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GEOGRAPHIC VARIATION IN RESPONSE OF PINE ENGRAVER, *Ips pini*, AND ASSOCIATED SPECIES TO PHEROMONE, LANIERONE

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Abstract—Lanierone strongly synergized the attraction of male and female *Ips pini* (Say) to ipsdienol in New York and Wisconsin. Synergy was only weakly significant in Montana and British Columbia and not significant in California. Catches of *I. pini* in ipsdienol-baited traps were increased 0% (i.e., nonsignificant) to 9942% by lanierone, with the highest increases in eastern North America. Lanierone had the least effect in California. The effects of lanierone on sex ratios of *I. pini* in trap catches varied significantly between regions. The addition of lanierone to ipsdienol-baited traps resulted in a general increase in male representation at nine of 12 sites. *Ips integer* (Eichhoff) was attracted to lanierone alone. Ipsdienol reduced the response of *I. integer*

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to lanierone. *Enoclerus lecontei* (Wolcott) (Cleridae) preferred traps baited with the combination of ipsdienol and lanierone. (*R*)-(−)-Ipsdienol was attractive to *E. sphegeus* (F.), *Thanasimus undatulus* (Say) (Cleridae), and *Temnochila chlorodia* (Mannerheim) (Trogositidae), while racemic ipsdienol was attractive to *E. nigrifrons* var. *gerhardi* Wolc. and *Thanasimus dubius* (F.). Lanierone had no effect on these species.

Key Words—*Ips pini*, *Ips integer*, Coleoptera, Scolytidae, aggregation pheromone, lanierone, ipsdienol, geographic variation, synergism, *Enoclerus lecontei*, Cleridae, kairomone.

INTRODUCTION

The pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae), uses the enantiomers of ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) as aggregation pheromones (Stewart, 1975; Birch et al., 1980; Lanier et al., 1980). In New York, males produce both (*R*)-(−)- and (*S*)-(+)-ipsdienol, and both sexes prefer blends with roughly equal ratios of (*R*)-(−)- and (*S*)-(+)-ipsdienol over blends enriched with either enantiomer (Lanier et al., 1980; Teale, 1990; Teale and Lanier, 1991). Males in California produce primarily (*R*)-(−)-ipsdienol (Stewart, 1975; Birch et al., 1980; Miller et al., 1989a; Seybold, 1992; Seybold et al., 1995). Californian beetles are attracted to (*R*)-(−)-ipsdienol (Birch et al., 1980; Seybold, 1992). Attraction to (*R*)-(−)-ipsdienol is interrupted by (*S*)-(+)-ipsdienol. In southeastern British Columbia, males produce primarily (*R*)-(−)-ipsdienol as in California (Miller et al., 1989a, 1996; Seybold et al., 1995), but both sexes are attracted to a broader range of enantiomeric mixtures [98:2 to 20:80 (*R*)-(−):(*S*)-(+), inclusively] (Miller et al., 1996) than Californian beetles (Birch et al., 1980; Seybold, 1992). In contrast to most other western populations, males in southwestern British Columbia produce ipsdienol with a mean enantiomeric composition of 34:66 (*R*)-(−):(*S*)-(+) (Miller et al., 1989a, 1996) and a pooled composition of 39:61 (*R*)-(−):(*S*)-(+) (Seybold et al., 1995). In this area, both sexes are attracted to intermediate enantiomeric mixtures [70:30 to 20:80 (*R*)-(−):(*S*)-(+), inclusively] (Miller, 1990; Miller et al., 1996). Beetles in Wisconsin prefer an enantiomeric mixture of 25:75 (*R*)-(−):(*S*)-(+) (Raffa and Klepzig, 1989).

Lanierone (2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one) has been identified as a third male-produced aggregation pheromone for *I. pini* in New York (Teale et al., 1991). Attraction of both sexes to racemic ipsdienol is synergized by lanierone. However, the role of lanierone may not be the same throughout the broad transcontinental range of *I. pini* (Teale et al., 1991; Seybold et al., 1992).

The high degree of geographic variation in the use of ipsdienol enantiomers as pheromones by *I. pini* suggests that similar variation may also exist for

lanierone. Lanierone was not detected in Porapak extracts from California (Seybold et al., 1992). Similarly, the effect of lanierone on the attraction of *I. pini* to ipsdienol is weaker in California than in New York (Seybold et al., 1992). Therefore we hypothesized that lanierone would not have the same effect on the response of *I. pini* to region-specific enantiomeric mixtures of ipsdienol throughout North America.

METHODS AND MATERIALS

Chemicals and Release Devices. Phero Tech Inc. (Delta, British Columbia) supplied the following types of polyvinyl, bubble-cap lures: (1) 1,3-butanediol (chemical purity, >98%); (2) racemic ipsdienol (chemical purity, 98%) in solution with 1,3-butanediol; (3) (*R*)-(−)-ipsdienol (chemical purity, 98%; optical purity, >97%) in solution with 1,3-butanediol; and (4) lanierone (chemical purity, 95%) in solution with 1,3-butanediol. The release rates of 1,3-butanediol, ipsdienol [racemic and (*R*)-(−)], and lanierone were approximately 2, 0.2, and 0.02 mg/day, respectively, at 24°C (determined by collection of volatiles on Porapak-Q). 1,3-Butanediol is not attractive to *I. pini* alone or in combination with ipsdienol (Miller, 1990).

Experimental Design. The same experiment was conducted at two sites in each of the following regions: (1) Syracuse, New York; (2) Madison, Wisconsin; (3) Missoula, Montana; (4) Kimberley, British Columbia; (5) Penticton, British Columbia; and (6) Hat Creek, California. At each site, 20 multiple-funnel traps (Lindgren, 1983) (Phero Tech Inc.) were set in five parallel lines of four traps per line. Each line of four traps constituted a replicate. Eight-unit funnel traps were used in California and New York, while 12-unit funnel traps were used in the remaining regions. Each trap was suspended by rope such that the bottom funnel of each trap was 0.5–1.0 m above ground level. No trap was within 1 m of any tree. Traps were set in the following types of stands: (1) lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, in British Columbia; (2) ponderosa pine, *P. ponderosa* Lawson, in Montana; (3) ponderosa and Jeffrey pines, *P. jeffreyi* Greville and Balfour, in California; and (4) red pine, *P. resinosa* Aiton, in New York and Wisconsin. Sites were at least 1 km apart while traps were spaced 10–15 m apart within sites.

The following treatments were randomly assigned within each replicate: (1) 1,3-butanediol control; (2) ipsdienol with 1,3-butanediol; (3) lanierone with 1,3-butanediol; and (4) ipsdienol and lanierone with 1,3-butanediol. (*R*)-(−)-Ipsdienol was used in California and southeastern British Columbia. Racemic ipsdienol was used in the remaining regions. For most regions, the choice of enantiomeric compositions for ipsdienol was based upon regional specificities in production and response by *I. pini* (Birch et al., 1980; Lanier et al., 1980;

Miller et al., 1989a, 1996; Raffa and Klepzig, 1989; Teale, 1990; Herms et al., 1991; Teale and Lanier, 1991; Seybold, 1992; Seybold et al., 1995). No previous studies had been conducted in Montana. We chose racemic ipsdienol, reasoning that populations in Montana would likely be similar to eastern populations.

The experiments were conducted in 1991 during the following periods: (1) July 18–31 in New York; (2) July 19–August 16 in Wisconsin; (3) July 12–29 in Montana; (4) June 27–July 11 in southeastern British Columbia; (5) July 3–August 17 in southwestern British Columbia; and (6) July 23–30 in California. Sexes of captured *I. pini* were determined, using declivital characters (Lanier and Cameron, 1969), for all beetles in small catches and for subsamples (N = 30–50) in large catches. Responses of associated beetle species were also recorded.

Statistics. Data were analyzed using the Systat statistical package ver. 5.02 (Systat, Inc., Evanston, Illinois). Trap catch data were transformed by $\ln(Y + 1)$ to remove heteroscedasticity and subjected to analysis of variance (ANOVA) using the following model factors: (1) replicate nested within site, (2) site, (3) ipsdienol, (4) lanierone, (5) site * ipsdienol, (6) site * lanierone, (7) ipsdienol * lanierone, and (8) site * ipsdienol * lanierone. For both male and female *I. pini*, differences in mean catches were determined using Tukey's HSD multiple-comparison test at $P = 0.05$ (experiment-wise). Proportional increases were calculated for each replicate by dividing the difference in catches between the two treatments by the catch in the trap baited with ipsdienol alone.

Synergism between lanierone and ipsdienol was indicated by satisfying three statistical conditions. First, synergism required a significant interaction between these two factors in the ANOVA, indicating that the proportional increases (not absolute increases) due to both factors were not additive. Secondly, one factor (lanierone) had to be benign while the second factor (ipsdienol) had to be active. Multiple comparison tests such as Tukey's HSD can affirm that one factor is benign and does not differ from the control and that a second factor is attractive relative to the control. Lastly, the combination of lanierone and ipsdienol had to result in significantly higher catches than ipsdienol alone.

Sex ratio data for *I. pini* captured in ipsdienol-baited traps were normalized by arcsine (Y), and analyzed by ANOVA using the following model factors: (1) replicate nested within site, (2) site, (3) lanierone, and (4) site * lanierone. Mean proportions of males in traps baited with ipsdienol were compared to those in traps with ipsdienol and lanierone using a two-sided t test. Differences in sex ratios among regions and among mean catches of other species were determined using Tukey's HSD multiple comparison test at $P = 0.05$ (experiment-wise).

RESULTS

Ips *Species.* In all regions, lanierone and ipsdienol had significant effects on trap catches of both sexes (all $P \leq 0.035$) (Table 1). The interaction between ipsdienol and lanierone had a significant effect on males in all regions except southeastern British Columbia. The interaction had no significant effect on females in California and southeastern British Columbia.

The effects of lanierone varied between regions (Figure 1), with the most pronounced effect apparent in New York and Wisconsin. In general, the preferred treatment for both sexes was the combination of ipsdienol and lanierone (Tables 2 and 3), with synergism apparent in most regions. Lanierone was not attractive in Montana, southwestern British Columbia, Wisconsin, and New York. However, when added to ipsdienol, lanierone increased trap catches 260–9942%. Lanierone did not increase catches of females in California. At one site in California, the addition of lanierone to ipsdienol resulted in a modest increase in trap catch of males.

Traps baited with lanierone alone did not generally catch more *I. pini* than control traps (Tables 2 and 3). The exception was in southeastern British Columbia where significantly more males and females were captured in traps baited with lanierone alone than in control traps. The mean proportional increases ranged from 107% to 878%.

Lanierone, or the interaction between lanierone and site, had significant effects on the sex ratio of *I. pini* caught in ipsdienol-baited multiple-funnel traps in five of the six regions (Table 4). In California, southeastern British Columbia and New York, the mean proportion of males was greater in catches to traps baited with lanierone and epsdienol than in those to traps baited with ipsdienol alone (Table 5). The effect was weakly significant at site A in southwestern British Columbia and site B in Wisconsin.

Sex ratios varied between regions for catches to traps baited with either ipsdienol alone or ipsdienol and lanierone (Table 5). In nine of 12 sites, the sex ratio in catches to traps baited with ipsdienol alone was generally female-biased (mean proportions of males ranging from 0.19 to 0.40). In Montana, the sex ratio was male-biased (mean proportions of males were 0.63 and 0.71 for sites A and B, respectively). The sex ratios in catches of traps baited with ipsdienol and lanierone were generally close to 1 : 1. The exceptions were in Montana (male-biased) and southwestern British Columbia (female-biased).

Lanierone was attractive to *I. integer* (Eichhoff) in Montana (Figure 2). Significantly more beetles were caught in traps baited with lanierone alone than in those baited with any other treatment. The response to lanierone was strongly inhibited by racemic ipsdienol (ANOVA, $P < 0.001$, $df = 1,24$). The mean catches to traps baited with ipsdienol and lanierone were not significantly dif-

TABLE 1. SIGNIFICANCE LEVELS FOR ANALYSES OF VARIANCE (ANOVAS) ON TRAP CATCHES OF MALE AND FEMALE *Ips pini* IN MULTIPLE-FUNNEL TRAPS BAITED WITH IPSDIENOL AND/OR LANIERONE

Region	Sex	Site (S)	Ipsdienol (Id)[a]	Lanierone (L)	S * Id[a]	S * L	L * Id[a]	S * L * Id[a]
California	Female	0.000	0.000	0.035	0.013	0.497	0.795	0.084
	Male	0.000	0.000	0.016	0.002	0.235	0.002	0.708
British Columbia (SW)	Female	0.371	0.000	0.000	0.170	0.165	0.001	0.863
	Male	0.134	0.000	0.000	0.625	0.584	0.000	0.120
British Columbia (SE)	Female	0.335	0.000	0.000	0.447	0.726	0.603	0.188
	Male	0.309	0.000	0.000	0.526	0.513	0.327	0.102
Montana	Female	0.252	0.000	0.002	0.045	0.376	0.001	0.081
	Male	0.005	0.000	0.000	0.225	0.474	0.000	0.370
Wisconsin	Female	0.915	0.000	0.000	0.082	0.734	0.000	0.033
	Male	0.052	0.000	0.000	0.318	0.004	0.000	0.042
New York	Female	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Male	0.000	0.000	0.000	0.000	0.000	0.000	0.000

[a] (*R*)-(−)-Ipsdienol in California and southeastern British Columbia, and racemic ipsdienol in remaining regions.

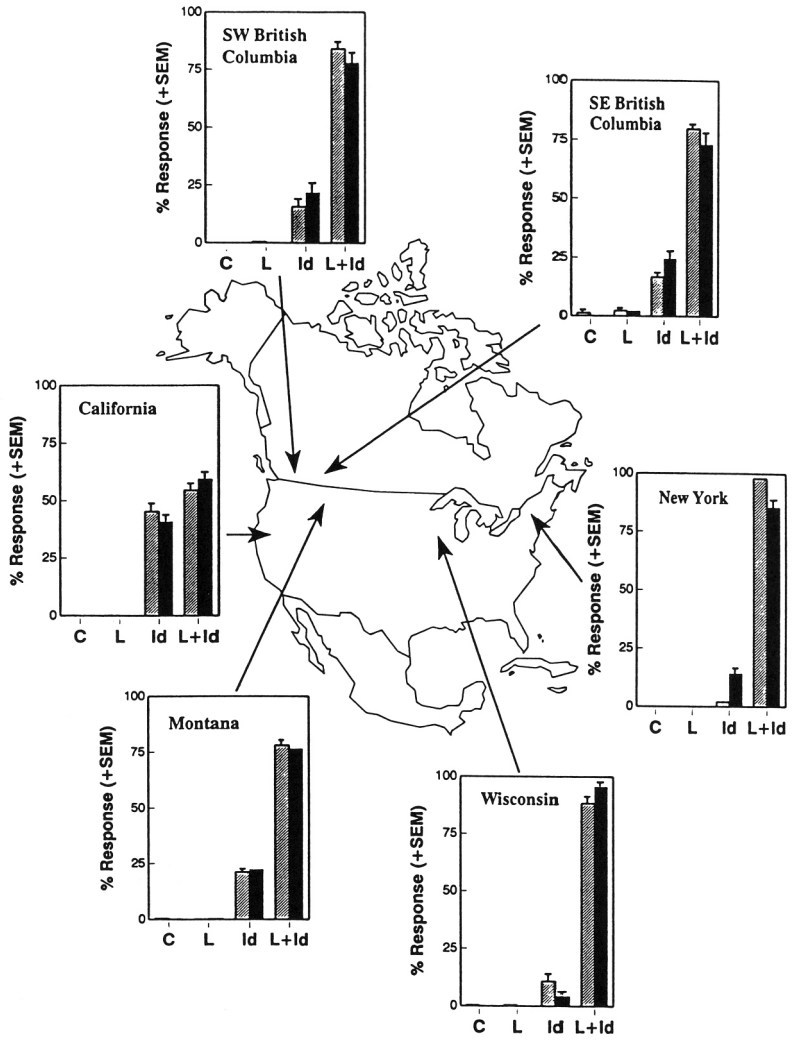


FIG. 1. Geographic variation in the response of *Ips pini* to multiple-funnel traps baited with lanierone (L) and/or ipsdienol (Id), relative to control traps (C). (*R*)-(−)-Ipsdienol was used in southeastern British Columbia and California. Racemic ipsdienol was used in the remaining regions. Data are presented as mean percentages of replicate catches for site A (striped bar) and site B (solid bar) in each region ($N = 5$).

TABLE 2. CATCHES OF MALE *Ips pini* IN MULTIPLE-FUNNEL TRAPS BAITED WITH IPSDIENOL AND/OR LANIERONE AT TWO SITES IN EACH OF SIX REGIONS IN NORTH AMERICA ($N = 5$)

Region and site	Mean (±SEM) number of male beetles[a]				
	Control	Lanierone	Ipsdienol[b]	Lanierone + Ipsdienol[b]	Increase (%)[c]
California					
A	0.4 ± 0.3 a	0.2 ± 0.2 a	129.9 ± 9.1 b	179.4 ± 5.6 b	
B	0.0 ± 0.0 a	0.0 ± 0.0 a	52.0 ± 19.8 b	94.2 ± 7.9 c	81
British Columbia (SW)					
A	0.0 ± 0.0 a	0.0 ± 0.0 a	10.9 ± 2.8 b	85.1 ± 23.1 c	681
B	0.0 ± 0.0 a	0.4 ± 0.3 a	17.1 ± 2.5 b	75.9 ± 13.9 c	344
British Columbia (SE)					
A	5.6 ± 2.6 a	13.8 ± 4.2 a	107.3 ± 15.1 b	714.0 ± 100.9 c	565
B	1.5 ± 0.5 a	12.2 ± 1.7 b	132.8 ± 30.1 c	542.7 ± 76.3 d	309
Montana					
A	3.9 ± 2.7 a	1.4 ± 0.4 a	188.0 ± 26.8 b	689.0 ± 37.2 c	266
B	4.3 ± 1.3 a	5.6 ± 1.4 a	252.9 ± 18.0 b	909.6 ± 87.3 c	260
Wisconsin					
A	1.2 ± 0.7 a	0.5 ± 0.2 a	19.5 ± 4.7 b	169.5 ± 34.9 c	769
B	0.0 ± 0.0 a	0.4 ± 0.3 a	6.4 ± 2.6 b	228.2 ± 32.3 c	3,466
New York					
A	0.2 ± 0.2 a	0.2 ± 0.2 a	83.3 ± 12.9 b	722.4 ± 132.7 c	767
B	0.0 ± 0.0 a	0.0 ± 0.0 a	2.4 ± 0.8 b	241.0 ± 22.3 c	9,942

[a] Means followed by the same letter within a row are not significantly different at $P = 0.05$ [Tukey's HSD multiple comparison test on data transformed by $\ln(Y + 1)$].

[b] (*R*)-(−)-Ipsdienol in California and southeastern British Columbia, and racemic ipsdienol in remaining regions.

[c] Percent increase of catches in traps baited with both ipsdienol and lanierone relative to those in traps baited with ipsdienol alone.

TABLE 3. CATCHES OF FEMALE *Ips pini* IN MULTIPLE-FUNNEL TRAPS BAITED WITH IPSDIENOL AND/OR LANIERONE AT TWO SITES IN EACH OF SIX REGIONS IN NORTH AMERICA ($N = 5$)

Region and site	Mean (±SEM) number of female beetles[a]				Increase (%)[c]
	Control	Lanierone	Ipsdienol[b]	Lanierone + Ipsdienol[b]	
California					
A	0.2 ± 0.2 a	0.8 ± 0.4 a	208.1 ± 16.7 b	219.8 ± 12.1 b	
B	0.0 ± 0.0 a	0.0 ± 0.0 a	95.6 ± 10.5 b	117.0 ± 4.0 b	
British Columbia (SW)					
A	0.0 ± 0.0 a	1.4 ± 0.8 a	27.7 ± 5.2 b	144.3 ± 29.8 c	421
B	0.2 ± 0.2 a	0.6 ± 0.3 a	41.1 ± 2.7 b	159.3 ± 28.1 c	288
British Columbia (SE)					
A	11.0 ± 6.9 a	22.8 ± 5.6 a	160.9 ± 18.7 b	576.0 ± 56.4 c	258
B	2.8 ± 1.0 a	17.6 ± 4.1 b	199.6 ± 33.0 c	472.9 ± 109.8 d	137
Montana					
A	2.7 ± 1.0 a	1.2 ± 0.4 a	104.0 ± 12.3 b	385.2 ± 14.5 c	270
B	3.3 ± 0.9 a	5.4 ± 1.6 a	102.1 ± 9.3 b	314.0 ± 68.9 c	208
Wisconsin					
A	0.4 ± 0.3 a	1.5 ± 0.4 a	23.3 ± 8.5 b	190.1 ± 25.9 c	716
B	0.0 ± 0.0 a	0.4 ± 0.3 a	17.4 ± 4.3 b	329.6 ± 33.3 c	1,794
New York					
A	0.0 ± 0.0 a	0.0 ± 0.0 a	7.8 ± 1.5 b	264.2 ± 28.9 c	3,287
B	0.0 ± 0.0 a	0.0 ± 0.0 a	138.1 ± 23.2 b	817.2 ± 200.6 c	492

[a] Means followed by the same letter within a row are not significantly different at $P = 0.05$ [Tukey's HSD multiple comparison test on data transformed by $\ln(Y + 1)$].

[b] (*R*)-(−)-Ipsdienol in California and southeastern British Columbia, and racemic ipsdienol in remaining regions.

[c] Percent increase of catches in traps baited with both ipsdienol and lanierone relative to those in traps baited with ipsdienol alone.

TABLE 4. SIGNIFICANCE LEVELS FOR ANALYSES OF VARIANCE (ANOVAS) ON SEX RATIOS IN CATCHES OF *Ips pini* IN IPSDIENOL-BAITED MULTIPLE-FUNNEL TRAPS IN SIX REGIONS OF NORTH AMERICA

Region	Site (S)	Lanierone (L)	S * L
California	0.287	0.001	0.495
British Columbia (SW)	0.886	0.051	0.328
British Columbia (SE)	0.866	0.001	0.800
Montana	0.033	0.562	0.567
Wisconsin	0.007	0.235	0.042
New York	0.017	0.001	0.023

TABLE 5. PROPORTION OF MALE *Ips pini* IN IPSDIENOL-BAITED MULTIPLE-FUNNEL TRAPS AT TWO SITES IN EACH OF SIX REGIONS IN NORTH AMERICA ($N = 5$)

	Mean (± SEM) proportion of males[a]		*t* test[b]	
Region and site	Ipsdienol[c]	Lanierone + ipsdienol[c]	*df*	*P*
California				
A	0.38 ± 0.02 abcd	0.45 ± 0.02 abc	7.8	0.015
B	0.35 ± 0.02 abcd	0.44 ± 0.02 abc	7.9	0.007
British Columbia (SW)				
A	0.26 ± 0.04 abc	0.36 ± 0.03 ab	7.5	0.068
B	0.29 ± 0.02 abcd	0.32 ± 0.02 a	7.6	0.258
British Columbia (SE)				
A	0.40 ± 0.02 c	0.55 ± 0.02 cd	7.6	.001
B	0.38 ± 0.03 c	0.55 ± 0.04 cd	7.1	0.017
Montana				
A	0.63 ± 0.06 ef	0.64 ± 0.02 de	4.7	0.996
B	0.71 ± 0.01 f	0.75 ± 0.05 e	4.2	0.456
Wisconsin				
A	0.50 ± 0.06 cde	0.46 ± 0.04 abc	7.5	0.517
B	0.23 ± 0.08 abcd	0.41 ± 0.03 abc	5.2	0.079
New York				
A	0.19 ± 0.06 a	0.48 ± 0.02 abc	5.1	0.003
B	0.38 ± 0.02 abcd	0.48 ± 0.02 abc	7.9	0.004

[a] Means followed by the same letter within a column are not significantly different at $P = 0.05$ [Tukey's HSD multiple comparison test on data transformed by arcsine(Y)].

[b] Two-sided test comparing proportions of males in traps baited with ipsdienol alone to those in traps baited with the combination of ipsdienol and lanierone.

[c] (*R*)-(−)-Ipsdienol in California and southeastern British Columbia, and racemic ipsdienol in remaining regions.

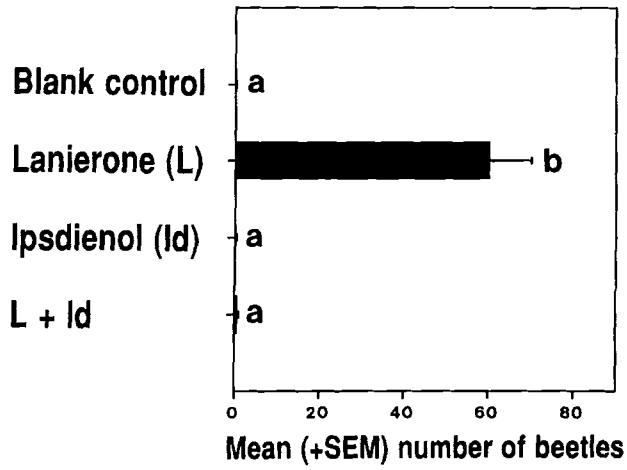


FIG. 2. Responses of *Ips integer* to multiple-funnel traps baited with lanierone and/or racemic ipsdienol in Missoula, Montana, from July 19 to 29, 1991 (N = 10). Means followed by the same letter are not significantly different at P = 0.05 [Tukey's HSD multiple comparison test on data transformed by $\ln(Y + 1)$].

ferent from the control traps (Figure 2). Racemic ipsdienol alone was not attractive. *Ips integer* were not caught at any other location.

Predators. Ipsdienol and or lanierone were attractive to six species of bark beetle predators. The preferred treatments for *Enoclerus lecontei* (Wolcott) (Cleridae) were the combination of lanierone and (*R*)-(−)-ipsdienol in California and southeastern British Columbia, and the combination of lanierone and racemic ipsdienol in Montana (Table 6). The attraction was synergistic in California (ANOVA, $P < 0.001$, $df = 1,24$) but not in southeastern British Columbia and Montana (ANOVA; $P = 0.616$ and $P = 0.554$, respectively; $df = 1,24$ and $df = 1,24$, respectively). (*R*)-(−)-Ipsdienol was attractive to *E. sphegeus* (F.) and *Temnochila chlorodia* (Mannerheim) (Trogositidae) in California and to *Thanasimus undatulus* (Say) (Cleridae) in southeastern British Columbia (Table 6). Similarly, *E. nigrifrons* var. *gerhardi* Wolc. and *Thanasimus dubius* (F.) in Wisconsin were attracted to racemic ipsdienol. None of these five species were affected by lanierone.

DISCUSSION

Our results are consistent with those of Teale (1990), Teale et al. (1991), and Seybold et al. (1992). Lanierone generally synergized the attraction of both male and female *I. pini* to the principal pheromones, (*R*)-(−)- and (*S*)-(+)-

TABLE 6. RESPONSES OF BANK BEETLE PREDATORS TO MULTIPLE-FUNNEL TRAPS BAITED WITH IPSDIENOL AND/OR LANIERONE

Species and region	Mean (±SEM) number of beetles[a]			
	Control	Lanierone	Ipsdienol[b]	Lanierone + ipsdienol[b]
Enoclerus lecontei				
California[c]	0.0 ± 0.0 a	0.2 ± 0.1 a	0.6 ± 0.2 a	6.3 ± 1.6 b
British Columbia (SE)[d]	0.8 ± 0.6 a	1.5 ± 0.3 ab	2.3 ± 0.4 b	6.9 ± 1.4 c
Montana[e]	8.6 ± 2.1 a	24.2 ± 4.9 b	76.2 ± 6.6 cd	156.2 ± 23.9 d
Enoclerus sphegeus				
California[c]	0.3 ± 0.2 a	0.4 ± 0.2 a	8.2 ± 1.6 b	6.3 ± 1.8 b
British Columbia (SE)[f]	2.0 ± 0.8 a	2.0 ± 1.1 a	6.2 ± 1.5 a	5.4 ± 1.8 a
Enoclerus nigrifrons				
Wisconsin[g]	0.1 ± 0.1 a	0.0 ± 0.0 a	2.8 ± 0.7 b	2.8 ± 1.4 b
Thanasimus dubius				
Wisconsin[g]	0.0 ± 0.0 a	0.0 ± 0.0 a	3.6 ± 1.7 b	3.2 ± 1.1 b
Thanasimus undatulus				
British Columbia (SE)[f]	1.6 ± 0.3 a	1.4 ± 0.4 a	6.4 ± 1.8 b	7.0 ± 1.1 b
Temnochila chlorodia				
California[c]	0.8 ± 0.5 a	1.3 ± 0.4 a	14.2 ± 0.9 b	11.4 ± 1.2 b

[a]Means followed by the same letter within a row are not significantly different at $P = 0.05$ [Tukey's HSD multiple comparison test on data transformed by $\ln(Y + 1)$].
[b](*R*)-(−)-Ipsdienol in California and southeastern British Columbia, and racemic ipsdienol in remaining regions.
[c]Collected between July 23 and 30, 1991 ($N = 10$).
[d]Collected between June 27 and July 11, 1991 ($N = 10$).
[e]Collected between July 12 and 29, 1991 ($N = 10$).
[f]Collected between June 27 and July 11, 1991 ($N = 5$).
[g]Collected between July 19 and August 16, 1991 ($N = 10$).

ipsdienol. By itself, lanierone was not very attractive. However, there were significant regional differences in the effects of lanierone on the responses of male and female *I. pini*.

Firstly, there was geographical variation in the mean proportional increases in catches to ipsdienol-baited, multiple-funnel traps due to the presence of lanierone. Synergism was most pronounced in both of the eastern regions. The mean proportional increases ranged from 492% to 9942% in eastern North America compared to a range of 0–681% in western regions. Lanierone had the least effect in California.

Secondly, southeastern British Columbia differed from other regions with respect to the effect of lanierone alone, and the interaction between ipsdienol and lanierone. This was the only region where lanierone was significantly attractive to *I. pini* by itself. The mean proportional increases in catches between control traps and those baited with lanierone alone ranged from 107% to 713%. Further, southeastern British Columbia was the only region where the interaction between ipsdienol and lanierone was not significant ($P = 0.603$ and $P = 0.327$ for females and males, respectively). The lack of statistical significances associated with the interaction terms for males and females indicate that the proportional increases in trap catches due to the presence of lanierone was the same with and without ipsdienol. This was not true of most of the other sites, which clearly demonstrated synergism between ipsdienol and lanierone in the attraction of *I. pini*.

Lastly, the differences in sex ratios in catches to traps baited with ipsdienol and lanierone, relative to those in traps baited with ipsdienol alone, were clearly significant in three regions. In California, New York, and southeastern British Columbia, proportionally more males than females were attracted to traps baited with the binary mixture, resulting in a reduction in female bias. Yet lanierone had little effect on catches of males and females in California. Previous results in New York demonstrated that an increase in the proportion of lanierone resulted in an increase in the proportion of males (Teale et al., 1991). In a separate trial in California, Seybold et al. (1992) found that sex ratio of *I. pini* caught in ipsdienol-baited funnel traps was not affected by lanierone. In the three sites in this study where the sex ratio was either 1 : 1 or slightly male biased, there were no significant differences in sex ratio between the two ipsdienol treatments. Further, the increase in male representation in trap catches varied between sites in eastern regions but not in western ones.

Presently, we cannot fully explain all these observed variations. However, the following factors may be important. Firstly, geographic variation in the use of (*R*)-(−)- and (*S*)-(+)-ipsdienol as a pheromone by *I. pini* is known to occur between populations in California and New York. Beetles in California produce and respond primarily to (*R*)-(−)-ipsdienol (Birch et al., 1980; Seybold, 1992).

Attraction to (*R*)-(−)-ipsdienol is interrupted by (*S*)-(+)-ipsdienol. The sympatric species *I. paraconfusus* Lanier uses (*S*)-(+)-ipsdienol as one of its pheromones (Silverstein et al., 1966; Wood et al., 1968). The pheromones of *I. pini* and *I. paraconfusus* are mutually inhibitory in California, presumably to ensure reproductive isolation (Birch and Wood, 1975; Birch and Light, 1977; Birch, 1978; Light and Birch, 1979; Birch et al., 1980). *Ips paraconfusus* is not present east of Oregon and California (Wood, 1982) and eastern *I. pini* use both enantiomers of ipsdienol as pheromones (Stewart, 1975; Lanier et al., 1980; Teale and Lanier, 1991).

The lack of a strong increase in attraction of *I. pini* to ipsdienol-baited traps in California, when lanierone is added, may be a consequence of similar species specificity in the use of pheromones to ensure reproductive and/or ecological separation. The role of lanierone in the aggregation behavior of *I. paraconfusus* has not been studied. Seybold et al. (1992) could not detect lanierone in another potential competitor, *Dendroctonus brevicomis* LeConte. *Ips integer* was strongly attracted to lanierone in Montana but not detected in California or British Columbia, even though both regions are well within its range (Wood, 1982).

Either lanierone is not important as a pheromone to *I. pini* in California or only a specific mixture of lanierone and (*R*)-(−)-ipsdienol is active. Evidence by Seybold et al. (1992) seems to support the first hypothesis. However, they suggest that selection pressure from predation by *E. lecontei* may have favored *I. pini* that no longer used lanierone as a pheromone, similar to the model proposed for differential chiral and seasonal responses between *I. pini* and its major predators in Wisconsin (Raffa and Klepzig, 1989; Raffa, 1991). Yet, in southeastern British Columbia and Montana both *E. lecontei* and *I. pini* responded strongly to the combination of ipsdienol and lanierone.

Regional differences in population levels may explain some of the variation. The mean replicate catches of *I. pini* per site were correlated with the mean proportion of males in catches to traps baited with ipsdienol alone and the mean proportion of males in catches to traps baited with ipsdienol and lanierone ($r = 0.623$ and $r = 0.781$, respectively; $P = 0.031$ and $P = 0.003$, respectively). In contrast, the mean replicate catches of males and females per site were not correlated with the mean proportional increases in catches of males and females, respectively, in ipsdienol-baited traps due to the presence of lanierone ($r = 0.220$ and $r = 0.249$, respectively; $P = 0.491$ and $P = 0.436$, respectively). We should note, however, that the mean replicate catches of beetles may not be a good estimator of population size since the regions varied in trapping period and duration, stand structure, and temperature regime. Unfortunately, we do not have a better estimator.

Regional differences in the timing of life history activities relative to the trapping periods might also explain some of the variation. In California, responses

of male and female *I. pini* to extracts of male frass were lowest during the winter months, presumably due to diapause-related decreased receptiveness and/or decreased pheromone production (Birch, 1974). In New York and Wisconsin, most *I. pini* respond to synthetic ipsdienol late in the season (Teale, 1990; Raffa, 1991; Teale and Lanier, 1991). However, in Wisconsin in 1990, catches of beetles in ipsdienol-baited funnel traps were as high in April as they were in June and August (Raffa, 1991).

We should expect changes in the operational sex ratio (Emlen and Oring, 1977) during the course of an attack as well. The sex ratio among *Dendroctonus ponderosae* Hopkins landing on lodgepole pine changes during the colonization period, with the proportion of females declining over time (Raffa and Berryman, 1983). In this study, the operational sex ratio is defined as the ratio of active, searching males to available, unattached females, thereby omitting those beetles that are occupied in activities under the bark such as mating or egg laying. Males initiate the attacks and then produce pheromones while they wait for females. This results in local depletions of males available for capture in pheromone-baited traps. *Ips pini* is a polygynous species, with males having three to four females per harem (Thomas, 1961). Once harems are fully established, the operational sex ratio should become male biased with two to three times as many females as males under the bark. If males exhibit defensive behaviors by remaining in the gallery after females have left, then once again we would expect female bias in the operational sex ratio. Therefore, variation in sex ratios between regions may be due to variation in sampling periods relative to the sequence of behaviors during attack. Beetles in some regions may have only begun to attack new host material while beetles in other regions may have been well into the last stages of the behaviors associated with attacks.

There are three additional factors that may contribute to regional differences in the operational sex ratio. Firstly, attacks by bark beetles on suitable hosts are sequential. Activities at one gallery may not be synchronous with activities in galleries farther up the trunk of a tree (see reviews in Coulson, 1979; Berryman, 1982). Secondly, if males have a propensity to disperse prior to attack, then this would result in additional depletion of males. The sex ratio among *I. pini* attacking new host material is generally female biased at 2 females/male (Schenk and Benjamin, 1969; Schmitz, 1972). Lastly, the male bias detected in Montana may have resulted from the use of racemic ipsdienol in this region. Past studies in British Columbia and California have demonstrated that the proportion of males captured in ipsdienol-baited traps is correlated with the proportion of (*S*)-(+)-ipsdienol used in the lures (Miller, 1990; Seybold, 1992). Males in Montana produce ipsdienol with an enantiomeric composition of 91:9 to 95:5 (*R*)-(−):(*S*)-(+) (Seybold et al., 1995). Male bias in trap catches might have been reduced if (*R*)-(−)-ipsdienol had been used instead of racemic ipsdienol.

Examples of geographic variation in the use of pheromones have commonly

been reported for Lepidoptera (Roelofs, 1980; Cardé and Baker, 1984). The European corn borer, *Ostrinia nubilalis* (Hübner) (Pyralidae), uses 11-tetradecenyl acetate (11–14:OAc) as a sex pheromone (Klun, 1968; Klun and Brindley, 1970; Klun and Robinson, 1971). Geographic variation is based on the relative proportion of the *E* and *Z* isomers of 11–14:OAc (Klun et al., 1973; Kochansky et al., 1975). Most populations in Europe and North America respond to a 3:97 *E*:*Z* ratio of 11–14:OAc, while populations in Italy, the Netherlands, and parts of northeastern United States respond preferentially to a 97:3 *E*:*Z* blend (Klun and Cooperators, 1975). Some areas, such as New York state, have discrete populations using only the 3:97 *E*:*Z* blend as well as hybrid populations (Roelofs et al., 1985). In California, at least three different populations of the western avocado leafroller, *Amorbia cuneana* (Walsingham) (Tortricidae), differ in the production of *E*:*Z* blends of the sex pheromone, (*E*,*E*/*Z*)-10,12-tetradecadien-1-ol acetate. Two populations respond to blends close to 1:1, while the third prefers blends with a higher *E*:*Z* ratio (Bailey et al., 1986). In New Zealand, brown-headed leafrollers, *Ctenopseustis obliquana* (Walker) (Tortricidae), exhibit two different pheromone population types. One uses an 80:20 mix of (*Z*)-8-tetradecenyl and (*Z*)-5-tetradecenyl acetates, while the other uses only (*Z*)-5-tetradecenyl acetate (Foster and Roelofs, 1987).

Our results and those of others (Birch et al., 1980; Lanier et al., 1980; Miller et al., 1989a, 1996; Raffa and Klepzig, 1989; Teale, 1990; Teale et al., 1991; Seybold, 1992; Seybold et al., 1992, 1995) with *I. pini* support the need for tests over its full geographical range. Pheromone blends differ substantially between areas, necessitating the use of regionally specific lures for pest management. Our results indicate that a blend of racemic ipsdienol and lanierone is optimal for most regions while (*R*)-(−)-ipsdienol by itself is sufficient for use in California. Regional pheromone specificity, and the need for tests throughout geographic ranges, may be important for other scolytid species as well. In laboratory bioassays, the southern pine beetle, *Dendroctonus frontalis* Zimmerman, collected from Georgia, Texas, and Virginia, showed marked preferences for pheromones from their respective regions (Berisford et al., 1990). A standard lure of frontalin, *trans*-verbenol, and turpentine was as attractive as the regional blends in Georgia and Texas, but not in Virginia.

Bark beetle pheromones are used as kairomones by predators (Borden, 1982; Dahlsten, 1982; Miller et al., 1989b; Payne, 1989). *Temnochila chlorodia*, *Enoclerus sphegeus*, *E. nigrifrons*, *Thanasimus undatulus*, and *T. dubius* were all attracted to ipsdienol. These results are consistent with those of Vité and Gara (1962), Wood et al. (1968), Furniss and Livingston (1979), Raffa and Klepzig (1989), Miller and Borden (1990), and Seybold et al. (1992). Similar research has demonstrated that *E. lecontei* is also attracted to ipsdienol (Wood et al., 1968; Miller and Borden, 1990). Our results further show attraction of *E. lecontei* to ipsdienol and lanierone in three different regions. As found by

Seybold et al. (1992), synergism occurred between ipsdienol and lanierone in attracting *E. lecontei* in California. Synergism did not occur in British Columbia or Montana. We suggest that synergism may reflect differential benefits for *E. lecontei* in California, compared to *E. lecontei* in British Columbia and Montana, resulting in a greater measure of prey specificity in California, relative to other regions.

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RESPONSE OF PREDATORY INSECT *Scolothrips takahashii* TOWARD HERBIVORE-INDUCED PLANT VOLATILES UNDER LABORATORY AND FIELD CONDITIONS[1]

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Abstract—We studied the response of a predatory thrips, *Scolothrips takahashii*, towards herbivore-induced plant volatiles emitted by Lima bean plants infested by two-spotted spider mites *Tetranychus urticae* (green form). Tests were conducted with a Y-tube olfactometer in the laboratory and with traps under field conditions. The odor of artificially damaged and uninfested Lima bean leaves was not more attractive than clean air in the Y-tube olfactometer. The predatory insects showed a greater preference for Lima bean leaves infested by the two-spotted spider mites than for either clean air or uninfested bean leaves. They showed the same preference towards infested leaves from which all spider mites and their visible products had been removed. Neither the spider mites themselves nor their products attracted the predators. In a satsuma mandarin grove, two traps with infested Lima bean plants as an odor source attracted 42 adult *S. takahashii* in 55 days, whereas no *S. takahashii* were trapped in two control traps with uninfested Lima bean plants during the same period. No *S. takahashii* were found during this period in the vicinity of either the sample traps or the control traps (5-m radius of each trap). These data showed that *S. takahashii* use herbivore-induced plant volatiles in their foraging behavior in natural ecosystems.

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Key Words—Tritrophic interactions, Lima bean plants, *Tetranychus urticae*, *Scolothrips takahashii*, herbivore-induced plant volatiles, Y-tube olfactometer, field trap.

INTRODUCTION

Several plant species infested by herbivorous arthropods are reported to emit characteristic volatiles that attract carnivorous natural enemies (predators and parasitoids) in response to herbivore damage (for review, Dicke, 1994; Takabayashi and Dicke, 1996; Turlings et al., 1993). One of the well-studied examples is a tritrophic system consisting of Lima bean plants, two-spotted spider mites *Tetranychus urticae*, and predatory mites *Phytoseiulus persimilis*: when Lima bean plants are infested by the spider mites, the plants start emitting herbivore-induced plant volatiles that attract the predatory mites (Dicke et al., 1990a,b; Takabayashi et al., 1995). The emission of the volatiles can be adaptive to plants when the volatiles recruit carnivores that reduce herbivore damage. The use of the volatiles is also beneficial to natural enemies when the volatiles enhance their foraging efficiency. Recently, some anthocirid predators [*Anthocoris nemorum*, *A. nemoralis* and Orius spp. (Heteroptera: Anthocoridae)] were reported to be attracted to pear trees infested by pear psylla under field conditions (Drukker et al., 1995). When such volatiles are beneficial to the signaler (plant) and a receiver (natural enemy), they are called herbivore-induced plant synomones (Vet and Dicke, 1992).

In the previous studies on plant–carnivore interactions through herbivore-induced plant volatiles, the interactions were mainly demonstrated under laboratory conditions using olfactometers and wind tunnels, where only sample and control volatiles were offered to test animals (Agelopoulos and Keller, 1994; Dicke and Sabelis, 1988; Dicke et al., 1990a,b; Mattiacci et al., 1994; Takabayashi and Dicke, 1992; Takabayashi et al., 1995; Sabelis and Baan, 1983; Turlings et al., 1990, 1993). However, there are many background volatiles in natural ecosystems, and it is not yet clearly demonstrated how such carnivores can detect herbivore-induced volatiles mixed with the background.

In this study, we investigate a tritrophic interaction of Lima bean plants, two-spotted spider mites, and predatory thrips *Scolothrips takahashii* Priesner (Thysanoptera: Thripidae) under both laboratory and field conditions. Spider mites are ravenous herbivores and important pests in many agricultural crops (Helle and Sabelis, 1985). They frequently overexploit their host plants in the absence of their carnivorous natural enemies. There are many effective natural enemies of spider mites, however, such as predatory mites and predatory insects (Chazeau 1985; McMurtry et al., 1970; Mori, 1967). Dicke and his coworkers have extensively studied the response of predatory mites to herbivore-induced

volatile attractants emitted by plants infested by spider mites (for review, see Dicke et al., 1990b; Dicke, 1994; Takabayashi et al., 1994). In contrast, nothing is known about the response of predatory insects towards plant volatiles induced by spider mite infestation. Here, we focus on the response of the predatory insects toward spider mite-induced volatiles. We also carry out a field investigation for spider mite species and their predator species to estimate the effectiveness of the volatiles in predator attraction in field conditions.

METHODS AND MATERIALS

Biology of Scolothrips takahashii. *Scolothrips takahashii* is a small insect (ca. 0.8–1.4 mm long), but it is considered to be an important natural enemy of some spider mite species, especially the genus *Tetranychus* (Nakagawa, 1987, 1988; Yamasaki et al., 1983). It preys on *T. urticae* (green form) on Japanese pear trees (Shimoda et al., unpublished data), *T. kanzawai* Kishida on grapevine trees (Ashihara et al., 1992), *T. kanzawai* on tea trees (Hamamura, 1986; Nakagawa, 1988) and other spider mite species in Japan. Both adult and larval stages of this insect feed on all stages of those spider mites (Nakagawa, 1987). Although the predator is also found on plants infested by spider mites of the genus *Panonychus* in orchards (Hamamura et al., 1984), it remains to be clarified whether or not this predator is an important natural enemy of those spider mites.

Insect Rearing. Scolothrips takahashii were collected from three sites: leguminous arrowroot plants [*Pueraria lobata* (Wild.) Ohwi] infested by *T. urticae* (red form), asparagus bean (*Vigna sinensis* Savi) infested by *T. phaselus* Ehara, and *Clerodendron trichotomum* infested by *T. kanzawai* at the Kuchinotsu Fruit Research Station in 1994. The predators were reared for about one year on *T. urticae* (green form) on Lima bean plants (*Phaseolus lunatus* cv. Sieva or Baby Lima) in a climate room at 20 ± 2°C, 50–70% relative humidity, and 16L:8D regime.

Plants. Lima bean plants (Sieva) were cultivated on wet rockwool-blocks (10 × 10 × 7.5 cm) in a climate room at 20 ± 2°C, 50–70% relative humidity, and 16L:8D photoperiod. Trifoliar leaves (ca. 10–15 cm long, ca. 5–10 cm wide) from the plants three to five weeks after germination were used for the laboratory experiments.

Response of Predatory Insects Toward Leaves Infested by T. urticae *in Y-Tube Olfactometer.* We used a Y-tube olfactometer (Takabayashi and Dicke, 1992) that was vertically fixed with a stand. Air (4 liters/min) was first cleaned through granular activated charcoal (1 liter in a glass bottle) and then sent to odor source bottles (a sample odor bottle and a control odor bottle). The air containing each odor was sent separately to either of the arms of the olfactometer

setup. Satiated *S. takahashii* females were individually introduced at a start point (downwind end) on a Y-shaped iron wire set in the tube, and their behavior on the wire was observed for 5 min. When the predator walked across the finish line set on the upwind ends of the Y-shaped wire, this was classified as a final choice. When the predator did not make a final choice within 5 min, it was classified as no choice. We switched the arm containing the sample odor and that containing control odor in the olfactometer after every five bioassays. The bioassay was performed in a climate room at 25 ± 2°C from the middle of February 1995 to the end of April 1996. In each experiment, we added a piece of moist cotton wool to both sample and control odor source. For details of the olfactometer setup, see Takabayashi and Dicke (1992).

Odor sets for the Y-tube olfactometer bioassays were as follows:

1. Uninfested leaves vs. clean air. Nine to 12 uninfested Lima bean leaves were used as the odor source.

2. Artificially damaged leaves vs. clean air. Nine to 12 Lima bean leaves were mechanically damaged by rubbing with carborundum (Silicon carbide: 400) on a wet cotton wool pad and then used as the odor source.

3. Leaves infested with *T. urticae* vs. clean air. Nine to 12 Lima bean leaves infested by more than 100 adult mites per leaf for three to seven days were used as the odor source.

4. Leaves infested with *T. urticae* vs. uninfested leaves. Nine to 12 Lima bean leaves either uninfested or infested by more than 100 adult mites per leaf for three to seven days were used as the odor source.

5. *Tetranychus urticae* and their products vs. clean air. All living spider mites and their visible products such as feces, exuviae, eggs, egg shells, dead bodies, and silk webs were carefully removed from the leaves (9–12 leaves). These products were used as the odor source.

6. Infested leaves without *T. urticae* and their products vs. clean air. We used 9–12 infested leaves from which the spider mites and their visible products had been removed as the odor source.

The data were subjected to a G test to determine significant differences from 50:50 distribution of the predatory insects in the olfactometer.

Response of Predatory Insects Toward Leaves Infested by T. urticae *Under Field Conditions.* We used four trap boxes for field experiments (Figure 1). We placed four uninfested Lima bean plants (three to five weeks after germination) in each of two control traps. In each of two sample traps, we placed four Lima bean plants (three to five weeks after germination) infested by more than 500 *T. urticae* in all stages of development per plant. All four trap boxes were placed in a satsuma mandarin grove (46 ares, 172 trees) in Kuchinotsu Fruit Tree Research Station on May 12, 1995 (Figure 2). No pesticides were used in the grove during the experimental period. The plants and sticky trap sheets

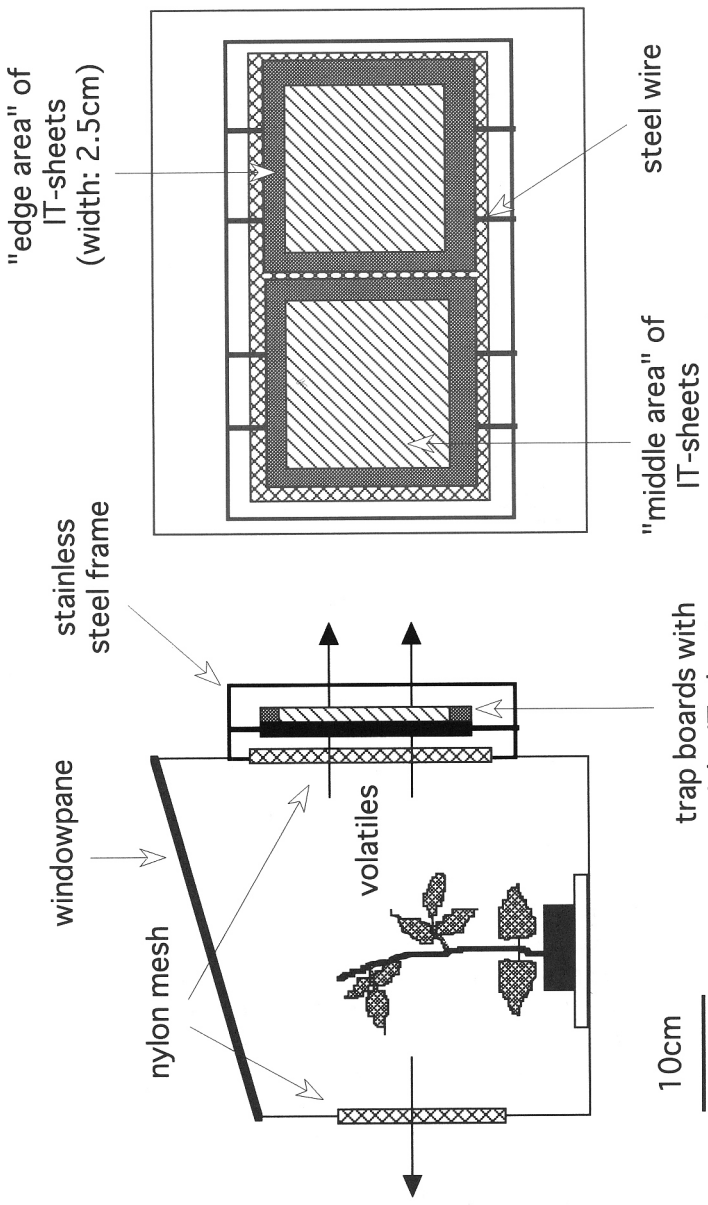


FIG. 1. Lateral view of a wooden trap box used for the field experiment. Four Lima bean plants infested by *Tetranychus urticae* were placed in a sample trap box and four uninfested Lima bean plants were placed in a control trap box. The plants and sticky trap sheets (IT-sheets) in a trap were exchanged every 7–11 days. The number of predators trapped on the sheets was counted under a binocular microscope in the laboratory.

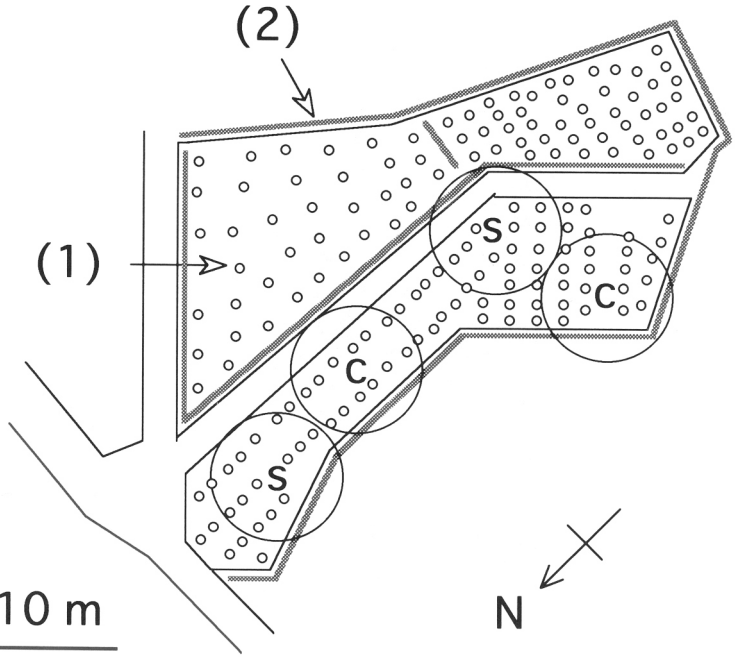


FIG. 2. A map of experimental satsuma mandarin orchard: (1) satsuma mandarin trees, (2) longleaf podocarp windbreak trees, S: sample traps, and C: control traps.

(IT-sheets, Nitto Denko Corporation) on a trap box were exchanged every 7–11 days. The number of predators trapped on a trap sheet was counted under a binocular microscope in the laboratory. The trap sheet was divided into two parts: edge area (2 cm margin of the sheet) and middle area (the rest of the sheet). The position of the predator trapped on the sheet was recorded. The experiment was carried out from May 12 to July 6, 1995.

Spider Mite and Predator Species Around Traps. The presence of spider mites and predators on plants around the sample and control traps in the satsuma mandarin groves was determined (Figure 2). We chose five mandarin trees, five longleaf podocarp windbreak trees (*Podocarpus macrophyllus*), and more than 50 weed plants (*Vicia sativa*), which were the predominant plant species within a 5-m radius of each trap (hereafter called experimental area). We placed a sticky sheet (IT-sheet: 20 × 20 cm) under each plant species and beat mandarin trees 200 times, longleaf podocarp trees 100 times, and weeds 50 times with plastic sticks (beating-trap method). With this method, the spider mites and their predators within each experimental area were collected on the sheet. They were counted under a binocular microscope in the laboratory.

We also chose 40 mandarin trees that were more than 5 m away from both the sample and control traps (hereafter called "outside the experimental area"). By the beating-trap method (beating 400 times per plant), we investigated the species of spider mites and their predators outside of the experimental area.

RESULTS

Response of Predators in Olfactometer

When the odor of uninfested Lima bean leaves and clean air were compared in the olfactometer, satiated *S. takahashii* females showed no preference to uninfested leaves over clean air (Figure 3a). The same was the case for the response to volatiles from artificially damaged leaves vs. clean air (Figure 3b).

When odor of Lima bean leaves infested with *T. urticae* and clean air were compared in the olfactometer, *S. takahashii* showed a significant preference toward odors from the infested leaves (Figure 3c). The predators also showed a preference to the infested leaf volatiles over uninfested ones (Figure 3d).

The predators showed no preference toward spider mites and their products vs. clean air (Figure 3e). The predators, however, preferred the infested leaves from which all spider mites and their products were removed over clean air (Figure 3f).

Response of Predators in the Field

During the 55-day experimental period, a total of 42 adult *S. takahashii* (20 females, 10 males, 12 unidentified sex) were trapped on the trap sheet of the sample traps (Figure 4). Most (35 adults, 83.3%) were collected from June 20 to July 6. Of 40 predators trapped, 33 (82.5%) were in the middle area of the sheets during the experimental period and the rest were in the edge area. The positions of predators trapped from May 19 to 26 were not recorded. In the control traps, not a single adult *S. takahashii* was trapped (Figure 4).

In addition to adult *S. takahashii*, the predatory beetle *Oligota kashmirica benefica* Naomi (Coleoptera: Staphylinidae) was trapped in both sample and control traps (sample traps: 2, control traps: 4). One adult *Orius* sp. was trapped in the sample trap.

No predatory mites were found on the trap sheet in either the sample or control traps. However, they were found on the infested Lima bean plants in the sample traps. From June 6 to July 6, *Amblyseius womersleyi* Schicha (Acari: Phytoseiidae), *A. eharai* Amitai et Swirski, and other *Amblyseius* mites were found on the infested plants. No predatory mites were found on the uninfested plants in the control traps.

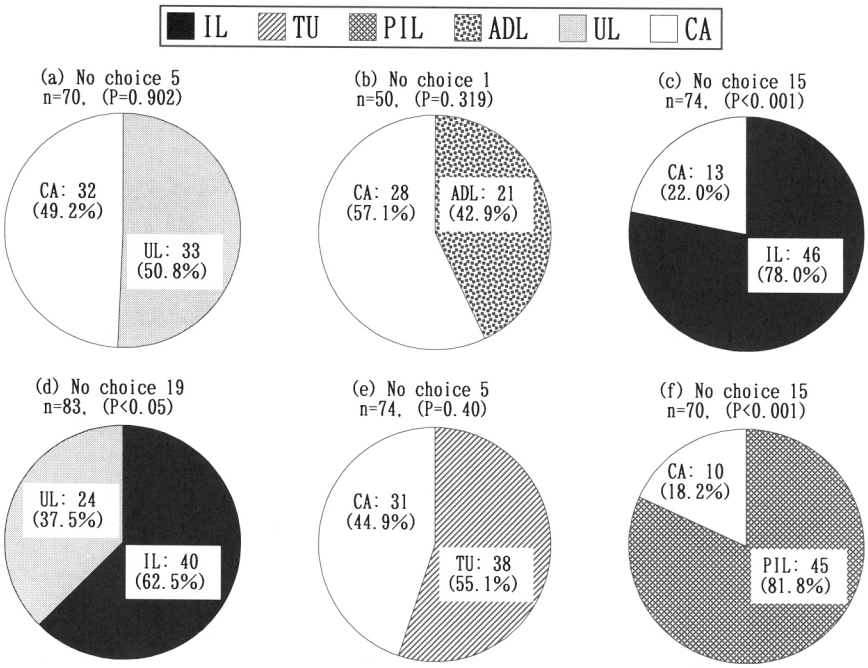


FIG. 3. Response of satiated *Scolothrips takahashii* females in a Y-tube olfactometer when offered: (a) uninfested leaves vs. clean air; (b) artificially damaged leaves (ADL) vs. clean air; (c) Lima bean leaves infested by *Tetranychus urticae* mites (IL) vs. clean air (CA); (d) IL vs. uninfested leaves (UL); (e) living spider mites and their visible products (TU) vs. clean air; or (f) infested leaves without the spider mites (PIL) vs. clean air. The *G* test was used to evaluate differences from 50:50 distribution over two olfactometer arms. Predators that did not reach the end of either olfactometer arm within 5 min (no choice) were excluded from statistical analyses.

Spider Mite and Predator Species Around Traps

Satsuma Mandarin Trees in Experimental Area. From the trees in both the sample and control trap areas, *P. citri* was trapped as the predominant spider mite species. The number of trapped *Panonychus citri* (McGregor) in both the sample and control trap areas was high on April 21 and had decreased considerably by May 15 (Figure 5). On July 7 especially, only a few spider mites were observed in both trap areas. No *S. takahashii* were trapped from the trees in the areas (Figure 5). *Oligota kashmirica benefica* was the predominant predatory insect species, and *A. eharai*, *A. sojaensis* Ehara, and *Agistemus* mites were the predominant predatory mice species in the areas.

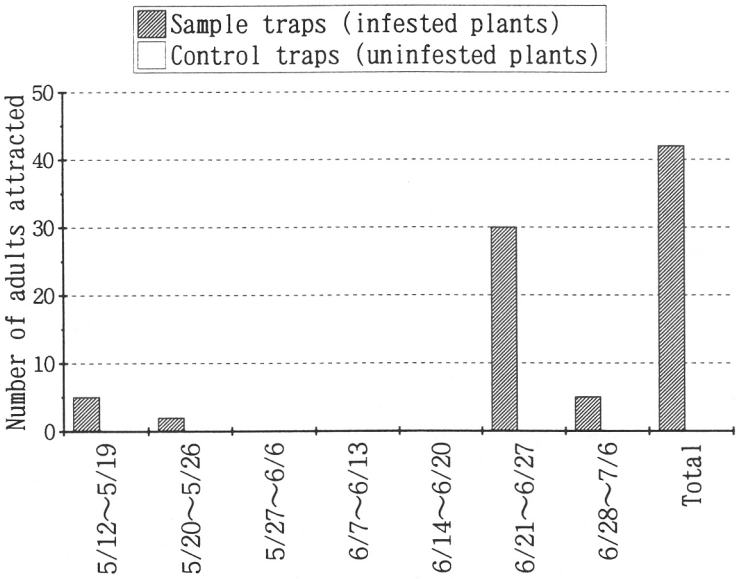


FIG. 4. The total number of *Scolothrips takahashii* adults caught by two sample traps containing Lima bean plants infested by *Tetranychus urticae* and by two control traps containing uninfested Lima bean plants from May 12 to July 6, 1995. The traps were placed within the satsuma mandarin grove at Kuchinotsu Fruit Tree Research Station on May 12, 1995. The plants and trap sheets in a trap box were changed every observation day.

Satsuma Mandarin Trees Outside Experimental Area. The dynamics of the population density of *P. citri* in the trees outside of the experimental area (more than 5 m away from each trap) resembled that of this species in the trees in the experimental area (Figure 6). The spider mites outside the experimental area were more abundant than those in the experimental area. Large numbers of predatory mite species were found. *Oligota kashmirica benefica* was the predominant predatory insect species (Figure 6). The presence of *S. takahashii* was recorded on April 21, May 12, and May 22, 1995 (Figure 6).

Longleaf Podocarp Windbreak Trees in Experimental Area. Although *P. citri* and *Tetranychus* spp. (*T. kanzawai* and other species) were found on trees in both the sample and control trap areas, their density was quite low during the investigation period (Figure 7). No *S. takahashii* were trapped from trees in the areas. *Amblyseius sojaensis* and *Agistemus* mites were the predominant predatory mice species in the areas.

