configuration of naturally produced **4.** 5-Nonanol and 3-hydroxy-4-methyl-5-nonanone also produced by WISW males were reported to be EAG active (Ramirez-Lucas et al., 1996a), but they did not elicit significant responses in our GC/EAD recordings.

The potential semiochemical role of EAD-active ketones **5–8** is not clear. Released together with EAD-active alcohols, they reduced attraction, suggesting a role as intra- or interspecific "spacing" pheromones. Attraction to alcohol pheromonal components and "antiaggregative" characteristics of the corresponding ketones has been well documented in the Douglas fir beetle, *Dendroctonus pseudotsugae* (Hopkins) (Rudinsky, 1973) and in the mountain pine beetle *D. ponderosae* (Hopkins) (Ryker and Yandell, 1983). Production of antiaggregative **5–8** may have been artifically induced by aeration of many confined weevils. Ketones **5–8** could also be intermediates in the biosynthesis of **1–4** as has been shown for other Coleoptera (Vanderwel and Oehlschlager,

1987). They could have been repellent because release rates differed significantly from natural rates.

Although blends of **2** plus **4** are attractive alone, attractiveness was strongly increased when combined with sugarcane. Antennally active sugarcane volatiles consisting of ethyl acetate, ethyl propionate, and ethyl butyrate exhibited kariomonal synergism in field experiments. However, none of these esters alone or in combination exhibited synergistic attraction equivalent to sugarcane, which suggests the presence of additional, as yet unknown sugarcane kairomones.

The same esters in fermenting tissues of African oil palm, *Elaeis guineensis* (Jacq.); coconut palm, *Cocos nucifera* L.; and cabbage palmetto, *Sabal palmetto* (Walter), also elicited antennal responses by *Rhynchophorus* weevils (Gries et al., unpublished). However, kairomonal synergism of ethyl butyrate in WISW; ethyl acetate in *R. cruentatus* (Giblin-Davis et al., 1994b), *R. palmarum* (Jaffé et al., 1993), and WISW; and ethyl propionate in *R. phoenicis* (Gries et al., 1994) and WISW did not approximate synergistic activity of host plant activity. Attraction of sugarcane tissue (in the presence of **2** and **4**) can be enhanced further by addition of ethyl acetate (Giblin-Davis et al., 1996). Antennal and behavioral activities of the same esters in *Metamasius* (WISW) and *Rhynchophorus* weevils indicate cross-generic recognition of the same kairomones in different plant tissues. As yet unknown kairomones in palm and sugarcane may be cross-generic attractants, whose identification may prolong the service life of traps currently used in semiochemical-based management of *Rhynchophorus* and *Metamasius* weevils (Oehlschlager et al., 1993).

Because WISW pheromone component **4** is attractive to APW and APW pheromone component **9** is not repellent to WISW, traps can be baited with **2, 4,** and **9** plus sugarcane to capture both weevils (Chinchilla et al., 1996). This tactic seems advantageous for lowering the incidence of red ring disease in those areas where both species are considered to vector the red ring nematode, *Bursaphelenchus cocophilus* (Chinchilla et al., 1996).

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EFFECT OF VISUAL CUES AND A MIXTURE OF ISOTHIOCYANATES ON TRAP CAPTURE OF CABBAGE SEED WEEVIL, *Ceutorhynchus assimilis*

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Abstract—The effect of trap design, trap color, and a mixture of isothiocyanates on the capture of *Ceutorhynchus assimilis* (Paykull) was studied in a series of field experiments. Unbaited yellow water traps, a yellow sticky box trap, and a yellow sticky card trap, mounted vertically, or at 45° to the vertical with the yellow card facing upwards were effective for capturing seed weevils, but a horizontal yellow sticky card trap caught few. White, green, and black traps were unattractive. During migratory periods, trap catch could be enhanced two to four times with a lure consisting of a mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl isothiocyanates, but this bait did not attract in a trap with ineffective design. The sticky card trap, mounted at 45° to the vertical and baited with the isothiocyanate mixture, may be useful for monitoring movement of *C. assimilis* during migratory periods. However, during the colonization phase, lures of either a mixture of isothiocyanates or of allyl isothiocyanate alone were not attractive.

Key Words—*Ceutorhynchus assimilis*, cabbage seed weevil, Coleoptera, Curculionidae, isothiocyanates, attractant, trap design, trap color.

INTRODUCTION

The cabbage seed weevil, *Ceutorhynchus assimilis* (Paykull) (Syn: *C. obstrictus*, Marsham) (Coleoptera: Curculionidae) is an oligophagous insect that feeds and develops on brassicaceous (= cruciferous) species. After overwintering in soil or leaf litter, the adults migrate in the spring to colonize flowering host

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plants and feed on pollen. Eggs are laid singly in young pods, and the larvae feed on the developing seeds, then leave the pods and pupate in the soil. New adults emerge in late summer and, after a short feeding period, disperse to overwintering sites. There is one generation per year (Bonnemaison, 1957).

C. assimilis is a pest of oilseed rape, *Brassica napus* L., in Europe (Bonnemaison, 1965; Winfield, 1992) and the Pacific Northwest, USA (Hill, 1991). Larval feeding reduces seed production. In addition, adult feeding and oviposition punctures in pods provide access points for another pest, the brassica pod midge, *Dasineura brassicae* (Sylvén, 1949), which is presently confined to Europe. The amount of damage caused by *C. assimilis* and the potential yield loss is variable because populations vary annually in both Europe (Winfield, 1992; Lane and Walters, 1994) and the United States (Brown et al., 1995). Weevil numbers should be monitored to determine when, and if, insecticide control is required.

Chemical stimuli are important in the orientation of phytophagous insects to host plants (Miller and Strickler, 1984), but visual stimuli, including silhouette, shape, and color, can also play a significant role (Prokopy and Owens, 1983; Miller and Strickler, 1984). Synergistic or additive interactions may occur between vision and odor (Todd et al., 1990; Judd and Borden, 1991; Green et al., 1994). Thus, design (e.g., shape and size), position, and color of traps, as well as chemical lures, must be considered when developing a semiochemical-based system for monitoring insects.

The response of *Ceutorhynchus assimilis* to brassicaceous plants and their odors has been investigated in field and laboratory studies. Field attraction to extracts of brassicaceous plants was first demonstrated by Görnitz (1953) and subsequently confirmed by Evans and Allen-Williams (1993). It was shown that weevils move upwind within the odor plume of a crop, probably by means of odor-mediated anemotaxis (Kjaer-Pedersen, 1992; Evans and Allen-Williams, 1993). In olfactometer studies, walking weevils also oriented to the odors of oilseed rape flowers and their extracts (Evans and Allen-Williams, 1989; Bartlet et al., 1993).

In early field studies (Ankersmit and van Nieukerken, 1954; Ankersmit, 1956; Dmoch, 1965), unbaited yellow shallow pan water traps (Moericke, 1953), placed on the ground, were used to trap seed weevils. More recently, unbaited water traps (e.g., Goos et al., 1976; Lechapt, 1980; Láska et al., 1986; Berger, 1987; Kocourek and Beránková, 1988; Šedivý and Kocourek, 1994) and unbaited sticky traps (Buechi, 1990; Büchs, 1993; Ekbom and Borg, 1993), mounted at crop height, have been used in monitoring or behavior studies. However, no comparisons of trap design were made in any of these studies.

The influence of trap color on *C. assimilis* catches has been investigated by several groups, but numbers trapped were often too low for statistical anal-

ysis. Goos et al. (1976) and Láska et al. (1986) trapped seed weevils in both yellow and white unbaited bowls. Buechi (1990) found that seed weevils preferred yellow to light green (turnip rape color) sticky traps. Dark green (rape color) sticky traps were the least attractive. In another study (Ekbom and Borg, 1993), yellow sticky traps caught 19 times more seed weevils than blue traps.

Many brassica-feeding insects perceive, and are attracted by, isothiocyanates (NCS) (Feeny et al., 1970, Finch and Skinner, 1982; Pivnick et al., 1991, 1994), which are produced by catabolism of glucosinolates. The latter are thioglucosides, characteristic of Brassicaceae (Fenwick et al., 1983). In *C. assimilis*, peripheral olfactory perception of isothiocyanates has been investigated with electroantennogram (Kozlowski, 1984; Evans and Allen-Williams, 1992; Blight et al., 1995) and single-cell (Blight et al., 1989, 1995) studies. By testing compounds at a physiologically discriminating level (Blight et al., 1989, 1995), it was shown that isothiocyanate perception is mediated by three types of olfactory cells that respond differentially to 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS. These cells, which comprised approximately one third of the total of recorded cells, also responded to allyl NCS, but only at unnaturally high concentrations.

Until recently (Blight et al., 1992), only allyl and phenyl NCS had been used in attempts to trap seed weevils. Finch (1977) reported that during the spring migration, allyl NCS-baited traps caught nine times as many seed weevils (mean of 183 per trap per day over three days) as unbaited water traps, where about 3 g/trap/day of allyl NCS were released (S. Finch, personal communication). However, using the same trap and lure release system, Free and Williams (1978) caught less than one weevil per trap per day in seedling spring rape adjacent to winter rape in May–June, and the number was not significantly different from the control. Lerin (1984) also found that the same trap baited with either allyl or phenyl NCS failed to catch significantly more weevils than unbaited traps in an experiment conducted during April.

Recently, walking weevils were shown to respond to a mixture of 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS in an olfactometer (Bartlet et al., 1993), and in initial field experiments (Blight et al., 1992; Smart et al., 1993), traps baited with a mixture of 3-butenyl, 4-pentenyl, 2-phenylethyl, and allyl NCS were attractive to *C. assimilis*.

In this paper we describe field-trapping experiments carried out to investigate aspects of the effects of visual cues (trap design and color) and isothiocyanate lures on attraction of *C. assimilis*. The aim was to find a convenient trap and lure that could be used to detect and monitor seed weevil populations. Water and sticky traps were directly compared, and trap color was examined in order to confirm that yellow is the most effective. Lures consisting of mixtures of isothiocyanates are described in detail, and the effectiveness of these during

the migratory and host colonization phases of the seed weevil is considered. The effect of allyl NCS, when released alone, was also examined, since its efficacy as an attractant was unclear from previous studies.

METHODS AND MATERIALS

Experimental Design. Five field experiments (A–E) were conducted on Rothamsted Farm, Harpenden, UK, during 1991–1992.

Randomized block (Latin-square) designs were used for all experiments. Traps in one replicate (i.e., row) of a block were set out in a straight line at 10-m spacing. Captured insects were removed at regular intervals and were identified and counted in the laboratory. Where feasible, blocks were rerandomized when a mean of at least five weevils per treatment had been captured in a replicate.

Analyses of variance (ANOVA) were performed on transformed total catch data. The transformation used was $x = \log_{10}(y + 1)$, where x and y were the transformed and untransformed counts, respectively. Following ANOVA, the transformed means were compared using Duncan's multiple range test (Duncan, 1955). These means were then transformed back, and these data are given in the Results (Tables 2–5, below).

Traps. Trap 1, a plastic bowl water trap (Just Plastics Ltd.; 21 cm diam. × 9 cm), was used in experiments A, D, and E (see Results). The bowls were brush painted with ICI Autocolour BS381/c P383 paints: canary yellow (BS0409), white (BS0101), black (BS0122), or grass green (BS0024). The traps were two-thirds filled with detergent solution (0.5% in water) and placed on wooden platforms. Lures were wired to a clear plastic 'cross bar', secured across the top of the bowl.

Trap 2, a Petri dish water trap (14 cm diam. × 1.7 cm), was used in experiments A and B. The dishes were painted canary yellow (as above) and filled with detergent solution (0.5% in water). Lures were wired above the trap to a piece of pipe that was glued to the center of the inside of the dish.

Trap 3, an open-ended sticky box trap (Smart et al., 1989) with sides measuring 10 × 20 cm (w × h), was used in experiment A. Yellow sticky cards (Oecos Ltd., 10 × 20 cm, coated with Oecotak A5) were clipped onto the four outer faces. The box was mounted over a post, and the lure was attached to the post above the box.

Trap type 4, an angled sticky card trap (20 × 10 cm), was used in experiment B. Each consisted of an oblong piece of grey plastic sheet (0.3-cm-thick ABS) with a 10-cm length of grey plastic pipe glued to the center of the lower surface of the sheet. The pipe was positioned such that each trap had one flat face that was either vertical, horizontal, or at an angle of 45° to the vertical

when the pipe was mounted on a metal post. A single yellow sticky card (10 × 20 cm) coated with Oecotak A5 was clipped to the flat face of each trap. The yellow faces of the 45°-angled and vertical traps pointed towards the (harvested) field. Lures were held in position above the traps with wire.

Traps angled at 45° were also used in experiment C. Single yellow or white sticky cards (10 × 20 cm) coated with either Oecotak A5 or Oecotak A10 adhesive were clipped to the flat face of each trap. They were positioned at the edge of a small area of flowering oilseed rape, with the colored faces inclined outwards from the rape.

All the traps were mounted on stakes or poles, approximately 1 m above ground level.

Chemicals. Nonane (99%), allyl NCS (94%), and 2-phenylethyl NCS (99%) were obtained from Aldrich Chemical Co. 3-Butenyl NCS (99%) and 4-pentenyl NCS (97%) were synthesized (Dawson et al., 1993).

Lures. Wick dispenser type 1, which was used in experiments A and B, consisted of a screw top glass vial (4 ml, Chromacol, code 4-SV) equipped with a pipe-cleaner wick, the tip of which just protruded through a hole drilled in the polyethylene screw cap. It was filled with a solution of allyl (18.5% w/w), 2-phenylethyl (1.85%), 3-butenyl (1.85%), and 4-pentenyl (1.85%) NCS in nonane. The release rates at 20°C were ca. 60 mg/day for allyl NCS and ca. 6 mg/day for each of the other three isothiocyanates. Nonane was dispensed alone as a control.

Polyethylene bag dispensers superseded the type 1 wick dispenser and were used in experiment D. Each isothiocyanate was applied to a piece of cellulose sponge that was sealed into a polyethylene bag. Separate bags were used for each compound. The quantities of chemical used, the sponge size, the gauge of the polyethylene and the release rates (at 20°C) are shown in Table 1.

TABLE 1. EXPERIMENT D: RELEASE RATES OF ISOTHIOCYANATES (NCS) FROM POLYETHYLENE BAGS

Compound (volume used, ml)[a]	Sponge[b] dimension (cm)	Gauge of polyethylene	Release (mg/day)
Allyl NCS (0.5)	2.5 × 2	1000	30
3-Butenyl NCS (0.1)	2.5 × 1	500	10
4-Pentenyl NCS (0.1)	2.5 × 1	250	10
2-Phenylethyl NCS (0.5)	2.5 × 2	100	6

[a]The compounds were sealed into individual polyethylene bags. Release rates were obtained by measuring weight loss after exposure in the field.

[b]Cellulose sponge (3 mm thick, J. Sainsbury plc, code S0865) was used.

Wick dispenser type 2 was used in experiment E to release allyl NCS in large amounts i.e., 470, 940, and 1880 mg/day. It consisted of a universal bottle (22 ml, 2.8 diam. × 8 cm) with a screw thread aluminum cap, through which a wick of woven cotton (0.5 cm diam.) protruded 0.5 cm. A hood made from a polyethylene cap prevented ingress of rain water. Release rates (at 20°C) are shown in Table 5 below.

RESULTS

Experiment A. The experiment was conducted in late summer on a site where oilseed rape had recently been harvested and from which weevils were dispersing. Two types of yellow water traps—a bowl and a Petri dish—were compared with a yellow sticky box trap. The traps were baited either with a mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS diluted in nonane, or with nonane alone. The chemicals were released from wick dispenser type 1.

The results are presented in Table 2. All three trap designs were equally effective, whether baited with the mixture of isothiocyanates or with nonane alone (factorial ANOVA, F statistic for trap type, $P = 0.40$). Traps baited with the isothiocyanate lure were more effective than the nonane-baited traps (F statistic for lure effect, $P < 0.001$), but the lure × trap type interaction was not significant (F statistic for interaction, $P = 0.56$).

Experiment B. This experiment was conducted at a similar time and site to experiment A. Yellow sticky card traps, mounted vertically, horizontally, or at

TABLE 2. EXPERIMENT A: *Ceutorhynchus assimilis* CAUGHT IN THREE TYPES OF YELLOW TRAP, BAITED WITH NONANE OR MIXTURE OF FOUR ISOTHIOCYANATES (NCS), AUGUST 11–19, 1991[a]

Trap	*C. assimilis* caught per replicate [mean (range)]	
	Nonane-baited	NCS baited[b]
Sticky box	6.2 (3–9)	21.6 (8–65)
Petri dish water trap	5.0 (1–16)	22.9 (10–176)
Bowl water trap	9.7 (3–17)	24.1 (12–41)

[a] A total of 707 weevils was caught in six replicates. Factorial ANOVA gave for trap effect: $F = 0.97$; $df = 2, 19$; $P = 0.40$; for lure effect: $F = 33.13$; $df = 1, 19$; $P < 0.001$. Means within each column were not significantly different. SE of a treatment mean was 12.1.

[b] The lure was a mixture of allyl, 3-butenyl, 4-pentenyl and 2-phenylethyl NCS. Allyl NCS was released at ca. 60 mg/day, and each of the other NCS at ca. 6 mg/day.

TABLE 3. EXPERIMENT B: *Ceutorhynchus assimilis* CAUGHT ON YELLOW STICKY CARD TRAPS INCLINED AT THREE DIFFERENT ANGLES AND BAITED WITH NONANE OR MIXTURE OF FOUR ISOTHIOCYANATES (NCS), AUGUST 13–SEPTEMBER 5, 1991[a]

Trap	*C. assimilis* caught per replicate [mean (range)]	
	Nonane-baited	NCS-baited[b]
Sticky card, vertical	5.8a (1–22)	21.1a (11–64)
Sticky card, 45° to vertical	4.8a (1–25)	9.7b (1–44)
Sticky card, horizontal	0.7b (0–2)	1.8c (0–4)
Petri dish water trap	3.8a (0–15)	13.4ab (3–43)

[a] A total of 645 weevils was caught in eight replicates. Factorial ANOVA gave for trap effect: $F = 22.49$; $df = 3, 42$; $P < 0.001$; for lure effect: $F = 28.84$; $df = 1, 42$; $P < 0.001$. Means within a column followed by different letters are significantly different (Duncan's multiple range test, $P = 0.05$). SE of a treatment mean was 3.2.

[b] The lure was a mixture of allyl, 3-butenyl, 4-pentenyl and 2-phenylethyl NCS. Allyl NCS was released at ca. 60 mg/day, and each of the other NCS at ca. 6 mg/day.

45° to the vertical were compared to the yellow Petri dish water trap. The traps were baited with the same lures as used in experiment A.

The results are presented in Table 3. There were large differences in the efficiencies of the traps (factorial ANOVA, F statistic for trap type, $P < 0.001$). Of the nonane-baited traps, the Petri dish and the sticky cards mounted vertically or at 45° were equally effective, but the horizontal sticky card caught negligible numbers of weevils. When baited with the isothiocyanate mixture, the Petri dish was again as attractive as the vertical or 45°-angled sticky traps, but the vertical sticky card was more attractive than the angled card (Duncan's multiple range test, $P = 0.05$). The isothiocyanate-baited horizontal sticky card trap remained ineffective.

The catch in each NCS-baited trap compared to the corresponding nonane-baited trap was enhanced two to four times in all instances (F statistic for lure effect, $P < 0.001$), but the lure × trap type interaction was not significant (F statistic for interaction, $P = 0.36$).

Experiment C. This test was carried out in summer alongside flowering oilseed rape, when seed weevils were colonizing the crop. The effectiveness of unbaited, yellow, and white sticky card traps, inclined at 45° and coated with either Oecotak A5 or Oecotak A10, were compared. (The two adhesives were designed to work over slightly different temperature ranges.)

Of the total of 452 weevils trapped in the four replicates of the experiment, 444 weevils (98.2%) were caught on the yellow traps. (ANOVA gave for color

effect: $F = 214.53$; $df = 1, 6$; $P < 0.001$). The two adhesives were equally effective (for adhesive effect: $F = 0.44$, $df = 1, 6$; $P = 0.53$).

Experiment D. This experiment also was sited alongside flowering oilseed rape in summer. The attraction to yellow, white, green, and black painted water bowls, either unbaited or baited with an isothiocyanate mixture, was compared. The lure consisted of a mixture of the same isothiocyanates as used in experiments A and B, but the compounds were released (more conveniently) from polyethylene bags and nonane was absent as diluent. There were differences in the release rates—the 3-butenyl and 4-pentenyl NCS components were released at 10 mg/day, compared to 6 mg/day in A and B, and release of allyl NCS was halved to 30 mg/day.

Results are displayed in Table 4. A total of 218 weevils was captured in eight replicates, but it was not possible to do a valid ANOVA because the distribution among the replicates was too uneven. However, clear results were obtained. Only the yellow bowls, which captured 208 weevils (95.4% of the total), were attractive. The addition of the isothiocyanate bait did not increase the numbers of weevils trapped in any of the colored bowls.

Experiment E. The test was conducted alongside flowering oilseed rape in mid-summer during the colonization phase. Yellow water bowl traps were used to investigate the effect of releasing large amounts of allyl NCS. The isothiocyanate was dispensed from wick dispenser type 2, with nonane as a diluent where appropriate. None of the allyl NCS-baited traps (Table 5) caught significantly more weevils than the nonane or blank traps (F statistic for treatment effect, $P = 0.50$).

TABLE 4. EXPERIMENT D: *Ceutorhynchus assimilis* CAUGHT IN COLORED BOWL WATER TRAPS UNBAITED OR BAITED WITH A MIXTURE OF FOUR ISOTHIOCYANATES (NCS), JUNE 12–JULY 8, 1992

	C. assimilis caught per replicate [mean (range)][a]	
Trap color	Unbaited	NCS-baited[b]
Yellow	5.4 (0–68)	5.8 (1–62)
White	0.0	0.2 (0–1)
Green	0.2 (0–1)	0.1 (0–1)
Black	0.1 (0–1)	0.2 (0–2)

[a]In eight replicates 218 weevils were caught, but the distribution among the replicates was too uneven to do a valid ANOVA.

[b]The lure was a mixture of allyl, 3-butenyl, 4-pentenyl and 2-phenylethyl NCS (see Table 1 for details).

TABLE 5. EXPERIMENT E: *Ceutorhynchus assimilis* CAUGHT IN YELLOW WATER TRAPS BAITED WITH LURES RELEASING LARGE AMOUNTS OF ALLYL ISOTHIOCYANATE (NCS), JUNE 12–JULY 2, 1992

Bait	Nominal release rate (mg/day) of allyl NCS	*C. assimilis* caught per replicate [mean (range)][a]
Blank (nonane)	—	4.6 (0–13)
Allyl NCS		
100%	1880	6.2 (1–13)
50% in nonane	940	7.3 (3–15)
25% in nonane	470	6.3 (2–12)
Control (no treatment)	—	4.6 (0–15)

[a]Traps caught 184 weevils in five replicates. There were no significant differences between any of the treatments (ANOVA gave $F = 0.90$; $df = 4, 24$; $P = 0.50$). SE of a treatment mean was 0.08.

DISCUSSION

Ceutorhynchus assimilis is a variable, unpredictable pest, for which a convenient, reliable monitoring system, incorporating traps, is required. The experiments reported here were part of a series carried out between 1991 and 1995 to examine the responses of *C. assimilis* (and pollen beetles, *Meligethes* spp., data not reported) to visual and odor cues, with the aim of developing an effective baited trap.

No information has been available on the comparative efficiences of various trap designs for the capture of *C. assimilis*. In some preliminary experiments (Smart and Blight, unpublished), we compared water and sticky traps with the boll weevil scout cone trap (Forey and Quisumbing, 1987), a delta trap (normally used to capture Lepidoptera), and a funnel trap similar to the Lindgren trap (Lindgren, 1983). The cone, delta, and funnel traps all caught negligible numbers of seed weevils and pollen beetles, as did water bowl traps placed on the ground, but the results of experiments A and B, reported here, suggested that yellow water traps and sticky traps, placed at crop height, are equally effective. Although water traps may be preferred for research, based on convenience, sticky traps are more practical for use in detection and monitoring and have been used successfully with other insect species (Muirhead-Thomson, 1991). The small sticky card trap (experiments B, C) was effective and simple to use.

When sticky cards mounted at three different angles were compared, the horizontal card was ineffective for catching seed weevils. This also applied to pollen beetles (Smart et al., 1993). In contrast, the horizontal Petri dish water

trap was effective, but this had a vertical yellow edge and yellow underside. These results were obtained in the late summer when the new generations were emerging from the soil at the experimental site, and the attractive yellow color on the upper surface of the horizontal card was not visible to insects on the ground. In the spring, when both species are dispersing from overwintering sites to rape fields, horizontal cards catch pollen beetles effectively (Smart and Blight, unpublished) and may therefore also be attractive to seed weevils at this time. An attempt was made to confirm this, but a conclusion was precluded because of low weevil numbers. However, because pollen beetles were trapped preferentially, in both spring and late summer, on 45°-angled compared to vertical traps (Blight and Smart, in preparation), the 45° trap configuration appears to be the best compromise for catching both species throughout the year. In an earlier study (Finch and Collier, 1989), four dipteran species, including the brassicaceous pests *Delia floralis* (turnip root fly) and *D. radicum* (cabbage root fly), were also caught efficiently on 45°-inclined, upwards facing traps.

It has been suggested (Prokopy and Owens, 1983) that a positive response to yellow is characteristic of most herbivorous insects, although there are exceptions to this (Kirk, 1984; Glinwood et al., 1993). Results from four earlier studies involving *C. assimilis* (see Introduction) indicated that only yellow, white, and light green (turnip rape color) traps attracted significant numbers of seed weevils. We found that only yellow was effective. However, attraction to a particular color may be influenced by the surroundings (Kirk, 1984), so that in some situations white and light green traps could be attractive.

In two experiments (A, B), the capture of seed weevils in yellow traps was increased two- to fourfold by adding a bait consisting of a mixture of 3-butenyl, 4-pentenyl, 2-phenylethyl, and allyl NCS. This was observed in the late summer when the new generation of weevils was dispersing. However, in another experiment (D), conducted in mid-summer when the weevils were colonizing the crop, a combined isothiocyanate bait of the same components, but with different release rates, was not effective. From our experience of the effects of various release rates on capture of oilseed rape pests, we do not believe that release rates were a factor in these results. Rather, the data indicate that seed weevils do not show a behavioral response to isothiocyanates during the colonization phase. This has now been confirmed in other field experiments (manuscript in preparation).

With some insect species, e.g., the onion fly, *Delia antiqua* (Judd and Borden, 1991), and the western corn rootworm, *Diabrotica virgifera virgifera* (Hesler and Sutter, 1993), synergistic interactions have been observed between visual (trap color and design) and semiochemical cues. This was not observed with *C. assimilis*. Addition of the isothiocyanate lure to traps of unsuitable design (experiment B and unpublished results) had no effect on numbers of weevils trapped, suggesting that visual cues are of primary importance in ori-

entation of this insect. However, it is also possible that visual and olfactory cues operate over different distances, as has been shown with the onion fly (Judd and Borden, 1991).

The failure of *C. assimilis* antennal cells to respond to allyl NCS when the compound was tested at a physiologically discriminating dose (Blight et al., 1989, 1995) could indicate that the original wild brassicaceous hosts did not contain sinigrin, the parent glucosinolate of allyl NCS. The isothiocyanate was included in the combined bait because there was some evidence in the literature that it attracts seed weevils when it is released in gram amounts from baited traps (Finch, 1977). However, in the present work, it was unattractive when released alone, in large amounts, during the colonization phase of the insect. This is in accord with the results of Free and Williams (1978) and Lerin (1984), who also must have been studying weevil populations during the colonization phase.

The results reported in this paper suggest that an isothiocyanate bait on a yellow sticky card trap, inclined at 45°, can be used to monitor *C. assimilis* during its dispersive phases. Experimental monitoring of seed weevils (and pollen beetles), which is in progress (Smart et al., 1996), has confirmed this. Although isothiocyanate baits do not attract seed weevils during the colonization phase, some other semiochemicals do. Details will be reported in another paper (Smart and Blight, submitted).

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LOCALIZATION AND MORPHOLOGY OF SEX PHEROMONE GLANDS IN SCARAB BEETLES

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Abstract—The sex pheromone glands of female *Anomala albopilosa albopilosa* have been localized by extracting various parts of the body and analyzing the extracts by GC-MS and by histological and morphological studies. Female-specific epithelial cells line the inner surfaces of anal plates and two apical sternites; these cells are connected through many pores to the cuticle surface. The sex pheromones of other rutelines, *A. cuprea* and *Popillia japonica* and a melolonthine species, *Heptophylla picea*, have been also detected in the extracts from the same abdominal parts. On the other hand, the source of *Holotrichia parallela* sex pheromone was confirmed to be a ball-shaped abdominal sac that is exposed during the calling behavior, and no trace of the semiochemicals has been found in the anal plates and two apical sternites.

Key Words—Pheromone gland, *Anomala albopilosa albopilosa*, *Anomala cuprea*, *Popillia japonica*, *Holotrichia parallela*, *Heptophylla picea*, Coleoptera, Scarabaeidae

INTRODUCTION

Scarab beetles in the genera *Holotrichia* and *Phyllophaga* (Scarabaeidae: Melolonthinae) display a typical calling behavior by extruding the tip part of the abdomen, exposing a ball-shaped sac (Leal et al., 1993b). The sex pheromones of a few species have been identified thus far from whole extracts of these abdominal tips (Leal et al., 1992, 1993b, 1996c; Leal, 1995, 1996; Zhang et al., 1997). Interestingly, the chemistry of the pheromones of these scarab species

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is remarkably different from that of beetles in the subfamily Rutelinae (Leal, 1995, 1996). While the former utilize amino acid derivatives, terpenoids, anisole, and other phenolic compounds, the sex pheromones of the latter are likely fatty acid derivatives, namely, 2-(*E*)-nonenol, methyl (*Z*)-5-tetradecenoate and a family of unusual lactones, 5-alkenyl-dihydrofuranones. Recently, the sex pheromone of a melolonthine, *Heptophylla picea*, has been identified as a possible fatty acid derivative, (*R,Z*)-7,15-hexadecadien-4-olide (Leal et al., 1996b). It would be interesting from an evolutionary viewpoint to get a better understanding of pheromone production in these subfamilies.

Hitherto, biosynthesis of scarab beetle sex pheromones remained a matter of speculation because, among other factors, little was known about the pheromone-producing glands in rutelines. In order to provide a basis for research on the biosynthesis of pheromones in these scarab beetles, we have conducted a study on the localization and morphology of pheromone glands in scarab beetles.

METHODS AND MATERIALS

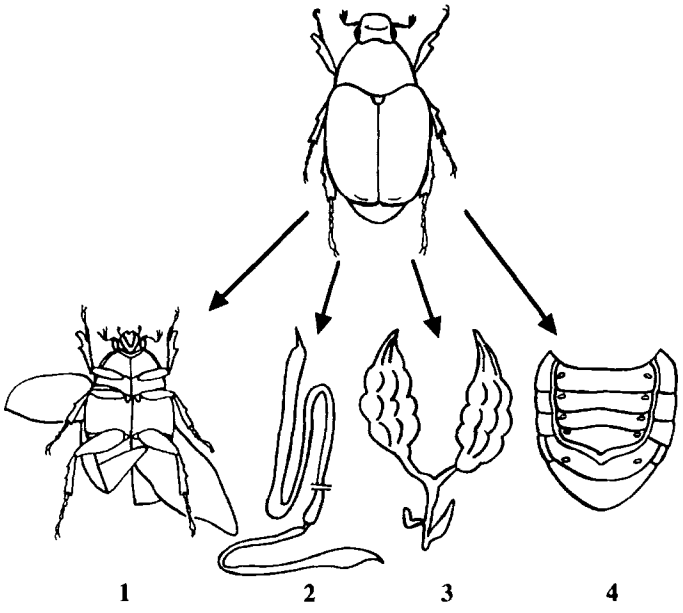
Insects. Eggs laid by a laboratory colony of *A. a. albopilosa* (Leal et al., 1996a) were transferred to wet sand in cups kept at 25°C. After hatching, grubs were transferred individually to ice cream cups filled with moist leaf mold and kept under a 16L:8D photoperiod (photophase 06:00–22:00 hr). Newly eclosed adults were separated individually and were also kept at 16L:8D in ice cream cups with lids (upper and lower diameters: 9 and 7 cm; height: 5 cm) and provided with an artificial diet (Leal, 1991) and moist peat moss. Test insects (two to three weeks after eclosion) were transferred to cups provided only with wet tissue paper 10 hr before they were dissected. The other scarab species utilized in this work were field-collected.