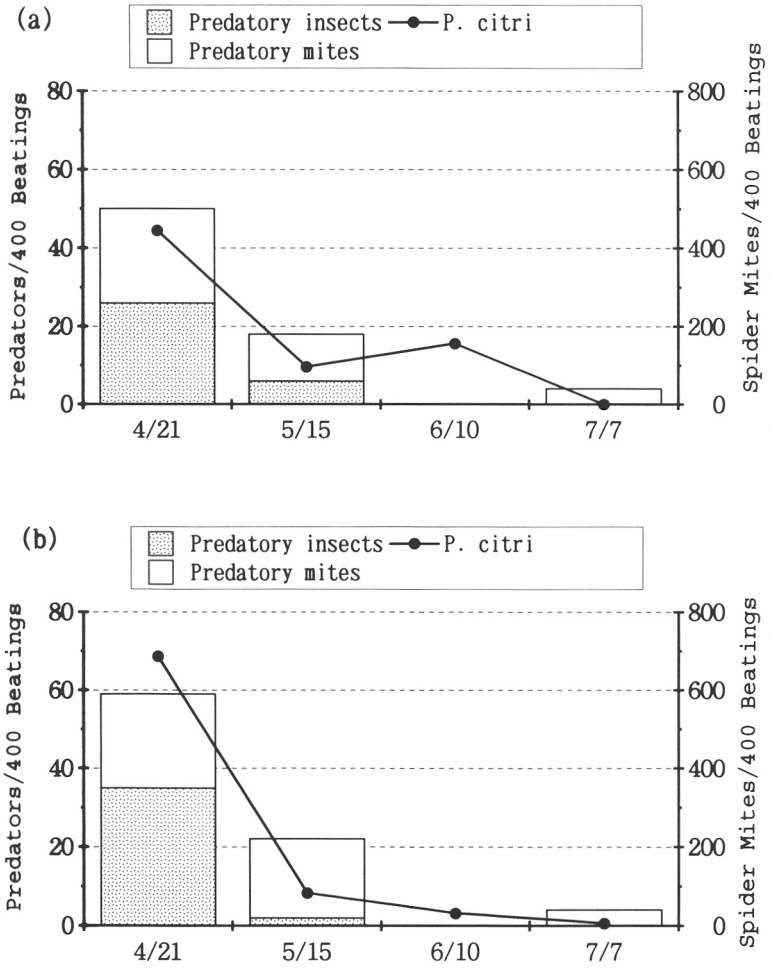


FIG. 5. Seasonal prevalence of *Panonychus citri* and their natural enemies in unsprayed satsuma mandarin trees in the experiment area within a 5-m radius from: (a) sample traps, and (b) control traps at Kuchinotsu Fruit Tree Research Station from April 21 to July 7, 1995.

Vicia sativa *Weeds in Experimental Area.* From the weeds in both the sample and control traps areas, *Tetranychus* spp. (mainly *T. kanzawai*) were trapped as the predominant spider mite species (Figure 8). The number of the spider mites trapped in both the sample and control trap areas remained high until June 10. On July 7, however, no spider mites were found in the areas

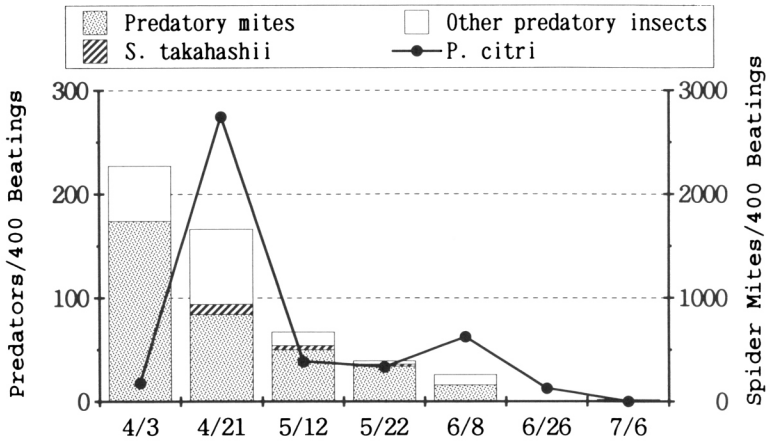


FIG. 6. Seasonal prevalence of *Panonychus citri* and their natural enemies in unsprayed satsuma mandarin trees from sample and control traps more than 5-m outside experimental area from April 3 to July 6, 1995.

because most of the weeds in the experimental grove had died. In contrast to longleaf podocarp windbreak trees, predatory insects (mainly *Orius* sp.) were more abundant than the predatory mites trapped in the areas. No *S. takahashii* were trapped in this area.

DISCUSSION

The results of the olfactometer experiments demonstrate that *S. takahashii* females are attracted to volatiles from Lima bean leaves infested by *T. urticae* mites. These volatiles appeared to originate from the leaves, because this predator did not respond to odors from the spider mites and their products, but they did respond to those from infested leaves from which spider mites and their products had been removed. The predators did not respond to the volatiles from uninfested leaves or to those from artificially damaged leaves. These results indicated that the volatiles attracting this predator are induced by spider-mite damage but not by mechanical damage. Sabelis et al. (1984) reported similar results in the interaction of the same plant species, the same herbivores, and the predatory mite *P. persimilis*. The predatory mites were attracted to the infested leaves themselves but not to the spider mites and artificially damaged leaves. They also showed that the predatory mites were slightly attracted to feces of the spider mites. In our study, the predatory thrips showed no response toward the volatiles emitted from the spider mites and their products.

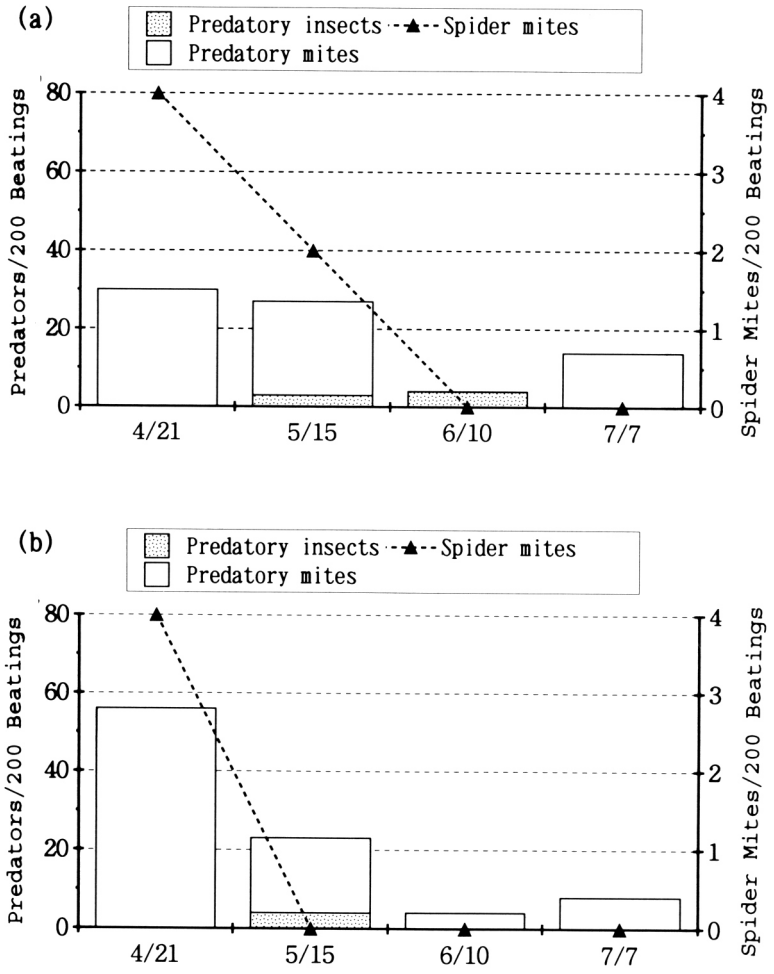


FIG. 7. Seasonal prevalence of spider mites and their natural enemies in longleaf podocarp windbreak trees in experiment area from: (a) sample traps, and (b) control traps from April 21 to July 7, 1995.

Field trap experiments also demonstrated that *S. takahashii* responded positively to volatiles emitted from the *T. urticae*-infested Lima bean plants in the satsuma mandarin orchard just as the predators did in the olfactometer. This is the first demonstration of the importance of the carnivore attractants under field conditions. The environmental conditions in the orchard are highly complex for the predatory insects, and there are several sources of background volatiles,

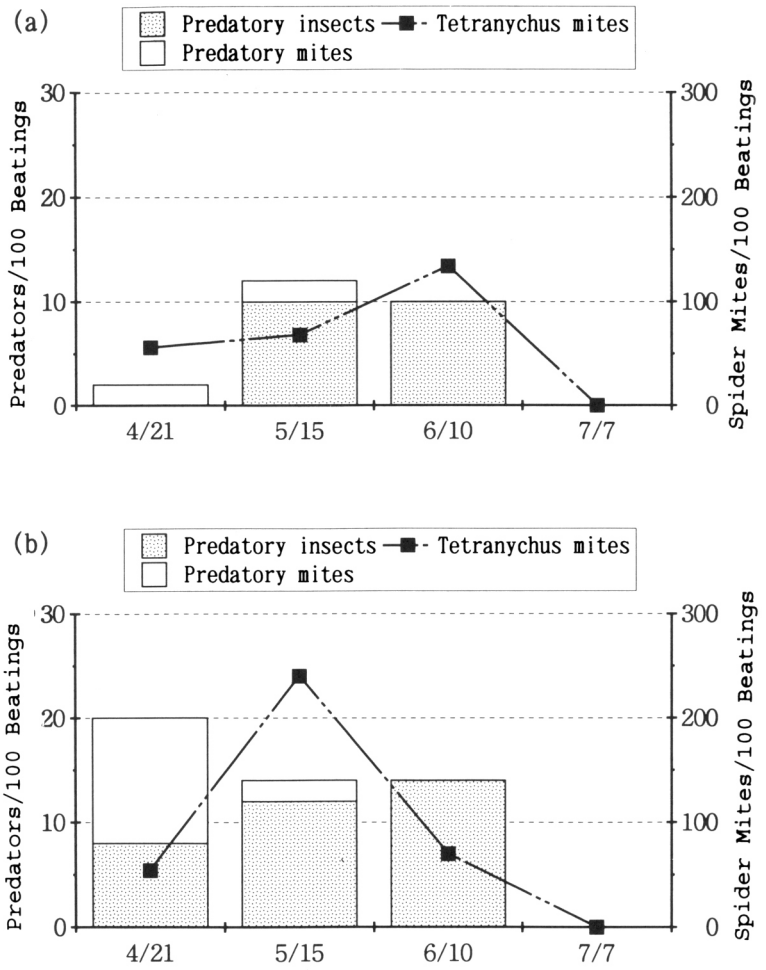


FIG. 8. Seasonal prevalence of spider mites and their natural enemies in *Vicia sativa* weeds in experiment area from: (a) sample traps, and (b) control traps from April 21 to July 7, 1995.

such as volatiles from *P. citri*-infested citrus trees and other plant species. Even under such heterogenous field conditions, the predators are able to locate the origin of the attractants from the bean plants.

Five components [(3*E*)-4,8-dimethyl-1,3,7-nonatriene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (*E*)-β-ocimene, methyl salicylate and linalool] are emitted by Lima bean plants in response to *T. urticae* damage and four of

them, but not (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, attract *P. persimilis* when offered separately in the olfactometer (Dicke et al., 1990a). In this study, we used the same Lima bean cultivar (cv. Sieva). Thus, the predatory thrips may use some of the five *T. urticae*-induced volatiles to locate the infested plants under laboratory and field conditions.

There is plant species specificity in the production of *T. urticae*-induced plant volatiles (Takabayashi et al., 1994). The preference of the predatory mites *P. persimilis* toward volatiles emitted by two different plants species infested by the same spider mites, *T. urticae*, was reported by Dicke et al. (1990c) to be subject to learning. In our orchard Lima bean plants had never been planted before. Thus, *S. takahashii* in this orchard has never experienced the specific odors emitted from Lima bean plants infested by *T. urticae* mites. In addition, we placed the infested plants into the trap boxes to prevent the attracted predators from contacting their prey spider mites. This means that the responding predatory insects had never experienced the plant volatiles emitted from this plant–prey combination in association with predation. Thus, the response of *S. takahashii* to the volatiles does not appear to be from learning but rather to be innate.

Most of the trapped *S. takahashii* individuals (82.5%) were trapped at the middle area of the trap sheets. In our preliminary observations, this predator could not move on the surface of the sticky trap sheet. Thus it was impossible for the predators to move from the edge area to the middle area on foot. These data indicate that the predators found in the middle area of the sheets were trapped as they flew against it.

Although *O. kashmirica benefica* was the predominant species of all the predatory insects in the experimental grove, only a few adults were caught in the sample traps. The predatory mites *Amblyseius womersleyi*, *A. eharai*, and other *Amblyseius* mites were found on infested Lima bean plants in the sample traps but not in the control traps. This, however, does not necessarily mean that the predatory mites were attracted. The predators may have visited both the sample and control traps and might have been arrested only in the sample traps.

During the experimental period (May 12–July 6, 1995), no *S. takahashii* were found on the predominant plant species in the experimental areas (satsuma mandarin trees, longleaf podocarpe windbreak trees, and *Vicia sativa* weeds). The presence of *S. takahashii* was recorded on the mandarin trees outside the experimental areas on April 21, May 12, and May 22, 1995. Probably, most of *S. takahashii* were from outside the experimental areas (at least 5 m away from the traps).

In the field experiment, 33 of 42 of *S. takahashii* were trapped from June 20 to July 6. During this period, the population of *P. citri* on the mandarin trees in the experiment area was quite small. The same was the case for the population of the spider mites on the mandarin trees outside the experimental areas. The

population density of *Tetranychus* spp. on the weeds and on the longleaf podocarpe windbreak trees was low in the experimental period. Thus, most of the food resources for the predatory insect in the experimental area were absent in this period. This may have caused the predator to search for alternative prey and could explain why most of the predators were trapped during this period.

In this paper, we showed the importance of herbivore-induced attractants in the tritrophic system of Lima bean plants, two-spotted spider mites, and predatory thrips under laboratory and field conditions. In particular, our results indicate that emission of volatiles from infested Lima bean plants was effective in luring the predatory thrips from a distance of more than 5 m in the orchard. *T. urticae*-induced volatiles from Lima bean plants, therefore, attract not only predatory mites but also predatory insects. The effectiveness of the attractants produced by infested Lima bean plants, however, appears to be influenced by environmental conditions such as the number of other plant species and their associated herbivore species. Further studies are needed on the effects of the attractants in complex ecosystems.

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CHEMICAL ANALYSIS OF DEFENSE SECRETIONS OF *Sipyloidea sipylus* AND THEIR POTENTIAL USE AS REPELLENTS AGAINST RATS

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Abstract—The defensive secretion of *Sipyloidea sipylus* was analyzed using a GC-MS technique. Five chemicals were identified (diethyl ether, acetic acid, benzaldehyde, limonene, and benzothiazole) and mixed in about the same ratio as detected in the secretion to determine the potential effectiveness of such a mixture as a repellent against lab rats.

Key Words—Stick insect, *Sipyloidea sipylus*, Phasmatidae, chemical defense, diethyl ether, benzothiazole, benzaldehyde, acetic acid, limonene, repellent, rat.

INTRODUCTION

Insect defense mechanisms can be classified into two different categories (Robinson, 1969): a primary defense system that reduces the likelihood of an attack (e.g., camouflage, mimicry, nocturnal behavior) and a secondary system that comes into play after an attack is initiated (e.g., antipredator displays, defensive chemical secretions, and active fighting). Stick insects are known to make use of both types of defense systems (Bedford, 1978; Carlberg, 1986a). Among the secondary defense mechanisms employed by the phasmatids, chemical secretions have been recorded for at least 14 species (Bedford, 1978; Smith et al., 1979; Chow and Lin, 1986; Ho and Chow, 1993). Scudder (1876) went as far as saying that secretory glands might be present in all of the phasmatids. The chemical composition of the defensive secretions of the following four stick

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insects have been analyzed to date: *Paradoxomorpha crassa* (Blanchard) (Schneider, 1934), *Anisomorpha buprestoides* (Stoll) (Meinwald et al., 1962), *Graeffea crouani* (Smith et al., 1979), and *Megacrania tsudai* Shiraki (Chow and Lin, 1986; Ho and Chow, 1993). Iridodial, nepetalactone, and actinidine have been identified as major components of the defensive secretions of those insects. In an attempt to identify potential useful insecticidal fumigants isolated from biochemical secretions present in different insect groups, Dettner et al. (1992) concluded that iridodial and actinidine compounds could potentially be used as insecticidal fumigants against other insects. Phasmatid defensive secretions can be used in two different ways. The chemicals can be dispersed as a cloud covering the body of the insect, making it unpalatable to potential predators, or the insect can eject a narrow jet of secretion directly towards the predator (Carlberg, 1986a).

Sipyloidea sipylus is a phasmatid originating from southeast Asia (Westwood, 1859), and both its primary and secondary defense mechanisms have been studied (Carlberg, 1981a,b, 1986b; Bouchard and Hsiung, 1996). Because it is thought to be more or less a ground living species, potential natural predators encountered by *S. sipylus* in the wild could include lizards, frogs, birds, other insects, as well as small mammals (Carlberg, 1986b). When threatened, *S. sipylus* sprays a body-covering chemical secretion on itself, and it was shown to possess a stronger chemical defense than *A. buprestoides* and *Extatasoma tiaratum* (MacLeay) when placed in the presence of the laboratory rat *Rattus norvegicus* (Berkenhout) (Carlberg, 1986b). The secretion comes from two oval glands that measure about 1.5 mm in length and are situated dorsally and laterally to the gut (Rabozzi and Dazzini, 1972). In this study, the defensive secretions of *S. sipylus* were obtained by restraining the motion of the insect for 30 min in a capped test tube. The chemical secretion was directly injected and analyzed by GC-MS in duplicate (Figures 1 and 2, and Table 1). Five of the chemicals identified were combined, in about the same ratio as detected in the secretion, to determine the potential effectiveness of such a mixture as chemical repellent against laboratory rats.

METHODS AND MATERIALS

Biological Material. Specimens of *S. sipylus* were reared on guava leaves (*Psidium guajava* L.) from a culture originally obtained from the Montreal Insectarium (Montreal, Canada). The defensive secretion (a colorless liquid) of an adult female *S. sipylus* was collected with a syringe, after keeping the insect motionless (30 min) in a capped test tube, and directly injected into a gas chromatograph–mass spectrometer (GC-MS).

GC-MS Analysis. A Hewlett-Packard GC–mass selective detector (5890 GC/5971B MSD) was used for the analysis. The GC column flow rate was

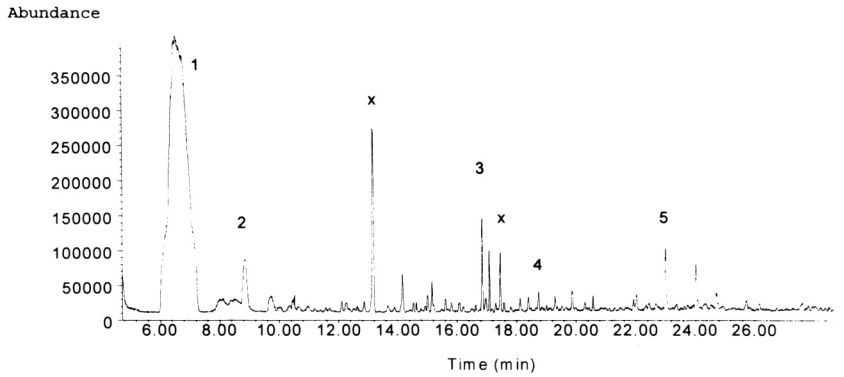


FIG. 1. GC-MS chromatogram of the defensive secretion of the stick insect. Peak 1, diethyl ether (70%), 2, acetic acid (3.2%); 3, benzaldehyde (2%); 4, limonene (0.5%); 5, benzothiazole (1.5%); x, column bleed.

0.8 ml/min for a split ratio of 92 : 1 and a septum purge of 3 ml/min. Capillary direct MS interface temperature was 180°C, and ion source temperature was 280°C. The ionization voltage was 70 eV, and the electron multiplier was 1682 V. The mass range analyzed was 30–300 amu. The column was a fused silica DB-5 column (30 m long × 0.25 mm ID × 25-μm film thickness; Supelco, Inc.). The column initial temperature was −5°C for 3 min and was increased to 50°C at a rate of 30°C/min; the temperature was immediately further increased to 270°C at a rate of 8°C/min and kept at 270°C for 5 min. Products were identified by mass spectral library search. The sample was analyzed in duplicate.

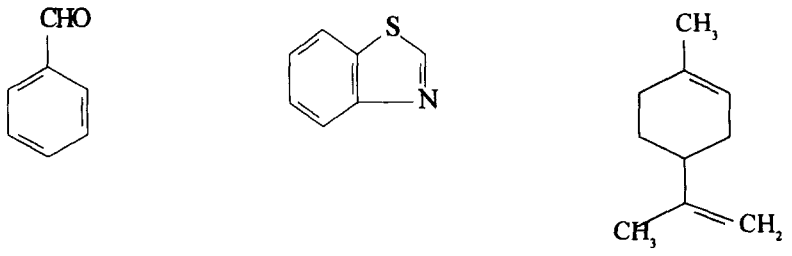


FIG. 2. Selected chemical structures identified by GC-MS in the defensive secretions of *Sipyloidea sipylus*.

TABLE 1. MASS SPECTROMETRIC DATA OF COMPOUNDS IDENTIFIED IN *S. sipylus*

Compound	*m/z* (% relative abundance)	Quality of match (%)[a]
Diethylether	31(100), 41(9), 42(2), 43(7), 44(2), 45(56), 46(1), 59(51), 60(2), 73(5), 74(38), 75(2)	91
Acetic acid	31(5), 40(3), 41(1), 42(13), 43(81), 44(5), 45(100), 60(68)	90
Benzaldehyde	32(1), 37(3), 38(4), 39(8), 40(2), 49(3), 50(22), 52(10), 53(1), 55(1), 61(1), 62(1), 63(2), 73(2), 74(9), 76(5), 77(100), 78(19), 105(90), 106(90), 107(7)	97
Limonene	39(13), 40(3), 41(31), 42(3), 43(8), 51(11), 53(30), 67(90), 68(100), 69(4), 77(35), 78(3), 79(59), 80(3), 91(29), 92(13), 93(73), 94(30), 107(23), 108(8), 121(1)	92
Benzothiazole	32(5), 37(2), 38(4), 39(4), 44(3), 45(7), 50(5), 51(3), 54(6), 57(1), 58(6), 61(2), 62(3), 63(9), 64(2), 69(17), 74(2), 75(2), 81(5), 82(12), 83(3), 84(3), 91(10), 108(33), 109(4), 135(100), 136(9), 137(5)	94

[a] With respect to authentic standards.

Rat Experiment. Eight male (weight 400–500 g) and eight female (weight 250–350 g) laboratory rats (*R. norvegicus*) of the strain CD were fed with rabbit chow pellets, and fresh water was provided daily. Rats were kept in pairs. One rat was placed in a circular arena (72 cm in diameter) and observed for 30 min. The arena was supplied with a layer of wood chip bedding as in the cages. Six holes, each 2.3 cm diameter, were drilled at equal distances from one another and 6.5 cm from the floor of the arena. For each of the holes in the wall, a vertical glass vial with the open end leading to the inside of a horizontal plastic bottle (2 × 2 × 5.4 cm) was tightly fitted into the holes to allow the rat inside the arena to insert its nose in the holes when investigating. For the control tests, the glass vials were either filled with distilled water (3 holes: 10 ml/hole) or Swiss cheese (3 holes: 10 g/hole). A visitation was recorded each time the nose of the rat went beyond one of the six circular openings in the wall of the arena. Two sets of control tests were conducted with four males and four females at approximately one-month intervals.

Tests with the chemical mixture were performed in the same manner as the controls, except that the 3 holes with distilled water were replaced with 8 ml of distilled water and 2 ml of the chemical mixture (referred to as the test mixture, 81% diethyl ether, 9.8% acetic acid, 6.5% benzaldehyde, 1.3% benzothiazole, and 0.6% limonene). The other three holes contained 10 g Swiss cheese. Visitations of rats to the holes were recorded. Five pairs of rats were used in experiments with the test mixture. Rats, like other vertebrates, move around and investigate (using mainly their sense of smell) to get information about their surroundings, especially in a new environment (Barnett, 1963). Rats were always given the option to investigate as much as they wanted, and two choices of holes were present in both the control and the test mixtures. Rats were fed ad libitum so they were not affected by a hunger drive to investigate the holes. Student's t test analysis was performed for each sex group for the control tests (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Chemical Composition. Table 1 lists the chemicals identified in *S. sipylus* and their mass spectral fragments. Although the analysis yielded more than 30 peaks, only the structures of five compounds could be confirmed with certainty (quality of match >90%). Repeated injections of sample yielded the same distribution of chemicals. The most abundant compound detected was diethyl ether, comprising 70% of the total volatile composition. Acetic acid (3.2%) was the second most abundant substance. Benzaldehyde, benzothiazole, and limonene came after with 2%, 1.5%, and 0.5%, respectively.

Diethyl ether has not previously been reported in such quantity in any insect species. Acetic acid has been found in the secretions of some Hemiptera, Coleoptera, and Hymenoptera species and one butterfly species (Blum, 1981). Benzaldehyde was found in a few species of Coleoptera and Hymenoptera (Blum, 1981) and was proven to be a particularly effective insecticide against *Drosophila* flies (Dettner et al., 1992). The fourth compound, benzothiazole, has not been previously recorded as an insect defensive secretion, unlike limonene, which was found to have a moderate insecticidal action against *Drosophila* flies (Dettner et al., 1992) and is present in termite and ant secretions (Blum, 1981).

Rat Experiments. In the control test involving rats, the mean number of visits to holes with distilled water and holes with cheese did not differ statistically for either male or female rats (Student's t test, $P = 0.05$; Table 2). However, the ranges were widely separated (especially for females). The specific rats that showed a high number of visits to cheese holes exhibited similar behavior for holes with distilled water, thus keeping a ratio of about 1 : 1 for the two types of holes. When the distilled water was replaced with the test mixture containing

the five chemicals selected previously, it was found that the mean number of visits by both male and female rats increased for cheese holes and greatly decreased for holes containing the test mixture when compared to the control (Table 2).

The number of visits by females was always greater than those by males in both series of tests. According to Table 2, the males seemed more affected by the chemical mixture than the females because only one or no visits were recorded for those holes within the 30-min observation. The females visited holes with the test mixture at least twice within the same period of time, and one female visited the holes with the test mixture nine times. This frequency, however, is still lower than the lowest number of visits for the cheese holes (12 times).

The choice of 30 min for the period of observation could be considered long, especially when dealing with volatile compounds. However, during the experiments with the cheese vs. the test mixture, both females and males decreased their mean number of visits to holes with the test mixture in the last 15 min of observations (females: 1.4, males: 0) as opposed to the first 15 min (females: 3.8, males: 0.4). The opposite would have been expected if the test mixture had lost its repellent properties due to evaporation. This decrease in the mean number of visitations during the second half of the 30-min period, when compared to the mean during the first 15 min, is highly consistent with what occurred in both sexes with the cheese and distilled water holes in the control test compared with the cheese holes and the test mixture. Therefore, we believe that the effect of the test mixture was retained during the 30-min period, despite

TABLE 2. VISITS BY *Rattus norvegicus* DURING CONTROL TESTS (CHEESE VS. DISTILLED WATER HOLES; N = 8 MALES AND 8 FEMALES) AND EXPERIMENTAL TESTS (CHEESE VS. TEST MIXTURE[a] [HOLES; N = 5 MALES AND 5 FEMALES) RECORDED FOR 30 MIN

Test	Sex	Holes	Mean	Range	S_d
Control	male	distilled water	4.75	3-6	1.05
	male	cheese	4.63	0-8	2.56
	female	distilled water	10.25	2-29	8.96
	female	cheese	8.63	1-20	6.37
Experimental	male	test mixture	0.40	0-1	0.55
	male	cheese	6.40	1-13	4.93
	female	test mixture	5.20	2-9	2.86
	female	cheese	16.80	12-23	7.53

[a]Test mixture: 8 ml of distilled water + 2 ml of the test chemical solution (81% diethyl ether, 9.8% acetic acid, 6.5% benzaldehyde, 1.3% benzothiazole, and 0.6% limonene).

the fact that a compound such as diethyl ether is highly volatile. Furthermore, accelerated evaporation of the different volatiles in the holes with the mixture plus distilled water (i.e., by being exposed to air currents or having a large surface area in contact with air) was avoided by providing only a small vertical hole (2.3 cm diameter) where the gas could escape from the container.

Sipyloidea sipylus was shown to have a good combination of primary and secondary defense systems when placed in the presence of vertebrate predators (Carlberg, 1986b; Bouchard and Hsiung, 1996). This stick insect can often avoid being eaten by laboratory rats because it covers itself with a chemical secretion that repels the predators (Carlberg, 1986b). After combining five of the chemicals found in the stick insect's defense secretions in about the same ratio as in that secretion, we found that the laboratory rats were negatively affected. In fact, male rats showed a mean number of visitations that was 16 times lower when compared to the visits to the cheese holes (mean = 0.40 investigations for the test mixture and 6.40 for the cheese) after 30 min during the experimental tests. The females seemed less affected by the test mixture, as shown by the higher number of visits compared to males, but the mean number of visits was over three times lower compared to visits to cheese holes (mean = 5.20 for the test mixture and 16.80 for the cheese holes). These results differ greatly from the 1 : 1 ratio of visits to the two types of holes (cheese and distilled water) available during the control test. Since no significant difference was observed between the number of visits to the distilled water and cheese holes in the control test, it could be concluded that the rats were not particularly attracted by either hole. However, when given the choice between cheese and holes with the test mixture, both male and female rats visited the cheese holes more often. It seems that the rats were repelled by the test mixture and chose not to investigate when coming close to those holes. The higher mean number of visits by females in both series of tests could be explained by a difference in the physiological state between male and females, for example, estrus (Barnett, 1963). This difference may also explain the greater standard deviations for the number of investigations among females than males. The test mixture did not elicit any noticeable physical response (for example, none of them jumped backwards or shook their heads when smelling the mixture; mainly it affected the number of visits.

Phasmatids in general have many potential predators, including birds, spiders, lizards, frogs, other insects (such as ants or praying mantis), as well as small rodents (Carlberg, 1986a,b; Bedford, 1978). The use of rats (*Rattus norvegicus* in this case) as bioassay tools is believed to be adequate for this kind of study because they are known to prey on stick insects (Carlberg, 1985a,b,c, 1986b) and can even destroy entire populations if introduced on islands. This was the case when ship rats (*Rattus* spp.) almost extirpated the stick insect *Dryococelus australis* (Montrouzier) from Lord Howe Island, Australia in the first part of this century (Carlberg, 1986a).

In conclusion, the combination of diethyl ether, acetic acid, benzaldehyde, limonene, and benzothiazole in about the same ratio as found in the defense secretions of the stick insect, *S. sipylus*, shows potential as a repellent against *R. norvegicus*. More experiments should be performed to investigate if individual chemicals affect rodents or if it is the combination that works best and at what concentrations.

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TROPANE ALKALOID DISTRIBUTION IN *Atropa baetica* PLANTS

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Abstract—Fifteen different tropane alkaloids and derivatives were identified by GC-MS in various plant organs of *Atropa baetica*. The main root and leaves displayed the largest number of tropane alkaloids, most of which were also present in lateral roots. In contrast, only five of these alkaloids appeared in stem tissue. Quantitative analysis by HPLC showed the presence of the two major tropane alkaloids, atropine and scopolamine, in all the samples studied. Atropine was more abundant, with the highest concentration in the main root (ca. 10.0 mg/g dry wt) followed by leaves (ca. 3.0 mg/g dry wt); scopolamine was present in highest concentration in the main root (0.6 mg/g dry wt) followed by leaves (0.4 mg/g dry wt). The lowest concentrations of these compounds were detected in stem tissue, followed by the lateral roots. The main root constitutes the major tropane alkaloid storage site; moreover, the distribution of these compounds does not appear to be organ dependent. These latter two characteristics are in contrast to closely related *Atropa* genera.

Key Words—*Atropa baetica*, main root, leaves, stem, tropane alkaloids.

INTRODUCTION

Atropa baetica Willk. is a herbaceous perennial plant belonging to the Solanaceae family. The plants have stems of 80–120 cm long, petiolated leaves arranged alternatively, with yellow flowers and black berry fruits, containing 30–50 seeds (Valdés, 1987). The species is endemic to southern Spain and northern Morocco. Ecological and biological aspects (distribution, phenology, reproduction, predators) of *A. baetica* have been reported (Herrera, 1987), and studies on seed germination and seedling develoment have also been published (Aparicio,

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1993), as well as a karyological examination (Silvestre, 1986). However, its population has decreased markedly, and it is presently classified as a rare endangered species (Hernández-Bermejo et al., 1994). Recently, an *in vitro* propagation method has been established for its rapid propagation (Zárate et al., 1997).

The plants contain various tropane alkaloids, including atropine (*dl*-hyoscyamine) and scopolamine, which possess anticholinergic and spasmolytic properties (Tyler et al., 1988); they are commonly used as an anaesthetic and spasmolytic and in eye surgery. These are a series of secondary metabolites that have been mainly described in the Solanaceae family and initially in the genus *Atropa* that act as feeding deterrents and antimicrobial defenses for the plants (Waterman, 1993). Compounds of this type have also been observed in other families e.g., Orchidaceae, Euphorbiaceae, Cruciferae, Convolvulaceae (Evans, 1979), and in the fungus *Amanita muscaria* (Willaman and Li, 1970), but their distribution is higher in the Solanaceae family.

These secondary compounds have been reported in several species of the family, including *Datura stramonium* (Fliniaux et al., 1993), *Hyocyamus albus* (Doerkschmitz et al., 1994), *Duboisia myoporoides* (Gritsanapan and Griffin, 1991), and particularly in *Atropa belladonna* (Dhar and Bhat, 1982; Ylinen et al., 1986), which has been widely cultivated and constitutes a major source of atropine and scopolamine. More recently, the tropane alkaloids of *A. acuminata*, an Asian species, have also been described (Harborne and Khan, 1993). Two other *Atropa* species (*A. caucasica* Kreyer and *A. komarovii* Blin et Shal) are known, but their alkaloid profile has not been determined. Records on the alkaloid content of *A. baetica* are rather scarce (Heltmann, 1979).

In this paper, we report the determination and quantification of tropane alkaloids from different mature tissues of *A. baetica*, by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS). A comparison of its tropane alkaloid profile with other *Atropa* genera, especially *A. belladonna*, has been drawn.

METHODS AND MATERIALS

Plant Material. Atropa baetica leaves and stems were collected from a mature field grown plant located in Grazalema Natural Park (Cádiz province, southern Spain), three months after flowering (the plant flowers in June–August), when fruit had matured and seed dispersal had occurred. Due to the low number of plants in the field, only one specimen was collected. The lateral and main root samples were harvested from various 14-month-old plants, following germination of seeds in a greenhouse. These plants were cultured in compost

no. 2 in daylight, with a photoperiod of 16 hr that was supplemented when required with artificial light using 400-W bulbs (mercury ballast fluorescent reflector, General Electric Co.) The temperature regime was 25–18 ± 2°C (day–night), except in the hot summer when temperatures reached over 40°C.

Extraction and Analysis of Tropane Alkaloids. Tropane alkaloid extraction was performed after lyophilizing the plant material for 24 hr as described by Kamada et al. (1986) in the procedure summarized in Figure 1. The final extracts were taken up in 1 ml MeOH (HPLC grade, Merck, Darmstadt, Germany), filtered through a 0.45-μm filter membranes (Lida Manufacturing Corp.), and kept at −20°C until required for analysis.

Thin-Layer Chromatography. For preliminary qualitative analysis, TLC was conducted employing silica gel 60 F_{254} plates (20 × 20 cm aluminum sheets) (Merck). The TLC plates were developed using two solvent systems: (1) ethyl acetate–2-propanol–28% ammonia (9:7:3), and (2) chloroform–acetone–methanol–28% ammonia (75:10:15:2) (Kamada et al., 1986). The tropane alkaloids were visualized under UV light and after spraying with Dragendorff's reagent. The tropane alkaloid standards atropine, scopolamine, and tropine (Sigma-Aldrich, Madrid, Spain) were used for identification by comparing their R_f values with those of the extracted samples.

High-Performance Liquid Chromatography. Quantitative determination of the main tropane alkaloids atropine and scopolamine was performed by HPLC employing a Waters system (Waters, Millipore Corp.) equipped with a 510 pump and a U6-K manual injector. The UV detector was a 481 λ_{max} and the column used was a 250- × 4.6-mm Hypersil ODS, packed with 5-μm particles (Sugelabor, Madrid, Spain).

The isocratic mobile phase was a mixture of 22% acetonitrile and a buffer containing 50 mM potassium dihydrogen orthophosphoric acid adjusted to pH 3.0 with orthophosphoric acid. Sample injection was 1–5 μl, and the analysis was performed at an initial flow rate of 1.0 ml/min for the first 4.75 min, increased to 1.5 ml/min to 14.50 min, and then back to 1.0 ml/min before a new sample was injected. Column temperature was 28–30°C and detection was conducted at 215 nm. The data were generated using a Data Module 730, employing atropine and (−)-scopolamine (Sigma-Aldrich) as standard samples.

Gas Chromatography–Mass Spectrometry. GC-MS was used for chemical identification of the different tropane alkaloids and derivatives present in the plant material. This was performed using a Fisons 8000 gas chromatograph (Fisons Instruments) equipped with a fused silica capillary column (30 m × 0.32 mm ID) coated with SPB-5 (1.0-μm film thickness) (Supelco) connected to a MD 800 (Fisons Instruments) quadrupole mass spectrometer operated in the electron impact (EI) mode. The operating conditions were as follows: temperature program 50 to 100°C at 30°C/min, 100 to 280°C at 15°C/min, and

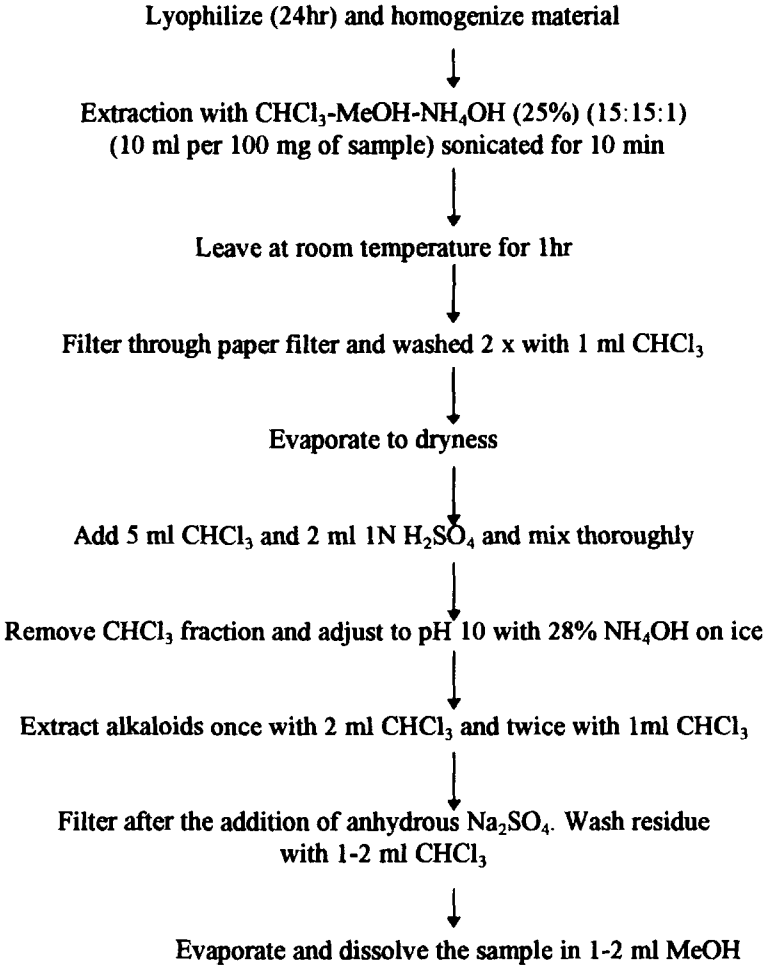


FIG. 1. Scheme of the tropane alkaloid extraction method described by Kamada et al. (1986) and used in this study.

then held at 280°C for 20 min, with the injector temperature at 250°C. Helium was used as the carrier gas. Spectra were obtained at 70 eV, and with the ion source at 250°C. Compounds were identified by comparison of their mass spectral fragmentation patterns with a Mass Spectra Data Base (Heller and Milne, 1980), NBS library (National Bureau of Standards), and mass spectra and GC retention times of standard compounds.

RESULTS AND DISCUSSION

The presence and identity of 15 different alkaloids and derivatives were determined in various plant parts studied. The main root and leaves displayed the largest alkaloid pattern with a total of 14 different compounds identified (Table 1). Lateral roots showed the majority of the same metabolites; however, only five of these components appeared in stem tissue. The lack of most of these compounds in the stem could be due to a dilution effect in this organ, since it functions as an organ of transport in moving these metabolites from their site of synthesis (roots) to the aerial parts.

Our data indicate that the distribution of these compounds in *A. baetica* does not seem to be organ specific. A larger number of alkaloids has been reported in roots of *A. acuminata*, (Harborne and Khan, 1993) and *A. belladonna* (Simola et al., 1988), with a much lower number of alkaloids in leaf, stem, fruit, and seed, which suggests a tissue-specific distribution of compounds in these two closely related species. The reports of Witte et al. (1987) on the closely related genera *Datura* indicated an alkaloid pattern that was organ dependent, with a wider spectrum of alkaloids in the roots than in the leaves.

The results of the quantitative analysis of the main tropane alkaloids (atropine and scopolamine) from different *A. baetica* mature plant tissues showed a

TABLE 1. TROPANE ALKALOIDS AND DERIVATIVES FOUND IN DIFFERENT *Atropa baetica* SAMPLES ANALYZED BY GC-MS

Compound	Main root	Lateral root	Leaf	Stem
Hygrine	+	+	+	–
Tropinone	+	+	+	–
Tropine	+	+	+	–
Pseudotropine	+	+	+	–
N-Methylpyrrolidinyl-hygrine A	+	+	+	–
N-Methylpyrrolidinyl-hygrine B	+	+	+	–
Cuscohygrine	+	+	–	–
3α-Phenylacetoxytropane	+	+	+	+
3α-Isobutyryloxytropane	–	–	+	–
Apoatropine	+	+	+	+
Atropine	+	+	+	+
Aposcopolamine	+	+	+	+
Scopolamine	+	+	+	+
6-Hydroxyapoatropine	+	–	+	–
6-Hydroxyhyoscyamine	+	+	+	–

significantly higher amount of atropine than scopolamine in all the samples studied (Figure 2). Results, although from different extraction and analytical methods, have been reported previously (Simola et al., 1988), showing higher amounts of atropine than scopolamine in leaves, seeds, and roots of *A. belladonna*. Likewise Harborne and Khan (1993) observed the same quantitative difference in these secondary products in *A. acuminata*. These data suggest a similarity between these three different but close species. We found that the maximum amount of atropine (ca. 10.0 mg/g dry wt) was present in the main root, which had a ca. 3.4-fold higher quantity of this compound than that recorded in leaves (ca. 3.0 mg/g dry wt). These data indicate a higher atropine accumulation in *A. baetica* main roots compared to published records for *A. belladonna* roots, although the leaf content was similar (Hartmann et al., 1986; Simola et al., 1988).

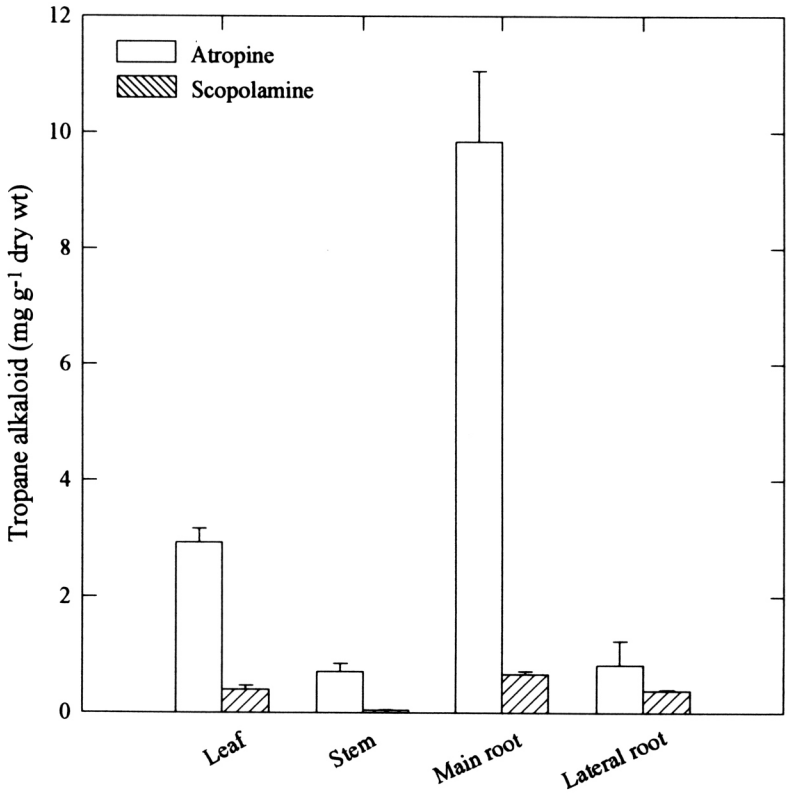


FIG. 2. Amount of tropane alkaloids (atropine and scopolamine) determined in different tissues of mature *Atropa baetica* plants. Each sample represents the mean of at least three replicates ± SE.

Scopolamine in the main root (0.6 mg/g dry wt) was only 1.7-fold higher compared to leaves (ca. 0.4 mg/g dry wt), and similar to that observed in the lateral roots. The lowest level of this alkaloid occurred in stem tissue. However, these results do not coincide with those of Heltmann (1979), who identified just three tropane alkaloids in *A. baetica*, and higher atropine amounts in leaves compared to roots. Heltmann (1979) did not report the presence of scopolamine in roots, but our results showed the appearance of this alkaloid in all fractions studied. This difference may have been due to the use of different extraction and analytical methods.

Several reports in the literature indicate that the site of tropane alkaloid synthesis in a number of *Atropa* species lies in the roots (Hartman et al., 1986). These metabolites are then transported and stored in the leaves as well as in other tissues (stems, fruits, seeds) where they act as feeding deterrents and antimicrobial defenses for the plants (Waterman, 1993). Our data show that *A. baetica* main root also constitutes the major storage site and had the highest amount of these alkaloids. These alkaloids may be transported to other plant organs providing potential deterrent and antimicrobial activities.

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BEHAVIORAL RESPONSE OF MALES TO MAJOR SEX PHEROMONE COMPONENT, (Z,Z)-5,25-HENTRIACONTADIENE, OF *Drosophila ananassae* FEMALES

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Abstract—To identify sex pheromone components in *Drosophila ananassae*, the cuticular hydrocarbons were analyzed and bioassayed. After silicic acid and two silver nitrate–silicic acid chromatographic steps, active fractions containing (*Z,Z*)-5,25- and (*Z,Z*)-4,26-hentriacontadiene as the most abundant components were obtained. Both hydrocarbons could elicit male courtship behavior. The former was the more predominant compound and elicited a higher courtship index value than the latter. Cuticular hydrocarbon differences between *D. ananassae* and its close relative, *D. pallidosa*, and their roles in species recognition are discussed.

Key Words—*Drosophila ananassae*, *Drosophila pallidosa*, sex pheromones, cuticular hydrocarbons, courtship behavior, species recognition.

INTRODUCTION

Drosophila ananassae and *D. pallidosa* are closely related members of the *ananassae* complex, which consists of 10 species (Tobari, 1993). *D. ananassae*

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is cosmopolitan, as are *D. melanogaster* and *D. virilis*, but *D. ananassae* is primarily distributed in tropical and subtropical regions. The other taxa of this complex, including *D. pallidosa*, are also found in tropical regions, but all of them are endemic forms. *D. pallidosa* is found in the Tongan and Fijian islands in Melanesia, where *D. ananassae* also exists (for a review, see Tobari, 1993). For a long time *D. ananassae* and *D. pallidosa* were thought to be one species comprised of light and dark forms, even though they were cytologically and ethologically distinguished (Futch, 1966; Spieth, 1966; Stone et al., 1966). Bock and Wheeler (1972) recognized the two as distinct species, with the dark identified as *D. ananassae*, and the light as *D. pallidosa*. The major morphological differences between the two species are the body color and the number of rows in the sex-comb. These similarities suggest that phylogenetic separation of *D. ananassae* and *D. pallidosa* must have been one of the more recent events of speciation in the *melanogaster* group (Bock and Wheeler, 1972).

D. ananassae and *D. pallidosa* do not exhibit postmating isolation such as hybrid inviability and sterility (Futch, 1966; Stone, 1966). This indicates that only sexual (ethological) isolation can prevent gene flow between these two close relatives (Futch, 1966, 1973; Spieth, 1966).

Cuticular hydrocarbons of females play an important role in stimulating and eliciting male courtship behaviors in *Drosophila* species (Antony and Jallon, 1982; Antony et al., 1985). Four hydrocarbons have been reported as major sex pheromones: (*Z*,*Z*)-7,11-heptacosadiene and 7-tricosene in *D. melanogaster* (Jallon, 1984; Antony et al., 1985); (*Z*)-7-tricosene in *D. simulans* (Jallon, 1984); (*Z*)-11-pentacosene in *D. virilis* (Oguma et al., 1992), and (*Z*,*Z*)-5,27-tritriacontadiene in *D. pallidosa* (Nemoto et al., 1994). These long-chain hydrocarbons are thought to act as contact pheromones, probably by stimulating chemoreceptors on the male foreleg or proboscis. Males actively court conspecific females having the characteristic hydrocarbons but generally do not court heterospecific females. A few exceptions exist: e.g., *D. melanogaster* males actively court the heterospecific flies, *D. affinis* and *D. rajasekari*, because they have predominantly (*Z*,*Z*)-7,11-heptacosadiene, the same hydrocarbon of *D. melanogaster* females (Scott and Tompkins, 1986; Scott and Jackson, 1989). Hydrocarbon differences between close relatives, however, cause mate discrimination and sexual isolation (Cobb and Jallon, 1990; Coyne et al., 1994; Coyne and Oyama, 1995). Recent work has focused on studying the genetic control of sex pheromone production (Ferveur, 1991; Ferveur and Jallon, 1993; Coyne, 1996; Doi et al., 1996).

We have reported previously on the *D. pallidosa* sex pheromone (Nemoto et al., 1994). In this report, we analyze the hydrocarbons of *D. ananassae* and identify a major sex pheromone component. We also examine male courtship activities toward females by *D. ananassae* or *D. pallidosa* to understand species recognition by males.

METHODS AND MATERIALS

Flies and Rearing. The *Drosophila ananassae* isofemale strain was obtained from Honolulu, Hawaii, in 1981. The wild-type *D. pallidosa* strain (NAN4), an isofemale line, was collected from Laotoka, Fiji. Flies were kept in 3- × 10-cm vials containing standard yeast–cornmeal–glucose medium under a 14L(07:00–21:00):10D cycle at 25 ± 1°C and 55 ± 5% relative humidity.

Flies were sexed within 12 hr of their emergence with no anesthesia. Males and females were kept separate (five to seven days in a food vial during which one transfer of the vial occurred) until hydrocarbon analysis or bioassay.

Bioassay. The bioassay and all other experimental procedures described below were similar to those in Nemoto et al. (1994). Briefly, males for bioassays were individually kept in vials for two days before an experiment. A given amount of female extract or synthetic hydrocarbon was applied to *D. melanogaster* males (decoys) that were previously washed three times with hexane. A decoy was introduced into a glassy assay chamber (15 mm ID × 3 mm height) with a test male whose behavior was observed for 5 min. To quantify the intensity of courtship activity by the test male, the percent of time that the male spent performing any and all courtship behaviors toward a decoy was determined as the courtship index (CI) (Tompkins et al., 1980). If a male courts for 2 min in the observation time (5 min), the CI is 40 [(2/5) × 100 = 40]. Furthermore, the percentage of males that showed any courtship behaviors was determined as the response.

Extraction of Cuticular Hydrocarbon. About 3000 virgin females were killed by freezing, soaked in hexane (2 ml) for 5 min, and agitated for 1 min. This extraction procedure was repeated three times and the three separate extracts were combined. The final extract was stored in a freezer until analysis.

Column Chromatography. The hexane extract was fractionated by three chromatographic steps to purify the pheromone. A SiO_2 column was developed by a series of hexane–ether mixtures (6 ml each) in a stepwise manner (100:0, 97:3, 95:5, 90:10, 0:100 by volume). Then, the sample was applied to $AgNO_3$/SiO_2 column 1 (10% w/w of $AgNO_3$), and it was developed with the same series of hexane–ether mixtures given above. The active fraction from column 1 was applied to $AgNO_3/SiO_2$ column 2 of the same percentage as the first one, and it was developed with a series of 10% benzene in hexane mixtures (2 ml each, total 30 ml).

Gas–Liquid Chromatographic Analysis. Hexane extracts were analyzed on a gas–liquid chromatograph (GLC) model 380FS (GL Science) equipped with a FFAP capillary column (0.25 mm ID × 25 m, film thickness 0.25 μm, Quadrex), and a flame ionization detector for peak detection. The temperature was programmed from 100°C to 250°C at 2.5°C/min. For determination of the cuticular hydrocarbon composition from a single fly, the fly was soaked 5 min

in 10 μl hexane containing 50 ppm docosane (C_{22}) as an internal standard. One microliter of this solution was injected into a cooled on-column injector (OCI-3, GL Science). A Fourier transform infrared absorption spectrum was measured by a FT/IR-5MP (Jasco) equipped with a PC9801VX personal computer (NEC). GC-MS analysis was performed as previously described (Nemoto et al., 1994), and double bond locations were determined by detecting diagnostic ions in the electron impact mass spectra (EI-MS) from dimethyl disulfide (DMDS) adducts (Carlson et al., 1989).

Chemicals. The alkadienes (*Z*,*Z*)-5,25-hentriacontadiene, (*Z*,*Z*)-4,26-hentriacontadiene, and (*Z*,*Z*)-5,27-tritriacontadiene were synthesized by a coupling reaction between the monoene units by the method of Nemoto et al. (1994).

RESULTS

Purification of Sex Pheromone Compounds. To detect pheromonal activity of the female extract, we observed behavioral responses of *D. ananassae* males in the presence of each of the column-chromatographic fractions applied to decoys. Crude extract did not elicit male courtship behavior at 0.1, 1, or 10 female equivalents (FE). Hexane eluent from the SiO_2 column, however, elicited all courtship behaviors except attempted copulation. The male response to the hexane eluent was 43% at 1 FE. After solvent evaporation, the active fraction was applied to $AgNO_3/SiO_2$ column 1. Male courtship was elicited by the 97:3 hexane–ether fraction. After purification on $AgNO_3/SiO_2$ column 2, the seventh and eighth fractions eluted with 10% benzene–hexane elicited male courtship. All active fractions were combined and used for structure elucidation.

Structure Elucidation. From EI-MS data (data not shown), the active fraction consisted of major peaks of 31 carbons (Figure 1A). We determined the double-bond locations by DMDS analysis; the major peak had double-bonds at the C-5 and C-25 positions. The diagnostic ions were as follows: 620 (M^+), 526 (M^+-94), 478 (M^+-142), 432 (M^+-88), 117 (A^+, C5 position), 131 (B^+, C6 position), 455 (M^+-A^+-48), 407 (M^+-A^+-48-48), 359 (M^+-A^+-48-48-48), 441 (M^+-B^+-48), 393 (M^+-B^+-48-48), and 345 (M^+-B^+-48-48-48). The (M-A) and (M-B) ions were not detected. The *Z* configuration was assigned by checking for lack of absorption around 975 cm^{-1} in the FT-IR spectrum of the hydrocarbon fraction. Synthetic (*Z*,*Z*)-5,25-hentriacontadiene was coinjected with the active fraction and was chromatographically identical to the predominant peak. Thus, we concluded that (*Z*,*Z*)-5,25-hentriacontadiene [(*Z*,*Z*)-5,25-$C_{31:2}$] elicited the male courtship behavior. Using the same procedure, we determined that the minor peak of the active fraction was (*Z*,*Z*)-4,26-hentriacontadiene [(*Z*,*Z*)-4,26-$C_{31:2}$].

Cuticular Hydrocarbons of D. ananassae. Cuticular hydrocarbon compo-

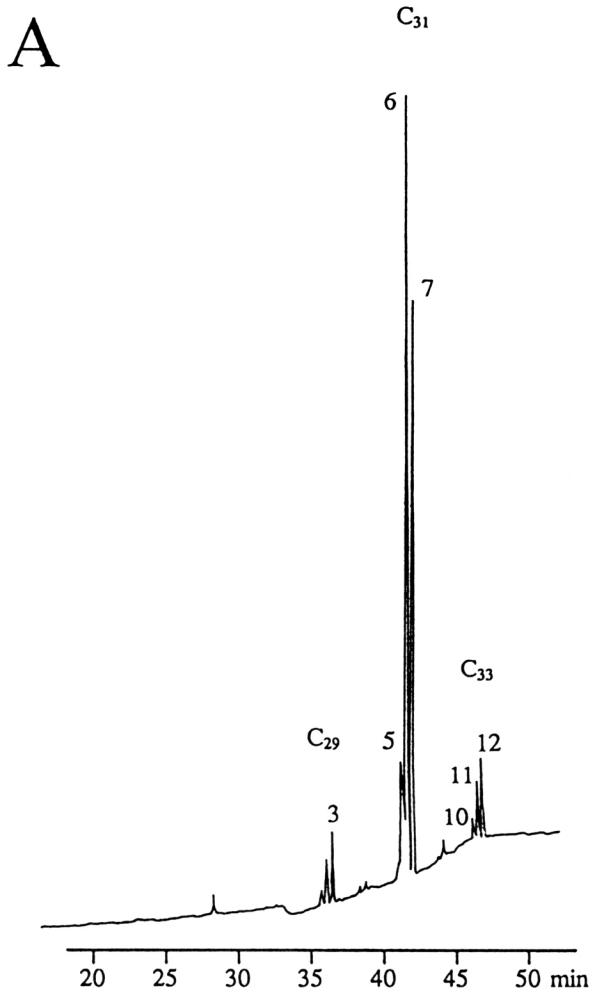


FIG. 1. Gas-liquid chromatograms of an active sex pheromone fraction from the second $AgNO_3/SiO_2$ column (A) and a microextract from one *D. ananassae* female (B). Temperature was programmed from 100°C to 250°C at 2.5°C/min. Peak numbers correspond to those of Table 1.

sitions are given in Table 1, and a chromatogram of the cuticular hydrocarbons of a single female *D. ananassae* is shown in Figure 1B. No qualitative differences were found between females and males (Table 1). The average amounts of total hydrocarbons were 2.84 ± 0.11 μg/female (N = 20) and 2.65 ± 0.14 μg/male (N = 14), respectively. The most abundant molecules in this species

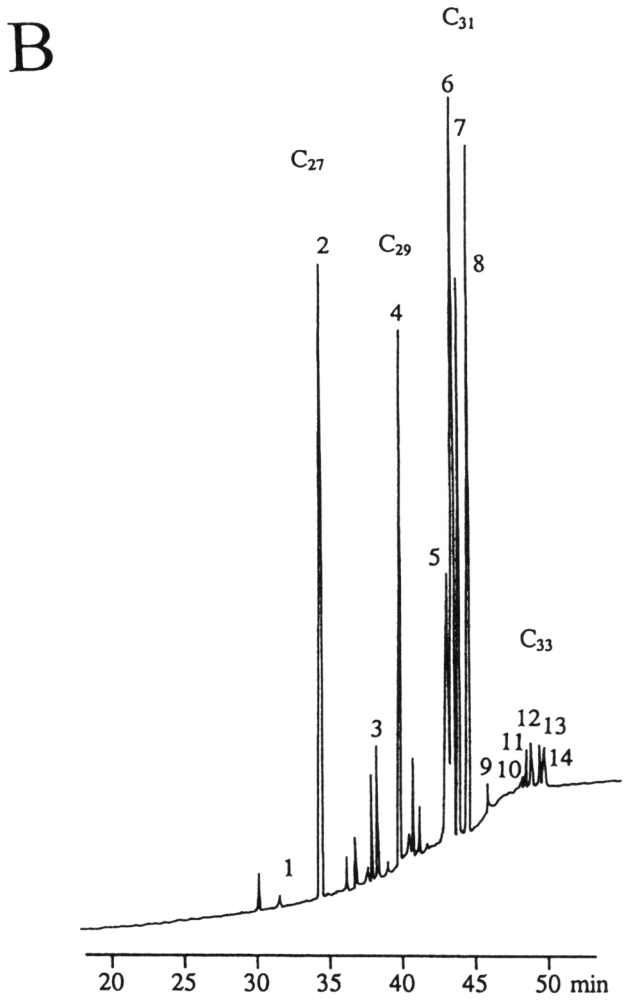


FIG. 1. Continued.

are 31 carbon dienes. The most predominant hydrocarbon in both sexes was (*Z*,*Z*)-5,25-$C_{31:2}$. The other peaks are primarily 27 to 32 carbon molecules. All the branched compounds are likely 2-methylalkanes since a 43 molecular mass weight was detected in the GC-MS data.

Bioassay. The crude extract did not elicit male courtship, although the hexane eluent containing only hydrocarbons did. *D. ananassae* males actively courted freeze-killed *D. ananassae* females (CI = 61). After removal of cu-

TABLE 1. CUTICULAR HYDROCARBONS OF MALE AND FEMALE *D. ananassae* AND FEMALE *D. pallidosa*

Hydrocarbons	*D. ananassae* female (N = 20) (μg ± SEM)	*D. ananassae* male (N = 14) (μg ± SEM)	*D. pallidosa* female (N = 18)[a] (μg ± SEM)	Peak[b]
C_{27}				
Branched	0.22 ± 0.01	0.20 ± 0.01	0.02 ± 0.01	2
Diene	0.16 ± 0.02	0.10 ± 0.01	0.13 ± 0.01	1
C_{29}				
Branched	0.32 ± 0.01	0.27 ± 0.01	0.34 ± 0.02	4
Diene	0.03 ± 0.01	0.02 ± 0.01		3
C_{31}				
Branched	0.06 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	9
Diene	0.21 ± 0.01	0.21 ± 0.01		5
	0.81 ± 0.03[c]	0.87 ± 0.05	0.07 ± 0.01	6
	0.34 ± 0.02[d]	0.32 ± 0.02	0.06 ± 0.01	7
Triene	0.38 ± 0.02	0.35 ± 0.02	0.04 ± 0.01	8
C_{33}				
Diene	0.03 ± 0.01	0.02 ± 0.01	0.17 ± 0.08	10
	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.02	11
	0.08 ± 0.01[e]	0.07 ± 0.01	0.58 ± 0.03	12
Triene	0.06 ± 0.01	0.04 ± 0.01	0.15 ± 0.01	13
	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	14
Others	0.05 ± 0.01	0.08 ± 0.01	0.01 ± 0.01	
Total	2.84 ± 0.11	2.65 ± 0.14	1.73 ± 0.09	

[a] After Nemoto et al. (1994).
[b] Numbers correspond to the numbers in the Figure 1.
[c] (Z,Z)-5,25-$C_{31:2}$.
[d] (Z,Z)-4,26-$C_{31:2}$.
[e] (Z,Z)-5,27-$C_{33:2}$.

ticular lipids including hydrocarbons by washing with hexane, they did not show courtship behavior (Table 2). These results confirmed that the pheromone was comprised of cuticular hydrocarbons from *D. ananassae* females. Neither dead nor washed *D. melanogaster* flies elicited male courtship; thus, there was no problem in using them as decoys.

Table 3 shows courtship responses and CI values to synthetic hydrocarbons. Both values clearly increased in a dose-related manner to increased FE of (Z,Z)-5,25-hentriacontadiene. Two FE gave the highest CI value (42), which was comparable to that of a dead female (61). (Z,Z)-4,26-$C_{31:2}$ also elicited male courtship, but CI values were significantly lower (Mann-Whitney U test, U =

TABLE 2. COURTSHIP RESPONSES AND COURTSHIP INDICES IN *D. ananassae* MALES

Source	*N*	Response (%)[a]	CI ± SEM[b]
D. ananassae			
Live female	20	100	95 ± 1
Dead female	20	95	61 ± 6
Washed female	20	0	0
Washed male	20	0	0
D. melanogaster			
Dead female	20	0	0
Dead male	20	0	0
Washed female	20	0	0
Washed male	50	2	0.6

[a]The percentage of males responding to the source.
[b]Mean ± SEM of courtship index (CI) in males responding to the source.