Protocol for Localizaiton of Putative Zones Involved in Sex Pheromone Emission. A. a. albopilosa adults were anesthesized with ether and dissected at the first half-hour of the scotophase (22:00–22:30 hr), when the highest amount of sex pheromone is released (Leal et al., 1996a).

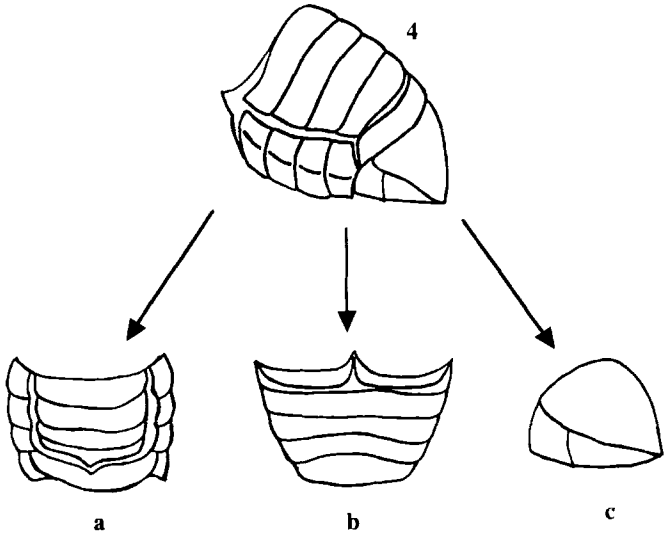
In preliminary tests, two females per experiment were dissected into four parts: (1) head, thorax, legs, and wings; (2) digestive system; (3) reproductive system, and (4) other abdominal tissues (Scheme 1). Subsequently, the abdomens of two females were further separated into three parts: (a) dorsal cuticle; (b) ventral cuticle, and (c) remaining abdominal tip (Scheme 2).

Each part of the specimen was dipped in hexane for 3 min, filtered, and concentrated to ca. 5 μl. The occurrence of the major sex pheromone constituent, buibuilactone (Leal et al., 1996a), was analyzed by GC-MS.

Beetles of the other species investigated were dissected at the time of peak



SCHEME 1.



SCHEME 2.

of pheromone production, extracted with hexane (ether for *H. parallela*), and the extracts were analyzed by GC-MS.

Analytical Procedures. Gas chromatography—mass spectrometry (GC-MS) was carried out either on an HP 5890 II Plus gas chromatograph linked to a mass selective detector MSD 5972 (Hewlett-Packard), a Mass Engine 5989B, or on a Hewlett-Packard gas chromatograph electron ionization detector GCD Series. Chromatographic resolution was achieved on HP-5MS capillary columns (30 m × 0.25 mm × 0.25 μm; Hewlett-Packard) BP20 (25 m × 0.25 mm × 0.25 μm; SGE). These columns were operated at 70°C for 1 min, temperature increased to 260°C at a rate of 10°C/min, and held at this temperature for 10 min.

Histological Studies. Soon after extraction, dorsal cuticle, ventral cuticle, and abdominal tip were fixed in Bouin's alcoholic solution for one day, cleared in xylene, and embedded in paraffin wax (mp 56°C). Sagittal and transverse sections (10 μm thick) were prepared and stained with Delafield's hematoxylin and counterstained with eosin Y. After histological analysis of the beetles used in the localization protocol, another group of males and females was also analyzed without any extraction procedure in order to preserve the cuticular wax layer.

SEM Observations. For observations on a scanning electron microscope (SEM), the abdominal tips of both male and female *A. a. albopilosa* were fixed in 70% ethanol and dehydrated in an ethanol series and acetone. After critical-point drying and sputter-coating with gold, the tips were examined on a JSM 6301 F SEM (Jeol, Tokyo, Japan).

RESULTS AND DISCUSSION

Localization of Sex Pheromone Glands. Preliminary extractions showed that the major sex pheromone constituent for *A. a. albopilosa*, buibuilactone (Leal et al., 1996a), was detected only in the abdominal tissues. Further separation of these abdominal tissues demonstrated that the putative sex pheromone glands were localized in the abdominal tip. The amount of buibuilactone obtained in the extract from abdominal tips was very high (ca. 52 ng/female), whereas trace amounts of the major sex pheromone constituents were detected in the dorsal (3.4 ng/female) and ventral (4.5 ng/female) parts.

Morphology and Histology of Pheromone Gland. One layer of secretory-type epithelial cells was observed in the inner surface of both anal plate and two apical sternites in *A. a. albopilosa* females, whereas male beetles lacked these structures. These epithelial cells are likely to release their volatile products through pores that appear only in the cuticular layer of female beetles adjacent to these glandular cells (Figure 1). The cuticle of both male and female beetles

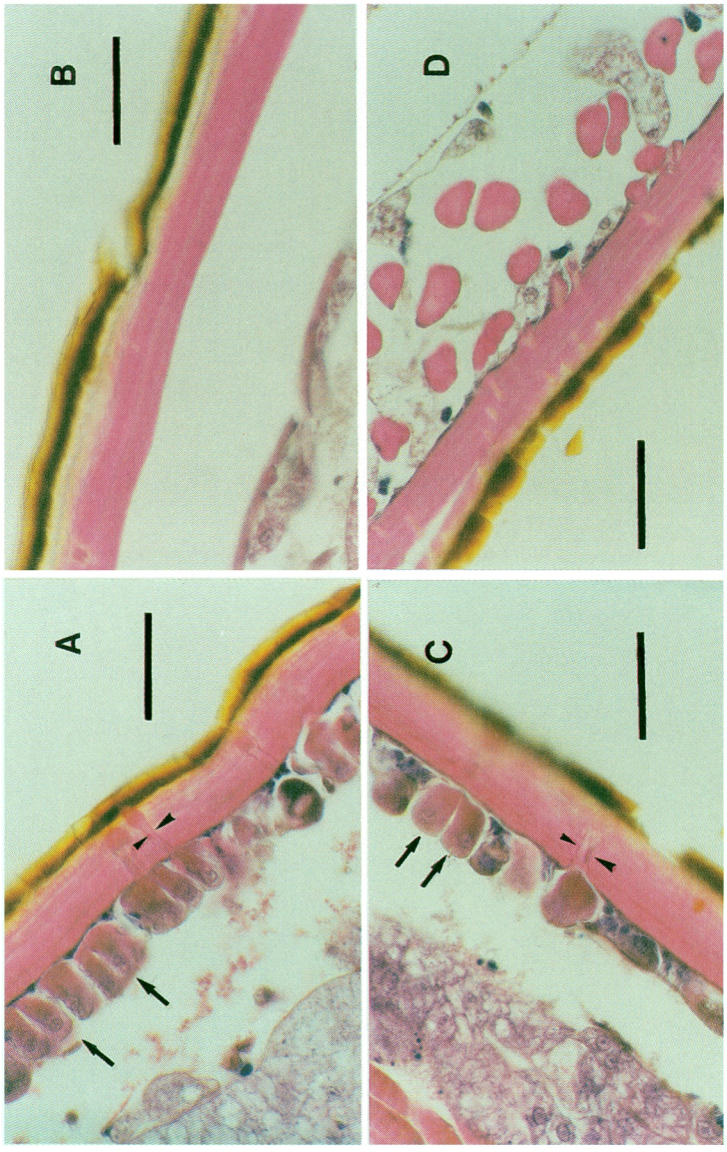


FIG. 1. Sections of abdominal tip of adult *A. a. albopilosa*. Arrows indicate epidermal (pheromonal) cells and arrowheads show ducts. Anal plates: transverse section in a female (A) and sagittal section in a male (B). Apical sternites: transverse section in a female (C) and sagittal section in a male (D). Bar = 50 μm.

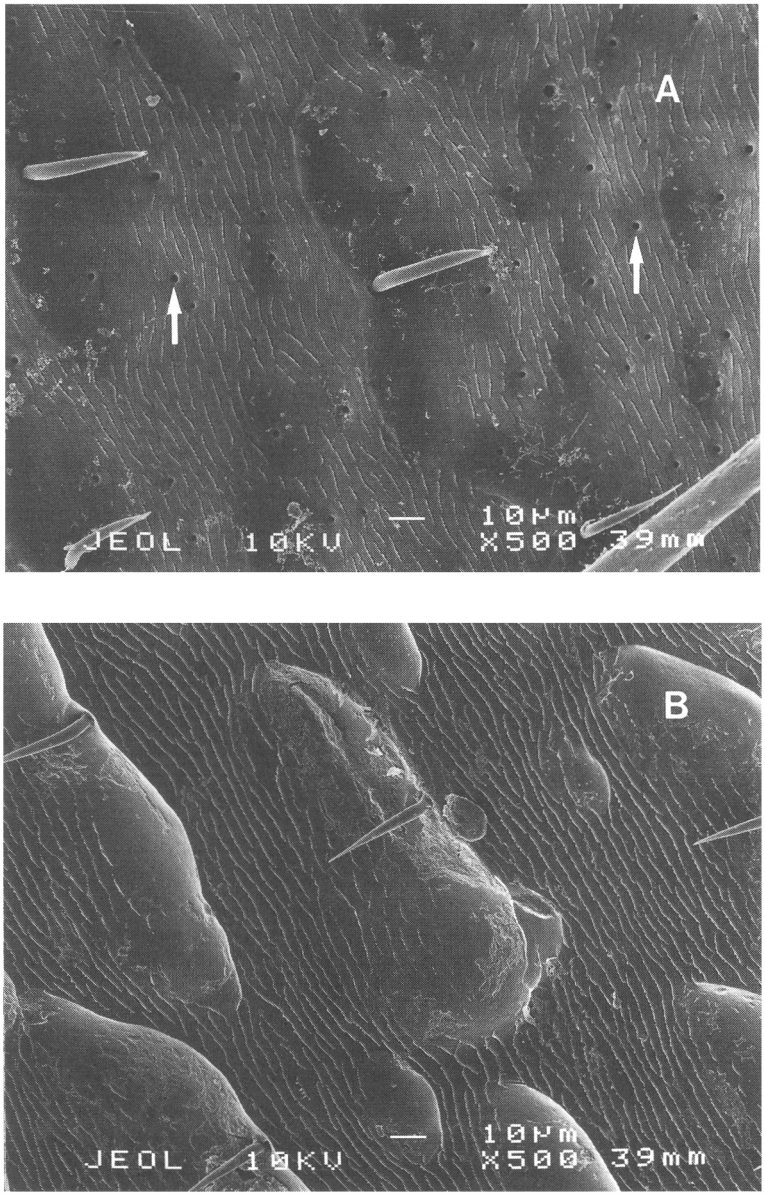
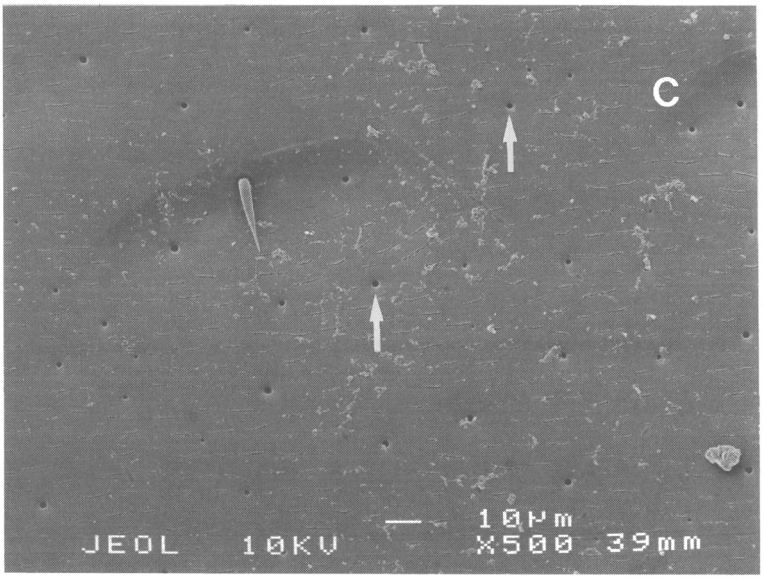


FIG. 2. Scanning electron micrographs of abdominal tip surfaces of adult *A. a. albopilosa*. Arrow indicates porelike structures. Anal plate surfaces of female (A) and male (B) and sternite surfaces of female (C) and male (D).



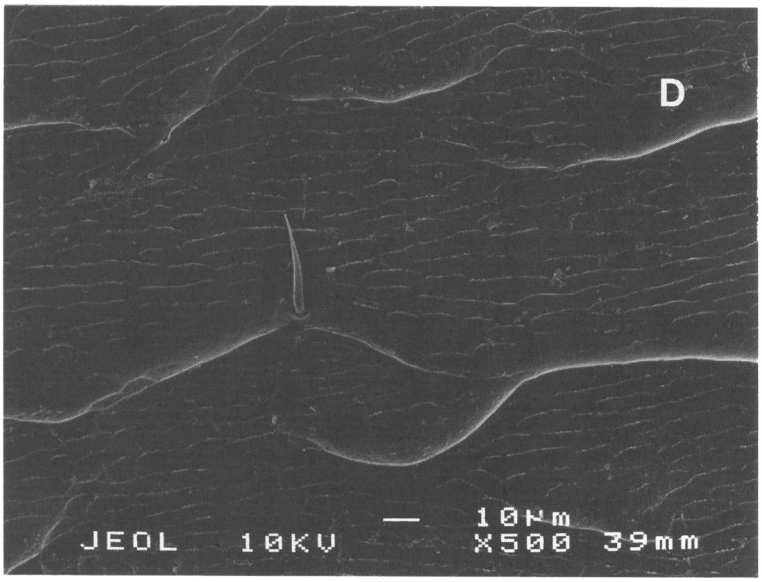


FIG. 2. Continued.

were covered by a brown wax layer (ca. 12 μm); this layer was removed by hexane utilized in the localization protocol.

SEM obsrvations showed porelike structures scattered on the anal plate (Figure 2a), and the two apical sternites (Figure 2c). Similar structures were absent in male anal plate (Figure 2b) and apical sternites (Figure 2d). Both the diameter (1–2 μm) and intervals between these openings were consistent with those observed by light microscope (Figure 3).

The detection of a sex pheromone constituent from female abdominal tips, combined with the observation of specialized secretory epithelium on the inner surface of this sclerite, provides sufficient evidence to conclude that this epithelium is the site of sex pheromone production in *A. a. albopilosa*. The cells were distributed on the inner surface of the anal plate and the two apical sternites of female beetles (Figure 3) and connected with the outside via many pores in the cuticle.

Extraction of the two apical sternites and anal plates of other species suggested that the localization of the pheromone glands may be a common feature in species belonging to the subfamily Rutelinae. The sex pheromone constituents of *A. cuprea* (Figure 4), *A. a. sakishimana*, and *Popillia japonica* were detected in extracts of the abdominal tip of females, but not from male beetles.

Interestingly, *Holotrichia* and *Phyllophaga* species (Melolonthinae) display a typical calling behavior by extruding the tip part of the abdomen and displaying a ball-shaped sac (Leal et al., 1992; Leal, 1996). The sex pheromone of *H. parallela* and a few *Phyllophaga* species have been identified from extracts of the whole abdominal tip (Leal et al., 1992, 1993b; Leal, unpublished data).

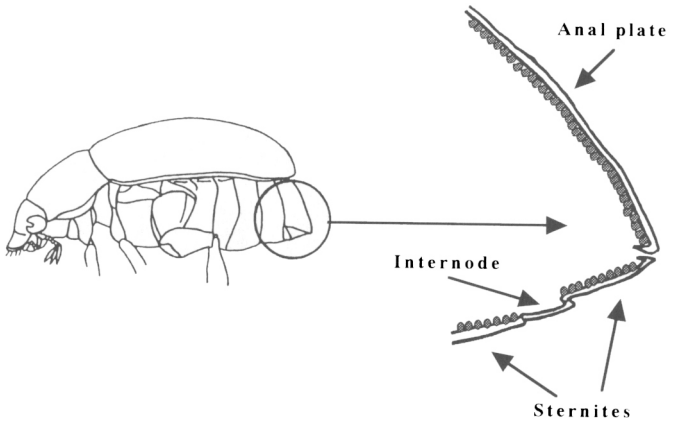


FIG. 3. Diagrammatic representation of abdominal tip of adult *A. a. albopilosa* showing the location of pheromone glands.

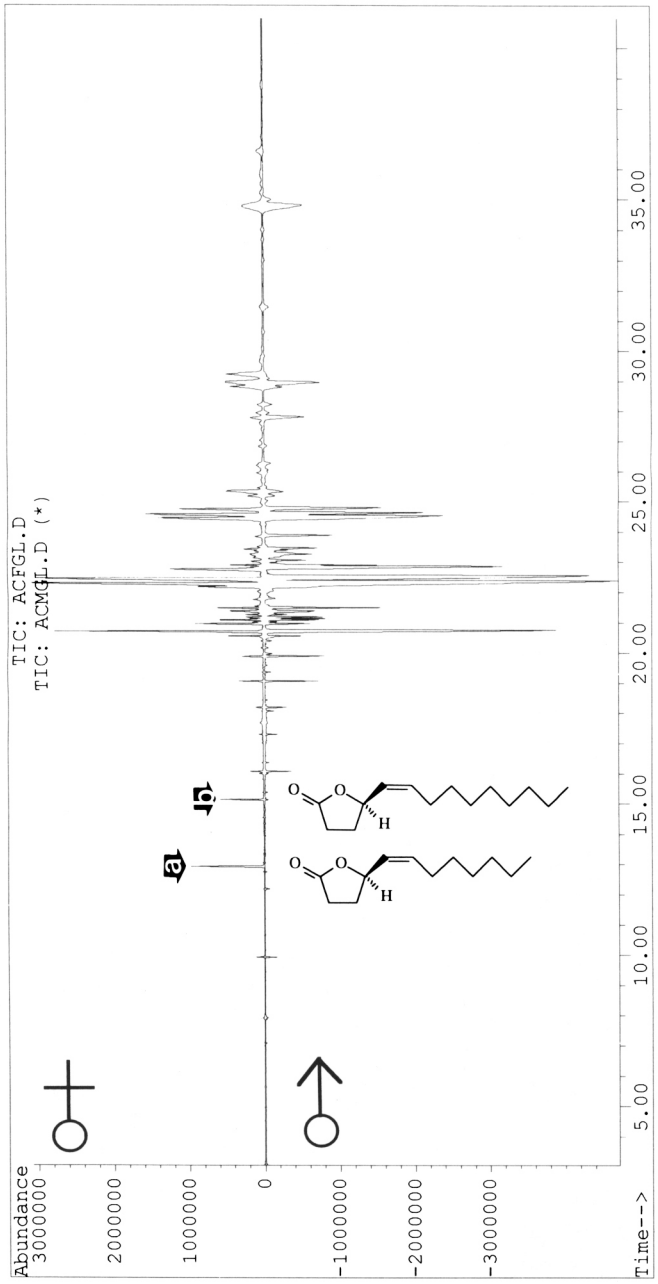


FIG. 4. Reconstructed total ion monitor profile of the abdominal tip extracts from *A. cuprea* female (top) and male (bottom). The two sex pheromone constituents, buibuilactone (a) and japonilure (b) (Leal et al., 1993a), were detected in the female extract. The minute peak in the male extract corresponding in retention time to japonilure (b) is due to a contaminant (phthalate ester).

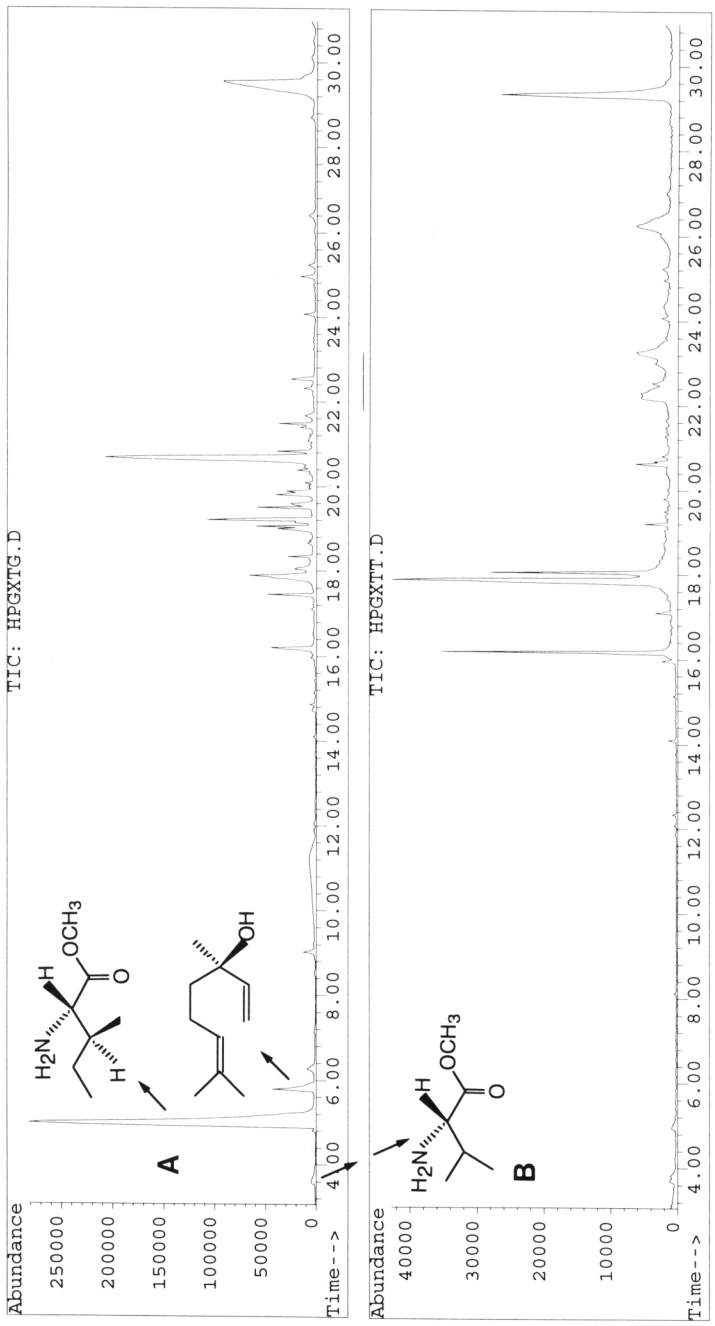


FIG. 5. Profiles of ether extracts (0.2 female-equivalent) from one single *H. parallela* female. (A) Abdominal sac and (B) anal plate plus the two apical sternites. The two sex pheromone constitutents, L-isoleucine methyl ester (5.01 min) and (*R*)-(−)-linalool (5.80 min), were detected only in the abdominal sac.

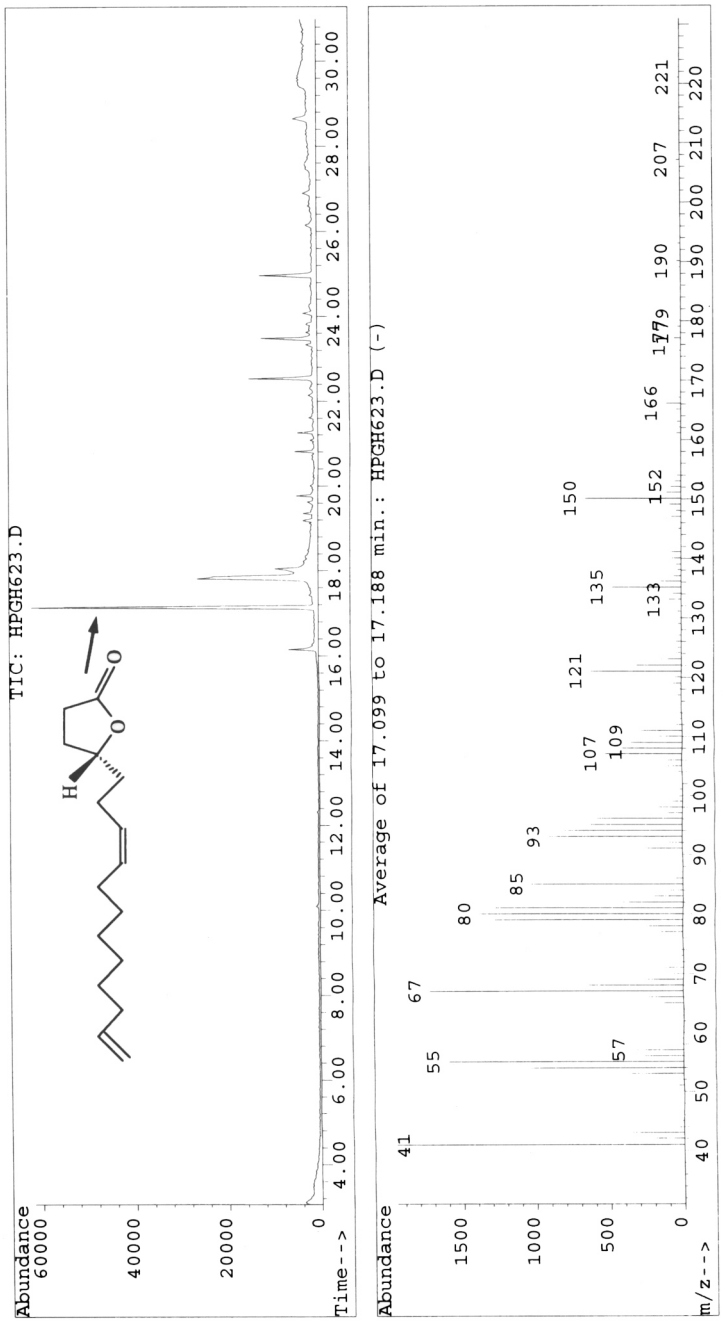


FIG. 6. Total ion monitor profile of the extract from abdominal tip (anal plate and the two apical sternites) of female *H. picea* and mass spectrum of the sex pheromone constituent (*R*,*Z*)-7,15-hexadecadien-4-olide that appeared at 17.15 min.

Reexamination of the pheromone gland localization in *H. parallela* showed that only the abdominal sac possesses the two sex pheromone constituents. Not even traces of the semiochemicals have been obtained from the extracts of the anal plate and the two apical sternites (Figure 5).

The sex pheromone glands of *Holotrichia* and *Phyllophaga* species are thus remarkably different from the epidermal pheromonal cells of rutelines with respect to localization as well as morphology. The chemical diversity of the sex pheromone in the two subfamilies Rutelinae and Melolonthinae is also remarkable (Leal, 1995, 1996). Recently, we have identified a sex pheromone for the yellowish elongate chafer, *Heptophylla picea* (Melolonthinae) (Leal et al., 1996b). This species displays a calling behavior similar to that of *Holotrichia* and *Phyllophaga* species, except for the fact that they do not display the abdominal tip. Intriguingly, the pheromone constituent of this melolonthine, (*R*,*Z*)-7,15-hexadecadien-4-olide, is likely to be a fatty acid derivative, a characteristic of the ruteline sex pheromones. In addition, the pheromone constituent was detected only in the anal plate and the two apical sternites of females (Figure 6), but not in the abdominal tip, which is similar to that of *H. parallela*. Thus, *H. picea* has some biological and biochemical features similar to those of species belonging to the Rutelinae. It would be interesting to address the question regarding the classification of *H. picea* in the Melolonthinae by studying the molecular phylogeny of these two subfamilies.

It has also been reported that dung beetles (Scarabaeidae: Scarabaeinae) have integumentary glands on the inner surface of sternites (Plout-Sigwalt, 1982, 1986). It was suggested that these cells release some vesicle substance via many pores on these cuticles. Tribe (1975) also indicated that *Kheper* dung beetles release pheromone components from pores located on their sternites, although in both cases the involvement of these cells in pheromone production has never been demonstrated. It is worth mentioning that the secretory glands in *A. a. albopilosa* differed from the putative pheromone glands of dung beetles in the localization as well as the morphology.

On the other hand, structures similar to the one we found in *A. a. albopilosa* have been described as sex pheromone glands of *Trogoderma* species (Coleoptera: Dermestidae). Secretory cells were located in the inner surface of the 7th abdominal sternite of females, but only on the ventral side (Hammack et al., 1973).

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RESPONSES OF *Dasineura brassicae* AND ITS PARASITOIDS *Platygaster subuliformis* AND *Omphale clypealis* TO FIELD TRAPS BAITED WITH ORGANIC ISOTHIOCYANATES

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Abstract—The responses of *Dasineura brassicae* and its parasitoids *Platygaster subuliformis* and *Omphale clypealis* to allyl and 2-phenylethyl isothiocyanates have been investigated using a new design of trap in winter oilseed rape. Traps baited with allyl isothiocyanate caught more male and female *D. brassicae* and more female *O. clypealis* than traps baited with 2-phenylethyl isothiocyanate or unbaited traps, whereas traps baited with 2-phenylethyl isothiocyanate caught more male and female *Platygaster subuliformis* than traps baited with allyl isothiocyanate or unbaited traps. The implications of these results for host-plant and oviposition-site location by *D. brassicae* and for host habitat and host location by the parasitoids are discussed, as is the potential for using these responses in integrated pest management strategies.

Key Words—*Dasineura brassicae, Platygaster subuliformis, Omphale clypealis,* parasitoids, pest management, oilseed rape, *Brassica napus,* Brassicaceae, glucosinolates, isothiocyanates, 2-phenylethyl isothiocyanate, allyl isothiocyanate, host location.

INTRODUCTION

Dasineura brassicae Winnertz (Diptera: Cecidomyiidae), the brassica pod midge, is an important summer pest of crops belonging to the family Brassicaceae (=

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Cruciferae), such as oilseed rape (*Brassica napus* L.), in Europe (Bromand, 1990; Alford et al., 1991). It is cited as being attacked by over 20 species of hymenopteran parasitoids, among them species of the genus *Platygaster* (Hymenoptera: Platygastridae) and *Omphale clypealis* (Thomson) (Hymenoptera: Eulophidae) (Williams and Walton, 1990; Alford et al., 1995).

Host location in phytophagous insects is mediated by a combination of visual and olfactory cues (Visser, 1986) and secondary plant chemicals provide orientation and feeding cues for specialized insect feeders, which are often specific to a restricted range of plants (Feeny, 1983). Parasitoids also often use plant-derived chemicals to locate host habitats (Vinson, 1981). The family Brassicaceae is characterized by secondary plant chemicals called glucosinolates (GS), which are stored in parenchymous tissues (Larsen, 1981); at least 16 aliphatic, aromatic, and indolylglucosinolates commonly occur in oilseed rape (Fieldsend and Milford, 1994). In damaged tissue, the enzyme myrosinase (a thioglucosidase) initiates catabolism of many glucosinolates to isothiocyanates (NCS) and sometimes to other compounds such as nitriles, oxazolinidethiones, thiocyanates, or cyanoepithioalkanes, depending on glucosinolate type and conditions (Fenwick et al., 1983). At least 20 species of specialist crucifer-feeding insects utilize glucosinolates or their volatile metabolites in host-plant location or recognition (Feeny, 1983; Bartlet, 1995).

This study investigated the responses of *D. brassicae* and its parasitoids, *P. subuliformis* and *O. clypealis*, to allyl NCS, which is a catabolite of allyl GS (2-propenyl), and 2-phenylethyl NCS, which is a catabolite of 2-phenylethyl GS (gluconasturtiin), using a new design of trap.

The 2-phenylethyl GS is present in the vegetative tissues of oilseed rape (Fieldsend and Milford, 1994), and 2-phenylethyl NCS is a component of the volatile profile from the plant and is perceived by a number of oilseed rape pests (Blight et al., 1992, 1995a). 2-allyl GS, although present in many Brassicaceae and in older cultivars of oilseed rape, is not present in the newer double-low cultivars (Milford et al., 1989) and consequently allyl NCS is not released from them. *Dasineura brassicae* has been reported to respond to 2-allyl GS in an olfactometer (Pettersson, 1976), which is surprising as glucosinolates are not very volatile, and males but not females are attracted to traps releasing allyl NCS (Lerin, 1984; Evans, 1991). The responses of the parasitoids of *D. brassicae* to isothiocyanates have not been investigated previously although allyl NCS is known to elicit responses from the parasitoids of other cruciferous pests (Read et al., 1970; Titayavan and Altieri, 1990; Pickett et al., 1991; Pivnick, 1993).

METHODS AND MATERIALS

Experimental Design. Traps, baited with allyl NCS or 2-phenylethyl NCS, at either a high or low release rate, or unbaited, were placed in winter oilseed

rape (cv. Falcon) at Rothamsted Farm, Hertfordshire, UK, from June 17 until August 5, 1994, when the crop was at growth stages 6, 2 to 6, 5, respectively (following Sylvester-Bradley, 1985). Twenty-five traps (five replicates of each treatment) were arranged in a 5 × 5 Latin square with a minimum distance of 10 m between traps and emptied weekly. Catches were stored in fresh 70% methanol and *D. brassicae, P. subuliformis* sp., and *O. clypealis* counted.

Traps. Selective flight traps, designed to catch small flying insects in alcohol, were used for this study because the sorting of water traps is time-consuming and small insects quickly deteriorate in them. A basic design, derived from Wilkening et al. (1981), was extensively modified to dispense volatiles in a crop environment. The trap (Figure 1) had three main parts: the fins (A), the funnel unit (B), and the collecting jar (C). The fins consisted of four sheets of polyvinyl chloride (PVC) hot-air welded onto PVC tubing and painted yellow (undercoat Dulux Off White, Imperial Chemical Industries plc, and Saffron Yellow gloss, Johnstone's Paints plc). The funnel unit was attached to the fins by two self-tapping screws. The collecting jar was partially filled with 70% methanol and connected to the funnel unit by screwing the universal tube into its cap. The trap was mounted on steel tubing attached to a wooden post. Its height was adjusted, to keep the top of the fins level with the top of the crop canopy, by sliding it up or down the tubing to which it was secured by tightening the nylon screw.

Dispensers. The dispensers used in this study were similar to those being developed for use in an NCS-baited monitoring trap for the oilseed rape pests, *Ceutorhynchus assimilis* Paykull (Coleoptera: Curculionidae), the cabbage seed weevil, and *Meligethes aeneus* Fabricius (Coleoptera: Nitidulidae), the pollen beetle (Smart et al., 1993). Each dispenser consisted of a cellulose sponge impregnated with the isothiocyanate (allyl NCS, 94% pure, from Lancaster Synthesis Ltd., or 2-phenylethyl NCS, 98% pure, from Avocado Research Chemicals Ltd.) sealed in a polyethylene bag; the dispenser was mounted inside the funnel unit slightly above the height of the fins. Each compound was tested at two release rates, obtained by using different sizes of sponge and/or different thicknesses of polyethylene bag. Rates were estimated in the laboratory over a 16-day period, at 15–25°C. Nominal release rates were: allyl NCS low release rate 12–13 mg/day, high release rate 40–53 mg/day; 2-phenylethyl NCS low release rate 1.4–2.0 mg/day, high release rate 10–13 mg/day. Because of their greater release rates, the allyl NCS dispensers were replaced after four weeks, whereas the 2-phenylethyl NCS dispensers were left unchanged throughout the experiment.

Data Analysis. Data were analyzed using a Poisson regression, adjusting for heterogeneity (McCullagh and Nelder, 1989), using Genstat 5 (Genstat 5 Committee, 1993). Overall treatment differences were assessed using an F test as the residual mean deviance was greater than 1, indicating departure from Poisson assumptions. Where the overall F test was significant ($P < 0.05$), three

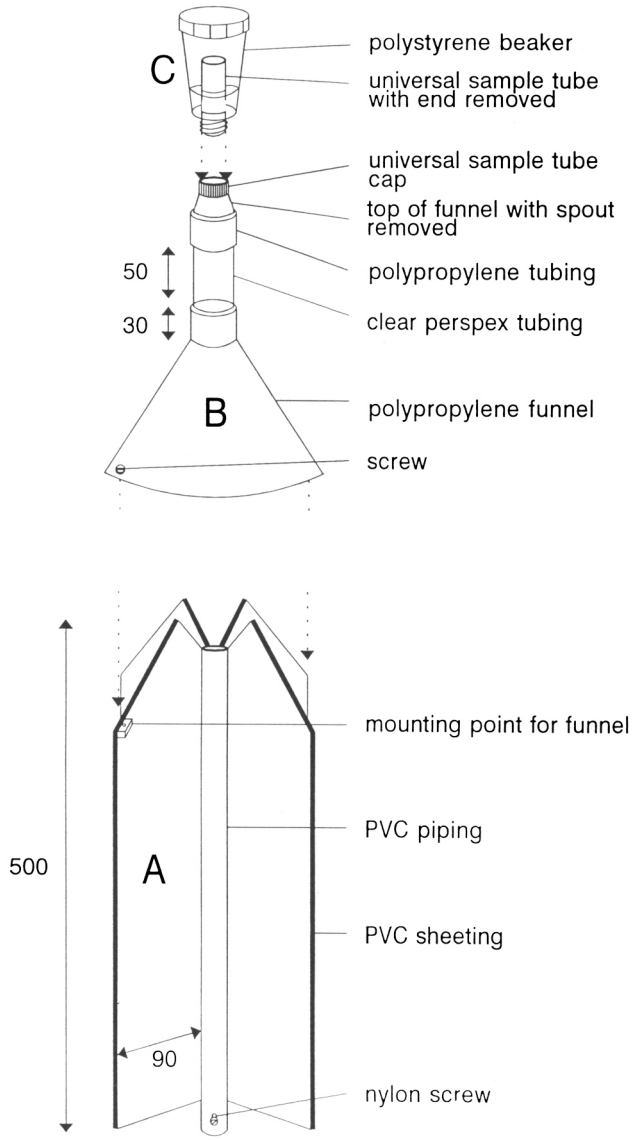

FIG. 1. Diagram showing construction of the flight trap (sizes in millimeters, not to scale).

orthogonal contrasts representing differences between the treatment means for allyl NCS vs. 2-phenylethyl NCS vs. control, allyl NCS high vs. allyl NCS low, and 2-phenylethyl NCS high vs. 2-phenylethyl NCS low were determined.

RESULTS

The traps caught *D. brassicae* and two of its parasitoids, *P. subuliformis* and *O. clypealis*. The *Platygaster* species reared from *D. brassicae* cocoons taken from this site were confirmed not to be *Platygaster oebalus* Walker, *Platygaster iolas* Walker, or *Platygaster nitida* Thomson, the three *Platygaster* species cited as parasitoids of *D. brassicae* (Williams and Walton, 1990), but *P. subuliformis* (identified by Peter Buhl; confirmed by Andrew Polaszek), probably a new species record for the UK (A. Polaszek, personal communication).

D. brassicae (*Table 1*). A total of 1354 male but only 10 female *D. brassicae* were caught in the traps during seven weeks; none were caught in the first two weeks and maximum numbers of males (838) were caught during week 6 (July 22–29). Differences between treatments were significant for males for each week that males were caught but were significant for females for the total period only. Traps baited with allyl NCS caught significantly more males and females than traps baited with 2-phenylethyl NCS or unbaited traps (male: $F = 405$; $df = 2,20$; $P < 0.001$; female: $F = 7.79$; $df = 2,20$; $P < 0.01$). Traps baited

TABLE 1. MEAN (±SE) NUMBERS OF *Dasineura brassicae, Platygaster subuliformis*, AND *Omphale clypealis* CAUGHT PER TRAP[a]

	Allyl NCS		2-Phenylethyl NCS			
Sex	High	Low	High	Low	Unbaited	F^b
D. brassicae						
Male	46.2 ± 2.96	223.0 ± 19.14	0.6 ± 0.40	0.4 ± 0.20	0.6 ± 0.40	256.55***
Female	0.6 ± 0.40	1.2 ± 0.49	0.2 ± 0.20	0	0	4.68**
P. subuliformis						
Male	8.4 ± 1.60	5.0 ± 1.30	341.2 ± 55.42	102.4 ± 40.35	5.6 ± 2.66	68.32***
Female	0.8 ± 0.37	0.4 ± 0.40	43.0 ± 6.14	8.0 ± 1.22	0.2 ± 0.20	76.99***
O. clypealis						
Male	0.4 ± 0.40	1.4 ± 0.73	0.2 ± 0.20	0.2 ± 0.20	0.2 ± 0.20	0.81 ns
Female	3.2 ± 1.07	7.2 ± 1.32	3.8 ± 1.74	3.0 ± 0.84	0.8 ± 0.60	2.42 ns

[a] $N = 5$/treatment. Traps were unbaited or baited with an isothiocyanate released at a high or low rate (June 17–August 5, 1994).
[b] Critical values for significance of tabulated F. $df = 4, 20$: **4.43 at $P < 0.01$, ***7.10 at $P < 0.001$.

with a low dose of allyl NCS caught significantly more male *D. brassicae* than those baited with a high dose of allyl NCS in all weeks except July 15–22, the week after the dispeners were replaced with new ones ($F = 216$; $df = 1,20$; $P < 0.001$).

P. subuliformis (*Table 1*). A total of 2313 male and 262 female *P. subuliformis* were caught during the seven weeks; few (1 male, 1 female) were caught during the first two weeks and maximum numbers (988 males, 128 females) were caught during week 4 (July 8–15). Differences between treatments were significant for both males and females for weeks 3–7 and overall. Traps baited with 2-phenylethyl NCS caught significantly more males and females than traps baited with allyl NCS or unbaited traps in all weeks (male: $F = 114$; $df = 2,20$; $P < 0.001$; female: $F = 116$; $df = 2,20$; $P < 0.001$). Traps baited with a high dose of 2-phenylethyl NCS caught significantly more males and females than traps baited with a low dose in most weeks and overall (male: $F = 45.7$; $df = 1,20$; $P < 0.001$; female: $F = 74.7$; $df = 1,20$; $P < 0.001$).

O. clypealis (*Table 1*). A total of 12 male and 90 female *O. clypealis* were caught in the traps during the seven weeks. None were caught during the first two weeks and maximum numbers (10 males, 64 females) were caught during weeks 6 and 7 (July 22–August 5). Differences between treatments (F) were significant only for females and only in week 7. During that week, traps baited with allyl NCS caught significantly more females than traps baited with 2-phenylethyl NCS or unbaited traps ($F = 6.7$; $df = 2,20$; $P < 0.01$). Low-dose allyl NCS traps caught significantly more females than high-dose allyl NCS traps ($F = 12.1$, $df = 1,20$; $P < 0.01$).

DISCUSSION

In this study, two parasitoids of *D. brassicae*, like the pest itself, respond to secondary plant chemicals from cruciferous plants in the field when these are presented in traps. This suggests that these chemicals may be used as cues to help these insects locate their hosts. Allyl NCS attracted male and, to a lesser extent, female *D. brassicae* and female *O. clypealis* whereas 2-phenylethyl NCS attracted both male and female *P. subuliformis*. The response of male *D. brassicae* to allyl NCS has been reported previously (Lerin, 1984; Evans, 1991) but this is the first time that females, albeit in small numbers, appear to respond to this chemical. It is also the first time that these parasitoids have been shown to respond to isothiocyanates and that any parasitoid has been shown to respond to isothiocyanates other than allyl NCS. The finding that species differ in their responses to different isothiocyanates concurs with previous studies of the responses of other cruciferous insects (Pivnick et al., 1992; Blight et al., 1995b).

The strong response of male *D. brassicae* to allyl NCS is surprising as mating can occur at emergence sites without cruciferous plants (Williams et al.,

1987) and is mediated by a female-produced sex pheromone (Williams and Martin, 1986; Isidoro et al., 1992). However, local movements of males towards sources of isothiocyanates could increase their chances of coming within range of calling females; in the parasitoid *Campoletis sonorensis*, males are inefficient at locating females, which also produce a sex pheromone, unless the host plant is also present (MacAuslane et al., 1990). The much greater numbers of male than female *D. brassicae* in traps reflects the greater flight activity of males in search of females for mating, and agrees with previous studies with other traps (Williams et al., 1987). Mated female *D. brassicae* may orient towards isothiocyanates to locate their cruciferous host plants for oviposition; for example, where crops are grown in rotation, they migrate from overwintering sites to cruciferous crops such as oilseed rape or mustard. Mated females need to locate areas of the pod wall of their host plants that have been punctured by *C. assimilis*, or damaged in some other way, through which to oviposit, and may orient towards locally high concentrations of isothiocyanates from the damaged tissues. The parasitoids are probably orienting towards isothiocyanates in a similar way to locate their host habitats, thereby increasing the chances of males finding females for mating and females locating eggs/larvae in which to oviposit. Some parasitoids are known to respond to odors released by the feeding activity of their hosts (Godfray, 1994). More behavioral work is needed to define the roles of these chemicals in the ecology of these insects.

Air entrainment extracts of oilseed rape contain over 50 different volatiles, including 3-butenyl, 4-pentenyl, 2-phenylethyl, and *sec*-butyl NCS (Tollsten and Bergstrom, 1988; Evans and Allen-Williams, 1992; Blight et al., 1992, 1995b), and some of the nitriles, terpenes, fatty acid derivatives, and aromatic compounds are known to attract rape pests (Bartlet, 1995). This study investigated the responses to two isothiocyanates only, selected because they have elicited responses from other cruciferous insects in the field and are readily available.

As nothing was known about the release rate to which these insects respond, two rates of each isothiocyanate were tested to give more information. The responses to the two different rates appeared to be dose dependent. *P. subuliformis* was attracted in greater numbers to the high release rate of 2-phenylethyl NCS whereas *D. brassicae* and, to a lesser extent, *O. clypealis* responded preferentially to the low release rate of allyl NCS. However, observations on differential response to the doses of allyl NCS may not be real.

In laboratory tests, the release rate of 2-phenylethyl NCS, by diffusion through polyethylene, did not vary much with changes in temperature, whereas the release of allyl NCS, for the same system, particularly at the high rate, was much more variable. We have calculated that, before the appearance of *D. brassicae* and *O. clypealis* in the field, the high-release allyl NCS dispenser was prematurely depleted due to the unexpectedly high temperature within the trap. During week 5, immediately after the allyl NCS dispensers were replaced, there

was no significant difference in the number of *D. brassicae* caught. This suggests that the lower overall catches in the high-release allyl NCS traps were not due to repellency. More *P. subuliformis* may have oriented to the higher rather than to the lower release of 2-phenylethyl NCS because the former provided a greater attraction to more chemical or a greater catch area than the latter.

The numbers of insects caught in the traps reflected their phenology and density on the crop; second-generation *D. brassicae* adults emerge from late June until early August, which probably explains why none were caught during the first two weeks of trapping. The life-histories of the parasitoids are poorly known; *P. oebalus* in Poland has three generations closely coinciding with those of *D. brassicae* (Czajkowska, 1978a,b) although emerging slightly earlier. The number of generations in *O. clypealis* is uncertain but probably more than one (J. Axelson, personal communication).

At present, there is no monitoring system for *D. brassicae* in oilseed rape. The decision to apply insecticide to control *D. brassicae* is based on the level of infestation by the seed weevil, *C. assimilis* and previous history of *D. brassicae* attack on the farm (Alford et al., 1991); it takes no account of the natural control that can be exerted by the parasitoids of the pest or the effect of pesticide application on their populations (Alford et al., 1995). Traps of this design baited with the appropriate isothiocyanate could perhaps be used to selectively monitor *D. brassicae, P. subuliformis,* and *O. clypealis* directly, as part of a more refined integrated pest management strategy that incorporates natural control by parasitoids.

The finding that these parasitoids respond to isothiocyanates may also lead to ways in which field parasitization rates could be enhanced by manipulation of the chemical environment of the crop. For instance, parasitism of the cabbage aphid, *Brevicoryne brassicae*, by *Diaeretiella rapae*, has been increased by application of an emulsion of allyl NCS to broccoli plants (Titayavan and Altieri, 1990).

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CUTICULAR HYDROCARBONS OF TERMITES OF THE BRITISH VIRGIN ISLANDS

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Abstract—A survey of the termites (Isoptera) of 17 islands of the British Virgin Island (BVI) complex yielded eight taxa belonging to three families. The Kalotermitidae include *Neotermes mona* (Banks), *Cryptotermes brevis* (Walker), *Procryptotermes corniceps* (Snyder), and an undetermined species of *Incisitermes*, likely *Incisitermes* nr *snyderi* (Light) or *I. incisus* (Silvestri). The only rhinotermitid collected is an undetermined species of *Heterotermes* (Froggatt). *Parvitermes wolcotti* (Snyder), *Nasutitermes costalis* (Holmgren), and *N. acajutlae* (Holmgren) comprise the Termitidae. Cuticular hydrocarbon mixtures were characterized for each of the taxa. Blends of abundant hydrocarbons are species-specific and can be used to identify a given taxon without the diagnostic castes, soldiers, or imagoes, although the species of *Incisitermes* were not separable on the basis of cuticular hydrocarbons.

Key Words—Cuticular hydrocarbons, chemotaxonomy, Isoptera, tropical termites, gas chromatography, mass spectrometry, Virgin Islands, Caribbean termites, olefins, methylalkanes.

INTRODUCTION

The termite fauna of the West Indies was summarized first by Banks (1919), who described termites collected from the larger islands, except Puerto Rico

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and other islands of the Puerto Rico Bank. Individual collections in the West Indies placed in the US National Museum were included in Snyder's compilation (Snyder, 1956). Scheffrahn et al. (1994) summarized the literature and unpublished records of the termites of the West Indies. From this survey it is clear that, until recently, little effort has been devoted to collecting the fauna of the Virgin Island complex, especially the British Virgin Islands (BVI).

The BVI are a complex of more than 50 land masses that are part of the Puerto Rico Bank. The BVI were apparently not intensively collected until M. S. Collins began systematic work in 1986 (Collins et al., 1997). In this paper we expand this work to include documentation of the cuticular hydrocarbon mixture of all termite taxa collected from the BVI. Characterization of the cuticular hydrocarbons of each taxon supports the species specificity of hydrocarbon mixtures for this region.

METHODS AND MATERIALS

Collection of Termites. Collecting periods of two to four weeks each were spent on Guana Island from 1986 to 1994, most often during the month of October. During those stays, short trips were made to other islands of the BVI complex. We attempted to sample termite colonies from every habitat that could be reached. Termite samples were bagged and brought to the laboratory on Guana Island where the termites were separated from soil, nest, and wood debris.

Samples of workers, soldiers, larvae, pseudergates, nymphs, or alates were placed in separate dishes or vials and dried. The number of termites in a sample varied by species; 15–20 nymphs or pseudergates of kalotermitids or up to 200 workers of the nasutes were dried. These samples were placed over a single incandescent light. Initially samples were dried in whatever vessel was available over whatever lamp was available in the guest cottages on Guana Island. From 1991 to 1994, we dried termite samples in 20-ml scintillation vials over a single 75-W, reflecting incandescent light. Vials were moved periodically in an attempt to make drying uniform (Haverty et al., 1996).

The amount of time required to completely dry termites varied slightly as a function of the number and size of the termites in the sample and the position of the vials over the bulb. With some of the kalotermitid species, drying was accelerated by decapitating termites. Internal hydrocarbons do not appear to affect characterization of cuticular hydrocarbons (Haverty et al., 1996). Once the termites were completely dry, specimens were placed in a vial that was tightly capped. Dried samples were returned to our laboratory in California for extraction and characterization of cuticular hydrocarbons. Concurrently, fresh (i.e., not dried) voucher samples from each collection were preserved in 85%

ethanol and deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC.

Species diagnoses were made primarily by M. S. Collins using keys, original references and descriptions, and by comparison with type and previously identified material. Much work needs to be done to develop usable keys for the Caribbean fauna, and new descriptions are needed for some species.

Extraction Procedure and Characterization of Cuticular Hydrocarbons. In this study cuticular lipids were extracted by immersing termites, as a group, in 10 ml of *n*-hexane for 10 min. After extraction, hydrocarbons were separated from other components by pipetting the extract through 4 cm of activated BioSil-A in Pasteur pipet mini-columns. An additional 5 ml of clean hexane was dripped through the BioSil-A. The resulting hydrocarbon extracts were evaporated to dryness under a stream of nitrogen and redissolved in 60 μl of *n*-hexane for gas chromatography—mass spectrometry (GC-MS) analyses. A 3-μl aliquot was injected into the GC-MS.

GS-MS analyses were performed on a Hewlett-Packard (HP) 5890 gas chromatograph equipped with a HP 5970B Mass Selective Detector interfaced with a computer and HP Chemstation data analysis software (HP59974J Rev. 3.1.2). The GC-MS was equipped with an HP-1, fused silica capillary column (25 m $\times$ 0.2 mm ID) and operated in split mode (with a split ratio of 8 : 1). Each mixture was analyzed by a temperature program from 200°C to 320°C at 3°C/min with a final hold of 11 or 16 min. Electron impact (EI) mass spectra were obtained at 70 eV.

n-Alkanes were identified by their mass spectra. Mass spectra of methylalkanes were interpreted as described by Blomquist et al. (1987) to identify methyl branch locations. Mass spectra of di- and trimethylalkanes were interpreted as described in Page et al. (1990) and Pomonis et al. (1980). Alkenes were identified by their mass spectra and/or retention times relative to *n*-alkanes. A typical alkene mass spectrum shows a molecular ion and a series of fragments at 14-mass-unit intervals (69, 83, 97), similar to those displayed by *n*-alkanes, less 2 mass units. Interpretation of the mass spectra of dienes and polyunsaturated hydrocarbons was extrapolated from this pattern, i.e., for each double bond, the molecular ion is decreased by 2 mass units.

Integration of the total ion chromatogram was performed using the HP Chemstation data analysis software. GC-MS peak areas were converted to percentage of the total hydrocarbon fraction. Summary statistics for percentages of each hydrocarbon for each taxon or geographic location of a taxon were computed using SAS (1990) to make comparisons.

In the text and tables, we use shorthand nomenclature to identify individual hydrocarbons or mixtures of hydrocarbons. This shorthand uses a descriptor for the location of methyl groups (X-Me), the total number of carbons (C_{XX}) in the hydrocarbon component excluding the methyl branch(es), and the number of

double bonds following a colon ($C_{XX:Y}$). Thus, pentacosane becomes *n*-C_{25}; 3-methylpentacosane becomes 3-MeC_{25}; 3,13-dimethylpentacosane becomes 3,13-DimeC_{25}; and pentacosadiene becomes $C_{25:2}$. Hydrocarbons are presented in the tables for each taxon in the order of elution on our GC-MS system.

RESULTS AND DISCUSSION

In their survey of the termites of the West Indies, Scheffrahn et al. (1994) listed a total of nine species in seven genera and three families found in the BVI. We characterized cuticular hydrocarbons for all of these species, although we were unable to differentiate *Incisitermes incisus* (Silvestri) and *I. snyderi* (Light) (or *I.* nr *snydreri*). Most of the specimens used for these analyses were collected on Guana Island, and incidentally from many other islands in the BVI complex (Table 1). Whenever possible, we used collections from locations in addition to Guana Island to include interisland variation. We summarize the relative proportions of each hydrocarbon for eight taxa; hydrocarbons are presented in order of elution within a hydrocarbon class (Table 2). Hydrocarbon mixtures for pseudergates or workers are discussed for each taxon within the three families. Comparison of castes within a taxon or island-to-island variation are presented separately for select taxa.

TABLE 1. SPECIES OF TERMITES COLLECTED FROM VARIOUS LOCATIONS IN BRITISH VIRGIN ISLANDS FOR CHARACTERIZATION OF CUTICULAR HYDROCARBONS

Species	Collection sites
Neotermes mona	Guana
Cryptotermes brevis	Oahu, Hawaii[a]
Procryptotermes corniceps	Guana, Lesser Jost Van Dyke, Great Camino, Great Thatch
Incisitermes species	Guana, Lesser Jost Van Dyke, Greater Jost Van Dyke, Eustatia, Scrub, Anegada
Heterotermes sp.	Guana, Tortola, Great Thatch
Parvitermes wolcotti	Peter
Nasutitermes costalis	Guana, Tortola
Nasutitermes acajutlae	Guana, Great Camino, Scrub, Eustatia, Virgin Gorda, Lesser Jost Van Dyke, Greater Jost Van Dyke, Great Thatch, Cooper, Necker, Tortola

[a] *Cryptotermes brevis* occurs only in structures. We were not able to collect a sample from a building or furniture. We received these from a colleague in Hawaii, where this species is quite common.

TABLE 2. RELATIVE QUANTITIES OF CUTICULAR HYDROCARBONS FROM PSEUDERGATES (LARVAE AND NYMPHS) OR WORKERS OF 8 TERMITE TAXA FROM BRITISH VIRGIN ISLANDS[a]

Hydrocarbon	Termite species[b]							
	N mon	C bre	P cor	I spp[c]	Het sp	P wol	N cos	N aca
n-Alkanes								
C_{23}	0	tr	0	0/+	0	0	0	+
C_{24}	tr	0	tr	tr/+	0	0	0	tr
C_{25}	+++	+++	+++	+++	0	0	++	+++
C_{26}	+++	++	++	++	tr	0	0	tr
C_{27}	+++	+++	+++	+++	+++	+++	++	++
C_{28}	tr	+	tr	0/+	++	++	+	tr
C_{29}	+	++	+	+	++	++	++	+
C_{30}	0	0	0	0	tr	0	0	0
C_{31}	0	tr	0	0	tr	0	0	tr
Internally branched methylalkanes								
12-; 11-; 10-MeC_{24}	+	0	0	0	0	0	0	0
13-; 11-MeC_{25}	+++	+	0	+	0	0	0	tr
13-; 12-MeC_{26}	+++	0	0	0	0	0	0	0
13-; 11-; 9-; 7-MeC_{27}	+++	tr	tr	0/tr	+++	0	+	tr
14-; 13-; 12-; 9-; 7-MeC_{28}	+	0	0	0/tr	+++	0	+	0
15-; 13-; 11-; 9-; 7-; 5-MeC_{29}	tr	0	0	tr/+	+++	+	+++	0
15-; 14-; 12-; 11-; 10-; 9-MeC_{30}	0	0	0	0	+	0	+	0
15-; 13-; 11-; 9-MeC_{31}	tr	0	0	0	+	0	+++	0
14-MeC_{32}	0	0	0	0	0	0	+	0
13-MeC_{33}	tr	0	0	0	0	0	+	0
15-; 13-MeC_{35}	tr	0	0	0/tr	0	0	0	0
12-MeC_{36}	tr	0	0	0	0	0	0	0

TABLE 2. Continued

Hydrocarbon	Termite species[b]							
	N mon	C bre	P cor	I spp[c]	Het sp	P wol	N cos	N aca
Internally branched methylalkanes (Continued)								
17-; 15-; 13-MeC_{37}	tr	tr	0	0/tr	tr	0	0	0
12-MeC_{38}	tr	0	0	0	0	0	0	0
15-; 13-MeC_{39}	+	tr	+	0/+	0	0	0	tr
12-MeC_{40}	+	0	0	0	0	0	0	0
15-; 13-C_{41}	++	tr	+	0	0	0	0	tr
12-MeC_{42}	tr	0	0	0	0	0	0	0
13-MeC_{43}	+	0	0	0	0	0	0	0
Terminally branched methylalkanes								
2-MeC_{23}	0	+	0	0/tr	0	0	0	0
3-MeC_{23}	0	++	0	0	0	0	0	0
2-MeC_{24}	++	+++	+++	0/+++	0	0	0	0
3-MeC_{24}	tr	+	tr	0	0	0	0	0
2-MeC_{25}	+++	++	+++	+++	0	0	0	0
3-MeC_{25}	+++	+++	+++	+++	0	0	0	0
2-MeC_{26}	++	+	+++	+/++	+	0	0	0
3-MeC_{26}	+	tr	tr	0/tr	tr	0	0	0
2-MeC_{27}	0	tr	+	tr/+	++	++	0	tr
3-MeC_{27}	+	+	++	0/+	++	+	tr	0
2-MeC_{28}	0	0	tr	0	+	+++	0	0
2-MeC_{29}	0	tr	0	0	0	++	0	0
3-MeC_{29}	0	0	0	0	0	++	tr	0
Dimethylalkanes								
3,X-$DimeC_{25}$	++	tr	0	0	0	0	0	0
11,15-$DimeC_{27}$	++	0	0	0	+++	0	++	0
9,17-$DimeC_{27}$	0	0	0	0	+++	0	0	0

5,15-DimeC_{27}	tr	0	0	0	0	0	0	0
3,13-DimeC_{27}	tr	0	0	0	0	0	0	0
11,15-; 12,16-DimeC_{28}	tr	0	0	0	0	0	++	0
9,X-DimeC_{28}	0	0	0	0	+++	0	0	0
13,17-; 11,15-DimeC_{29}	0	0	0	0	0	0	+++	0
9,19-; 9,17-DimeC_{29}	0	0	0	0/+	+++	0	0	0
7,21-DimeC_{29}	0	0	0	0	+++	0	0	0
5,X-DimeC_{29}	tr	0	0	0	tr	++	0	0
3,21-DimeC_{29}	0	0	0	0	tr	0	0	0
11,15-; 12,16-; 13,17-DimeC_{30}	0	0	0	0	0	0	+++	0
9,X-DimeC_{30}	0	0	0	0	++	0	0	0
13,17-DimeC_{31}	0	0	0	0	0	0	+++	0
9,21-DimeC_{31}	0	0	0	0	++	0	0	0
3,X-DimeC_{31}	tr	tr	0	0	0	0	0	0
14,18-DimeC_{32}	0	0	0	0	0	0	+	0
11,21-; 13,21-; 15,19-DimeC_{33}	0	0	0	0/tr	0	0	+	0
13,X-; 11,23-DimeC_{35}	tr	0	0	0/+	0	0	0	0
12,24-DimeC_{36}	0	0	0	0/tr	0	0	0	0
17,X-; 15,X-; 13,X-DimeC_{37}	+	0	0	0/+	0	0	0	0
12,16-DimeC_{38}	tr	0	0	0	0	0	0	0
11,15-DimeC_{39}	++	0	0	0	0	0	0	0
12,16-DimeC_{40}	tr	0	0	0	0	0	0	0
13,17-DimeC_{41}	++	0	0	0	0	0	0	0
12,16-DimeC_{42}	tr	0	0	0	0	0	0	0
13,17-DimeC_{43}	+	0	0	0	0	0	0	0
Trimethylalkanes								
9,13,17-TrimeC_{27}	0	0	0	0	0	0	+	0
11,15,19-TriMeC_{29}	0	0	0	0	0	0	+	0
7,13,21-TrimeC_{29}	0	0	0	0	tr	0	0	0
5,11,21-TrimeC_{29}	0	0	0	0	tr	0	0	0
3,11,21-TrimeC_{29}	0	0	0	0	tr	0	0	0
13,17,21-; 11,15,21-TrimeC_{33}	0	0	0	0/tr	0	0	0	0
13,17,23-; 11,15,19-TrimeC_{35}	0	0	0	0/tr	0	0	0	0
13,17,21-TrimeC_{37}	0	0	0	0/tr	0	0	0	0

TABLE 2. Continued

Hydrocarbon	Termite species[b]							
	N mon	C bre	P cor	I spp[c]	Het sp	P wol	N cos	N aca
Olefins								
$C_{23:1}$	0	0	0	0	0	0	0	+
$C_{24:1}$	0	0	0	tr	0	0	0	0
$C_{25:1}$	0	0	++	+++	0	0	0	+
$C_{25\ 2}$	0	0	0	+	0	0	0	0
$C_{26:1}$	0	0	0	tr	0	0	0	0
$C_{27\ 1}$	0	0	0	+++	0	0	tr	+
$C_{27:2}$	0	0	0	tr	0	0	0	0
$C_{27\ 3}$	0	0	0	0/tr	0	0	0	0
$C_{29:1}$	0	tr	0	0/tr	0	0	0	0
$C_{31:2}$	0	0	0	0/+	0	0	0	0
$C_{31\ 1}$	0	tr	0	0/+	0	0	0	0
$C_{33\ 2}$	0	tr	0	0/tr	0	0	0	0
$C_{33\ 1}$	0	tr	0	0/tr	0	0	0	0
$C_{35\ 2}$	0	++	tr	0	0	0	0	0
$C_{35\ 1}$	0	tr	0	0	0	0	0	0
$C_{36\ 2}$	0	+	0	0	0	0	0	0
$C_{37\ 3}$	0	+	+	0	0	0	0	0
$C_{37\ 2}$	0	+++	++	0	0	0	0	0
$C_{37\ 1}$	0	++	tr	0	+	0	0	+
$C_{38\ 3}$	0	0	tr	0	0	0	0	0
$C_{38\ 2}$	0	++	+	0	0	0	0	0
$C_{38\ 1}$	0	0	0	0	0	0	0	+
$C_{39\ 5}$	0	0	0	0	0	0	0	+
$C_{39\ 4}$	0	0	0	0	0	0	0	++
$C_{39\ 3}$	0	++	++	0/tr	0	0	0	0

$C_{39:2}$	0	+++	+++	0/+	0	0	0	+
$C_{39:1}$	0	++	++	0/tr	tr	0	0	+++
$C_{40:5}$	0	0	+	0	0	0	0	0
$C_{40:3}$	0	+	0	0	0	0	0	0
$C_{40:2}$	0	++	++	0	0	0	0	0
$C_{40:1}$	0	0	0	0	0	0	0	+++
$C_{41:5}$	0	0	0	0	0	0	0	+++
$C_{41:4}$	0	0	0	0	0	0	0	+++
$C_{41:3}$	0	+++	+++	0/+	0	+++	0	0
$C_{41:2}$	0	+++	+++	0/++	0	0	0	++
$C_{41:1}$	0	++	+++	0/+	0	0	0	+++
$C_{42:3}$	0	+	++	0	0	0	0	0
$C_{42:2}$	0	+	+	0/tr	0	0	0	0
$C_{42:1}$	0	0	tr	0	0	0	0	++
$C_{43:6}$	0	0	0	0	0	0	0	tr
$C_{43:5}$	0	0	0	0	0	0	0	tr
$C_{43:4}$	0	0	+	0	0	+++	0	+++
$C_{43:3}$	0	++	++	0/+++	0	+++	0	0
$C_{43:2}$	0	++	++	+++	0	0	0	+
$C_{43:1}$	0	++	0	0/++	0	0	0	+++
$C_{44:2}$	0	0	0	0/tr	0	0	0	0
$C_{45:4}$	0	0	0	0/+	0	+++	0	0
$C_{45:3}$	0	++	0	0/++	0	+++	0	0
$C_{45:2}$	0	++	0	0/++	0	0	0	0
$C_{45:1}$	0	0	0	0/+++	0	0	0	++

[a] Relative proportions of the total hydrocarbon mixture for each species. +++ = >3.0%; ++ = 1.0–3.0%; + = 0.3–0.99%; and tr = <0.3%; 0 = not detected.