48, $P < 0.05$ for one FE; $U = 79$, $P < 0.01$ for two FE). Hence, (Z,Z)-5,25-$C_{31:2}$ appears to be the major sex pheromone component of *D. ananassae*.

Sex Pheromones and Species Recognition. To determine how hydrocarbon or sex pheromone differences contribute to mate recognition, we investigated male courtship behavior towards females of *D. ananassae* or *D. pallidosa* (Table 4). Since freeze-killed females elicited male courtship, the attractiveness of those females was due to cuticular hydrocarbons rather than female behavior. Signif-

TABLE 3. COURTSHIP RESPONSES AND COURTSHIP INDICES TO SYNTHETIC HYDROCARBONS IN *D. ananassae* MALES

Hydrocarbon	*N*	Amount (μg)	Dose (FE)	Response (%)[a]	CI ± SEM[b]
(Z,Z)-5,25-$C_{31\,2}$	36	0.5	0.5	19	9 ± 2
	40	1	1	45	21 ± 4
	20	2	2	55	42 ± 9
(Z,Z)-4,26-$C_{31:2}$	20	0.25	0.5	0	0
	20	0.5	1	15	4 ± 1
	20	1	2	25	3 ± 3

[a]The percentage of males responding to the source.
[b]Mean ± SEM of courtship index (CI) in males responding to the source.

TABLE 4. FEMALE OR SEX PHEROMONE RECOGNITION BY *D. ananassae* AND *D. pallidosa* MALES

Source	Males					
	D. ananassae			*D. pallidosa*		
	N	Response (%)[a]	CI ± SEM[b]	*N*	Response (%)	CI ± SEM
Females						
D. ananassae	20	95	61 ± 6	20	65	11 ± 2
D. pallidosa	20	70	18 ± 4	20	90	67 ± 7
Synthetic hydrocarbons						
(Z,Z)-5,25-$C_{31:2}$						
(1 μg)	40	45	21 ± 3	20	20	4 ± 1
(Z,Z)-5,27-$C_{33.2}$						
(1 μg)	20	0	0	60	48	35 ± 5

[a]The percentage of males responding to the source.
[b]Mean ± SEM of courtship index (CI) in males responding to the source.

icant conspecific and heterospecific courtship activities were observed (Mann-Whitney U test, $U = 234$, $P < 0.001$ for *D. ananassae* males; $U = 217$, $P < 0.001$ for *D. pallidosa* males).

D. ananassae males never responded to the synthetic sex pheromone [(Z,Z)-5,27-$C_{33:2}$] of *D. pallidosa*. *D. pallidosa* males showed only a weak response to (Z,Z)-5,25-$C_{31:2}$, and a significant difference was observed between the CI value of (Z,Z)-5,25-$C_{31:2}$ (CI = 4) and that of (Z,Z)-5,27-$C_{33:2}$ (CI = 35, $U = 95$, $P < 0.05$). Males could recognize conspecific females by their hydrocarbons.

DISCUSSION

Functions of (Z,Z)-5,25-hentriacontadiene. Since (Z,Z)-5,25-$C_{31:2}$ elicited all courtship elements, it is considered the major sex pheromone component of *D. ananassae*. This compound is a very long chain alkadiene. One female equivalent of the synthetic hydrocarbon elicited a CI value of one third that of a dead female. The response to 1 FE (45%) coincided with that of one FE of hexane eluent (43%). (Z,Z)-4,26-$C_{31:2}$ was also found in the active fractions, but elicited only slight male courtship behavior by itself. The response and the CI value were low in all tested doses. However, this hydrocarbon may have

some undetermined role in male courtship, possibly as a synergist, for example.

(Z,Z)-11-Octadecenyl acetate and (Z)-11-eicosenyl acetate have been identified as the aggregation pheromones of *D. ananassae* (Schaner et al., 1989). They are more effective when they are mixed with food. They are not found in the cuticle, but are present in the ejaculatory bulb of sexually mature male flies and are transferred to the female during copulation. In nature, flies are attracted by these volatile pheromones and food odors to mating, feeding, and oviposition sites. After aggregation, male flies perhaps recognize conspecific females by the nonvolatile (Z,Z)-5,25-$C_{31:2}$, which probably stimulates contact chemoreceptors on the forelegs of males.

Hydrocarbon Differences and Species Recognition Between D. ananassae *and* D. pallidosa. Cuticular hydrocarbon differences between *D. ananassae* and *D. pallidosa* were noteworthy with respect to C_{31} and C_{33} carbons: *D. ananassae* predominantly possesses the former (63% of total cuticular hydrocarbons), and *D. pallidosa* the latter (57%). However, except for the C_{31} and C_{33} carbons, both species had almost the same ratios of the other cuticular hydrocarbons. The ratios of the major sex pheromone to total hydrocarbon composition were almost the same (29% for *D. ananassae* and 33% for *D. pallidosa*). Furthermore, since neither qualitative nor quantitative differences between males and females were observed in either species (Nemoto et al., 1994), they could be called sexually monomorphic species for cuticular hydrocarbons. Male-male courtship (homosexual courtship) of these species was frequently observed in the laboratory. A brief functional antimating pheromone, such as shown in *D. melanogaster* (Scott, 1986; Scott et al., 1988), may not be contained in the cuticular hydrocarbons of *D. ananassae* and *D. pallidosa*.

The only significant difference in cuticular hydrocarbon composition between *D. ananassae* and *D. pallidosa* is the difference in major sex pheromone compounds. Difference in cuticular hydrocarbons contribute to sexual isolation between close relatives (Coyne et al., 1994; Coyne and Oyama, 1995). In the present study, males clearly recognized their own freeze-killed females, so attractiveness of females to males does not depend on species-specific female behaviors. Our results indicate that a major sex pheromone compound, not morphological differences, is a key factor in male recognition between *D. ananassae* and *D. pallidosa*. Weak courtship behavior seen to heterospecific freeze-killed females was probably due to a minor presence of heterospecific sex pheromone or to minor compounds that elicited weak male courtship.

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RESPONSE OF MALE DOMESTIC RABBITS (*Oryctolagus cuniculus*) TO INGUINAL GLAND SECRETION FROM INTACT AND OVARIECTOMIZED FEMALES

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Abstract—European rabbits (*Oryctolagus cuniculus*) of both sexes possess a variety of skin glands important in chemical communication. These include sizable inguinal glands, the strong-smelling secretion from which accumulates in conspicuous pouches of skin on either side of the genital opening. To test the hypothesis that this secretion advertises sexual receptivity in females, inguinal gland secretions from nine intact and six ovariectomized females were presented on dummy rabbits or in a simultaneous choice paradigm, and the responses of nine stud males to these secretions were recorded. Although the males clearly perceived the stimuli, no differences in responses to secretions from the intact and ovariectomized does were observed, and no differences were found in the size, weight, or secretory activity of the does' glands. It is concluded that inguinal gland secretion plays little role in communicating sexual receptivity in female rabbits and that ovarian steroids play little part in regulating inguinal gland activity.

Key Words—Chemical communication, inguinal gland secretion, female rabbit, ovariectomy, sexual attraction.

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INTRODUCTION

Chemical signals play an important part in regulating the social life of the European rabbit (*Oryctolagus cuniculus*). In both sexes, urine, feces, and secretions from a variety of skin glands all appear to have specific communicatory functions (Bell, 1985; Hudson and Distel, 1995; Martínez-Gómez et al., 1997). While investigating the role of chemical communication in the reproductive biology of the female rabbit, we became particularly interested in the possible communicatory significance of the inguinal glands.

These conspicuous glands have been anatomically, histologically, and chemically well described by several authors (Buschke, 1933; Coujard, 1947; Montagna, 1950; Mykytowycz, 1966; Kühnel and Wrobel, 1967a,b, 1969; Wales and Ebling, 1971; Goodrich and Mykytowycz, 1972; Goodrich, 1983; Martínez-Gómez et al., 1977). Briefly, they lie close to a hairless pouch of skin on either side of the rectum and penis or vagina, and are composed of two easily distinguishable parts—a superficial, whitish sebaceous gland and an adjacent, deeper-seated, brownish sudoriferous gland. Secretion is discharged into the pouch where it accumulates as a brownish, and to the human nose, strong-smelling substance.

Although it is this secretion that gives rabbits their characteristic odor (Montagna, 1950; Mykytowycz, 1966; Goodrich and Mykytowycz, 1972), its actual communicatory function remains unclear. From the few behavioral reports available it seems likely to be involved in sexual recognition and signaling sexual receptivity (Southern, 1948; Myers and Poole, 1962; Mykytowycz, 1966; Hesterman and Mykytowycz, 1982b), although it has also been suggested to play a role in signaling territorial possession and social rank (Mykytowycz and Dudzinski, 1966) and in the recognition of group members (Hesterman and Mykytowycz, 1982a,b; Hesterman et al., 1984).

It was the aim of the present study to investigate experimentally the hypothesis that inguinal gland secretion is involved in signaling sexual receptivity in rabbits by testing the response of stud males to secretions from the inguinal glands of intact and ovariectomized females.

METHODS AND MATERIALS

Animals. A total of 24 mature, sexually experienced chinchilla-breed rabbits from the Tlaxcala colony weighing between 4.5 and 6.0 kg were used: nine intact males, nine intact females, and six females that had been ovariectomized at least five months prior to the start of the study. Animals were kept in individual stainless-steel cages (50 × 60 × 40 cm) in the same colony room on a 16L:8D cycle (lights on at 06:00 hr) and at 20 ± 2°C. They were given 200

g food/day (Purina rabbit chow), and water ad libitum. A daily cycle of 16 hr light was chosen as representative of the long-day conditions characteristic of the summer breeding season for rabbits in much of Europe, and because this has been found to be suitable for maintaining laboratory rabbits in permanent estrus throughout the year (Hudson and Distel, 1990).

Behavioral Testing. The response of the nine males to various odor stimuli was tested by placing them individually in a wire mesh arena 1 m in diameter and 40 cm high, which was located in a separate room from the colony. To minimize habituation and possible learning effects, animals were only tested for 3 min once every third day. Before each test they were allowed 5 min to habituate to the arena. The same odorants were presented in two separate tests: on a dummy rabbit and in a simultaneous choice paradigm. During both kinds of test the behavior of the animals was recorded using a video camera mounted above the arena.

The rabbit was a life-size, washable, stuffed cloth imitation of a chinchilla-breed rabbit (Figure 1, top). Double-headed cotton wool Q-tips bearing the odorant and attached to disposable, adhesive Velcro strips were fastened beneath the tail of the dummy in the anogenital region. During the 3 min test the frequency and duration of the following behaviors were recorded: sniff anogenital area of dummy; sniff head or body, jump over, circle, urinate on or near, mount (attempt to copulate with), lie beside, or bite, scratch, rip, or kick at dummy; move around and sniff at arena; remain immobile; chin-mark anogenital area, head, or body of dummy or of arena. Some of these behaviors were so seldom observed that only those reported in the results section were finally considered (cf. Figures 3 and 4 below).

In the simultaneous choice paradigm the test animal was confined within one quadrant of the arena by a right-angled mesh divider (Figure 1, bottom). Two Q-tips, each bearing a different stimulus, were fixed to the mesh wall 40 cm apart and 25 cm from the floor at approximate head height for the test animal. During the 3-min test the frequency and duration with which animals sniffed at or chin-marked each of the odor stimuli, explored the arena, or remained immobile were recorded. The position of the two Q-tips was swapped between each test.

Animals were fed and cleaned at 09:00 hr, and testing was carried out between 11:00 and 13:00 hr.

Odor Stimuli. Three main kinds of stimuli were used: inguinal gland secretion from intact does in estrus, secretion from ovariectomized does, and untreated Q-tips. Inguinal secretion was obtained by wiping both ends of a Q-tip in the inguinal pouches of the donor female immediately before each test. Stimuli were presented to animals according to the scheme in Table 1. Males were tested in groups of three, with each group tested in a different stimulus condition on each of three test days. The sequence in which groups and stimulus conditions were

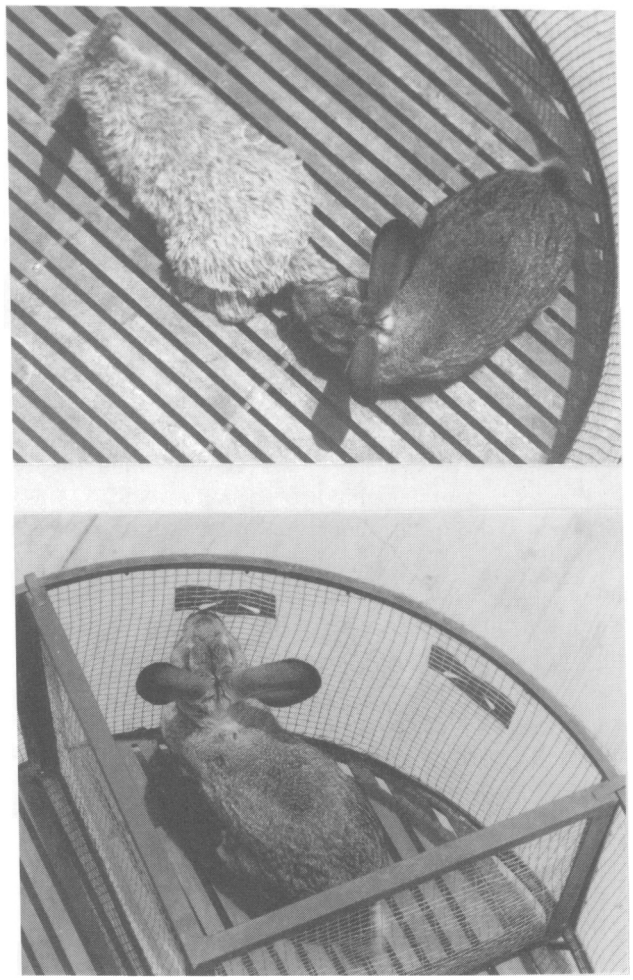


FIG. 1. Arena used for testing the response to odor stimuli presented on Q-tips attached either to the anogenital region of a dummy rabbit (top) or to the arena wall in a simultaneous choice paradigm (bottom).

tested was changed each day so as to control for possible order effects. To ensure an adequate amount of each stimulus, no donor was used more than once on any one day.

Morphology. At the end of the study six randomly selected intact donors and the six ovariectomized animals were killed by an overdose of urethane

TABLE 1. SEQUENCE IN WHICH MALE SUBJECTS WERE TESTED IN EACH STIMULUS CONDITION

	Dummy rabbit			Simultaneous choice		
Test ♂	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
1	1. Blank	2. Intact	3. Ovx[a]	1. Blank vs Intact	2. Blank vs Ovx	3. Intact vs Ovx
2		♀ 4,5,6	♀ 01,02,03	♀ 1,2,3	♀ 01,02,03	♀ 4,5,6 &
3						01,02,03
4	2. Ovx	3. Blank	1. Intact	2. Blank vs Ovx	3. Intact vs Ovx	1. Blank vs Intact
5	♀ 01,02,03		♀ 7,8,9	♀ 01,02,03	♀ 7,8,9	♀ 7,8,9
6					04,05,06	
7	3. Intact	1. Ovx	2. Blank	3. Intact vs Ovx	1. Blank vs Intact	2. Blank vs Ovx
8	♀ 1,2,3	♀ 04,05,06		♀ 4,5,6 &	♀ 1,2,3	♀ 04,05,06
9				04,05,06		

[a]Ovx = ovariectomized.

injected into the marginal ear vein, and the inguinal glands were removed. After carefully dissecting away connective tissue, both parts of the left and right inguinal gland of each animal were weighed and the length recorded (Figure 2).

Statistical Analysis. Tests of statistical significance were performed using an InStat program (Version 2, GraphPad Software, Inc., San Diego). Both in the test with the dummy and in the simultaneous choice test, behavioral responses from the three stimulus conditions were compared using the Friedman analysis of variance combined with Dunn's post hoc multiple comparisons test. In the simultaneous choice test, within-session responses directed to the two Q-tips were compared using the Wilcoxon signed-ranks test corrected for ties. The weight and size of inguinal glands from intact and ovariectomized females were compared by Student's t test, and the relationship between body weight and gland weight was tested with the Pearson correlation coefficient (Zolman, 1993). As secretion from intact donors was expected to elicit the highest number of responses, the Wilcoxon tests were one-tailed. An alpha value of 0.05 was taken as the level of significance throughout.

RESULTS

Gland Secretion. In tests with the dummy, the animals were generally active and did not appear inhibited by the test conditions. On average they spent

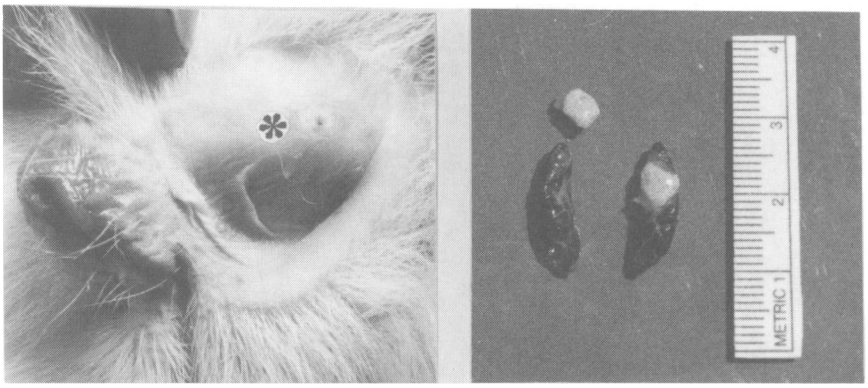


FIG 2. Left: The inguinal gland pouch of an adult female rabbit. To the left is the vaginal opening and to the right of the asterisk is an inguinal gland pore. Right: Freshly dissected inguinal glands from an adult female rabbit. The long, dark structures are the sudoriferous glands and the pale, spherical structures are the more superficially located sebaceous glands. Although the two compartments normally lie immediately adjacent to each other (right), they can be readily separated for individual measurement (left). The scale refers to the right photograph only.

about one third of the time sniffing at and apparently investigating the dummy and at least another third moving around, sniffing at, and chinning the arena (Figures 3 and 4).

Presenting them with inguinal gland secretion from either intact or ovariectomized females had little visible effect on their behavior. As shown in Figures 3 and 4, there was no significant difference in either the frequency or duration of any behavior pattern compared to the blank baseline condition, except for the frequency of chinning the arena. In the presence of secretion from either intact or ovariectomized donors, the test animals chinned the arena significantly more often than in the baseline condition ($F_r = 7.8$, $N = 9$, $k = 3$, $P < 0.02$).

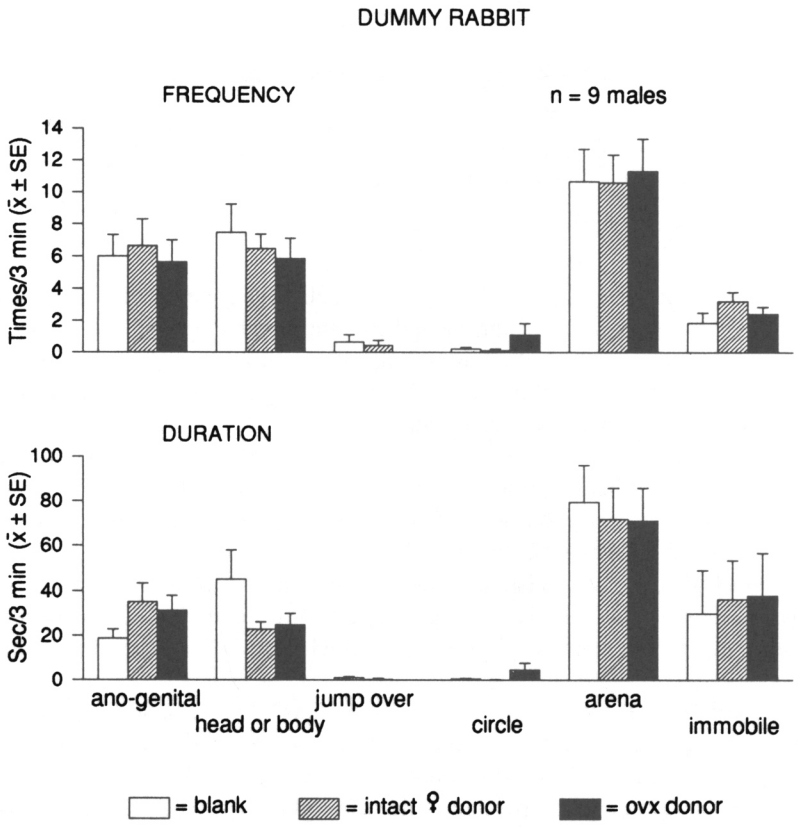


FIG. 3. Mean frequency (above) and duration (below) with which male rabbits performed various behaviors in the presence of blank Q-tips or Q-tips bearing inguinal gland secretion from intact or ovariectomized females presented on a dummy rabbit in a 3-min test. Behavioral categories shown are sniff anogenital region of dummy, sniff head or body of dummy, jump over dummy, circle dummy, sniff arena, or remain immobile.

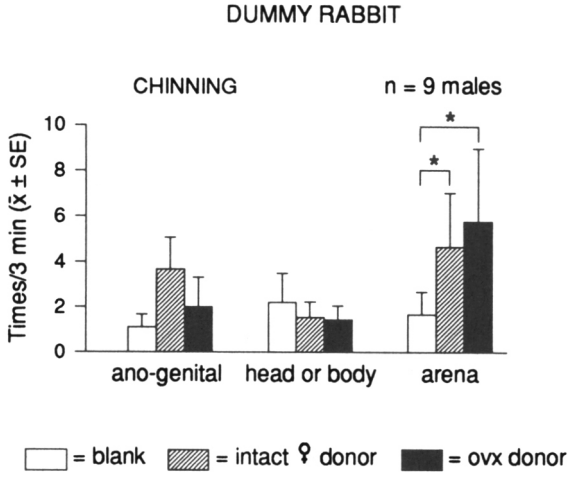


FIG. 4. Mean frequency of chin marking by male rabbits in the presence of blank Q-tips or Q-tips bearing inguinal gland secretion from intact or ovariectomized females presented on a dummy rabbit in a 3-min test. Animals and test sessions are the same as for Figure 2; $*P < 0.05$.

In the simultaneous choice test, the animals also appeared calm and spent about half the test time sniffing at and moving quietly around the arena. As shown in Figure 5, they sniffed at and also often licked the Q-tips from donor animals more often than the blank stimuli, although only significantly so for secretion from the ovariectomized animals ($T = 2$, $N = 7$, $P < 0.02$). They also spent significantly longer sniffing at stimuli from intact or ovariectomized donors than at the blank stimuli ($T = 2$, $N = 7$, $P < 0.02$ and $T = 0$, $N = 7$, $P < 0.008$, respectively), and significantly longer investigating stimuli from ovariectomized than intact does ($T = 3$, $N = 7$, $P < 0.04$).

Although there was also a tendency for animals to chin Q-tips bearing secretion more often than blank Q-tips (Figure 6), tests of significance were not possible due to the generally low scores, which resulted in a large number of ties.

Gland Size. Table 2 gives the measurements for the glands from the six ovariectomized and six intact donors. The glands were rather large, quite uniform in size, and could be easily identified, removed, and cleaned of connective tissue (Figure 2). Gross inspection under the light microscope failed to reveal any differences in general appearance such as color, texture, or shape between glands from the two groups of does. Furthermore, inspection of the inguinal pouches at the time the glands were removed revealed substantial quantities of strong-smelling secretion in all animals. At least to the several human noses

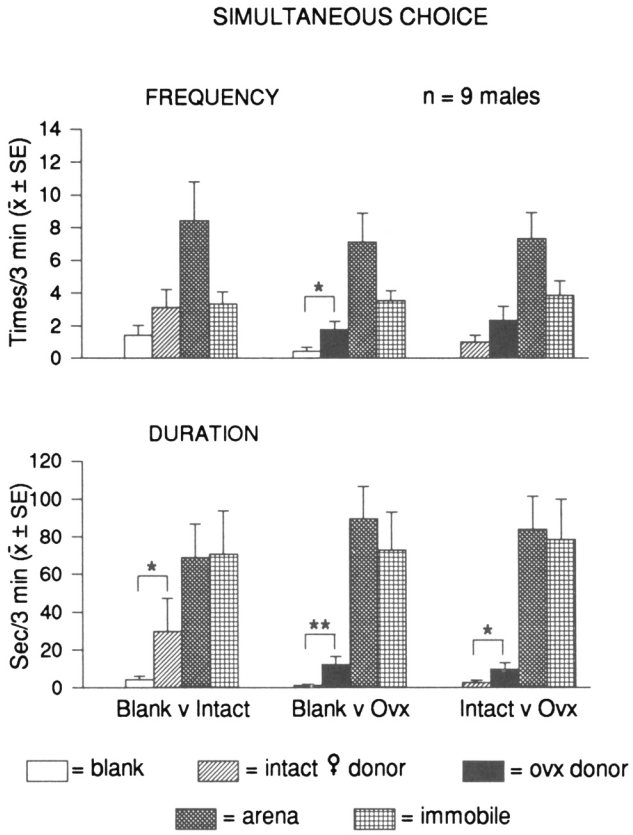


FIG. 5. Mean frequency (top) and duration (bottom) with which male rabbits performed various behaviors in a 3-min simultaneous choice test when presented with either a blank control stimulus versus inguinal gland secretion from an intact female, the control stimulus versus inguinal secretion from an ovariectomized female, or inguinal secretion from an intact female versus secretion from an ovariectomized female; $*P < 0.05$, $**P < 0.01$.

present, no difference in the odor of secretion from intact and ovariectomized does could be detected.

Since no significant differences in size or weight were found between the left and right sebaceous or sudoriferous compartments for either group of animals, data from the two sides were combined. Doing so failed to reveal a significant difference in the total weight or length of either compartment between the two groups. Although the ovariectomized does were somewhat heavier, they were not significantly so, and when the weights of all 12 animals were compared

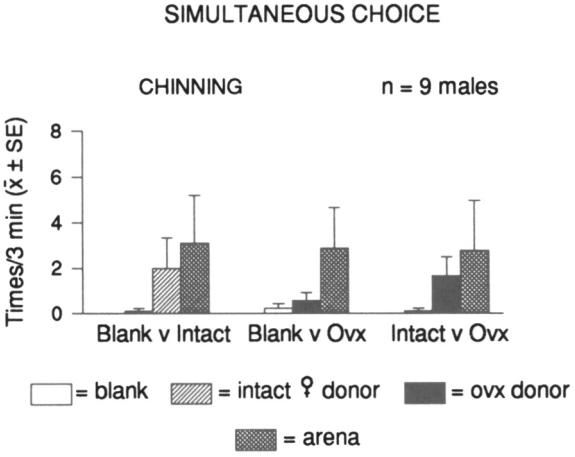


FIG. 6. Mean frequency of chin marking by male rabbits in the same series of simultaneous choice tests as for Figure 5.

TABLE 2. SIZE[a] OF THE TWO INGUINAL GLAND COMPARTMENTS IN INTACT AND OVARIECTOMIZED RABBITS

Donor ♀	Body Weight (kg)	Sebaceous		Sudoriferous	
		Weight (mg)	Length (mm)	Weight (mg)	Length (mm)
Intact	4.77	25	4.8	156	19.8
(N = 6)	(0.10)	(2.2)	(0.2)	(15)	(0.5)
Ovx	5.19	33	4.8	135	18.6
(N = 6)	(0.22)	(3.3)	(0.2)	(4.2)	(0.4)

[a] Means (± SE) are given.

with their gland weights, no significant correlation was found between body weight and weight of either the sebaceous ($r = -0.07$) or sudoriferous ($r = -0.12$) compartments.

DISCUSSION

The results of this study fail to support the hypothesis that in female rabbits inguinal gland secretion is involved in sexual attraction or signaling sexual

receptivity. Although the test males were able to perceive the odor, as indicated by greater inspection times, in the test with the dummy they rarely demonstrated any of the behaviors typically associated with courtship, such as mounting, jumping over, circling, or urinating on or near the doe (Southern, 1948), nor did they differentiate between secretions from intact and ovariectomized does, for example, by spending more time investigating secretions from intact females, as would be expected in the case of a sexual signal (cf. Doty and Dunbar, 1974).

Although the test situation was quite artificial, under very similar conditions rabbits of both sexes clearly differentiate between chin-gland secretions from donors of different reproductive state, and they respond most strongly to estrous females and reproductively active males (Soares and Diamond, 1982; Hudson and Vodermayer, 1992). Furthermore, inguinal gland secretion could always be obtained in sufficient amounts to cover the Q-tips. Collected secretion was used immediately. The method of collection was similar to that described by Hesterman and Mykytowycz (1982a) and found by them to be effective in eliciting aggressive behavior towards unknown conspecifics. Moreover, failure by the males to differentiate between secretions from the two groups of does is consistent with the lack of difference in the size or weight of the does' glands and with the large quantities of strong-smelling secretion present in their inguinal pouches. This contrasts with the clear effects of gonadectomy on the morphology and secretory activity of the chin glands (Wales and Ebling, 1971; Mykytowcyz, 1965; Cerbón et al., 1966). Since neither inguinal gland compartment appeared to be affected by ovariectomy, these probably function as a unit.

Although somewhat unexpected, these results are consistent with several previous reports suggesting the relative unimportance of ovarian steroids in regulating inguinal gland function; lack of effect of ovariectomy on gland size or morphology (Mykytowycz, 1966), lack of change in gland size or function during the female sexual cycle (Mykytowycz, 1966), and the presence of well-developed, active glands in immature animals (Montagna, 1950; personal observations).

Returning to the hypothesis proposed in the introduction, it now seems unlikely that in female rabbits inguinal gland secretion is involved in sexual attraction or in signaling sexual receptivity. Since does have a variety of other means for communicating reproductive state (Bell, 1985), it would make sense that this secretion, which is not associated with the marking behaviors typically used for signaling territoriality or reproductive state, has another function. The secretion may be involved in individual recognition (Hesterman and Mykytowycz, 1982a,b; Hesterman et al., 1984).

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PREDATOR RESPONSES TO SEQUESTERED PLANT TOXINS IN BUCKEYE CATERPILLARS: ARE TRITROPHIC INTERACTIONS LOCALLY VARIABLE?

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Abstract—Recent studies demonstrate that generalist insect herbivores may be more subject to predation than specialists and suggest that this pattern is related to the availability and utilization of plant-derived natural products in diets of the latter. This intuitively attractive hypothesis has seldom been tested under natural conditions, however. A well-described plant-insect system, the buckeye butterfly, *Junonia coenia*, and its iridoid glycoside-containing host plants, was used to test the hypotheses: (1) that larval sequestration of iridoids reduces predation in the field, and (2) that this protection varies among populations. Fifth-instars reared in the laboratory on leaves of two host-plant species, *Kickxia elatine* (Scrophulariaceae) or *Plantago lanceolata* (Plantaginaceae), or on artificial diets were attached to monofilament tethers and set out into four natural populations. Only larvae fed on the locally available host plant and artificial diet-fed caterpillars were used at each site. Despite high overall predation, significantly more leaf-fed caterpillars survived than did artificial diet-fed caterpillars in *Plantago* sites, but the trend was not significant in *Kickxia* sites. The results reflect the differences in iridoid glycoside availability in these two host-plant species. They indicate that predators exert a strong selective force on this insect, that sequestered iridoid glycosides can be effective deterrents to predation, and that host-plant choices by ovipositing females and feeding larvae have consequences for mortality from natural enemies. This protection, however, is not absolute, and depends on local predator abundance and/or selectivity. The ecological interactions between buckeye caterpillars, their host plants, and their natural enemies are locally variable in nature.

Key Words—Aucubin, catalpol, iridoid glycosides, chemical defense, sequestration, tritrophic interactions, diet breadth, host range, predation.

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INTRODUCTION

Recent research in the evolution of insect diet breadth has demonstrated that specialized herbivorous insects generally may be less vulnerable to predation than generalists (Bernays and Graham, 1988; Bernays, 1988; Bernays and Cornelius, 1989; Dyer and Floyd, 1993; Dyer, 1994). One mechanism that may contribute to this pattern is the sequestration of plant toxins for use in insect chemical defense (for reviews see Duffey, 1980; Blum, 1981; Bowers, 1990, 1993), which may be more common in specialists than generalists. Tests of the efficacy of these putative defenses, however, often use captive vertebrate predators that may behave abnormally (Sargent, 1967; Keiper, 1969; Jarvi et al., 1981) or offer allelochemicals to potential vertebrate and invertebrate predators in unnatural forms such as liquid solutions (Bowers and Larin, 1989; De la Fuente et al., 1994/1995), artificial prey (Duncan and Sheppard, 1965; Morrell and Turner, 1970; Lea and Turner, 1972; Gittleman et al., 1980), or previously killed prey (Brower, 1958a–c; Brower and Brower, 1964; Coppinger, 1969, 1970; Bowers, 1980, 1981; Bernays and Cornelius, 1989). In addition, these studies typically focus on one or a few predator species, particularly birds, despite observations that invertebrate predators and parasitoids also are important sources of mortality in nature (Bernays and Graham, 1988; Bernays, 1988; Bernays and Cornelius, 1989; Morris, 1972; Dempster, 1983; Steward et al., 1988; Stamp and Bowers, 1992). Even studies that use live prey may present prey items to predators under unnatural circumstances such as Petri-dish predation trials (e.g., Denno et al., 1990) or forced encounters with foraging predators (e.g., Dyer and Floyd, 1993).

While these studies demonstrate that sequestered plant toxins are potentially important determinants of predation rates, extending their conclusions to more natural situations requires several important assumptions: (1) that the chosen predator(s) is either an important agent of selection under natural conditions or representative of all naturally occurring predators; (2) that the experimental conditions (i.e., mode of presentation of the prey to predators and the array of possible predator responses) are either similar to the natural situation or irrelevant to the outcome of predation trials; and (3) that the interactions between predator and prey are consistent among localities. In many situations, these assumptions are not justified. For example, captive predators usually cannot engage in the full array of behaviors that influence their decisions to accept or reject a particular prey item. In addition, predators normally encounter prey in a complex environment of sensory cues produced by the prey themselves, their host plants, and myriad other biotic and abiotic sources, many of which are altered or absent in these types of experiments and vary among sites in nature. Predators also generally have a wide range of alternative foods available. All of these factors may influence the detectability or acceptability of prey. Exper-

iments under natural field conditions with freely foraging predators are, therefore, better able to evaluate whether or not the protection of putative chemical defenses is realized in nature.

One of the many specific examples of plant chemical sequestration thought to function in insect defense is the accumulation of iridoid glycosides by a variety of herbivorous insects including Lepidoptera, Hymenoptera, Homoptera, and Coleoptera (Bowers, 1991). Butterflies in the family Nymphalidae are especially well studied in this regard, but the available studies of vertebrate predation on iridoid glycoside-sequestering butterflies emphasize behavioral questions of their palatability to birds rather than the levels of predator-inflicted mortality in nature. Bowers (1980), studying captive blue and grey jay predators attacking freeze-killed adult butterflies as prey, found that adult *Euphydryas phaeton,* for example, are unpalatable to jays if they are reared on *Chelone glabra* but palatable if they are fed *Plantago lanceolata.* These two host plants contain the same two sequesterable iridoid glycosides (aucubin and catalpol), but in different amounts (Bowers and Puttick, 1986; Bowers et al., 1992). Bowers and Farley (1990) found a range of palatabilities among freeze-killed adults of four toxin-sequestering butterfly species offered to captive grey jays and attributed this interspecific variability to differences in both the types and amounts of sequestered allelochemicals. They did not, however, measure the iridoid glycoside content of the prey insects used in their experiment, but relied instead on data from a previous study (Gardner and Stermitz, 1988) and other independently collected data. Those data are characterized by high levels of intraspecific variation, such that species overlapped considerably in iridoid glycoside content.

The evidence that sequestered iridoid glycosides deter invertebrate predators is somewhat tenuous. Stamp (1992), for example, found differential attack rates by vespid wasps on caterpillars of a generalist arctiid (*Spilosoma congrua*) and a specialist nymphalid (*Junonia coenia*) in an experimental garden, but both species sequester iridoid glycosides (Bowers, personal communication). The differences in predator behavior, therefore, cannot be unambiguously attributed to sequestration. In addition, De la Fuente et al. (1994/1995) found that laboratory colonies of predacious ants (*Camponotus floridanus*) almost never attacked live larvae of *J. coenia* regardless of their iridoid glycoside content, implying that sequestration was not an important defense against that predator. Finally, Dyer (1994) studied the response of naive predatory thatch ants (*Formica rufa planipiles*) to *J. coenia* caterpillars varying in iridoid glycoside content under field conditions and found that the ants' responses varied between streamside and moraine sites. These study sites, however, lacked naturally occurring populations of both the caterpillars and their host plants, and caterpillars were exposed to predators on bare soil (Dyer, personal communication; Camara, personal observation).

Taken together, these studies suggest that iridoid glycosides sequestered

by larval and adult nymphalid butterflies are distasteful to birds and may deter some invertebrate predators. However, the efficacy of these compounds in live larvae vis-à-vis predators that cooccur with them and encounter them in a natural manner is still unresolved.

The research reported here tests the hypotheses that the sequestration of host-plant-derived iridoid glycosides by the larvae of the buckeye butterfly (*Junonia coenia* Hübner, Nymphalidae) reduces predation in naturally occurring populations subject to freely foraging predators and that these effects are consistent among sites. Larvae of the buckeye butterfly feed on plants in four families, all of which contain iridoid glycosides (Bowers, 1984). The caterpillars of this species sequester two iridoid glycosides, aucubin and catalpol, in concentrations as high as 20% of their dry biomass but eliminate them immediately prior to pupation (Bowers and Collinge, 1992; Bowers and Garrigan, unpublished data). The observation that the larvae have the ability to eliminate or metabolize iridoid glycosides prior to pupation demonstrates that this species has the metabolic machinery to rid itself of these compounds. That they do not do so as larvae, however, together with Dyer's (1994) observations of ant predators and Bowers and coworkers studies of captive birds (Bowers, 1980; Bowers and Farley, 1990), argues that sequestration is probably adaptive and may function to deter predation.

Field experiments were conducted with tethered caterpillars to find the answer to three questions about the effects of host-plant chemistry on larval predation: (1) Do host-plant-derived allelochemicals protect *J. coenia* larvae from predation in natural populations? (2) Does the degree of protection depend upon the species of host plant on which larvae feed? (3) Are these effects consistent across different populations of *J. coenia* that use the same host-plant species?

METHODS AND MATERIALS

Insect Collection and Rearing. Larvae used in this experiment were raised from the eggs of about two dozen adult female buckeye butterflies collected from an open, intermittently mown field in Discovery Park (Sacramento, California) on September 2 and 3, 1994. Females were held in a greenhouse at the University of California, Davis, in mesh cages in groups of about 10, hand-fed dilute honey–water solutions daily, and provided with cut leaves of *Plantago lanceolata* held in aquapics as oviposition substrate. Eggs laid on the leaves were collected every few days and gently stripped from the leaves into Petri dishes, where they were allowed to hatch in controlled environment chambers set for a photoperiod of 15L:9D and a constant temperature of 25°C. Dishes were checked daily for neonate larvae and the newly hatched larvae were split

each day into three groups. Each was randomly assigned to one of three diet treatments: freshly collected leaves of *Plantago lanceolata*, fresh leaves of *Kickxia elatine*, or artificial diet (Table 1). While the iridoid glycoside content of the leaves used as food in this experiment was not quantified, previous studies have shown that *Plantago* leaves contain 2–10% dry weight total iridoid glycosides with aucubin two to four times as abundant as catalpol (Adler *et al.*, 1994). *Kickxia* leaves, in contrast, contain 2–5% total iridoid glycosides by dry weight, almost all of which is catalpol (Camara, unpublished data). Artificial diets contain approximately 5% powdered *Plantago* leaves and therefore approximately 1/20th the iridoid glycoside content of leaves. All larvae were checked daily and food was supplied *ad libitum*.

Development of larvae was monitored, and those that were preparing for the fourth and final larval molt were removed from the cultures to individual small cups where they molted but were not fed. This procedure ensured that all larvae were similar in size and at the same developmental stage. For the field experiments, each larva was attached to a 0.5-m length of light (4-lb test) monofilament fishing line using super glue. The tethering ensured that larvae did not wander off and that recovered larvae could be correctly assigned to their diet treatment. The trailing end of the tether was marked with color-coded tape, and large nails were pushed through the tape and into the soil at the base of host plants. To test the secureness of the tethers, 10 tethered larvae were held

TABLE 1. CONSTITUENTS OF ARTIFICIAL DIET

Constituent	Amount
Water (ml)	105
Plantago powder (g)	2.35
Agar (g)	0.88
Linseed oil (ml)	0.47
Formalin (ml)	0.24
Wesson salts (g)	1.18
Sucrose (g)	2.97
Wheat germ (g)	7.68
Casein (g)	3.91
Torula yeast (g)	2.35
Alphacel (g)	0.80
Cholesterol (g)	0.16
Methyl paraben (g)	0.14
Vanderzant vitamin mix (g)	0.94
Ascorbic acid (g)	0.24

in the incubators by taping their tethers to the incubator racks and allowing them to feed in open Petri dishes. Over a three-day period, much longer than the experimental trials, only one larva escaped its tether during the third day.

Cultures of larvae were fed daily and molting and premolting caterpillars were isolated in the morning. The same evening, after larvae had completely molted, they were glued to their tethers and held unfed in a cold room overnight. The following day, the tethered larvae were brought into the field in their individual containers and dispersed onto naturally occurring host plants at about 1-m intervals. A representative sample of the larvae in each group was frozen for chemical analysis using gas chromatography.

Field Assays of Predation. Larvae were set out in two field sites in which *Plantago* was the only available host plant (Lagoon Valley Park and Suisun City) and two in which *Kickxia* was the only host (Vaca Valley and West Sacramento). At each site, only larvae raised on that site's host plant or on artificial diet were used because these two plants very seldom cooccur in the Central Valley and naturally occurring larvae would never have the opportunity either to feed on both host plants or to feed on one but bask or rest on the other. Experiments were all performed over a 10-day period from September 19 to 28, 1994. Due to variable availability of caterpillars, unequal numbers of caterpillars were set out at different sites and on different days (Table 2).

In all trials tethered caterpillars were censused for survivors 24 hr after placing them in the field. At this time, the tethers from which caterpillars had

TABLE 2. DATES AND LOCATIONS OF EXPERIMENTAL TRIALS

Date	Site	Host plant	Plant-fed (*N*)	Diet-fed (*N*)
Sept. 19	Lagoon Valley Park	*Plantago*	38	18
	Vaca Valley	*Kickxia*	25	15
Sept. 20	Lagoon Valley Park	*Plantago*	24	18
	Vaca Valley	*Kickxia*	28	16
Sept. 21	West Sacramento	*Kickxia*	30	29
	Suisun City	*Plantago*	27	27
Sept. 22	West Sacramento	*Kickxia*	29	14
	Suisun City	*Plantago*	27	14
Sept. 24	Suisun City	*Plantago*	28	23
Sept. 26	West Sacramento	*Kickxia*	29	29
Sept. 27	Suisun City	*Plantago*	27	29
Sept. 28	West Sacramento	*Kickxia*	28	23
	Suisun City	*Plantago*	28	24
Total			368	279

disappeared were also examined. In most instances, it was obvious that the caterpillar had been consumed rather than escaped either because a patch of glue-contaminated larval integument remained attached to the tether or because predators were still actively consuming the caterpillars. In addition, during qualitative surveys of the larvae soon after they were set out, many predation events were observed in which caterpillars struggled to escape both the predators and their tethers, but never succeeded.

Preliminary observations of tethered and untethered caterpillars fed all three diets revealed only one obvious differences in behavior. Attacked caterpillars initially thrashed their heads and regurgitated when attacked by the ants. Some then dropped from the plants. Attacking ants fell with them and more were recruited to the struggle on the ground after the caterpillars had dropped. Some, but not all, tethered caterpillars were unable to drop all the way to the ground because their tethers caught on the plants, but this appeared to have little effect on the results since, once discovered and attacked, caterpillars were unable to escape by dropping. Caterpillars that were not being attacked by predators tended not to move about, but rather to bask or feed on the host plant on which they were placed.

Diet Effects on Larval Chemistry and Size. The iridoid glycoside content of the random sample of approximately 15 caterpillars in each diet treatment was quantified using gas chromatography. Each larva was ground to a fine powder with a glass rod and some fine sand in a 15-ml test tube and then extracted overnight in 5 ml of methanol. After this initial extraction, undissolved solids were filtered from the extract and washed with an additional 9 ml of methanol (3×3 ml). The solvent was then evaporated to dryness at 50°C. The remaining residue was redissolved in 5 ml of distilled water and an internal standard (phenyl-β-D-glucoside) was added. This mixture was partitioned three times against 5 ml of diethyl ether to remove nonpolar contaminants and reevaporated to dryness. Finally, the resulting residues were redissolved in 1 ml methanol, and a 0.1 ml subsample was removed, dried, derivatized using Tri-Sil Z (Pierce), and analyzed using a Hewlett Packard 5890 gas chromatograph (Belofsky et al., 1989).

Statistical Analyses. All statistical analyses were performed using SAS (SAS Institute, 1987). Differences in larval iridoid glycoside content among the diet treatments were analyzed using multivariate analysis of variance in which the angular-transformed proportions of larval dry weight consisting of aucubin and catalpol were the scalar response variables. One-way univariate analysis of variance and Scheffe's multiple comparison procedure were used to test for univariate effects of diet on each chemical measure as well as for dry weight (i.e., size) differences among the diet treatments.

Survival rates of caterpillars were analyzed using contingency table analyses of the numbers of survivors. Percentages are presented to facilitate inter-

pretation because sample sizes are unequal, but tests were done on frequencies. These analyses tested several null hypotheses. The first was that survival was independent of whether the larvae were fed host-plant foliage or artificial diets. This analysis pooled data from both host plants and sites to test for overall effects of leaf versus artificial diets. Separate tests of this hypothesis were performed for each host plant, still pooling the sites within host plant species. The second null hypothesis tested was that rates of predation were independent of the host-plant species onto which the larvae were deployed. Because any overall host-plant difference in predation could be due to chemical protection or to different predator encounter rates, separate comparisons of host-plant-specific survival rates were performed for leaf-fed caterpillars and artificial diet-fed caterpillars. If differences in host-plant-associated survival were a function of defensive chemistry, then any differences in survivorship would appear only in the comparison of leaf-fed caterpillars, whereas different encounter rates should impact both leaf- and artificial diet-fed comparisons. Finally, the null hypothesis that the rates of predation were independent of sites within host-plant species was tested by performing separate analyses for each host plant.

RESULTS

Size of Larvae and Iridoid Glycoside Content. Larval diet treatments produced no significant differences in the dry weight of the newly molted fifth-instar caterpillars used in this experiment (Figure 1A; $F = 2.45$, $df = 2, 42$, $P > 0.05$; sample sizes: AD, 14; KX, 16; PL, 15). In contrast, there were marked differences in iridoid glycoside content among the diet treatments (Figure 1B). MANOVA indicated a significant multivariate effect of host-plant species on larval chemistry (Wilk's lambda = 0.0038, $F = 312.1$, $df = 4, 82$, $P < 0.001$), and univariate ANOVAs revealed significant diet effects on both sequestered iridoid glycosides (aucubin; $F = 609.7$, $df = 2, 42$, $P < 0.001$; catalpol; $F = 147.1$, $df = 2, 44$, $P < 0.001$). Scheffe's multiple comparison procedure showed significant differences between all treatment pairs for both iridoid glycosides. Furthermore, these differences mirrored availability in the diets. Caterpillars fed artificial diets were very nearly iridoid glycoside-free; those fed *Kickxia* contained approximately 5% sequestered iridoid glycosides, almost entirely catalpol; and those fed *Plantago* contained about 16% sequestered iridoid glycosides, approximately two thirds of which was catalpol.

Foliage vs. Artificial Diet Effects on Predation. Predation was intense on both types of caterpillars. Overall, only 9.7% of caterpillars survived. Despite these high rates of disappearance, considering both host plants and all sites simultaneously, there was a significant difference in predation rates between caterpillars fed leaves and those fed artificial diets with only about 6% of the

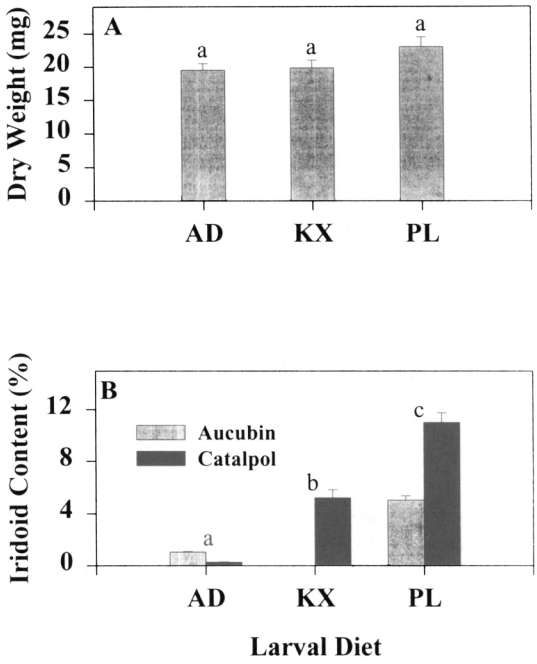


FIG. 1. Comparisons among diet treatments (AD = artificial diet, KX = *Kickxia elatine*, PL = *Plantago lanceolata*) of (A) larval dry weight at the fourth larval molt and (B) larval iridoid glycoside content at the fourth larval molt. Error bars are standard errors of the diet means. Bars with different letters are significantly different. (See Results for statistical details.)

diet-fed larvae and 12% of the leaf-fed larvae surviving the 24-hr trials (Table 3, comparison 1). Considering each host-plant species separately, but pooling the two sites for each, there was a significant effect of larval food treatment for *Plantago* versus artificial diet (Table 3, comparison 2) with survival of leaf-fed larvae approximately five times that of diet-fed larvae. On *Kickxia*, there was no significant difference in survival between leaf- and artificial diet-fed caterpillars (Table 3, comparison 3).

Hostplant and Site Effects on Predation. Predation rates also depended on the host-plant species onto which caterpillars were deployed. Larvae in *Kickxia* sites were more likely to survive than those in *Plantago* sites, regardless of whether they fed on leaf material (Table 3, comparison 4) or artificial diet (Table 3, comparison 5).

Furthermore, for both host-plant species, levels of predation depended significantly on the site at which the experiment was performed (Table 3, com-

TABLE 3. RESULTS OF CONTINGENCY TABLE ANALYSES COMPARING SURVIVAL OF LARVAE IN FIELD[a]

Comparison	Categories compared (*i*)	(*j*)	% Survival (*i*)	% Survival (*j*)	χ^2	*df*	*P*
A. Among diets							
1	AD	PL + KX	6.45	12.33	6.03	1	*
2	AD	PL	1.34	6.84	5.97	1	*
3	AD	KX	12.31	17.98	1.84	1	ns
4	KX	PL	15.58	4.42	22.87	1	***
5	KX	PL	5.73	0.72	13.83	1	***
B. Among Sites							
6	LV	SC	0	6.22	6.38	1	*
7	VV	WS	0	21.43	21.32	1	***

[a]Comparison 1 pools *Plantago*- and *Kickxia*-fed larvae into a single category (leaf-fed) and also pools the data from the two sites for each resulting diet category. Comparison 2 uses only the data from *Plantago* sites whereas comparison 3 uses only data from the *Kickxia* sites. Comparisons 4 and 5 use only the leaf-fed and artificial diet-fed caterpillars, respectively, but pool the two sites for each host-plant species. Comparisons 6 and 7 test for differences among sites with the same host-plant species available and pools the leaf-fed and artificial diet-fed larvae within sites. See Table 2 for sample sizes and dates. AD = artificial diet, PL = *Plantago*, KX = *Kickxia*; *$P < 0.05$, **$0.05 < P < 0.01$, ***$P < 0.001$; *Plantago* sites: LV, Lagoon Valley Park; VV, Vaca Valley; *Kickxia* sites: SC, Suisun City; WS, West Sacramento.

parisons 6 and 7). In fact, no larvae survived at one of the *Plantago* sites (Lagoon Valley Park) and one of the *Kickxia* sites (Vaca Valley).

DISCUSSION

Observations suggest that the high rates of predation were largely due to a single predator, the introduced Argentine ant (*Iridomyrmex humilis*), which was observed attacking many caterpillars within 30–45 min after they were placed in the field. Although several attacks by vespid wasps were observed, predation by vertebrates was not. This cannot be ruled out, however, as birds and small mammals had access to the tethered caterpillars, but were unlikely to attack while being watched. Although it is possible that such high rates of predation are anomalous, other studies also suggest that ant predation is extremely high on lepidopteran larvae (reviewed in Montllor and Bernays, 1993). The summer of 1994 was a dry one in the Central Valley: host plants were in poor condition and natural populations of buckeye larvae were sparse in comparison to the

previous year (personal observation). Predators, therefore, may have experienced reduced prey availability, and this could, in turn, affect the degree to which they exhibit selectivity. The data support the hypothesis that natural enemies have the potential to exert strong selective pressure on the larvae of *J. coenia* if the mortality they inflict discriminates among phenotypic variants. Predators did discriminate between larvae that had fed on artificial diets and those fed leaf material. Overall, leaf-fed caterpillars were approximately twice as likely to survive than were their artificial-diet-reared conspecifics (Table 3). Given the minimal size difference and substantial difference in allelochemical content of these groups (Figure 1), together with data from previous studies that iridoid glycoside sequestration may deter predators, these data suggest that iridoid glycoside sequestration also may be responsible for differences in survival.

This defense, however, is far from perfect. For each of the two host-plant species studied, all caterpillars were consumed at one of the two sites regardless of their diet or iridoid glycoside content. The interactions among host plants, their herbivores, and the natural enemies of these herbivores are variable, even over very small geographic scales. This confirms the finding of Dyer (1994), who found differences in the responses of thatch ants (*Formica rufa planipiles* Creighton) to sequestered iridoid glycosides among sites close to a stream (wet) and atop a glacial moraine (dry) at the base of the eastern Sierra Nevada mountains in northern California. As recently advocated by Thompson (1994), the evolution of these interactions, therefore, must be viewed as a geographic mosaic in which the selection pressures on the plant-derived defenses of herbivores vary among populations, even over relatively small spatial scales.

The differences in rates of predation between *Kickxia* and *Plantago* sites where some larvae did survive are similar for both leaf-fed and artificial diet-fed caterpillars, so the levels of sequestered iridoid glycosides cannot completely explain the higher survival at *Kickxia* sites. The fact that diet-fed caterpillars also survive better when set out on *Kickxia* (Table 3) argues that the abundance of predators, their ability to locate caterpillars, or both are lower on that host plant. In this regard, the more complex, branching morphology of *Kickxia* may provide another form of protection from predators that the simple rosette morphology of *Plantago* does not.

The choice of a host plant by an ovipositing female has consequences beyond its suitability as food. The survival of offspring depends upon a variety of factors, and host-plant chemistry may provide reliable cues for some, but not all of them. Ovipositing females show strong individual- and population-level preferences for these two host-plant species in greenhouse tests (Camara, 1997), and iridoid glycosides are known to be oviposition stimulants for buckeye butterflies (Bowers, 1984; Pereyra and Bowers, 1988). Larvae that feed on one host-plant species in nature are able to accumulate higher concentrations of iridoid glycosides on their normal host than are larvae that feed on a different

species in nature (Camara, 1997). The overall pattern found here of decreased survival of larvae fed an iridoid glycoside-free diet argues that these differences in oviposition preference and iridoid sequestration ability have evolved in response to predation on larvae.

In summary, this study demonstrates three important points. First, the impact of natural enemies, especially ants, is sufficient to exert strong natural selection on any aspect of caterpillar foraging that decreases predation. Second, the decision to oviposit on a particular host-plant species or in a particular host-plant population impacts survival for reasons other than food quality. Host plants are sources of not only nutrients but also defensive chemicals. Finally, the efficacy of this defense depends on many factors that must be studied further, but probably include the distribution of predators among sites and the detectability of prey on particular host-plant species.

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VOLATILE ALLELOCHEMICALS RELEASED BY CRUCIFER GREEN MANURES[1]

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Abstract—Several members of the crucifer family (Brassicaceae), including white mustard (*Brassica hirta* Moench), brown mustard [*B. juncea* (L.) Coss], black mustard [*B. nigra* (L.) Koch], leafy turnip (*B. campestris* L.), rapeseed (*B. napus* L.), and garden cress (*Lepidium sativum* L.) were examined for their potential as allelopathic green manure crops. Hemp sesbania [*Sesbania exaltata* (Raf.) Rydb. Ex A. W. Hill] germination and fresh weight was inhibited by chopped leaf tissues of all green manures tested, including wheat (*Triticum aestivum* L.), when added to a sandy loam soil. Wheat seed germination was inhibited only by *B. nigra*, *B. hirta*, and *L. sativum*, although none of the treatments reduced fresh weight of germinated seedlings. The major volatiles released by chopped plants were determined by solid-phase microextraction sampling and identified by gas chromatography–mass spectrometry (GC-MS). Volatiles included allyl isothiocyanate (allyl-ITC), 3-butenyl isothiocyanate, benzyl isothiocyanate (benzyl-ITC), *cis*-3-hexen-1-ol, and *trans*-2-hexenal. These compounds, together with methyl-ITC (methyl-ITC), β-phenylethyl-ITC, benzaldehyde, β-ocimene, and α-farnesene were tested for inhibition of seed germination of several crop and weed species when applied as volatiles. Of these, allyl-ITC and methyl-ITC were the most inhibitory, completely inhibiting the germination of all species at a headspace gas concentration of 1 ppm in airtight glass containers. Selecting mustard green manures that release high levels of allyl-ITC would appear to be optimal for allelopathic activity, and plants that produce high levels of benzyl-ITC also appear promising.

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[1] Propietary names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Key Words—*Brassica campestris, Brassica hirta, Brassica juncea, Brassica napus, Brassica nigra, Lepidium sativum*, allyl isothiocyanate, benzyl isothiocyanate, methyl isothiocyanate, volatiles, phytotoxicity, allelopathy.

INTRODUCTION

The Brassicaceae, or crucifer family, is a large plant family that includes important food crops, ornamentals, and weeds (Lawrence, 1951). Crucifers have generated much interest for their potential as green manures or soil amendments for the suppression of weeds (Brown and Morra, 1995; Grossman, 1993; Boydston and Hang, 1995), soil pathogens (Papavizas, 1996; Lewis and Papavizas, 1971; Papavizas and Lewis, 1971; Ramirez-Villapudua and Munnecke, 1988; Muelchen et al., 1990; Mayton et al., 1996), nematodes (Mojtahedi et al., 1991, 1993), and insects (Brown et al., 1991). The pesticidal activity of crucifers has been thought to be due primarily to glucosinolates and their degradation products. Glucosinolates are a class of glucose- and sulfur-containing compounds whose biologically active degradation products are produced when the plant cells are ruptured and the glucosinolates present in vacuoles are hydrolyzed by the enzyme myrosinase (β-thioglucosidase glucohydrolase; EC 3.2.3.1) (VanEtten and Tookey, 1983). These products include substituted isothiocyanates (ITC), nitriles, thiocyanates, and oxazolidinethiones, which vary depending on the side-chain substitution, pH, and iron concentration (Cole, 1976; Daxenbichler and VanEtten, 1977; Fenwick et al., 1983; Uda et al., 1986; Borek et al., 1994, 1995). Several volatile ITCs, including allyl (2-propenyl)-ITC (allyl-ITC), 2-phenylethyl-ITC, and benzyl-ITC have been shown to be toxic to a wide range of organisms at relatively low molar concentrations (Wolf et al., 1984; Bialy et al., 1990; Lazzeri et al., 1993; Williams et al., 1993). Because ITCs are relatively insoluble in water, their activity would be in the vapor phase as volatiles. In addition to glucosinolate degradation products, other volatiles would be expected to be released when crucifer green manures are incorporated into the soil, notably products of the lipoxygenase pathway, which include (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate (Gardner, 1995). These compounds were among the major volatiles produced by disrupted tissues of several crucifers (Wallbank and Wheatley, 1976; Tollsten and Borgström, 1988), and they have been shown to be toxic as volatiles to plants (Gardner et al., 1990; Bradow, 1991), fungi (Hamilton-Kemp et al., 1992; Doehlert et al., 1993; Gamliel and Stapleton, 1993; Vaughn and Gardner, 1993; Zeringue et al., 1996), and bacteria (Croft et al., 1993; Deng et al., 1993).

We are examining several members of the Brassicaceae as green manure crops to suppress weeds, including white mustard (*Brassica hirta* Moench),

brown mustard [*B. juncea* (L.) Coss], black mustard [*B. nigra* (L.) Koch], leafy turnip (*B. campestris* L.), rapeseed (*B. napus* L.), and garden cress (*Lepidium sativum* L.). Our objectives were to evaluate their potential to suppress weed emergence and growth when green tissues were incorporated into soil, to determine the major volatiles released by chopped tissues, and to determine the relative toxicities of these volatiles against several bioassay species.

METHODS AND MATERIALS

Plant Material. Crucifers examined were *B. juncea* Greenwave, *B. juncea* Florida Broadleaf, *B. campestris* L. Seven Top, *B. napus* L. Jupiter, *B. hirta* Moench Martigeña, and *L. sativum* L. Curly Cress. *B. nigra* seed used was a wild population collected near Peoria, Illinois. Wheat (*Triticum aestivum* L. Cardinal), which has shown little or no weed suppression in field tests, was used as a green manure control. Plants were grown in a growth chamber at a 16 hr 26°C day/8 hr 21°C night for 30 days before leaf and stem tissues were harvested.

Germination and Growth of Wheat and Hemp Sesbania in Soil with Incorporated Plant Tissues. Green manure plants grown in the growth chamber were harvested, chopped into 0.5-cm^2 pieces, and uniformly mixed into soil (Onarga sandy loam; Typic Argiudoll) at a rate of 0.05 g of plant material/ g soil. The plant–soil mixture was added to 200-ml cups, and 10 seeds of either wheat or hemp sesbania (species that we have found to be good indicators of suppressive activity in bioassays) were added and covered with approximately 2 cm of the mixture. Nonamended soil was used as the control. Each cup received 10 ml of a solution containing 1 g/liter thiabendazole (TBZ; 10 mg/cup) to inhibit soil fungi, which were found to inhibit soil germination of the test species. Additional water was added to bring the cups to field capacity, and the plants were placed in the growth chamber at the same conditions as before. Cups were watered daily to field capacity, with the soil becoming dry near the surface between waterings. Germination and fresh weight (aboveground tissue only) were measured 10 days after planting. Each green manure treatment was replicated five times per species, and the experiment was repeated. Treatment means were separated using Fisher's protected LSD test at the 5% level (StatView, Abacus Concepts, Berkeley, California).

Volatile Analysis. Five grams of plant material were shredded with a food chopper and placed rapidly in 2.4-liter glass desiccators equipped with 3-mm holes through which rubber septa were fitted. After 15 min, a solid-phase microextraction (SPME) device (Supelco, Inc., Bellefonte, Pennsylvania) with a 100-μm polydimethylsiloxane coating was inserted through the septa and allowed to absorb volatiles for 1 min. Absorbed compounds were desorbed at

200°C onto the inlet injector for 0.5 min on a Hewlett-Packard (HP) 5890 Series II gas chromatograph (Hewlett-Packard Co., Palo Alto, California) equipped with a flame ionization detector, and peaks were analyzed by an HP 3396 integrator. The column used was a fused silica DB-1 capillary (0.25-μm film thickness, 15 m × 0.25 mm ID; J & W Scientific, Folsom, California). Mass spectra were produced by introducing samples through a HP 6890 gas chromatograph connected to a HP 5973 mass selective detector. The column used was a HP-5MS capillary (30.0 m × 0.25 mm ID, 0.25-μm film thickness). Compounds were identified by comparison of mass spectra with pure standards, with a library data base, and/or with previously published spectra for glucosinolate degradation products (Spencer and Daxenbichler, 1980).