[b] N mon = *Neotermes mona*; C bre = *Cryptotermes brevis*; P cor = *Procryptotermes corniceps*; I spp = *Incisitermes* species; H sp = *Heterotermes* species; P wol = *Parvitermes wolcotti*; N cos = *Nasutitermes costalis*; N aca = *Nasutitermes acajutlae*.

[c] *Incisitermes* spp. displayed a wide range of hydrocarbon mixtures. For example, 0/+++ would denote the range from absent to above 3%.

Kalotermitidae

We characterized the cuticular hydrocarbons of Kalotermitidae identified as *Neotermes mona* (Banks), *Cryptotermes brevis* (Walker), *Procryptotermes corniceps* (Snyder), and *Incisitermes* spp. Species of the family Kalotermitidae, although commonly known as drywood termites, differ widely in their moisture requirements. The fauna of the BVI includes species at both ends of the moisture-dependence spectrum. In the BVI we feel that *N. mona* is dependent upon a high constant environmental moisture supply, usually obtained by inhabiting living trees, whereas *C. brevis*, the "furniture termite," is capable of living without access to free water and is unable to thrive when exposed to sustained presence of free water (Collins, 1969; Williams, 1977).

Neotermes mona (*Banks*). This is the largest termite of the area. We found it on the relatively moist, north slope of Guana Island. The bulk of the colony developed excavations in living, as well as dead, wood. This species was once thought to be endemic to Mona Island, but its range was recently extended west through the Dominican Republic to the Turks and Caicos archipelago (Scheffrahn et al., 1990; Jones et al., 1995).

The cuticular hydrocarbon mixture of *N. mona* reflected a general pattern seen in most of the termite species examined thus far in the West Indies. Cuticular hydrocarbons occurred in two distinct groups: early eluting compounds (24–29 carbons in the parent chain) and late eluting compounds (37–43 carbons in the parent chain) (Table 3; Figure 1). In *N. mona* the early eluting compounds predominate, representing over 90% of the total hydrocarbon in nymphs and pseudergates (Figure 1). The hydrocarbon mixtures of pseudergates and nymphs were very similar to the one alate sample (Table 3).

n-Alkanes present were *n*-C_{24}, *n*-C_{25}, *n*-C_{26}, *n*-C_{27}, *n*-C_{28}, and *n*-C_{29}. The most abundant were *n*-C_{25} and *n*-C_{27}, comprising about 13% and 12% of the total hydrocarbons, respectively. Slightly lower amounts were seen in the alate sample. The other *n*-alkanes accounted for about 5% of the total hydrocarbons.

We identified isomeric mixtures of internally branched monomethylalkanes with parent chains ranging from C_{24} to C_{43}, except for C_{30}, C_{32}, and C_{34}. Positions of methyl branches ranged from C-10 to C-15. Internally branched monomethylalkanes were the most abundant class of hydrocarbons produced by *N. mona*, representing about 42% of the total hydrocarbon. One isomeric mixture, 13-; 11-MeC_{25}, accounted for 22–26% of the total hydrocarbon (Table 3; Figure 1).

2- and 3-Methylalkanes were identified for C_{24} to C_{29}. These terminally branched monomethylalkanes comprised approximately 17–20% of the total hydrocarbon. Internally branched dimethylalkanes constituted $<7\%$ of the total cuticular hydrocarbon fraction of *N. mona*. There was only one type of internally branched dimethylalkane, those with three methylene groups separating the

TABLE 3. RELATIVE QUANTITIES (MEAN, STANDARD DEVIATION, AND RANGE) OF CUTICULAR HYDROCARBONS OF PORTIONS OF COLONIES OF *Neotermes mona* (BANKS) FROM BRITISH VIRGIN ISLANDS[a]

Hydrocarbon	Larvae, nymphs, and pseudergates		Alates (mean)
	Mean ± SD	Range	
n-C_{24}	0.20 ± 0.17	0–0.30	0.24
12-; 11-; 10-MeC_{24}[b]	0.92 ± 0.31	0.68–1.27	1.18
2-MeC_{24}	1.74 ± 0.92	1.19–2.80	1.74
3-MeC_{24}	0.06 ± 0.10	0–0.17	0
n-C_{25}	13.00 ± 4.14	8.72–16.98	11.45
13-; 11-MeC_{25}[b]	21.39 ± 5.32	18.06–27.52	25.85
2-MeC_{25}	7.25 ± 0.66	6.79–8.01	7.58
3-MeC_{25}	4.78 ± 1.92	3.34–6.96	4.89
n-C_{26}	4.04 ± 1.41	2.41–4.97	3.00
3,13-$DimeC_{25}$	1.19 ± 0.35	0.82–1.51	1.38
13-; 12-MeC_{26}[b]	4.33 ± 1.06	3.11–4.95	5.17
2-MeC_{26}	2.34 ± 0.42	1.90–2.73	1.71
$C_{27:1}$ + 3-MeC_{26}[c]	0.47 ± 0.46	0–0.91	0.97
n-C_{27}	11.94 ± 6.20	7.65–19.06	9.55
13-; 11-MeC_{27}[b]	9.48 ± 2.95	7.72–12.89	11.51
11,15-$DimeC_{27}$; 2-MeC_{27}[d]	4.60 ± 1.31	3.40–6.00	4.79
3-MeC_{27}	0.35 ± 0.34	0–0.69	0.71
5,15-$DimeC_{27}$	0.06 ± 0.11	0–0.19	0
n-C_{28}	0.09 ± 0.15	0–0.27	0
3,13-$DimeC_{27}$	0.07 ± 0.12	0–0.21	0
12-MeC_{28}	0.56 ± 0.56	0–1.13	0.81
12,16-; 11,15-$DimeC_{28}$[e]	0.11 ± 0.19	0–0.33	0
n-C_{29}	0.66 ± 0.65	0–1.31	0.84
13-; 11-MeC_{29}[b]	0.25 ± 0.43	0–0.75	0.44
13,17-$DimeC_{29}$	0.10 ± 0.17	0–0.30	0
3-MeC_{29}	0.07 ± 0.12	0–0.21	0
5,17-$DimeC_{29}$	0.04 ± 0.08	0–0.13	0
15-; 13-MeC_{31}[b]	0.05 ± 0.08	0–0.14	0
3,7-$DimeC_{31}$	0.03 ± 0.06	0–0.10	0
13-MeC_{33}	0.06 ± 0.11	0–0.18	0
13-MeC_{35}	0.07 ± 0.13	0–0.22	0
13,17-$DimeC_{35}$	0.07 ± 0.13	0–0.22	0
12-MeC_{36}	0.06 ± 0.11	0–0.19	0
13-MeC_{37}	0.81 ± 0.40	0.53–1.26	0.57
13,17-; 11,15-$DimeC_{37}$[e]	0.38 ± 0.66	0–1.15	0
12-MeC_{38}	0.28 ± 0.30	0–0.59	0.20
12,16-$DimeC_{38}$	0.12 ± 0.20	0–0.35	0
13-MeC_{39}	0.94 ± 0.30	0.59–1.12	0.53
11,15-$DimeC_{39}$	1.51 ± 0.41	1.26–1.98	0.76

TABLE 3. Continued

Hydrocarbon	Larvae, nymphs, and pseudergates		Alates (mean)
	Mean ± SD	Range	
12-MeC$_{40}$	0.53 ± 0.06	0.46–0.57	0.30
12,16-DimeC$_{40}$	0.22 ± 0.39	0–0.67	0.20
13-MeC$_{41}$	1.25 ± 0.62	0.59–1.81	0.77
13,17-DimeC$_{41}$	1.60 ± 0.80	0.84–2.44	1.10
12-MeC$_{42}$	0.07 ± 0.12	0–0.21	0
12,16-DimeC$_{42}$	0.11 ± 0.19	0–0.33	0
13-MeC$_{43}$	0.77 ± 0.21	0.53–0.89	0.72
13,17-DimeC$_{43}$	0.98 ± 1.41	0–2.23	1.04

[a]The data from this table are derived from separate samples taken in 1991 and 1992 from the same colony on Guana Island. Samples include combinations of large larvae, nymphs, and/or pseudergates. The single alate sample was extracted in hexane before drying. No soldiers are included in the hydrocarbon samples.
[b]An isomeric mixture. These monomethylalkanes coelute.
[c]This alkene and monomethylalkane coelute.
[d]This monomethylalkane and two isomers of this dimethylalkane coelute.
[e]An isomeric mixture. Two or more isomers of these dimethylalkanes coelute. Distinct, separate isomers can be distinguished from mass spectra.

methyl branches. Terminally branched dimethylalkanes were not abundant (<1.4% of the total hydrocarbon) and were trivial except for 3,13-DimeC$_{25}$. No trimethylalkanes were found. Only one alkene, C$_{27:1}$, was found in *N. mona*.

Cryptotermes brevis (*Walker*). The phragmatic heads of the soldiers, the presence of piles of dry fecal pellets in infested buildings, and the paper-thin outer surface of furniture or wood containing large colonies of *C. brevis* are characteristic of this termite. This species has never been reported in the BVI from habitats other than structural timber, furniture, and objects of art not exposed to moisture. This habitat limited our ability to collect samples in the BVI. Because *C. brevis* is very common in Hawaii, we were able to obtain a sample from Oahu, Hawaii, so that we could present the cuticular hydrocarbon mixture of this now circumtropical species.

C. brevis clearly reflects the general pattern of hydrocarbon mixtures of drywood termites of the West Indies. In this species hydrocarbons occurred in two groups: the early-eluting compounds consisted almost exclusively of *n*-alkanes and terminally branched monomethyl alkanes, and late-eluting compounds were primarily olefins (Table 2; Figure 2).

n-Alkanes present were *n*-C$_{23}$, *n*-C$_{25}$, *n*-C$_{26}$, *n*-C$_{27}$, *n*-C$_{28}$, *n*-C$_{29}$, and *n*-C$_{30}$. As in *N. mona*, *n*-C$_{25}$ and *n*-C$_{27}$ were the most abundant, comprising

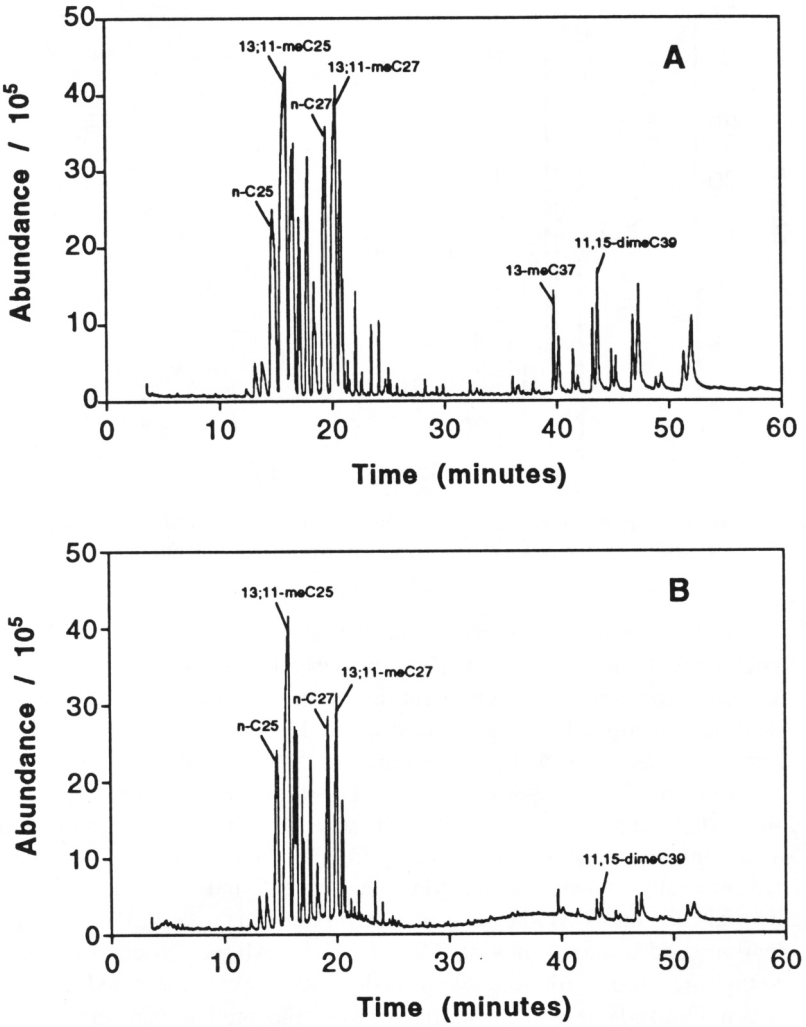

FIG. 1. Total ion chromatogram of the cuticular hydrocarbons from *Neotermes mona* from Guana Island. A = pseudergates, nymphs, and larvae; B = alates.

about 13% and 12%, respectively, of the total hydrocarbons from the sample of larvae, nymphs and pseudergates. All of the other *n*-alkanes combined represented no more than 5.5% of the total hydrocarbons.

Only isomeric mixtures of internally branched monomethylalkanes with parent carbon chains of C_{25}, C_{27}, C_{37}, C_{39}, and C_{41} were found. The early eluting components (C_{25} and C_{27}) of this class of hydrocarbons represented

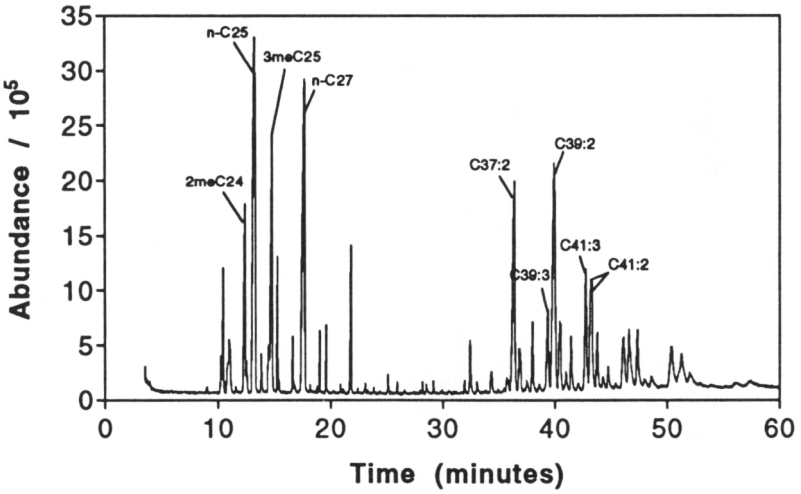

FIG. 2. Total ion chromatogram of the cuticular hydrocarbons from nymphs, pseudergates, and large larvae of *Cryptotermes brevis* from Honolulu, Hawaii.

$< 1.0\%$ of the total hydrocarbon. The later eluting components (C_{37}, C_{39}, and C_{41}) coeluted with a diene and could not be separately quantified.

2- and 3-Methylalkanes were identified from C_{23} to C_{29}. These terminally branched monomethylalkanes comprised about 17% of the total hydrocarbon. In *C. brevis* the 2- and 3-methylalkanes almost always occurred in pairs. When the parent chain of these hydrocarbons contained an even number of carbons, the 2-methylalkane predominated; when the parent chain contained an odd number of carbons, the 3-methylalkane was more abundant (Figure 2).

No internally branched dimethylalkanes were found in our sample. Terminally branched dimethylalkanes were not very abundant ($< 0.3\%$ of the total hydrocarbon) and occurred only at C-25 and C-31. All were found to have the first methyl branch on carbon 3. No trimethylalkanes were identified.

Alkenes, alkadienes, and alkatrienes were the predominant class of cuticular hydrocarbons, representing approximately 51% of the total hydrocarbons. The number of carbons ranged from 31 to 45 (Table 2).

Procryptotermes corniceps (*Snyder*). *P. corniceps* is moderately abundant in the BVI (Collins et al., 1997). Darlington (1992) and Krishna (1962) reported an extention of the known range of *P. corniceps* to Antigua, Guadeloupe, Jamaica, Montserrat, and Puerto Rico; Scheffrahn et al. (1990) added Turks and Caicos Islands. Soldiers of *P. corniceps* are distinctive with proportionally long, strongly curved, sharply pointed mandibles. The heads of the soldiers slope steeply in front and have short hornlike projections from the frons near the outer edge of the bases of the mandibles (Krishna, 1962).

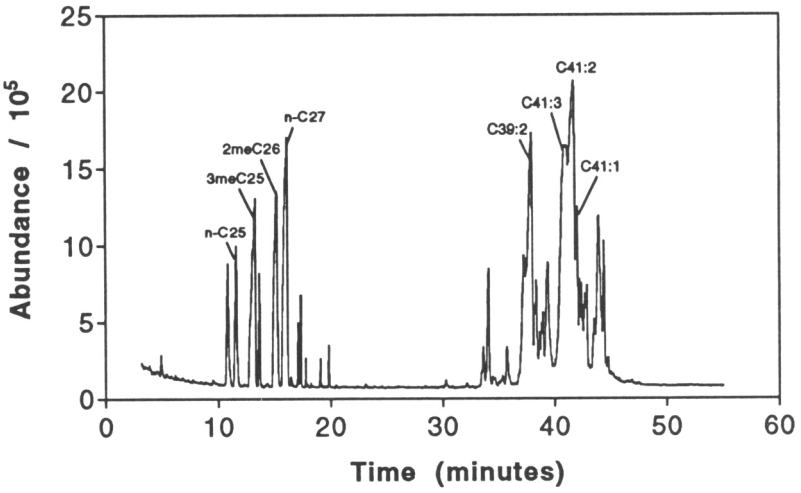

FIG. 3. Total ion chromatogram of the cuticular hydrocarbons from 20 pseudergates of *Procryptotermes corniceps* from Great Camino.

The cuticular hydrocarbon mixture of *P. corniceps* was very similar in gross comparison to that of *C. brevis*. The early-eluting components were almost exclusively *n*-alkanes and terminally branched monomethylalkanes, and the late-eluting compounds were primarily olefins (Table 2; Figure 3).

n-Alkanes present were *n*-C_{24}, *n*-C_{25}, *n*-C_{26}, *n*-C_{27}, *n*-C_{28}, and *n*-C_{29}. The most abundant hydrocarbons were *n*-C_{25} and *n*-C_{27}, comprising about 6.3% and 11.7% of the total hydrocarbon, respectively. All of the other *n*-alkanes represented $<2.5\%$ of the total hydrocarbons.

Internally branched monomethylalkanes were not common in *P. corniceps*. 13-MeC_{27} occurred in trivial amounts. The isomeric mixture of 15-; 13-MeC_{39} coeluted with $C_{40:2}$. In most samples 15-; 13-MeC_{41} made up an average of about 0.9% of the total hydrocarbons. 2- and 3-Methylalkanes were identified from C_{24} to C_{28}. These terminally branched monomethylalkanes comprise about 23% of the total hydrocarbon. As in *C. brevis* the 2- and 3-methylalkanes almost always occurred in pairs. When the parent chain of these hydrocarbons contained an even number of carbons, the 2-methylalkane predominated; when the parent chain contained an odd number of carbons, the 3-methylalkane was more abundant (Figure 3). Di- and trimethylalkanes were not detected.

As with *C. brevis*, the alkenes, alkadienes, and alkatrienes were the predominant class of cuticular hydrocarbons, comprising over 55% of the total hydrocarbons. The number of carbons ranged from 25 to 43. One sample contained a significant amount of pentacosadiene ($>15\%$ of the total hydrocarbons); this compound was not seen in any of the other samples.

Incisitermes *spp.* Members of the genus *Incisitermes* are the most common kalotermitds found in the BVI. Collins et al. (1997) and Scheffrahn et al. (1994) list *I. snyderi* (or *I.* nr *snyderi*) from all of the islands in the BVI sampled by M. S. Collins, except for Eustatia, and *I. incisus* from Beef Island, Eustatia, Guana Island, and Virgin Gorda. Because of the morphological variation and the uncertainty of the taxonomy of *Incisitermes* in the BVI, we were unable to unambiguously assign specimens used for hydrocarbon characterization to a specific taxon within *Incisitermes* (Collins et al., 1997).

Specimens identified as *I.* nr *snyderi* were the most common found in the BVI. They live in sound, dead trees of many species, as well as in structural timber throughout much of its range (Light, 1993; Harris, 1961). Specimens identified as *I. incisus* were collected in the mangrove swamps fringing Beef Island and in trees on the wetter side of Guana Island. No records of attack on buildings were available for *I. incisus*.

After comparing the cuticular hydrocarbons of over 20 colonies of *Incisitermes* from the BVI including samples from Eustatia identified as *I. incisus*, we concluded that the mixtures of the cuticular hydrocarbons of these samples were as variable as the morphology of the termites. The general "patterns" of these chromatograms varied from one similar to *N. mona*, where >95% of the hydrocarbons have 23–29 carbons (Figure 4), to one similar to the kalotermitids found in drier conditions, where a significant proportion (>30%) of the hydrocarbons were olefins with 33 or more carbons (Figure 5). We also found *Incisitermes* specimens with hydrocarbon mixtures that were intermediate to these extremes (Figure 6). Because of this broad variation, we report the extremes and discuss the classes of hydrocarbons in general terms, describing the range that we observed (Table 2).

n-Alkanes present were *n*-C_{23}, *n*-C_{24}, *n*-C_{25}, *n*-C_{26}, *n*-C_{27}, *n*-C_{28}, and *n*-C_{29}. The most abundant ones were *n*-C_{25} and *n*-C_{27} in all *Incisitermes* samples. Internally branched monomethylalkanes were rare and were found for C_{25}, C_{27}, C_{28}, C_{29}, C_{35}, C_{37}, and C_{39}; they were not detectable in most of the samples. Methyl branches were found on carbons, 7, 9, 11, 13, 15, and 17, occasionally in isomeric mixtures. The 2- and 3-methylalkanes were identified for C_{24}–C_{27}, with 2-MeC_{25} being the most abundant. Of the 3-methylalkanes, 3-MeC_{25} was the most abundant. These terminally branched monomethylalkanes comprised from about 16–32% of the total hydrocarbon.

Di- and trimethylalkanes were rare in *Incisitermes* from the BVI. Most of the dimethylalkanes were late-eluting (carbon numbers in the parent chain from 33 to 37). The dimethylalkanes were all internally branched with the first methyl branch occurring on 9, 11, or 13 carbon. The position of the first methyl branch tended to be more internal as the parent chain length increases. The second methyl group was usually separated from the first by nine methylene units.

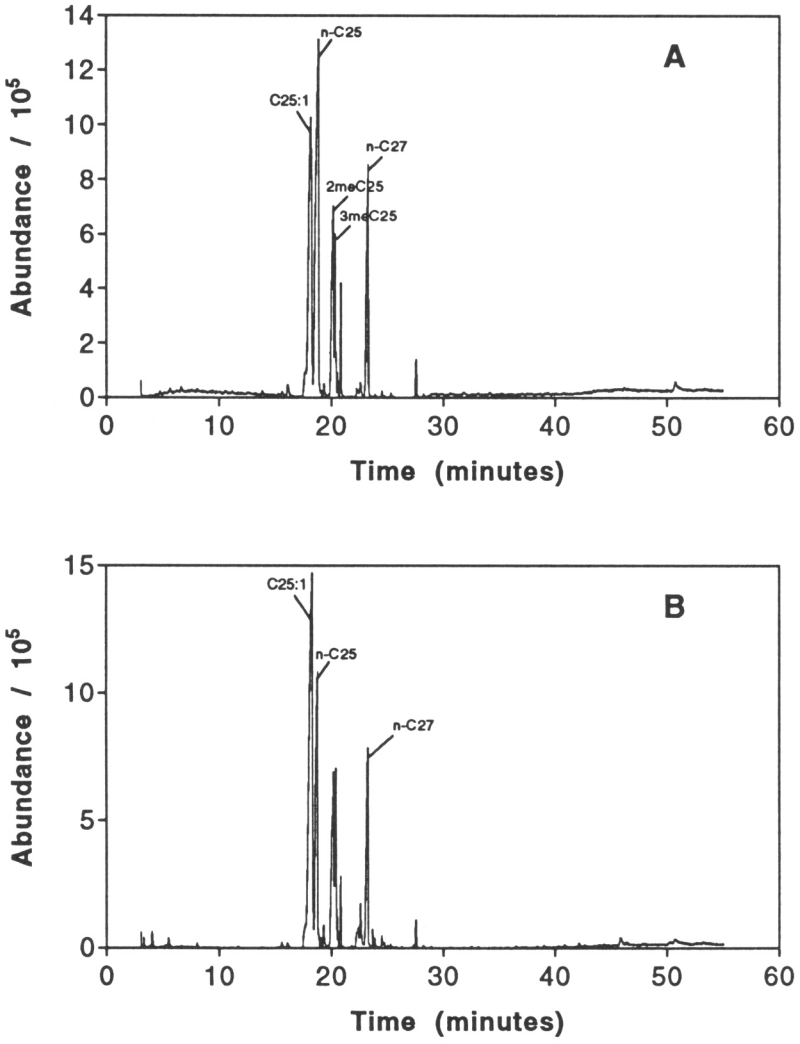

FIG. 4. Total ion chromatogram of the cuticular hydrocarbons from pseudergates from two separate colonies of *Incisitermes* from Guana Island.

Trimethylalkanes were found at C_{33}, C_{35}, and C_{37} in very few samples and were trivial in abundance when present.

The unsaturated components constituted about 30–60% of the total hydrocarbon. $C_{25:1}$ was the predominant olefin comprising as much as 25% of the

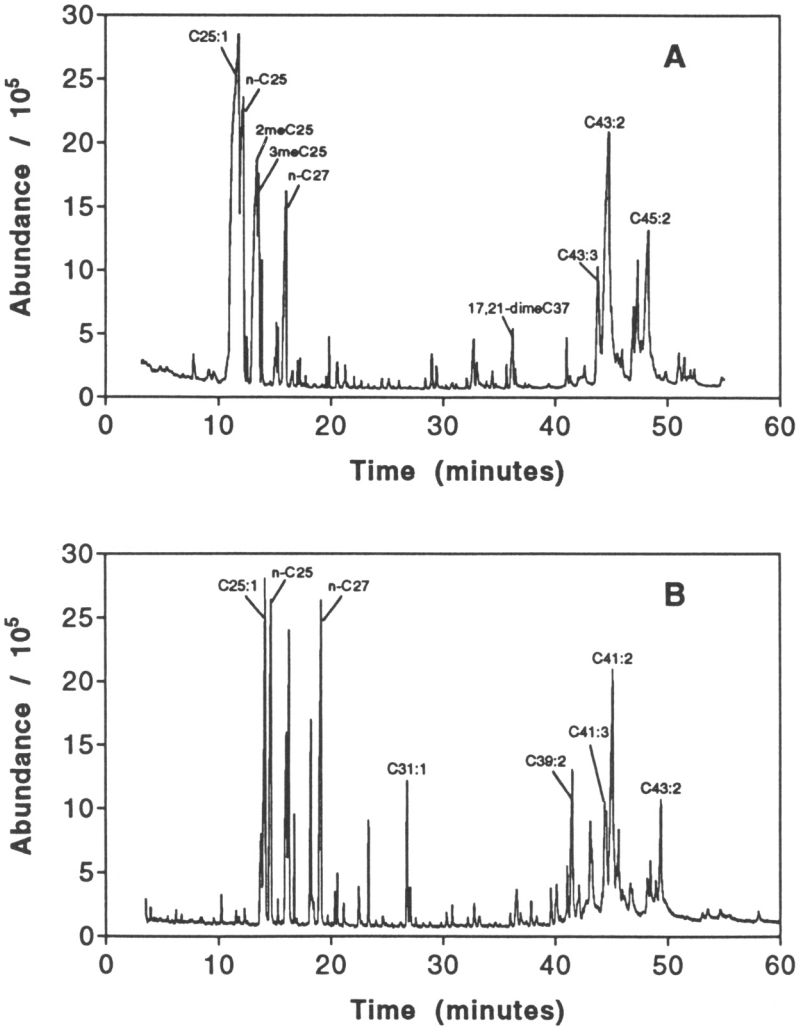

FIG. 5. Total ion chromatogram of the cuticular hydrocarbons from pseudergates of *Incisitermes* from Scrub Island (A) and Anegada (B).

total (Figures 4–6). Late-eluting olefins were either totally absent (Figure 4) or constituted 25–35% of the total hydrocarbons (Figures 5 and 6).

The results of our studies of *Incisitermes* from the BVI were equivocal. Cuticular hydrocarbons have proven useful in discriminating species of many groups of termites (Haverty et al., 1988, 1990, 1991, 1992; Howard et al., 1978, 1982, 1988; Thorne and Haverty, 1989; Thorne et al., 1993), but they

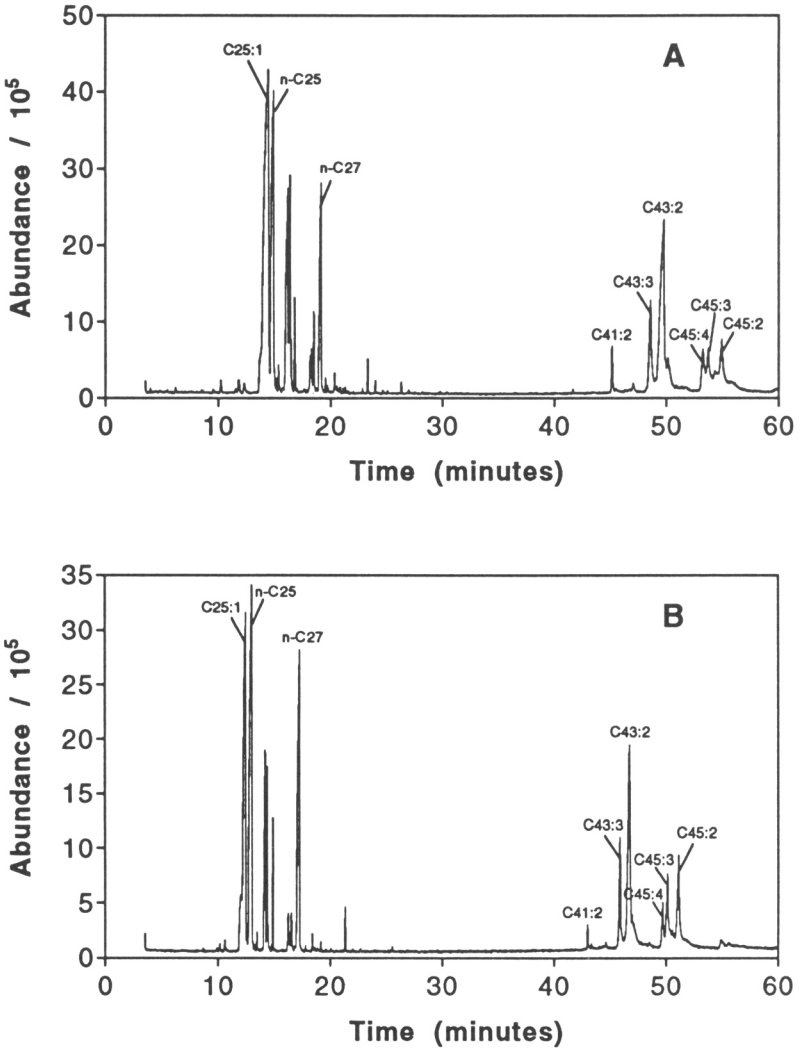

FIG. 6. Total ion chromatogram of the cuticular hydrocarbons from pseudergates and/or nymphs of *Incisitermes* from Guana Island (A) and Tortola (B).

were not diagnostic characters for sorting taxa in *Incisitermes* from the BVI. We saw no distinct differences between samples identified as *I. incisis* and many of those identified as *I.* nr *snyderi*. Further collections from the US Virgin Islands, the Greater Antilles, and mainland North America will be necessary to resolve the taxonomy of this refractory genus.

Rhinotermitidae

Thus far, only one taxon from this family has been collected in the British Virgin Islands.

Heterotermes *sp.* Samples of this genus are so morphologically variable that Snyder questioned whether the genus comprises a single, highly variable species or a complex of closely related species (Scheffrahn et al., 1994). There are three described species recorded from this region: *H. tenuis* (Hagen), *H. convexionotatus* (Snyder), and *H. cardini* (Snyder). Until the taxa are resolved, members of this genus collected in the BVI will be referred to as *Heterotermes* sp.

The cuticular hydrocarbon mixture of *Heterotermes* sp. from the BVI included mostly compounds with 26–31 carbons in the parent chain (Table 2; Figure 7). Late-eluting compounds accounted for only about 1% of the total hydrocarbons. Mono- and dimethylalkanes with 27 or 29 carbons in the parent chain accounted for >76% of the total. *Heterotermes* sp. from the BVI had a mixture of hydrocarbons very similar to that of *H. aureus* (Snyder) from the Sonoran Desert near Tucson, Arizona (Haverty and Nelson, unpublished observations).

n-Alkanes present were *n*-C_{26}, *n*-C_{27}, *n*-C_{28}, *n*-C_{29}, *n*-C_{30}, and *n*-C_{31}. *n*-C_{27} was the most abundant normal alkane comprising about 4.5% of the total hydrocarbon. The others combined represented <4% of the total. We identified

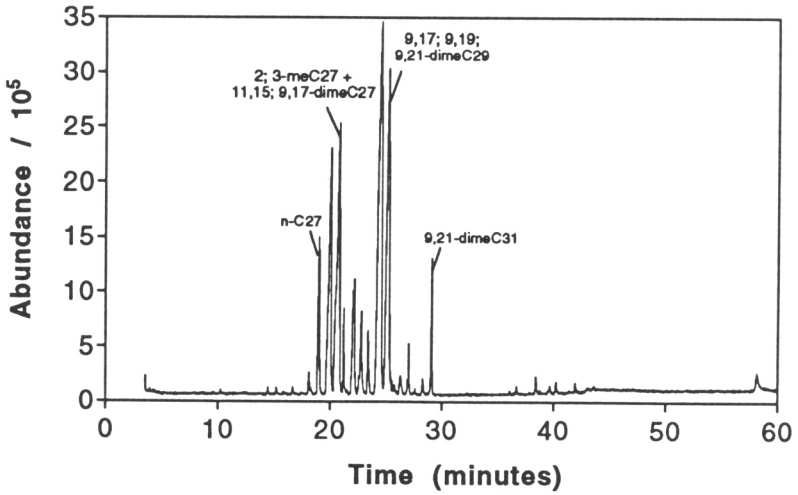

FIG. 7. Total ion chromatogram of the cuticular hydrocarbons from workers of *Heterotermes* sp. from Guana Island.

isomeric mixtures of internally branched monomethylalkanes with parent carbon chains ranging from C_{27} to C_{31}, and C_{37}. This class of compounds was very abundant, representing nearly 44% of the total. Two isomeric mixtures, 13-; 11-; 9-; 7-MeC_{27} and 13-; 11-; 9-; 7-MeC_{29}, were predominant and accounted for >37% of the total hydrocarbon. We identified 2- and 3-methylalkanes for C_{26} and C_{27}. Only the 2-methyl isomer occurred at C_{28}. Terminally branched monomethylalkanes comprise only 1.1% of the total hydrocarbon.

Internally branched dimethylalkanes predominated and accounted for over 45% of the total hydrocarbon fraction. The most abundant dimethylalkanes occurred in isomeric mixtures with 27 or 29 carbons in the parent chain (Figure 7). Only one terminally branched dimethylalkane (3,21-DimeC_{29}) was identified, and it coeluted with 7,13,21-TrimeC_{29} in trace amounts. Various isomers of trimethylnonacosane were detected, but in trivial amounts. Only two olefins were present, $C_{37:1}$ and $C_{39:1}$, constituting <1% of the total hydrocarbon.

Termitidae

Three species of termitids, belonging to two genera, were collected on Guana Island and nearby islands of the BVI complex.

Parvitermes wolcotti (*Snyder*). *P. wolcotti* is a small nasute that forages in dead wood or on the ground in areas with fairly dense tree cover on Guana Island, BVI (Collins et al., 1977). We were not able to collect large samples until a fortuitous collection of 173 workers from a colony on Peter Island allowed us to document the hydrocarbon mixture of this species. The pattern of the hydrocarbon mixture of *P. wolcotti* was an inverse of *N. mona* in that nearly 80% of the hydrocarbons were late-eluting olefins with 41–45 carbons (Figure 8).

n-Alkanes present were *n*-C_{27}, *n*-C_{28}, and *n*-C_{29}. In total the *n*-alkanes comprised only 6.5% of the total hydrocarbon. One internally branched monomethylalkane was detected, 5-MeC_{29}, and represented only 1% of the total hydrocarbon. 2- and 3-Methylalkanes were identified for C_{27} and C_{29}, but 2-MeC_{28} was the most abundant internally branched monomethylalkane (Figure 8). The five compounds in this group comprised over 11% of the total hydrocarbons. One dimethyalkane was noted (as isomeric mixture 5,19-; 5,17-DimeC_{29}) and amounted to <1.7% of the cuticular hydrocarbon fraction. No trimethylalkanes were observed.

Unsaturated components comprised the predominant class of hydrocarbons in *P. wolcotti*, making up nearly 80% of the total hydrocarbons present. All of the olefins had an odd number of carbons (41, 43, and 45) and possessed three or four double bonds.

Nasutitermes costalis (*Holmgren*). *N. costalis* is the less common of the two carton nest-building nasutes found in the BVI. It occurs primarily in wetter

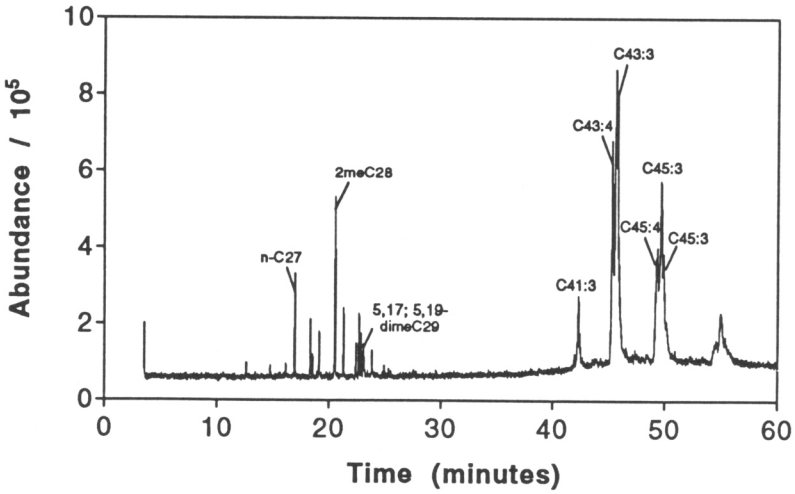

FIG. 8. Total ion chromatogram of the cuticular hydrocarbons from workers of *Parvitermes wolcotti* from Peter Island.

localities in the BVI; Tortola and Guana Island are the only islands where this species has been found (Collins et al., 1997; Scheffrahn et al., 1994). *N. costalis* occurs on many of the islands in the Greater and Lesser Antilles, from Cuba south to Trinidad and Tobago (Snyder, 1949; Araujo, 1977; Scheffrahn et al., 1994). The relative scarcity of *N. costalis* in the BVI may be related to the lower moisture availability on most of the islands of the complex. Krecek (1970) found that *N. costalis* distribution patterns and nest composition on Cuba indicated a relatively higher moisture demand than that shown by the other common nasute, *N. rippertii* (Rambur).

All of the cuticular hydrocarbons of *N. costalis* had parent chains ranging from 25 to 33 carbons. Those with 29–31 carbons in the parent chain comprised over 88% of the total hydrocarbon mixture (Figure 9).

n-Alkanes present were *n*-C_{25}, *n*-C_{27}, *n*-C_{28}, and *n*-C_{29}. *n*-C_{29} was the most abundant, comprising 1.9% of the total hydrocarbon. The other three *n*-alkanes represented only 3.2% of the total hydrocarbons.

We identified isomeric mixtures of internally branched monomethylalkanes with parent carbon chains ranging from C_{27} to C_{33}. Positions of methyl branches ranged from C-9 to C-15. Methyl branches located on even-numbered carbons were found only when the parent chain of the hydrocarbon had an even number of carbons, while branches on odd-numbered carbons were found to occur on hydrocarbons with either odd or even numbers of carbons in the parent chain. Internally branched monomethylalkanes were one of the most abundant classes

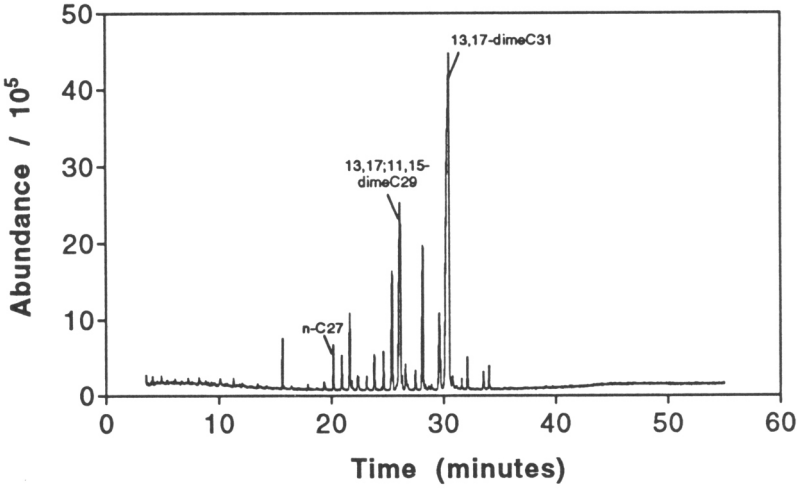

FIG. 9. Total ion chromatogram of the cuticular hydrocarbons from workers of *Nasutitermes costalis* from Tortola.

of hydrocarbons produced by *N. costalis*, representing about 15% of the total hydrocarbon. Two isomeric mixtures, 15-; 13-; 11-MeC_{29} and 15-; 13-; 11-MeC_{31}, accounted for nearly 80% of this class of hydrocarbon.

3-Methylalkanes were identified for C_{27} and C_{29}. 2-MeC_{27} coeluted with 11,15-$DimeC_{27}$. These terminally branched monomethylalkanes comprised at least 0.45% of the total hydrocarbons, but certainly less than 3.0%.

Internally branched dimethylalkanes were the predominant hydrocarbon class and constituted 78% of the total cuticular hydrocarbon fraction of *N. costalis* (Figure 9). All of these internally branched dimethylalkanes had three methylene groups separating the methyl branches. Dimethylalkanes had carbon numbers in the parent chain ranging from 27 to 33. Dimethylalkanes with 29 and 31 carbons in the parent chain accounted for nearly 64% of the total hydrocarbon complement. Terminally branched dimethylalkanes were not encountered.

Only two trimethylalkanes were identified. Each had three methylene groups between the methyl branches. Combined, they totaled $< 1\%$ of the total hydrocarbon. $C_{27:1}$ was the only olefin found and represented only 0.24% of the total hydrocarbon.

Nasutitermes acajutlae *(Holmgren). N. acajutlae* is the most conspicuous, and apparently the most abundant, species of termite in the BVI complex. Colonies of this species construct enormous nests (up to 1.5 m in diameter, up to 2.0 m in height) composed of dark to silvery brown, delicate, friable, parch-

mentlike outer walls enclosing the variously sized, heavier-walled cells of the carton matrix. Nests are usually ellipsoidal or irregularly rounded.

N. acajutlae was recently resurrected as a species morphologically distinguishable from *N. nigriceps* (Thorne et al., 1994). Termites in the *N. acajutlae/N. nigriceps* complex range from Mexico south into South America, then east and north through the Caribbean. Members of the two species have a tolerance for wide variations in moisture availability and use a variety of foods and nesting sites. Mature nests and individuals of *N. acajutlae* are larger than those of *N. costalis*. Soldiers of *N. acajutlae* have reddish to dark brown heads; alates are relatively large and chestnut brown in color (Thorne et al., 1994).

N. acajutlae was found on every island surveyed and has been found on even the smallest of islands, such as Carrot Rock (Scheffrahn et al., 1994; Collins et al., 1997). We obtained hydrocarbon samples of this species from 11 islands (Table 1). We extensively sampled workers and soldiers from 13 colonies on Guana Island, and collected alates when we encountered them (Haverty et al., 1996). To further assess interisland variability, we sampled *N. acajutlae* from diverse habitats on Tortola in 1994.

We identified 33 hydrocarbons from workers, 45 from soldiers, and 43 from alates of *N. acajutlae* (Haverty et al., 1996). The hydrocarbons found in all three castes aggregated into two distinct groups. The early-eluting components were primarily *n*-alkanes, methyl-branched alkanes, and a few normal alkenes. The late-eluting compounds consisted almost exclusively of unsaturated components, with chain lengths of 37–45 carbons and one to six double bonds, and a few monomethyl alkanes in trace amounts (especially in alates). Soldiers had considerably greater proportions of the early-eluting compounds (23–29 carbons) than did workers or alates (Figures 10 and 11). Whereas workers and alates had an average of 88–96% of the cuticular hydrocarbons with 33 or more carbons, soldiers had an average of about 69% of these late-eluting compounds. The predominant class of hydrocarbons was the olefin fraction, comprising greater than 89% of the total hydrocarbon component in workers and alates and about 76% in soldiers.

n-Alkanes present ranged from *n*-C_{23} to *n*-C_{33}. Usually *n*-C_{25} and *n*-C_{27} were the most abundant for workers, soldiers, and alates. The *n*-alkanes were least abundant in alates (1.8–4.3% of total), moderately abundant in workers (4–7.3% of total), and most abundant in soldiers (17.4–18.8% of the total hydrocarbon) (Figures 10 and 11).

Internally branched monomethylalkanes were nearly always encountered, in trivial amounts in workers (0.4–0.9% of total), and in significant amounts in soldiers (about 5.5% of total). Internally branched monomethylalkanes were usually present in alates, but accounted for only 0.4–1.7% of the total hydrocarbon. Only trivial amounts of 2- and 3-methylalkanes occurred in workers and soldiers. Alates usually had a significant component of 2-methylalkanes, com-

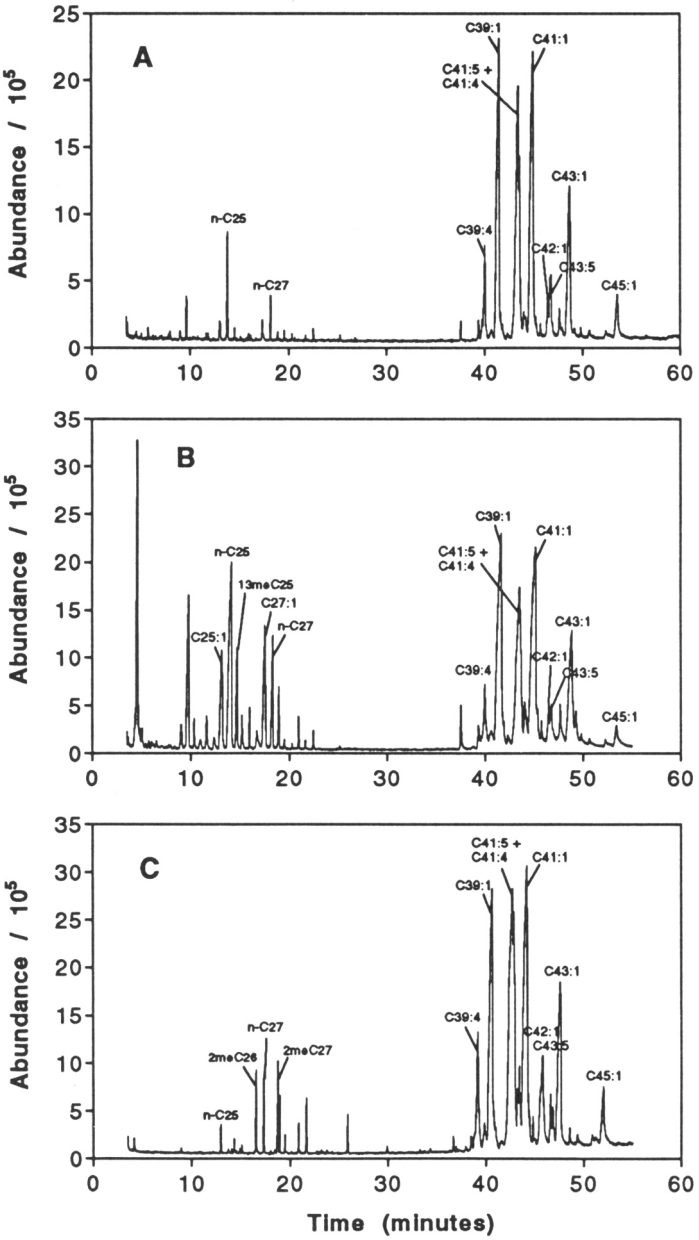

FIG. 10. Total ion chromatogram of the cuticular hydrocarbons from *Nasutitermes acajutlae* from Guana Island. A = workers, B = soldiers, C = alates.

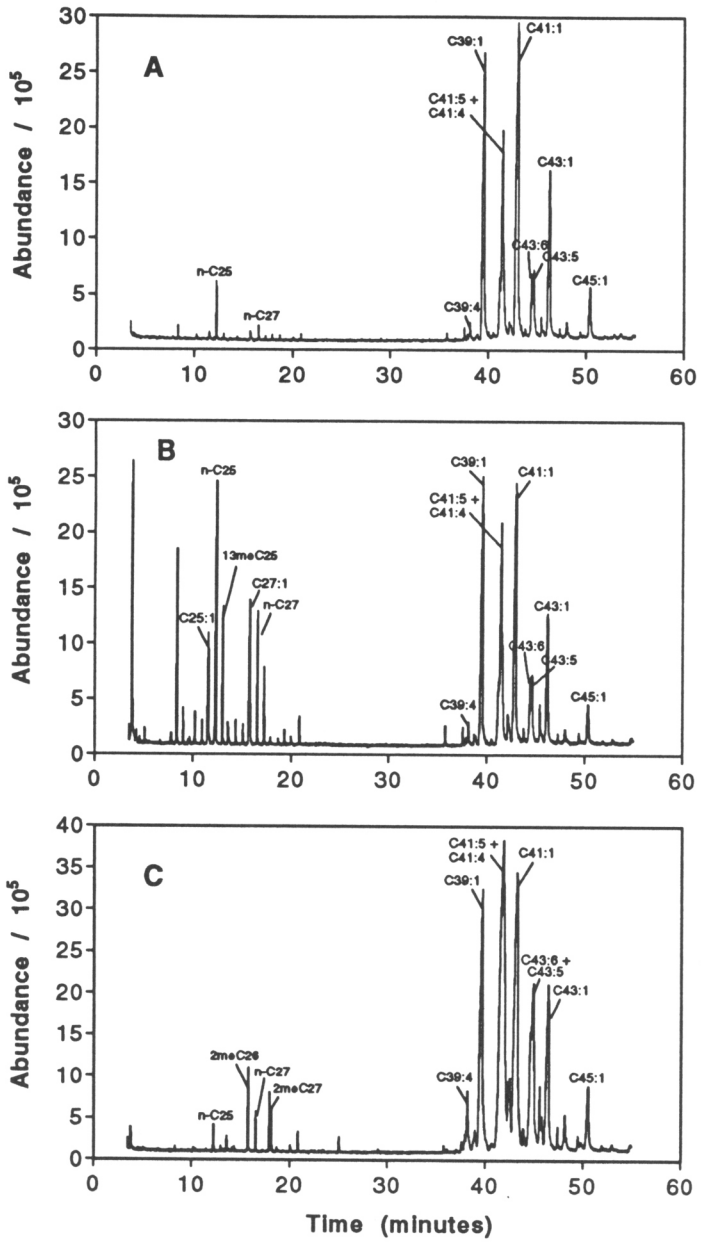

FIG. 11. Total ion chromatogram of the cuticular hydrocarbons of *Nasutitermes acajutlae* from Tortola, BVI. A = workers, B = soldiers, C = alates.

prising 1.8–4.6% of the total hydrocarbon, and were always found in the early-eluting constituents (Figures 10 and 11). No dimethyl- or trimethylalkanes were found in workers or soldiers; only 5,17-DimeC_{25} was found in alates in trivial amounts.

The unsaturated component was the paramount class of hydrocarbons in the cuticular lipids of *N. acajutlae.* Olefins comprised an average of 90–96% of the total hydrocarbon in workers and alates. Since soldiers contained a larger proportion of early-eluting *n*-alkanes, the olefin component amounted to about 76% of the total hydrocarbon in this caste. For all castes, $C_{39:1}$, $C_{41:5}$, $C_{41:4}$, $C_{41:1}$, and $C_{43:1}$ accounted for at least 70% of the total olefin (Haverty et al., 1996).

Intercaste Variation in N. acajutlae. We observed consistent intercaste differences in hydrocarbon mixtures of *N. acajutlae.* Workers and alates produced proportionally more olefins than did soldiers. Conversely, soldiers made proportionally more of the early-eluting hydrocarbons, such as *n*-alkanes and monomethylalkanes, than did workers and alates.

Cuticular hydrocarbons of workers, soldiers, and alates were not qualitatively identical. Soldiers had some early eluting compounds (11-; 9-MeC_{23}, 3-MeC_{23}, $C_{24:1}$, 11-MeC_{24}, 3-MeC_{25}, $C_{26:1}$, and 13-; 12-; 11-MeC_{26}) that were not found in workers or alates from Guana Island or Tortola (Tables 4 and 5; Figures 10 and 11). Samples of alates from both Guana Island and Tortola included hydrocarbons (5-MeC_{25}, 2-MeC_{25}, 5,17-DimeC_{25}, 2-MeC_{26}, and 3-MeC_{27}) not found in either workers or soldiers (Tables 4 and 5). Alates from Guana Island contained some abundant hydrocarbons ($C_{40:5}$, 15-MeC_{43}, and $C_{45:2}$) and trace hydrocarbons not seen in workers or soldiers from the same island (Table 4). Alates, workers, and soldiers from Tortola have these latter hydrocarbons as well as significant amounts (0.6–0.7%) of $C_{45:5}$, which was not seen in the Guana Island samples (Tables 4 and 5). Alates from both Guana Island and Tortola were also missing a few hydrocarbon components ($C_{25:1}$, $C_{27:1}$, and 13-; 11-MeC_{27}) that were commonly observed in workers and soldiers (Tables 4 and 5). $C_{40:1}$ and $C_{42:1}$ were not detected in alates from Guana Island, but were frequently found in workers and soldiers (Table 4).

$C_{43:6}$ was detected only in alates from Guana Island that were collected in 1993; however, this hydrocarbon was found in all castes collected on Tortola in 1994. It is curious that in preliminary work $C_{43:6}$ was also seen in workers, soldiers, and alates from specimens taken in 1994 from some of the same colonies on Guana Island that we sampled in 1993 (Haverty, Thorne, and Nelson, unpublished observations).

These obvious year-to-year differences could, in fact, represent distinct annual variation. They may be an artifact resulting from variations in handling of samples (for example, minor differences in drying technique or storage before and after processing). Whether these differences are real or an artifact of pro-

TABLE 4. RELATIVE QUANTITIES (MEAN AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS OF SAMPLES OF WORKERS, SOLDIERS, AND ALATES OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND[a]

Hydrocarbon	Workers (mean ± SD)	Soldiers (mean ± SD)	Alates (mean ± SD)
$C_{23:1}$	0.32 ± 0.32	0.41 ± 0.17	0.00 ± 0.00
C_{23}	0.97 ± 0.33	4.04 ± 0.91	0.08 ± 0.04
11-; 9-MeC_{23}[b]	0.00 ± 0.00	0.51 ± 0.15	0.00 ± 0.00
3-MeC_{23} + $C_{24:1}$[c]	0.00 ± 0.00	0.18 ± 0.15	0.00 ± 0.00
C_{24}	0.22 ± 0.22	0.66 ± 0.19	0.00 ± 0.00
11-MeC_{24}	0.00 ± 0.00	0.37 ± 0.32	0.00 ± 0.00
$C_{25:1}$	0.68 ± 0.27	2.96 ± 0.69	0.00 ± 0.00
C_{25}	3.05 ± 1.14	9.16 ± 1.28	0.55 ± 0.14
13-; 11-MeC_{25}[b]	0.16 ± 0.17	1.98 ± 0.43	0.12 ± 0.03
5-MeC_{25}	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01
2-MeC_{25}	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.03
3-MeC_{25} + $C_{26:1}$[c]	0.00 ± 0.00	0.57 ± 0.13	0.00 ± 0.00
5,17-$DimeC_{25}$	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.01
C_{26}	0.26 ± 0.27	0.59 ± 0.16	0.14 ± 0.02
13-; 12-; 11-MeC_{26}[b]	0.00 ± 0.00	0.42 ± 0.35	0.00 ± 0.00
2-MeC_{26}	0.00 ± 0.00	0.00 ± 0.00	1.42 ± 0.16
$C_{27:}$	0.86 ± 0.42	3.88 ± 0.63	0.00 ± 0.00
C_{27}	1.90 ± 1.60	3.67 ± 0.92	1.44 ± 0.28
13-; 11-MeC_{27}[b]	0.07 ± 0.11	1.14 ± 0.21	0.00 ± 0.00
5-MeC_{27}	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.04
2-MeC_{27}	0.15 ± 0.13	0.14 ± 0.09	1.67 ± 0.29
3-MeC_{27}	0.00 ± 0.00	0.00 ± 0.00	0.72 ± 0.13
C_{28}	0.15 ± 0.19	0.13 ± 0.12	0.30 ± 0.08
2-MeC_{28} + $C_{29:1}$[c]	0.00 ± 0.00	0.19 ± 0.10	0.53 ± 0.11
C_{29}	0.73 ± 0.56	0.56 ± 0.25	1.08 ± 0.25
5-MeC_{29}	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.05
2-MeC_{29}	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.03
3-MeC_{29}	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.05
C_{30}	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.04
C_{31}	0.06 ± 0.15	0.02 ± 0.04	0.58 ± 0.08
C_{33}	0.00 ± 0.00	0.01 ± 0.03	0.12 ± 0.03
$C_{35:1}$	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.06
13-MeC_{35}	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.06
$C_{37:1}$	0.40 ± 0.17	0.65 ± 0.17	0.40 ± 0.14
$C_{38:}$	0.53 ± 0.20	0.58 ± 0.13	0.47 ± 0.07
$C_{39:5}$[d]	0.36 ± 0.33	0.28 ± 0.25	0.00 ± 0.00
$C_{39:4}$[d]	2.00 ± 0.73	1.33 ± 0.61	3.89 ± 0.78
$C_{39:2}$	0.43 ± 0.48	1.29 ± 0.56	1.14 ± 0.11
$C_{39:1}$	20.01 ± 2.20	15.77 ± 2.79	14.40 ± 1.26
15-MeC_{39}	0.04 ± 0.12	0.36 ± 0.27	0.00 ± 0.00
$C_{40:5}$	0.00 ± 0.00	0.00 ± 0.00	0.90 ± 0.35
$C_{40:1}$	2.51 ± 0.42	2.89 ± 0.62	0.00 ± 0.00

TABLE 4. Continued

Hydrocarbon	Workers (mean ± SD)	Soldiers (mean ± SD)	Alates (mean ± SD)
$C_{41:4}$ + $C_{41:5}$[e]	18.77 ± 3.81	11.01 ± 3.92	21.72 ± 2.41
$C_{41:2}$	1.21 ± 0.63	1.50 ± 0.49	3.31 ± 0.20
$C_{41:1}$	24.43 ± 2.74	17.42 ± 3.52	20.24 ± 1.18
15-MeC_{41}	0.13 ± 0.23	0.46 ± 0.24	0.76 ± 0.15
$C_{42:1}$	1.60 ± 0.65	1.99 ± 1.03	0.00 ± 0.00
$C_{43:6}$ + $C_{43:5}$[f]	4.35 ± 1.37	2.41 ± 1.09	6.24 ± 1.11
$C_{43:2}$	0.83 ± 0.52	1.14 ± 0.43	2.63 ± 0.27
$C_{43:1}$	10.40 ± 1.61	7.66 ± 1.48	10.28 ± 1.09
15-MeC_{43}	0.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.11
$C_{45:2}$	0.00 ± 0.00	0.00 ± 0.00	0.32 ± 0.11
$C_{45:1}$	2.41 ± 0.82	1.69 ± 0.64	3.26 ± 0.87

[a] Three subsamples of 100 workers from each of 13 colonies. Four subsamples of ca. 4 ml of soldiers from each of 13 colonies. Two subsamples of 25–31 alates from four colonies.
[b] An isomeric mixture. These monomethylalkanes coelute.
[c] This monomethylalkane and the olefin coelute.
[d] These two isomers did not completely resolve in alates. Therefore, the areas for the two isomers were summed for alates only.
[e] These two isomers did not completely resolve. Therefore, the areas for the two isomers were summed.
[f] $C_{43:6}$ was identified only in alates. There were two isomers of $C_{43:5}$ that did not completely resolve. Therefore, the areas for the two isomers were summed.

tocol, we must consider them when evaluating minor variations in hydrocarbon mixtures for taxonomic studies of termites.

Island-to-Island Variation in N. acajutlae. Our collections of *N. acajutlae* were much more extensive, both in numbers of samples and geographic coverage (Table 1), than for any other termite taxon in the British Virgin Islands. We observed qualitative differences between samples of soldiers from Guana Island and Tortola, two islands separated by a channel approximately 2 km wide (Tables 4 and 5). Soldiers from collections on Guana Island did not have $C_{40:5}$ or $C_{45:2}$, whereas soldiers from Tortola possessed small amounts of these olefins. We did not have samples of soldiers from other islands, and our alate collections from other islands were limited. However, comparison of hydrocarbon mixtures from workers indicated a range in variation, both qualitative and quantitative, possible within one species (Tables 4 to 6).