Effect of Volatiles on Crop and Weed Seed Germination. The five volatiles found in the highest concentrations by SPME were allyl-ITC, 3-butenyl-ITC, benzyl-ITC, *cis*-hexen-1-ol, and *trans*-2-hexenal. Other volatiles examined were β-phenylethyl-ITC, benzaldehyde, β-ocimene, and α-farnesene, which have been reported as major volatiles released from either intact and disrupted tissues of several *Brassica* species (Tollsten and Bergström, 1988). These compounds were tested for inhibition of seed germination of corn (*Zea mays* L.), soybeans [*Glycine max* (L.). Merr.], wheat, rapeseed, cucumber (*Cucumis sativus* L.), alfalfa (*Medicago sativa* L.), and dandelion (*Taraxacum officinale* Weber in Wiggers) when applied as volatiles, with the commercial soil fumigant methyl-ITC included as a reference standard. Ten seeds of each species were placed in 9-cm petri dishes on Whatman No. 1 filter paper saturated with sterile distilled water. These dishes were placed in 2.4-liter desiccator flasks containing a 9-cm filter paper disk to which test compounds were added on a volume compound–headspace volume basis at rates of 1.0, 5.0, and 10.0 ppm. Each flask was fitted with a septum allowing the headspace volatiles to be sampled by SPME. Flasks were placed in darkness in a growth chamber at 25°C for four days, after which germination was scored. Each treatment was replicaed three times and the experiment was repeated.

RESULTS AND DISCUSSION

Germination and Growth of Wheat and Hemp Sesbania with Incorporated Green Manures. Hemp sesbania and wheat were markedly different in their response to soil-incorporated green manure tissues (Table 1). Hemp sesbania emergence and growth were reduced by every green manure treatment, including incorporated wheat. In several of the green manure treatments (*B. campestris* Seven Top, *B. hirta* Martigeña, *B. juncea* Greenwave, *B. nigra* and *L. sativum* Curly Cress) hemp sesbania seedling emergence, and hence growth, was zero. Conversely, wheat emergence was suppressed to a much lower extent, and only

TABLE 1. EMERGENCE AND GROWTH OF WHEAT AND HEMP SESBANIA IN SOIL WITH MACERATED GREEN MANURE LEAF AND STEM TISSUES[a]

	Hemp sesbania (% of control)		Wheat (% of control)	
Green manure	Emerged seedlings	Fresh weight	Emerged seedlings	Fresh weight
None	100.0a	100.0a	100.0a	100.0cd
B. campestris				
Seven Top	0.0d	0.0d	84.8c	105.8c
B. hirta Martigeña	0.0d	0.0d	78.2d	91.6e
B. juncea				
Florida Broadleaf	4.4c	4.7c	100.0a	126.7a
B. juncea Greenwave	0.0d	0.0d	93.6b	97.2d
B. napus Jupiter	4.2c	3.0c	93.6b	112.7b
B. nigra	0.0d	0.0d	84.8c	105.8c
Lepidium sativum				
Curly Cress	0.0d	0.0d	69.4e	95.0de
Triticum aestivum				
Cardinal	72.2b	61.3b	100.0a	108.6bc

[a]Means within a column followed by the same letter are not different at $P = 0.05$ according to Fisher's protected LSD test.

the *B. hirta* Martigeña treatment significantly inhibited growth relative to the control, with growth promotion occurring with incorporated *B. juncea* Florida Broadleaf and *B. napus* Jupiter tissues. Boydston and Hang (1995) found similar results when they incorporated leaf tissues of rapeseed or potato into a sandy loam soil. Hairy nightshade (*Solanum sarrachoides* Sendtner) seedling emergence and growth were inhibited by both rapeseed and potato tissues, but longspine sandbur [*Cenchrus longispinus* (Hack.) Fern.] emergence and growth were only reduced by the rapeseed treatment. The authors observed that hairy nightshade was suppressed by the addition of all of the biomasses tested, while longspine sandbur was not. The physical impacts of a biomass acting as a mulch may have contributed to inhibition in field plots, as Putnam et al. (1983) found that popular excelsior (which had been found to have no adverse effect on plants in the greenhouse), although not affecting weed seed germination, reduced weed biomass in field plots, possibly by shading and/or cooling of the soil.

Volatiles Released by Macerated Tissues. Although there were substantial differences between the volatiles collected by SPME from the different green manures, there were also several similarities (Table 2). With the exception of *B. campestris* and wheat, one volatile accounted for >50% of the total volatiles collected. In all cases, the dominant compounds were either products of glu-

TABLE 2. RELATIVE CONCENTRATIONS OF MAJOR VOLATILES RELEASED BY MACERATED GREEN MANURE LEAF AND STEM TISSUES

Species	Volatile	% of total volatiles collected by SPME[a]
B. campestris Seven Top	*cis*-3-hexen-1-yl acetate	27.2
	3-butenyl isothiocyanate	21.5
	cis-3-hexen-1-ol	19.8
B. hirta Martigeña	benzyl isothiocyanate	84.0
	cis-3-hexen-1-ol	8.7
B. juncea Florida Broadleaf	allyl isothiocyanate	67.7
	trans-2-hexenal	8.6
B. juncea Greenwave	allyl isothiocyanate	67.3
	trans-2-hexenal	17.7
B. napus Jupiter	*cis*-3-hexen-1-yl acetate	78.5
	cis-3-hexen-1-ol	13.9
B. nigra	allyl isothiocyanate	54.4
	cis-3-hexen-1-yl acetate	12.7
Lepidium sativum Curly Cress	benzyl isothiocyanate	56.9
	benzaldehyde	7.0
Triticum aestivum Cardinal	*trans*-2-hexenal	26.6
	cis-3-hexen-1-ol	20.2
	cis-3-hexen-1-yl acetate	11.2

[a] Values represent the mean percentages of three replicates. Only compounds comprising >5% of total volatiles are presented.

cosinolate degradation (allyl-, 3-butenyl-, and benzyl-ITC) or were lipoxygenase pathway metabolites [(*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate]. Allyl-ITC was the major volatile from both of the brown mustard cultivars as well as the black mustard tissues, while benzyl-ITC was the major compound released from white mustard and garden cress. Tollsten and Bergström (1988) found a similar volatile profile, although in their study the volatiles from macerated leaf tissues were captured on a porous polymer (Porapak Q) instead of SPME. Although β-hydroxy-substituted glucosinolates have been reported in several of the species examined (Daxenbichler et al., 1991) and are especially high in rapeseed (Brown et al., 1991), their degradation products (oxazolidinethiones) were not detected with SPME sampling, probably due to low volatilities for these compounds.

Inhibition of Seed Germination by Volatiles. Methyl-ITC and allyl-ITC were the most inhibitory compounds tested, with I_{50} values of less than 1 ppm for all of the species tested (Table 3). 3-Butenyl-ITC was as toxic as methyl-

TABLE 3. I_{50} VALUES FOR INHIBITION OF GERMINATION OF SEEDS EXPOSED TO GREEN MANURE VOLATILES

Volatile	I_{50} (ppm)[a]						
	Soybean	Corn	Wheat	Rapeseed	Dandelion	Alfalfa	Cucumber
Methyl-ITC	<1	<1	<1	<1	<1	<1	<1
Allyl-ITC	<1	<1	<1	<1	<1	<1	<1
3-Butenyl-ITC	<1	<1	<1	>5	<1	<1	<1
Benzyl-ITC	>10	>10	>10	>10	<1	<1	>10
2-Phenylethyl-ITC	>10	>10	>10	>10	>10	>10	>10
Benzaldehyde	>5	>10	>5	>5	<1	>1	>10
trans-2-Hexenal	>1	>5	>5	>1	<1	>1	>10
cis-3-Hexen-1-ol	>5	>5	>5	>5	>1	<1	>10
cis-3-Hexen-1-yl acetate	>5	>5	>1	>1	<1	>1	>5
β-Ocimene	>10	>10	>10	>10	>10	>10	>10
α-Farnesene	>10	>10	>10	>10	>10	>10	>10

[a] I_{50} values represent the mean of three replicates.

ITC and allyl-ITC to every species except rapeseed, for which the I_{50} value was >5 ppm. This may be due to metabolism of 3-butenyl-ITC by the rapeseed, as it has been shown to contain the parent 3-butenyl glucosinolate (Daxenbichler et al., 1991; Brown et al., 1991). The much lower levels of activity shown by benzyl-ITC and 2-phenylethyl-ITC were somewhat surprising. 2-Phenylethyl-ITC was the most phytotoxic isothiocyanate tested against wheat seed germination (Bialy et al., 1990), while benzyl-ITC constitutes the major volatile released by both *B. hirta* Martigeña and *L. sativum* Curly Cress, the most inhibitory green manure treatments to wheat seed germination used in this study. The decreased activities of these two compounds may be due to their lower volatilities (therefore decreasing the headspace concentrations) in comparison to methyl-ITC and allyl-ITC. The lipoxygenase pathway volatiles, *trans*-2-hexenal, *cis*-3-hexen-1-ol, and *cis*-3-hexen-1-yl acetate, were intermediate in activity, exhibiting the lowest activity against cucumber, a species that has been reported to possess high lipoxygenase activity (Matsui et al., 1992; Avdiushko et al., 1994; Gardner, 1995). Both α-farnesene and β-ocimene, volatiles reportedly released from intact leaves of *Brassica* spp. (Tollsten and Bergström, 1988), were the least phytotoxic compounds tested, with I_{50} values of >10 ppm for all species tested.

Volatiles released by crushed leaves of *B. juncea* and *B. nigra* inhibited seed germination of lettuce (*Lactuca sativa* L.), barnyardgrass [*Echinochloa*

crus-galli (L.) Beauv.], and wheat (Oleszek, 1987). In our study, the major volatile compound released by both species was allyl-ITC. Although benzyl-ITC was less toxic than allyl-ITC to seeds in the glass desiccators, two of the green manures with the most activity (*B. hirta* and *L. sativum*) released benzyl-ITC as their primary volatile. The lower activity of benzyl-ITC in the desiccator study can be explained by its much lower volatility [allyl-ITC has a vapor pressure of 5 mm Hg at 25°C while benzyl-ITC has a vapor pressure of < 1 at this temperature (Jordan, 1954)]. Perhaps in the soil–plant mixture higher levels of benzyl-ITC were reached as compared to inside the desiccators. Teasdale and Taylorson (1986) reported that methyl-ITC was more toxic to large crabgrass [*Digitaria sanguinalis* (L.) Scop.] when applied to soil containing crabgrass seeds rather than when the seeds were directly exposed to a methyl-ITC solution. Another possibility is a synergy of several different compounds, which, as Einhellig (1987) has pointed out, appears to be the norm in allelopathic interactions.

While it appears from our data that commercially acceptable suppression of weeds using crucifer green manures is unlikely, their use may allow reduced or delayed herbicide use in conventional systems and may be especially helpful for organic production systems where tillage is the major weed control method. We have observed that crucifer green manures can delay weed seed germination and emergence in field trials. Selecting crucifer green manures with higher allyl and benzyl glucosinolate levels than those tested in this study may provide adequate weed suppression if combined with tillage and (reduced) use of herbicides.

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FEEDING DETERRENCE AND TOXICITY OF NEEM TRITERPENOIDS

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Abstract—The neem tree, *Azadirachta indica*, produces a plethora of triterpenoids. One of these is azadirachtin, a complex compound with powerful insecticidal properties. The biosynthesis of azadirachtin starts with a steroid precursor and proceeds via two levels of structural complexity, i.e., furan ring formation and C-ring opening, after which further modifications yield azadirachtin. Antifeedant and toxic properties of azadirachtin against insects were compared with those of various compounds from the two lower levels of structural complexity. To larvae of the moth *Spodoptera littoralis*, several compounds from the lower structural levels showed strong antifeedant activity, comparable to that of azadirachtin. Nymphs of the locust *Schistocerca gregaria* in antifeedancy tests were more sensitive to azadirachtin than to the other compounds tested. To *Spodoptera littoralis*, *Schistocerca gregaria*, and the bug *Oncopeltus fasciatus*, azadirachtin was very toxic, exerting severe growth and molt disrupting effects. In contrast, compounds from the lower structural levels than azadirachtin showed virtually no toxicity to these three insects. Thus, although several simpler triterpenoid structures other than azadirachtin have insect antifeedant activity, the combination of strong antifeedant and toxic properties of azadirachtin may provide the more important chemical protection of the neem tree. Our data reveal no explicit link between antifeedant activity and toxicity of individual neem triterpenoids.

Key Words—*Azadirachta indica*, azadirachtin, chemical defense, feeding deterrence, neem tree, toxicity, triterpenoids.

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INTRODUCTION

The neem tree, *Azadirachta indica*, is thought to have originated in southern Asia (India, Burma), but has been introduced throughout tropical and subtropical regions (Benge, 1989). Neem belongs to the Meliaceae (mahogany family), placed within the Rutales, an order characterized by widespread occurrence of bitter triterpenoids (Das et al., 1987; Frohne and Jensen, 1992). Diverse parts of the neem tree contain a plethora of triterpenoids (Jones et al., 1989). A complex neem triterpenoid, azadirachtin, is one of the most powerful plant-derived insecticides known (Jacobson, 1989a; Schmutterer, 1990; Mordue (Luntz) and Blackwell, 1993). Despite its potent insecticidal properties, azadirachtin is remarkably nontoxic to many other forms of life (Jacobson, 1989b).

Azadirachtin shows three specific modes of action in insects, as reviewed in Mordue (Luntz) and Blackwell (1993). First, it has strong antifeedant activity due to its effects on chemoreceptors. Second, it affects ecdysteroid and juvenile hormone titers through a blockage of morphogenetic peptide hormone release, resulting in severe growth and molting aberrations. Third, it has direct detrimental and histopathological effects on most insect tissues, e.g., muscles, fat body, and gut cuticular epithelial cells. These distinctive properties with respect to insects, together with the apparent lack of effects on many other taxa, suggest that the biosynthetic pathway of azadirachtin evolved under selective pressures by herbivorous insects.

The steroid tirucallol is considered to be the precursor of neem triterpenoids (Ley et al., 1993; Hansen et al., 1994). From tirucallol, the biosynthesis of azadirachtin is thought to proceed via two levels of biosynthetic complexity (Figure 1) (Ley et al., 1993; Hansen et al., 1994). First, four atoms are lost from the side chain, and the remainder are cyclized to form a furan ring. This gives rise to limonoids such as azadirone and azadiradione. Subsequently, the C-ring is opened and C-*seco*-limonoids such as nimbin and salannin are produced. The in vivo conversion of radiolabeled tirucallol into the C-*seco*-limonoid nimbolide in the leaves of the neem tree has been reported (Ekong et al., 1971). The synthesis of azadirachtin requires further rearrangements and oxidations (Figure 1), and it is one of the most highly oxidized triterpenoids within the Rutales (Das et al., 1987). A biosynthetic pathway has been proposed in which azadirone is converted into salannin, and the latter into azadirachtin (Hansen et al., 1994).

To investigate the biological relevance of the large variety of neem triterpenoids, we determined the toxic and antifeedant properties against insects of compounds from the lower levels of biosynthetic complexity described above, and we compared the properties of these compounds with those of azadirachtin. Although the effects of azadirachtin and many of its structural analogs on several

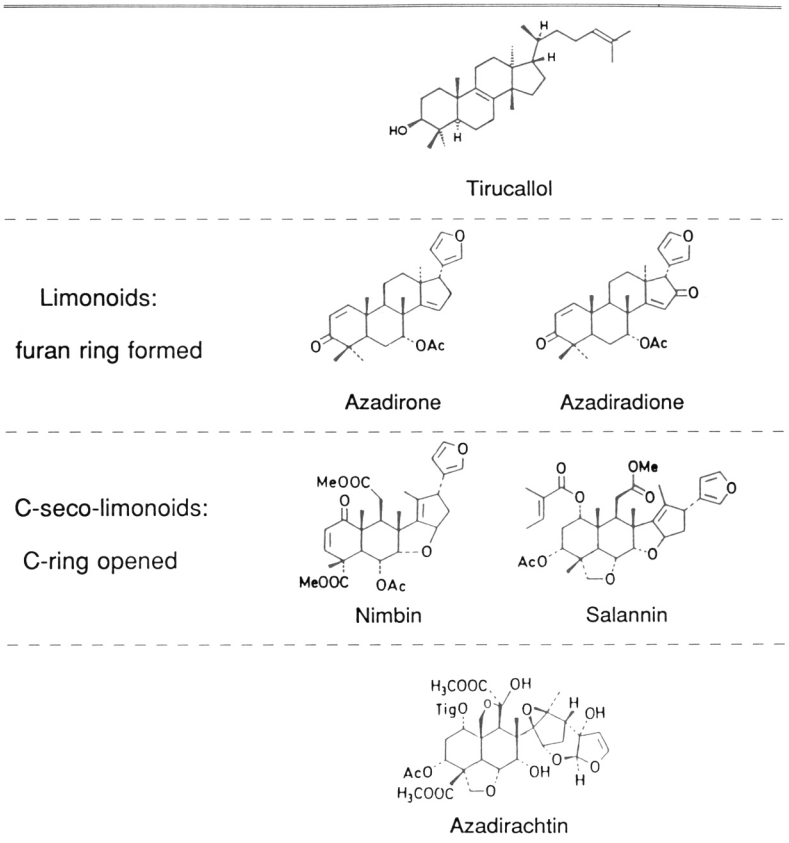


FIG. 1. Neem triterpenoids of increasing biosynthetic and structural complexity.

insect species have been well documented (Rembold, 1989a; Blaney et al., 1990; Simmonds et al., 1990), the properties of the structurally simpler compounds have been far less systematically studied.

For our studies we used the milkweed bug, *Oncopeltus fasciatus* Dallas (Hemiptera); the cotton leafworm, *Spodoptera littoralis* Boisduval (Lepidoptera); and the desert locust, *Schistocerca gregaria* Forskal (Orthoptera). *Oncopeltus fasciatus* has been demonstrated to be susceptible to endocrine disruption. *Spodoptera littoralis* and *Schistocerca gregaria* are polyphagous insects, originating from Africa and the Near East (Brown and Dewhurst, 1975), and the Indian subcontinent (Bernays and Chapman, 1978, 1987), respectively. In this paper, we compare the effects of azadirone, azadiradione, nimbin, salannin, and

azadirachtin on these three insects. The compounds were tested at concentrations that encompass levels in the neem tree.

METHODS AND MATERIALS

Insects. Oncopeltus fasciatus was obtained from Blades Biological, Cowden, Edenbridge, Kent, and reared on cracked sunflower seeds at 27°C (12L:12D). *Spodoptera littoralis* eggs were obtained from NRI, Chatham, London, from an original Canary Islands culture. Larvae were reared on an artificial diet at 27°C (12L:12D or 16L:8D). *Schistocerca gregaria* were reared in the Department of Zoology, University of Aberdeen, under 16L:8D conditions at 27°C as previously described (Nasiruddin and Mordue (Luntz), 1994).

Toxicity Assays. Molt-inhibiting effects of neem triterpenoids to *Oncopeltus* were determined after topical application in 1 μl acetone to 10 (0 to 1-day-old) fifth instars per treatment as described in Isman et al. (1990). Abbott's formula was used to correct for control mortality (Busvine, 1971).

Toxicity to *Spodoptera* was monitored after topical application of the compounds dissolved in acetone to newly molted fifth instars (0.5-μl aliquot, seven larvae per treatment), or to newly molted final instars (2-μl aliquot, seven larvae per treatment). Application was on the dorsothoracic segments. Furthermore, toxic effects were determined after the compounds, dissolved in ethanol, were directly injected with a 10-μl Hamilton syringe into the hemolymph of newly molted final instars (1-μl aliquot, 10 larvae per treatment). Injection was in the abdomen, between abdominal segments 4 and 5. In all cases, controls were treated or injected with carrier solvent only.

Toxic effects to *Schistocerca* were determined after topical application of compounds dissolved in acetone to the ventral surface of the abdomen of newly molted second instars of around 55 mg fresh weight (2-μl aliquot, 10 nymphs per treatment). Controls were treated with solvent only, and Abbott's formula was used to correct for control mortality (if any) (Busvine, 1971).

Antifeedancy Assays. The antifeedant activity of neem triterpenoids to *Spodoptera* was determined by the protocol for choice tests as described in Blaney et al. (1990). Individual, final instars (five per treatment) were offered two dried glass-fiber discs (2.1 cm diam., Whatman GF/A) that had previously received 100 μl of a 50 mM sucrose solution, and additionally 120 μl ethanol (control disc) or 120 μl of an ethanolic solution (0.02–2000 μM) of triterpenoid (treated disc).

For the determination of antifeedant activity in choice tests with *Schistocerca*, nymphs of both sexes (5–10 per treatment), two to three days into the final instar, were starved as described previously (Allan et al., 1994). Subsequently, after checking whether individual nymphs had produced fecal pellets,

they were each offered two dried glass-fiber discs (2.5-cm diam., Whatman GF/A) that had previously received 150 μl of a 50 mM sucrose solution, and additionally 150 μl ethanol (control disc) or 150 μl of an ethanolic solution (0.0012–1200 μM) of triterpenoid (treated disc). The test was further performed according to Blaney et al. (1990).

RESULTS

Toxicity of Neem Triterpenoids

Effects with Oncopeltus. Development-disrupting effects of neem triterpenoids to *Oncopeltus* were studied by topical application of the compounds in a broad concentration range to newly molted final instars. At day 10 after treatment, deviations from normal development into the adult phase were assessed. Figure 2 shows that azadirachtin (Az) completely blocked development at concentrations of 75 pmol/insect or higher. At 75 pmol/insect, nymphs showed no

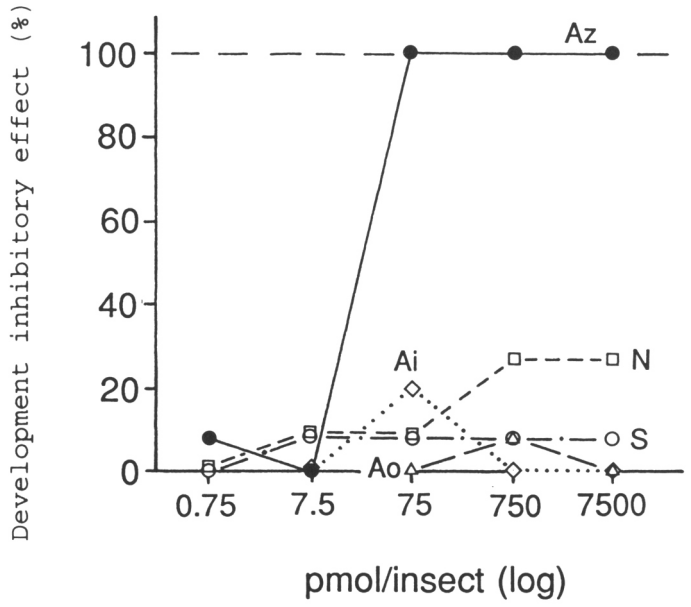


FIG. 2. Development-disrupting effects of neem triterpenoids to *Oncopeltus fasciatus*. Final instars were topically treated with the compounds, and deviations from normal development into the adult phase were assessed. Az, azadirachtin; S, salannin; N, nimbin; Ai, azadiradione; Ao, azadirone.

molt, died during the molt, or molted abnormally. At higher concentrations, all nymphs stopped molting and remained permanent nymphs. In contrast, such severe toxic effects were not observed with neem compounds of less structural complexity than azadirachtin (Figure 2). At the highest concentrations, nimbin treatment resulted in an occasional death after the molt or an abnormal molt.

Effects with Spodoptera. The toxicity of neem triterpenoids to *Spodoptera* was investigated in three ways. First, fifth instars were topically treated with 10 nmol/insect of compound. During the subsequent transition from late fifth to young sixth (final) instar, controls (C) as well as larvae treated with triterpenoids simpler than azadirachtin all increased in weight by about 100%, whereas azadirachtin-treated larvae increased in weight by only about 40% (Figure 3A). Furthermore, azadirachtin-treated larvae went into pupation one to two days later than larvae treated otherwise. In a second experiment, the compound (5 nmol/insect) was injected directly into the hemolymph of final instar larvae. Under all conditions, increases in weight of the larvae during the following few days of development were comparable. However, the majority of azadirachtin-injected larvae died immediately after the onset of pupation, whereas these severe effects were not observed with less complex neem triterpenoids (Figure 3B, hatched bars). Finally, when final instars were topically treated with 25 nmol/insect of compound, the majority of azadirachtin-treated insects showed abnormal molts (Figure 3B, dark bars). Azadirachtin treatment resulted in blockage of development in the pupal stage, death during the molt into the adult phase, or emergence of adults with deformed wings. Again, the effects of the less complex neem triterpenoids were minor and comparable to the controls (Figure 3B).

Effects with Schistocerca. As in the other two species, toxic properties were observed with azadirachtin but not with the less complex triterpenoids. Second instars of *Schistocerca* were topically treated with compounds in a broad concentration range, and the growth of the nymphs was assessed seven days later. Figure 4A shows that at the highest concentrations applied, azadirachtin stopped growth of nymphs, whereas nymphs treated with the same concentrations of less complex triterpenoids showed growth similar to controls. Figure 4B shows that the highest concentrations of azadirachtin almost completely blocked development of the nymphs into the third instar. At an azadirachtin concentration of 3000 pmol/insect, nymphs died during the molt, or showed no or abnormal molts, and at a concentration of 30,000 pmol/insect, about half of the nymphs died before the molt, while almost all others did not enter the molt and remained as second instars. These dramatic effects were not observed with the less complex triterpenoids (Figure 4B).

Thus, in *Oncopeltus*, *Spodoptera*, and *Schistocerca*, strong toxic effects were observed only with azadirachtin (Figures 2–4).

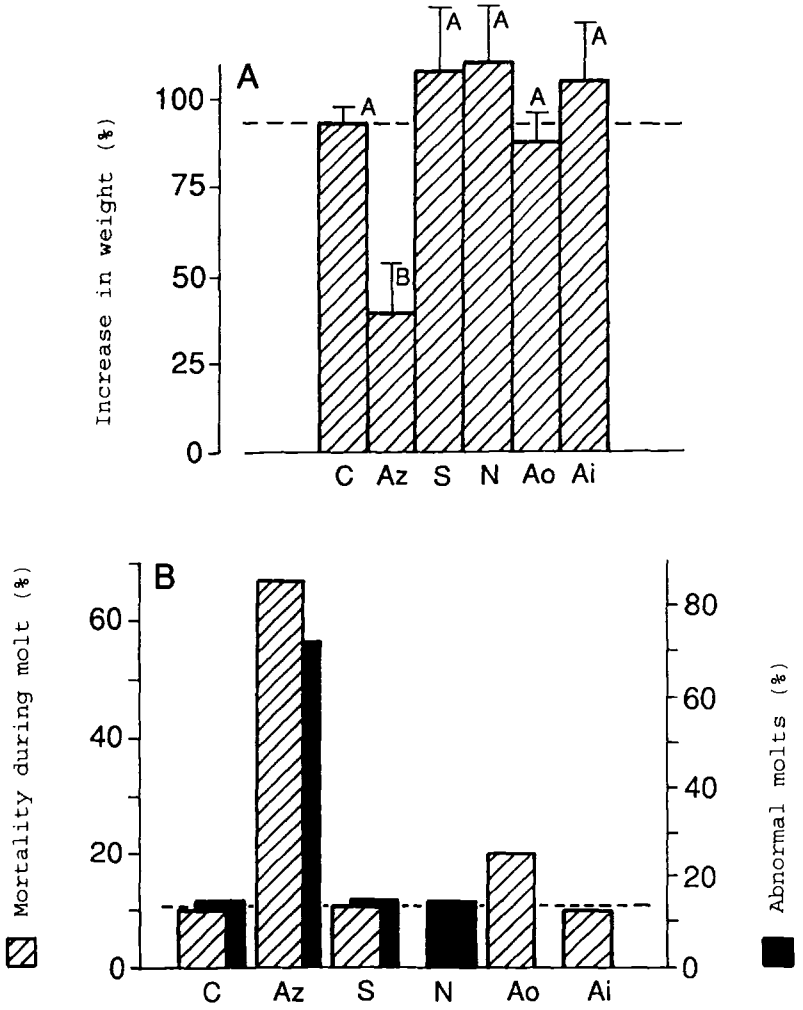
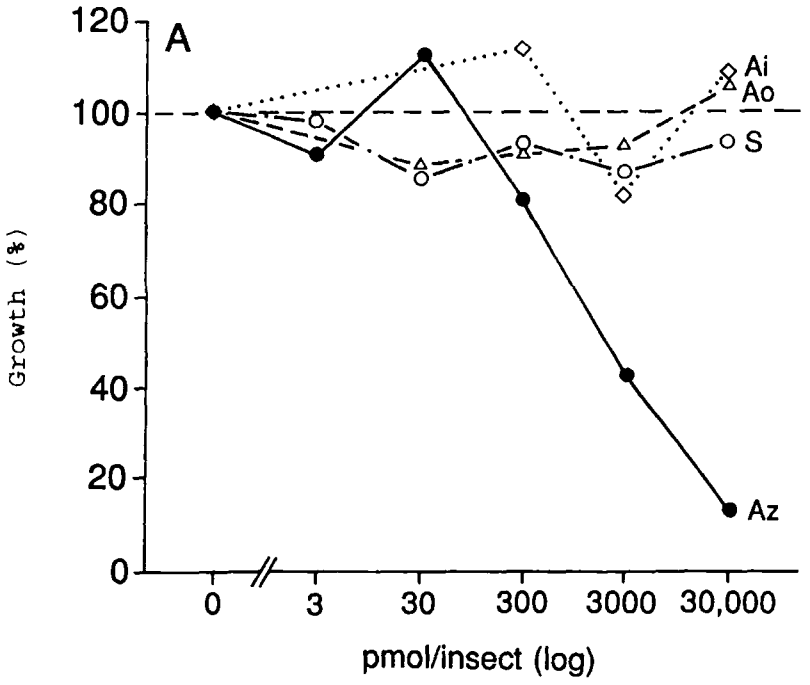
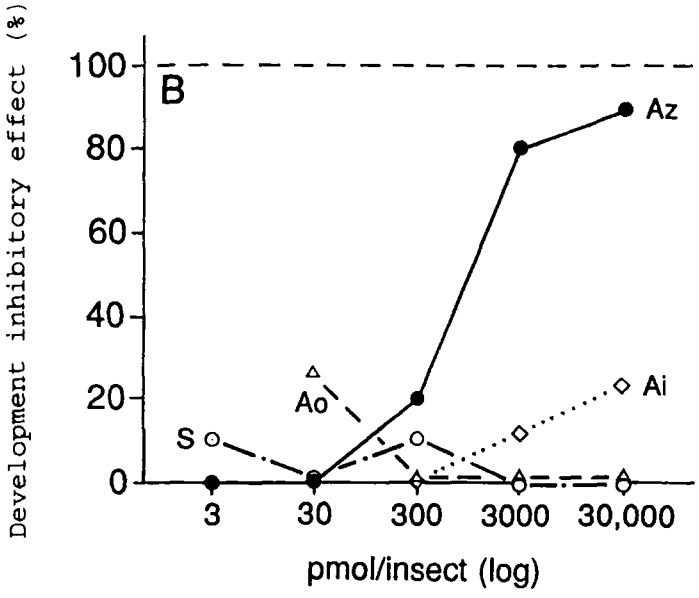


FIG. 3. Toxicity of neem triterpenoids to *Spodoptera littoralis*. Triterpenoid designations as in Figure 2: C, control. (A) Neem triterpenoids were topically applied to fifth instars (10 nmol/insect), and increases in weight (means ± SE) during transition to the next instar were determined. All treatments except azadirachtin formed a statistically homogeneous group (indicated with A above bars ($\alpha = 0.05$)), but azadirachtin treatment resulted in a significantly smaller increase in weight ($P < 0.05$) than all other treatments (indicated with B above bar). (B) Hatched bars: neem triterpenoids were injected into the hemolymph of final instars (5 nmol/insect), and mortalities during the molt were determined. Dark bars: neem triterpenoids were topically applied to final instars (25 nmol/insect), and abnormal molts were determined.


A
120
100
80
60
40
20
0
Growth (%)
Ai
Ao
S
Az
0
3
30
300
3000
30,000
pmol/insect (log)


B
100
80
60
40
20
0
Development inhibitory effect (%)
Az
Ai
Ao
S
3
30
300
3000
30,000
pmol/insect (log)

Antifeedancy of Neem Triterpenoids

Despite their lack of toxicity, several of the simpler neem triterpenoids strongly deterred feeding by *Spodoptera* larvae. Figure 5 shows antifeedant indices determined over a broad concentration range of compounds when final instars were subjected to choice tests (values near 100% mean strong preference of control material over treated, while values near 0% indicate a lack of antifeedant activity). Azadirone, from the first level of biosynthetic complexity, and nimbin, from the second level, showed antifeedant activity as strong as azadirachtin. Salannin had somewhat less deterrent activity, and the larvae displayed indifference towards azadiradione (Figure 5). Larvae ate almost only from control material in the azadirachtin, azadirone, nimbin, and salannin tests, but ate about equal amounts of control and treated material in the azadiradione tests (Figure 6).

In *Schistocerca*, azadirachtin differed in its antifeedant properties from the other neem triterpenoids. When final instars of *Schistocerca* were subjected to choice tests, they were strongly deterred by azadirachtin over a very broad concentration range (Figure 7). Salannin showed antifeedant activity down to 12 μM, but lost activity at lower concentrations, whereas compounds from the first biosynthetic level showed activity only at very high concentration (Figure 7). Thus, at concentrations lower than 12 μM, *Schistocerca* was deterred by azadirachtin alone (Figure 7). In contrast, *Spodoptera* was deterred by several neem triterpenoids in addition to azadirachtin at those low concentrations (Figure 5).

DISCUSSION

We compared the activity of neem triterpenoids of increasing structural complexity in feeding and development bioassays with insects. Table I summarizes the results.

The pattern of developmental toxic properties of neem triterpenoids was remarkably similar among the insect species, but feeding deterrency varied with species.

FIG. 4. Growth (A) and development (B) disrupting effects of neem triterpenoids to *Schistocerca gregaria*. Triterpenoid designations as in Figure 2. Second instars were topically treated with the compounds, and growth as well as deviations from normal development were monitored. The 100% growth of controls in panel A corresponds to a 2.9-fold increase in weight, and the 100% level of inhibition of development in panel B indicates that none of the nymphs became normal third instars.

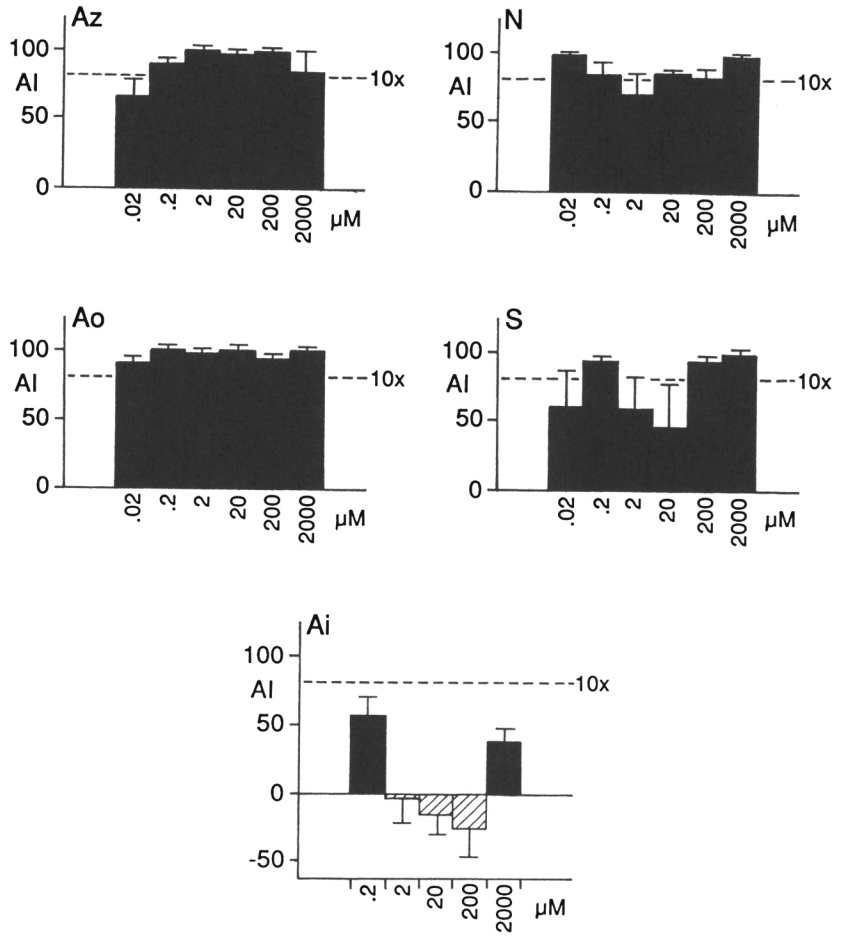


FIG. 5. Antifeedant activity of neem triterpenoids to *Spodoptera littoralis*. Triterpenoid designations as in Figure 2. Antifeedant indices (AI) were determined over a range of concentrations of neem triterpenoids in choice tests with final instars. The antifeedant index is defined as $[(C - T)/(C + T)]\%$, where C and T represent the mass eaten of the control and the treated disc, respectively. The AI is indicated at the point at which larvae would eat 10 times more from the control disc than from the treated disc (dashed line). Means ± SE are shown. Calculations with the Student-Newman-Keuls test showed that in the azadirachtin, salannin, and nimbin tests, for each compound the means from all concentrations formed a statistically homogeneous group ($\alpha = 0.05$), that in the azadirone tests the means from all concentrations except the lowest one formed a homogeneous group, and that in the azadiradione tests the means from the 2–200 μM concentrations formed a homogeneous group.

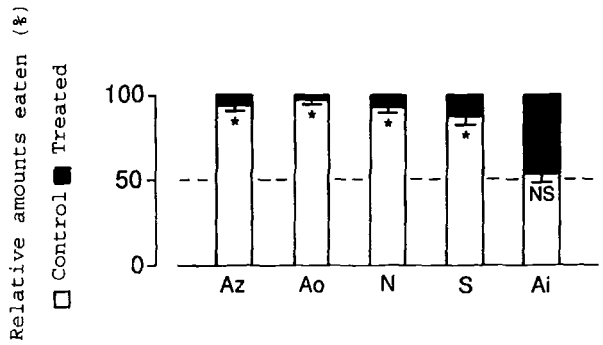


FIG. 6. Relative amounts eaten of control and treated material in choice tests with *Spodoptera littoralis*. Triterpenoid designations as in Figure 2. For each compound, data from all concentrations shown in Figure 5 were pooled. Means ± SE are shown. The statistical significance of differences between control and treated data is indicated: *, $P < 0.005$; NS, not significant ($\alpha = 0.05$) (*Wilcoxon matched-pairs tests*).

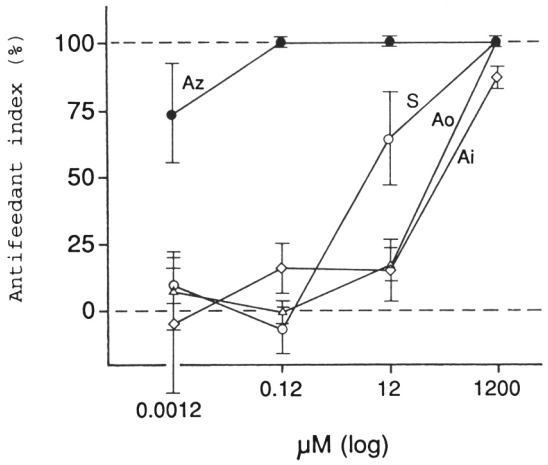


FIG. 7. Antifeedant activity of neem triterpenoids to *Schistocerca gregaria*. Triterpenoid designations as in Figure 2. Antifeedant indices were determined in choice tests with final instars. For the definition of the antifeedant index, see legend to Figure 5. Means ± SE are shown. Calculations with the Student-Newman-Keuls test showed that in the azadirachtin tests, means from all concentrations formed a statistically homogeneous group ($\alpha = 0.05$); in the salannin tests, the means from the two lowest concentrations formed a homogeneous group, and in the azadirone and azadiradione tests, for both compounds means from the three lowest concentrations formed a homogeneous group.

TABLE 1. RELATIVE TOXICITY AND ANTIFEEDANT ACTIVITY OF NEEM TRITERPENOIDS OF INCREASING COMPLEXITY

	Oncopeltus	*Spodoptera*		*Schistocerca*	
	Toxicity	Toxicity	Antifeedant activity	Toxicity	Antifeedant activity
Limonoids:					
furan ring formed					
Azadiradione	−	−	−	±	−
Azadirone	−	−	+ + +	−	−
C-*seco*-Limonoids:					
C-ring opened					
Nimbin	±	−	+ + +	ND[a]	ND
Salannin	−	−	+ +	−	±
Azadirachtin	+ + +	+ + +	+ + +	+ + +	+ + +

[a] ND: not determined.

Antifeedancy of Neem Triterpenoids. Although *Schistocerca* showed relative insensitivity to most compounds, *Spodoptera* was deterred by several of the less complex neem triterpenoids as effectively as by azadirachtin. In *Spodoptera*, strong antifeedant activity was observed with azadirone from the first level of biosynthetic complexity. There are several other examples within the Meliaceae of feeding-deterrent triterpenoids that belong to this first level of biosynthetic complexity (for example, the limonoids). *Azadirachta indica* and various *Melia* species produce compounds from the first biosynthetic level that belong to the vilasinin group, of which several are as potent antifeedants as azadirachtin in tests with *Epilachna varivestis* (Kraus et al., 1993). Although azadiradione was inactive against *Spodoptera*, this compound and its derivatives, as well as the related compound gedunin, show moderate antifeedant activity against *Popillia japonica* and *Epilachna varivestis* (Schwinger, 1985). Khivorin, produced in *Khaya nyasica* is as effective as azadirachtin in antifeedancy tests against *Epilachna* (Kraus et al., 1993), and it appears to be the only antifeedant present in *K. nyasica* (Keller, 1988).

The antifeedancy of compounds is continued at the second level of structural complexity. *Spodoptera* was deterred by both nimbin and salannin (this study and Blaney et al., 1990). Considerable to strong antifeedant activity of nimbin and nimbin-type compounds and of salannin and analogs has been observed also in numerous other insect species (Warthen et al., 1978; Warthen, 1979; Schwinger, 1985; Kraus et al., 1993).

Strong deterrence by azadirachtin has been shown in many insect species (Mordue (Luntz) and Blackwell, 1993) and was also observed in our studies with *Spodoptera* and *Schistocerca*. Thus, antifeedancy seems to be a common characteristic of triterpenoids from diverse levels of structural complexity along the pathway to azadirachtin.

Toxicity of Neem Triterpenoids. In contrast to antifeedancy, there appears to be discontinuity in the toxic properties of the triterpenoids along the pathway. As expected, azadirachtin showed strong toxic effects in *Oncopeltus*, *Spodoptera*, and *Schistocerca*, predominantly disrupting the progression of the insects through the next molt. In all three insects, all compounds from the two lower levels of biosynthetic complexity had no, or hardly any toxic action. None were able to radically disrupt development of the insects as azadirachtin did. Low toxicity of salannin has been reported also in *Spodoptera frugiperda*, *Heliothis virescens*, *Epilachna varivestis*, and *Locusta migratoria* (Rembold, 1989b; Simmonds et al., 1990; unpublished results A. J. Mordue (Luntz)). Based on preliminary experiments, Schwinger (1985) concluded that toxicity of the neem triterpenoids is confined to azadirachtin and its derivatives.

Observations described here indicate that compounds from the two lower biosynthetic levels primarily have antifeedant activity, whereas the more complex compound azadirachtin possesses a combination of antifeedant and toxic properties. Antifeedancy may be insufficient for chemical defense of neem trees, and the combination of toxicity and antifeedancy of azadirachtin provides more protection. Antifeedancy may be a relatively weak defense, since it has been shown that in a no-choice situation insects may eat substantial amounts of deterrent compounds (Blaney et al., 1990). Moreover, insects can rapidly habituate to compounds that are antifeedant at first, but otherwise harmless (Bernays and Graham, 1988).

Antifeedancy versus Toxicity in Spodoptera. Our experiments show that with respect to *Spodoptera*, neem triterpenoids can be antifeedant without having any appreciable toxicity. There are other examples of insect species that are deterred by plant natural products that cause no toxicity. Grasshoppers are deterred by the alkaloid gramine, condensed tannins, and cyanogenic glycosides, but no deleterious effects in the insects were observed after ingestion of these compounds (Boys, 1981; Bernays et al., 1981; Bernays, 1983). Likewise, plant secondary compounds deterrent to the graminivorous *Locusta migratoria* and to the specialist coleopteran *Hypera brunneipennis* caused no postingestion toxic effects (Bernays, 1990; Bernays and Cornelius, 1992). Cucurbitacins, cardenolides, and alkaloids that deterred larvae of *Pieris rapae* had no adverse effects on these insects (Usher and Feeny, 1983). As discussed by Bernays and Chapman (1987) and Bernays and Graham (1988), an explanation for such poor deterrence–toxicity relationships may be that the compounds function mainly as signals for insects to distinguish between host and nonhost plants. The choice

of the right host may be of paramount importance in terms of food, mate-finding, and protection against predators and parasitoids.

Changes in Antiinsect Activity Along the Biosynthetic Pathway. In coumarin biosynthesis in the family Apiaceae (Umbelliferae), stepwise changes in coumarin structure have been shown to yield compounds with different modes of antiinsect action and concomitant efficacy in the chemical defense of umbellifers (Berenbaum, 1983). Our data suggest that the antiinsect activity of neem triterpenoids also changes along the biosynthetic pathway, and this change in properties of compounds may point to the adaptive flexibility of the chemical defense of the neem tree.

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ODOR PREFERENCES OF WEANLING AND MATURE MALE AND FEMALE PINE VOLES

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Abstract—Olfactory cues from conspecifics have strong influences on behavior. Both female and male juveniles are attracted to familiar odors while adults show preferences for odors of opposite-sex conspecifics. Females show an increased preference for male odors as they mature, but no study has examined whether change with age is seen in males. In this experiment, responses of weanling and mature male and female pine voles (*Microtus pinetorum*) to familiar odor cues from their family versus those of an unfamiliar, opposite-sex conspecific were examined. Voles were tested in a three-chambered cage with end chambers containing the stimuli. There was a significant difference in odor preference between weanling and mature females. Weanling females preferred odor cues from their families, and mature females preferred the odors of unfamiliar mature males. The pattern was similar in males but the effect was not as strong. The results support the hypothesis that responses to odor cues change with age in pine voles. At least for females, the change in response to odor cues may be involved in the timing of reproduction and thus dispersal.

Key Words—Vole, rodent, olfactory preferences, conspecific odors, sex differences, age effects, sexual maturation, reproductive suppression, dispersal.

INTRODUCTION

Olfactory cues have strong influences on the behavior of many small mammals, particularly rodents. Odors may be involved in communicating information about reproductive condition or dominance status, as well as in mate choice, individual recognition, spacing, or defense (Richmond and Conway, 1969;

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Mykytowycz, 1974; Richmond and Stehn, 1976; Johnston, 1980; Brown and Macdonald, 1985; Ferkin, 1989; Hurst, 1989; Ferkin and Rutka, 1990; Drickamer, 1992a). Many rodents mark their territories with odoriferous compounds from urine, feces, or scent glands (for review see Halpin, 1986). These scent marks may function as signals to conspecifics of territorial boundaries or as orientation cues, allowing animals to recognize their home area (Mykytowycz, 1974; Bronson, 1976; Hurst, 1987). In either case, individuals respond differentially to olfactory cues from their own social group as compared to those from conspecifics (Brown, 1979; Halpin, 1986), and preferences for odors of one's own group versus conspecifics can change with age. Juvenile house mice (*Mus musculus*) may use scent marks deposited by their parents to remain within their natal territory and are extremely cautious when they approach odors from unfamiliar conspecifics (Hurst, 1987). Similarly, male and female meadow voles (*Microtus pennsylvanicus*) spend more time with odors from a familiar than with an unfamiliar adult (Ferkin, 1989).

The attraction of juveniles to familiar odors may function to keep them close to natal nests, within the safety of their own territory (Hurst and Nevison 1994). Dispersal by subadults occurs in a number of rodent species, and olfactory cues may be involved in dispersal (emigration and immigration). Hurst (1987) has postulated that loss of the aversive response to conspecific odors may be a factor involved in emigration of subadult mice. This hypothesis predicts that odors from unfamiliar opposite-sex conspecifics should be avoided and family odors should attract weanlings.

Previous studies with house mice show that attraction to stimuli from males changes as females mature (Coppola and O'Connell, 1988; Drickamer, 1989a). Prepubertal female house mice avoid male odors but, after puberty, are attracted to them (Drickamer, 1989a). Drickamer (1989a, b, 1992b) and Coppola and O'Connell (1988) have suggested that this change in odor preference allows females to control the degree to which they are exposed to puberty-accelerating stimuli. Although female pine voles (*Microtus pinetorum*) are not reproductively activated by olfactory stimuli alone, olfactory cues, in conjunction with physical contact with an unfamiliar male, are needed to activate reproduction (Solomon et al., 1996). Therefore, the changing preferences for olfactory stimuli from family versus those from opposite-sex conspecifics also may regulate reproduction in female pine voles.

The same pattern is seen in males as in females. Adult, but not juvenile, males appear to be attracted to odors from females. Juvenile male house mice do not spend much time investigating odors from unfamiliar breeding females (Hurst, 1990), but adult meadow and prairie voles (*M. ochrogaster*) prefer urine, feces, and anogenital odors from females to those of males (Ferkin et al., 1994; Ferkin and Johnston, 1995). Although these data are suggestive, no previous study has examined whether or not odor preferences of males change with age.

This experiment was designed to test the hypothesis that olfactory stimuli from the family versus those from an opposite-sex conspecific differ in their attractiveness to female and male pine voles and that these preferences change with age. The hypothesis predicts that family odors should be preferred by weanlings as compared to odors from an unfamiliar opposite-sex conspecific. As individuals get older, the cost of delayed reproduction increases (Creel and Waser, 1997). If the only way to reproduce is to disperse and establish an independent nest, then older individuals should prefer cues from opposite-sex conspecifics to those of family members. We expected that mature males and females would display strong preferences for cues from unfamiliar, opposite-sex conspecifics.

METHODS AND MATERIALS

Voles used in this experiment were descendants of animals from the North Carolina State University colony and a small number of animals from an apple orchard in Henderson County, North Carolina. Food (Purina Rodent Breeder chow) and water were provided ad libitum. Animals were maintained on a 14L:10D schedule in a climate-controlled environment with a temperature of 25 ± 3°C. Lights came on at 06:00 hr.

Breeding pairs were housed in a 36 × 30 × 18-cm plastic cages with paper bedding (Cell Sorb, A & W Products) and cotton (Nestlets, Ancare Corp.) for nesting material. Female and male pine voles do not breed prior to 56–84 or 42–56 days of age, respectively (Schadler and Butterstein, 1979; Lepri and Vandenbergh, 1986). Therefore, offspring were removed from their home cages for testing before or after the earliest age of reproduction, i.e., when they were either 24–27 (N = 14 females and 16 males) or 95–109 (N = 16 females and 16 males) days of age. In the latter case, where offspring remained with their families beyond 28 days of age, all pups from subsequent litters were removed when they reached 24–28 days of age. All test subjects were sexually naive; pine voles do not mate with family members even if left in the same cage past weaning (Solomon and Vandenbergh, 1994).

Test subjects were removed from their family cage at the previously mentioned age and placed in a choice apparatus for 48 hr. The choice apparatus consisted of a Plexiglas box (61 × 22 × 20 cm) with a screen-wire lid and was divided into three equal-sized compartments, separated by removable Plexiglas partitions. Each partition had a hole, 2.2 cm in diameter, allowing the test subject access to all chambers. Food and water were located in the central (neutral) chamber. Equal amounts of nest material were placed in the two stimulus (end) chambers.

One stimulus chamber contained the quantity of bedding that filled a

250-ml beaker from an unfamiliar, sexually inexperienced, mature, opposite-sex conspecific. Stimulus animals were housed alone in 18 × 29 × 12.5-cm cages with ad libitum food and water in an adjacent room under identical environmental conditions to those provided for test subjects. Cages of stimulus animals were bedded with ground corn cob and provided with a Nestlet for nest material. The stimulus animal's cage was cleaned one week prior to the test so odor cues would accumulate in the bedding. The other stimulus chamber contained an equivalent amount of bedding from the test subject's family. Family cages also were cleaned one week prior to testing and bedded with ground corn cob. The central (neutral) chamber contained clean bedding.

In the test apparatus, the side on which odors were located was alternated to avoid position effects (preference for either the right or left chamber irrespective of odor source). After each trial, the choice apparatus was cleaned with unscented detergent (Quatricide 20, Pharmacal Research Labs) and water.

Male and female test subjects were given simultaneous access to odor sources from their family and an unfamiliar, sexually inexperienced, mature opposite-sex conspecific to determine whether they would preferentially nest with one stimulus odor. Tests began when the test subject was gently placed in the central chamber. Preference was measured by location of the nest and time spent with each stimulus. The nest location was examined after 48 hr because, in a previous study with prairie voles, Solomon (1993) concluded that short tests were insufficient to detect a preference using data from nest location. A clear choice was defined as a vole moving nest material to only one chamber (Solomon, 1993). In addition, a time-lapse video camera was used to record the amount of time spent in each chamber during the first 24 hr of the test period. Two red lights (25 W) were used to illuminate the test apparatus during the dark portion of the cycle so filming could continue uninterrupted for 24 hr.

Time spent with odor sources during the first 15 min of the test was analyzed to test Hurst and Nevison's (1994) hypothesis that a response to an unfamiliar odor may be a response to novelty. If this is the case, then we would expect to see a preference for the novel odor during the first 15 min of the test.

Time spent by weanling and mature voles with each stimulus source during the first 15 min and 24 hr of the test was analyzed with two-tailed Mann-Whitney U tests. The proportion of voles of each age group that built nests in each of the two stimulus chambers were compared with a Fisher exact test, with the expectation that there would be a 50:50 distribution of nests in the two end chambers. The possibility of position effects was examined for each age group with binomial tests (Howell, 1982).

RESULTS

There was a significant difference in the time spent with family versus male bedding in weanling as compared to mature females after 24 hr ($z = 2.45$,

$P = 0.0142$, $N = 14$ weanling and 16 mature females). During the 24-hr test, weanling females spent more time with family odors than male odors, and mature females spent much more time with male than family odors (Figure 1a). Females did not show position effects; neither weanling nor mature females displayed a preference for the right or left chambers ($P = 0.1796$ and $P = 0.8036$, respectively). After 48 hr, there was a statistically significant difference in nest location between weanling and mature females (Fisher exact test, $P = 0.0023$; Table 1). Weanling females built nests in family-scented bedding while mature females built their nests on the side with male odors.

Neither weanling nor mature females showed a significant preference for the novel (male) odor as compared to family odor during the first 15 min of the test ($z = 1.54$, $P = 0.1241$, and $z = 1.60$, $P = 0.1093$, respectively). Weanling females spent 5.86 ± 1.35 min and 2.34 ± 0.81 min (mean $\pm$ SE) with the male and family odors, respectively. Mature females spent 5.31 ± 0.96 min and 3.03 ± 0.65 min with the male and family odors, respectively.

Males showed the same pattern as females, although the effect was not as strong; weanling and mature males differed in the time spent with odor sources during a 24-hr test, but the difference was of borderline statistical significance ($z = 1.92$, $P = 0.0546$, $N = 16$ weanling and mature males; Figure 1b). Males did not show position effects; neither weanling nor mature males displayed a preference for the right or left chambers ($P > 0.95$ and $P = 0.4545$, respectively). There was no difference in the side in which males of different age classes built nests after 48 hr (Fisher exact tests, $P = 0.6765$ and $P = 0.5279$, respectively; Table 1).

Neither weanling nor mature males showed a significant preference for the novel (female) odor as compared to family odor during the first 15 min of the test ($z = 1.29$, $P = 0.1977$ and $z = 1.79$, $P = 0.0736$, respectively). Weanling males spent 6.35 ± 1.46 min and 3.47 ± 1.06 min with the female and family odors, respectively. Mature males spent 4.80 ± 0.88 and 2.62 ± 0.58 min with the female and family odors, respectively.

Odor preferences of males and females followed the same pattern within age classes. There was no significant difference in time spent with the odor of a member of the opposite sex versus family in weanlings ($z = 0.125$, $P = 0.9008$) or mature ($z = 0.339$, $P = 0.7345$) individuals.

DISCUSSION

The results from this experiment show that weanling female pine voles were more attracted to family odors than to male odors. In contrast, mature females spent significantly more time with male odors than family odors. The nest locations after 48 hr also show the same pattern. Although not all females built nests in one of these two chambers, more weanling females built nests in family bedding, and more mature females built nests in male bedding.

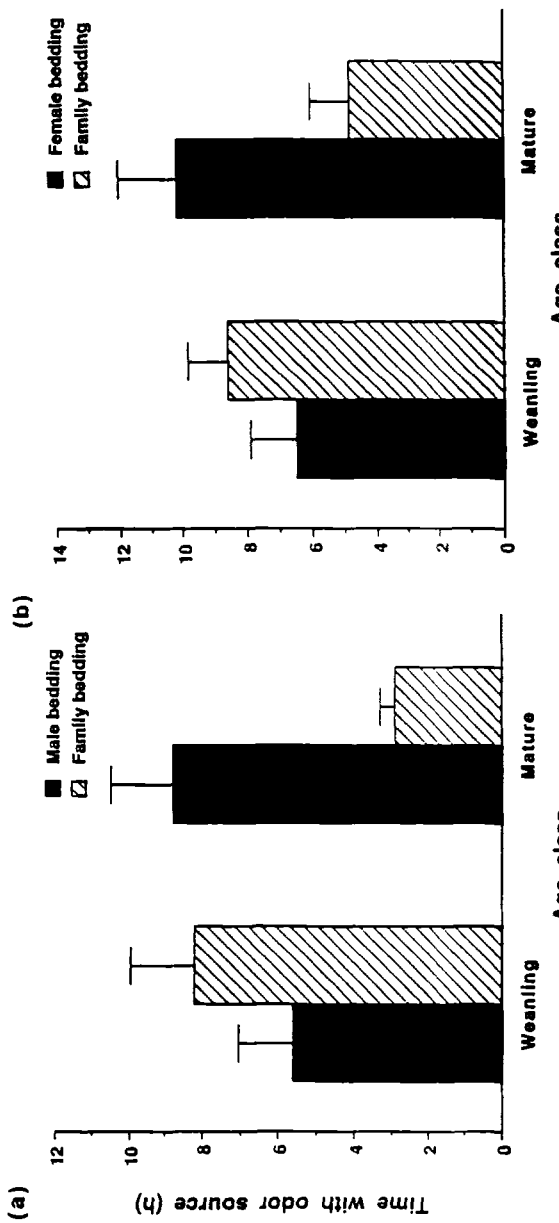


FIG. 1. Time spent (hr) by weanling and mature female (a) and male (b) pine voles with olfactory cues from an unfamiliar, mature male (or female) versus those from their family. Test subjects were housed with their family until the test began. For females, there was a significant difference between age classes ($P = 0.01$) but for males, the effect was borderline ($P = 0.05$). There were 14 and 16 weanling and mature females, respectively, and 16 males of each age class.

TABLE 1. NUMBER OF VOLES BUILDING NESTS IN EACH OF TWO END CHAMBERS IN TEST APPARATUS[a] AFTER 48 HOURS

Odors from	Opposite-sex	Family	*P*
Weanling female (N = 14)	0	6	0.0023
Mature female (N = 16)	7	1	
Weanling male (N = 15)	4	3	0.5279
Mature male (N = 16)	7	6	

[a]One end chamber contained bedding soiled by an opposite-sex conspecific and the other by the individual's family.

Previous studies with house mice show that attraction to stimuli from males changes as females mature; this may allow females to control their reproductive physiology (Coppola and O'Connell, 1988; Drickamer, 1989a, b, 1992b). The mechanism for this control may be that the motivational state of females changes with age (Drickamer, 1989a). Young females may prefer to spend time with familiar cues associated with maternal care, and mature females may be sexually motivated. The change in preference may be a developmental response associated with changes in maturation of the neuroendocrine system (but see Holekamp, 1986).

In some species, such as cotton-top tamarins (*Saguinus oedipus*), levels of reproductive hormones increase with age even in females that remain in their family group (Tardif, 1984; Ziegler et al., 1987). Additionally, Creel and Waser (1990) have noted that it is more difficult to suppress reproduction in female dwarf mongooses (*Helogale parvula*) as they get older. In female pine voles, the change observed in odor preference may reflect a maturational change that is associated with sexual motivation. If suppression of reproduction in female pine voles becomes more difficult to maintain as females get older, these older females that are "released" from suppression would be expected to show preferences for odors of opposite-sex individuals. Studies are currently in progress to determine if the degree of reproductive suppression differs in female pine voles of different ages.

Olfactory cues in conjunction with physical contact with an unfamiliar, opposite-sex conspecific are needed to activate reproduction in female pine voles (Solomon et al., 1996). Therefore, the changing preferences for olfactory stimuli from family versus those from opposite-sex conspecifics may regulate reproduction. Preferences for opposite-sex conspecifics, i.e., potential males, may trigger or facilitate reproductive dispersal, and these olfactory cues may couple sexual motivation to dispersal. Hurst (1987) has postulated that loss of the

aversive response to conspecific odors may be a factor involved in emigration of subadult mice.

The results of this experiment suggest that young females remain philopatric, and disperse only when mature. The attraction of juvenile females to familiar odors may keep them close to natal nests, within the safety of their natal territory (Hurst and Nevison, 1994), and delay dispersal. When mature, the preference by females for family odors wanes. This lack of preference for family odors suggests that dispersal may be the preferred option at this age. Coppola and O'Connell (1988) suggested a similar function of changing attraction to grouped female versus male odor cues in house mice. There are few field data to address this prediction for pine voles, but the data that exist indicate that 94% of dispersing females may have switched nests as adults. Sixteen of 17 females were caught at a new nest when they were classified as adults in terms of body mass and pelage (Solomon and Vandenbergh, unpublished observations).

Male pine voles showed a pattern similar to females. The preferential attraction of mature males to odor cues from unfamiliar adult females was similar to, although not as strong as, the preference of mature females for male odors. Male preferences could not be detected by examining the locations of nests after 48 hr. In contrast to the results for female pine voles, the pattern of nest location was not indicative of where males spent their time.

Although mature males spent about the same amount of time with odors from opposite-sex conspecifics as did mature females, the relative preference (expressed as the difference between time spent with odors from the opposite-sex versus family) was not as strong. This result may reflect a difference in the mechanism of reproductive suppression in males and females. In Damaraland mole rats, *Cryptomys damarensis*, nonbreeding males appear to be physiologically similar to breeding males, although physiological differences are found in breeding versus nonbreeding females (Bennett, 1994). Additionally, Kerr (1994) did not find a difference in the degree of maturity of gametes in male pine voles housed with family members as compared to those isolated from their family. If, as it appears, males are not physiologically suppressed by the presence of family members, they may not show avoidance of odors from their family when they are mature.

Mature males and females preferred the novel odor of an unfamiliar opposite-sex conspecific to the odor of the family with which they had been living. These voles had been exposed to family odors for approximately 50 more days than were weanlings, yet the increased experience did not result in a preference for these odors. Although one possible explanation for these results is that the mature voles were habituated to familiar odors, neither males nor females spent more time with either familiar or novel odors during the first 15 min of the test. Thus, the lack of a short-term preference for the novel odor during the first

15 min indicates that preferences seen in this experiment are long-term choices instead of short-term responses to novelty, as proposed by Hurst and Nevison (1994).