Workers from islands more distant from Tortola and Guana tended to have more of the terminally branched monomethylalkanes (Tables 4–6; Figures 10–12). Two samples in particular, those from Scrub Island and Great Camino, displayed hydrocarbon mixtures more similar to soldiers (Figure 12); however,

TABLE 5. RELATIVE QUANTITIES (MEAN AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS OF SAMPLES OF WORKERS, SOLDIERS, AND ALATES OF *Nasutitermes acajutlae* (Holmgren) FROM TORTOLA[a]

Hydrocarbon	Workers (mean ± SD)	Soldiers (mean ± SD)	Alates (mean ± SD)
$C_{23:1}$	0.00 ± 0.00	0.38 ± 0.13	0.00 ± 0.00
C_{23}	0.47 ± 0.16	3.99 ± 0.56	0.08 ± 0.01
11-; 9-MeC_{23}[b]	0.00 ± 0.00	0.66 ± 0.11	0.00 ± 0.00
3-MeC_{23} + $C_{24:1}$[c]	0.00 ± 0.00	0.32 ± 0.06	0.00 ± 0.00
C_{24}	0.07 ± 0.08	0.65 ± 0.05	0.01 ± 0.03
11-MeC_{24}	0.00 ± 0.00	0.46 ± 0.10	0.00 ± 0.00
$C_{25:1}$	0.50 ± 0.18	3.23 ± 0.46	0.00 ± 0.00
C_{25}	2.11 ± 0.59	8.41 ± 0.86	0.57 ± 0.20
13-; 11-MeC_{25}[b]	0.27 ± 0.09	2.52 ± 0.38	0.07 ± 0.06
5-MeC_{25}	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.03
2-MeC_{25}	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.03
3-MeC_{25} + $C_{26:1}$[c]	0.00 ± 0.00	0.62 ± 0.07	0.00 ± 0.00
5,17-DimeC_{25}	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.02
C_{26}	0.06 ± 0.09	0.49 ± 0.08	0.08 ± 0.02
13-; 12-; 11-MeC_{26}[b]	0.00 ± 0.00	0.35 ± 0.09	0.00 ± 0.00
2-MeC_{26}	0.00 ± 0.00	0.00 ± 0.00	1.04 ± 0.24
$C_{27:1}$	0.54 ± 0.17	3.47 ± 0.56	0.00 ± 0.00
C_{27}	0.74 ± 0.24	3.25 ± 1.00	0.47 ± 0.10
13-; 11-MeC_{27}[b]	0.08 ± 0.10	1.08 ± 0.26	0.00 ± 0.00
2-MeC_{27}	0.24 ± 0.15	0.12 ± 0.09	0.61 ± 0.17
3-MeC_{27}	0.00 ± 0.00	0.00 ± 0.00	0.45 ± 0.10
C_{28}	0.13 ± 0.10	0.00 ± 0.00	0.07 ± 0.02
2-MeC_{28}	0.07 ± 0.10	0.00 ± 0.00	0.12 ± 0.02
$C_{29:1}$	0.00 ± 0.00	0.13 ± 0.11	0.00 ± 0.00
C_{29}	0.33 ± 0.09	0.62 ± 0.43	0.41 ± 0.14
C_{31}	0.04 ± 0.07	0.02 ± 0.04	0.28 ± 0.05
C_{33}	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.05
$C_{37:1}$	0.24 ± 0.12	0.38 ± 0.09	0.09 ± 0.02
$C_{38:1}$	0.39 ± 0.08	0.36 ± 0.09	0.26 ± 0.02
$C_{39:5}$	0.25 ± 0.15	0.15 ± 0.12	0.42 ± 0.08
$C_{39:4}$	1.36 ± 0.67	1.02 ± 0.46	2.60 ± 0.69
$C_{39:2}$	0.29 ± 0.29	0.59 ± 0.18	0.87 ± 0.10
$C_{39:1}$	18.06 ± 1.64	12.66 ± 1.68	12.40 ± 0.28
$C_{40:5}$	0.66 ± 0.30	0.44 ± 0.24	1.44 ± 0.29
$C_{40:1}$ + $C_{41:4}$ + $C_{41:5}$[d]	20.77 ± 3.01	18.98 ± 3.25	30.53 ± 2.19
$C_{41:2}$	1.43 ± 0.15	1.60 ± 0.22	3.39 ± 0.26
$C_{41:1}$	24.21 ± 2.06	15.27 ± 1.84	18.13 ± 0.29
15-MeC_{41}	0.39 ± 0.29	0.46 ± 0.25	0.83 ± 0.04
$C_{43:6}$	2.55 ± 0.76	2.05 ± 0.36	3.18 ± 0.64
$C_{43:5}$[e]	5.77 ± 1.19	4.07 ± 1.14	7.18 ± 0.90
$C_{43:2}$	1.12 ± 0.32	1.39 ± 0.15	2.17 ± 0.19
$C_{43:1}$	10.69 ± 0.80	6.77 ± 0.58	8.04 ± 0.34

TABLE 5. Continued

Hydrocarbon	Workers (mean ± SD)	Soldiers (mean ± SD)	Alates (mean ± SD)
15-MeC_{43}	0.20 ± 0.19	0.21 ± 0.10	0.43 ± 0.12
$C_{45:5}$	0.69 ± 0.37	0.59 ± 0.28	0.66 ± 0.31
$C_{45:2}$	0.19 ± 0.15	0.33 ± 0.06	0.26 ± 0.07
$C_{45:1}$	3.09 ± 0.31	1.94 ± 0.09	2.52 ± 0.36

[a] Three subsamples of 200 workers from each of seven colonies. Four subsamples of about 4 ml of soldiers from each of six colonies. Two subsamples of 25–31 alates from three colonies.
[b] An isomeric mixture. These monomethylalkanes coelute.
[c] This monomethylalkane and the olefin coelute.
[d] These three isomers did not completely resolve. Therefore, the areas for the isomers were summed.
[e] There were two isomers of $C_{43:5}$ that did not completely resolve. Therefore, the areas of the two isomers were summed.

TABLE 6. RELATIVE QUANTITIES (MEAN AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS OF SAMPLES OF WORKERS AND ALATES OF *Nasutitermes acajutlae* (HOLMGREN) FROM BRITISH VIRGIN ISLANDS, EXCLUSIVE OF GUANA ISLAND AND TORTOLA[a]

Hydrocarbon	Workers (mean ± SD)	Alates (mean ± SD)
C_{23}	0.63 ± 0.47	0.15 ± 0.31
C_{24}	0.08 ± 0.15	0.00 ± 0.00
2-MeC_{24} + $C_{25\cdot1}$[b,c]	0.32 ± 0.75	0.00 ± 0.00
$C_{25:1}$[c]	1.73 ± 4.45	0.00 ± 0.00
C_{25}	3.65 ± 2.47	0.67 ± 0.83
13-; 11-MeC_{25}[d]	0.27 ± 0.21	0.00 ± 0.00
2-MeC_{25}	0.56 ± 1.59	0.03 ± 0.06
3-MeC_{25} + $C_{26\cdot1}$[b]	0.48 ± 1.29	0.00 ± 0.00
C_{26}	0.20 ± 0.36	0.00 ± 0.00
2-MeC_{26}	0.32 ± 0.60	0.78 ± 0.50
$C_{27\cdot1}$	0.36 ± 0.44	0.00 ± 0.00
C_{27}	1.78 ± 1.22	0.51 ± 0.29
13-; 11-MeC_{27}	0.14 ± 0.18	0.00 ± 0.00
2-MeC_{27}	0.48 ± 0.42	0.63 ± 0.45
3-MeC_{27}	0.04 ± 0.13	0.34 ± 0.23
C_{28}	0.10 ± 0.12	0.04 ± 0.07
C_{29}	0.42 ± 0.31	0.38 ± 0.12
C_{31}	0.00 ± 0.00	0.09 ± 0.10

TABLE 6. Continued

Hydrocarbon	Workers (mean ± SD)	Alates (mean ± SD)
$C_{37:1}$	0.11 ± 0.13	0.05 ± 0.10
$C_{38:1}$	0.29 ± 0.21	0.24 ± 0.16
$C_{39:5}$	0.00 ± 0.00	0.19 ± 0.25
$C_{39:4}$	1.53 ± 1.00	2.08 ± 1.73
$C_{39:3}$	0.16 ± 0.28	0.24 ± 0.48
$C_{39:2}$	0.13 ± 0.43	0.63 ± 0.48
$C_{39:1}$	20.92 ± 4.61	18.18 ± 2.68
15-MeC_{39}	0.07 ± 0.23	0.21 ± 0.42
$C_{40:1}$ + $C_{41:4}$ + $C_{41:5}$[e]	22.28 ± 5.61	30.18 ± 3.38
$C_{41:3}$	0.24 ± 0.79	0.00 ± 0.00
$C_{41:2}$	0.83 ± 1.07	2.81 ± 1.93
$C_{41:1}$	27.65 ± 5.53	26.30 ± 2.54
15-MeC_{41}	0.17 ± 0.34	0.14 ± 0.28
$C_{42:X}$[f]	0.00 ± 0.00	0.25 ± 0.31
$C_{42:1}$	0.77 ± 0.43	0.79 ± 0.55
$C_{43:5}$[g]	3.49 ± 1.14	4.86 ± 0.99
$C_{43:3}$	0.23 ± 0.77	0.00 ± 0.00
$C_{43:2}$	0.81 ± 1.71	0.76 ± 0.55
$C_{43:1}$	8.73 ± 2.67	8.50 ± 1.89

[a] Mean for workers is derived from 11 samples from colonies of *N. acajutlae*: three from Necker Is. and one each from Great Camino, Scrub Is., Eustatia, Virgin Gorda, Lesser Jost Van Dyke, Greater Jost Van Dyke, Great Thatch, and Cooper. Mean for alates is derived from four samples from colonies of *N. acajutlae*: one each from Lesser Jost Van Dyke, Greater Jost Van Dyke, Great Thatch, and Necker Is.

[b] This monomethylalkane and this olefin coelute.

[c] Two separate isomers of $C_{25:1}$ occur in the samples from Great Camino and Scrub Island. This abundant, second isomer (maximum value of 15.1% in the Great Camino sample) was found only in these two samples.

[d] An isomeric mixture. These monomethylalkanes coelute.

[e] These three isomers did not completely resolve. Therefore, the areas for the three isomers were summed.

[f] An isomeric mixture. The exact number of double bonds is difficult to determine. In some instances two or three peaks were present with identical spectra. The areas for all isomers of the same olefin were summed.

[g] This olefin had two isomers that did not completely resolve. The areas for both isomers were summed.

examination of the extracted voucher specimens confirmed that only workers were extracted in these samples. The single sample from Great Camino displayed some distinct differences from samples collected on other islands. The later-eluting isomer of $C_{25:1}$ was present in great abundance, its peak area exceeding that of *n*-C_{25} (Figure 12B). This sample from Great Camino was the only one in which two isomers of $C_{43:3}$ were detected in measurable amounts.

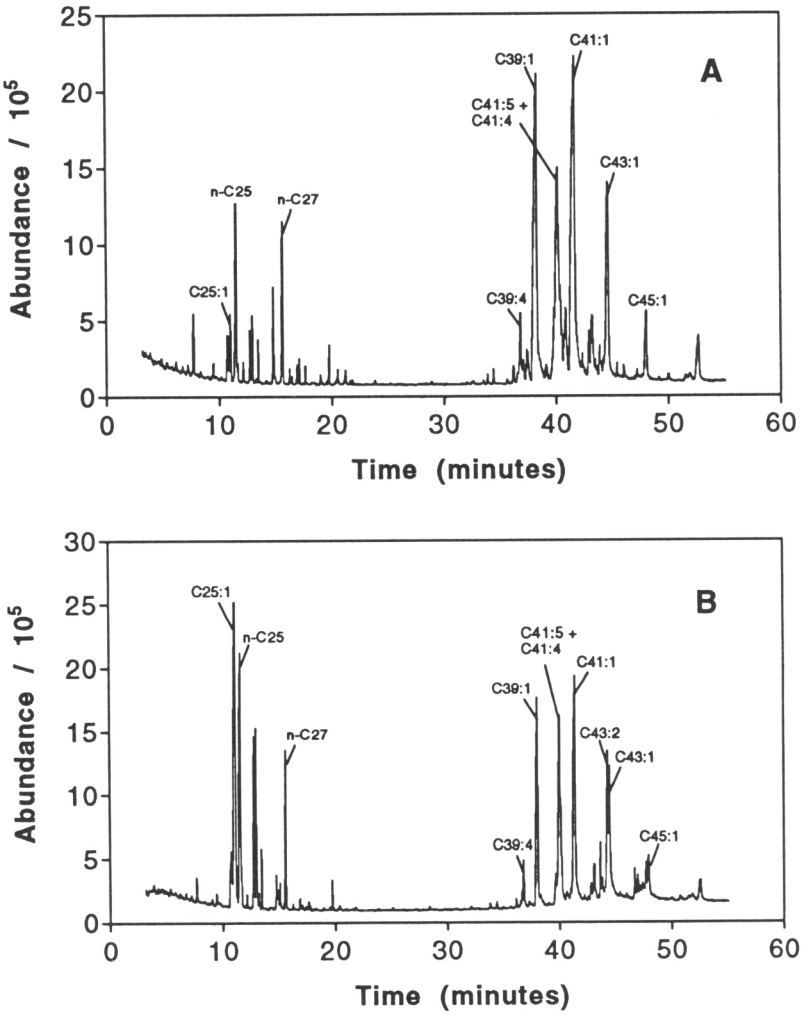

FIG. 12. Total ion chromatogram of the cuticular hydrocarbons from workers of *Nasutitermes acajutlae* from (A) Scrub Island and (B) Great Camino.

The retention time of $C_{43:2}$ in this sample indicated that this compound was a different isomer of $C_{43:2}$ than was seen in other samples of *N. acajutlae*. In the Great Camino sample, $C_{43:2}$ was not completely resolved from $C_{43:1}$; this is true for $C_{45:2}$ and $C_{45:1}$ as well (Figure 12B). The existence of island-to-island differences in hydrocarbon mixtures within one species should alert us to exercise caution when evaluating the use of cuticular hydrocarbons in termite taxonomy.

TABLE 7. DIAGNOSTIC CUTICULAR HYDROCARBONS (>1.0% OF TOTAL HYDROCARBON) FROM PSEUDERGATES (LARVAE AND NYMPHS) OR WORKERS OF 8 TERMITE TAXA FROM BRITISH VIRGIN ISLANDS[a]

Hydrocarbon	Termite species[b]							
	N mon	C bre	P cor	I spp[c]	H sp	P wol	N cos	N aca
C_{25}	+++	+++	+++	+++	0	0	++	+++
C_{26}	+++	++	++	++	tr	0	0	tr
C_{28}	tr	+	tr	tr	++	++	+	tr
C_{29}	+	++	+	+	++	++	++	+
13-; 11-MeC_{25}	+++	+	0	+	0	0	0	tr
13-; 12-MeC_{26}	+++	0	0	0	0	0	0	0
13-; 11-; 9-; 7-MeC_{27}	+++	tr	tr	tr	+++	0	+	tr
14-; 13-; 12-; 9-; 7-MeC_{28}	+	0	0	0	+++	0	+	0
15-; 13-; 11-; 9-; 7-; 5-MeC_{29}	tr	0	0	0/+	+++	+	+++	0
15-; 13-; 11-; 9-MeC_{31}	tr	0	0	0	+	0	+++	0
2-MeC_{24}	++	+++	+++	+++	0	0	0	0
2-MeC_{25}	+++	++	+++	+++	0	0	0	0
3-MeC_{25}	+++	+++	+++	+++	0	0	0	0
2-MeC_{26}	++	+	+++	+/++	+	0	0	0
3,X-$DimeC_{25}$	++	tr	0	0	0	0	0	0
11,15-$DimeC_{27}$	++	0	0	0	+++	0	++	0
9,17-$DimeC_{27}$	0	0	0	0	+++	0	0	0
9,X-$DimeC_{28}$	0	0	0	0	+++	0	0	0
13,17-; 11,15-$DimeC_{29}$	0	0	0	0	0	0	+++	0
9,19-; 9,17-$DimeC_{29}$	0	0	0	0/+	+++	0	0	0
7,21-$DimeC_{29}$	0	0	0	0	+++	0	0	0
11,15-; 12,16-; 13,17-$DimeC_{30}$	0	0	0	0	0	0	+++	0
11,15-$DimeC_{39}$	++	0	0	0	0	0	0	0
13,17-$DimeC_{41}$	++	0	0	0	0	0	0	0
$C_{25:1}$	0	0	++	+++	0	0	0	+
$C_{27:1}$	tr	0	0	+++	0	0	tr	+
$C_{37:2}$	0	+++	++	0	0	0	0	0
$C_{39:2}$	0	+++	+++	0/+	0	0	0	+
$C_{39:1}$	0	++	++	tr	tr	0	0	+++
$C_{40:1}$	0	0	0	0	0	0	0	+++
$C_{41:5}$	0	0	0	0	0	0	0	+++
$C_{41:4}$	0	0	0	0	0	0	0	+++
$C_{41:3}$	0	+++	+++	0/+	0	+++	0	0
$C_{41:2}$	0	+++	+++	0/++	0	0	0	++
$C_{41:1}$	0	++	+++	0/+	0	0	0	+++
$C_{43:4}$	0	0	+	0	0	+++	0	+++
$C_{43:3}$	0	++	++	0/+++	0	+++	0	0
$C_{43:2}$	0	++	++	+++	0	0	0	+
$C_{43:1}$	0	++	0	0/++	0	0	0	+++

TABLE 7. Continued

Hydrocarbon	Termite species[b]							
	N mon	C bre	P cor	I spp[c]	H sp	P wol	N cos	N aca
$C_{45:4}$	0	0	0	0/+	0	+++	0	0
$C_{45:3}$	0	++	0	0/++	0	+++	0	0
$C_{45:1}$	0	0	0	0/+++	0	0	0	++

[a] Relative proportions of the total hydrocarbon mixture for each species. +++ = >3.0%; ++ = 1.0-3.0%; + = 0.3-0.99%; and tr = <0.3%; 0 = not detected.

[b] N mon = *Neotermes mona*; C bre = *Cryptotermes brevis*; P cor = *Procryptotermes corniceps*; I spp = *Incisitermes* species; H spp = *Heterotermes* species; P wol = *Parvitermes wolcotti*; N cos = *Nasutitermes costalis*; N aca = *Nasutitermes acajutlae* (from Guana Island).

[c] *Incisitermes* spp. displayed a wide range of hydrocarbon mixtures. For example, 0/+++ would denote the range from absent to above 3%.

Taxonomic and Biogeographic Value of Cuticular Hydrocarbon Profiles. One of the objectives of our studies of the termite fauna of the British Virgin Islands was to begin to build a library of cuticular hydrocarbon profiles correlated with species determinations based on morphological characters. Much of this work will be published separately as in-depth studies of individual taxa (genera or species complexes) from the Caribbean Basin. BVI termite species that were readily identifiable on the basis of morphological characters of the soldiers or alates also had diagnostic cuticular hydrocarbon mixtures. Using only the consistently abundant hydrocarbons, one could unambiguously identify species based on characterization of the hydrocarbons of workers, larvae, pseudergates, or nymphs without the sometimes rare soldiers and alates needed for morphological diagnoses (Table 7). Separation of closely related taxa has been demonstrated for species of *Nasutitermes* from the Caribbean Basin and *Zootermopsis* from western North America using the consistently abundant hydrocarbons (Haverty et al., 1988, 1992).

Cuticular hydrocarbons may eventually help resolve one of the more difficult taxonomic problems among the termite species of the British Virgin Islands, i.e., separation of species within the genus *Incisitermes*. *Incisitermes* from the Cayman Islands identified as *I. tabogae* (Snyder) possess a distinct cuticular hydrocarbon mixture clearly separable from the *Incisitermes* examined from the BVI (Haverty et al., unpublished observations). Similarly, the taxonomy of *Heterotermes* might be clarified if consistent hydrocarbon mixtures can be used to presort specimens for morphological study.

CONCLUSIONS

All classes of hydrocarbons are seen among the eight termite taxa characterized from the BVI. All taxa have normal alkanes present in their cuticular hydrocarbon mixture: n-C_{25} and n-C_{27} are the most abundant. Internally branched monomethylalkanes are not commonly seen or are present in very small amounts relative to all other hydrocarbons; only *Neotermes mona* and *Heterotermes* sp. incorporate these components in relatively large quantities. Terminally branched monomethylalkanes are much more common in most of the species, but are present only in trace amounts in *Nasutitermes costalis* and *N. acajutlae*. Dimethylalkanes are present in relatively large quantities only in *N. mona*, *Heterotermes* and *N. costalis*, species with rather high moisture requirements, and are absent or present only in trace amounts in the other taxa. Trimethylalkanes are quite rare; they are completely absent in six of the taxa, present in trace amounts in *Incisitermes* and in small amounts in *N. costalis*.

In general, olefins are the most common of the hydrocarbons found in the termites of the BVI. Early-eluting alkenes are abundant in *Incisitermes* spp., but absent or rare in all other taxa. Late-eluting olefins, especially those with 39, 41, and 43 carbons, are quite abundant for many of the species. These late-eluting olefins have one to six double bonds. The positions of these double bonds were not determined, but for the purposes of this paper we feel it is not essential to know their location.

Polyunsaturated hydrocarbons are common in the termites of the BVI. This degree of unsaturation is not common in termites we have sampled from temperate or subtropical locations. For the termites that live in above-ground nests in dry habitats or entirely within dry wood, cuticular hydrocarbon mixtures consist of generally larger molecules, reflecting the moisture demands of this habitat (Collins et al., 1997; Hadley, 1980, 1985). Termites that live within wood on live trees or in situations with more available moisture generally have a hydrocarbon mixture composed mostly of lower-molecular-weight components with carbon numbers ranging from 23 to 33 (Collins et al., 1997).

Consistently abundant hydrocarbons can be used as taxonomic characters for separating the termites of the BVI. Variation in hydrocarbon components was shown for *N. acajutlae* from different islands, but the differences were relatively minor. *Incisitermes* presented the greatest challenge; the variation in cuticular hydrocarbons was as great as that of soldier morphology.

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IDENTIFICATION OF MALE PECAN WEEVIL PHEROMONE

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Abstract—The pecan weevil, *Curculio caryae* is a serious economic pest of pecans (*Caryae illinoensis*). In late summer, the weevil attacks maturing nuts and damages them when making feeding and/or oviposition punctures. The larvae leave the nut and burrow into the soil, remaining there for two to three years before emerging as adults to commence another cycle. This present work has resulted in the identification of the male pecan weevil pheromone as a mixture of four components; I as both the *cis* and *trans* isomers of 2-propenyl-1-methyl-cyclobutaneethanol [also identified as (1*R*,2*S*)−(+ and −)-grandisol], II [(*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\beta}$-ethanol], III [(*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde], and IV [(*E*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde]. They are synthesized by the male pecan weevil, but not by the female, in the ratio 7:16:3:3 of I, II, III, and IV, respectively. These same compounds were earlier identified as the pheromone of the male boll weevil, *Anthonomus grandis* (Boh.), in which they were isolated from frass in the ratio 6:6:1.5:1.5. However, only the (+) isomer of grandisol was synthesized by male boll weevil. In laboratory tests, 80% of female pecan weevils were attracted to a synthetic formulation based on the ratio found in male pecan weevils, while only 28% of the females were attracted to a synthetic formulation based on the ratio found in boll weevil frass. The attraction of males to these synthetic formulations was minimal (14, 4, and 2%, respec-

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tively). Live males and their extracts were also attractive to females, but males did not respond to male or females. Preliminary field tests demonstrated that females were attracted to males and the synthetic pecan weevil formulation, but not to the synthetic boll weevil formulation.

Key Words—Pecan weevil, pheromone.

INTRODUCTION

The pecan weevil, *Curculio caryae* (Horn), attacks maturing pecan [*Caryae illinoensis* (Wangenh) Koch] fruit in late summer and damages them by making feeding and oviposition punctures. The larvae, upon completing development in the nut, leave through a small hole cut in the shell and burrow into the soil. The weevils remain there for a period of two to three years before emerging as adults to commence another cycle (Raney and Eikenbary, 1968; Van Cleave and Harp, 1971).

Van Cleave and Harp (1971) reported that field-caged female pecan weevils attracted more weevils of both sexes than did caged male weevils. Polles et al. (1977) reported on field studies in which wing-type traps, each baited with six live females and pecan nutlets for food, captured 85 pecan weevils, 73% of them males. The same number of traps baited with males captured 56 weevils, 66% males; blank traps captured 55 weevils, 65% males. Polles et al. (1977) also baited traps with one or more of the components of the pheromone of the boll weevil *Anthonomus grandis grandis* Boheman (Tumlinson et al., 1969) on the premise that related insects may biosynthesize and respond to related compounds. A total of 23 pecan weevils, 87% of them male, were captured with the four-component mixture, grandlure. With (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol (component I alone), the captures totaled 7 (86% males); with (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol (II), there were 15 (100% males); and with (*Z*)- and (*E*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (III and IV), there were 10 (90% males). However, the blank captured about as many as did individual components (10 weevils, 70% males). Therefore, none of these tests was sufficiently comprehensive to permit statistical evaluations, and in no instance was further work reported.

In 1973, Mody et al. (1973) bioassayed fractions obtained from volatile oils of each sex of pecan weevils in field tests. Primarily males were trapped with female fractions, and primarily females were trapped with male fractions. GLC-MS was used to identify a number of volatile components from the male and female oils, but on the basis of their structures, none appeared capable of accounting for the attractancy of either sex.

Hedin et al. (1979) showed that male and female pecan weevils were

attractive to their opposite sex using a newly developed laboratory bioassay. Extracts of males attracted females and vice versa. (*Z*)-3,3-Dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol was identified as present in a weevil extract and shown to possess some attractiveness to both sexes in preliminary field bioassays.

The study of the behavior of this insect has been handicapped for several reasons. Efforts to shorten or eliminate the two to three-year diapause have been unsuccessful. No large-scale laboratory rearing system has been developed. Efforts to obtain adults from larvae by maintaining them in soil beds have resulted in poor yields and poor insect vigor. The collection of adults emerging from the soil in the late summer has not been reliable because it is largely dependent on precipitation, which may be erratic. Consequently, the search for more definitive chemical information has been stymied until now. This present report details results of a number of analyses and bioassays to identify the male pecan weevil pheromone.

METHODS AND MATERIALS

Procurement of Insects and Collection of Extracts and Washings for GLC-MS Analysis. Two trapping sites were established for obtaining the weevils to isolate the weevil pheromone. The first site was located near the Samuel Robert Noble Foundation Red River Demonstration Farm, Burneville, Oklahoma. The second trapping site was located on the Horticultural Research Station, Stillwater, Oklahoma. Weevils were also collected from cone emergence traps (Eikenbary et al., 1978) placed under infested pecan trees (Raney and Eikenbary, 1968; Boethel et al., 1976; Eikenbary et al., 1978).

Insect extracts and washings for bioassay and chemical work were obtained using the following procedures. After capture, individual weevils were placed in vials and transported to the laboratory, sexed, and then transferred to feeding chambers (200-ml glass jars) with immature pecans for five days. Individual pairs of males and females were then placed in 28-ml glass jars with Teflon lids. In two tests, male/female washings were collected by rinsing the chamber after removal of the pair with methylene chloride (ca. 1 ml; Test A) or alternatively hexane (Test B), after the males had mounted and assumed the mating posture. The washings were then transferred to an amber glass bottle, combined with the other replicates, and stored at −20°C.

In four other tests, pairs were placed in mating chambers and observed until excitation occurred, but males were not allowed to mount or assume a mating posture. The individuals were separated and males (tests C and D) and females (tests E and F) were washed with 1 ml of methylene chloride. Replicate washings were combined and stored at −20°C. The summary of collections

was as follows: A—68 M/F, 5 days old, in methylene chloride; B—68 M/F, 5 days old, in hexane; C—68 males, 5 days old, in methylene chloride; D—68 males, 5 days old, in methylene chloride (replicate); E—68 females, 5 days old, in methylene chloride; and F—68 females, 5 days old, in methylene chloride (replicate).

Procurement of Pheromone Components. The four components, a mixture of *cis*- and *trans*-2-isopropenyl-1-methyl-cyclobutaneethanol [also identified as (1*R*,2*S*-(+ and −)-grandisol) I, [(*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\beta}$-ethanol] II, [(*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde] III, and [(*E*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde] IV, originally synthesized by Tumlinson et al. (1969, 1971), were obtained from MTM Chemicals, Columbus, Ohio.

Synthetic Pheromone Formulations. Formulations were based on the following rationales. The ratio of the four boll weevil components in boll weevil frass was found to be 6:6:1.5:1.5 of I, II, III, and IV, respectively (Tumlinson et al., 1969, 1971). The relatively greater cost of I led to the use of the formulation 3:4:1.5:1.5, which was found to be adequately attractive in field tests and has been used since then as the commercial boll weevil sex pheromone formulation. In the male pecan weevil, these pheromone components were found to be present in a ratio of 7:16:3:3 (see Results and Discussion).

Formulations based on the ratios found by analyses of the previously described washes and extracts of live pecan weevils and the previously published ratio of components found in boll weevil frass (Tumlinson et al., 1969, 1971) were dispersed in laminates prepared by Hercon Environmental Corporation, Emigsville, Pennsylvania, so that 2.5-cm-square sections contained 10 mg of the prescribed four-component mixtures. Hercon Luretape is a laminated, three-layered plastic dispenser; a reservoir layer contains the active ingredients that are sandwiched between two outer permeable layers of polyvinyl chloride film. The inner layer is also a polyvinyl chloride film. Laminates of the commercial boll weevil formulation were also procured from Hercon. In the synthetic formulation, both the (+) and (−) isomers of I are present. Pecan weevils synthesize both isomers (see Results and Discussion), while the boll weevil sysnthesizes only the (+) isomer (Tumlinson et al. 1969, 1971).

Laboratory Bioassays. Laboratory tests were done to determine the relative attractiveness of the synthetic formulations of: (1) the pecan weevil pheromone, (2) the commercial boll weevil pheromone, (3) a synthetic formulation based on the ratio of components in boll weevil frass, and (4) live pecan weevil males to both sexes of the pecan weevil. The bioassays were carried out in a choice olfactometer in which the airflow was calibrated at 25 ml/min (Hardee et al., 1967). The candidate pheromone lure or live males were placed in one arm of the olfactometer with no lure in the other arm. Virgin, unfed males or females were introduced into the olfactometer between 0900 and 1400 hr, which had

been determined to be the hours of maximum mating activity of the pecan weevil (Collins, unpublished data). Responses of the insects were recorded after 1 hr. For each candidate pheromone tested, 100 replications were employed, except for the boll weevil frass formulation where 50 replications were employed. The data were analyzed using the chi-square procedure (SAS Institute, 1991).

Field Methods. To test the effectiveness of the pecan weevil pheromone in the field, a site at the OSU Horticulture Research Station near Sparks, Oklahoma, containing native trees was chosen. The experimental design was a randomized complete block with a 2 × 3 factorial treatment. Two trap types, boll weevil traps and Tedders traps (Tedders and Wood, 1995) were placed in six 1-ha blocks. Three treatments—synthetic pecan weevil pheromone, synthetic boll weevil pheromone—and checks were employed per replication with 1 ha separating each treatment. Data were analyzed using a split plot analysis with time as the subplot.

Extractions, Fractionations, and Gas Chromatography Mass Spectral Analysis of Insects. A 1- × 30-cm column equipped with a bulb and frit was filled with a slurry of 6 g of Baker silica gel (60–200 mesh, 3405-05) in hexane (Optima, Fisher Scientific). Previous to application of the extract to the column, the insects that had been stored at −20°C under hexane or hexane—methylene chloride were ground with a Polytron homogenizer. The entire contents were applied to the column, which was then washed first with 125 ml hexane and then with 125 ml of methylene chloride. Each fraction was concentrated to 1 ml or less for GLC-MS. The fractions were monitored using silica gel TLC plates that were chromatographed with 50% methylene chloride in hexane and visualized in an iodine chamber. It was determined, as expected, that the hexane eluate consisted mostly of hydrocarbons, and a separate column chromatographic test demonstrated that the boll weevil grandlure components were not eluted with hexane but with methylene chloride. Nevertheless, several GLC-MS analyses were performed on both the hexane and methylene chloride fractions.

All GLC-MS analyses (except for one) were performed with a DB-1 column (J & W Scientific, Folsom, California; 30 m × 0.32 mm ID × 0.25-μm layer thickness), injection temperature 280°C, transfer line 300°C, program was: 45°C, 2 min hold, 15°C/min to 300°C, 5 min hold; split ratio 15:1.

A chiral column, Beta-Dex 120 (Supelco, Inc., Bellefonte, Pennsylvania, 30 m × 0.25 mm ID × 0.25-μm thickness), operated at a column temperature of 150°C, injection temperature 180°C, detector 250°C, FID, split ratio 80:1, was employed to separate the (+) and (−) isomers of component I. The columns were interfaced to a Hewlett-Packard HP-5989 quadruple mass spectrometer operated in the EI mode. Spectral interpretations were supported by the NIST/EPA/MSDC Mass Spectral Database 1A PC Version 3.0 (Lias and Stein, 1990), and the HP 59944C MS Chem System Version 8.05 (1992).

RESULTS AND DISCUSSION

Chemical Analyses. GLC-MS analyses conducted in 1993 on six collections of male, female, and mixed body washings and extracts gave 28 significant maxima, of which structural assignments were made for 19 (Table 1). Unambiguous mass spectral data for the presence of the four boll weevil pheronome components (Figure 1) were obtained from male collections C and D while male/female collections A and B (see Methods and Materials) contained only the more prevalent components I and II. Female collections E and F did not contain any of these constituents (Table 1).

Figure 2 is a mass chromatogram of male collection C. Mass spectra of the four small maxima between 6.5 and 7.2 min were those of the four boll weevil sex pheromones. From the total ion count, the content from 68 males was determined to be 0.29 μg/insect of I + II + III + IV. The distribution was approximately 0.07 μg I, 0.16 μg II, 0.03 μg III, and 0.03 μg IV. The chromatogram of male collection D included four maxima of apparent masses M+ 152–156 intermingled with those of the four grandlure components. Two of these were also present in collection C, but not in either of the female collections E and F. Figures 3 and 4 present mass spectra for these four maxima from collection D at I_k 1190, 1195, 1210, and 1295. While these spectra could not be matched using the available data bases, they have been included as a possible help to some future investigation on the assumption that other terpenoids could also contribute in some way to a behavioral response by the pecan weevil.

Figure 5 includes chromatograms of synthetic grandlure (A), male pecan weevil collection C (B), and boll weevil frass (C) that were obtained with a chiral GLC column. Compound I exists in the boll weevil and its frass only as (1*R*,2*S*)-(+)-grandisol. We later showed that the antipode (−)-grandisol, although not synthesized by the male boll weevil, is also active (Mori et al., 1978). Both (+) and (−) isomers are present in the synthetic grandlure (8.04 and 8.19 min, Figure 5A) and in the male pecan weevil (8.32 and 8.47 min, Figure 5B), while the boll weevil frass contains the (+) isomer at 8.04 min (Figure 5C). Mass spectral data confirmed the identities of these maxima even though the retention volumes were slightly different. While compounds II, III, and IV were poorly separated by this chiral column, the mass spectral data obtained with the DB-1 column (Figure 2) were definitive.

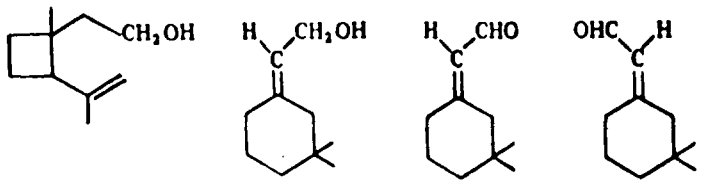
Results of Laboratory Bioassays. Olfactometer data were analyzed using a chi-square (SAS Institute, 1987) on all possible pairwise combinations. Significant differences were observed between all combinations at $P < 0.05$ except one. The data on percent attractiveness are summarized in Table 2.

In these tests, 43% of pecan weevil females responded to males, but no males responded to males or to females. The response of females to the synthetic

TABLE 1. GLC-MS ANALYSES OF METHYLENE CHLORIDE FRACTIONS FROM WASHINGS OF PECAN WEEVILS

I_k	MW	Compound	A (♂, ♀)	B (♂, ♀)	C (♂)	D (♂)	E (♀)	F (♀)
815	100		x	x	x	x	x	x
850	100	1-methyl-cyclopentanol	x	x	x			
865	100	3-hexanol	x	x	x	x	x	x
880	94	phenol	x	x	x	x	x	x
955	100	3-hexanone	x		x			x
962	98	2-hexenal						x
1005	142	*n*-decane	x	x	x	x	x	x
1080	142	*n*-nonanal	x	x	x	x		x
1190		*m/e* 112			x	x		
1195	(154)	*m/e* 135				x		
1200	154	Cpd I[a]	x	x	x	x		
1210	152/154	*m/e* 125				x		
1215	154	Cpd II[a]	x	x	x	x		
1225	152	Cpd III[a]			x	x		
1235	152	Cpd IV[a]			x	x		
1240	154	(*Z*)-2-decenal				x		
1250		*m/e* 142	x		x			
1280	152	thujone				x	x	
1282	158	1-decanal				x		
1295		*m/e* 127			x	x		
1295	156	*m/e* 113	–	–	x	x	–	–
1350	182	2-dodecenal						
1425	220	∝-caryophyllene oxide				x		
1510	250		x	x	x	x		x
1520	222					x		
1610	222	bulnesol		x		x		
1690	228	myristic acid				x	x	
2010	268	oleyl alcohol					x	x

[a] I [*cis*- and *trans*-2-isopropenyl-1-methylcyclobutaneethanol], II [(*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\beta}$-ethanol], III [(*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde], and IV [(*E*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde].



(I) (II) (III) (IV)

FIG. 1. Structures of the four boll weevil sex pheromone components. See Table 1 for nomenclature.

formulation based on the ratio of components present in pecan weevil males (7:16:3:3 of I, II, III, and IV, respectively) was 80%, while only 14% of males responded to this formulation (Table 2). The response of females to the commercial boll weevil pheromone (3:4:1.5:1.5) was somewhat less: 60% of females and 4% of males. Finally, the response of females to the formulation based on boll weevil frass (6:6:1.5:1.5) was significantly lower: 28% of females and 2% of males. In our earlier work (Hedin et al., 1979) we had established that females were not attractive to other females.

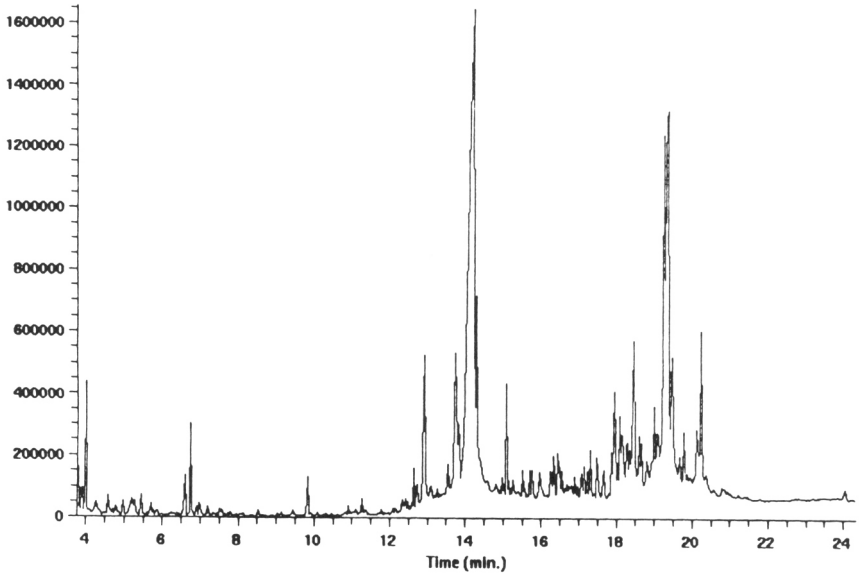


FIG. 2. GLC-MS of male pecan weevil collection C. Note the four boll weevil pheromone components at 6.5–7.2 min; their total content is 0.29 μg/insect.

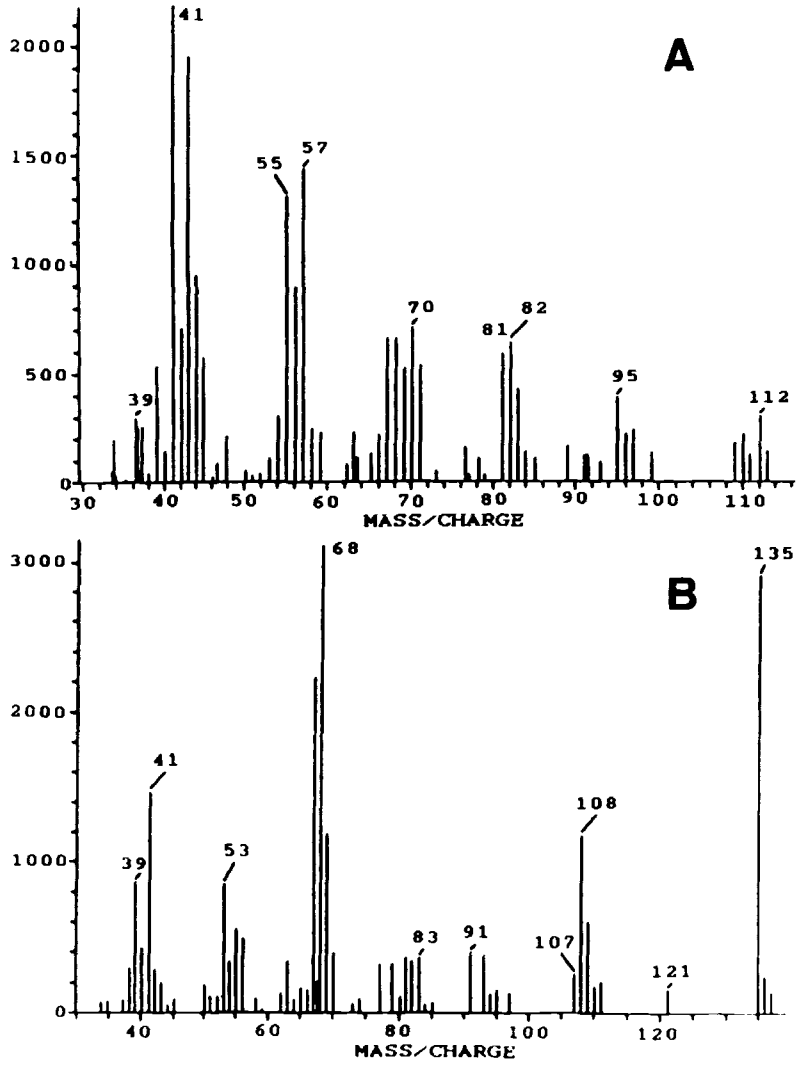


FIG. 3. Mass spectral fragmentation patterns for two male pecan weevil maxima from collection D at I_k 1190 (A) and I_k 1195 (B); see Table 1.

These chemical analyses showed that the ratio of the four boll weevil components synthesized by male pecan weevils is significantly different from that synthesized by male boll weevils. The bioassays show that the response of female pecan weevils to the synthetic formulation based on the ratio in male pecan weevils is significantly stronger than that to the formulation based on the

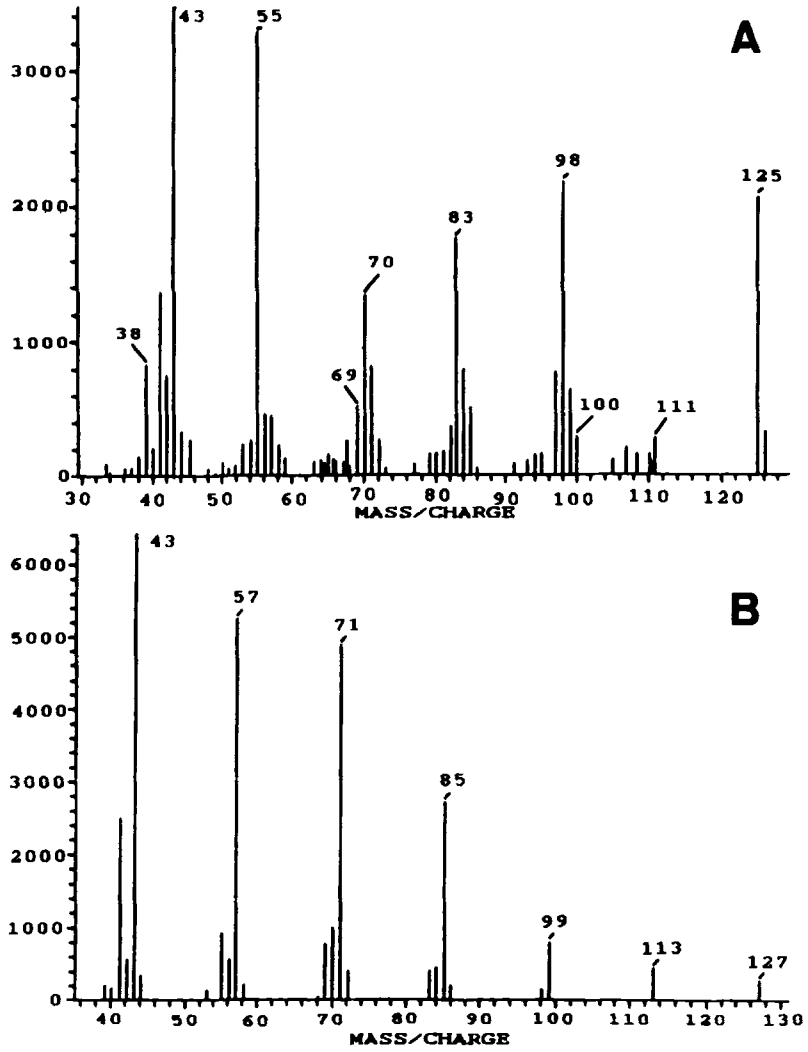


FIG. 4. Mass spectral fragmentation patterns (see Table 1) for two male pecan weevil maxima from collection D at I_k 1210 (A) and I_k 1295 (B).

ratio in boll weevil frass. Therefore, the pecan weevil pheromone, although consisting of the same four components, is unique because of the difference in the ratio of components synthesized by males and responded to by females. The low response by males to the formulations and not at all to females or males (Table 2) demonstrates that the males are the primary attractive sex, and females

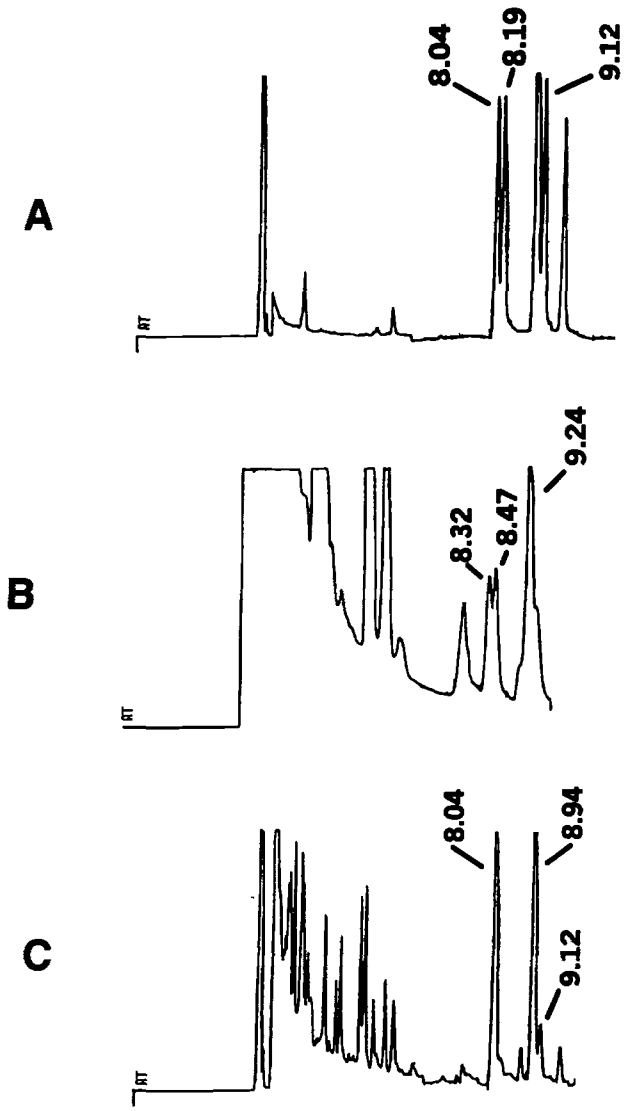


FIG. 5. GLC separations of synthetic grandlure (A), male pecan weevil collection C (B), and boll weevil frass (C). Note that only (+)-grandisol (8.04 min) is present in boll weevil frass (C) while both (+)- and (−)-grandisol are present in male pecan weevil and synthetic grandisol.

TABLE 2. OLFACTOMETER TESTS OF RELATIVE ATTRACTIVENESS OF THREE SYNTHETIC PHEROMONE FORMULATIONS AND VIRGIN FEMALE AND MALE PECAN WEEVILS[a]

Sex responding	Pecan weevil pheromone 7:16:3:3	Boll weevil pheromone 3:4:1.5:1.5	Boll weevil frass 6:6:1.5:1.5	Live males	Live females
Female (%)	80 a	60 b	28 d	43 c	
Males (%)	14 e	4 f	2 f	0 f	0 f

[a]Numbers followed by the same letter are not significantly different (chi-square $P = 0.05$).

are the primary responders. Because the male pecan weevil synthesizes both the (+) and (−) isomers of I, and female pecan weevils respond to the synthetic formulations that include both isomers, it is presumed that both isomers are either attractive or at least not repellent to the female.

Although these four components were first identified for the boll weevil pheromone, one or more of the structures and variations thereof have since been reported as pheromone components for a number of insects. In this sense, the finding that these components are pheromones in the pecan weevil, a phyllogenetically related insect, is not surprising.

Preliminary Field Experiments. The tests showed that synthetic formulations based on the male pecan weevil pheromone ratio were significantly more effective than synthetic formulations based on the commercial boll weevil ratio or unbaited traps ($P > 0.05$). A t test grouped the control and the boll weevil pheromone together with a mean of 0.55 and 0.88 females per trap, respectively. Traps employing the pecan weevil pheromone captured a mean of 5.00 females per trap with a LSD of 1.67. Additional field trials are planned to further evaluate the synthetic pecan weevil pheromone. Information from these trials may then lead to the use of the pheromone as an indicator of pecan weevil densities in the field. In addition, it is conceivable that if pheromone attractiveness and trap efficiency can be enhanced, their combination may provide a partial control alternative in insecticide sensitive urban environments.

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EFFECTS OF CARBON DIOXIDE, WATER SUPPLY, AND SEASONALITY ON TERPENE CONTENT AND EMISSION BY *Rosmarinus officinalis*

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Abstract—*Rosmarinus officinalis* L. plants were grown under carbon dioxide concentrations of 350 and 700 μmol/mol (atmospheric CO_2 and elevated CO_2) and under two levels of irrigation (high water and low water) from October 1, 1994 to May 31, 1996. Elevated CO_2 led to increasingly larger monthly growth rates than the atmospheric CO_2 treatments. The increase was 9.5% in spring 1995, 23% in summer 1995, and 53% in spring 1996 in the high-water treatments, whereas in low-water treatments the growth response to elevated CO_2 was constrained until the second year spring, when there was a 47% increase. The terpene concentration was slightly larger in the elevated CO_2 treatments than in atmospheric CO_2 treatments and reached a maximum 37% difference in spring 1996. There was no significant effect of water treatment, likely as a result of a mild low water treatment for a Mediterranean plant. Terpene concentration increased throughout the period of study, indicating possible age effects. The most abundant terpenes were α-pinene, cineole, camphor, borneol, and verbenone, which represented about 75% of the total. No significant differences were found in the terpene composition of the plants in the different treatments or seasons. The emission of volatile terpenes was much larger in spring (about 75 μg/dry wt/hr) than in autumn (about 10 μg/dry wt/hr), partly because of higher temperature and partly because of seasonal effect, but no significant difference was found because of CO_2 or water treatment. The main terpene emitted was α-pinene, which represented about 50% of the total. There was no clear correlation between content and emission, either quantitatively or qualitatively. More volatile terpenes were proportionally more important in the total emission than in total content and in autumn than in spring.

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Key Words—Terpenes, α-pinene, camphor, borneol, cineole, emission, carbon-based secondary compounds, biomass, nitrogen, water, carbon dioxide concentrations.

INTRODUCTION

Plant terpenoid concentrations are generally in the range of 1–2% dry weight, but they can reach as much as 15–20% of the dry weight of a plant (Ross and Sombrero, 1991; Langenheim, 1994). The annual rate of monoterpene emission from vegetation is estimated at 120–150 $\times$ 10^{12} grams carbon per year (g C/yr), representing 0.1–0.3% of global net primary productivity (Lerdau et al., 1995). Terpenes emitted by plants are significant sources of ozone formation and one of the principal factors regulating oxidative capacity of the atmosphere (Singh and Zimmerman, 1992).

The great ecological interest of plant terpenoids is reflected in the several reviews of the ecological chemistry and role of terpenoids (Harborne, 1991; Gershenzon and Croteau, 1991; Lerdau and Peñuelas, 1993; Langenheim, 1994; Gershenzon, 1994). Defensive and infochemical roles have persistently been highlighted in the plant relationships with other parts of the plant, other plants, animals, and microorganisms (Langenheim, 1994; Bruin et al., 1995; Peñuelas et al., 1995a, 1996a). Thus, changes in terpene concentrations and emission rates in plants are of great ecological interest. In spite of this importance in ecology and atmopheric chemistry, little is known of the factors controlling terpene concentration and emission.

Rising atmospheric CO_2 concentrations may increase the concentration of carbon-based secondary compounds, including terpenes, through increased photosynthetic carbon gain and carbon excess, as expected from carbon nutrient balance or growth differentiation balance hypotheses (Bryant et al., 1983; Herms and Matson, 1992; Lambers, 1993; Peñuelas et al., 1996b). Our recent compilation of data from 17 species (Peñuelas et al., 1997) showed that at elevated CO_2 concentrations there were, on average, 14% larger carbon-based secondary compound concentrations in the species studied. However, terpenes (mevalonic pathway) seemed to be less responsive than phenolics (shikimic acid pathway), and there was wide variation in the production of carbon-based secondary compounds with respect to studies, species, and compounds (Peñuelas et al., 1997). This may indicate that allocation to secondary compounds, especially terpenoids, is not completely governed by changes in CO_2 availability, and it may be closely regulated biochemically (Lavola and Julkunen-Tiitto, 1994).

Seasonal variations in terpene concentrations and emissions have been reported by several authors (Yokouchi and Ambe, 1984; Lerdau et al., 1995; Zou and Cates, 1995) but variations are still not well known. In addition, there

is little information on the effect of other factors such as water stress characteristic of summer conditions in Mediterranean ecosystems, in which terpenes are very abundant (Enders, 1994).

In order to test the possible increase of terpenoid content and emission under increased atmospheric CO_2 concentrations and also the role of seasonality and water availability characteristic of Mediterranean conditions, we analyzed terpenoid content and emission of a very abundant, typical Mediterranean plant, *Rosmarinus officinalis* L., growing at different CO_2 concentrations and different water supplies throughout the year.

METHODS AND MATERIALS

Plants. Assays were conducted in multitunnel-type greenhouses located at Cabrils (Catalonia, NE Spain) (2°30′E, 41°45′N). Cuttings of *Rosmarinus officinalis* L. plants from the same clone were used in the assay in order to reduce genetic variation. Plants were grown in 12-liter pots filled with a sandy-clay soil. They were fertilized with Nitrophoska 12-12-17 every three months. They were grown for two years at atmospheric CO_2 concentrations of 350 μmol/mol (atmospheric CO_2) and at an elevated level of 700 μmol/mol (elevated CO_2) in two greenhouses, each having an area of 77 m^2. The CO_2 concentration in each of the two greenhouses was monitored by an infrared gas analyzer and maintained within an accuracy of ± 50 μmol/mol during day and night. The accuracy decreased in summer months when greenhouse roofs were open for 6 hr around midday (no samples were taken in summer). Two irrigation treatments were established. Highly watered plants received 2 liters of tap water per plant per week in spring, summer, and autumn, and 1 liter in winter, and in the low-water treatment, plants received 1 and 0.5 liters, respectively. The CO_2 treatments were exchanged between greenhouses every year in order to minimize potential greenhouse effects. Greenhouse temperature averaged 2–5°C warmer than that outside. The cuvette temperatures when sampling were 22–23°C on June 21 and 22–23°C on November 7, 1995, and 30–31°C on May 31, 1996.

Sampling and Terpene Analysis. Plants were sampled on March 15, June 21, and November 7, 1995, and on May 31, 1996, between 8 and 10 AM solar time. We collected the leaves of 24 plants (six per treatment) and took them in black plastic bags with a moist paper inside to the laboratory, where they were extracted in pentane (Hall and Langenheim, 1986); dodecane was used as an internal standard.

The leaf monoterpene contents were monitored with a gas chromatograph–mass spectrometer (GC-MS) (Hewlett-Packard HP59822B, Palo Alto, California) containing a 30-m × 0.25-mm Supelco SPB-5 fused silica column. After sample injection, the initial temperature (80°C) was increased at 4°C/min

to 220°C and maintained at 220°C for 5 min. Flow rate of the helium carrier gas was 1 ml/min. The identity of monoterpenes was confirmed with GC-MS and by comparison with standards from Fluka (Chemie AG, Buchs, Switzerland).

Nitrogen. Carbon and nitrogen leaf concentrations were analyzed with a Carlo Erba NA1500 Analyser (Milan, Italy), using the standard configuration for those determinations.

Emission Sampling. Terpene emission was sampled on June 21 and November 7, 1995, and on May 31, 1996. A continuously stirred transparent dynamic 15-liter cuvette with an inlet and outlet was used to enclose branches for 3 min. Outgoing air was sampled at a flux of 200 ml/min. The outgoing air terpenes were adsorbed in 4-mm-diam. tubes filled with 300 mg of Carbotrap C, 200 mg of Carbotrap B, and 125 mg of Carbosieve S-III from Supelco as adsorbents between plugs of quartz wool. The tubes had previously been conditioned for 10 min at 300°C with a stream of purified helium. The same process was also conducted without plants as control.

Thermal Desorption–GC Analysis. Thermal desorption was conducted with a thermal desorption unit 890/891 (Supelco, Bellafonte, Pennsylvania) with a preparation temperature of 220°C for 6 min, desorption temperature of 220°C for 8 min, transfer temperature of 230°C, and valve temperature of 230°C.

The leaf volatile monoterpenes were monitored with the previously described gas chromatograph–mass spectrometer containing a 30-m × 0.25-mm Supelco SPB-5 fused silica column as described above.

Statistical Analyses. Statistical analyses were done using a three-way ANOVA (two CO_2 levels × two watering levels × three or four sampling times (repeated measures) × six replicates for biometric measurements and three replicates for emission measurements). The minimum significant difference was calculated and Tukey's test was used to determine significant differences at the 0.05 level between means. All analyses were conducted using SYSTAT 5.2 (SYSTAT Inc., Evanston, Illinois) and StatView 4.5 (Abacus Concepts Inc., Berkeley, California) statistical program packages.

RESULTS

Terpene Concentration. Elevated CO_2 led to increasingly larger monthly growth rates (measured as leaf biomass increase per month) than the atmospheric CO_2 treatments in the high water treatments (9.5% in spring 1995, 23% in autumn 1995, and 53% in spring 1996, $P < 0.001$ in ANOVA). In low water treatments the growth response to elevated CO_2 was constrained until the second year (spring 1996), when there was a 47% increase (Figure 1). Leaf nitrogen concentration declined significantly in elevated CO_2 treatments (1.92% dry

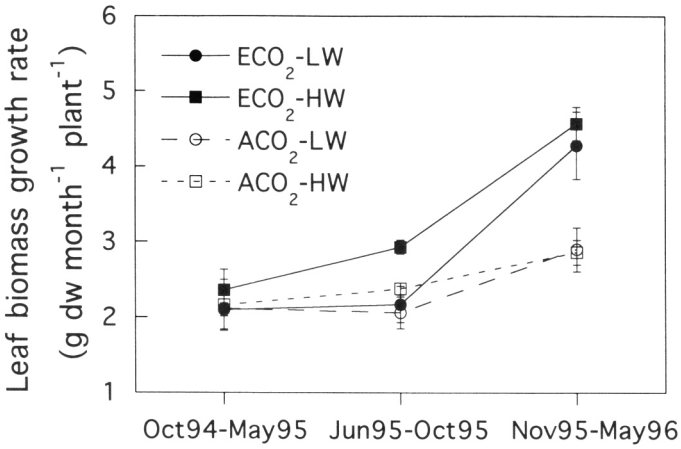


FIG. 1. Monthly growth rates of *Rosmarinus officinalis* leaf biomass under CO_2 treatments of 350 and 700 μmol/mol (ACO_2 and ECO_2) and under two levels of irrigation [high water (HW) and low water (LW)] throughout the study.

weight ± 0.02 SE versus 2.08% dry weight ± 0.03 SE for atmospheric CO_2, $P < 0.05$ in ANOVA).

There were slightly larger terpene concentrations under elevated CO_2 treatments than under atmospheric CO_2 treatments (Figure 2). The increase was up to 37% in the second year ($P < 0.1$). Therefore, the total amount of terpenes

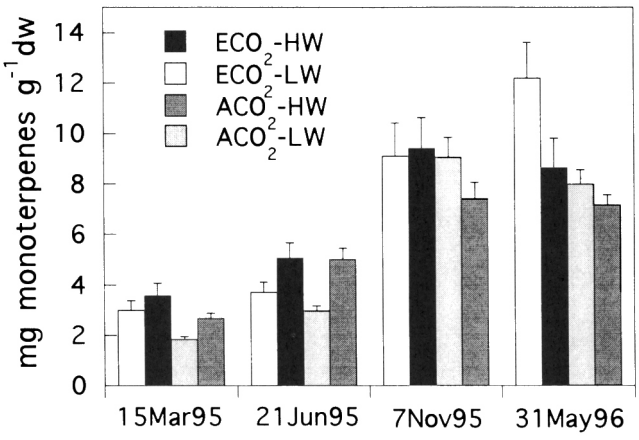


FIG. 2. Total terpene concentration of *Rosmarinus officinalis* plants under CO_2 treatments of 350 and 700 μmol/mol (ACO_2 and ECO_2) and under two levels of irrigation [high water (HW) and low water (LW)] at different sampling dates.

per plant increased significantly under elevated CO_2 treatments mainly through increased plant biomass (Figures 1 and 2).

There was no significant trend in water treatment effects. The terpene concentrations did not show any clear seasonal trend but increased throughout the study (Figure 2), indicating a possible age effect.

The most abundant terpenes were α-pinene, cineole, camphor, borneol, and verbenone, representing about 75% of the total (Figures 3 and 4). Other monoterpenes also found in *R. officinalis* are shown in Figures 3 and 4. No significant differences were found in the terpene composition among different treatments or seasons.

Terpene Emission. The main terpenes emitted were α-pinene, camphene, β-pinene, myrcene, Δ^3-carene, *p*-cymene, cineole, γ-terpinene, and linalool in spring and α-pinene, camphene, myrcene, Δ^3-carene, and *p*-cymene in autumn. Thus, in autumn emissions the more volatile monoterpenes and especially α-pinene (it reached about 50%) were proportionally more important than in summer (Figures 3 and 4). The less volatile monoterpenes such as γ-terpinene, linalool, and camphor presented lower percentages (Figures 3 and 4), and the least volatile borneol, verbenone, and 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol were not emitted in significant amounts. Therefore, no qualitative match was found between content and emission.

There was no direct quantitative relationship between terpene concentration and emission rates. The emission of volatile terpenes was much larger in spring 1996 at about 75 μg/g dry wt/hr than in autumn 1995 (about 10 μg/g dry wt/hr) partly because of higher temperatures and partly because of seasonal effects, as shown by the fact that much higher emission rates were found on June 21, 1995, than on November 7, 1995 (about 50 μg/g/hr compared with about 10 μg/g/hr, respectively, Figure 4) in spite of the lower concentrations of terpenes accumulated in the leaves (Figure 2) and even though sampling temperatures were the same.

No significant effect of CO_2 treatment in the emission of either individual or total terpenes (Figures 3, 4, and 5) was found.

DISCUSSION

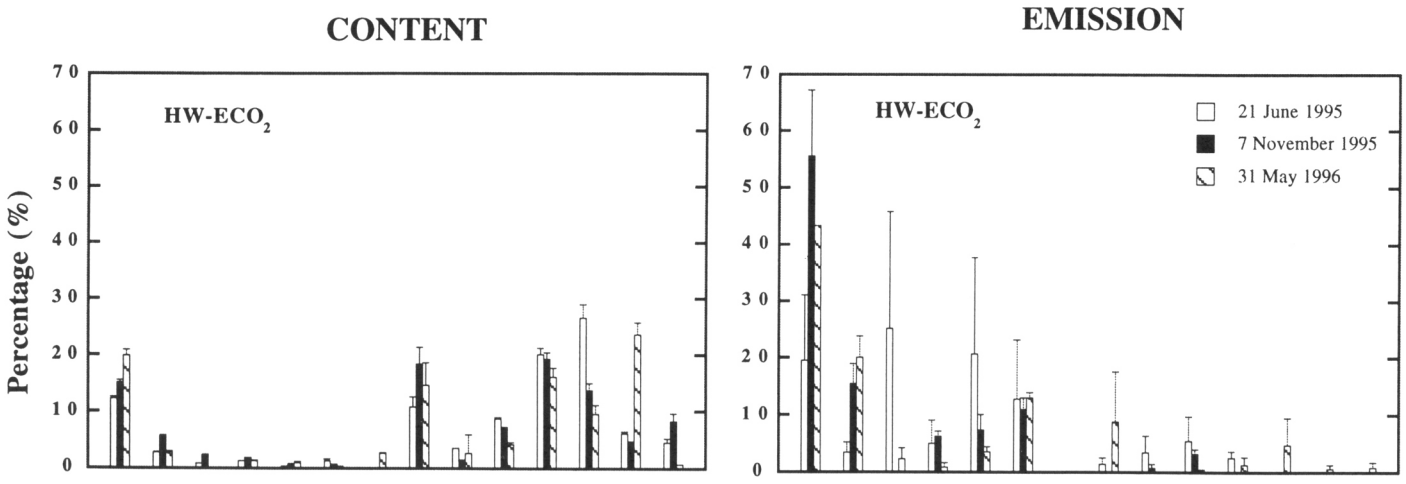
Terpene Content. The terpene content of *R. officinalis* plants was between 0.2 and 1% dry weight, in agreement with previously reported values (Ross and Sombrero, 1991). Results show that even though the pool size and composition of volatile terpenes in plants is under genetic control (Seufert et al., 1995), it is also influenced by environmental factors such as CO_2 supply (slight increases in concentration and clear increases in total amount per plant because of increased plant biomass), seasonality (larger contents in autumn), and age (larger contents

in older plants). Water availability did not have a clear effect in this study, likely because the low water treatment was too mild for this Mediterranean plant.