In the current study, we were able to control the amount of bedding provided to test subjects but could not control the quantity of odors in the bedding. In particular, bedding from the family may have contained a larger quantity of odor cues because there were more animals in the family cage. The cages containing family members were larger than those that housed the opposite-sex stimulus animal in an attempt to control the quantity of odors to some extent. The bedding from the family cage did not always look more soiled than the bedding from the stimulus vole. Even if there was a difference in the concentration of odors in the two types of bedding, odor concentration per se was unlikely to have been the factor that determined preference because we found that the mature voles spent more time with the potentially less concentrated odors.

Changes in attraction to odor cues from different types of conspecifics may be a widespread response in rodents that influences reproduction and thus dispersal in males and females. A developmental change may be the mechanism that results in the change in preference for stimuli from members of the opposite sex as compared to those from the family in female and male pine voles. This preference for sexually related stimuli over those from the family may be the stimulus that induces emigration of mature individuals from their natal nests. Studies using other rodent species are needed to test the generality of these responses.

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OPTIMIZATION OF PHEROMONE DISPENSERS FOR DIAMONDBACK MOTH *Plutella xylostella*

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Abstract—We have investigated the effects of pheromone dispenser design, pheromone release rate, and the products of Z11-16:Ald decomposition on the attractiveness of the pheromone blend (Z)-11-hexadecenyl acetate, (Z)-11-hexadecenal, and (Z)-11-hexadecen-1-ol) in traps to *Plutella xylostella* males. Rubber minidispensers (K-50) were shown to have an active exposure time of at least two months and in delta traps to be capable of monitoring a population of *P. xylostella* throughout the summer in Estonia. Pheromone release rates between 8 and 17 ng/hr are recommended for maximum trap catches. The attractive blend contained 15–35% of Z11-16:Ac. Decomposition products of Z11-16:Ald inhibited the activity of the pheromone blend when more than 50% of the aldehyde had decomposed.

Key Words—Lepidoptera, Plutellidae, (Z)-11-hexadecenyl acetate, (Z)-11-hexadecenal, *Plutella xylostella* L., diamondback moth, sex pheromone, dispenser, cabbage, pheromone evaporation rate.

INTRODUCTION

P. xylostella L. (Lepidoptera: Plutellidae), the diamondback moth, a worldwide pest of cruciferous crops has, in recent years, caused economic damage to cabbage and some other cruciferous plants, such as oilseed rape, in Estonia. Measures to control the pest are needed. Pheromone methods could be used to monitor the moths and indicate the need for control. A local population of *P. xylostella* appears to occur, at relatively low densities, in Estonia and adjacent

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areas of Russia (Kopvillem, 1965; Makarova et al., 1990). There is one generation per year. How it overwinters successfully in temperatures below 0°C for five to six months (November–April) is not clear but *P. xylostella* appears to overwinter in Canada and Aomori (Hokkaido, Japan), where snowcover lasts for up to three months (Dosdall, 1994; Kimura, 1987). In Estonia, pheromone traps usually start catching male *P. xylostella* in early June, when cabbage plants are transplanted to fields. Normally population densities are low during early growth of cruciferous plants, and the moths cause only superficial damage to leaves of cabbage. Maximum densities occur in early August. Occasionally, perhaps due to long-distance migration, the moths reach outbreak densities and cause extensive crop damage. In Estonia such outbreaks can occur each decade (French and White, 1960; Kopvillem, 1965).

Female moths produce a pheromone that attracts males, and Tamaki et al. (1977) identified (*Z*)-11-hexadecenyl acetate (Z11-16:Ac) and (*Z*)-11-hexadecenal (Z11-16:Ald) as the main active components. Koshihara and Yamada (1980) found that the attractiveness of mixtures of Z11-16:Ac and Z11-16:Ald to males was synergized by (*Z*)-11-hexadecen-1-ol (Z11-16:OH).

Different research groups have used different ratios of the main pheromone components during tests or in pheromone monitoring. In Taiwan, Chow et al. (1977) demonstrated that in field bioassays with doses of 10 μg/dispenser, the best Z11-16:Ac to Z11-16:Ald ratio (Ac/Ald ratio) was 1:1 to 3:1. Blend ratios of 8:2 to 6:4 Ac/Ald had different activities in Taiwan depending upon climatic conditions. Blends of 6:4 and 5:5 lured more male moths (Maa, 1987). A 3:7 Ac/Ald mixture was used in field tests in Canada (Chisholm et al., 1983), whereas a 1:1 mixture was used in the United Kingdom (Macaulay et al., 1986). Our experiments in Siberia showed that mixtures consisting of more than 50% of Z11-16:Ald were less active than the 1:1 mixture (Krivokhizhin et al., 1985). In field tests the amount of pheromone blend used per dispenser has varied from 10 to 100 μg (Koshihara and Yamada, 1980; Chisholm, 1983; Chen et al., 1990; Macaulay et al., 1986). On rubber dispensers the pheromone has a rather short effective period and replacement is recommended every 15–21 days (Liu et al., 1990; Chisholm et al., 1983). In laboratory experiments, male moths responded optimally to a narrow range of active blend concentrations (Kawasaki, 1984).

The influence of pheromone dispenser design, pheromone release rate, and decomposition products of Z11-16:Ald on the attractiveness of pheromone baited traps to *P. xylostella* males in Estonia is the subject of this report.

METHODS AND MATERIALS

Chemicals. A 1:1:0.01 mixture of Z11-16:Ac, Z11-16:Ald, and Z11-16:OH was used as the attractant. The chemicals were purchased from KV

Flora, Tartu, Estonia, and purified by distillation or column chromatography. The *cis/trans* purity of the compounds was >97%. The ratio of components in blends was confirmed by gas chromatography (column OV-1, 0.25 mm × 25 m).

Dispensers. Dispensers were prepared from red natural rubber. Cylindrical Feroflor (F-1000, F-500, and F-50) dispensers from Flora (outside diameter 8.6 mm, inner diameter 5 mm, length 18 mm) were used as standard. Smaller dispensers were disks cut from Feroflor (S-20) or segments of disks (K-50). Details of the dispensers are given in Table 1. Dispensers were impregnated with the chemicals in a rotary evaporator with a mixture of hexane and toluene as solvent. Impregnated dispensers were stored for at least five days before field tests.

Determination of Evaporation Rate from Dispensers. Evaporation rates for Z11-16:Ac and Z11-16:Ald from F-1000 dispensers were measured in the laboratory at different temperatures and air speeds. The amount of pheromone components in dispensers was determined by extraction after chopping the dispensers into 80-mg pieces and extraction for 24 hr with 2 ml of diethyl ether. Tridecyl acetate was used as an internal standard, and the extracts were analyzed by GC on an OV-1 capillary column (Vilegzhanina et al., 1984).

The evaporation rate of Z11-16:Ac and Z11-16:Ald from dispenser K-50 in traps in the field exposed for different lengths of time was determined by extraction of the remaining pheromonal components and their determination by GC, as noted above.

Calculation of Evaporation Rate from Dispensers. The pheromone components are released from the dispensers by a first-order evaporation loss (McDonough and Butler, 1983; Môttus et al., 1993). The integral form of the first-order process is

$$c_t = c_0 \times e^{-kt} \tag{1}$$

TABLE 1. MAIN CHARACTERISTICS OF DISPENSERS USED IN FIELD EXPERIMENTS

Dispenser type	Attractant amount (μg)	Dispenser weight (mg)	Surface factor (s_f) (mm^2/mg)	Evaporation rate constant	
				Z11-16:Ald/day	Z11-16:Ac/day
F-1000	1000	800	1.2	1.0×10^{-2}	2.0×10^{-3}
F-500	500	400	1.35	1.1×10^{-2}	2.25×10^{-3}
F-50	50	800	1.20	1.0×10^{-2}	2.0×10^{-1}
K-50	50	40	1.8	1.52×10^{-2}	2.6×10^{-3}
S-20	20	160	1.5	1.27×10^{-2}	2.5×10^{-3}

where c_0 is the amount of compound at the beginning of evaporation, c_t is the amount of compound present at time t in the dispenser, and k is the evaporation rate constant. Constant k characterizes the evaporating compound, but is dependent on the temperature, wind speed, and surface structure of the dispenser. Half-life time ($t_{1/2}$) is the time needed for evaporation of half of the chemical loaded into the dispenser. The value of $t_{1/2}$ may be calculated from the formula

$$t_{1/2} = \ln 2 \times k^{-1} \tag{2}$$

Values of k for many pheromone components have been published (McDonough and Butler, 1983; McDonough et al., 1989; Môttus et al., 1993). The value of k is not dependent on concentration of pheromone in the dispenser, but is dependent on the surface area factor s_f, where

$$s_f = S/V \tag{3}$$

In this case S is the surface area of the dispenser and V is the volume of the dispenser. Equation 3 shows that the evaporation process is limited by the surface area of the dispenser. The specific gravity of different rubber septa dispensers is close to 1.0, and in our experiments we used

$$s(f) = S/G \tag{3a}$$

instead of formula (3).

In formula 3a, G is the weight of the dispenser in milligrams. Evaporation constants measured for dispensers having $s(f)$ equal to 1 may be used as a standard (K_{st}). Evaporation rate constants for dispensers with different surface constants may be calculated by equation 4.

$$k(s) = k(st) \times s(f) \tag{4}$$

Equation 4 is correct in cases where evaporation of pheromones is not limited by diffusion processes. Our experiments showed that this assumption is correct if s_f does not differ very greatly (Môttus et al., 1996).

Decomposition of Z11-16:Ald. Light from a medical UV lamp (125 W) at room temperature was used to irradiate weighted amounts of Z11-16:Ald (about 200 mg) for 1–2 hr/day in air in a small quartz test tube until the required proportion (30% or 50%) had decomposed. Decomposition was followed by CG. The mixture of decomposed products and residual Z11-16:Ald was used to prepare the K-20D and K-50D dispensers; these consisted of 20 or 50 μg of 1:1 Ac/Ald, respectively, plus decomposition products or 3 μg (S-20) or 12 μg (K-50) of Z11-16:Ald.

Field Tests. The series of eight field tests were all conducted in fields of cabbage in Jõgeva or near Tartu in eastern Estonia during 1993–1995. Plastic delta traps (Atrakon AA, produced by KV Flora, Tartu) with a sticky base area

of 310 cm^2 were placed at a height of 0.6 m, with 8–12 m between traps or from the edge of the field. Five replicates of each dispenser were tested.

Statistical Analyses. The numbers of moths caught in the traps were subjected to Student's t test or to one-way ANOVA to determine the statistical significance between the mean values. Calculations were performed by using Origin (version 3.0) or Microsta programs. The program Origin was used for linear regression analysis of evaporation rate constants.

RESULTS

Effect of Attractant Dose on Attractivity. In all four field tests comparing dispenser design (F, S, or K) and attractant dose (20, 50, and 1000 μg), K-50 dispensers attracted more males than the other designs or loadings. K-50 and F-50 dispensers attracted significantly more ($P < 0.05$) males per trap per day than F-1000 dispensers over 40 days (Figure 1). K-50 dispensers also attracted

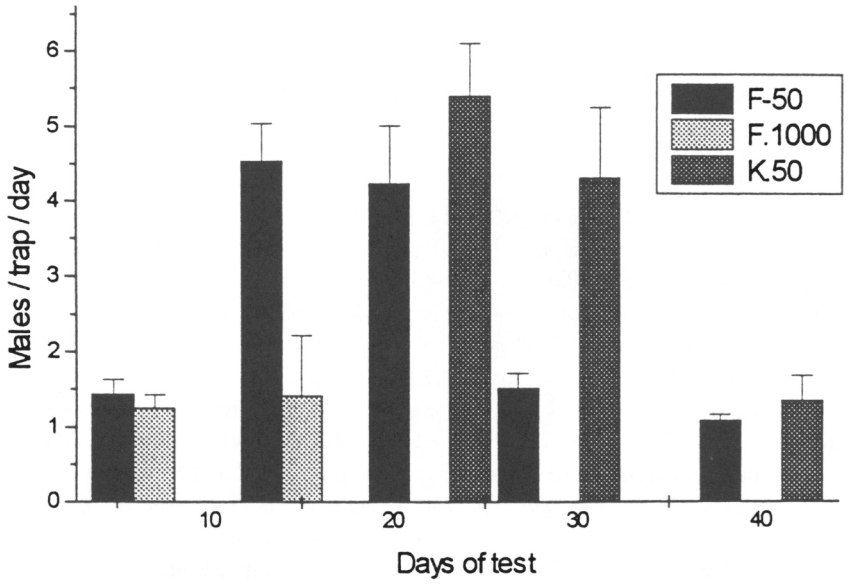


FIG. 1. Mean numbers of male *Plutella xylostella* caught by traps ($N = 5$ per treatment) baited with Feroflor dispensers (F = cylinder, K = segment) loaded with 50 μg or 1000 μg pheromone (1:1:0.01 of Z11-16:Ac, Z11-16:Ald, Z11-16:OH) in field tests at Jõgeva started on June 16, 1993. Traps baited with F-1000 dispensers were replaced by new traps baited with K-50 dispensers on June 30. Traps with K-50 dispensers caught significantly more ($P < 0.05$) than other traps.

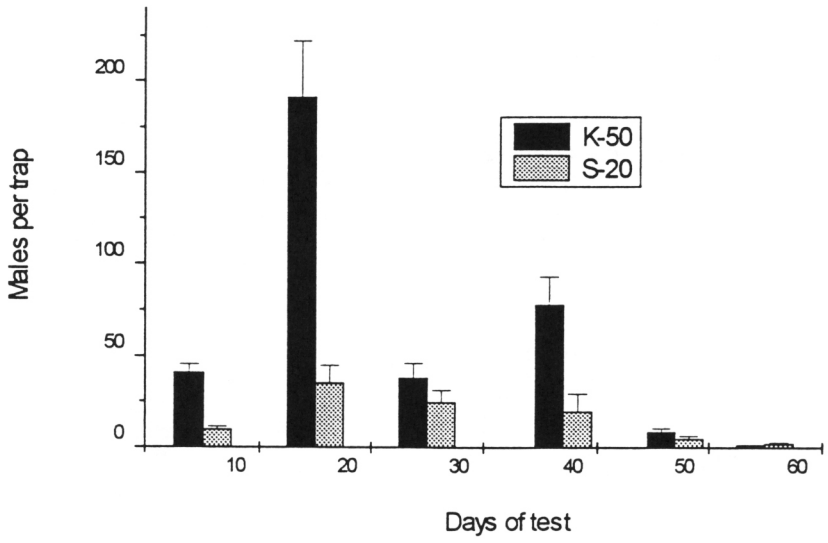


FIG. 2. Mean numbers of male *Plutella xylostella* caught by traps ($N = 5$ per treatment) baited with Feroflor dispensers (K = segment, F = cylinder) loaded with 20 or 50 μg pheromone (1:1:0.01 of Z11-16:Ac, Z11-16:Ald, Z11-16:OH) in field tests near Tartu started on June 14, 1994. Differences for days 1–20 and 30–40 are significant ($P > 0.05$).

more males than S-20 dispensers for the first 40 days of a 60-day trial (Figure 2), more than F-500 dispensers for 60 days (Figure 3), and more than S-20 dispensers for the first 55 days of a 62-day trial (Table 2).

Effect of Exposure Time on Dispenser Attractivity. In two experiments, K-50 dispensers retained their attractivity over a period of about two months of exposure. K-50 dispensers put out at the beginning of a trial attracted as many males over a 61-day period as K-50 dispensers replaced at least every two weeks (Table 3), and K-50 dispensers already exposed for 45 days continued to attract as many males as fresh K-50 dispensers for an additional 30 days (Figure 4).

Effect of Z11-16:Ald Decomposition Products on Dispenser Attractivity. Two experiments in which the attractivity of fresh dispensers was compared with that of dispensers containing aldehyde decomposition products showed that at high concentrations decomposition products diminished dispenser attractivity, but S-20D dispensers with 30% decomposed aldehyde were no less attractive than fresh S-20 dispensers over a 56-day field test (Table 4). K-50 dispensers with >50% decomposed aldehyde caught significantly fewer males during the first eight days and during days 19–23 of a 50-day test (Figure 5).

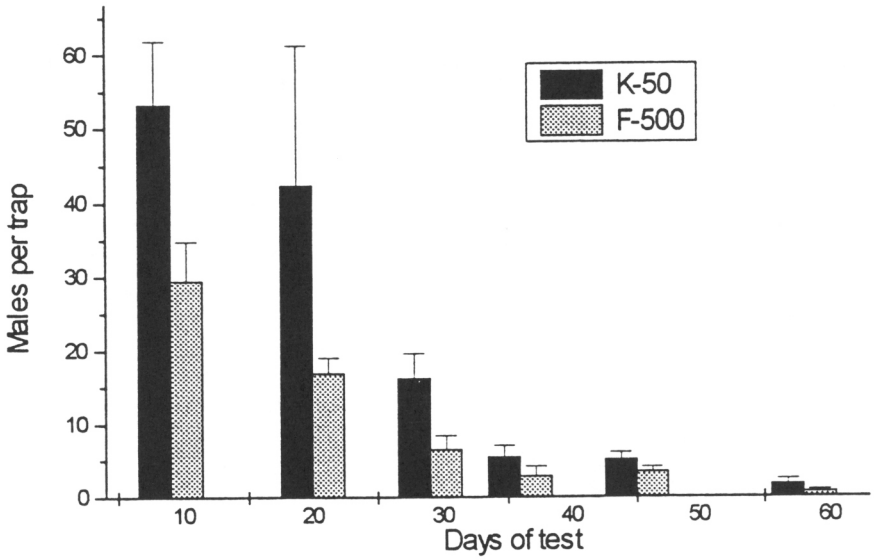


FIG. 3. Mean numbers of male *Plutella xylostella* caught by traps (N = 5 per treatment) baited with Feroflor dispensers (K = segment, F = cylinder) loaded with 50 or 500 μg pheromone (1:1:0.01 of Z11-16:Ac, Z11-16:Ald, Z11-16:OH) in field tests near Tartu started on June 10, 1995.

TABLE 2. MEAN NUMBERS OF MALE *Plutella xylostella* CAUGHT BY TRAPS (N = 5 per TREATMENT) BAITED WITH FEROFLOR DISPENSER (K = SEGMENT, S = DISK) LOADED WITH 20 OR 50 μg PHEROMONE (1:1:0.01 OF Z11-16:Ald, Z11-16:Ac, AND Z11-16:OH)[a]

Dispenser type	Days					
	0-11	12-25	26-32	33-45	46-55	56-62
K-50	40.4 ± 5.0	191.2 ± 31.4	37.6 ± 19.0	77.8 ± 34.4	8.6 ± 5.1	1.2 ± 1.1
S-20	11.2 ± 1.2	41.2 ± 8.7	29.2 ± 13.3	23.4 ± 18.8	5.6 ± 3.1	2.4 ± 1.9

[a]Field test at Jõgeva started on July 7, 1994. Differences are significant for the first 25 days ($P > 0.05$).

TABLE 3. MEAN NUMBERS OF MALE *Plutella xylostella* CAUGHT BY TRAPS (N = 5 PER TREATMENT) BAITED WITH FEROFLOR DISPENSER (K-50 TYPE) LOADED WITH 20 OR 50 μg PHEROMONE (1:1:0.01 OF Z11-16:Ald, Z11-16:Ac, AND Z11-16:OH) AND LEFT OUT CONTINUOUSLY OR CHANGED AT LEAST EVERY 2 WEEKS[a]

Test dates	Days from start (N)	Test length (days)	Dispensers replaced every two weeks	Dispensers not changed from start of test
June 10–25	14	(14)	124.2 ± 17.7	99.8 ± 5.5
June 26–July 2	21	(7)	62.0 ± 13.5	59.0 ± 7.7
July 3–10	29	(8)	43.4 ± 4.6	31.8 ± 4.8
July 11–17	36	(7)	10.6 ± 1.4	13.4 ± 4.1
July 18–23	42	(6)	21.6 ± 3.0	23.4 ± 5.4
July 24–August 11	61	(19)	8.6 ± 1.1	9.0 ± 0.6

[a]Field test near Tartu, started on July 10, 1995. Differences are not significant ($P > 0.05$).

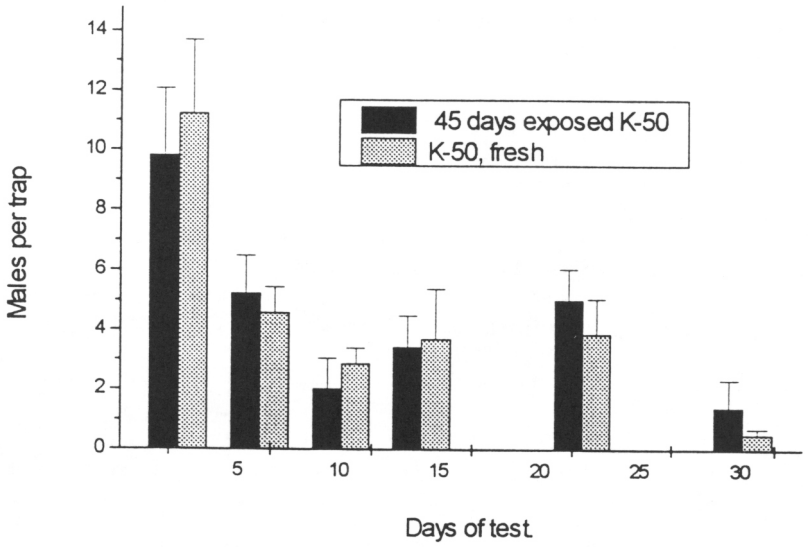


FIG. 4. Mean numbers of male *Plutella xylostella* caught by traps (N = 5 per treatment) baited with Feroflor dispensers (K = segment) loaded with 50 μg pheromone (1:1:0.01 of Z11-16:Ac, Z11-16:Ald, Z11-16:OH) in field tests near Tartu started on August 1, 1995. Differences are not significant ($P > 0.05$).

TABLE 4. MEAN NUMBERS OF MALE *Plutella xylostella* CAUGHT BY TRAPS (N = 5 PER TREATMENT) BAITED WITH FEROFLOR DISPENSER (DISK TYPE) LOADED WITH 20 μg FRESH PHEROMONE (1:1:0.01 OF Z11-16:Ald, Z11-16:Ac, AND Z11-16:OH) OR 20 μg PHEROMONE WITH 30% Z11-16:Ald DECOMPOSED[a]

Dispenser type	Days				
	0-11	12-25	26-32	33-45	46-56
S-20	1.2 ± 0.5	6.2 ± 1.0	4.1 ± 1.0	3.3 ± 1.1	1.1 ± 1.0
S-20D	1.2 ± 0.6	5.6 ± 0.9	4.2 ± 0.8	3.8 ± 0.8	1.1 ± 0.9

[a]Field tests near Tartu, started on June 16, 1994.

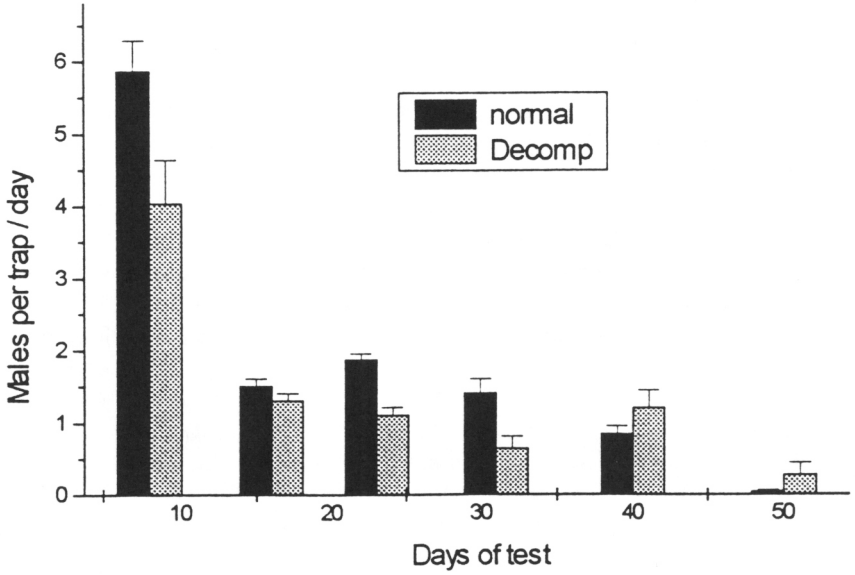


FIG. 5. Mean numbers of male *Plutella xylostella* caught by traps (N = 5 per treatment) baited with Feroflor dispensers K-50 loaded with 50 μg pheromone (1:1:0.01 of Z11-16:Ac, Z11-16:Ald, Z11-16:OH) or with the dispensers with 50 μg pheromone plus >25 μg decomposed aldehyde (K-50D) in field tests near tests near Tartu, started on June 10, 1995. Differences are not significant at P = 0.03. Differences are significant at P = 0.06 for the first eight days.

TABLE 5. RATE OF LOSS OF 11-16:Ald FROM FEROFLOR DISPENSERS LOADED WITH 50 μg OF PHEROMONE (1:1:0.01 of Z11-16:Ald, Z11-16:Ac, AND Z11-16:OH) IN FIELD TEST[a]

Days of exposure	Z11-16:Ald left in dispensers (%)	Calculated Z11-16:Ald for k = 0.0152/day	Evaporation rate of Z11-16:Ald (ng/hr)	Calculated % of Z11-16:Ac in evaporated mixture
10	90.0	85.9	17.0	13.2
20	68.0	73.8	14.8	15.2
32	63.5	61.5	12.9	17.4
41	58	53.6	11.3	19.8
50	39.5	46.8	10.1	22.2
77	26	31.0	8.3	26.9
78	32	30.6	7.4	30.2
98	25	22.5	6.4	34.2

[a]Near Tartu started on July 10, 1995.

Rate of Evaporation of Z11-16:Ald in the Field. The evaporation rate constant (k^{Ald}), calculated by equation 1 from data on residual amounts of Z11-16:Ald following field exposure of K-50 dispensers for 0–98 days (Table 5) was 0.0152 per day (correlation constant R = 0.97968, SD = 0.1115). The half-life of Z11-16:Ald was calculated by equation 2 to be 46 days.

The rate of evaporation of Z11-16:Ac from dispensers is too low to measure in the field, but the data on the residual quantities of Z11-16:Ac/Z11-16:Ald over the 0–78 days of exposure of K-50 dispensers (Table 6) were

TABLE 6. CHANGE IN RATIO OF Z11-16:Ald TO Z11-16:Ac IN FEROFLOR DISPENSERS LOADED WITH 50 μg OF FRESH PHEROMONE (1:1:0.01 OF Z11-16:Ald, Z11-16:Ac, AND Z11-16:OH) IN ATRAKON AA TRAPS EXPOSED IN THE FIELD FOR 0–78 DAYS

	Days of exposure							
	0	10	20	28	32	41	56	78
Z11-16:Ald to Z11-16:Ac ratio	1.01	0.91	0.70	0.80	0.65	0.58	0.47	0.35

TABLE 7. CALCULATED RELEASE RATES OF Z11-16:Ald AND Z11-16:Ac AND PHEROMONE BLEND (Σ) FROM DISPENSERS (S = DISKS; K = SEGMENTS; F = CYLINDER) LOADED WITH 20, 50, OR 500 μg OF PHEROMONE (1:1:0.01 OF Z11-16:Ald, Z11-16:Ac, AND Z11-16:OH) DURING 85 DAYS OF EXPOSURE

Days of exposure	Dispenser S-20 (ng/hr)			Dispenser F-50 (ng/hr)			Dispenser K-50 (ng/hr)			Dispenser F-500 (ng/hr)		
	Ald	Ac	Σ	Ald	Ac	Σ	Ald	Ac	Σ	Ald	Ac	Σ
5	5.0	0.89	5.9	9.9	2.06	12.0	14.7	2.7	17.4	99	21	120
15	4.4	0.87	5.2	9.0	2.02	11.0	12.6	2.6	15.2	90	20	110
25	3.9	0.85	4.7	8.1	1.98	10.1	10.8	2.5	13.4	81	20	100
35	3.4	0.83	4.2	7.3	1.94	9.3	9.3	2.5	11.8	73	19	93
45	3.0	0.82	3.8	6.6	1.9	8.5	8.0	2.4	10.4	66	19	85
55	2.6	0.79	3.4	6.0	1.87	7.9	6.9	2.3	9.2	60	19	89
65	2.3	0.78	3.1	5.4	1.82	7.3	5.9	2.3	8.2	54	18	73
75	2.0	0.77	2.8	4.9	1.79	6.7	5.1	2.2	7.3	49	18	67
85	1.8	0.75	2.5	4.5	1.76	6.2	4.4	2.2	6.5	45	17	62

used to calculate the evaporation rate constant for Z11-16:Ac (k^{Ac}) using the formula derived from equation 1.

$$k^{Ac} = \ln D/t - k^{al} \tag{5}$$

where D is the ratio of amounts Z11-16:Ac/Z11-16:Ald determined from their peak areas on GC chromatograms. The calculated mean value for k^{Ac} is 0.0026 per day ($E_r \pm = 0.0014$). Calculated release rates using the release rate constants (k^{Ald} and k^{Ac}) given in Table 1 of Z11-16:Ac and Z11-16:Ald are summarized in Table 7. Table 8 shows the amount of Z11-16:Ac remaining in different disks or cylinders following field exposure of dispensers loaded with 20 or 50 μg pheromone.

DISCUSSION

The experiments and calculations demonstrated that the attractivity and active period of exposure of pheromone dispensers for *P. xylostella* may be influenced by three factors. The first is the evaporation rate of the active blend. Our calculations confirmed that evaporation of components under field conditions follows the first-order process of equation 1. In accordance with the first-order process, the evaporation rate declines during exposure of dispensers.

TABLE 8. CONTENT OF Z11-16:Ald REMAINING IN DIFFERENT DISPENSERS (S = DISK; F = CYLINDER) FOLLOWING FIELD EXPOSURE OF DISPENSERS LOADED WITH 20 OR 50 μg PHEROMONE (1:1:0.01 OF Z11-16:Ald, Z11-16:Ac, AND Z11-16:OH) FOR 95 DAYS

	Dispenser S-20		Dispenser F-50		Dispenser K-50	
Days of exposure	Release rate (ng/hr)	Z11-16:Ac remaining (%)	Release rate (ng/hr)	Z11-16:Ac remaining (%)	Release rate (ng/hr)	Z11-16:Ac (%)
5	5.9	15.2	12	17.2	17.4	15.4
15	5.2	16.1	11	18.4	15.2	17.1
35	4.2	19.7	9.3	21	11.8	21
55	3.4	23.3	7.9	23.7	9.2	25.5
75	2.8	27.3	6.7	26.7	7.3	30.6
95	2.3	31.6	5.8	30	5.9	36.1

Field tests with a 1:1:0.01 mixture of Z11-16:Ac, Z11-16:Ald, and Z11-16:OH demonstrated that dispenser K-50 with an initial rate of evaporation of 17.4 ng/hr had higher activity than dispensers S-20 and F-50 with initial evaporation rates of 5.9 and 20 ng/hr. K-50 dispensers exposed in the field for 45 days prior to a test were as active as fresh dispensers for 20 days. The release rate of K-50 on day 55 of exposure was 9.2 ng/hr, which was less than from F-50 during the first month of exposure. Evidently, the release rate is very important, but it is not the only factor influencing the activity of dispensers.

Temperature influences evaporation rate. Mean evaporation rate constants for field conditions were used in this work. A change in temperature of 1°C caused a 12% change in release rates (Môttus et al., 1993), while a temperature rise of 12°C caused a fourfold increase in release rate. As a result, most of the pheromone in a trap (more than 70%) evaporates during the daytime, when the moths are inactive. The higher pheromone concentration and surface factor of K-50 in comparison with S-20 and F-50 may be essential. In July, at the beginning of our test, when days were longer and warmer, K-50 was about four times more active than S-20. In August, when daytime temperatures were lower and nights longer, K-50 exceeded S-20 by 1–1.5 times only. This suggests that with a low release rate, activity depends on the surface factor of the dispensers, and minidispensers with greater surface factor seem to have some advantages. We calculate that the minimum mean release rate of pheromone blends is about 8–10 ng/hr. Higher doses of pheromone can result in decreased activity (Macaulay et al., 1986), and this was confirmed by our experiments. F-500 and F-1000 were less active than K-50. The initial evaporation rate of F-500 exceeded that

of K-50 by 6.9 times and was equal to 119.7 ng/hr. After 55 days of exposure, evaporation velocity of the attractant was equal to 78.7 ng/hr or 4.2 times the initial release rate of K-50. Evidently, there is no need to use release rates greater than 20 ng/hr for *P. xylostella* dispensers. Our findings concur with the results of laboratory experiments by Kawasaki (1984), who also demonstrated a narrow optimum range for concentrations of *P. xylostella* pheromone.

A second factor influencing attractivity of pheromone dispensers is the ratio of components in the blend. The ratio of Z11-16:Ac and Z11-16:Ald evaporating from dispensers changes with time. The initial Ac/Ald ratio is dependent on evaporation rate constants. The rate of evaporation of Z11-16:Ald diminishes during the exposure of dispensers more rapidly than the evaporation velocity of Z11-16:Ac. Due to the long half-life of 270 days for Z11-16:Ac, its release remains practically constant for the first two to three months.

In the K-50 dispenser, the content of Z11-16:Ac changed most (by 15%) during 90 days of exposure. Other dispensers had a smaller surface factor and rate constant k, which caused smaller changes in the evaporated blend (12.7% for F-50 and F-500). The blend released from K-50 dispensers consisted of 36% of Z11-16:Ac on day 95 of exposure. This corresponds to a 1:3.3 mixture of Z11-16:Ald and Z11-16:Ac in dispensers. Consequently, large changes in the Ac/Ald ratio in dispensers cause small changes in the Ac/Ald ratio in evaporated pheromone. This phenomenon probably explains why rather different mixtures of pheromone components have been described as active in field tests (see Introduction).

The third factor that influences attractivity of pheromone dispensers is the decomposition of Z11-16:Ald. Our experiments suggest that when more than 50% of the Z11-16:Ald had decomposed, the decomposition products reduced the activity of the pheromone blend, but these aspects need more detailed investigations. The inhibition may be connected to some abiotic or biotic factors and may not occur in all conditions. Addition of BHT, an inhibitor of radical decomposition processes, to the *P. xylostella* pheromone dispenser (Macaulay et al., 1986) is known to have a positive effect.

The decrease of activity of dispensers may have been caused by other factors than the Z11-16:Ald decomposition products. Our earlier laboratory experiments demonstrated that higher wind speeds will increase the evaporation constant by up to four to five times (Môttus et al., 1993). This shows the effect of the porous structure of the dispenser surface on evaporation. Air in the pores is saturated by vapors of the evaporating components. Different decomposition processes, together with high daytime temperature, may exhaust the stock of aldehyde on the surface, especially in pores. Decomposition processes may also enhance the content of more stable Z11-16:Ac in the evaporated blend. Smaller dispensers with larger surface areas will have large quantities of components on the surface and may be less sensitive to decomposition processes. Z11-16:Ald

is the pheromone component of many economically significant pest insects (e.g., *Heliothis viriscens* and *Heliothis armigera*), but Shaver et al. (1982) demonstrated that decomposition products have no inhibiting effect on the activity of the pheromone blend for *H. virescens*.

CONCLUSIONS

1. Rubber minidispensers (K-50) have active exposure times of at least two months in Estonia and may be used in delta traps to monitor *P. xylostella* throughout the summer in Estonia.

2. Pheromone release rates between 8 and 17 ng/hr are recommended for dispensers to achieve maximum trap catches. The blend evaporated from dispensers must contain 15–35% of Z11-16:Ac in order to be attractive.

3. When present in high concentrations, decomposition products of Z11-16:Ald inhibit the activity of the pheromone blend for *P. xylostella*. Decomposition of 50% of Z11-16:Ald inhibits attractivity by 35%.

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Sitona lineatus HOST-PLANT ODORS AND THEIR COMPONENTS: EFFECT ON LOCOMOTOR BEHAVIOR AND PERIPHERAL SENSITIVITY VARIATIONS

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Abstract—Adult *Sitona lineatus* L., (Coleoptera: Curculionidae) feed on the leaves of various species of leguminous plants, and females lay in the vicinity of pea plants, the roots of which are eaten by the larvae. A study of the volatiles from several legumes and of the response of *S. lineatus* individuals to these substances was undertaken using two complementary techniques: behavioral, to study the locomotory orientation; and electrophysiological, using electroantennography (EAG). Four volatile compounds, *cis*-3-hexen-1-ol, 2-hexenal, *cis*-3-hexen-1-yl acetate, and 3-octanone, were identified from pea, vetch, clover, and lucerne, by coupled gas chromatography–mass spectrometry (GC-MS) and coupled gas chromatography–infrared spectrometry (GC-IR). After emergence from July to mid-November, the imago display high response to the odor of pea and some other leguminous plants. A second period of enhanced sensitivity was observed during crop colonization from March to May. High EAG response to pea odor and *cis*-3-hexen-1-yl acetate was correlated with periods of enhanced locomotory orientation during these months. Experimental results indicate that *cis*-3-hexen-1-yl acetate could play a key role in discriminating pea among other acceptable leguminous species.

Key Words—Pea weevil, olfaction, EAG, locomotory behavior, green odors, *cis*-3-hexen-1-yl acetate, host odor, *Sitona lineatus*, Coleoptera.

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INTRODUCTION

Sitona lineatus L. [Coleoptera: Curculionidae (Brachyderinae)], which consumes leguminous plants exclusively, is a common and harmful species on cultivated pea that is distributed across the Euro-Siberian zone up to Mongolia, the Near and Middle East, and North Africa. This weevil recently has been introduced into the USA and Canada (Hoffman, 1963). Emergence takes place from July to September. In the spring, the adults move away from wild legumes such as clover, on the leaves of which they have fed, in order to colonize crops of protein-rich peas and to lay their eggs. The larvae reach the roots of peas and eat the nodules (the only food of first instars). Early location of fields by adults may be associated with volatile substances emitted by the host plants. This study analyzed locomotory responses of individuals of both sexes and olfactory sensitivity to odors of leguminous plants and their component odors.

METHODS AND MATERIALS

Origin of Weevils and Plants. Adult weevils were collected at the ITCF field experimental station at Boigneville in the Essonne region of France throughout the year preceding each set of experiments. Plants used for analysis of volatile substances and for behavioral or physiological tests were cultivated at 18°C, 60 ± 10% humidity, and photoperiod of 10L: 14D. Plant species were chosen according to the degree of weevil attack under natural conditions. *Pisum sativum* L., pea (variety Solara), is the preferred host plant; *Vicia sativa* L., common vetch (variety Valor), is very often attacked even when peas are present; *Medicago sativa* L., lucerne, is regularly attacked in the field; and *Trifolium repens* L., white clover, is consumed before the overwintering period but is not attacked during the reproductive period.

Analysis of Odors of Plants: Trapping of Volatile Molecules. Preliminary experiments showed that intact foliage emitted only a small quantity of volatiles. Hence, 100 g of young plants (about 20 cm high) were picked, immediately chopped, and subjected to headspace air trapping. Chopped foliage was put into a glass flask (2 liters) at room temperature. An air current of 250 ml/min from a pump (Gilian LFS 113DC) carried the volatiles to a cartridge of 30 mg of Tenax GC (60–80 mesh, 4 mm diameter, 20 mm in length) for 1 hr (Auger et al., 1989). Components collected on the cartridge were eluted with 1 ml of diethyl ether.

Chromatography of Isolated Volatiles. Separation of trapped volatiles was accomplished with a Hewlett-Packard (HP5890 II) chromatograph (GC) equipped with a split-splitless injector in splitless mode and a capillary column (20 m × 0.22 mm) coated with methyl silicon film HP1 (thickness 0.33 μm). The injec-

tion temperature was 200°C, and oven temperature was programmed from 45°C to 280°C at 2°C/min. The carrier gas was helium at 0.5 ml/min), and 1 μl was injected.

Separated compounds were identified by combined gas chromatography–mass spectrometry (GC-MS) and gas chromatography–infrared spectrometry (GC-IR) (Auger and Ferary, 1994; Ferary et al., 1996). The mass spectrometer was a Mass Engine (Hewlett Packard HP 5989A) used in electronic impact mode at 70 eV. The transfer line was maintained at 250°C, the source at 200°C, and the quadrupole at 100°C. The infrared spectrometer was the Digilab FTS 45A (Bio-Rad) equipped with a Tracer direct deposition interface. The line was maintained at 250°C, and the spectra were obtained in real time by the accumulation of four scans at a resolution of 8 cm^{-1}.

Olfactometry and Electroantennography. Behavioral bioassay in a tubular olfactometer and electroantennography (EAG) were used to record responses of insects. We determine the periods of adult insects responses to pea volatile during the year and their sensitivity to various components of the odors.

Behavioral Test: The Olfactometer. The tubular olfactometer and handling methods were the same as described by Chaïbou (1993) for *Caryedon serratus* and by Lextrait et al. (1994, 1995) for *Callosobruchus maculatus*. The plant odor source (3–4 leaves of the entire aerial part of *P. sativum*) was contained in a 250-ml bubble flask placed downstream from electronically-controlled electrovalve. In the case of identified pure compounds, the odor source was placed in a glass tube (6 cm × 0.6 cm ID). Pure compounds tested were *cis*-3-hexen-l-ol, *cis*-3-hexen-l-yl acetate, and 3-octanone. Each was deposited (5 μl) on a 5-mm × 30-mm piece of Whatman No. 3 paper. Odor containing air puffs of 800-msec duration were separated by puffs of pure air lasting 200 msec, and the rate of airflow was 3.5 liters/min, i.e., a speed of 0.185 m/sec. The behavioral response of individual weevils was assessed by the three criteria of no response, antennal movements, or attraction, and the insects were classified according to their locomotory displacement towards the odor source (Landon, 1995).

Physiological Test: Electroantennography. Electroantennographic recordings (EAG) were carried out on intact insects of various ages according to the technique described by Pouzat (1974) and modified thereafter (Lextrait et al., 1995). The odor delivery system was composed of two concentric tubes arranged so that the central tube delivered the odor and the peripheral tube provided clean air. The antenna was subjected to a clean air current that was interrupted during stimulation by a puff of odor-laden air of 800-msec duration, at the same speed as the clean air (Pierre, unpublished). The airflow was 600 ml/min, and the speed at the outlet was 30 cm/sec. The host-plant stimulus was presented as described above and pure compounds were diluted (10^{-3} ml) in paraffin oil and 5 μl deposited on a piece of filter paper.

Statistical Analyses. The number of individuals in the three response categories were subjected to statistical analysis using chi-square contingency tables. Means were compared using Student's t test (Sokal and Rohlf, 1969) with STATITCF software.

RESULTS

Characterization of Volatile Emissions. Four volatile compounds (99% of all detected compounds) were identified in the *P. sativum* sample as *cis*-3-hexen-1-ol, 2-hexen-1-al, *cis*-3-hexen-1-yl acetate, and 3-octanone. *cis*-3-Hexen-1-yl acetate represented about 87% of the total of volatile compounds. Three other legumes, *T. repens*, *M. sativa*, and *V. sativa*, show the same four substances, but the relative proportions of the compounds were very different (Table 1).

Behavioral Study: Response to Pea Odor. In August, the response of emerging adults to pea odor was weak (Figure 1). Response increased during the summer migration period (end of summer, beginning of autumn), and then decreased toward the end of the overwintering period in January. In February–March it began to increase sharply and then decreased again in June towards the end of the insect's life. The second period of high response towards host-plant odor is synchronized with the early growth of pea, which generally takes place at the beginning of April in the region studied.

The response of insects according to their behavior during the period of pea colonization (mid-March to the end of May) and outside this period (June to mid March), was analyzed by a chi-square test (Table 2). The response of insects to pea odor differs during these two periods (chi-square of contingency table = 37.28, $P < 0.001$).

The criteria that contribute the most to the chi-square value were determined by calculating the chi-square in each case and the probability associated with it

TABLE 1. PERCENTAGE OF FOUR PRINCIPAL VOLATILE COMPOUNDS OF FOUR LEGUMINOUS PLANTS

Volatiles	*Pisum sativum*	*Vicia sativa*	*Medicago sativa*	*Trifolium repens*
cis-3-Hexen-1-ol	12	35	24	35
2-Hexenal	1	4	9	3
cis-3-Hexen-1-yl acetate	87	48	17	10
3-Octanone	0	13	50	52

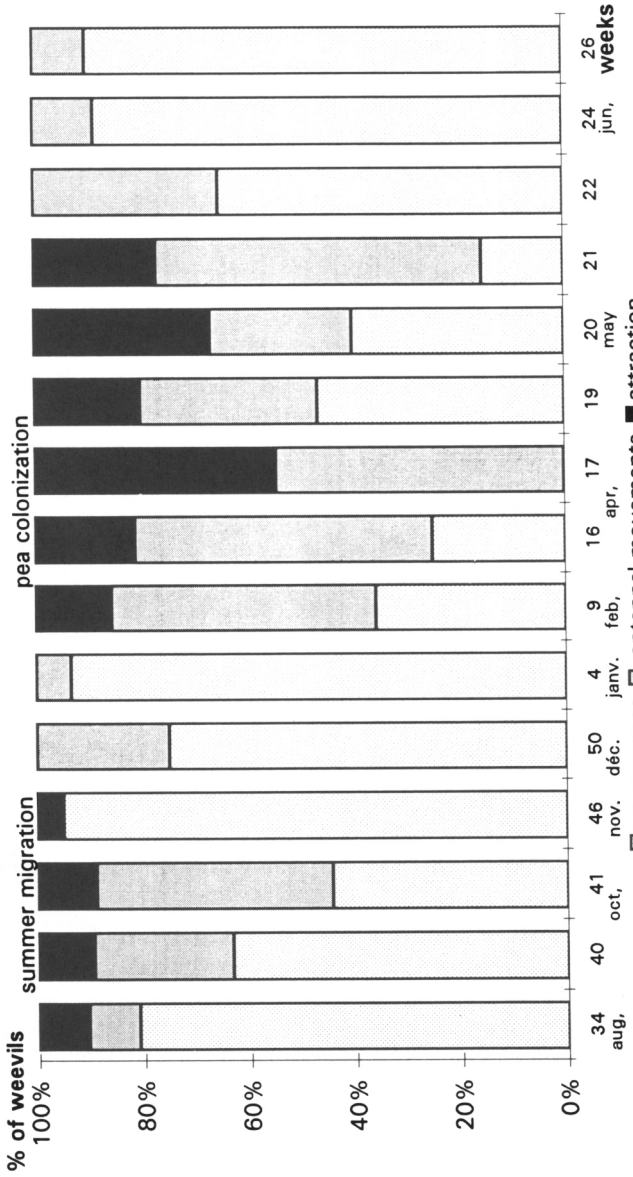


FIG. 1. Responsiveness of weevils to pea odor during the year. Results from 225 weevils belonging two successive generations.

TABLE 2. NUMBER OF WEEVILS OF BOTH SEXES IN THREE CATEGORIES OF RESPONSE TO PEA ODOR IN TUBULAR OLFACTOMETER WITH CHI-SQUARE AND ASSOCIATED PROBABILITIES IN PARENTHESES

	No response	Antennal movements	Attraction
June to mid-March	140	16	12
Chi^2 (P)	2.6 (0.10)	1.28 (0.26)	4.36 (0.04)
Mid-March to the end of May	17	24	16
Chi^2 (P)	9.32 (0.002)	4.45 (0.03)	15.15 (0.0001)
Total chi^2 of contingency table: 37.28			

(Table 2); 65% of the global chi-square comes from the two criteria "no response" and "attraction" during the colonization period. Individuals in the "attraction" category are proportionally more numerous than during the rest of the year ($P = 10^{-4}$), and individuals in the "no response" category are proportionally fewer ($P = 0.002$).

Response to Pea Odor Components. The results of behavioral tests in the olfactometer are based upon all males and females collected during the year. The odor profiles of the legumes show that *cis*-3-hexen-1-yl acetate could be the substance that enables weevils to discriminate between pea and other legumes. The distribution, according to their behavioral response, during the year, of 163 individuals subjected to olfactory stimulation with *cis*-3-hexen-1-yl acetate is indicated in Figure 2 (global chi-square = 22.72; $P < 0.01$). In the overwintering period (mid-November to mid-January), the number of "no response" individuals is higher than the number of individuals displaying "antennal movements" during the rest of the year (chi-square = 4.06; $P = 0.04$). Thus, response to the odor of *cis*-3-hexen-1-yl acetate is lower (chi-square = 4.79; $P < 0.03$).

cis-3-Hexen-1-ol on its own does not trigger a directional locomotory response in the insects. Individuals that respond to *cis*-3-hexen-1-yl acetate alone display an identical response to the mixture of *cis*-3-hexen-1-yl acetate and *cis*-3-hexen-1-ol in the same proportions as in the pea (Landon, 1995).

Electroantennography: Response to Pea Odor. Aerial parts of the pea (leaves and stems) were used as a source of odor to stimulate the antenna of individuals of the 1993–1994 and 1994–1995 generations (74 insects). In order to establish more precisely the limits of the period of response towards the host plant, we determined the time intervals during which measurements were homogenous. On a given date, the variability of the responses of the same insect to identical stimulations by host-plant odor (separated by 2 min) was very low.

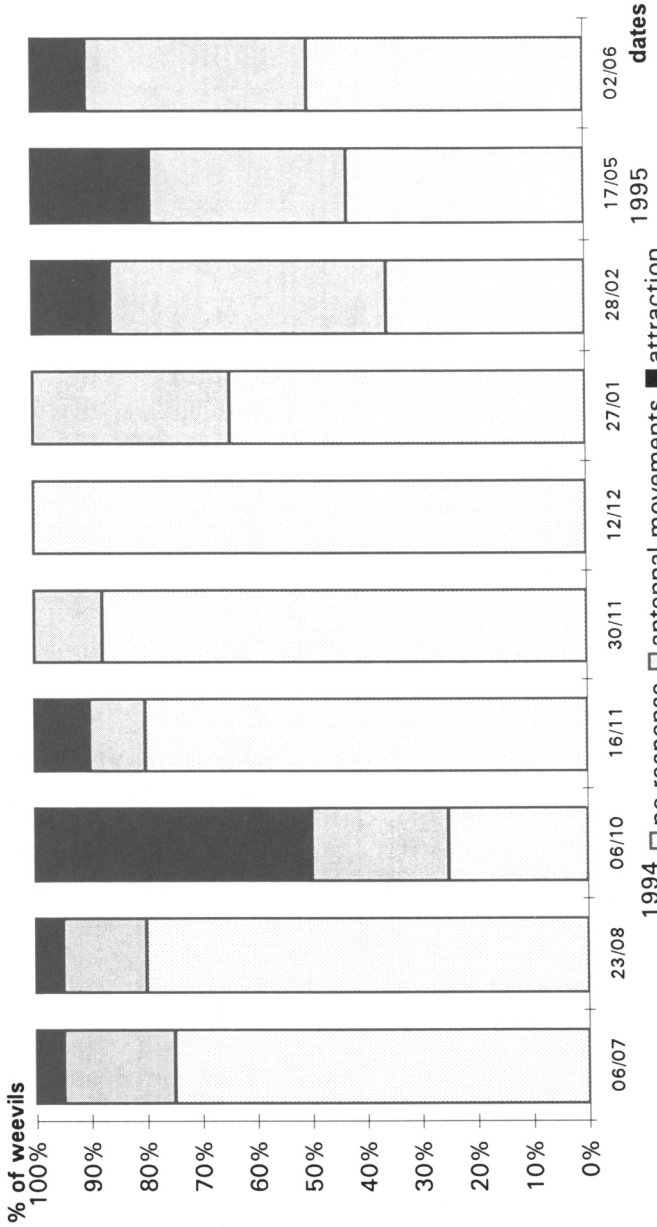


FIG. 2. Responsiveness of 163 weevils of the 1994–1995 generation according to their reaction to *cis*-3-hexen-1-yl acetate.

TABLE 3. COMPARISON OF AVERAGE RESPONSES TO HOST PLANT ODOR IN ELECTROANTENNOGRAPHY IN FOUR PERIODS OF HOMOGENOUS RESPONSES[a]

Period	Mean ± SEM (mV)	Probabilities of equality between periods			
		1	2	3	4
1: Mid-January to mid-March	0.46 ± 0.08	1			
2: Mid-March to end of June	0.19 ± 0.06	6×10^{-3}	1		
3: July to mid-November	0.44 ± 0.08	0.86	0.023	1	
4: December to beginning of January	0.03 ± 0.03	2×10^{-4}	0.05	4×10^{-3}	1

[a]Probabilities of equality between the 2 periods are indicated for each comparison (from 1 to 4).

However, there was great variability between individuals. Responses for a given date were thus compared to responses obtained on other dates that took into account the variability of individual measurements (Table 3). The data for Table 3 reveal four groups of homogenous responses during the year corresponding to four distinct periods, labeled 1–4. Two periods of high responsiveness alternate with two periods of reduced responsiveness. The average responses obtained during each period indicate that at the two periods of high responsiveness (labeled 1 and 3 in Table 3), the physiological responses of the insects are not significantly different ($P = 0.86$) but are significantly different from those of periods 2 and 4 showing reduced responsiveness. During period 2, which corresponds to the overwintering of the species, the insects display the weakest response to pea ($P = 0.05$) (Table 3).

Responsiveness to Volatiles from Pea. EAGs from 102 insects subjected to stimulation by *cis*-3-hexen-1-yl acetate were recorded. This substance evokes an EAG response throughout the life of the insect. Four groups of responses were observed corresponding to the four periods of Table 4. EAG responses during periods 1 and 3 (periods of the highest responsiveness) do not differ from each other ($P = 0.47$) but differ significantly from those of periods 2 and 4 (periods of reduced responsiveness). Responses during periods 2 and 4 do not differ from each other ($P = 0.72$). During these four periods the average of EAG amplitudes obtained by stimulation with *cis*-3-hexen-1-yl acetate are higher than those evoked by the odor of green parts of host plants (Table 3).

The average amplitudes of the EAG responses to *cis*-hexen-1-ol are 0.7 mV during the winter (October–February), 0.5 mV in April, and 0.25 mV in May. Variability of the results does not allow discrimination to be made between periods of particular responsiveness during the year. The responses to *cis*-3-

TABLE 4. COMPARISON OF AVERAGE RESPONSES TO *cis*-3-HEXEN-1-yl ACETATE BY ELECTROANTENNOGRAPHY IN FOUR PERIODS OF HOMOGENOUS RESPONSES[a]

	Mean ± SEM (mV)	Probabilities of equality between periods			
		1	2	3	4
1: Mid-January to mid-March	1.35 ± 0.11	1			
2: Mid-March to end of June	0.79 ± 0.09	2×10^{-4}	1		
3: July to mid-November	1.51 ± 0.17	0.47	4×10^{-4}	1	
4: December to beginning of January	0.73 ± 0.02	10^{-3}	0.72	8×10^{-4}	1

[a]Probabilities of equality between 2 periods are indicated for each comparison (from 1 to 4).

hexen-1-ol in mixture with *cis*-3-hexen-1-yl acetate in the same proportions as in the volatiles from the green parts of pea do not differ from those obtained with *cis*-3-hexen-1-yl acetate alone.

DISCUSSION

Hans (1959) concluded that color of plants did not play a role in location of a crop, but he was unable to demonstrate an olfactory mechanism. According to Moeller (1974), the location of crops by *S. lineatus* is primarily a visual discrimination between different cultivated pea species, after which olfactory stimuli intervene. Our results indicate that pea volatiles contribute to location (Landon, 1995). Individuals of both sexes respond to the major volatile compound of the pea (*cis*-3-hexen-1-yl acetate). In the laboratory, males display higher locomotory activity than females in response to this compound. Our field observations at the end of the winter indicate a higher proportion of precociously active males relative to females.

Behavioral tests in an olfactometer show two periods of high insect response to host plants. The first corresponds to the feeding period (soon after emergence to the end of November). The second (in February–April) encompasses the growth period of the larval host plant and the oviposition period, with further adult feeding. The beginning of imaginal life (from July to October–November) is devoted to feeding in order to build body reserves. During this period the behavioral response to host-plant odor is clearly correlated with high olfactory responsiveness. The December to mid-January period is characterized by a complete lack of behavioral and EAG responsiveness to host-plant odor. A period of sharply increasing olfactory and behavioral responsiveness to host-plant odor

occurs during colonization of the growing host plant. The end of imaginal life is marked by a more or less regular decrease of these parameters. The coincidence of the antennal (EAG) and behavioral (locomotory) responsiveness is fairly good for the two first periods. Because of differences in climate in successive years, the correlation during the two last periods is less good, but the peak of responsiveness in EAG and behavior is clearly related to host-plant colonization. The variation in antennal responsiveness is a likely component of the change in behavior, but the latter might also be associated with differences in central nervous system integrative activity. Whenever a clear behavioral reaction is observed, antennal receptor responsiveness is high. A perfect correlation between these two biological responses is not to be expected for various reasons, one of which is the variability between individuals that creates background noise.

The quantity of *cis*-3-hexen-1-yl acetate deposited on the filter paper (5 μl) is fairly high. This might explain why the EAG response to this substance is always higher than the response to intact pea odor. The behavioral response of October 1994 to this substance might partly be explained in the same way.

In the overwintering period, the drop in responsiveness is brief, probably in relation to the fact that this species only seems to display a reproductive quiescence and not a true diapause. In diapausing *Culex pipiens* L., host-searching behavior disappears and there is a drop in responsiveness of antennal receptors to lactic acid, while other receptors are not affected (Bowen et al., 1988). In other insects, periods of behavioral response and nonresponse towards the host plant have been observed to be linked to satiation. In *Leptinotarsa decemlineata* Stal., for example, the state of satiation modifies positive anemotaxis triggered by host-plant odor (Thiery and Visser, 1995), indicating that the state of satiation is a regulatory factor in orientation behavior towards the host plant. In *Locusta*, the anemotaxic response conditioned by food odors may be under the control of a stimulatory/inhibitory center depending on the state of satiation; however, the physiology of such a stimulatory center has not yet been described (Kennedy and Moorhouse, 1969). These insects are certainly very different from *S. lineatus*. The time scale is different. For the latter, peripheral and behavioral variations cannot be attributed to a satiation phenomenon since it happens after the feeding period following emergence but lasts at least six weeks (during which insects do not feed). Variations in adult emergence that spread over July to August might explain the long duration of the first period of high olfactory responsiveness, and clear behavioral response to pea odor as compared with the second, much shorter period at the end of winter–beginning of spring. The former correspond to an intense feeding period during which nutritive reserves are built up, and the latter to the period of colonization (i.e., oviposition) of the larval host plant.

Among the three legumes (*P. sativum*, *V. sativa*, and *M. sativa*) tested in April 1993 during the colonization period, (Landon, 1995), only pea is attrac-

tive. *cis*-3-Hexen-1-ol is present in *P. sativum* and *M. sativa* (Table 1) and is more abundant in *M. sativa* (99% versus 1% in pea). It is therefore unlikely that this substance accounts for the locomotory behavior of *S. lineatus*, at least at this period of the year. The two most abundant compounds are *cis*-3-hexen-1-yl acetate and *cis*-3-hexen-1-ol. The sensitivity of the former varies during the year, but the sensitivity to *cis*-3-hexen-1-ol is so variable between insects on a given date, all during the year, that no conclusion can be drawn about possible variation of responsiveness to this compound. *cis*-3-Hexen-1-ol, which stimulates the olfactory receptors, has no effect on locomotory behavior, and when associated with *cis*-3-hexen-1-yl acetate (in the proportions found in the pea) it does not modify the effects of the acetate on EAG or behavior (see Landon, 1995). The mixture of the two most abundant molecules emitted by the pea does not improve the insect response, but the possibility of attraction by a more complex mixture cannot be ruled out. In fact, behavioral tests and EAG with pure substances were conducted with concentrations that were certainly superior to those delivered by the host plant (compare EAG amplitudes with pea odor and with the pure substances). This indicates that the experiments with the pure substances or with mixtures should be repeated and carried out with much lower concentrations, which are likely closer to those emitted by the host plant. It would be interesting to compare chemical analyses of different vegetative stages, as performed by Wallbank and Wheatley (1976) in cauliflower (*Brassica oleracea* L.), which is attacked by *Delia brassicae* L. and in which *cis*-3-hexen-1-yl acetate is present in the small leaves that surround the immature heart of the young plants.

The principal substance responsible for attraction of *S. lineatus* in the tubular olfactometer is probably *cis*-3-hexen-1-yl acetate. This product is present in other legumes, such as the broad bean (*V. faba*) (Blight et al., 1991), and in other groups of plants, including celery (*Apium graveolens* L.), parsley (*Petroselinum crispum* Mill.) (Guerin and Visser, 1980), and potato (*Solanum tuberosum* L., Dejong and Visser, 1988). The capturing technique used here enables only comparison of relative proportions of the odor components. The proportion of *cis*-3-hexen-1-yl acetate was variable, depending on the legume, but consistently higher in pea than in other legumes tested. *Vicia sativa*, which ranks second in quantity found, has about half that in pea. The food preference of *S. lineatus* seems to be correlated with this high proportion (Landon et al., 1995). Location of host plants in response to what is termed the "green odor" has been shown in *L. decemlineata* (Visser, 1979) and the carrotfly *Psila rosae* (Guerin and Visser, 1980).

Electroantennographic responses from *Rynchaenus quercus* (Curc.) induced by emissions from oak, its host, and from nonhost plants do not differ, although the host odor is known to contain *cis*-3-hexen-1-yl acetate (Kozlowski and Visser, 1981). In this case, it is not the presence or absence of a stimulating signal

that enables host-plant recognition, but the composition of the volatile mixture. Pea or vetch, which emit higher proportions of *cis*-3-hexen-1-yl acetate, are preferred by the weevil, while clover is left alone. However, the latter, even though it emits little of this substance, serves as a winter food and is highly colonized at the end of the summer. In the absence of peas, the weevils concentrate on the available plants that emit this molecule. In the spring, when peas germinate, the insects leave the clover and move to peas and vetch emitting a higher amount of *cis*-3-hexen-1-yl acetate. The synchronization between phenology of the host plant, variation in behavioral responsiveness, and in peripheral olfactory sensitivity are particularly interesting. It is, to our knowledge, the first time that this relationship has been described in a phytophagous insect.

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EXPOSURE TO PREDATOR ODOR REDUCES LOCOMOTOR ACTIVITY LEVELS IN ADULT MALE RATS: LACK OF EFFECT OF HIPPOCAMPAL LESION

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Abstract—The hippocampus has been implicated in mediating responses to predators. Involvement of the hippocampus was tested in the present study in which a multivariate analysis of the locomotor activity of intact and hippocampectomized (destruction of dentate and CA1 cells) laboratory rats, *Rattus norvegicus*, was conducted following exposure to a predator odor. Levels of various activity variables were monitored in an automated activity monitoring system following a brief 3-min exposure to 2-propylthietane (weasel odor), caproic acid (goat odor), toluene (organic solvent), and a no-odor condition in hippocampal-lesioned (using colchicine) and sham-operated control male rats. Rats of both groups displayed reduced levels of total distance, movement time, vertical activity, and number of movements following exposure to weasel odor and toluene in comparison to the no-odor condition. Exposure to goat odor resulted only in reduced levels of vertical activity and number of movements. There were no differences in activity levels between hippocampal-lesioned and sham-operated rats during baseline activity recording or following exposure to any of the odors. However, hippocampal-lesioned rats spent less time in the center of the activity boxes than sham-operated control rats across all conditions. This study demonstrates that laboratory rats show marked changes in locomotion in response to the odor of a predator and suggests that an intact hippocampus is not essential for mediating these responses.

Key Words—2-Propylthietane, dentate gyrus, Digiscan activity monitoring system, predator odor, predation, hippocampus.

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INTRODUCTION

The threat of predation elicits a variety of behavioral and physiological responses in animals. Under predation risk, laboratory mice (*Mus musculus domesticus*) and rats (*Rattus norvegicus*), along with various species of wild rodents, display decreased nondefensive and increased defensive behaviors (Blanchard et al., 1990, 1995; Ylonen, 1994). In the short-term these include changes in feeding- and reproduction-related behaviors, decreased pain sensitivity (i.e., analgesia), and altered levels of locomotor activity (Blanchard and Blanchard, 1972; Kavaliers and Colwell, 1991, 1995; Perrot-Sinal et al., 1996; Ylonen and Ronkainen, 1994).

Exposure to predator odor has been used to simulate predation risk in field and laboratory studies. Field voles (*Microtus agrestis*) avoided traps tainted with either weasel (*Mustelus nivalis*) or red fox (*Vulpes vulpes*) anal gland and fecal odors, respectively (Dickman and Doncaster, 1984; Stoddart, 1976). Similarly, bank voles (*Clethrionomys glareolus*) showed reduced activity and avoided tubes simulating underground tunnels that predators, including the red fox, had passed through (Jedrzejewski et al., 1993). Meadow voles (*M. pennsylvanicus*) avoided traps treated with the principal component of fox feces, 2,5-dihydro-2,4,5-trimethyl thiazoline (Sullivan et al., 1988). In a laboratory investigation, adult male breeding meadow voles (*Microtus pennsylvanicus*) displayed reduced levels of various measures of locomotor activity following a brief exposure to trimethyl thiazoline but not to various pungent control odors (Perrot-Sinal et al., 1996).

Recent electrophysiological studies have confirmed that the rat hippocampus responds to olfactory stimulation and, more importantly for the present study, to the odor of a potential predator (Heale et al., 1994). The results of this study showed that fast-wave activity from the dentate gyrus of the rat hippocampal formation was obtained following exposure to components of anal scent gland secretions of the weasel (2-propylthietane) and the fox and to organic solvents such as toluene, but not to aversive nonpredator odors (Heale et al., 1994).

The fast-wave responses of the hippocampus to predator odor may be involved in olfaction related antipredator behavioral responses (Heale et al., 1994). However, whether there were any accompanying predator-associated changes in behavior, such as locomotor activity, was not determined by Heale et al. (1994). Additionally, an earlier study reported that hippocampectomized rats exhibited decreased defensive freezing reactions in response to the presence of a cat (Blanchard and Blanchard, 1972). However, whether these responses were mediated only by olfactory components of the exposure is unclear.

The first objective of the present study was to obtain a multivariate assessment of locomotor activity in laboratory rats following exposure to the same compounds that elicited fast-wave activity in the hippocampus (i.e., 2-propyl-

thietane and toluene). The second goal was to directly examine the role of the hippocampus in mediating changes in locomotor activity levels following exposure to the odor of a predator. Locomotor activity levels were monitored following brief exposure to 2-propylthietane, to an organic solvent (toluene), and to a component of the scent of a nonpredatory animal, the goat (caproic acid), in hippocampal lesioned and sham-operated male rats. Since it is generally recognized that any single measure of locomotor activity will be confounded with other aspects of behavior (Ossenkopp and Kavaliers, 1996; Ossenkopp and Mazmanian, 1985; Whishaw et al., 1983), multiple locomotor activity variables were measured. The automated Digiscan activity monitoring system provides such a multivariate examination of locomotor activity (Ossenkopp and Kavaliers, 1996). Various locomotor activity variables obtained with the Digiscan system have been shown to be reliable (Ossenkopp and Kavaliers, 1996; Ossenkopp et al., 1987), and valid (Sanberg et al., 1983, 1984, 1987).

METHODS AND MATERIALS

Subjects and Surgery

Adult male Long Evans rats ($N = 20$) weighing 350–500 g were used. Animals were housed individually in hanging wire mesh cages in a colony room maintained at about 21°C under a 12L:12D photoperiod with lights on at 06:00 hr. Food (Agway Rat Chow) and water were available ad libitum.

Eight rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and injected with colchicine (0.5 μg in a 0.5-μl injection; Sigma Chemical Co.) by using a stereotaxic instrument and a 26-gauge cannula. Injections of colchicine were made bilaterally at three sites in the dentate gyrus of the hippocampus selected in accordance with a stereotaxic atlas (Paxinos and Watson, 1986). The coordinates used were: (1) 2.3 mm posterior to the bregma, 3.6 mm ventral to the bregma, and 0.7 mm lateral to the midline; (2) 4.2 mm posterior to the bregma, 3.6 mm ventral to the bregma, and 2.2 mm lateral to the midline; and (3) 5.6 mm posterior to the bregma, 4.0 mm ventral to the bregma, and 3.5 mm lateral to the midline. Injections were made over a period of 6 min, and the cannula was raised only after a further delay of 5 min to allow diffusion into the tissues. No injections were made in the ventral or temporal extremity of the hippocampus in order to avoid the possibility of damage to the entorhinal cortex. Twelve sham-operated control rats were anesthetized and burr holes were drilled through the skull but no injections were made.

Experimental Apparatus

Activity Monitors. The automated Digiscan activity monitoring system consisting of six Digiscan Animal Activity Monitors (Omnitech model

RXY2CM-16, Omnitech Electronics Inc., Columbus, Ohio) was used. Activity test chambers consisted of clear Plexiglas boxes (40 cm × 40 cm × 30 cm) that had a grid of infrared beams mounted horizontally every 2.54 cm along the perimeter (16 infrared beams along each side) and 4.5 cm above the floor. Additionally, vertical activity sensors were located along two sides of the box, approximately 10 cm above the floor. The six monitors were connected to a Digiscan Analyzer (Omnitech model DCM-8) that transmitted the activity data to a microcomputer. Five activity variables, either calculated directly by the Digiscan Analyzer or derived from the measured variables, were examined during a given time sample: (1) total distance (centimeters), the horizontal distance traveled by an animal; (2) movement time (seconds), amount of time the animal spent engaged in movement; (3) number of movements, number of movements made by an animal; (4) vertical activity (arbitrary units), total number of beam interruptions that occurred in the vertical sensors; and (5) center time (seconds), amount of time the animal spent in the center of the testing box (animal's center of gravity more than 6.35 cm away from any wall).

Odor Exposure Apparatus. Rats were exposed to the various odors in a varnished wooden box that was divided into six equal compartments (32 × 15 × 15 cm each). The box had a Plexiglas front, to which odor-soaked cotton swabs were attached, and a Plexiglas lid that was closed during exposure.

Procedure

Baseline Activity Monitoring. At mid-photoperiod, each rat was placed individually into an activity monitor and the activity measures were accumulated in 5-min time bins over a 1-hr session (total of 12 time samples). Baseline activity was monitored in this manner on two consecutive days.

Activity Monitoring Following Odor Exposure Conditions. Sham-operated control rats were divided into two groups of six animals, while hippocampal-lesioned rats were divided into two groups of four animals. One group of rats was tested each day for four consecutive days beginning on the day following the second baseline determination. Each group received all four odor exposure conditions within the same day. One of the sham-operated control groups and one of the hippocampal-lesioned groups received the odors in the following order: (1) toluene (an organic solvent, aversive odor), (2) caproic acid (scent of wet goat, nonpredatory animal control odor), (3) control cotton swab (no odor), and (4) 2-propylthietane (2-PT, principal component of weasel anal gland secretion, predator odor). The second group of sham-operated control rats and the second group of hippocampal-lesioned rats received the odors in the following order: (1) control cotton swab, (2) 2-PT, (3) toluene, and (4) caproic acid. For each odor exposure, cotton swabs, saturated with one of the odor solutions, were attached with masking tape inside the Plexiglas front of each compartment

of the exposure box. Once all cotton swabs were in place, one animal was placed into each of the compartments and the lid was closed. Following each odor exposure (3 min), animals were removed from the compartments, placed in their home cages, and transported to an adjacent room where activity was monitored. This process required approximately 1–2 min. Activity was monitored in the same manner as during the baseline recording (see above).

Following each odor exposure/activity monitoring condition, rats were returned to their home cages and allowed free access to food and water. The time between conditions was 20–30 min. The wooden odor exposure box was thoroughly washed using very hot water delivered under high pressure following each odor exposure condition. No soap or other scented cleaning agent was used. The Plexiglas activity monitors were thoroughly cleaned with soap and water and rinsed with baking soda and water between each activity recording session.

Data Analyses

Initial analyses were performed on differences in activity levels between the two groups following each odor exposure during the 60 min of activity monitoring. These analyses revealed that activity levels decreased across the hour in a similar fashion for both groups. The effects of the various odor exposure conditions on subsequent activity were greatest during the initial 20 min of activity monitoring. Thus, results will be presented for this time period only. A mixed design analysis of variance (ANOVA) was used to analyze the data. Group (hippocampal-lesioned; sham-operated) was the between-subject variable while condition (second day of baseline activity monitoring and four odor exposure conditions) and time (four time samples) were the within-subject factors. Since no effects of time were noted (i.e., no interaction between time and group or time and condition), the data were simplified by averaging activity across the 20-min test session for each activity variable separately. The effect of presentation order on total distance was investigated using a mixed design ANOVA by including order (order 1; order 2) as the between-subject factor and condition as the within-subject factor. Posthoc comparisons were performed using Tukey's procedure, and all hypothesis tests used $\alpha = 0.05$ as the criterion for significance.

RESULTS

Histology. All rats had confirmed lesions of CA1 cells and dentate cells throughout most of the length of the hippocampus. No damage was sustained to the entorhinal or perirhinal cortical areas in any animal. Details of the his-

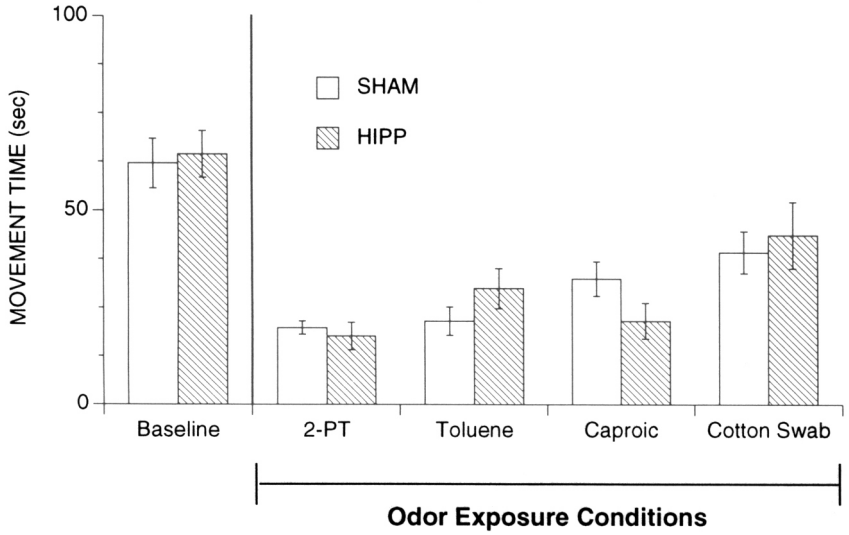


FIG. 1. Average center time during 20 min by hippocampal-lesioned (HIPP, $N = 8$) and sham-operated control (SHAM, $N = 12$) male rats during baseline activity monitoring and following exposure to 2-propylthietane (2-PT, weasel odor), an organic solvent (toluene), a nonpredatory animal odor (goat, caproic acid), and a no-odor control condition (cotton swab).

tological analyses can be found elsewhere (Heale et al., 1996) since the rats (hippocampal-lesioned and sham-operated controls) used in the present study had served as subjects in an investigation two to three months prior.

Locomotor Activity. The ANOVA for presentation order did not reveal a main effect of order ($P = 0.768$) or an interaction ($P = 0.235$) between order and condition for total distance traveled. Thus, presentation order of the odor conditions did not appear to affect the results.

Figure 1 shows that hippocampal-lesioned rats spent more time in the center of the activity monitoring boxes than sham-operated rats across all conditions (main effect of group $F_{1,18} = 7.023$, $P = 0.016$).

Main effects of condition were found for total distance $F_{4,72} = 21.059$, $P < 0.001$; Figure 2), movement time $F_{4,72} = 23.490$, $P < 0.001$; Figure 3), number of movements $F_{4,72} = 19.282$, $P < 0.001$; Figure 4), and vertical activity $F_{4,72} = 25.670$, $P < 0.001$; Figure 5). Posthoc comparisons revealed that levels of all of these variables were lower following all of the odor exposure conditions compared with baseline ($P < 0.01$ for all variables). In addition, posthoc comparisons revealed that total distance and movement time were greater following the cotton swab control condition compared with 2-PT ($P < 0.01$) and toluene ($P < 0.05$). The number of movements and vertical activity also

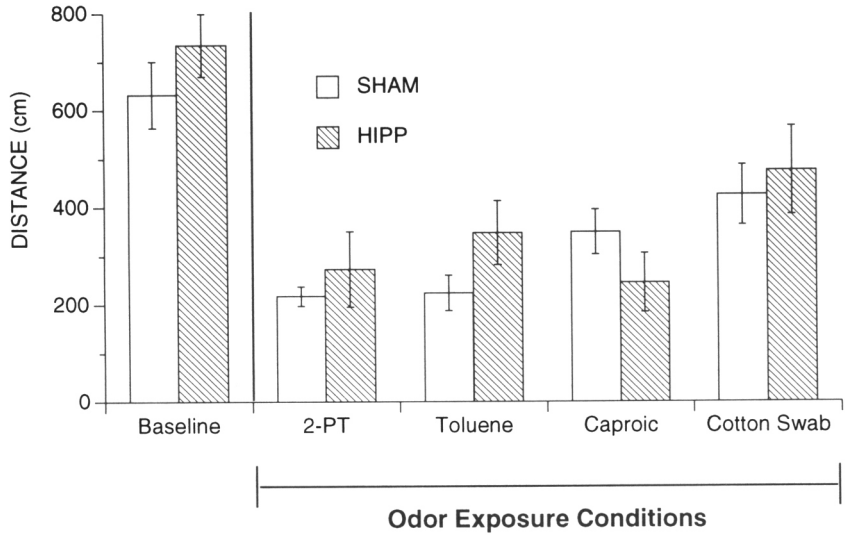


FIG. 2. Average distance traveled during 20 min by hippocampal-lesioned (HIPP, $N = 8$) and sham-operated control (SHAM, $N = 12$) male rats during baseline activity monitoring and following exposure to 2-propylthietane (2-PT, weasel odor), an organic solvent (toluene), a nonpredatory animal odor (goat, caproic acid), and a no-odor control condition (cotton swab).

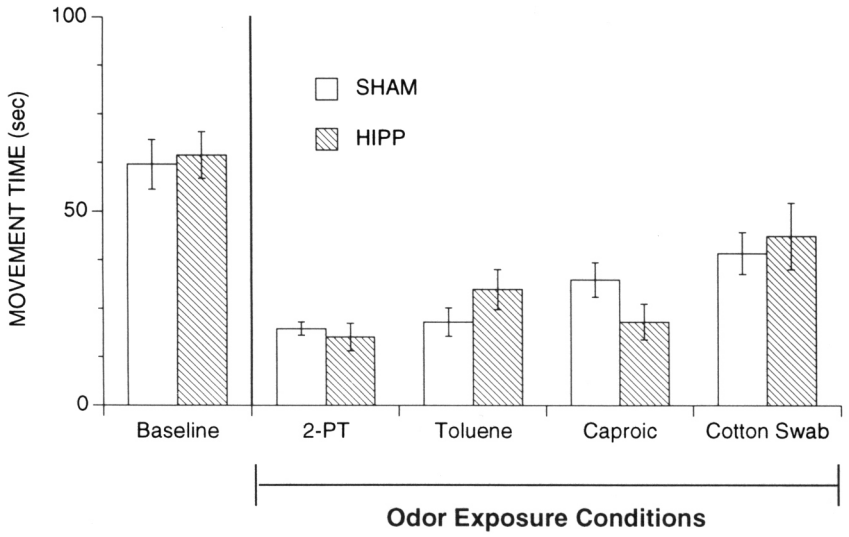


FIG. 3. Average movement time during 20 min by hippocampal-lesioned (HIPP, $N = 8$) and sham-operated control (SHAM, $N = 12$) male rats during baseline activity monitoring and following exposure to 2-propylthietane (2-PT, weasel odor), an organic solvent (toluene), a nonpredatory animal odor (goat, caproic acid), and a no-odor control condition (cotton swab).

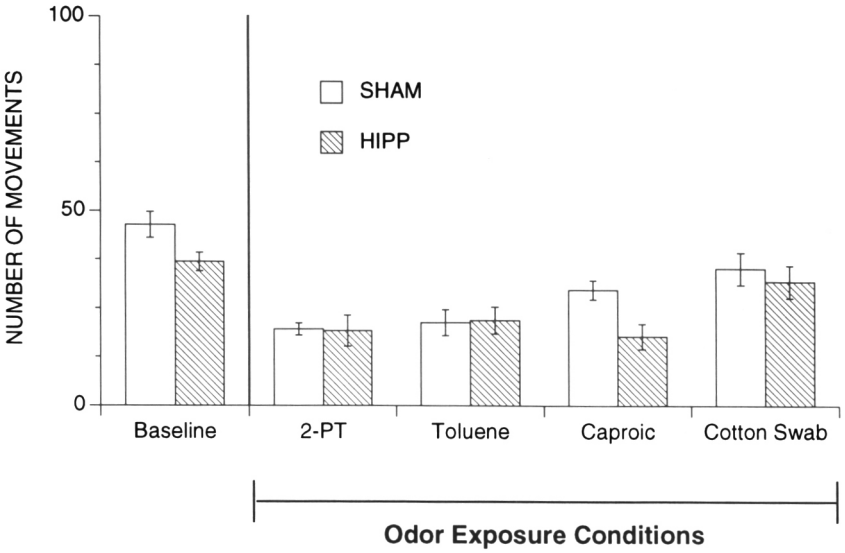


FIG. 4. Average number of movements made during 20 min by hippocampal-lesioned (HIPP, $N = 8$) and sham-operated control (SHAM, $N = 12$) male rats during baseline activity monitoring and following exposure to 2-propylthietane (2-PT, weasel odor), an organic solvent (toluene), a nonpredatory animal odor (goat, caproic acid), and a no-odor control condition (cotton swab).

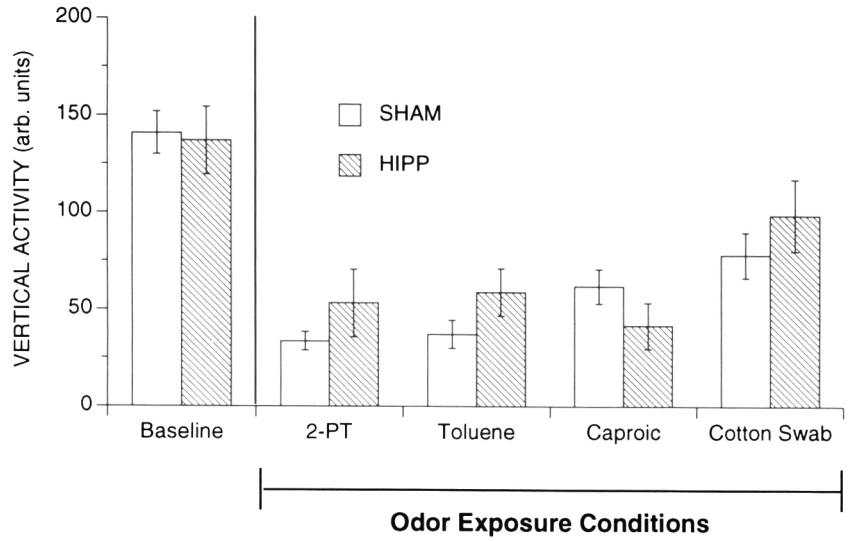


FIG. 5. Average vertical activity during 20 min by hippocampal-lesioned (HIPP, $N = 8$) and sham-operated control (SHAM, $N = 12$) male rats during baseline activity monitoring and following exposure to 2-propylthietane (2-PT, weasel odor), an organic solvent (toluene), a nonpredatory animal odor (goat, caproic acid), and a no-odor control condition (cotton swab).

were higher following the cotton swab control condition compared with 2-PT ($P < 0.01$), toluene ($P < 0.01$), and goat odor ($P < 0.05$). No differences between hippocampal-lesioned and sham-operated control rats were found.

DISCUSSION

Male laboratory rats displayed a reduction in levels of all locomotor activity variables measured following exposure to predator odor (2-PT) and toluene compared with a no-odor condition. Exposure to a nonpredatory animal control odor (caproic acid) resulted in a reduction in levels of some of the activity variables monitored. There were no differences in activity levels between hippocampal-lesioned and sham-operated rats during baseline activity recording or following exposure to any of the odors. However, hippocampal-lesioned rats did spend less time in the center of the activity boxes than sham-operated control rats across all conditions.

The reduction in levels of activity following exposure to the odor of a predator (2-PT) is consistent with past work from our laboratory, which found that adult breeding male meadow voles showed decreased levels of various measures of locomotor activity following exposure to trimethyl thiazoline (fox odor), compared with a no-odor condition (Perrot-Sinal et al., 1996). However, to date, the present results apparently provide the first detailed multivariate assessment of the effects of predator odor on the locomotor activity of laboratory rats. Previously, rats have been shown to respond to a cat exposure with increased freezing behaviors (Blanchard and Blanchard, 1972). The present findings also are consistent with past field work in microtine rodents showing that these animals avoid traps and tunnels in their environment that have been treated with components of predator odors (Dickman and Doncaster, 1984; Stoddart, 1976; Sullivan et al., 1988).

The present finding of a similar behavioral response following exposure to both 2-PT and toluene is consistent with previous electrophysiological work in the rat. It has been shown that olfactory stimulation with 2-PT, trimethyl thiazoline, and toluene elicited bursts of 15- to 30-Hz fast waves in the dentate gyrus of the male rat hippocampus (Heale et al., 1994). Other odorous substances such as ammonia and caproic acid (goat odor) were ineffective in comparison (Heale et al., 1994).

In the present study, however, we demonstrated that in addition to altered activity levels following 2-PT exposure, adult rats showed reduced levels of activity following exposure to toluene and a nonpredatory animal odor (caproic acid). This is somewhat inconsistent with the results from our past meadow vole study, which noted that activity levels were not altered following exposure to other pungent odors (i.e., butyric acid and extract of orange) (Perrot-Sinal et al., 1996). However, the discrepancy between the results from the meadow vole

study and the present findings likely is due to the use of butyric acid and extract of orange as control odors in the previous study and caproic acid in the present investigation. We did find qualitative differences in the behavioral response following exposure to 2-PT and caproic acid, and these are more consistent with a selective reduction of activity in response to the odor of a predator. These shifts in activity following exposure to a nonpredatory odor likely reflect responsiveness to a salient, biologically relevant novel odor.

It is possible, since odor exposure conditions were presented within the same day and only in two different presentation orders, that the order of presentation of the conditions influenced the results. Since no differences were noted between groups based on the order of presentation, we think it is unlikely that the results are due to an order effect. Although we can not be certain that some sort of stimulus generalization did not occur using the present design, this seems unlikely since we previously have noted a reduction of activity selectively following predator odor using a similar procedure (i.e., all conditions given in one day) (Perrot-Sinal et al., 1996). Future study in this area, however, might be improved by administering one odor per day.

In the present investigation, lesioning the granule cells of the dentate gyrus in the rat hippocampus did not affect the locomotor response to various odors, including predator odor. As previously mentioned, electrophysiological work has shown that the dentate gyrus displayed fast-wave activity selectively following exposure to odors such as predator odor and toluene (Heale et al., 1994). The present findings, however, do agree in part with the results of a recent investigation of the role of the hippocampal formation in the olfactory control of feeding behavior. The results of that study suggested that lesioning the dentate gyrus does not alter the normal feeding response of rats, which includes avoiding foods scented with weasel odor or toluene (Heale et al., 1996). However, lesioned animals did show an abnormal aversion to various odors including 2-PT, cadaverine (rotting flesh odor), butyric acid, and caproic acid compared with intact controls (Heale et al., 1996).

The present finding of similar basal levels of locomotor activity between hippocampal-lesioned and sham-operated rats is inconsistent with some past studies (O'Keefe and Nadel, 1978) but consistent with others (Blanchard and Blanchard, 1972). Differences in type and extent of lesion as well as measures of activity used could account for this discrepancy. In particular, it has been suggested that increases in locomotor activity are noted when damage is sustained to the posterior (entorhinal) cortex (Bender et al., 1968), which did not occur in the present study.

The present study did find that hippocampal-lesioned rats spent significantly more time in the center of the open field testing apparatus than sham-operated rats. This center-time activity variable has been used previously as a negative thigmotaxis measure (Perrot-Sinal et al., 1996). Thigmotaxis is best described

as wall-hugging behavior and is observed in most species of small rodent following placement in a novel, potentially threatening environment (Treit and Fundytus, 1989). Thigmotaxis is part of an animal's natural repertoire of defensive behaviors and is reduced by anxiolytic drugs such as chlorpromazine (Treit and Fundytus, 1989). Thus, the hippocampal-lesioned animals in the present study may have been displaying less defensive or "fear" behaviors in response to the open field compared with sham-operated rats. This is consistent with the finding of reduced defensive freezing behaviors in hippocampal-lesioned rats following exposure to a cat (Blanchard and Blanchard, 1972). An alternative explanation for the difference in center time between hippocampal-lesioned and sham-operated rats may involve differences in the patterning of behavior. Past work on the open-field behavior of hippocampal-lesioned rats has demonstrated that hippocampectomized animals often adopt a stereotyped pattern of behavior that includes going back to the same areas over and over again (Kimble, 1963).

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IDENTIFICATION OF SEX PHEROMONE COMPONENTS OF NETTLE CATERPILLAR, *Setothosea asigna*

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Abstract—Gas chromatographic-electroantennographic detection (GC-EAD) analyses of female nettle caterpillar, *Setothosea asigna*, pheromone gland extracts revealed seven antennally active compounds. Based on their retention indices on three fused silica columns (DB-5, DB-23, and DB-210), these compounds were hypothesized and, through comparative GC, GC-EAD and GC-mass spectrometry with authentic standards, confirmed to be Δ10-undecenal, dodecanal, (*E*)-9-dodecenal (*E*9-12:Ald), (*Z*)-9-dodecenal, (*E*)-9-dodecen-1-ol, (*E*)-9,11-dodecadienal (*E*9,11-12:Ald), and (*E*)-9,11-dodecadienol. *E*9-12:Ald and *E*9,11-12:Ald were most abundant in female *S. asigna* pheromone extracts. In field trapping experiments in Palembang, Indonesia, synthetic *E*9-12:Ald and *E*9,11-12:Ald at a 1:1 ratio, but not singly, attracted *S. asigna* males. Attractiveness of these two aldehydes could not be enhanced further through the addition of their corresponding alcohols and/or other aldehydic candidate pheromone components. Use of *E*9-12:Ald and *E*9,11-12:Ald for pheromone-based monitoring of *S. asigna* populations will require lure formulations that minimize pheromone degradation by ultraviolet radiation and atmospheric oxidation.

Key Words—*Setothosea asigna*, nettle caterpillar, Limacodidae, Lepidoptera, sex pheromone, (*E*)-9-dodecenal, (*E*)-9,11-dodecadienal, oil palm, *Elaeis guineensis*.

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INTRODUCTION

Larvae of the nettle caterpillar, *Setothosea asigna* (Van Eecke) (Lepidoptera: Limacodidae) are important defoliators of oil palm, *Elaeis guineensis* Jacq., plantations in Southeast Asia. During severe outbreaks, *S. asigna* causes complete defoliation of mature palms and severely reduces fruit bunch production in the following year (Wood et al., 1973). Various applications of chemical and biological agents to control this lepidopteran pest have been reported (Wood et al., 1976; Wood, 1987; Chung et al., 1995), but little is known about its behavioral and chemical ecology. Zagatti et al. (1993) identified five compounds produced by *S. asigna* females and field tested four of them in several but not all possible combinations. Traps baited with a blend of two components, (*E*)-9-dodecenal (*E*9-12:Ald) and (*E*)-9,11-dodecadienal (*E*9,11-12:Ald), were attractive to males, but Zagatti et al. (1993) did not conclude that this blend represented the complete pheromone. We report the results of an exhaustive study on the identity and bioactivity of all candidate pheromone components for *S. asigna*.

METHODS AND MATERIALS

Experimental Insects. S. asigna pupae were collected in commercial oil palm plantations in Medan, Indonesia, kept in filter paper-lined Petri dishes, and placed in a screen house with a natural 12L:12D photoperiod. Emergent males and females were sexed and held in separate Petri dishes to avoid mating. Because 24-hr observations of fifty 1-, 2-, or 3-day-old females revealed that they began calling behavior ~30 min after sunset, pheromone glands were extracted ~1 hr into the scotophase. Pheromone glands were extracted for 5 min in HPLC-grade hexane, and gland extracts and *S. asigna* pupae (for rearing of adults) were sent to Simon Fraser University. Aliquots of one female equivalent (FE) of pheromone extract were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Figure 1) (Arn et al., 1975) on fused silica columns (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-23, or DB-210 (J&W Scientific, Folsom, California). For these recordings an antenna from a male moth was suspended between two glass capillary electrodes filled with a saline solution (Staddon and Everton, 1980). Antennal responses were amplified using a custom-built amplifier with a passive low-pass filter and a cutoff frequency of 10 khz. The area under each antennal response in the EAD trace was compared to that of an induced known voltage. Coupled GC–mass spectrometry in electron impact mode employed a Varian Saturn II ion trap fitted with the DB-5 column described above.

Test Chemicals. (*E*)-9- and (*Z*)-9-Dodecenyl acetates were purchased from

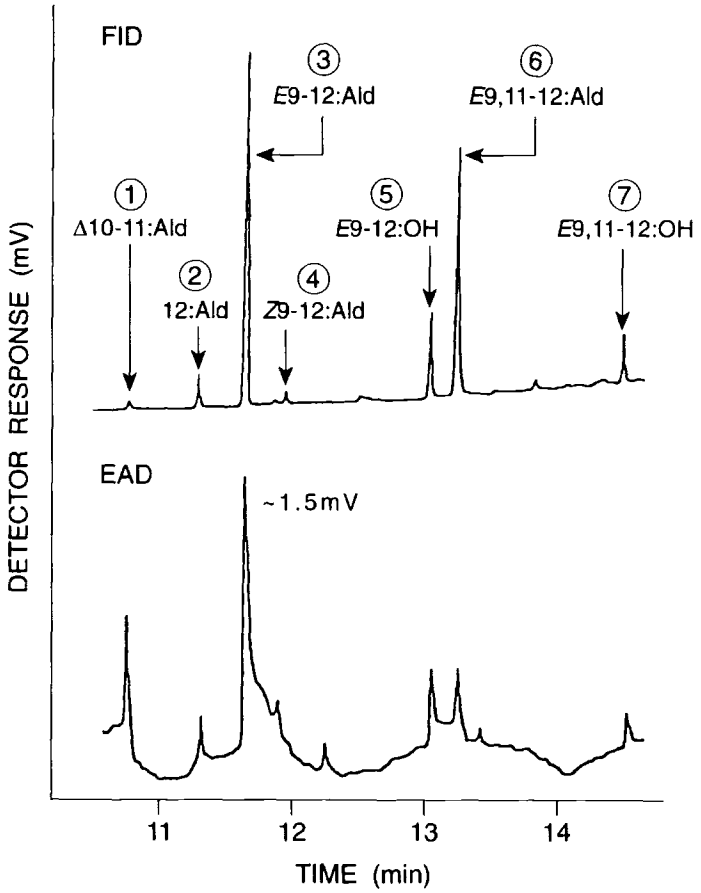


FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD: male *S. asigna* antenna) responses to one female equivalent of female *S. asigna* pheromone gland extract, chromatographed on a DB-23 column: 1 min at 50°C, 10°C/min to 200°C. Retention indices of EAD-active compounds **1**–**7** on columns coated with DB-23, DB-5, and DB-210 are as follows: DB-23: **1** = 1734; **2** = 1788; **3** = 1822; **4** = 1834; **5** = 1971; **6** = 1992; **7** = 2138; DB-5: **1** = 1313; **2** = 1413; **3** = 1404; **4** = 1410; **5** = 1469; **6** = 1442; **7** = 1501; DB-210; **1** = 1623; **2** = 1715; **3** = 1720; **4** = 1740; **5** = 1662; **6** = 1830; **7** = 1767.

Bedoukian (Danbury, Connecticut), converted to (*E*)-9-dodecen-1-ol (*E*9–12:OH) and (Z)-9-dodecen-1-ol (Z9–12:OH), and then oxidized with pyridinium chlorochromate (PCC) to (*E*)-9-dodecenal (*E*9–12:Ald), and (*Z*)-9-dodecenal (Z9–12:Ald), respectively. Dodecenol was purchased from Aldrich

(Milwaukee, Wisconsin) and converted with PCC to dodecanal (12:Ald). Δ10-Undecenal was available in our laboratory from previous work. Synthesis of *E*9,11-12:Ald (Figure 2) employed coupling of 3-sulfolene (**I**) with *O*-tetrahydropyrano-8-iodo octanol (**II**) (Yamada et al., 1983). Thermal decomposition of the coupling product (**III**) and subsequent removal of the tetrahydropyrane (THP) protective group yielded 47% (based on **II**) of (*E*)-9,11-dodecadienol (**IV**), which was oxidized with PCC to (*E*)-9,11-dodecadienal (**V**). Spectroscopic data of **IV** and **V** were consistent with those previously reported (Ando et al., 1985a,b). All test chemicals were >95% chemically pure. Butylated hydroxy toluene (1%) was added to monoene and diene aldehyde solutions to minimize (oxidative) degradation.

Field Experiments. Field experiments (Figure 3) were conducted in a commercial oil palm plantation in Palembang, Indonesia. Self-made sticky delta traps (Gray et al., 1984) were suspended from oil palms ~1.5–2 m above ground in randomized complete blocks, with traps and blocks at 15- to 20-m intervals. Traps were baited with rubber septa (Sigma Chemical Co., St. Louis, Missouri) impregnated with candidate pheromone components in HPLC-grade hexane. The number of captured *S. asigna* males was recorded at the end of each experiment. The first experiment tested the two major candidate pheromone components *E*9-12:Ald and *E*9,11-12:Ald alone and in combination (Figure 3, experiment 1). Experiment 2 tested *E*9-12:Ald and *E*9,11-12 Ald alone and in combination at different ratios (Figure 3, experiment 2). Experiment 3 tested

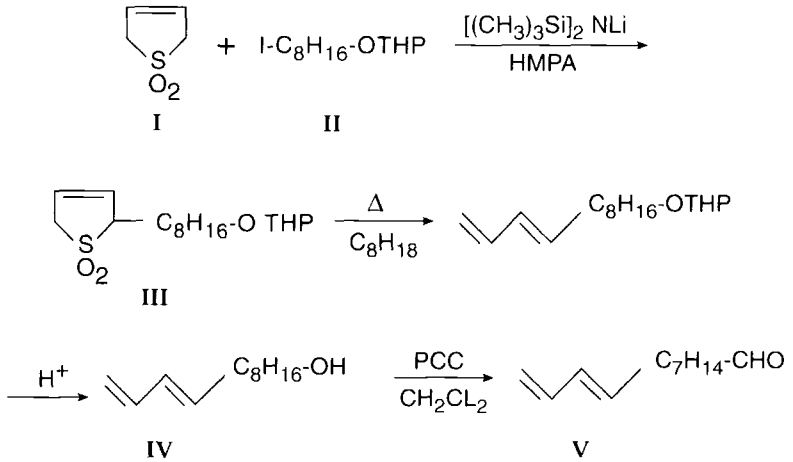


FIG. 2. Scheme for the synthesis of (*E*)-9,11-dodecadienal.

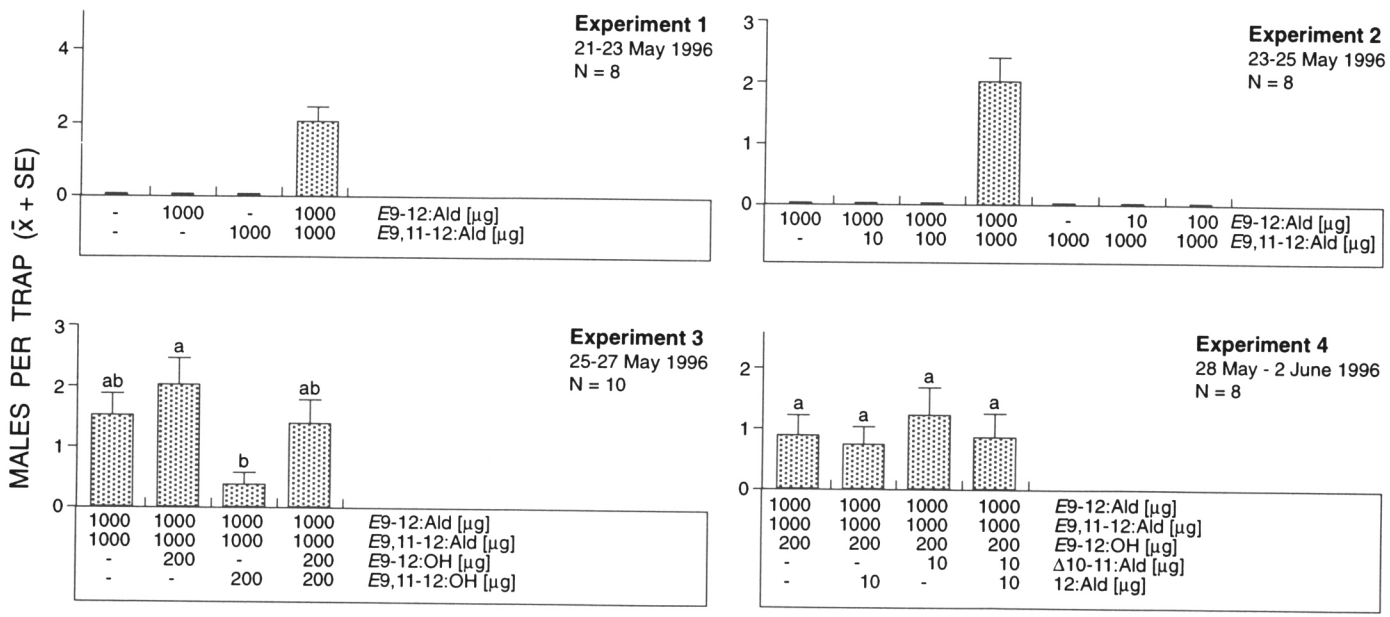
*E*9-12:Ald plus *E*9,11-12:Ald alone and in binary and ternary combinations with their corresponding alcohols (Figure 3, experiment 3). Experiments 4–6 tested *E*9-12:Ald plus *E*9,11-12:Ald alone and in combinations with other EAD-active (Figure 1) candidate pheromone components (Figure 3, experiments 4–6). Experiment 7 tested *E*9-12:Ald plus *E*9,11-12:Ald at increasing doses (Figure 3, experiment 7). A final experiment tested attractiveness of *E*9-12:Ald plus *E*9,11-12:Ald versus that of virgin *S. asigna* females (Figure 3, experiment 8). Each female in experiment 8 was confined in a screened, cylindrical cage (3 × 5 cm) in the middle of the trap and was sustained with a sugar-water-drenched cotton ball.

Voucher speciments of captured male moths will be deposited in the entomological collection at Simon Fraser University.

Statistical Analysis. Various treatments in several experiments did not attract any male moths. Despite transformation, trap catch data were thus not normally distributed and were analyzed using nonparametric analyses of variance (Friedman's test) followed by the Student-Newman-Keul's test (Zar, 1984; SAS/STAT User's Guide, 1988, SAS Institute Inc., Cary, North Carolina). In all analyses, $P < 0.05$ levels of significance were used.

RESULTS

GC-EAD analyses of female *S. asigna* pheromone extract revealed seven EAD-active compounds (Figure 1). Based on their retention indices (Van den Dool and Kratz, 1963) on three fused silica columns (DB-5, DB-23, and DB-210), the most EAD-active and abundant compounds **3** (~3 ng/FE) and **6** (~1.5 ng/FE) were hypothesized, and through comparative GC-MS with authentic standards confirmed, to be *E*9-12:Ald (**3**) and *E*9,11-12:Ald (**6**). GC and GC-EAD characteristics of compounds **1, 2, 4, 5** and **7** in pheromone extracts (Figure 1) were consistent on three columns (see above) with Δ10-11:Ald (**1**), 12:Ald (**2**), *Z*9-12:Ald (**4**), *E*9-12:OH (**5**), and *E*9,11-12:OH (**7**). In field experiments, *E*9-12:Ald and *E*9,11-12:Ald in combination, but not singly, attracted *S. asigna* males (Figure 3, experiment 1). A 1:1 ratio of *E*9-12:Ald and *E*9,11-12:Ald was more attractive to *S. asigna* males than was any other blend ratio tested (Figure 3, experiment 2). The attractiveness of *E*9-12:Ald plus *E*9,11-12:Ald could not further be enhanced by addition of their corresponding alcohols (Figure 3, experiment 3) and/or other candidate aldehyde pheromone components (Figure 3, experiments 4–6). Increasing the dose of *E*9-12:Ald plus *E*9,11-12:Ald resulted in increased numbers of males captured (Figure 3, experiment 7). Blends of *E*9-12:Ald plus *E*9,11-12:Ald attracted more *S. asigna* males than did unmated females (Figure 3, experiment 8).


MALES PER TRAP (x̄ + SE)
Experiment 1
21-23 May 1996
N = 8
- 1000 - 1000 E9-12:Ald [µg]
- - 1000 1000 E9,11-12:Ald [µg]
Experiment 2
23-25 May 1996
N = 8
1000 1000 1000 1000 - 10 100 E9-12:Ald [µg]
- 10 100 1000 1000 1000 1000 E9,11-12:Ald [µg]
Experiment 3
25-27 May 1996
N = 10
ab a b ab
1000 1000 1000 1000 E9-12:Ald [µg]
1000 1000 1000 1000 E9,11-12:Ald [µg]
- 200 - 200 E9-12:OH [µg]
- - 200 200 E9,11-12:OH [µg]
Experiment 4
28 May - 2 June 1996
N = 8
a a a a
1000 1000 1000 1000 E9-12:Ald [µg]
1000 1000 1000 1000 E9,11-12:Ald [µg]
200 200 200 200 E9-12:OH [µg]
- - 10 10 Δ10-11:Ald [µg]
- 10 - 10 12:Ald [µg]

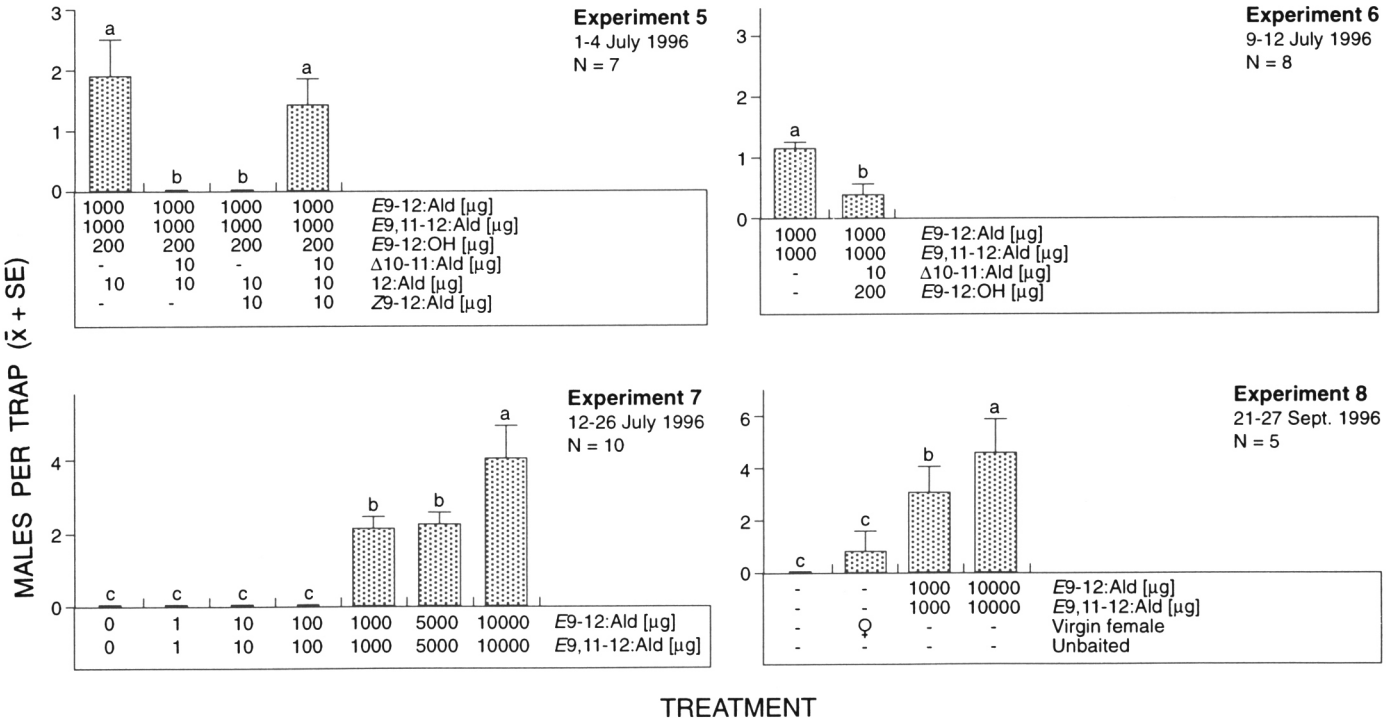


FIG. 3. Mean number and standard error (SE) of *S. asigna* males captured in experiments 1–8 in delta traps baited with candidate pheromone components. For each experiment, bars with the same superscript are not significantly different. Student-Newman-Keul's test, $P < 0.05$.

DISCUSSION

Evidence that *E*9–12 : Ald and *E*9,11–12 : Ald are essential components of the *S. asigna* pheromone blend includes: (1) identical retention and mass spectrometric characteristics of female *S. asigna*-produced **3** (Figure 1) and authentic *E*9–12 : Ald as well as female-produced **6** (Figure 1) and authentic *E*9,11–12 : Ald; (2) greatest abundance of *E*9–12 : Ald and *E*9,11–12 : Ald in *S. asigna* pheromone gland extracts (Figure 1); and (3) attraction of *S. asigna* males to *E*9–12 : Ald plus *E*9,11–12 : Ald in field experiments (Figure 3, experiments 1 and 2).

Although the effluvia of calling *S. asigna* females were not analyzed, the similar abundance of *E*9–12 : Ald and *E*9,11–12 : Ald in gland extracts (Figure 1) and the strongest attraction of males to a 1 : 1 blend of these two aldehydes (Figure 3, experiment 2) suggest that *S. asigna* females release *E*9–12 : Ald and *E*9,11–12 : Ald at similar quantities. Although present in pheromone gland extracts and EAD-active (Figure 1), corresponding *E*9–12 : OH and *E*9,11–12 : OH did not enhance attraction of males to the aldehyde blend (Figure 3, experiments 3–7) and are apparently not part of the *S. asigna* pheromone. However, field-trapping experiments evaluate only attraction of male moths to a pheromone lure and do not determine additional and/or different functional roles of candidate pheromone components. It is conceivable that the presence of either one or both of the alcohols is required during courtship or mating behavior. Alternatively, the alcohols may be biosynthetic precursors of corresponding aldehydes, as demonstrated in heliothine moths (Teal and Tumlinson, 1986).

Although limacodid moths are economically important oil palm defoliators, their chemical communication has hardly been studied (Arn et al., 1992). *E*9–12 : Ald and *E*9,11–12 : Ald constitute the first pheromone components identified in the Limacodidae. Our findings confirm "initial results" by Zagatti et al. (1993) and provide essential further information pertaining to the analysis, field testing, and competitive attractiveness of (candidate) pheromone components. Neither *E*9–12 : Ald nor *E*9,11–12 : Ald has been reported as a pheromone component in other species of the Lepidoptera. Traps baited with *E*9,11–12 : Ald plus *Z*9,11–12 : Ald captured 250 males of *Tortricidia testacea* (Packard) (Chisholm and Reed, 1985), but it remains unknown whether *T. testacea* females indeed produce these compounds and whether attraction of males requires the presence of the *Z* isomer.

Production of *E*9,11–12 : Ald by female *S. asigna* and production of homologous, pheromonal (*E*)-11,13-tetradecadienal (*E*11,13–14 : Ald) by female *Acleris minuta* (Robinson) (Schwarz et al., 1983), *A. variana* (Fern.) (Gries et al., 1994), and *A. gloverana* (Walsingham) (Gray et al., 1996) suggest similar pheromone biosyntheses in these tortricid and limacodid moths. We hypothesize

that *E*11,13-14:Acyl is a biosynthetic intermediate in pheromone glands of *S. asigna* females that undergoes β-oxidation, reduction, and oxidation to pheromonal *E*9,11-12:Ald.

With $<1\%$ of palms infested with *S. asigna* in 1996 (Syed, 1996), relatively few males were captured during all experiments (Figure 3, experiments 1-8). The greater number of males captured in traps baited with synthetic pheromone than in traps baited with a virgin female (Figure 3, experiment 8) indicates competitive attractiveness of the pheromone lure. To implement long-term pheromone-based monitoring of *S. asigna* populations, lure formulations must minimize pheromone degradation by ultraviolet radiation and atmospheric oxidation. The currently developed Metered Semiochemical Timed Released System (MSTRS) (Baker et al., 1996) may prove ideal for use of aldehydic sex pheromones in tropical environments.

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RESPONSE AND ADAPTATION TO ANALOGS OF DISPARLURE BY SPECIALIST ANTENNAL RECEPTOR NEURONS OF GYPSY MOTH, *Lymantria dispar*

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Abstract—Reception and adaptation of antennal receptors in response to (+)-disparlure [(+)-D], the sex attractant pheromone of the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae)], its enantiomer, (−)-D, and analogs of disparlure in which the epoxide group is replaced with various three-membered rings [(±)-cyclopropyl (2HC), (±)-difluorocyclopropyl (2FC), (±)-dichlorocyclopropyl (2ClC), and (±)-azirdinyl (AZ), (+)-AZ, and (−)-AZ] as well as a mixture of 1,1-epoxy analogs were investigated using electroantennograms. Antennal receptors of male moths were more responsive (5 μg stimulus load) to (+)-D than to (−)-D; while responses to each of the analogs were significantly less and did not differ from each other. Antennal receptors of female moths did not respond significantly to any of the compounds at this stimulus load. Dose–response curves revealed greater sensitivity of male antennae to (+)-D than (−)-D. Among the cyclopropane analogs and 1,1-epoxy mixture (1,1E), the order of sensitivity was: 2FC > 2ClC > 2HC > 1,1E. Antennae were least sensitive to the aziridinyl analogs; however, (+)-AZ was more active than (−)-AZ, while an intermediate response was elicited by the racemate. Males exposed to (+)-D adapted to (+)-D, while responses to (+)-D were not significantly affected by exposure to (−)-D, 2FC or (+)-AZ. After exposure to hexylacetate, males showed adaptation to (+)-D or (−)-D, but not to the other odorants. Preliminary field data correlate well with knowledge obtained in our electrophysiological studies and demonstrate the usefulness of such studies. These results indicate that novel approaches to the design and synthesis of potential agonists of disparlure

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receptors are necessary before substantial effects on the activity of disparlure are realized.

Key Words—Analogs, semiochemicals, adaptation, receptor, neurophysiology, olfaction gypsy moth, *Lymantria dispar*.

INTRODUCTION

Since introduction of the gypsy moth, *Lymantria dispar*, into the United States in 1869 and its attainment of pest status (Plimmer et al., 1982), research on it and other potential lymantriid pests has intensified. Characterization of the female-produced sex attractant of the gypsy moth, disparlure (*cis*-7,8-epoxy-2-methyl-octadecane), by Bierl et al. (1970) provided a tool not only for use in population surveys but also for disruption of orientation of males to females (Schwalbe et al., 1983). Subsequently, the (+)-enantiomer of disparlure [(7*R*,8*S*)-epoxy-2-methyl-octadecane = (+)-disparlure = (+)-D] was shown to be attractive, while its antipode, (−)-D, was somewhat inhibitory (Vité et al., 1976).

Elucidation of the chemical structure of disparlure provided the framework for synthesis and testing of analogs as possible agonists, antagonists, or synergists of pheromone responses. Field testing of a number of synthetic epoxides related to disparlure clearly showed that movement of the epoxide or methyl group resulted in substantial loss of attractiveness of the resulting molecule (Bierl et al., 1972; Adler et al., 1972; Sarmiento et al., 1972; Schneider et al., 1974). Replacement of the epoxide group by another group or groups resulted in some compounds that were attractive to gypsy moths in the field, but none was as attractive as disparlure (Sheads et al., 1975).

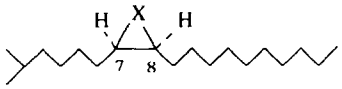
We report results of electrophysiological studies of antennal receptor responses of *L. dispar* to disparlure and analogs of disparlure in which the oxygen of the epoxide group of disparlure is replaced by carbon [cyclopropane (= CP) analogs] or nitrogen [aziridine (= AZ) analogs]. A mixture of 1,1-epoxy analogs previously shown to be active in field test (Sheads et al., 1975) was also tested. Adaptation experiments were done to determine effects of selected analogs on disparlure receptors. A green leaf volatile, hexyl acetate, was also tested in the adaptation studies because it enhances behavioral responses of moths to sex pheromones (Dickens et al., 1993a; Light et al., 1993). These studies provide the bases for field studies and may facilitate design of other potential agonists of disparlure receptors.

METHODS AND MATERIALS

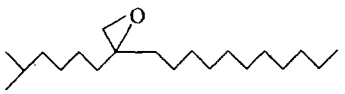
Insects. Pupae of *Lymantria dispar* were obtained from a laboratory colony maintained at the USDA, ARS, Insect Biocontrol Laboratory, Beltsville Agri-

cultural Research Center, Beltsville, Maryland. Pupae were sexed and placed into containers in an incubator at 26°C and a photoperiod of 16L:8D until emergence. Electrophysiological recordings were made from antennae of adults within one to three days after emergence.

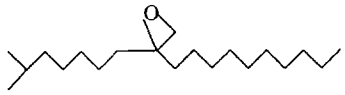
Odorant stimuli. Odorant stimuli used in experimental studies are represented in Figure 1. The enantiomers of disparlure [(+)-D, (7*R*,8*S*)-epoxy-2-methyl-octadecane **1**, and (−)-D, (7*S*,8*R*)-epoxy-2-methyl-octadecane **2**] were greater than 98% optical purity (Oliver and Waters, 1995). Other compounds studied included racemic cyclopropane (2HC), dichlorocyclopropane (2ClC), and difluorocyclopropane (2FC) analogs **5**, **4**, and **3**; the enantiomeric aziridine analogs [(+)-AZ and (−)-AZ] **6** and **7** as well as the racemic mixture (AZ); and the mixture of 1,1-distributed epoxides **8** and **9** (1,1E). Hexyl acetate (99%) used as the standard was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.



1. X = O, 7R, 8S [(+)-disparlure]
2. X = O, 7S, 8R [(-)-disparlure]
3. X = CF_2, 7R, 8S + 7S, 8R
4. X = CCl_2, 7R, 8S + 7S, 8R
5. X = CH_2, 7R, 8S + 7S, 8R
6. X = NH, 7R, 8S
7. X = NH, 7S, 8R



8



9

EAG Activity: 1 > 2 >> 3 > 4 > 5, 8+9 > 6 >7

FIG. 1. Structures of compounds used in electrophysiological and behavioral experiments: **1**; (+)-disparlure [(7*R*,8*S*)-epoxy-2-methyl-octadecane], the principal sex attractant of the gypsy moth, **2**; (−)-disparlure, an inhibitor of behavioral responses to (+)-disparlure, **3**; difluorocyclopropyl; **4**, dichlorocyclopropyl; and **5**, cyclopropyl, are disparlure analogs; **6**, (+)-aziridinyl and **7**, (−)-aziridinyl analogs; **8** + **9** mixture of 1,1-epoxide analogs. Stimulatory effectiveness of the compounds is (**1** > **2** >> **3** > **4** > **5**, **8** + **9** > **6** > **7**).

Chemical Syntheses. Gas chromatography (GC) was performed on Shimadzu instruments equipped with flame ionization detectors and either a 60-m SPB 608 or a 30-m DB-1 capillary column. Compositions of mixtures are as reported by Shimadzu CR501 Chromatopac recording integrators and are uncorrected for response or other factors. Mass spectra (MS) were recorded with a Finnigan Incos 50 GC-MS with a source block temperature of 150°C.

(±)-1-Decyl-2-(2-methylhexyl)-cyclopropane **5** was prepared from 2-methyl-7-octadecene 90.4% (*Z*) and 9.6% (*E*) and diiodomethane/tetraethylaluminum according to the procedure of Maruoka et al. (1985) and was purified by flash chromatography on silver nitrate-impregnated silica gel with hexane. The product after chromatography contained (by GC) 88.6% of **5**, 9.7% of the *trans* isomer, and 1.7% of (*E*)-2-methyl-7-octadecene. The mass spectrum of **5** showed ions (abundance) at *m/z*, (%): 280, M+ (1), 252 (1), 125 (6), 111 (19), 98 (10), 97 (39), 85 (16), 84 (24), 83 (53), 82 (15), 81 (10), 71 (29), 70 (42), 69 (80), 68 (15), 67 (20), 57 (83), 56 (95), 55 (75), 54 (16), 43 (100), 42 (14), and 41 (76).

For (±)-2-decyl-3-(2-methylhexyl)-1,1-dichlorocyclopropane **4**, a solution of 2-methyl-7-octadecene [ca. 0.55 g, 90.4% (*Z*) and 9.6% (*E*)] in chloroform was stirred and cooled to −30°C, and a two-phase mixture prepared by dissolving potassium hydroxide (3 g) in 40% aqueous tetrabutylammonium hydroxide (10 ml) was added dropwise from an addition funnel while a second 5-ml portion of chloroform was added dropwise from a second addition funnel (extremely vigorous initial reaction!). The thick mixture was allowed to slowly warm to room temperature, stirred for 1.5 days at room temperature, and then was thoroughly partitioned between 2 N hydrochloric acid and hexane. The hexane solution was washed well with water then with aqueous sodium carbonate, dried with magnesium sulfate, and concentrated. The residue (729 mg) was flash chromatographed on silica gel with cyclohexane (essentially no retention) to give 687 mg (ca. 95%) of **4** as a clear oil that consisted (by GC) of 90.8% *cis*-**4**, 7.7% *trans*-**4**, 0.4% unreacted (*Z*)-olefin, and 1.2% unreacted (*E*)-olefin. The mass spectrum of **4** showed ions (abundance) at *m/z* 123 (10), 109 (10), 97 (11), 95 (13), 83 (19), 82 (13), 81 (19), 79 (12), 71 (12), 70 (21), 69 (51), 57 (49), 56 (54), 55 (39), 43 (100), 42 (11), and 41 (58).

For (±) 2-decyl-3-(2-methylhexyl)-1,1-difluorocyclopropane **3**, the general procedure of Crabbé et al. (1973) was modified slightly in that sodium chlorodifluoroacetate was added in small portions as a solid from a solids addition funnel to a solution of 2-methyl-7-octadecene in refluxing diglyme. The reaction was monitored by removing aliquots for GC analysis and was continued until essentially all of the olefin had been consumed. Because of the relative unreactivity of 2-methyl-7-octadecene, rather large excesses of sodium chlorodifluoroacetate had to be employed. The olefin employed in this reaction had been purified by repeated flash chromatography on silver nitrate-impregnated silica

gel (hexanes followed by 2.5% ethyl acetate in hexanes), and the product was flash chromatographed on silica gel with petroleum ether to give **3** as a 99:1 *cis–trans* mixture. The mass spectrum of **3** showed ions (%) at *m/z* 149 (2), 135 (3), 121 (5), 109 (7), 97 (12), 96 (15), 95 (15), 85 (13), 84 (9), 83 (21), 82 (21), 81 (16), 77 (10), 71 (11), 70 (23), 69 (42), 68 (10), 67 (13), 57 (54), 56 (49), 55 (36), 43 (100), 42 (13), and 41 (59).

The mixture of 1,2-epoxy-2-(5-methylhexyl)tridecane and 1,2-epoxy-2-(6-methylheptyl)dodecane, **8** and **9**, the 1,1-disubstituted epoxides, was prepared as described by Sheads et al. (1975) and used as an unresolved mixture (two geometric isomers, four diastereomers).

Aziridines **6** and **7** (ca. 98% enantiomeric purity) were synthesized from **2** and **1**, respectively, as described previously (Oliver and Waters, 1995). Although no direct determinations of their enantiomeric purities were made, the conversions involve inversion of configuration at each carbon of the three-membered ring, and the enantiomeric composition is believed to reflect that of the starting disparlure samples [had inversion not been realized at one carbon, a *trans*-aziridine, not the enantiomerically opposite *cis*-aziridine, would have been obtained (Oliver and Waters, 1995)]. The (7*R*,8*S*) aziridine **6** was prepared from (7*S*,8*R*)-**2** synthesized here, which had been determined to contain about 2.2% of **1** (Oliver et al., 1996), and the (7*S*,8*R*) isomer **7** was prepared from a sample of **1** that contained about 1.5% of **2**. It should be noted that when **6** and **7** are referred to as (+)-AZ and (−)-AZ, respectively, the (+) and (−) are used to facilitate comparison of absolute configurations to those of (+)- and (−)-disparlure and do not necessarily indicate direction of rotation of polarized light, which has not been determined.

The cyclopropane analogs **3**, **4**, and **5** (all racemic) were prepared by addition of the appropriate carbene to (*Z*)-2-methyl-7-octadecene as described above. The stereochemistry of the products closely reflects that of the olefin employed, and since **4** and **5** were made from olefin containing about 10% of the *E* isomer, the predominantly *cis*-cyclopropanes contained about 10% of their *trans*-isomers. The olefin used for the preparation of **3** was more rigorously purified, and the resulting difluorocyclopropane was approximately 99% *trans*.

Electrophysiological Techniques. Electroantennograms (EAGs) were used to compare the efficacy of the homologous analogs on antennal pheromone receptors in male moths (Kaissling, 1995) and to elucidate potential presence of receptors in females. EAGs were recorded from adult males and females using a modification of an earlier technique (Schneider, 1957; Dickens et al., 1993b). An excised antenna was fixed between two glass capillary electrodes filled with 0.1 M NaCl. Ag–AgCl wires in the glass capillaries connected the preparation to the recording instruments: a Grass P-16 preamplifier, a Tektronix 5111A analog storage oscilloscope, and a Alpken 310 stripchart recorder.

Experimental Protocol. Three experiments were performed. The objective

of the first experiment was to determine the relative responsiveness of both male and female antennal preparations to an elevated stimulus load (5 μg) of all experimental compounds. Thus, EAGs to 5-μg stimulus loads were recorded for both sexes. This experiment was replicated six times for both sexes.

In a second experiment, the responsiveness of male antennal receptors to each odorant was determined by recording EAGs to serially increasing stimulus loads of the experimental odorants. Six replicates for both sexes were recorded for this experiment.

The third experiment determined the effects of exposure of male moths for five hours to volatiles emanating from 50 μg of (+)-D, (−)-D, 2FC, and (+)-AZ analogs of disparlure (5 μl of 10 μg/μl experimental odorant in hexane solvent) on EAGs to (+)-D and the plant odor, hexyl acetate [stimulus load = 0.05 μg (+)-disparlure or 100 μg hexyl acetate; these stimulus loads were chosen as they elicit similar EAG depolarizations in male moths prior to adaptation (ca. 0.5 mV)]. The adapting odorants were selected based on their high activity relative to the other analogs in the first two experimental series. A control consisted of exposure of the moth to an equivalent amount (5 μl) of the hexane solvent alone. During the 5-hr exposure, individual moths were placed in disposable plastic cups (4 cm diameter × 4 cm height) with paper lids and held in the dark at ambient temperature (ca. 20°C). Ten replicates were recorded for each adapting odorant in this experiment. Prior to exposure to the adapting odorant, one antenna was amputated from a male moth and EAGs were recorded to (+)-D and hexyl acetate. Following the 5-hr exposure, the remaining antenna was amputated and EAGs recorded within less than 3 min in response to the same odorants.