The increase in plant biomass with increasing carbon dioxide concentration followed documented responses for C_3 plants under agriculturally optimal conditions (Kimball, 1983; Poorter, 1993; Peñuelas et al., 1995b). This fact, together with the slight increase in terpene concentration, increased the total amount of terpenes in each plant under elevated CO_2. Nitrogen concentration decreased as is common in plants grown in high CO_2 environments (Peñuelas and Matamala, 1990; Lincoln, 1993). Thus, results tended to follow the pattern suggested by hypotheses that higher C/N ratios determine higher carbon-based secondary-compound concentrations (Bryant et al., 1983; Herms and Matson, 1992). Other authors did not find an influence of CO_2 supply (Lincoln and Couvet, 1989; Roth and Lindroth, 1994). Because of this, Lincoln and Couvet (1989), Johnson and Lincoln (1990), and Lavola and Julkunen-Tiitto (1994) stated that allocation to defense is closely regulated biochemically and not very sensitive to carbon supply. However, results from our study reveal that an enriched CO_2 atmosphere can slightly alter the foliar terpene concentration and largely alter the terpene plant content. This is important because a survey of recorded ecological effectiveness of terpenes shows it to be concentration- or dosage-dependent (Harborne, 1991). For example, biological reaction of insects to terpenes characteristically is one of attraction or stimulation at low concentrations, with the terpenes becoming increasingly repellent or inhibitory as the concentration increases (Lovett et al., 1989).

The terpene concentration did not present a clear seasonal trend but increased throughout the study. There was a larger concentration the second year of the study and the α-pinene and verbenone relative contents were increased, indicating a possible effect of stage of development and age. Kotzias et al. (1992) found little seasonal change in relative proportions, but other reports have shown seasonal modifications (Gershenzon and Croteau, 1991; Zou and Cates, 1995) and developmental stage effects (Lerdau et al., 1995). In the literature it is considered unclear whether the observed larger terpene concentrations at the end of summer are caused by enhanced biosynthesis of terpenes or indirectly by reduced growth (Seufert et al., 1995). In this study both causes seem to have a role because there were parallel increases in biomass and concentrations, but during summertime, low water treatments presented lower growth and a higher increase in concentration than high water treatments.

Terpene Emission. Emission of terpenes was not different in the different CO_2 treatments. Thus, the extra carbon only seemed to significantly affect the plant biomass and slightly effect the terpene concentration. Water availability (irrigation) did not show a clear effect. Plants receiving high water emitted slightly larger quantities than plants receiving low water in the first year, but this was reversed the second year. Increases in emissions through increased


CONTENT
EMISSION
Percentage (%)
HW-ECO2
HW-ECO2
21 June 1995
7 November 1995
31 May 1996
0
10
20
30
40
50
60
70

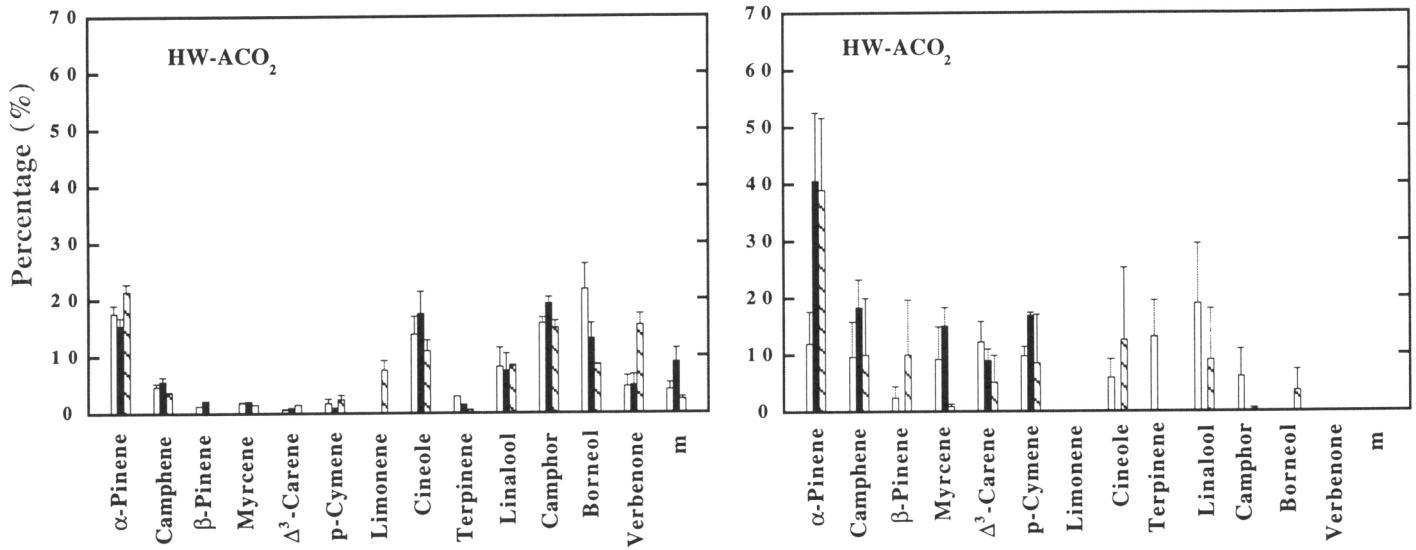
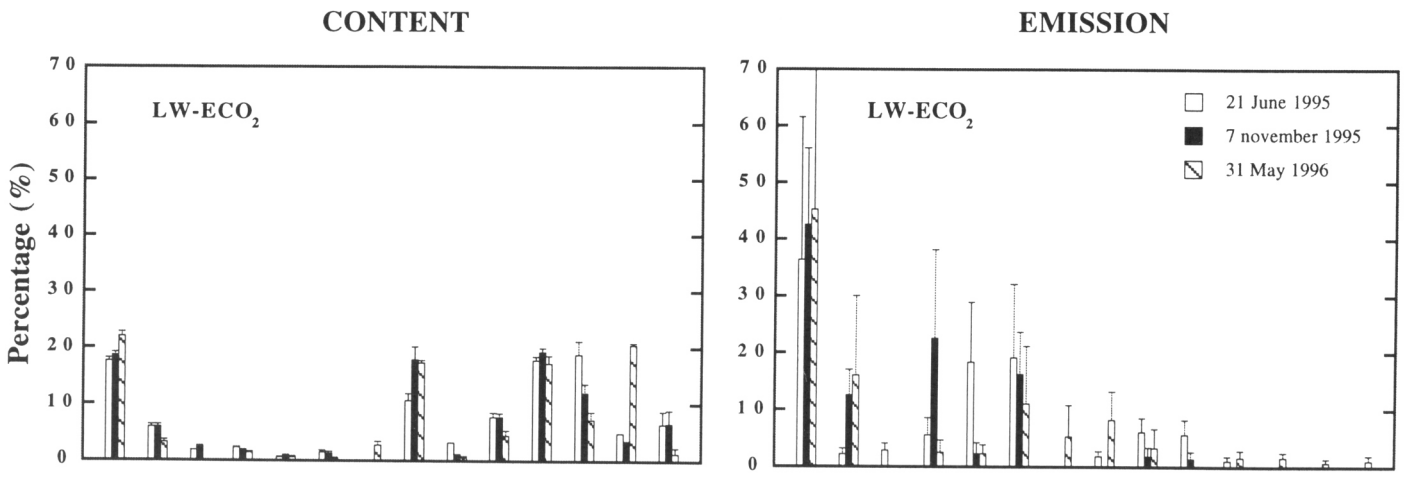


FIG. 3. Relative concentration and relative emission of different terpene components of *Rosmarinus officinalis* expressed as percentage of total under different CO_2 treatments of 350 and 700 μmol/mol (ACO_2 and ECO_2) and under high water treatments (HW) throughout the study. (m: 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol).


CONTENT
EMISSION
Percentage (%)
LW-ECO2
LW-ECO2
21 June 1995
7 november 1995
31 May 1996
0
10
20
30
40
50
60
70

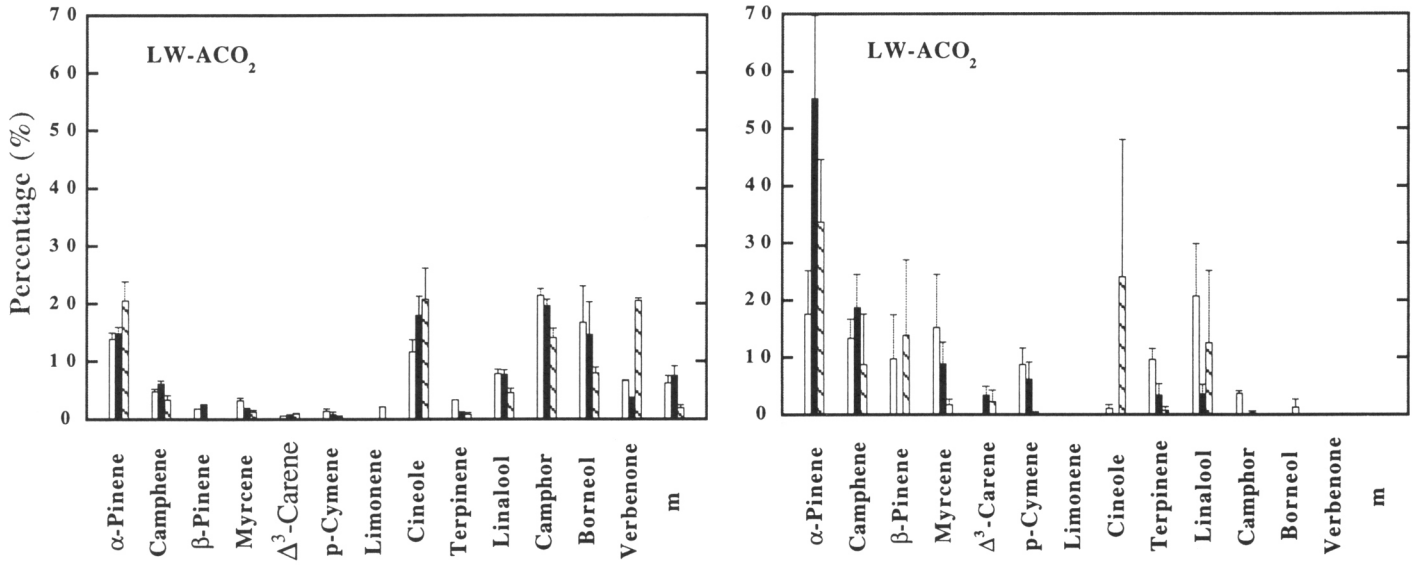


FIG. 4. Relative concentration and relative emission of different terpene components of *Rosmarinus officinalis* expressed as percentage of total under different CO_2 treatments of 350 and 700 μmol/mol (ACO_2 and ECO_2) and low water treatments (LW) throughout the study. (m: 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol).

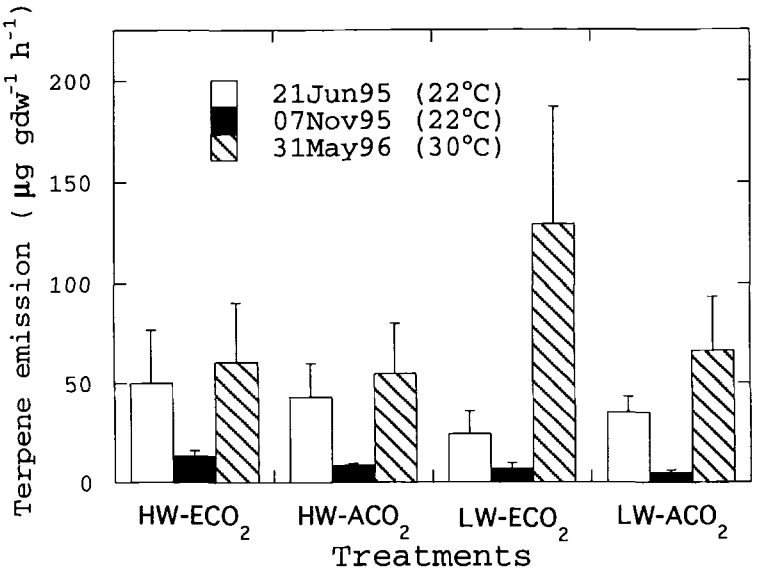


FIG. 5. Total terpene emission rates of *Rosmarinus officinalis* plants under different CO_2 treatments of 350 and 700 µmol/mol (ACO_2 and ECO_2) and under two levels of irrigation [high water (HW) and low water (LW)] throughout the study.

water supply have been described in *Mentha piperita* (Croteau, 1977), and emissions from wet branches of *Pseudotsuga menziesii* were an order of magnitude higher than those of dry branches (Lamb et al., 1985). However, the low water treatment in this study may be too mild for this Mediterranean plant.

Terpene emission in the plants is quite large (about 75 µg/g/hr on May 31, 1996) and is in the range reported by other authors for more northern vegetation species (Lerdau et al., 1995). It is somewhat larger than reported in other species of the same Mediterranean vegetation (20 µg/g/hr; Enders, 1994). The approximately 75 µg/g/hr emitted on May 31, 1996, represented approximately 0.75% of the pool and the 10 µg/g/hr emitted in early autumn represented 0.1% of the pool. This pool proportion is much larger than the reported values in the literature. Less than 5% of the pool is emitted per year in *Pseudotsuga menziesii* (Schindler and Kotzias, 1989). For *Salvia mellifera*, Tyson et al. (1974) calculated the volatization loss of carbon from the terpenes pool as 0.06% per month. The rest would be volatized if not catabolized during mineralization of the litter.

For a given pool size and diffusive pathway, the emission rate of volatile terpenes is controlled mainly by temperature-induced changes in the vapor pressure of the compound of interest and other factors related to temperature (Tingey

et al., 1991). In this study, terpene emission rates showed large seasonal changes that are likely linked in great part to changes in temperature. Maximum rates were found on May 31, 1996, when sampling temperature was highest (30–31°C). Although the basal emission rates (rates at a prescribed temperature) seem to be determined by terpene concentration (Lerdau et al., 1995), in this study emission rates were larger on June 21, 1995, than on November 7, 1995, even though the temperature was the same (22–23°C) and even though concentrations on November 7, 1995, were larger than on June 21, 1995, indicating that seasonality has other effects apart from those due to changes in temperature and concentration. The lack of positive relationship between terpene concentration and emission suggests that variations in emission due to temperature are larger than concentration-induced variations in terpene vapor pressure gradients from the leaf to the atmosphere.

Comparing pools and emissions of the aromatic shrubs, it becomes evident that there was no matching, as reported by Seufert et al. (1995). There was no quantitative or qualitative match between concentration and emission. The most volatile terpenes (α-pinene, camphene, myrcene or p-cymene) were proportionally more abundant in the total emission than in the total plant content, and the less volatile terpenes, such as camphor, borneol, verbenone, or 1,7,7,-trimethylbicyclo[2.2.1]heptan-2-ol were more abundant in the total plant content. Knowledge of concentration is not sufficient to predict relative emission rates, which could simplify development of emission inventories, unless temperature and other meteorological data are also considered, as was suggested by Lerdau et al. (1995), and unless the volatility of different terpenes is also considered.

In summary, plant biomass was largely increased by exposure at 700 μmol/mol CO_2. Terpene concentration also was increased slightly by the high CO_2 level, but there was no effect of high CO_2 on emission rates. There was no effect of mild water stress on the concentration or emission of terpenes, but there was a seasonal trend in concentration and emission linked to temperature and age of plants. Terpene emissions were not directly related qualitatively or quantitatively to total terpene content in the plants. The less volatile terpenes dominated concentration in the plants, and the most volatile terpenes dominated emission, especially in autumn when α-pinene represented about 50% of the total emissions. Further studies on the effects of all these environmental factors should be conducted to help in understanding the biogenic emission role in chemical atmospheric science (and especially to figure out the dynamics of ozone formation), in interorganism relationships, and in plant protection against extreme temperature and drought conditions.

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ALFALFA LEAF SAPONINS AND INSECT RESISTANCE

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Abstract—Dried alfalfa (*Medicago sativa*) leaf tissue incorporated in artificial diet to give a final concentration of 0.5 or 1.6 mg/g fresh weight of saponins significantly inhibited growth and development of larvae of the European corn borer (*Ostrinia nubilalis*). Saponin fractions isolated from root or shoot tissues of alfalfa also inhibited growth when incorporated at equivalent concentrations but had little effect on development. Root saponins were somewhat more harmful than shoot saponins.

Key Words—Alfalfa, European corn borer, *Medicago*, *Ostrinia nubilalis*, saponins.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a major forage crop in all temperate agricultural regions and is becoming increasingly important as a source of supplementary dietary protein for human consumption. Plant species in general defend themselves from herbivores by producing toxic phytochemicals. The important constitutive defenses of alfalfa plants include a complex mixture of about 30 saponin glycosides (Berrang et al., 1974; Tava et al., 1993). High levels of these substances are associated with antinutritional, even toxic, effects when alfalfa leaf protein preparations containing them are included in the diet of monogastric animals. Hemolysis of red blood cells, for example, may result (Small et al., 1990). However, the plant breeder selecting for minimal digestive problems for

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domestic animals and humans for whom the crop is grown may also be reducing natural resistance to other herbivores such as insects. Reduction of saponins to low levels is certainly desirable from the former point of view, but possibly disastrous from the latter. Thus, the breeder must strike a delicate balance between improved nutritional qualities and potentially lower yields.

To gain further insight into the role of saponins as defense compounds against herbivorous insects, the present study examined the feeding behavior of a polyphagous insect, *Ostrinia nubilalis* (Hubner) in response to various levels of saponins in alfalfa leaf tissue or isolated from alfalfa tissue, incorporated into artificial diets. Although this insect is called the European corn borer (ECB), because it is a major pest of corn (maize), it is also a serious pest of potato, green pepper, and winter wheat and attacks a wide variety of weed species in agricultural fields. It is not yet a pest of alfalfa. Our hypothesis is that saponins are a major barrier to herbivory by ECB on this economically important forage species.

METHODS AND MATERIALS

Plant Material. Three cultivars of alfalfa (*Medicago sativa* L.), selected for their differing levels of saponins, were obtained from Dr. E. Small, Agriculture Canada, Ottawa; namely, var. Azmafa with low saponin content (0.2%, dry wt basis), var. Macsel with medium saponin content (1.0%), and var. Algonquin with high saponin content (3.2%) (Small et al., 1990). The plants were grown in a greenhouse at the Central Experimental Farm, Ottawa, and harvested at the blossom stage. Harvested shoots were dried to constant weight at 60°C, and leaf tissues were then manually separated from stem tissue. Plants of cultivar Kleszczewska were field grown in Poland and both roots and shoots were harvested for isolation of saponin fractions.

Isolation of Saponins. Two fractions of saponins were obtained from the Polish variety of alfalfa, one from the shoots and one from the roots. Shoots and roots were extracted separately by refluxing with boiling aqueous ethanol, 80%, to obtain the so-called cholesterol-precipitable saponins from the shoots (Jurzysta, 1981) and a mixture of total saponins from the roots (Oleszek et al., 1990).

Insect Culture. European corn borers (*Ostrinia nubilalis* Hubner), originally obtained from the Agriculture Canada Research Station in London, Ontario, were reared in the laboratory at the University of Ottawa on artificial diet (Guthrie et al., 1985). For the first experiment, dried, powdered alfalfa leaf tissue was added to the diet in amounts equal to the dry chemical constituents, giving a final ratio of tissue of about 5% of the fresh weight of the diet cube and final concentrations of saponins equal to 0.1, 0.5, and 1.6 m/g fresh weight. Fifteen

second instars of uniform size were weighed and placed individually on moistened diet cubes (control) or on diet cubes containing dried tissue. The containers were incubated in a controlled environment cabinet maintained at 85% relative humidity and at 26°C–19°C day–night temperature. Larvae were weighed after 1, 8, 13, 19, and 23 days on the diet. Times of pupation and adult emergence were noted, weights of pupae and newly emerged adults were determined, and adults were sexed. For the second series of experiments, alfalfa saponin fractions were incorporated in the diet at the rates of 0.1 and 0.5 mg/g fresh weight and 30 newly hatched first instars were placed on individual cubes containing diet alone (control) or alfalfa saponins. Larvae were weighed at regular intervals and life-cycle data were obtained as described above.

Statistics. Weight data were subjected to a one-way analysis of variance

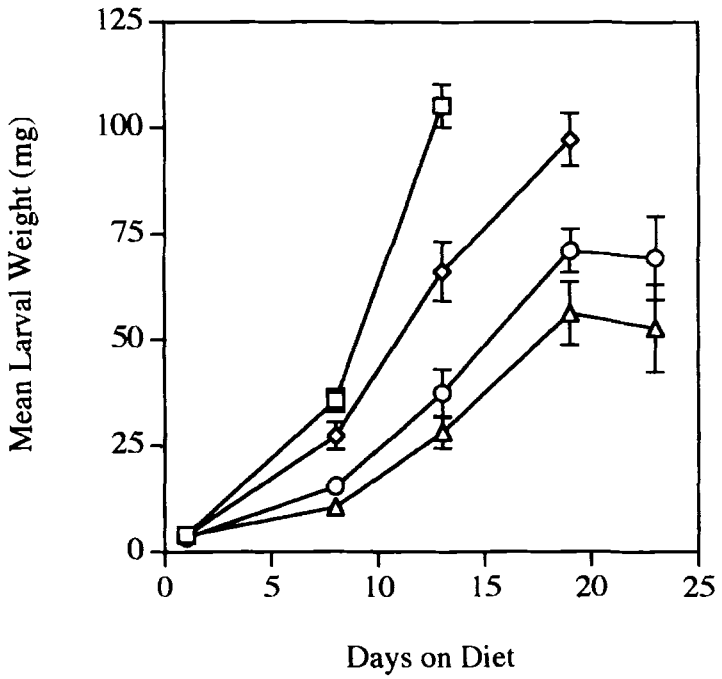

FIG. 1. Growth, expressed as mean larval weight, of European corn borer, *Ostrinia nubilalis* Hubner, feeding on artificial diets containing 5 g/100 g fresh wt of dried alfalfa leaf tissues of differing saponin contents. Final concentrations of saponins were 0.1 mg/g (◇), 0.5 mg/g (○), or 1.6 mg/mg (△). Control larvae (□) were fed on diet only. Vertical bars represent SE. Of 15 initial second instars, 83%, 53%, and 60%, respectively, survived, in contrast to 100% survival of control insects.

(SAS) followed by a comparison of the means if significant variation was found (LSD test). Development time data were analyzed by the Kruskal-Wallis test.

RESULTS

Bioassays with Alfalfa Genotypes of Varying Saponin Content. It is evident from Figure 1 that growth of second instars feeding on diet containing dried leaf tissue from three alfalfa varieties was reduced in direct proportion to the concentration of saponins in the tissue. Viability of the larvae was also affected, with only half (53% and 60%, respectively) surviving to pupation when fed diets containing 0.5 or 1.6 mg/g fresh weight of saponins in dried alfalfa tissue. Time to pupation (Table 1) was significantly increased ($P = 0.05$) in larvae fed these higher amounts of saponins. All but one of the control insects and half of insects feeding on cv. Azmafa tissue had pupated by day 19, whereas the mean time for pupation of the other treated insects was 25 days. Mean weights of fifth instars and pupae (Table 1) of these insects were significantly lower ($P = 0.05$), but duration of the nine-day pupal period was not significantly affected by feeding on saponins. Only 60% of treated pupae survived to the adult stage as compared to 85% of control pupae. Total time from second instar to adult

TABLE 1. DEVELOPMENTAL RESPONSES OF *O. nubilalis* SECOND INSTARS TO INCORPORATION OF DRIED ALFALFA TISSUE IN ARTIFICIAL DIET[a]

Saponin content of diet (mg/g)	Mean wt of 5th instar (mg)	Mean days to pupation	Mean pupal wt (mg)	Mortality (%)
None (control)	105.2a (5.1) $N = 15$	17.5a (0.3) $N = 15$	86.0a (1.7) $N = 15$	0
0.1	87.4b (4.4) $N = 13$	20.6b (0.6) $N = 13$	76.3ab (1.2) $N = 13$	13
0.5	77.3b (2.7) $N = 8$	24.5c (1.5) $N = 8$	58.0bc (0.8) $N = 8$	47
1.6	69.5b (5.6) $N = 10$	25.0c (1.0) $N = 10$	55.7bc (1.4) $N = 10$	33

[a]SE in parentheses; N indicates sample size; values followed by the same letter are not significantly different.

emergence, about 30 days, was not significantly ($P = 0.05$) affected but the mean weight of adults, 50 mg for females and 30 mg for males, decreased with ingestion of saponins to about 70% of normal. The final sex ratio (female-male) of adults was 8:5 for control insects, 5:6 for insects fed 0.1 mg/g, 3:2 for those fed 0.5 mg/g, and 2:3 for those fed 1.6 mg/g.

Bioassays with Saponins Extracted from Alfalfa. Two preliminary tests were made with insects fed on diet containing 10 mg/g fresh weight of either root or shoot saponins. In the first, all neonate larvae (120 total) died within 48 hr of placement on the diet cubes containing saponins, whereas the 60 larvae on control diet developed normally. In the second test, second instars fared somewhat better in that half (12) in the treated groups survived for seven days. However, no weight gain was made, and all the test larvae were dead by nine days. Two subsequent tests used much smaller amounts of saponins: 0.1 and 0.5 mg/g fresh weight, respectively. A slight, but not significant, inhibition of

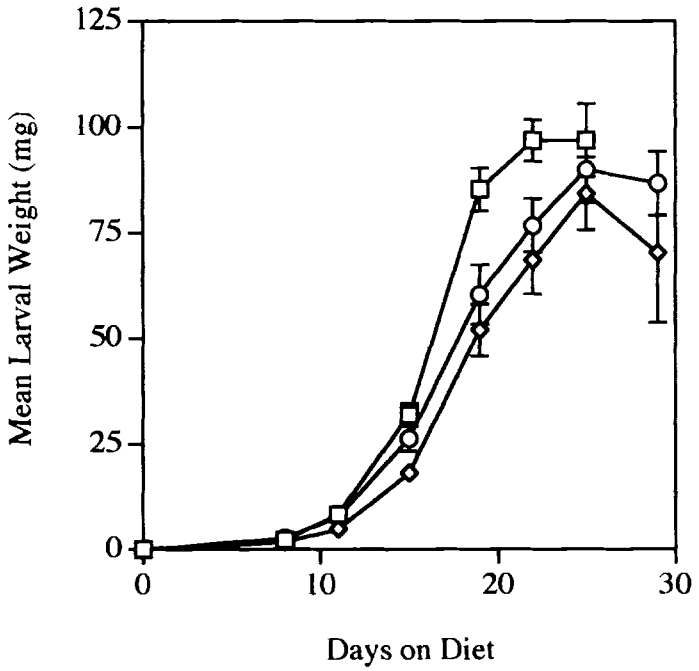

FIG. 2. Growth, expressed as mean larval weight, of European corn borer, *Ostrinia nubilalis* Hubner, feeding on artificial diet containing 0.5 mg/g fresh wt of saponins extracted from alfalfa root (◇) or shoot (○). Control larvae fed on untreated diet only (□). Vertical bars represent SE.

TABLE 2. EFFECT OF ALFALFA SAPONINS ON DEVELOPMENTAL PARAMETERS OF FIRST INSTARS OF *Ostrinia nubilalis* (ECB)[a]

Saponin conc. (mg/g)	Mean wt of 5th instar (mg)	Pupal wt (mg)	Days to pupation
None (control)	101.9a (3.8) $N = 23$	77.25a (3.2) $N = 22$	25.1a (0.6) $N = 23$
0.5 (root)	95.4a (4.6) $N = 18$	79.5a (3.3) $N = 16$	28.1ab (0.6) $N = 18$
0.5 (shoot)	104.9a (4.4) $N = 25$	79.5a (3.7) $N = 21$	28.7a (1.0) $N = 25$

[a]SE in parentheses; N indicates sample size; values followed by the same letter are not significantly different.

weight increase relative to control was observed when neonates were fed 0.1 mg/g of shoot or root saponins (data not shown). Growth was significantly ($P = 0.05$) less when 0.5 mg/g was fed (Figure 2), with a consequent delay in the mean time to pupation of three to four days (Table 2). Root saponins were somewhat more inhibitory than shoot saponins. Mean weights of fifth instars and mean pupal weights, however, were not significantly ($P = 0.05$) different, but the mean pupal period and therefore the time to the adult stage were significantly ($P = 0.05$) increased (Table 3) for males fed 0.5 mg/g of root saponins. Nevertheless, mean adult weights (Table 3) of the survivors were not significantly ($P = 0.05$) different from those of control insects. Sex ratios were unaffected (Table 3).

DISCUSSION

Incorporation of dried tissue of three varieties of alfalfa into diet adversely affected development of ECB, inversely in proportion to saponin content for cv. Azmafa and Macsel. A threefold increment in saponins of cv. Algonquin, however, did not result in any further significant reduction in growth over that obtained with cv. Macsel. This may have resulted from qualitative differences in the saponins of these two varieties. It has been established that alfalfa cultivars can differ widely in the kinds of saponins produced (Berrang et al., 1973; Tava et al., 1992). The saponins of cv. Algonquin may not be as toxic as those of cv. Macsel.

TABLE 3. EFFECT OF ALFALFA SAPONINS ON ADULTS OF *Ostrinia nubilalis* (ECB)[a]

Saponin conc. (mg/g)	Mean days to adult stage		Mean wt of adult (mg)		Sex ratio
	F	M	F	M	F/total
None (control)	34.7a (0.8) $N = 6$	31.3a (1.0) $N = 4$	46.9a (5.5) $N = 6$	32.2a (2.7) $N = 4$	0.55
0.5 (root)	36.2a (1.4) $N = 6$	37.1b (0.5) $N = 8$	48.7a (3.8) $N = 6$	30.2ab (3.9) $N = 8$	0.43
0.5 (shoot)	35.3a (1.5) $N = 6$	33.6ab (1.6) $N = 5$	53.3ab (3.2) $N = 6$	34.5a (3.0) $N = 5$	0.55

[a]SE in parentheses; N indicates sample size; values followed by the same letter are not significantly different.

Not only do saponins vary from cultivar to cultivar but distribution within the tissues may also vary, with roots generally having both a higher content and a higher proportion of toxic constituents. Oleszek et al. (1992) found that a zanhic acid tridesmoside of low biological activity is the major constituent of shoot saponins. Root saponins, on the other hand, are composed mainly of glycosides of medicagenic acid with high biological activity (Oleszek et al., 1990). A further factor to take into consideration in comparing results with dried tissues and isolated saponins is that other constituents of alfalfa leaves, which also may vary widely in amounts and types and which would not accompany the saponin fraction, e.g., some of the abundant flavonoids (Classen et al., 1982), may contribute to growth inhibition by dried tissue preparations.

That the presence of saponins in alfalfa is associated with resistance to some insect pests and fungal pathogens has been shown by other studies (e.g., Pederson et al., 1976; Sutherland et al., 1982; Tava et al., 1992). The present study indicates that the saponins of alfalfa are natural barriers to feeding by a generalist insect and further suggests that their reduction as endogenous constituents as a result of breeding programs may produce a crop that is susceptible to a generalist pest such as the ECB that is already well established in croplands.

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