Odorants used to elicit EAGs were serially diluted in hexane and were delivered as 5-μl aliquots of an appropriate serial dilution placed on a piece of Whatman No. 1 filter paper (8 × 18 mm). The filter paper was inserted into a glass tube (80 mm long × 5 mm ID) and oriented toward the preparation with the outlet 1 cm from the antenna. The hexane solvent was allowed to evaporate for 15 sec prior to use of the odor cartridge. Each stimulation lasted 1 sec. Airflow was 1 liter/min as measured by a flowmeter. The time between stimuli was 2–3 min. Five microliters of the hexane solvent served as a control after evaporating for 15 sec from the filter paper. In order that responses from different preparations might be compared, hexyl acetate (100 μg stimulus load) was used as a standard. Hexyl acetate was selected from several green leaf volatiles (Visser et al., 1979) since responses to it at this stimulus load were reliable. Stimulation with the standard preceded or followed the response to each experimental stimulus. Responses are represented as percent responses of the mean of the two nearest responses to the standard.

Statistical Analyses. Electroantennograms were evaluated by analysis of

variance (Ostle, 1963) and comparisons were made between treatments using Duncan's multiple range test (Duncan, 1955).

RESULTS

Responsiveness of Sexes to Disparlure and Analogs. Electroantennogram (EAG) responses to the standard (hexyl acetate, stimulus load = 100 μg) were nearly 10-fold greater in males (0.46 mV $\pm$ SE = 0.1 mV) than in females (0.04 mV $\pm$ SE = 0.01 mV). Relative to the standard, EAGs of male and female gypsy moths to 5-μg stimulus loads of the enantiomers of disparlure and their analogs were significantly different (Figure 2A and B). For male moths, (+)-D elicited the largest EAGs, which were significantly larger than those elicited by (−)-D ($P < 0.05$). Responses to both disparlure enantiomers were significantly greater than those elicited by the analogs, which were not significantly different from each other. EAGs elicited from females by either the disparlure enantiomers or their analogs were not signifcantly different (Figure

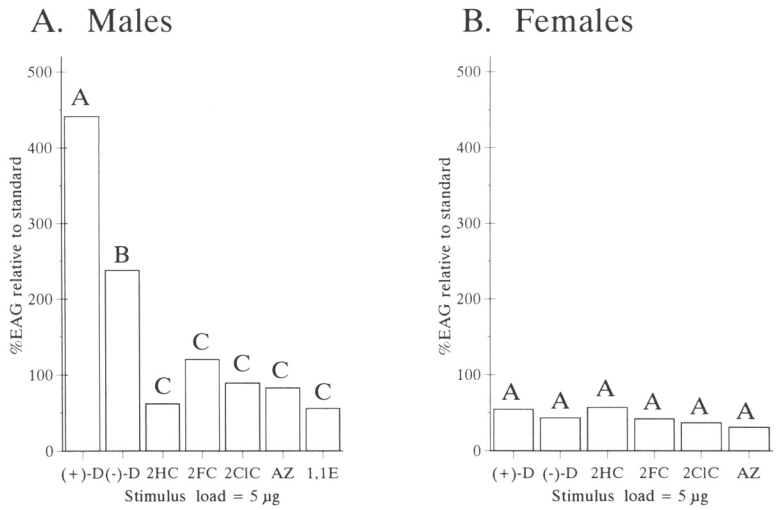


FIG. 2. Mean EAGs ($\pm$SE) of male (A) and female (B) gypsy moths to 5-μg stimulus loads of the enantiomers of disparlure [(+)-D and (−)-D] and several analogs of the epoxide group of disparlure: ($\pm$)-cyclopropyl disparlure (2HC), ($\pm$)-difluorocyclopropyl disparlure (2FC), ($\pm$)-dichlorocyclopropyl disparlure (2ClC), ($\pm$)-aziridinyl disparlure (AZ), and a mixture of 1,1-epoxide (1,1E) analogs of disparlure (tested on male only). Note that mean responses of males and females to standard were 0.46 mV and 0.04 mV, respectively. Bars with different letters are significantly different ($P > 0.05$).

2B). The millivolt responses of females to each compound were significantly less than responses elicited from male antennae ($P < 0.05$).

Sensitivity of Antennal Receptors of Males to Disparlure and Analogs. While the shapes of the dose–response curves for the enantiomers of disparlure were similar, male antennae were at least 100 times more sensitive to (+)-D than to (−)-D [threshold for (+)-D = 0.0005 μg, (−)-D = 0.005 μg] (Figure 3A). Dose–response curves constructed from EAGs of males to cyclopropyl (2HC), difluorocyclopropyl (2FC), and dichlorocyclopropyl (2ClC) analogs were parallel to those for the disparlure enantiomers but shifted closer to the abscissa (Figure 3B). The dose–response curve obtained for the 1,1-epoxide mixture (1,1E) (Figure 3D) was similar to those obtained for the cyclopropyl analogs. Threshold responses were elicited by the 0.5-μg stimulus load for 2FC and 2ClC, but only by 5 μg for 2HC and 1,1E. Relative stimulus loads necessary to elicit equivalent responses for (+)-D and its analogs were estimated as: (+)-D = 1, 2FC = 100, 2ClC = 500, and 2HC, 1,1E = 1000. Dose–response curves for the aziridine enantiomers were steeper than those for the other compounds tested. EAGs elicited by (+)-AZ [conformation corresponding to (+)-D] were greater than those elicited by (−)-AZ, while intermediate responses were given to racemic AZ (Figure 3C). Significant EAGs were recorded for the aziridine analogs only at the 5-μg stimulus load, which was at least 10-fold higher in threshold than all other analogs.

Adaptation Studies. The adaptation studies revealed differing effects on EAGs to the test stimuli, (+)-D, and hexyl acetate resulting from adaptation of males to (+)-D and the analogs (Figure 4). Stimulus loads for (+)-D and hexyl acetate (0.05-μg and 100-μg stimulus load, respectively) were chosen as they elicit similar EAG depolarizations in male moths prior to adaptation (ca. 0.5 mV). Previous adapting exposure to (+)-D significantly diminished EAGs to (+)-D by nearly 0.3 mV (60%) ($P < 0.05$) (Figure 4A). Although exposure to (−)-D reduced EAGs to (+)-D, exposure to (−)-D, (−)-2FC, or (+)-AZ did not significantly affect the magnitude of the EAG depolarization elicited by (+)-D. With the exception of (+)-D, the effects of these latter odorants did not differ from the control.

Surprisingly, exposure to both (+)-D and (−)-D significantly reduced EAGs to hexyl acetate (Figure 4B) ($P < 0.05$). Neither 2FC nor (+)-AZ reduced postexposure EAGs to hexyl acetate. Although postexposure EAGs to hexyl acetate following these latter odorants may have been enhanced slightly, they were not significantly different from the control.

DISCUSSION

Responsiveness and Sensitivity to Disparlure and Analogs. Dose–response curves for the enantiomers of disparlure observed in our study differ slightly

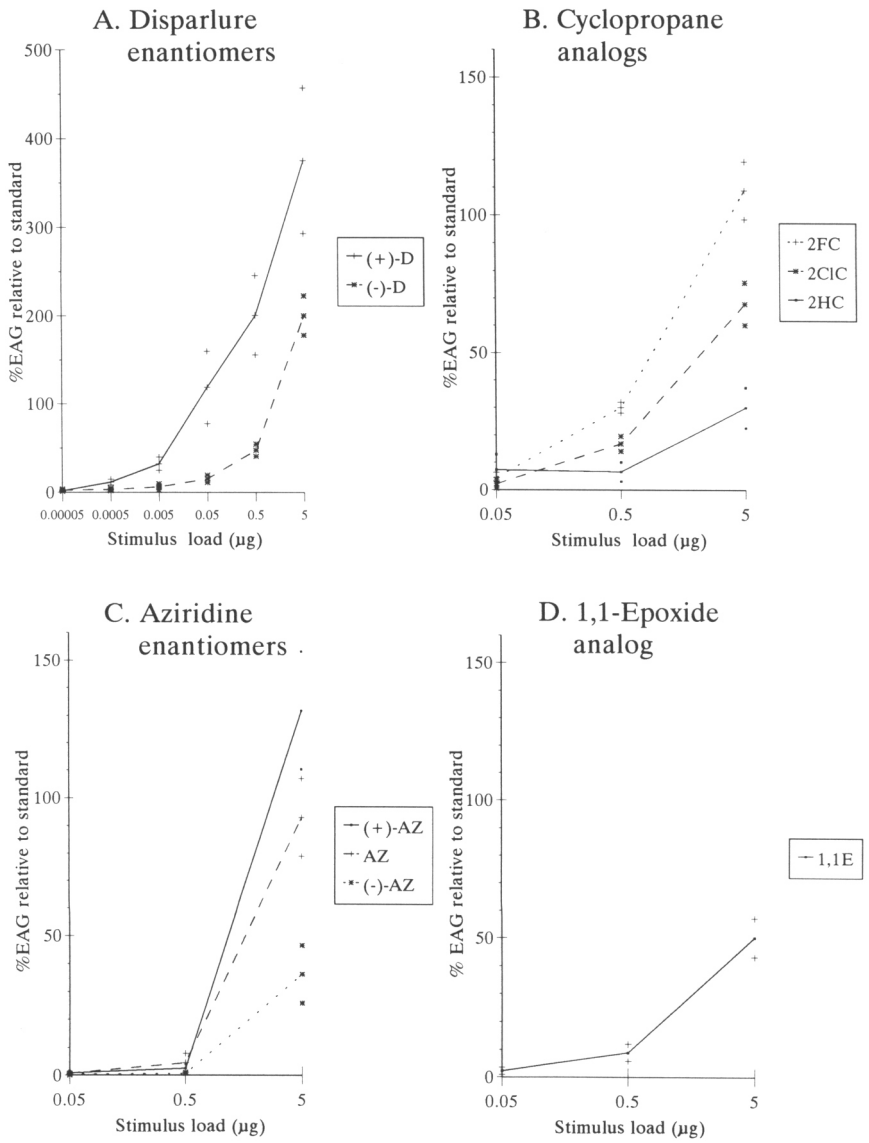


FIG. 3. Dose–response curves constructed from EAGs of male gypsy moths to (A) the enantiomers of disparlure [(+)-D and (−)-D]; (B) cyclopropane analogs [(±)-cyclopropyl disparlure (2HC), (±)-difluorocyclopropyl disparlure (2FC), (±)-dichlorocyclopropyl disparlure (2ClC)]; (C) the enantiomers [(+)-AZ and (−)-AZ] and racemate (AZ) of the aziridinyl dispalure, and (D) a mixture of 1,1-epoxy (1,1E) analogs of disparlure. Standard errors are represented by symbols above and below the same symbol designating the means.

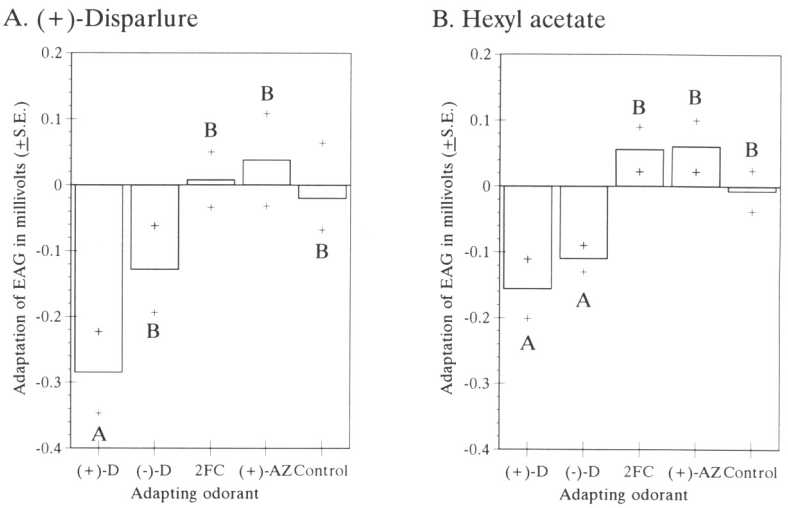


FIG. 4. Adaptation in millivolts of EAGs to (A) (+)-disparlure [(+)-D, 0.05-μg stimulus load], and (B) hexyl acetate (100 μg stimulus load), by exposure of male moths for 5 hr to 50 μg of (+)-D, (±)-difluorocyclopropyl disparlure (2FC), aziridinyl disparlure [(+)-AZ], or hexane solvent control. Stimulus loads for (+)-D and hexyl acetate were chosen as they elicit similar EAG depolarizations in male moths prior to adaptation (ca. 0.5 mV). Bars with different letters are significantly different ($P > 0.05$). Standard errors are represented by plus signs.

from those observed in a previous study (Yamada et al., 1976). In the previous study, EAGs to (−)-disparlure were only a small percentage of those elicited by the (+)-enantiomer. For example, EAGs to 1 μg/μl (−)-D were ca. 33% of the EAG to (+)-D at the same concentration, while in our study responses to (−)-D were greater than 50% of those elicited by (+)-D at the same dose. These differing observations may be a result of different stimulation techniques used and/or different populations of moths. It should also be noted that (−)-disparlure used in the previous study was contaminated with 5.8% of the (+)-enantiomer. Our dose–response curves more closely resemble those obtained in another study with single sensillum recordings (Hansen, 1984). Both the previous study and ours showed threshold responses to stimulus loads of between 0.001 μg and 0.005 μg for (+)-D, and 0.01 μg and 0.05 μg for (−)-D. Our study revealed separate receptor neurons for (+)-D and (−)-D within the long sexually dimorphic trichoid sensilla on male antennae. Although the disparlure enantiomers elicited small but significant EAGs from female moths, the lack of significantly different EAGs for the pheromone relative to the other odorants indicates a lack or low number of specific receptors for the pheromone, as was previously hypothesized (Yamada et al., 1976; Schneider et al., 1977).

Similarity in shapes of dose–response curves elicited by the disparlure enantiomers and the cyclopropane (C) analogs may be indicative of their interaction with the same receptors (Kaissling, 1971). Greater activity for dihalogenC analogs relative to the cyclopropyl analog (2HC) may be explained by inductive effects of the halogen atoms on neighboring carbons that mimic the electron-withdrawing effects of oxygen in the epoxide pheromone. The fact that the difluoroC (2FC) analog was more active than the dichloroC (2ClC) analog may be accounted for by the smaller size of the fluorine atom compared to chlorine, thus providing less steric hindrance for 2FC. The greater effectiveness of the 2FC analog relative to the 2HC analog is explained principally by inductive effects since the atomic radii of fluorine and hydrogen are similar.

Greater EAG activity of (+)-AZ relative to (−)-AZ correlates with the relationship of these analogs with (+)-D and (−)-D. Nitrogen (nitrile) analogs of aldehyde pheromone components of the boll weevil and the southwestern cornborer moth were active in electrophysiological studies and slightly active in field tests with the boll weevil (Ujváry and Dickens, 1991).

Both racemic AZ and 1,1E of disparlure were previously synthesized and tested in the field (Sheads et al., 1975). While racemic AZ was somewhat attractive, 1,1E was nearly as attractive as racemic disparlure, although insect captures in unbaited traps were not reported. When combined with disparlure, no effect on the attractiveness of disparlure was noted for either racemic AZ or 1,1E. The authors stated that "it would be interesting to determine whether electroantennogram responses of the compounds would parallel captures of insects in the field". Our current results show antennal receptors of male gypsy moths to be 1000-fold less sensitive to 1,1E than (+)-D and perhaps even less sensitive to racemic AZ. Thus, our results do not correlate with the observations made in the earlier study.

The low sensitivity of antennal receptors for the cyclopropyl, aziridinyl, and 1,1-epoxy analogs of disparlure may be indicative of the stringent requirements for interaction of related compounds with disparlure receptors. This low sensitivity of disparlure-sensitive neurons was also apparent in single sensillum recordings in which only a few spikes were elicited by 2FC even at the 5-μg stimulus load (Dickens, unpublished). We realize that comparisons made between EAGs elicited by the compounds at different stimlus loads are imprecise due to possible differences in the number of molecules evaporating from the filter paper as a result of differing volatilities of the compounds and inherent molecular properties causing differential binding to the filter paper. It was suggested in a recent study with another difluorinated pheromone analog that the retarded evaporation rate from filter paper observed for the analog was caused by interaction of flurorine with the hydroxyl groups of cellulose (Klun et al., 1996). Of the compounds considered here, 2ClC (**4**), (+)-AZ (**6**), and (−)-AZ (**7**) are the least volatile, but we lack a formula for estimating the amounts of material

actually volatilizing from the paper. For purposes of comparison, the order of elution from a nonpolar GC column is **3** > **5** > **1** = **2** > **6** = **7** > **8** + **9** > **4**. The order of activity observed for the compounds in EAG studies was **1** > **2** >> **3** > **4** > **5** > **8** + **9** >> **6** > **7**. Thus differences in volatility alone do not explain substantial differences in EAGs observed in our study.

Adaptation Studies. Interaction of the disparlure enantiomers and their analogs with receptor processes for (+)-D were further revealed by the adaptation experiments. Adaptation of responses to (+)-D by prolonged exposure to it was expected, and provided a test of the experimental procedure. The fact that exposure to (−)-D did not cause adaptation to (+)-D was not totally unexpected since specialized neurons housed in the same trichoid sensilla respond selectively to either (−)-D or (+)-D (Hansen, 1984). Thus, processes for the transport and interaction of the two molecules with their receptors may not significantly overlap, even though under certain conditions both molecules may bind to the same pheromone binding protein in the sensillar lymph (Vogt et al., 1989). The lack of an effect of 2FC and (+)-AZ analogs on postexposure responses to (+)-D further reflect their ineffectiveness in interactions with receptor processes for the pheromone.

Adaptation to hexyl acetate by (+)-D and (−)-D was unexpected. Receptor neurons for plant volatiles are housed in different sensilla than those housing neurons responsive to the pheromone (Kaissling, 1971). Processes for transport and interaction of such molecules with responsive dendrites may differ, as, for example, the localization of pheromone binding proteins and general odorant binding proteins (GOBPs) in different sensilla (Laue et al., 1994). However, sex pheromones in saturniid moths may bind general odorant binding proteins in females (Vogt and Riddiford, 1981; Vogt, 1987; Vogt et al., 1991). This may indicate a degree of flexibility in this and possibly other perireceptor events. In any case, the physical or biochemical processes responsible for our results remain unclear and will require further experimentation.

The relative lack of activity of the three-membered ring analogs of disparlure in our electrophysiological studies was recently verified in field tests in which 2FC was unattractive alone and had no significant effect on the number of male moths responding to (+)-D or (±)-D (Mastro et al., unpublished). The lack of activity of 2FC of the other compounds tested in our electrophysiological studies reflect the specificity of receptors for (+)-D and (−)-D, as previously reported for a number of analogs used in other studies (Bierl et al., 1972; Adler et al., 1972; Sarmiento et al., 1972; Schneider et al., 1974). These results indicate that novel approaches to the design and synthesis of potential agonists or antagonists of disparlure receptors are necessary before substantial effects on the activity of disparlure are realized.

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CHEMICAL BASIS FOR *Aspilia* LEAF-SWALLOWING BY CHIMPANZEES: A REANALYSIS

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Abstract—Thiarubrine A, an antimicrobial and nematocidal 1,2-dithiin polyyne has been reported to be a constituent of *Aspilia* leaves swallowed whole by chimpanzees. *Aspilia* samples were analyzed for the presence of thiarubrine A and other sulfur-containing polyynes using high-performance liquid chromatography (HPLC) and an antifungal bioassay. Thiarubrines A, B, D, and E and their corresponding thiophenes were not present in the leaves of three *Aspilia* species including *A. mossambicensis* and *A. rudis*, which are swallowed by chimpanzees at Mahale Mountains National Park and Gombe National Park, Tanzania. Antifungal activity, indicative of the presence of thiarubrines, was not detected in *Aspilia* leaf samples. Small amounts of thiarubrines A and B, and thiophenes A and B were found in *A. mossambicensis* roots. Analysis of three North American taxa found high concentrations of thiarubrines and thiophenes in the roots of *Eriophyllum lanatum* and *Chaenactis douglasii*, and the roots and leaves of *Ambrosia chamissonis*.

Key Words—*Aspilia*, chimpanzees, 1,2-dithiins, thiarubrines, zoopharmacognosy, antifungal bioassay, *Candida albicans*.

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INTRODUCTION

Animals may deliberately ingest plants containing secondary metabolites that have beneficial effects on health, a behavioral process that has been termed zoopharmacognosy (Janzen, 1978; Rodriguez and Wrangham, 1993). Examples of plant consumption for the purpose of self-medication have been suggested to be the ingestion of *Vernonia amygdalina* Del. (Asteraceae) pith (Huffman and Seifu, 1989; Ohigashi et al., 1994) and the swallowing of whole leaves by chimpanzees (*Pan troglodytes*) (Wrangham and Nishida, 1983; Rodriguez et al., 1985; Wrangham and Goodall, 1989). In the latter behavior, chimpanzees carefully select, fold, and swallow whole leaves of three *Aspilia* (Asteraceae) species and over 15 other plant taxa. The nutritional contribution of such leaves, which are found intact and undigested in chimpanzee feces following bouts of leaf-swallowing, appears to be minimal.

The peculiar method of ingesting *Aspilia* leaves, and the possibility that such leaves contain pharmacologically active substances, prompted investigation of the phytochemistry of *Aspilia* species. Rodriguez et al. (1985) reported that thiarubrine A (**1**) (Figure 1), a 1,2-dithiin polyyne with potent in vitro antibiotic, antifungal, and nematocidal activity (Towers et al., 1985; Constabel and Towers, 1989), was present in high concentrations in *A. mossambicensis* Oliv. and *A. pluriseta* O. Hoffm. leaves. In light of the biological activity of thiarubrine A, and the incidence of parasite infection in chimpanzees (Myers and Kunst, 1972), *Aspilia* leaf-swallowing was hypothesized to have an anthelmintic function (Rodriguez et al., 1985). Other possible functions of this anomalous plant use have been suggested to be fertility regulation, based on the presence of uterostimulatory kaurene diterpenes in *A. mossambicensis* (Page et al., 1992), reduction of intestinal pain (Wrangham, 1995) and intestinal nematode expulsion (Huffman et al., 1996).

Despite the isolation of thiarubrine A from one collection of *Aspilia* leaves, other researchers have been unable to replicate this finding. High-performance liquid chromatographic (HPLC) analysis of *A. mossambicensis* leaves collected in Mahale in 1991 and 1993–1994 from plants utilized by chimpanzees, as well as from greenhouse-grown specimens, failed to detect thiarubrine A (Page et al., 1992; Huffman et al., 1996). As a result of the uncertainty surrounding the presence of this compound in *Aspilia* leaves, the impact of thiarubrine A on chimpanzee health and the role of plant chemicals in mediating leaf-swallowing is unclear.

In this study we attempt to determine if thiarubrine A is present in *Aspilia* leaves using an improved HPLC assay for the detection and quantification of thiarubrine A and related sulfur-containing polyynes. In addition, we employ

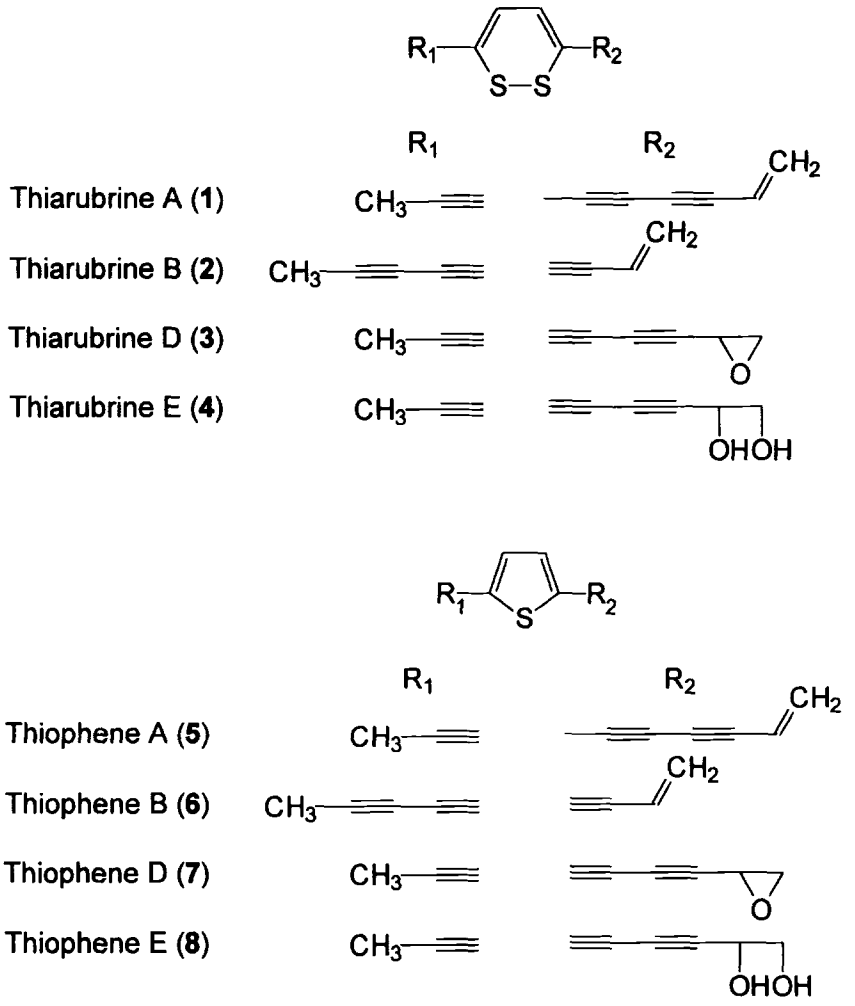


FIG. 1. Structure of thiarubrines and thiophenes detected and quantified by HPLC.

an antifungal bioassay for toxicity to *Candida albicans*, a human pathogenic yeast that is extremely sensitive to thiarubrines. We describe the application of both assays to extracts of fresh and dried samples of *Aspilia* species from four locations in East Africa as well as other asteraceous taxa known to contain thiarubrines.

METHODS AND MATERIALS

General Experimental Procedures. Plant samples were collected with the appropriate permission from each host country. Samples were collected in Canada by J. E. Page and G. H. N. Towers, in Tanzania by M. A. Huffman, in Uganda by G. H. N. Towers, and in Kenya by V. Smith and M. A. Huffman. Samples received minimal exposure to light after collection, and all procedures were performed in dim light to minimize the photoconversion of 1,2-dithiins. All solvents were HPLC grade (Fisher) except for reagent-grade MeOH or EtOH used in field-extractions. Water was Milli-Q plus (Millipore). The HPLC system consisted of a Waters 600 pump controller, Waters 996 photodiode array detector, and Waters 717 plus autosampler controlled by Millenium 2.1 software. A 3.9-mm × 150-mm (4 μm) C_{18} column (Waters NovaPak) equipped with NovaPak precolumn was used for HPLC analyses. UV-VIS spectra were obtained on a Pye-Unicam 8720 scanning spectrophotometer in MeCN. NMR spectra were recorded in acetone-D_6 on a Bruker AMX-500 spectrometer except for compound **7**, which was recorded in $CDCl_3$ on a Bruker AC-200E spectrometer. The residual acetone (δ 2.08 ppm) or $CHCl_3$ (δ 7.24 ppm) signals were used as references.

Field-Extracted Plant Material. Samples of *A. mossambicensis* were collected in Mahale Mountains National Park, Tanzania in December 1993 through February 1994. Nine leaf samples were collected from *A. mossambicensis* plants concurrently with direct observations of leaf-swallowing and five from plants that showed indirect evidence of leaf-swallowing, including discarded stalks with missing leaves. The age and size of the leaves sampled, as well as their location on the plant, were closely matched to leaves consumed by the chimpanzees. Leaves of an unidentified *Aspilia* sp. (#1) were collected in Gombe Stream National Park, Tanzania from a plant whose leaves the male chimpanzee Freud was observed to swallow. Plant identifications were verified by S. Mathenge (Department of Botany, University of Nairobi, Kenya) and E. Knox (East African Herbarium, National Museums of Kenya). Voucher specimens are deposited in the Herbarium of the University of Nairobi and the East African Herbarium, National Museums of Kenya. No voucher specimen was available for the Gombe *Aspilia* sp. (#1), which was preserved in EtOH.

Additional samples were collected during November 1995 through February 1996. Samples of *A. mossambicensis*, *A. rudis*, and an unidentified *Aspilia* sp. (#2) were obtained at Gombe; *A. mossambicensis* was collected in Nanyuki, Kenya, and one sample of *A. mossambicensis* leaves was collected at Mahale from a plant from which a chimpanzee was observed to swallow leaves. Voucher specimens are deposited in the East African Herbarium, National Museums of Kenya (Mahale and Gombe specimens), and in the Herbarium of the University

of Nairobi and the East African Herbarium, National Museums of Kenya (Nanyuki specimens).

Samples of the plant material of interest were collected, accurately weighed (3.0 g) and placed in amber vials containing 20 ml MeOH. Smaller samples (2.0 g or 2.6 g) were collected when the plant material was limited.

Dried Plant Material. A bulk collection of *A. mossambicensis* leaves was made at Mahale in January 1996 from plants known to be frequented by chimpanzees. Leaves and roots of *A. mossambicensis* and *A. africana* Pers. C. D. Adams were collected in the Kampala District of Uganda in November 1995 and identification confirmed by T. Katenge (Makerere University Herbarium, Uganda). Plants of *A. mossambicensis* were grown in the greenhouse of the Botany Department, University of British Columbia, from seeds collected at Mahale. *Ambrosia chamissonis* (Less.) Greene samples were collected at Iona Island and Comox, British Columbia, Canada, in June 1995 and May 1996, respectively. *Eriophyllum lanatum* (Pursh) Forbes was collected on Hornby Island, British Columbia, in May 1996 and *Chaenactis douglasii* (Hook.) H. & A. was collected near Princeton, British Columbia, in August 1996. Voucher specimens of the greenhouse-grown *A. mossambicensis* and the North American taxa are deposited in the University of British Columbia, Herbarium. Material for extraction was ground to a fine powder, and 1.0 g samples accurately weighed. If plant material was limited, <1.0 g samples were prepared.

Sample Preparation. The MeOH extracts of field-extracted samples were decanted off and the plant material washed with 3 ml of MeOH. The extracts were combined, passed through a C_{18} cartridge (Waters) and the volume adjusted to 25.0 ml. A 15.0-ml aliquot was dried in vacuo and the residue resuspended in MeCN to yield a solution of 1 g fresh weight (FW)/ml concentration. This solution was filtered through a 0.45-μm filter (Gelman) and analyzed by HPLC.

Dry plant samples were extracted with 3×25 ml CH_2Cl_2. Three rounds of extraction effectively removed $>99\%$ of the thiarubrines as determined by triplicate extraction of an *Ambrosia chamissonis* root sample monitored spectrophotometrically at 490 nm. The combined extracts were evaporated to dryness and the residue resuspended in 2.0 ml MeCN to yield a solution of 0.5 g dry weight (DW)/ml. The solution was filtered through a 0.45-μm filter (Gelman) and analyzed by HPLC.

For assessment of antifungal activity, a 500-μl aliquot of the MeCN solutions was dried by a stream of N_2 and reconstituted in 500 μl MeOH.

Standards. Thiarubrines A (**1**), B (**2**), D (**3**), and E (**4**) (Figure 1) were isolated from MeOH extracts of *Ambrosia chamissonis* roots collected near Centennial Beach, Tsawassen, Canada, using silica vacuum liquid chromatography and normal-phase and reversed-phase preparative HPLC following established procedures (Balza and Towers, 1993). Thiophenes A (**5**), B (**6**), D (**7**),

and E (**8**) (Figure 1) were isolated from the same source and by photoconversion of the corresponding thiabrines. The purity of all standards was >99% by HPLC. Standards were identified spectroscopically (UV-VIS and ^{1}H NMR; ^{13}C NMR when published data were available) by comparison with literature values (Balza and Towers, 1993; Gomez-Barrios et al., 1992). The concentration of thiarubrine (ϵ = 3000 at visible maximum 483–490 nm) and thiophene (ϵ = 31,500 at UV-A maximum 324–340 nm) solutions was determined spectrophotometrically.

Chromatographic Conditions and Standard Curves. Analysis was performed using a step-gradient elution system with the following conditions: preinjection (55:45, water–MeCN), 0 min (30:70), 10 min (10:90), 15 min (0:100), 32 min (55:45). Flow rate was 1 ml/min with detection over the wavelength range 200–600 nm and specific detection at 340 nm. Injection volume was 20 μl. Thiarubrine and thiophene peaks in samples were identified on the basis of retention time and comparison of UV-VIS spectra with those of authentic standards as determined by photodiode array detection. Quantification of thiarubrines and thiophenes was performed at 340 nm using peak area by comparison to standard curves derived from replicate injections of solutions ranging in concentration from ~1000 μg/ml to ~0.1 μg/ml for thiarubrines and ~125 μg/ml to ~0.1 μg/ml for thiophenes (Table 1). The correlation coefficient values (r^2) for all compounds exceeded 0.99 (Table 1). Six consecutive injections of a solution of **1** (100 μg/ml) gave a deviation in area of 0.8%. For **1**, the coefficient of variation for 5 independent analyses of a sample of *Ambrosia chamissonis* roots was 3.5%.

TABLE 1. RETENTION TIME AND CALIBRATION VALUES OF THIARUBRINE AND THIOPHENE STANDARDS

Compound	Retention time (min)[a]	Range (μg/ml)	r^2
Thiarubrine A (**1**)	16.1 ± 0.04	999.4–0.1	0.999
Thiarubrine B (**2**)	15.5 ± 0.08	992.6–0.1	0.999
Thiarubrine D (**3**)	10.9 ± 0.06	1011.0–0.1	0.999
Thiarubrine E (**4**)	5.3 ± 0.02	1021.8–0.1	0.999
Thiophene A (**5**)	14.3 ± 0.04	125.4–0.02	0.998
Thiophene B (**6**)	13.6 ± 0.20	123.2–0.03	0.999
Thiophene D (**7**)	9.8 ± 0.03	129.2–0.03	0.999
Thiophene E (**8**)	4.1 ± 0.03	114.6–0.06	0.999

[a] Mean ± standard deviation (N = 14).

Detection Limits. Detection limits for the HPLC assay were less than 50 μg/ml for thiarubrine and thiophene standards. To determine the practical detection limit of **1** in test solutions where interfering peaks are present, dried *A. mossambicensis* leaves (5.0 g), which previous analysis had shown were devoid of **1**, were finely ground and extracted with CH_2Cl_2. The extract was dried in vacuo and taken up in 10 ml MeCN. After spiking with 200 μg/g (based on 5.0 g extracted weight) of **1**, the solution was serially diluted to yield solutions ranging in concentration from 200 μg/g DW to 0.78 μg/g DW. Aliquots (500 μl) were dried with N_2 and resuspended in 500 μl of MeOH for determination of the detection limit of the antifungal bioassay.

Antifungal Assay. Antifungal activity was determined using the broth dilution method modified from Towers et al. (1985). A 48-hr culture of *C. albicans* (UBC #54) grown at 37°C in Sabouraud dextrose broth (SDB) was diluted to $\sim 1.0 \times 10^4$ cells/ml. MeOH solutions of plant extracts were diluted 100-fold and with a 200-μl aliquot transferred to a well in row 2 of a 96-well microtitre plate (8 lanes × 12 rows) (Costar). MeOH was used as a solvent (negative) control. A MeOH solution of **1** (114 μg/ml) was tested as a reference compound. Solutions were diluted in a series of nine twofold dilutions with 100 μl SDB, while row 12 served as an untreated control and received 100 μl SDB. A 100-μl aliquot of diluted *C. albicans* culture was transferred to each well of rows 2–12. Row 1 received growth medium only (200 μl SDB). Plates were incubated in the dark at 37°C for 24 hr and the optical density of each well read at 600 nm using an ELISA reader (Titertek Multiskan) zeroed on culture medium (row 1).

Solutions were screened for activity in single experiments in which one lane was used for each solution. Solutions that showed activity (>30% inhibition of growth in the first well) were retested in two experiments of three replicate lanes each. Minimum inhibitory concentration (MIC) values were determined visually as the lowest dilution that resulted in a clear well and the values from each experiment averaged.

RESULTS AND DISCUSSION

The structures of the sulfur-containing polyynes, thiarubrine A (**1**), thiarubrine B (**2**), thiarubrine D (**3**), thiarubrine E (**4**), thiophene A (**5**), thiophene B (**6**), thiophene D (**7**), and thiophene E (**8**), are shown in Figure 1.

HPLC Assay. The HPLC assay allowed for the detection and quantification of thiarubrine A (**1**) and three related 1,2-dithiin polyynes, thiarubrine B (**2**), thiarubrine D (**3**), and thiarubrine E (**4**), known to occur in the Asteraceae. Exposure of thiarubrines to visible light results in the formation of bicyclic photointermediates that convert to the corresponding thiophenes (Block et al.,

1996). Due to the potential for photoconversion during sample collection and transit, the HPLC assay included the detection and quantification of thiophenes **5**, **6**, **7**, and **8**, which are the photoproducts of the thiarubrines of interest. All 1,2-dithiin or thiophene polyynes included in the analysis exhibit distinct UV-VIS spectra, and their ambiguous detection in extracts was on the basis of retention time and UV-VIS spectroscopy by photodiode array detection. A chromatogram of a standard mixture of compounds (**1–8**) is illustrated in Figure 2A.

Previous studies of thiarubrine chemistry have relied on isocratic HPLC methods, which have been unable to separate thiarubrines with polar side chains (**3** and **4**) and those with diyne-ene side chains (**1** and **2**) in a single chromatographic run (Balza and Towers, 1993; Norton et al., 1993; Huffman et al., 1996). Our use of gradient elution resulted in resolution of all major thiarubrines and thiophenes. Extraction efficiency for dried plant material was >99% for thiarubrines determined by spectrophotometry. Although field extraction probably resulted in somewhat less efficient extraction of thiarubrines, the MeOH extracts that resulted contained most of the soluble pigments and subsequent reextraction was deemed unnecessary.

Antifungal Assay. Thiarubrines are toxic to many microorganisms and exhibit particularly potent activity against the pathogenic yeast *Candida albicans* (Towers et al., 1985; Bierer et al., 1995; Ellis et al., 1995). Based on the sensitivity of *C. albicans* to thiarubrines, we developed an antifungal bioassay for use as a complementary method for detection of thiarubrines in crude plant extracts. Although compounds other than thiarubrines may be expected to be toxic to *C. albicans* the bioassay detected antifungal activity only in plant samples that were shown to contain thiarubrines by HPLC. The bioassay was not used to quantify thiarubrines, nor was it able to detect thiophenes that lack activity against *C. albicans* in the absence of UV light (Towers et al., 1985). Solvent controls (MeOH) did not inhibit *C. albicans* growth at the concentrations used in the assay. Bioassay of a solution of pure thiarubrine A (**1**) as a reference compound showed it had an MIC of 0.6 μg/ml.

Detection Limits. The sensitivity of the HPLC assay and antifungal bioassay were assessed using an extract of dried *A. mossambicensis* leaves from Mahale that was shown by prior analysis to lack thiarubrines or antifungal activity. The extract was spiked with a known amount of **1** and serially diluted with additional extract. Analysis of the resulting solutions showed that detection limit for the HPLC assay were 6 μg/g DW Bioassay of the spiked solutions showed that a concentration of 25 μg/g DW of **1** resulted in >90% inhibition of *C. albicans* growth in the well containing the highest concentration of test solution.

Rodriguez et al. (1985) estimated that *Aspilia* leaves contained 5 mg of thiarubrine A per leaf based on the analysis of a chloroform extract of dried

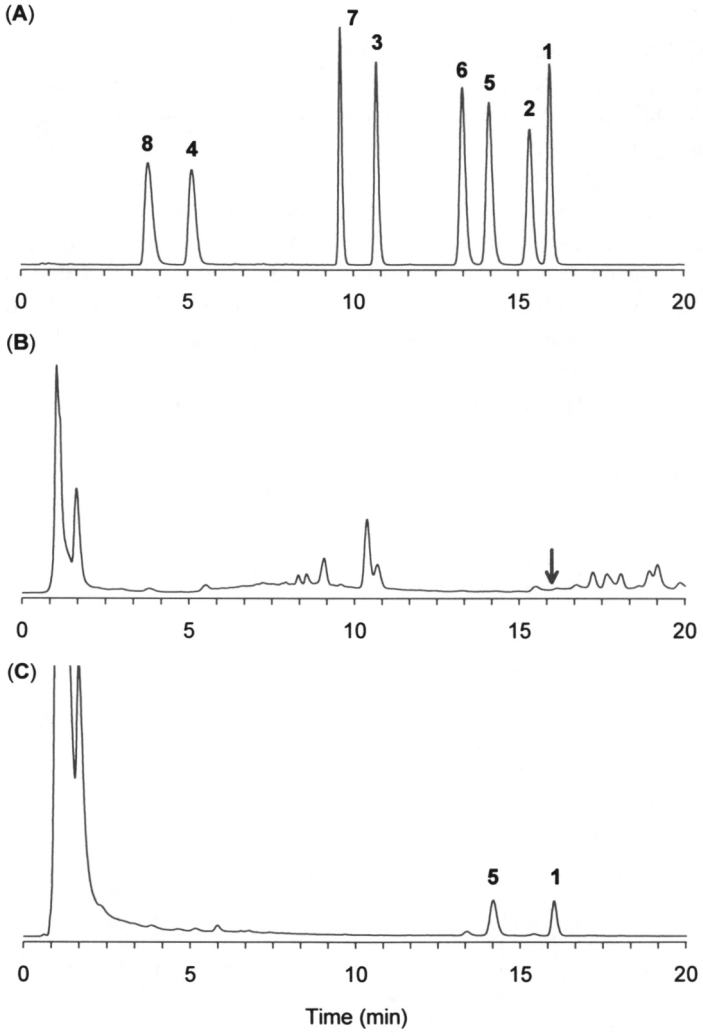


FIG. 2. Chromatogram of (A) Injection of standard mixture of thiarubrines (**1–4**) and thiophenes (**5–8**). Chromatogram obtained from (B) analysis of field-extracted *Aspilia mossambicensis* leaves collected at Mahale from a plant from which the chimpanzee Iwan was observed to swallow nine distal leaves on February 7, 1994. Peaks at ~10.8 min and ~15.5 min were shown not to be thiarubrines by UV-VIS spectroscopy. Arrow indicates the retention time of thiarubrine A (**1**). Chromatogram (C) of field-extracted *A. mossambicensis* roots collected at Gombe on February 2, 1996. Detection: 340 nm. A.U. full-scale: 0.25.

leaves. This amount was later revised to 100 μg/leaf (Rodriguez and Wrangham, 1993). Calculation using an average dry leaf weight of 0.092 g ($N = 28$) for young leaves of cultivated *A. mossambicensis* reveals that the HPLC assay and the antifungal bioassay should be capable of detecting levels <3 μg/leaf. For confirmatory purposes, we spiked fresh *A. mossambicensis* leaves from plants in cultivation with 100 μg **1** per leaf. The leaves were lyophilized, processed using the methodology developed for dry plant material, and analyzed in the HPLC and *C. albicans* assays. A comparison of chromatograms obtained from analysis of spiked and unspiked leaves (Figure 3) clearly shows that 100 μg **1** per leaf was well within the detection limit of the HPLC assay. As well, the spiked sample had significant activity in the antifungal assay (Table 3 below).

Analyses. Table 2 summarizes the results of the HPLC and bioassay analysis of field-extracted *Aspilia* leaf, stem, and root samples. The results of the

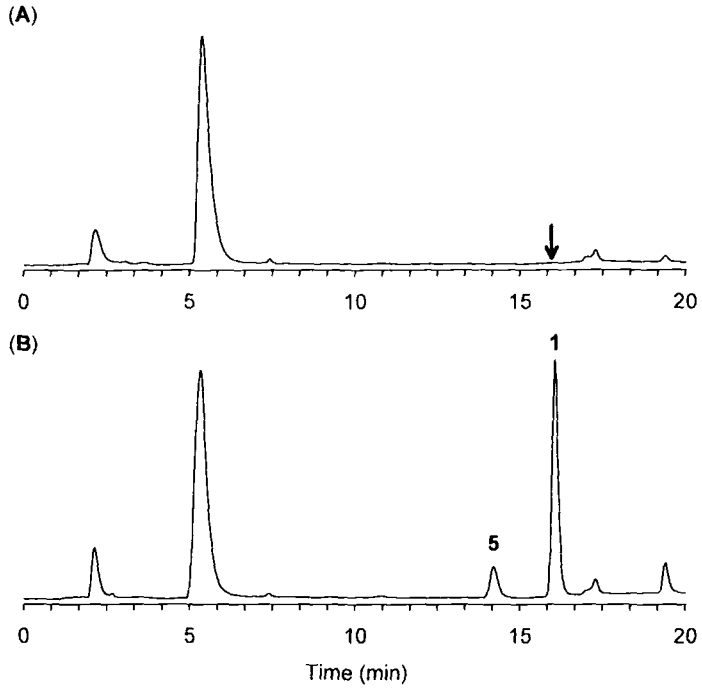


FIG. 3. Chromatograms obtained from analysis of (A) an extract of *Aspilia mossambicensis* leaves from greenhouse-grown specimens and (B) an extract of *A. mossambicensis* leaves from the same source spiked with 100 μg thiarubrine A (**1**) per leaf. Arrow indicates the retention time of thiarubrine A (**1**). The peak at ~5.5 min was shown not to be a thiarubrine (i.e., **4**) by UV-VIS spectroscopy. Detection: 340 nm. A.U. full-scale: 0.25.

TABLE 2. THIARUBRINE AND THIOPHENE CONTENT AND ANTIFUNGAL ACTIVITY OF FIELD-EXTRACTED *Aspilia* spp.

Sample	Location (date)	Total thiarubrines (μg/g FW)[a]	Total thiophenes (μg/g FW)[a]	MIC (mg FW/ml)[b]
A. mossambicensis	Mahale (1993–94)			
Leaves (N = 13)[c]		ND[d]	ND	inactive[e]
Stems (N = 2)		ND	ND	inactive
Roots (N = 2)		1.0 ± 0.4 (1[f])	1.1 ± 0.01 (**5**,**6**)	3.1 (inactive–6.3)
A. mossambicensis	Mahale (1995–96)			
Leaves (N = 1)[c]		ND	ND	inactive
A. mossambicensis	Gombe (1995–96)			
Leaves (N = 1)		ND	ND	inactive
Roots (N = 1)		9.1 (**1**,**2**)	2.7 (**5**,**6**)	0.6
A. mossambicensis	Nanyuki (1995–96)			
Leaves (N = 3)		ND	ND	inactive
A. rudis	Gombe (1995–96)			
Leaves (N = 1)		ND	ND	inactive
Aspilia sp. (#1)	Gombe (1993–94)			
Leaves (N = 1)[c]		ND	ND	inactive
Aspilia sp. (#2)	Gombe (1995–96)			
Leaves (N = 3)		ND	ND	inactive
Roots (N = 1)		ND	ND	inactive

[a] Mean ± standard error.
[b] Mean (range).
[c] Leaf samples collected after direct or indirect observations of leaf-swallowing behavior.
[d] ND, not detected.
[e] Inactive, no inhibition of *Candida albicans* growth at highest concentration (5 mg FW/ml) tested.
[f] Numbers in parentheses refer to compounds present in sample.

analysis of dried samples of *Aspilia* species and three North American asteraceous taxa, *Ambrosia chamissonis* (Less.) Greene, *Eriophyllum lanatum* (Pursh) Forbes, and *Chaenatis douglasii* (Hook.) H. & A. are shown in Table 3. Thiarubrines and thiophenes have been isolated from the latter three species. Their inclusion in the study served both to validate the methods of extraction and analysis and to compare the levels of thiarubrines in *Aspilia* species with those of other plants.

Analysis of Aspilia *Samples.* A total of 27 samples (19 fresh and 8 dried) of *Aspilia* leaves representing collections of *A. mossambicensis*, *A. rudis*, and *A. africana*, from four sites in East Africa and from plants in cultivation in North America, contained no detectable thiarubrine A (**1**), thiophene A (**5**) or any related thiarubrines or thiophenes. The absence of these compounds is illus-

TABLE 3. THIARUBRINE AND THIOPHENE CONTENT AND ANTIFUNGAL ACTIVITY OF *Aspilia* spp. AND OTHER ASTERACEAE TAXA

Sample	Location (date)	Total thiarubrines (μg/g DW)[a]	Total thiophenes (μg/g DW)[a]	MIC (μg DW/ml)[b]
A. mossambicensis	Mahale (1995–96)			
Leaves (N = 2)		ND[c]	ND	inactive[d]
A. mossambicensis	Uganda (1995)			
Leaves (N = 2)		ND	ND	inactive
Roots (N = 1)		ND	ND	inactive
A. mossambicensis	U.B.C. (1996)			
Leaves (N = 2)		ND	ND	inactive
Spiked leaves (N = 1)		125.1 (**1**[e])	5.2 (**5**)	468.8
Roots (N = 2)		164.4 ± 60.6 (**1,2,3,4**[b])	10.0 ± 0.6 (**5,6,7,8**)	58.6 (39.1–78.1)
A. africana	Uganda (1995)			
Leaves (N = 2)		ND	ND	inactive
Roots (N = 1)		ND	12.0 (**5,6**)	inactive
Chaenactis douglasii	Princeton (1996)			
Aerial parts (N = 2)		ND	ND	inactive
Roots (N = 1)		1396.4 (**1,2**)	104.6 (**5,6**)	19.5
Eriophyllum lanatum	Hornby Is. (1996)			
Aerial parts (N = 2)		ND	ND	inactive
Roots (N = 2)		214.1 ± 5.0 (**1,2**)	6.8 ± 0.1 (**5,6**)	97.7 (78.1–117.2)
Ambrosia chamissonis	B.C. (1996)			
Leaves (N = 3)		1442.9 ± 639.4 (**1,2**)	388.1 ± 131.9 (**5,6**)	74.9 (29.3–156.3)
Stems (N = 1)		3694.3 (**1,2,3,4**)	538.5 (**5,6,7,8**)	4.9
Roots (N = 3)		491.7 ± 50.3 (**5,6,7,8**)	48.5 ± 3.0 (**5,6,7,8**)	14.6 (4.9–19.5)
Thiarubrine A (**1**)		—	—	0.6
MeOH		—	—	inactive

[a] Mean ± standard error.
[b] Mean (range).
[c] ND, not detected.
[d] Inactive, no inhibition of *Candida albicans* growth at highest concentration (2500 μg DW/ml) tested.
[e] Numbers in parentheses refer to compounds present in sample.

trated by Figure 2B, which shows a chromatogram obtained by analysis of a field-extracted sample of *A. mossambicensis* leaves collected at Mahale from a plant from which chimpanzees swallowed leaves. Furthermore, *Aspilia* leaf samples showed no detectable antifungal activity.

Ten of the *A. mossambicensis* leaf samples from Mahale and one sample of leaves of an unidentified *Aspilia* sp. (#1) from Gombe were collected from plants from which chimpanzees were observed to swallow leaves, immediately after observations of leaf-swallowing were made. The complete absence of thiarubrines and antifungal activity in these samples underscores the finding that chimpanzees do not acquire significant levels of thiarubrines when they ingest *Aspilia* leaves.

Two samples of *A. mossambicensis* stem and one sample of *A. pluriseta* stem included in the analysis were found to be devoid of thiarubrines or antifungal activity.

Several of the field-extracted root samples contained low but detectable levels of thiarubrines and their thiophenes. Two samples of *A. mossambicensis* roots from Mahale contained 0.6 and 1.4 μg/g FW of **1**, respectively, and small amounts of thiophenes **5** and **6**. Antifungal activity was found only in the sample containing the higher concentration of **1**. Moderate levels of **1** and **2** (total 9.1 μg/g FW), and **5** and **6** (total 2.7 μg/g FW) were found in *A. mossambicensis* roots from Gombe (Figure 2C), which also showed activity in the bioassay (MIC 0.6 mg FW/ml). Neither thiarubrines nor thiophenes were found in a single root sample of *Aspilia* sp. (#1) from Gombe.

Of the dry root samples analyzed, *A. mossambicensis* roots from Uganda did not contain thiarubrines or thiophenes. Thiophenes **5** and **6** (total 12.0 μg/g DW) were present in a Ugandan sample of *A. africana*, a species that has not previously been shown to contain 1,2-dithiins or thiophenes (NAPRALERT, 1996). The roots of both *Aspilia* species from Uganda lacked antifungal activity.

Root samples from greenhouse-grown *A. mossambicensis* plants contained moderately high concentrations of **1**, **2**, **3**, and **4** as well as their corresponding thiophenes, and showed antifungal activity. Thiarubrine D (**3**) and thiarubrine E (**4**) and their corresponding thiophenes (**7** and **8**) have not previously been reported from *Aspilia* species. Total mean thiarubrine and thiophene levels were 164.4 μg/g DW and 10.0 μg/g DW, respectively. These quantities are substantially lower than the 2.03 mg/g DW of **1** and **2** reported in *A. mossambicensis* roots by Lopéz et al. (1989) but similar to the 230 μg/g DW found by Norton et al. (1993).

Analysis of North American Taxa. Sulfur-containing polyynes have been previously reported to be present in several *Ambrosia* species (Ellis et al., 1995) and in *C. douglasii* and *E. lanatum* (Norton et al., 1985). The pattern of thiarubrine distribution observed in *Aspilia* samples (i.e., thiarubrines present in the

roots but absent from leaves and stems) was found for two of the North American taxa sampled. The aerial parts (leaves and stems) of both *C. douglasii* and *E. lanatum* lacked thiarubrines or thiophenes, and above-ground plant material of both species was inactive in the bioassay. Cosio et al. (1986) were also unable to detect thiarubrines or thiophenes in the leaves and stems of *C. douglasii*. High levels of thiarubrines and potent antifungal activity were found in *C. douglasii* roots with total amounts of thiarubrines (**1** and **2**) of 1396.4 μg/g DW and thiophenes (**5** and **6**) and 104.6 μg/g DW. *E. lanatum* roots contained five-fold lower levels of **1** and **2** (total 214.1 μg/g DW) and **5** and **6** (total 6.8 μg/g DW) compared with *C. douglasii*.

Analysis of *Ambrosia chamissonis* found high concentrations of thiarubrines and thiophenes in leaves, stems, and roots (Table 3). It is worth noting that Ellis (1993) found the opposite distribution of thiarubrines and thiophenes, with higher levels in roots compared to leaves, from the distribution shown by this study. *Ambrosia chamissonis* extracts showed high activity in the antifungal assay with mean MIC values of 74.9, 4.9 and 14.6 μg DW/ml for leaves, stems, and roots, respectively.

CONCLUSIONS

Our results demonstrate that thiarubrine A (**1**) is not present in detectable concentrations in the leaves of *Aspilia* species swallowed by chimpanzees. This finding does not support the hypothesis that the basis for leaf-swallowing is the ingestion of pharmacologically active [i.e., nematocidal (Rodriguez et al., 1985)] plant chemicals. Recently, Messner and Wrangham (1996) reached a similar conclusion when they reported that in vitro tests on extracts of *Rubia cordifolia* L. (Rubiaceae) leaves, which are swallowed by chimpanzees at Kibale, showed a lack of antiparasitic activity against *Strongyloides* nematode larvae. More importantly, absolutely no sign of nematocidal activity has been noted in detailed observations of nematode (*Oesophagostomum stephanostomum*) expulsion directly associated with leaf-swallowing at Mahale using *A. mossambicensis* or any other species (Huffman et al., 1996). Such adult nematodes were always motile at the time of expulsion, and even when left in situ with leaf and dung, remained alive and motile for up to four days thereafter. It is worth noting that chemicals in swallowed leaves may function to repel or reduce the motility of parasites, rather than directly acting as nematocides.

In response to this evidence and to the growing list of plant species from diverse taxa that chimpanzees and other African apes are known to swallow, factors other than plant chemicals have been put forward as important determinants of leaf-swallowing behavior. All leaves swallowed by chimpanzees share a common physical characteristic; the presence of trichomes and a rough leaf surface (Huffman et al., 1993; Huffman and Wrangham, 1994). As an

alternative explanation for leaf-swallowing, Huffman et al. (1996) have hypothesized that such physical characteristics may function to trap and expel parasites.

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EXTRACTION, ISOLATION, AND COMPOSITION OF OVIPOSITION-DETERRING SECRETION OF CABBAGE SEED WEEVIL *Ceutorhynchus assimilis*

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Abstract—Behavioral bioassays have confirmed that the oviposition-deterring secretion of the cabbage seed weevil, *Ceutorhynchus assimilis* Payk., can be isolated from glass tubes marked by the weevil and from extracts of its dissected seventh urotergite. Analysis of the secretion by gas chromatography—mass spectrometry showed that it contained iso- and *n*-alkanes, dimethylalkanes, alkenes, fatty acids, 15-nonacosanone, 15-nonacosanol, and cholesterol. The oviposition-deterring properties of the secretion are associated with a more polar fraction, isolated by liquid chromatography from an extract of the seventh urotergite.

Key Words—Oviposition-deterring pheromone, cabbage seed weevil, *Ceutorhynchus assimilis*.

INTRODUCTION

Oviposition-deterring pheromones (ODPs) or markers inhibit multiple oviposition into or onto hosts and have been found in many insect groups where larval

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development is confined to a limited food resource (Prokopy et al., 1984). Behavioral observations of cabbage seed weevil (*Ceutorhynchus assimilis* Payk., synonym *C. obstrictus* Marsham) females ovipositing on oilseed rape (*Brassica napus* L.) pods provide evidence for such a marker in this species (Kozlowski et al., 1983). Each egg deposition is followed by specific and fixed behavior in which the female brushes the tip of her abdomen from side to side against the surface of the pod while walking up and down it. A female visiting a marked pod rejects it for oviposition, and this deterrent effect is specifically associated with the brushing behavior (Ferguson and Williams, 1991).

Oviposition-deterring pheromones that derive from egg-laying adults function both as pheromones (i.e., influencing the behavior of other conspecific individuals) and as behavior organizers, by which females regulate their own egg-laying behavior to distribute eggs optimally between hosts. Females are more likely to encounter hosts on which they themselves have already oviposited than to encounter a host used by another female, and therefore it is in the role of behavior organizer that the chief benefit of ODP accrues to reproductive success (Roitberg and Mangel, 1988). The optimum persistence of ODP in a particular species depends on the ecological and ethological factors that influence the time that a female remains in a host patch (Roitberg and Mangel, 1988). Seed weevil ODP remains active for only about 2 hr (Ferguson and Williams, 1991; Kozlowski, 1991).

Following oviposition, a female weevil readily brushed a tube placed over the distal portion of the pod; when transferred to a clean pod, this brushed tube carried the deterrent effect with it (Kozlowski et al., 1983). Morphological and histological studies (M. Solinas, unpublished observations) indicate that the dorsal part of the eighth abdominal segment forms the brush used in ODP deposition; on withdrawal, the brush comes into contact with the invaginated and sclerotized cuticular surface of the seventh urotergite (VII UTG) with which glandular cells are associated. This paper reports studies to isolate the oviposition deterrent either from glass tubes marked by female weevils following oviposition or from VII UTG excised from females, in order to determine the pheromone's chemical properties and analyze the secretion containing it.

METHODS AND MATERIALS

Insects. Seed weevils of the overwintered generation were collected from crops of oilseed rape every two weeks and maintained on cut racemes of rape as described by Ferguson and Williams (1991). The weevils were immobilized by chilling to allow sex determination, and only the females were used. New generation insects were collected as larvae emerging from cut rape plants collected from the field and were allowed to pupate in trays of soil maintained at

17°C for four to five weeks. Imagos were killed for preparation of extracts 24 hr after they had emerged from the soil.

Extraction of Rape Pod Cuticular Wax. Rape pods (13–16 mm long) were immersed in a mixture of pentane and ether (1 : 1) for 10 min, and the flask containing them was placed in an ultrasonic bath for a further 2 min. The pods were filtered off and the solvent evaporated almost to dryness under nitrogen.

Isolation of ODP from Marked Glass Tubes. A glass tube (15 mm × 2.2 mm OD) with a sealed end was fitted over a rape pod (13–16 mm long excluding stigma and style), leaving about 4 mm of the proximal end of the pod exposed. A female weevil was allowed to oviposit into the pod and brush the glass tube with her abdomen. Immediately after brushing, the outside surface of the tube was washed with one of three solvents [pentane, pentane–ether (1 : 1) or methanol–acetone (1 : 1)] to extract ODP. This procedure was slow, and so a second method was developed to obtain material more quickly and with less risk of contamination.

Isolation of ODP from Weevil Seventh Urotergites. The VII UTG was dissected from female weevils and extracted in hexane or hexane—ether (1 : 1). An aqueous extract of 300 VII UTG was also prepared by grinding them with a glass rod in 160 μl water and placing them in an ultrasonic bath for several minutes. The cloudy solution obtained was centrifuged and filtered to remove solid matter and then freeze-dried overnight to give a white residue to which acetone (300 μl) was added. After placing the mixture in an ultrasonic bath for several minutes to dissolve the residue, it was centrifuged and the supernatant removed by pipette.

Preparation of Extracts for Bioassay. Nonpolar solvents disrupt cuticular waxes when applied to pods, rendering them unsuitable for weevil oviposition. However, extracts of marked glass tubes made in a 1 : 1 mixture of pentane and ether were successfully bioassayed in a sevenfold excess of acetone. This solvent mixture was adopted as standard, and pentane was later substituted with hexane for ease of handling. To prepare extracts for bioassay, those in organic solvents were evaporated almost to dryness under nitrogen and those in water were freeze-dried. The residue was dissolved in acetone—hexane (or pentane)—ether (14 : 1 : 1) and presented in 10 μl at a rate of 4–20 (usually 10) weevil equivalents.

Behavioral Bioassay. The bioassay was similar to that described by Ferguson and Williams (1991). Oilseed rape stems (50–80 mm long) were stripped of leaves and all but one pod (13–16 mm long), which was left at the uppermost end. Pairs of stems with size-matched pods were presented contiguous to each other in a Y or T configuration and supported in a water-saturated porous block. The pods of each pair were treated with either test or control extract (10 μl) 10 min before the introduction of a female weevil, to allow time for the solvent to evaporate. In bioassays of extracts of marked tubes, controls were extracts of unmarked tubes similarly treated; in other experiments pods were treated with

solvent only. In each experiment a female weevil was placed on the petiole of one pod of each pair, facing away from the stem, and its behavior during four or (in experiments 3, 7, and 9–26) six pod visits was observed. For each pod, the number of weevil visits, ovipositions, and pod rejections (defined as the number of visits without egg-laying before the pod was used for oviposition or before the end of the test) was recorded. Tests were accepted only if the weevils oviposited, visited both pods, and completed four or six pod visits within an hour. Experiments were conducted at 17°C and usually the response of a minimum of 10 weevils was tested (each on a fresh pair of pods).

Statistical Analysis. The numbers of weevils that oviposited first in test and control pods were analyzed for evidence of oviposition deterrence in the test extract using a one-sided exact binomial test with null hypothesis H_0: $P \leq 0.5$, where P is the proportion of weevils ovipositing first in the control pod. The exact probability is calculated from:

$$P = \text{Prob}(X \geq x) = \sum_{r=x}^{n} \binom{n}{r} \left(\frac{1}{2}\right)^n$$

where n is the number of weevils tested and x is the number ovipositing first in the control pod. The null hypothesis, which represents no evidence of test extract activity, was rejected if $P < 0.05$. Sometimes, although the null hypothesis was accepted, there was a large difference in the mean number of times weevils rejected test and control pods for oviposition. These data were not analyzed statistically because the numbers of visits recorded were insufficient; however, large differences were also taken as evidence of test-extract activity.

Separation into Acidic, Basic, and Neutral Components. A hexane—ether (1 : 1) extract (6 ml) of 800 dissected VII UTG was shaken with 2 M sodium bicarbonate solution (3 × 2 ml) and washed with water (2 ml). The bicarbonate extracts were combined, acidified with 2 M hydrochloric acid, and extracted with ether (3 × 6 ml) to give the acid fraction, which was washed with water (2 × 2 ml), dried over anhydrous magnesium sulfate, and evaporated almost to dryness. The original hexane—ether (1 : 1) VII UTG extract from which the acidic components had been removed was extracted with 2 M HCl (3 × 2 ml) to remove any basic components and the organic phase washed with water (2 × 2 ml). The aqueous 2 M HCl extracts were combined, neutralized at 0 °C with 2 M NaOH and extracted with ether (3 × 6 ml) to give the basic fraction. The ethereal solution of the basic fraction was washed with water (2 × 2 ml), dried over anhydrous magnesium sulfate, and evaporated almost to dryness. The remaining hexane—ether solution containing the neutral components was dried over anhydrous magnesium sulfate and evaporated almost to dryness.

Gas Chromatography–Mass Spectrometry (GC-MS). Electron-impact mass spectra were obtained on a VG-70-250 double-focusing mass spectrometer oper-

ated at 70 eV. Gas chromatography (GC) was performed on a Hewlett-Packard 5890 gas chromatograph equipped with an on-column injector and a Durabond DB1 fused silica capillary column (0.1-μm film thickness, 30 m $\times$ 0.32 mm ID from J & W Scientific, Folsom, California). Samples (0.5 μl) were injected on-column at 30°C and the column held isothermally at 30°C for 5 min and then programmed at 5°C/min up to 320°C.

Preparative Gas Chromatography Isolation. An extract of 200 VII UTG in acetone (15 μl) was prepared and aliquots (1 μl) were injected into a Hewlett-Packard gas chromatograph fitted with the fused silica column described above and operated with the same temperature program. The ion source was removed from the mass spectrometer and replaced by a small vial containing 2 ml hexane. The end of the fused silica column that previously fed into the ion source was inserted into the vial to collect the effluent. To prevent the sample being retained on the short length of column inside the cold ion-source chamber, a short length of deactivated fused silica (0.32 mm ID) was joined to the end of the DBI capillary column. When all of the extract had been injected, two further injections of acetone (1 μl) were made; all effluent was collected in the same vial and the hexane solution was prepared for bioassay as above.

Liquid Chromatography. A small silica column was prepared by slurry packing a drawn-out Pasteur pipet with LiChroprep Si 60 silica (50 mg in hexane of 15- to 20-μm particle size, E. Merck, Darmstadt, Germany). An extract of 200 VII UTG in hexane was applied to the column, which was then eluted successively with 3 ml each of hexane, ether, and methanol.

Reverse-phase high-pressure liquid chromatography (HPLC) was performed on a 5 μ Spherisorb ODS column (25 cm $\times$ 4.2 mm) in methanol. A freeze-dried aqueous extract of VII UTG (970 glands) was dissolved in methanol (300 μl) and 20-μl aliquots injected onto the HPLC column through a loop-injector and eluted with methanol at 1 ml/min. Extracts of VII UTG in either water or methanol had little absorption in the ultraviolet region so two fractions were collected for behavioral bioassay and analysis, the first fraction consisted of the first 5 min of eluent collected from the column and the second fraction of the next 14 min.

Liquid Chromatography–Mass Spectrometry (LC-MS). Fractions from liquid chromatography of VII UTG extracts were evaporated almost to dryness, dissolved in acetonitrile–water (1 : 1), injected directly by loop injection into a Micromass Autospec mass spectrometer, and analyzed by atmospheric pressure chemical ionization (APCI) mass spectrometry.

RESULTS AND DISCUSSION

Extraction and Bioassay of ODP. Bioassay results proved that oviposition-deterring activity could be extracted from glass tubes marked by weevils by

rinsing them with pentane–ether (1 : 1); most weevils oviposited first in the control pod and rejected the pod with ODP extract more frequently than the control (experiment 1, Table 1). This confirms that the deterrence is caused by a chemical rather than by a physical mark made on the pod during brushing behavior (Kozlowski et al., 1983; Ferguson and Williams, 1991). An extract of VII UTG from egg-laying females in hexane—ether (1 : 1) also deterred oviposition strongly, and the observed behavioral response was indistinguishable from that to marked glass tube extract (experiment 2, Table 1). Moreover, an extract of VII UTG from nonovipositing, sexually immature day-old imagos did not deter oviposition (experiment 3, Table 1). The VII UTG encloses both the eighth and ninth abdominal segments when at rest (Jeannel, 1949). The reproductive and alimentary tracts both open onto the ninth abdominal segment, and both have been shown to be the sources of oviposition deterrents in other insects (Behan and Schoonhoven, 1978; Prokopy et al., 1982). Histological and bioassay studies demonstrating that ODP is produced in *C. assimilis* by glandular cells associated with the VII UTG are being published elsewhere (Ferguson et al., in preparation). The VII UTG is a readily dissected, convenient, and perhaps more productive source of ODP, which is likely to be less contaminated with plant-derived chemicals than glass tubes fitted over pods.

Oviposition-deterring activity could be extracted from marked glass tubes or from the VII UTG in a variety of solvents with a wide range of polarity (experiments 5–9, Table 1). Furthermore, when ODP extracts in hexane–ether or in hexane were shaken with water, there was evidence of deterrent activity in both aqueous and organic phases. Usually both phases were less active than an untreated hexane–ether or hexane extract, but they always elicited more pod rejections than did controls (experiments 10–13, Table 1). This partitioning implies that ODP has both polar and nonpolar characteristics and may explain why almost all fractions tested showed some sign of activity. The ODP was stable in pentane—ether (1 : 1) solution, retaining activity for two years when stored at −20°C (experiment 4, Table 1), and it was of low enough volatility to be recovered from aqueous extracts by freeze drying (experiment 9, Table 1). Extracts made from VII UTG or from marked glass tubes were both sufficiently active to give a response in the bioassay at a concentration of four weevil equivalents per test pod (experiments 5 and 7, Table 1).

Acidity. There was little evidence of any behavioral response to acidic, basic, or neutral fractions of ODP extract presented singly (experiments 14–16, Table 2); nor was a combination of acidic and basic fractions active (experiment 17, Table 2). However, combinations of either or both of these with the neutral fraction deterred oviposition (experiments 18–20, Table 2). These results cannot be explained in terms of the acidity or basicity of a single active component or in terms of combinations of three active components (acidic, basic, and neutral).

TABLE 1. WEEVIL RESPONSES TO EXTRACTS OF MARKED GLASS TUBES AND SEVENTH UROTERGITES IN DIFFERENT SOLVENTS

Exp.	Test extract	Extraction solvent	No. of weevil equivalents per test (N)	No. of weevils tested (n)	No. of weevils ovipositing first in control pod (x)	P = Prob ($X \geq x$)[a]	Rejections (mean ± SE) of	
							Test pod	Control pod
1	marked tubes	pentane–ether 1 : 1	20	16	15	<0.001	1.9 ± 0.06	0.1 ± 0.09
2	VII UTG	hexane–ether 1 : 1	10	12	11	0.003	2.6 ± 0.13	0.7 ± 0.28
3	VII UTG day-old sexually immature imagos	hexane–ether 1 : 1	4	10	5	0.623	0.2 ± 0.13	0.3 ± 0.15
4	Marked tubes. Extract stored for 2 yr at −20°C	pentane–ether 1 : 1	10	9	8	0.020	1.8 ± 0.22	0.1 ± 0.11
5	marked tubes	pentane	4	23	17	0.017	1.3 ± 0.17	0.04 ± 0.04
6	marked tubes	pentane–ether 1 : 1	10	10	10	0.001	2.0 ± 0	0 ± 0
7	VII UTG	hexane–ether 1 : 1	4	10	9	0.011	1.8 ± 0.40	0 ± 0
8	marked tubes	methanol–acetone 1 : 1	20	10	10	<0.001	1.9 ± 0.10	0.2 ± 0.20
9	VII UTG (freeze-dried extract)	water	10	11	11	<0.001	2.5 ± 0.31	0.2 ± 0.12
10	Organic fraction obtained by partitioning of marked tube extract between hexane/ether and water	hexane–ether 1 : 1	12	10	7	0.172	1.8 ± 0.44	0.3 ± 0.21
11	Aqueous fraction from above partitioning	water	12	10	9	0.011	2.4 ± 0.40	0.5 ± 0.34
12	Organic fraction obtained by partitioning of VII UTG extract between hexane and water	hexane	10	10	7	0.172	2.1 ± 0.48	0.3 ± 0.21
13	Aqueous fraction from above partitioning	water	10	11	8	0.113	2.1 ± 0.46	0.5 ± 0.25

[a]The probability P = Prob ($X \geq x$) is based on the binomial distribution with the assumption that under the null hypothesis pods are equally likely to be chosen (see text for formula).

TABLE 2. WEEVIL RESPONSES TO ACIDIC, BASIC, AND NEUTRAL FRACTIONS OF ODP

Exp.	Test extract (10 weevil equivalents)	No. of weevils tested (N)	No. of weevils ovipositing first in control pod (x)	P = Prob ($X \geq x$)[a]	Rejections of (mean ± SE)	
					Test pod	Control pod
14	VII UTG, acidic fraction	11	6	0.500	1.3 ± 0.45	0.3 ± 0.19
15	VII UTG, basic fraction	11	7	0.274	0.9 ± 0.34	0.1 ± 0.09
16	VII UTG, neutral fraction	10	5	0.623	0.8 ± 0.29	0 ± 0
17	VII UTG, acidic + basic fractions	13	7	0.500	0.9 ± 0.37	0.1 ± 0.08
18	VII UTG, acidic + neutral fractions	10	9	0.011	2.4 ± 0.31	0.2 ± 0.13
19	VII UTG, basic + neutral fractions	11	10	0.006	2.3 ± 0.30	0.1 ± 0.09
20	VII UTG, acidic + basic + neutral fractions	10	9	0.011	2.3 ± 0.33	0.1 ± 0.10

[a]See Table 1 footnote.

However, the results are consistent with the observation that the ODP is soluble in both water and organic solvents, and so activity may have been lost during the preparation of the ether extracts of the three fractions due to partitioning with the aqueous phase. The concentration of ODP in any one fraction may have been too low to elicit a behavioral response in the bioassay, whereas a combination of the neutral fraction with either of the other two may have exceeded the response threshold.

GC-MS Analyses. Analysis of ether–pentane washes of the sealed tubes by GC-MS (Figure 1) showed that only a few nanograms of a large number of compounds had been deposited on the tubes. Nevertheless, it was possible to identify the majority of the components, including several minor components that were phthalate plasticizer contaminants. All of the components, except the dimethylalkanes were identified by comparison of their mass spectra with library spectra (Lias and Stein, 1990) or by comparison with spectra published in the literature cited. Two of the major components, 15-nonacosanone and nonacosane, together with a minor component, 15-nonacosanol, are typical components of cuticular waxes of *Brassica* species (Evershed et al., 1992); this was confirmed by analyzing ether–pentane washes of rape pods using the same GC-MS conditions, and these compounds were found to be present in approximately the same ratio as in the active extract. The hydrocarbons were identified by GC-MS analyses of a hexane extract of active material that had been eluted from a small silica column with hexane. The dimethylalkanes were characterized by their (M − 15) ions and four prominent ions arising from cleavage at the sites of two methyl groups. Furthermore, strong even-mass ions at 1 amu less than those arising from cleavage at the methyl groups are observed and are due to rearrangement of the molecular ion by hydrogen transfer from a secondary position to the cleavage site generating a stable cyclic ion (McCarthy et al., 1968; Pominis et al., 1978, 1980; Doolittle et al., 1995).

The major components of the secretion were 13,17-dimethylhentriacontane (M − 15 at *m/z* 449 with cleavage ions at *m/z* 197, 225, 267, 295 and rearrangement ions at *m/z* 196 and 224 and at *m/z* 266 and 294), 13,17-dimethyltritriacontane (M − 15 at *m/z* 477 with cleavage ions *m/z* 197, 253, 267, 323 and rearrangement ions *m/z* 196 and 252 and at *m/z* 266 and 322), 13,17-dimethylpentatriacontane (M − 15 at *m/z* 505 with cleavage ions at *m/z* 197, 267, 281, 351 and rearrangement ions at 196 and 280 and at *m/z* 266 and 321) and 13,17-dimethylheptatriacontane (M − 15 at *m/z* 533 with cleavage ions at *m/z* 197, 267, 309, 379 and rearrangement ions at *m/z* 196 and at *m/z* 266 and 378) and are not present in the rape pod cuticle. Some of these hydrocarbons were found in hexane extracts of undissected *C. assimilis* (Richter and Krain, 1981).

To obtain a cleaner, purer sample of the active material, extracts were made in hexane–ether from the VII UTG. When concentrated to a small volume

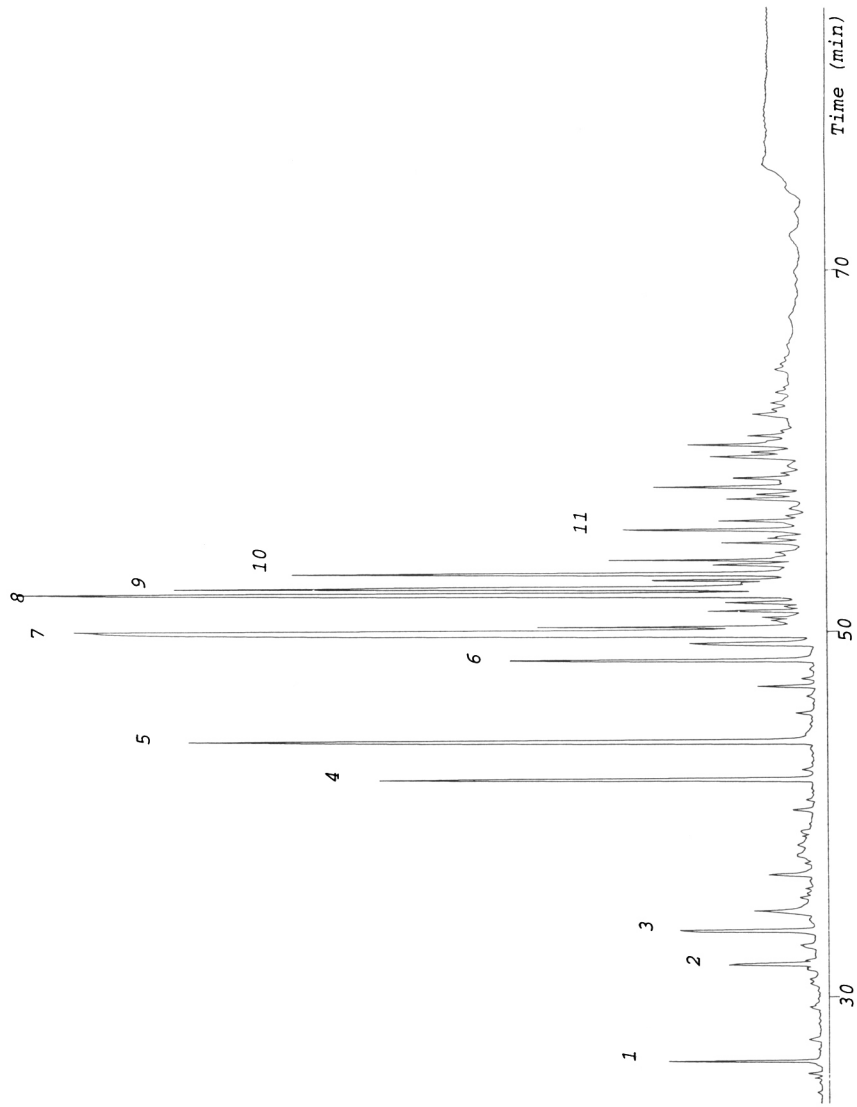


FIG. 1. Total ion chromatograms from GC-MS of extracts of glass tubes marked with ODP by weevils. Peaks 1–5 are phthalates, peak 6, squalene; 7, nonacosane; 8, 15-nonacosanone; 9, 15-nonacosanol; 10, 13,17-dimethylhentriacontane; 11, 13,17-dimethyltritriacontane; 12, 13,17-dimethylpentatriacontane; and 13, 13,17-dimethylheptatriacontane.

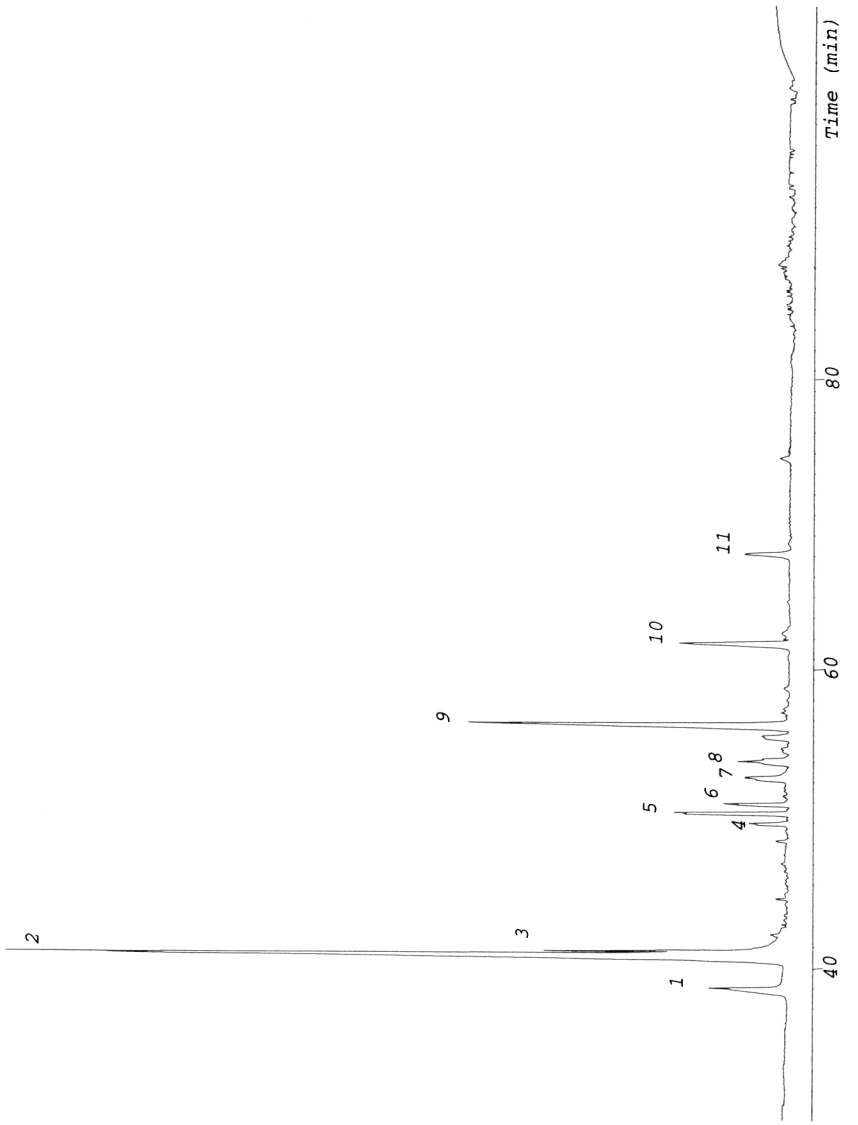


FIG. 2. Total ion chromatogram from GC-MS of an extract of weevil seventh urotergite glands. Peaks are: 1, palmitic acid; 2, linolenic, linoleic, and oleic acids; 3, stearic acid; 4, isononacosane; 5, *n*-nonacosane; 6, branched chain nonacosane; 7, 15-nonacosanone; 8, cholesterol; 9, 13,17-dimethylhentriacontane; 10, 13,17-dimethyltriacontane; and 11, 13,17-dimethylpentatriacontane.

TABLE 3. WEEVIL RESPONSES TO CHROMATOGRAPHIC FRACTIONS OF EXTRACT CONTAINING ODP

Exp.	Test extract	No. of weevil equivalents per test	No. of weevils tested (N)	No. of weevils ovipositing first in control pod (x)	P = Prob ($X \geq x$)[a]	Rejections of (mean ± SE)	
						Test pod	Control pod
21	Total eluate of preparative GC of VII UTG extract	13	10	7	0.172	1.7 ± 0.45	0.1 ± 0.10
22	Combined fractions from silica column chromatography of VII UTG extract eluted with hexane, ether and methanol	11	11	9	0.033	1.8 ± 0.35	0.2 ± 0.12
23	Hexane fraction of silica column chromatography of VII UTG extract	10	10	4	0.828	0.6 ± 0.22	0.1 ± 0.10
24	Ether fraction of silica column chromatography of VII UTG extract	10	12	7	0.387	1.3 ± 0.40	0.2 ± 0.1
25	Methanol fraction of silica column chromatography of VII UTG extract	10	11	9	0.033	1.6 ± 0.39	|0 ± 0|
26	Total methanolic eluate of ODS reverse-phase HPLC of VII UTG extract	15	10	10	0.001	2.9 ± 0.23	1.4 ± 0.4

[a]See Table 1 footnote.

for GC-MS analysis, the VII UTG extracts become cloudy and insoluble, so they were evaporated almost to dryness and redissolved in a few microliters of acetone. Analysis of this extract showed that a mixture of lipids, similar to those from the glass tubes marked with ODP by the weevil, were present (see Figure 2) but in different proportions and without some of the contaminants. However, the rising baseline and poor resolution of the GC chromatogram suggested that one or more components may be involatile or thermally unstable. The very thin film of the stationary phase (0.1 μm) in the GC column, chosen to allow components of low volatility to pass through, involves some sacrifice of sample loading capacity, resulting in a broadening of the GC peaks.

To discover whether the oviposition marker was decomposing on the GC column, the VII UTG extract was injected on-column under the same conditions as the GC analysis above and the GC effluent was collected in hexane. When the GC effluent was bioassayed as before, test pods were rejected more than controls (experiment 21, Table 3), suggesting that the oviposition deterrent was thermally stable under the conditions of the GC analysis. However, because several injections were required to obtain sufficient material for bioassay and because the film of the stationary phase was very thin (0.1 μm), the acetone solvent used for injection may have washed some of the active material through the column even though it had not vaporized sufficiently to give a discrete GC peak. Therefore, although the oviposition marker appeared to be thermally stable under these conditions, it was too involatile to give an identifiable GC peak.

Liquid Chromatography and Mass Spectrometry. Since not all of the components of the ODP extract were amenable to GC-MS analysis, preliminary experiments were performed to isolate the ODP by liquid chromatography. Oviposition deterrent activity was found in the total eluate from the small silica column loaded with VII UTG extract and eluted successively with hexane, ether, and methanol (experiment 22, Table 3). When the different solvent eluates were tested separately, only the methanol fraction was strongly active (experiments 23–25, Table 3), confirming that the ODP has polar properties. Oviposition deterrent activity was also found in the total eluate from an ODS reverse-phase HPLC column loaded with VII UTG extract and eluted with methanol (experiment 26, Table 3). Analysis of the eluate by mass spectrometry on the direct insertion probe yielded no useful information. However, when analyzed by atmospheric pressure chemical ionization mass spectrometry, a major ion *m/z* 679 (MH^+) was observed, consistent with a polar compound of low volatility.

Identification and synthesis of the ODP may provide a means for controlling seed weevil egg-laying in crops. The ODP could also be used to improve control of the brassica pod midge (*Dasineura brassicae* Winn.), which largely depends upon the seed weevil for oviposition sites.

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VOLATILES FROM *Psylla*-INFESTED PEAR TREES AND THEIR POSSIBLE INVOLVEMENT IN ATTRACTION OF ANTHOCORID PREDATORS

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Abstract—Previous work showed that anthocorid predators aggregate around gauze cages containing *Psylla*-infested trees in a pear orchard. Because anthocorids responded to odor from *Psylla*-infested leaves in a laboratory test, it was hypothesized that these aggregative responses in the field were triggered by olfaction of compounds associated with *Psylla* injury. We present chemical analyses of volatiles from damaged and undamaged plants and studies on behavioral responses of anthocorid predators to compounds released by damaged plants. Leaf headspace volatiles from clean and *Psylla*-infested pear trees were collected on Tenax and identified by GC-MS after thermodesorption. Twelve volatiles were found exclusively in headspace samples from *Psylla*-infested leaves. Six were present in significantly higher quantities in samples from infested leaves: the monoterpene, (*E*,*E*)-α-farnesene, the phenolic, methyl salicylate, and the green leaf compounds, (*Z*)-3-hexen-1-yl acetate, (*Z*)-3-hexen-1-ol, 1-hexyl-acetate, and 1-penten-3-ol. These compounds are known to be produced by plants, and damage by pear psyllids seems to trigger their emission. Blend composition varied and was partly correlated with tree or leaf age and degree of *Psylla* infestation. To study whether compounds associated with leaf injury elicit olfactory responses in anthocorid predators, apple-extracted (*E*,*E*)-α-farnesene, synthetic methyl salicylate, and (*Z*)-3-hexen-1-yl acetate were offered in a Y-tube olfactometer to field-collected adult *Anthocoris* spp. Significant positive responses were found to both the

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monoterpene and the phenolic, but not to the green leaf volatile. The results lend support to the hypothesis that predator attraction to herbivore-infested pear trees is mediated by herbivory-induced plant volatiles.

Key Words—Leaf volatiles, synomones, pear tree, *Psylla*, *Anthocoris*, induced response, tritrophic interaction, attraction, GC-MS, olfactometer.

INTRODUCTION

A plant may defend itself against herbivores by promoting the effectiveness of the herbivores' antagonists, a phenomenon called extrinsic or indirect defense (Price et al., 1980). One way to achieve this is by production of herbivory-induced volatiles in the plant. Irrespective of their origin, these volatiles are beneficial to both predator and plant, as they enable the predator to locate its prey, which consequently alleviates herbivore pressure on the plant. Such volatiles are termed synomones (sensu Dicke and Sabelis, 1988) and are reported for several acarine and insect predator–prey and parasitoid–host systems on plants (e.g., Dicke et al., 1993; Takabayashi and Dicke, 1996). In these systems, the origin of the volatiles is based on general plant biochemical pathways and the existence of systemic responses to damage. Evidence for a role of herbivory-induced plant volatiles in plant defense has been inferred from the following laboratory observations: (1) predators or parasitoids respond to odors from leaves infested with their prey or hosts (e.g., Sabelis and Van de Baan, 1983; Turlings et al., 1990; McCall et al., 1993); (2) headspace volatiles of herbivore-infested plants differ from those of clean and mechanically damaged plants (Dicke et al., 1990a; Turlings and Tumlinson, 1992; Takabayashi et al., 1994); (3) specific components elicit responses of the herbivore's antagonists (Dicke et al., 1990a; Turlings et al., 1991).

Recently, field evidence for synomone-mediated attraction of predators to herbivore-infested plants was obtained in a system consisting of pear trees (*Pyrus communis* L.), pear psyllids (*Psylla pyri* L. and *P. pyricola* Forster), and anthocorid predators [*Anthocoris nemorum* L., *A. nemoralis* (Fabricius), and various *Orius* spp.] (Drukker et al., 1995). In an orchard with a very low density of pear *Psylla*, significantly more anthocorid predators were attracted towards cages containing trees heavily infested with pear *Psylla*, than towards cages with uninfested trees. These predators migrate into the orchard from surrounding hedgerows where they feed on other homopteran prey (Drukker et al., 1992; Scutareanu et al., 1993; Scutareanu, Lingeman, and Sabelis, unpublished data). One of the hypotheses explaining the aggregative response to prey density is mediation by herbivory-induced plant volatiles (Drukker et al., 1995).

To test this hypothesis derived from field experiments, we previously performed laboratory experiments to demonstrate the *Psylla*-induced emission of

pear volatiles. We provided evidence for the involvement of several specific compounds in predator attraction. The evidence is based on Y-tube olfactometer tests in which individual predators were offered the choice between clean air and air from *Psylla*-infested leaves or air from uninfested leaves (Drukker and Sabelis, 1990; Drukker, unpublished results). In this paper, we focus on additional evidence: chemical analysis of headspace volatiles from damaged and undamaged pear trees and behavioral analysis of the predator response to single herbivory-associated components.

METHODS AND MATERIALS

Young potted pear trees of the variety Conference, grafted on either quince or pear root stocks and purchased from commercial nurseries, were kept uninfested or were infested with *P. pyricola* (Homoptera, Psyllidae) collected in a pear orchard (Watergraafsmeer, Amsterdam, The Netherlands).

Two experiments were carried out in successive years: in the first, variability due to leaf age (incorporating seasonal effects) was taken into account; in the second, variability due to time since *Psylla*-colonization was considered.

Experiment 1. In late March 1993, one 4-year-old tree (tree 2, Figure 1) was placed in a cage in a climate room (ca. 20°C; 60–70% relative humidity; 17L:7D). Field-collected twigs with several dozen *P. pyricola* adults and eggs were supplied to infest the tree. In July, when the *Psylla* population had reached high density, two additional trees of similar age (not yet infested; trees 3 and 4, Figure 1) were transferred from the field to the same cage (tree 3 in the first week of July, tree 4 in the last), which was now placed in another climate room (ca. 23°C; 65–70% relative humidity; 16L:8D). As a control, one outdoor tree of the same age was kept uninfested throughout the experiment (tree 1, Figure 1). In early May, shortly after the trees had started to flush, one sample of 10 young leaves (tree 1) and two samples of 9 leaves each (tree 2) were collected (total fresh weight of leaf blades: 3.2, 0.9, and 1.1 g, respectively; mean infestation level of the samples: 0, 31.3, and 40.6 nymphs per leaf, respectively). In late August, shortly after the flush had stopped, samples of 10 or 9 mature leaves were taken from trees 1, 3, and 4 (total fresh weight: 4.7, 1.9, and 2.3 g, mean infestation level: 0, 1.8, and 96.6 nymphs per leaf, respectively). All leaves were sampled randomly and used for headspace analysis of volatile compounds. Fresh weight and number of nymphs were assessed immediately after collection of volatiles.

Although control tree and treated trees were under different environmental conditions, weekly inspections showed that the control tree was free of psyllids and any other herbivores throughout the season. To what extent, if any, this tree had suffered from herbivory in previous years is unknown.

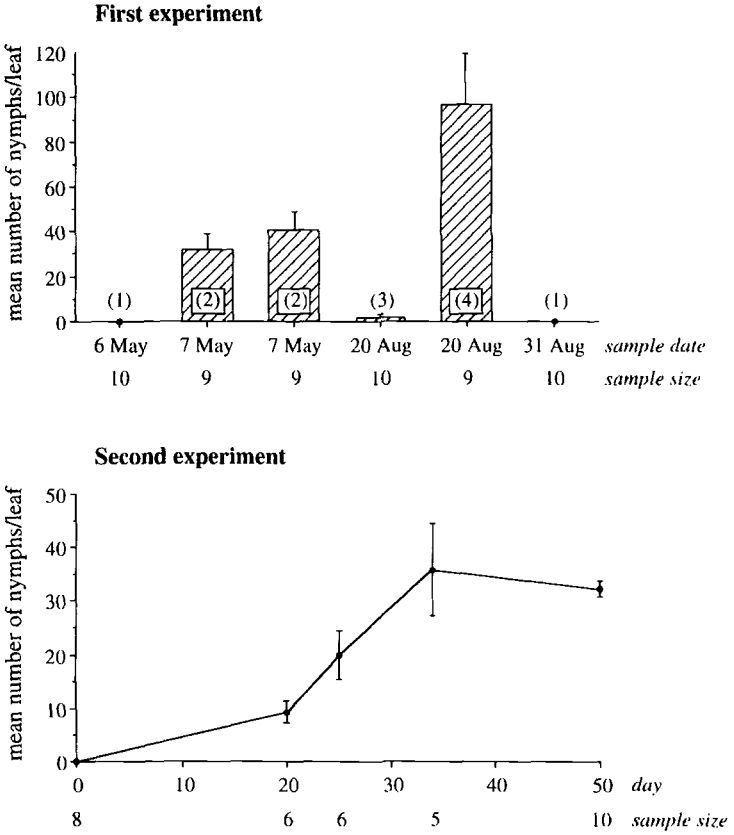


FIG. 1. The abundance (mean number ± standard error) of *Psylla pyricola* nymphs on leaves. First experiment: tree 1 is uninfested; trees 2, 3, and 4 are infested. All samples were used for headspace sampling (GC-MS analysis). Second experiment: day 0, before infestation; day 20, first nymphs present; day 25, 5 days after first presence of nymphs; day 50, 30 days after first presence of nymphs. Samples collected on these days were used for assessment of nymph density and for headspace sampling. On day 34 only nymphs were counted. "Sample size" refers to numbers of leaves.

Experiment 2. In early January 1994, two 1-year-old trees were placed in separate cages in a climate room (ca. 23°C; 65–70% relative humidity; 16L:8D). After the release in late January of several dozen *P. pyricola* adults, one tree gradually became infested (as shown in Figure 1, experiment 2); the other tree was kept uninfested (not shown in Figure 1) to serve as a control. At regular intervals (specified in Figure 1, experiment 2), random samples of 5–10 young

leaves were taken from each tree for analysis of headspace volatile compounds (total leaf blade fresh weights: 1.2–4.0 g for clean leaves, 1.2–2.7 g for infested leaves). The samples from the infested tree were also used for monitoring *Psylla* infestation. The first headspace sample (day 0) was taken just before release of psyllids; the second (day 20) shortly after the first larvae and honeydew were observed on leaves (infestation level 9.3 nymphs per leaf); the third (day 25) and fourth (day 50) were taken when levels of infestation had increased (20.0 and 31.6 nymphs per leaf, respectively). Weighing of leaves took place after collection of volatiles.

Collection and Identification of Leaf Volatiles. Fresh leaves (blades and petioles), cut just prior to headspace sampling, were put into a 500-ml glass jar. Incoming air was purified by drawing it through silica gel and activated charcoal (both 400 ml). Volatiles were trapped on Tenax adsorbant (90 mg) packed in a 160-mm × 4-mm-ID glass tube (Chrompack). Airflow was ca. 100 ml/min, and sampling time was 120 min (except once in experiment 1, tree 2, where sampling time was 30 min). The Tenax tubes were closed and stored in the dark at room temperature until they were subjected to thermodesorption.

Adsorbents were first released from the Tenax by thermodesorption at 250°C for 10 minutes with a helium flow of 10 ml/min. Desorbed compounds were cryofocused in a cold trap at −90°C (M-16200, Chrompack) and subsequently analyzed on a Supelcowax-10 capillary column (60 × 0.25 mm-ID, 0.25-mm film thickness). The temperature program of the gas chromatograph was 40°C (4 min), rising to 140°C at 3°C/min and then to 270°C at 6°C/min. The column was connected to a Finnigan MAT95 mass spectrometer, operated in the 70 eV EI ionization mode with scanning from mass 24 to 400 at 1.05 sec cycle time.

Compounds were identified by comparison of mass spectra with those in the Wiley Library (McLafferty and Staufer, 1989) and our own specialized library of natural products (M. A. Posthumus, Organic Chemistry, Wageningen) and by comparison of retention times with our home-built data base of retention indices based on authentic samples. Quantification was based on the average response of a mixture of 10 selected natural compounds run in a separate trial (1000 counts corresponded to 70 ng in all samples, except the two samples taken on day 50, experiment 2, where 1000 counts corresponded to 200 ng).

Olfactometer Bioassays. Experiments were carried out to determine whether anthocorids respond to two volatiles, (*E*,*E*)-α-farnesene and methyl salicylate, found abundantly in headspace samples of infested trees, but not at all or only in minute quantity in samples of uninfested trees. Attractiveness of these two compounds was compared to (*Z*)-3-hexen-1-yl acetate, found in both infested and uninfested trees.

A Y-tube olfactometer was used (cf. Sabelis and Van de Baan, 1983; Dicke et al., 1990a). The two upper arms of the Y-tube were connected to tubes containing the stimuli. One of the tubes contained the experimental stimulus—

a hexane solution of the volatile administered on filter paper. The other contained the control stimulus—the solvent on filter paper without the test chemical. Prior to testing, the hexane was allowed to evaporate for 3 min (a pretest had shown that more than 99.9% of the solvent evaporated within this period). After five runs, tubes with experimental and control stimuli were reversed. The basal arm of the Y-tube was connected to an air pump with a valve ensuring a constant air flow of 0.25–0.35 m/sec in both upper arms. In the center of the Y-tube, a Y-shaped metal wire served to guide the insects. Predators were released one at a time on the downwind end of the wire, from where they could walk to the junction where the two odor plumes met. At this point, the predator could choose to go towards either of the odor sources. A trial was ended when the predator stepped off the wire and reached the end of either of the upper arms, or 5 min after the start of a test. In the former case, the predator's behavior was interpreted as preferential for the stimulus concerned, in the latter, it was interpreted as not preferential for either of the stimuli. When the predator sat motionless for more than 1 min on the glass beyond the end of the wire, its behavior was also interpreted as preferential for the stimulus.

Tests were performed with 0.24 mg of (*E*,*E*)-α-farnesene (isolated from apple extracts, provided by TNO, Delft, The Netherlands), 2.3 mg of synthetic methyl salicylate (Aldrich), and 5 mg of synthetic (*Z*)-3-hexen-1-yl acetate (Aldrich). Odor sources were renewed every hour, except farnesene which was renewed every half hour. Hexane (100 μl) was used as solvent for (*E*,*E*)-α-farnesene and methyl salicylate. (*Z*)-3-Hexen-1-yl acetate was administered without solvent. The concentrations of chemicals offered in the olfactometer were rough estimates of concentrations that anthocorids could encounter in the field (see Results—Olfactometer bioassays).

Anthocoris nemorum males and females were collected in August and September 1994 and 1995 from pear trees in an experimental orchard at Lienden, and *A. nemoralis* males and females from pear trees in a commercial orchard at Goes, The Netherlands. All predators were kept at 4°C and were starved at 25°C for at least 2 hr prior to testing.

Statistical Tests. Chemical samples were compared using the Mann-Whitney U test for equal or unequal sample sizes (Siegel, 1956). Responses of anthocorids in the olfactometer were analyzed with a two-tailed binomial test (null hypothesis: both odor sources are equally attractive).

RESULTS

Composition of Volatile Blends. Representative gas chromatograms (Figure 2A and B) illustrate the differences of the volatile blends from uninfested and infested leaves. For further analysis we selected compounds based on potential

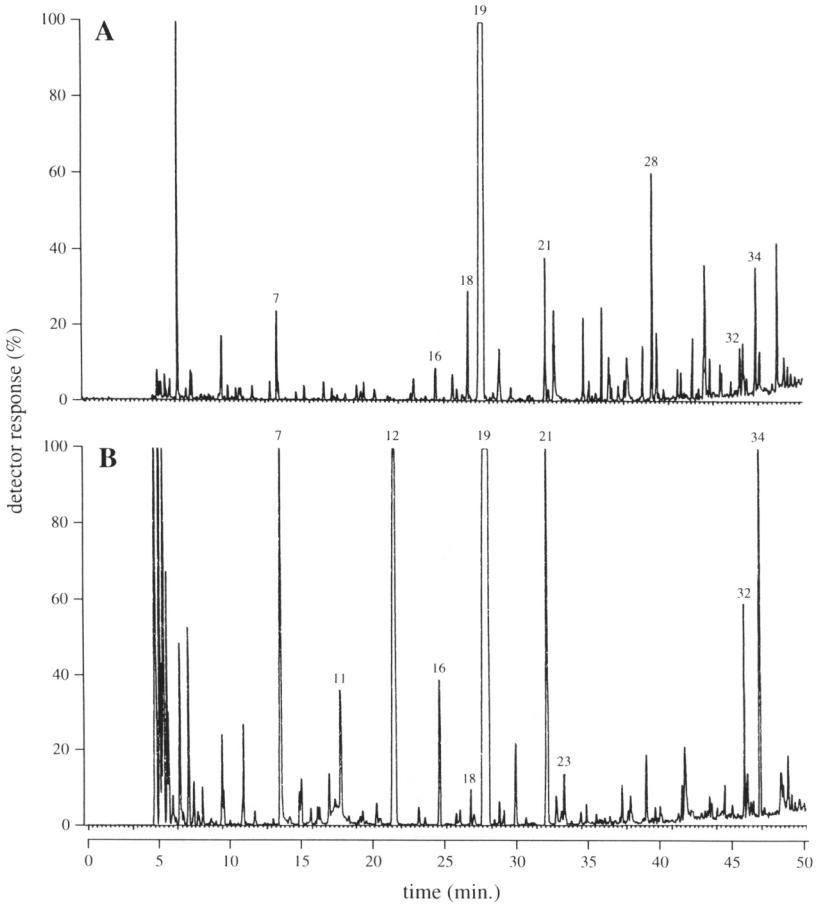


FIG. 2. Representative gas chromatograms of headspace volatiles sampled from pear tree leaves (first experiment). Temperature program of the gas chromatograph was 40°C for 4 min, then rising to 140°C at 3°C/min, then to 270°C at 6°C/min. A: uninfested young leaves (May); B: heavily infested young leaves (May). Peak numbers correspond to the compounds listed in Table 1.

plant origin and presence in two or more samples. We subsequently focused on compounds that were damage-related and hence could potentially mediate plant–predator communication. For this we used a third criterion: difference in frequency between infested and uninfested leaf samples exceeding three and/or difference in (mean) abundance between infested and uninfested leaf samples exceeding 25 ng. The purpose of the third criterion was merely to serve as a

TABLE 1. HEADSPACE VOLATILES COLLECTED FROM CLEAN PEAR LEAVES AND LEAVES WITH VARIOUS LEVELS OF *Psylla*-INFESTATION IN MAY AND AUGUST (EXPERIMENT 1)[a]

Volatile	Volatile amount (ng/hr/g fresh leaf)					
	May (young leaves)			August (old leaves)		
Nymphs/leaf:	0	31	41	0	1.8	97
Tree:	1	2	2	1	3	4
1. 2-butanone	2.84		16.73			
2. 2-methylbutanal			5.07			9.43
3. 3-methylbutanal			5.14			9.43
4. 3-pentanone			34.59			
5. 1-penten-3-one			46.42			
6. butyl acetate	1.46					
7. hexanal	7.19	23.21	235.30	2.21	3.58	32.21
8. 2-pentenal			18.62			
9. 3-pentanol		13.21	19.82			
10. 1-butanol	2.38	64.88	27.36			10.60
11. 1-penten-3-ol			63.63			41.03
12. (*E*)-2-hexenal		31.34	691.03			155.48
13. limonene	1.68	trace		0.48	3.47	
14. (*E*)-β-ocimene					36.30	5.88
15. 1-pentanol	2.02		8.16			21.66
16. 1-hexyl acetate	3.83	30.49	77.76			9.76
17. octanal*	3.18	27.44	6.03			
18. (*E*)-4,8-dimethyl-1,3,7-nonatriene	13.38		15.36		107.05	88.92
19. (Z)-3-hexen-1-yl acetate	302.14	2080.04	1289.35	63.15	417.03	436.67
20. 1-hexanol	1.86	24.23	42.48			49.28
21. (Z)-3-hexen-1-ol	19.76	269.70	266.98	12.67	52.38	197.87
22. nonanal*	16.42	87.08	23.31			
23. (*E*)-2-hexen-1-ol			22.42			78.81
24. unknown (43, 79, 80)	8.68		16.08			14.40
25. 3-hexen-1-yl butanoate	9.09					
26. 1-octen-3-ol		47.94			9.52	81.95
27. decanal*	5.25	155.18	17.18			
28. linalool	20.66		9.19		2.57	4.17
29. 1-octanol	6.65					4.35
30. β-caryophyllene	3.18				5.70	
31. α-copaene					3.43	
32. α-farnesene	3.41	19.65	72.79	1.86	37.01	113.63
33. δ-cadinene					3.52	
34. methyl salicylate	13.53	254.12	202.73		51.75	7.47
35. caproic acid		110.12	22.29	12.28		
36. benzyl alcohol	3.07		20.30			18.52
Total amounts (ng/hr/g fresh weight)	452	3239	3276	93	734	1392
Total number of compounds	22	16	27	6	13	21

[a]Comparison using Mann-Whitney U test ($N_1 = N_2 = 3$) for each compound showed significant differences between leaf samples in May and August at the $P = 0.05$ level, indicated by an asterisk.

conservative selection of compounds; the discriminators '3' and '25' are thus arbitrary. In the 14 samples, a total of 42 compounds satisfied the first and second criteria, 36 of which were found in experiment 1 (Table 1), 29 in Experiment 2 (Table 2), whereas 10 compounds met the third criterion (summarized in Table 3). All 10 compounds were more abundant, and eight were more frequent in samples from infested leaves. For six, the differences in abundance were statistically significant (Table 3), namely, 1-penten-3-ol, 1-hexyl-acetate, (*Z*)-3-hexen-1-yl acetate, (*Z*)-3-hexen-1-ol, (*E*,*E*)-α-farnesene, and methyl salicylate (Table 3). Two compounds, 3-hexen-1-yl-butanoate and linalool, were on average more abundant in samples from uninfested leaves, although not statistically significant. The frequency of linalool, however, was higher in infested than in uninfested leaf samples.

Considering the proportion of each compound in the blend, it appears that the green-leaf compounds (*Z*)-3-hexen-1-yl acetate and (*Z*)-3-hexen-1-ol are the most abundant (Tables 1 and 2). They make up 73% (42.8–97.5%) of the blend in infested leaves and as much as 90.5% (63.7–98.9%) in uninfested leaves.

Variability Due to Leaf Age (Experiment 1). The number and total amount of compounds found in young leaves (May) was higher than in old leaves (August), both uninfested and infested (Table 1; normalized chromatograms in Figure 3). Three aldehydes, consistently present in May (both in uninfested and infested leaves), were absent in August—octanal, nonanal and decanal. 3-Pentanol was present in infested plants in May but not in August. (*E*)-β-Ocimene was found in infested plants in August, but was absent in May. Hexanal and methyl salicylate were more abundant in May, whereas (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-α-farnesene were more abundant in August. Methyl salicylate was detected in high quantities in infested young leaves, but in much lower absolute and relative values in heavily infested, old leaves (August) (Table 1, Figure 3).

Both in May and in August, amounts of volatiles increased with the degree of infestation (Table 1). Some compounds showed a consistent quantitative increase from uninfested to highly infested leaves, e.g., hexanal and (*E*,*E*)-α-farnesene. Methyl salicylate and (*E*)-β-ocimene were most abundant in August in the headspace of the mildly infested leaves and absent or scant in the samples from uninfested and highly infested trees. These results are consistent with those obtained in experiment 2, when only young leaves were analyzed (Table 2).

Variability Due to Time Since Colonization (Experiment 2). Both the total number and amount of volatiles found in infested plants (experiment 2, Table 2) increased over time until day 25, then slightly decreased but remaining higher than before infestation. In uninfested plants, the number of compounds remained the same, but the total amounts decreased consistently (Table 2). Most compounds steadily increased after the onset of infestation. Five of them {(*Z*)-3-hexen-1-yl acetate, (*Z*)-3-hexen-1-ol, methyl salicylate, benzyl alcohol, and an

TABLE 2. HEADSPACE VOLATILES COLLECTED FROM *Psylla*-INFESTED PEAR LEAVES BEFORE AND AFTER 20, 25, AND 50 DAYS OF INFESTATION, COMPARED TO UNINFESTED TREE (EXPERIMENT 2)[a]

	Volatile amount (ng/hr/g fresh leaf)							
	Days after start (mean no. nymphs/leaf) in uninfested tree				Days after start (mean no. nymphs/leaf) in infested tree			
Volatile	0 (0)	20 (0)	25 (0)	50 (0)	0 (0)	20 (9.3)	25 (20.0)	50 (32.3)
1. 3-pentanone	0.02		trace			0.02	0.08	0.19
2. 1-penten-3-one	0.05		trace	0.01		0.03	0.10	0.76
3. butyl acetate*			0.01			0.02	0.03	0.06
4. hexanal	0.09		0.01	0.01		0.07	0.07	0.68
5. 2-pentenal*						trace	0.04	0.07
6. 3-pentanol*			0.05	0.12		0.19	0.92	1.20
7. 1-butanol	0.19	0.05	0.04			0.09	0.06	0.09
8. unknown (66, 96)*						0.01	0.14	0.09
9. 1-penten-3-ol*						0.04	0.19	0.50
10. (*E*)-2-hexenal								1.82
11. limonene	0.26	0.02	0.02	0.03		0.11	0.08	0.42
12. 1-hexyl acetate*						0.37	1.72	0.93
13. (*Z*)-3-hexen-1-yl acetate*	61.21	62.93	51.10	30.35	24.82	147.41	538.16	223.59
14. anisole			0.02	0.17		0.01		0.20
15. (*Z*)-3-hexen-1-ol*	9.53	4.12	1.00	0.44	1.92	9.74	81.83	46.13
16. nonanal	0.13		0.02	0.01				
17. unknown (43, 79, 80)*	0.31	0.48	0.45	0.31	0.09	0.62	1.71	0.56
18. 3-hexen-1-yl butanoate	0.21	0.09	0.02		0.07	0.30	2.16	0.10
19. 3-hexen-1-yl 2-methylbutanoate		0.01				0.09	0.41	
20. linalool	0.06					0.02	0.07	
21. β-caryophyllene	0.10		0.08	0.02		0.13	0.09	
22. α-copaene			0.05	0.17				
23. 5-ethyl-2(5H)-furanone							0.14	0.80
24. α-farnesene							0.28	2.03
25. δ-cadinene		0.02	0.03	0.11			0.09	0.15
26. methyl salicylate*	1.24	0.10	0.01		0.51	3.92	6.51	25.93
27. ethyl salicylate							0.02	0.11
28. benzyl alcohol*					0.07	0.16	0.29	0.31
29. 3-hexen-1-yl benzoate*						0.22	0.53	0.32
Total amounts (ng/hr/g fresh weight)	73	68	53	32	27	164	636	307
Total number of compounds	13	9	17	12	6	21	25	24

[a]Comparison with a Mann-Whitney-U test ($N_1 = 4$, $N_2 = 3$) for each compound showed significant differences between infested and uninfested leaf samples at the $P = 0.05$ level, indicated by an asterisk.

TABLE 3. SUMMARY OF COMPOUNDS COLLECTED FROM CLEAN AND *Psylla*-INFESTED PEAR LEAVES; RESPONSE BY TWO SPECIES OF ANTHOCORID PREDATORS TO SOME CHEMICALS[a]

Volatile	Identified in						Response by	
	Psylla pyricola infested leaves			Uninfested leaves			*Anthocoris nemoralis*	*Anthocoris nemorum*
	ng/hr/g	frequency	%GCarea	ng/hr/g	frequency	%GCarea		
1. hexanal	42	7	1.5	1.4	5	0.6		
2. 2-pentenal	2.7	4	0.1	0	0	0		
3. 1-penten-3-ol	15*	5	0.7*	0	0	0.0		
4. (*E*)-2-hexenal	126	4	4.7	0	0	0.0		
5. 1-hexyl-acetate	17*	6	0.7*	0.6	1	0.1		
6. (*E*)-4,8-dimethyl-1,3,7-nonatriene	30	3	3.0	1.9	1	0.4		
7. (*Z*)-3-hexen-1-yl acetate	733†	7	62.2	85	7	83.8	0	
8. (*Z*)-3-hexen-ol	132†	7	10.1	7	7	6.7		
9. (*E*,*E*)-α-farnesene	35*	6	2.3	0.8	2	0.4	+	+
10. methyl salicylate	79*	7	4.8*	2.2	5	0.9	+	+

[a] Amounts are expressed in ng/hr/g fresh weight of leaves estimated from peak areas of gas chromatogrammes (means of seven leaf samples), frequencies are per seven leaf samples, %GCarea express the proportion of each compound in the total blend of volatiles (means of seven leaf samples). Comparison using Mann-Whitney U test ($N_1 = N_2 = 7$) for each compound showed significant differences between infested and uninfested leaf samples at the $P = 0.05$ and 0.01 level, indicated by an asterisk and a dagger, respectively.

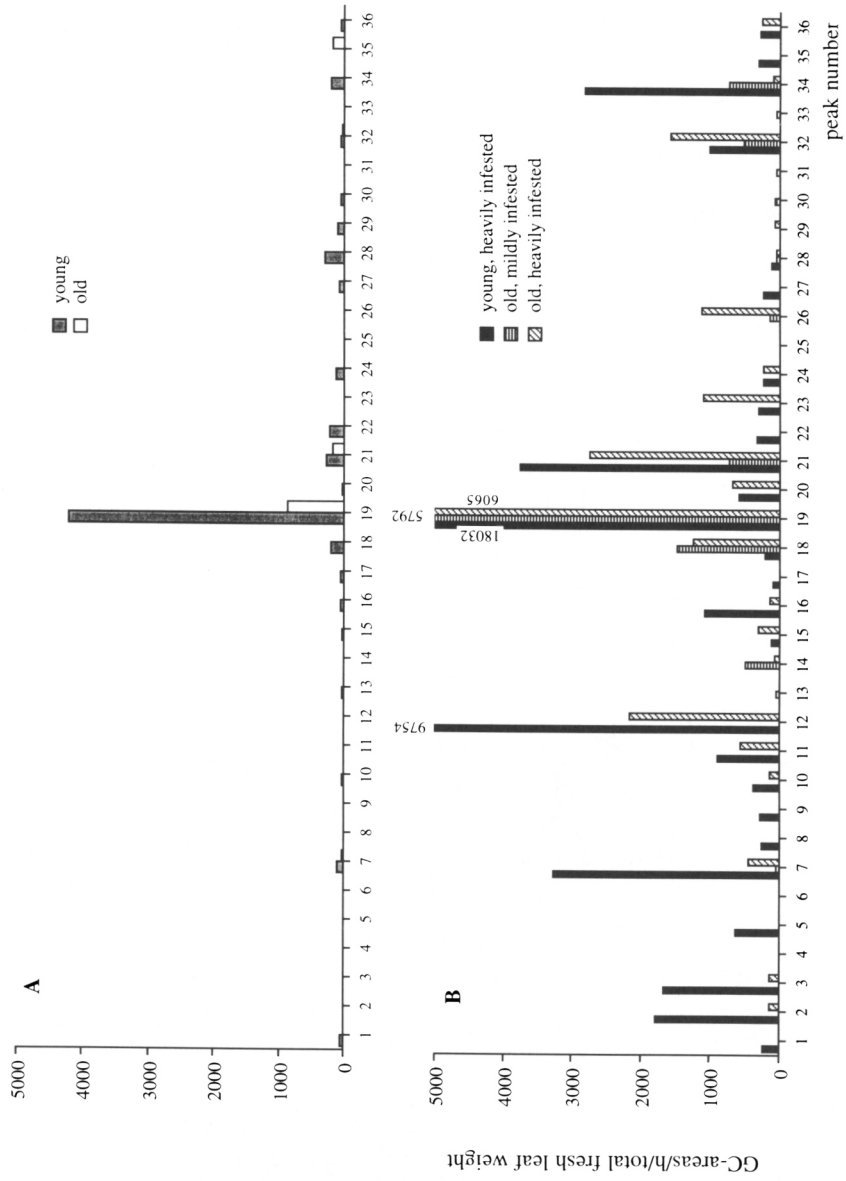


FIG. 3. Normalized GC peak areas of the main volatile compounds in young and old leaves of the 4-year-old pear trees related to the degree of *Psylla* infestation (first experiment). A: uninfested tree 1; B: infested trees 2, 3, and 4. (Peak numbers as in Table 1.)

unidentified compound [*m/z* 43 (100%), 79 (75%), 80 (81%)]} were present before the tree was inoculated with *Psylla*. Seven compounds {butyl acetate, 2-pentanal, 3-pentanol, unknown [*m/z* 66 (81%), 96 (100%)], 1-penten-3-ol, 1-hexyl-acetate, and 3-hexen-1-yl-benzoate} were detected after the first larvae and honeydew were observed on leaves, i.e., day 20. For these 5 + 7 = 12 compounds there was a significant difference between the three samples from the infested tree and the four samples from the uninfested tree. From day 25 onwards, four additional compounds were found [5-ethyl-2(5H)-furanone, (*E*,*E*)-α-farnesene, δ-cadinene, and ethyl salicylate]. On day 50, only one additional compound [(*E*)-2-hexenal] was detected. The relationship between the progression of *Psylla* infestation and the emission of volatile compounds from young leaves of a 1-year-old pear tree is depicted in Figure 4.

Interexperimental Variability. There were no consistent differences between 1-year-old trees in 1994 (Table 2) and 4-year-old trees in 1993 (Table 1) with respect to number of volatiles in infested or uninfested plants. Total amounts, however, were consistently higher in 4-year-old trees than in 1-year-old trees. Some compounds from 1-year-old trees were absent in samples from 4-year-old trees [isobutyl acetate, unknown (66, 96), anisole, 3-hexen-1-yl butanoate, 3-hexen-1-yl methylbutanoate, 5-ethyl-2(5H)-furanone, ethyl salicylate, and 3-hexen-1-yl benzoate]. Others were exclusively present in samples from 1-year-old trees [2-butanone, 2- and 3-methylbutanal, (*E*)-β-ocimene, 1-pentanol, octanal, (*E*)-4,8-dimethyl-1,3,7-nonatriene, 1-hexanol, nonanal, (*E*)-2-hexen-1-ol, 1-octen-3-ol, decanal, and 1-octanol]. Caution, however, should be taken when attributing the observed differences to differential tree age, since trees also differed with respect to season and year of investigation and in the rootstock upon which they were grafted—quince (kwee-C) for 4-year-old trees and seedlings for 1-year-old trees.

Olfactometer Bioassays. In the Y-tube olfactometer, both *Anthocoris nemoralis* and *A. nemorum* showed positive responses to methyl salicylate (80% and 83%, respectively) and (*E*,*E*)-α-farnesene (77% and 71%, respectively) that significantly deviated from the 50% expected under the null hypothesis (Table 4). (*Z*)-3-Hexen-1-yl acetate, a compound not indicative of *Psylla*-infestation, did not elicit a positive response (only 36% of *A. nemoralis* adults responded positively, $P = 0.3$).

Quantitative results of the chemical analysis can be compared with amounts of compounds administered in the olfactometer. Evaporation of compounds from the filter paper was determined in a pretest: 65% (1.5 ± 0.4 mg, $N = 5$) of methyl salicylate and 76% (2.7 ± 0.4 mg, $N = 5$) of (*Z*)-3-hexen-1-yl acetate evaporated in 30 min in the Y-tube (wind speed: 0.3 liter/sec). Evaporation of (*E*,*E*)-α-farnesene was not measured, but because this compound has a boiling point between that of the other two compounds, evaporation rate is assumed to have an intermediate value. For methyl salicylate, the concentration in the odor

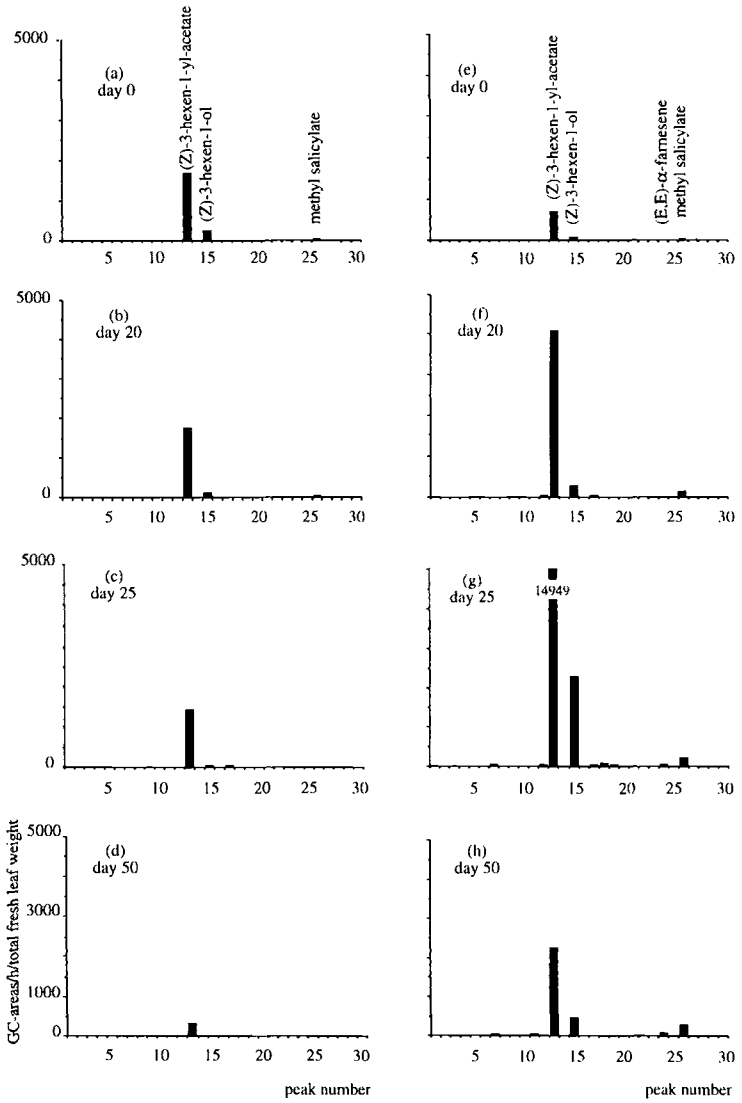


FIG. 4. Normalized GC peak areas of the main volatile compounds in young leaves of the 1-year-old pear trees related to the development of *Psylla* infestation (second experiment). a–d: uninfested tree, e–h: infested tree. Days 0, 20, 25, and 50 as in Figure 1. (Peak numbers as in Table 2.)

TABLE 4. RESPONSE IN *Y*-TUBE OLFACTOMETER BY ORCHARD-CAUGHT *Anthocoris nemoralis* AND *A. nemorum* ADULTS TO (*E*,*E*)-α-FARNESENE, METHYL SALICYLATE, AND (*Z*)-3-HEXEN-1-YL ACETATE TESTED VERSUS CLEAN AIR (TWO-TAILED BINOMIAL TEST; H_0: $P(+) = P(-) = 0.5$)

Experimental (+) stimulus	Species	$n(+)$	$n(-)$	$n(0)$	$\frac{n(+)}{n(+)+n(-)}$	P
0.24 mg (*E*,*E*)-α-farnesene	*A. nemoralis*	49	15	6	0.77	0.00002
	A. nemorum	50	20	8	0.71	0.0004
2.3 mg methyl salicylate	*A. nemoralis*	41	10	5	0.80	0.00001
	A. nemorum	39	8	7	0.83	0.000006
5 mg (*Z*)-3-hexen-1-yl acetate	*A. nemoralis*	8	14	8	0.36	0.3 (ns)

plume in the olfactometer was calculated to be 2.78 μg/liter (mean value over 30 min). In the airstream from the sampled infested leaves, the concentration was 6.58 ng/liter/leaf (6 liters sampled, on average 79 ng/hr/g fresh weight (Table 3), leaves weighing ca. 0.5 g). Therefore, ca. 420 infested leaves would be needed to cause the same concentration as 2.3 mg on filter paper (one single orchard tree will have many more than 420 leaves!). Likewise, the amounts of (*E*,*E*)-α-farnesene and (*Z*)-3-hexen-1-yl acetate used are equivalent to ca. 80 infested leaves.

DISCUSSION

Olfactometer bioassays carried out with two of the volatile compounds that are correlated with *Psylla*-infestation in pear trees elicit a response in anthocorid predators, namely methyl salicylate and (*E*,*E*)-α-farnesene. Because these compounds are of plant origin, it may be inferred that their production is induced by *Psylla* injury. These chemical signals may be plant–predator synomones because upon herbivore attack plants may benefit from attracting predators, and predators profit from responding to prey-related signals (Dicke and Sabelis, 1988).

We did not make further attempts to investigate specificity with respect to the damaging agent. Previous claims (Dicke et al., 1990a; Turlings et al., 1990; Mattiacci et al., 1994) that mechanical damage does not induce the production of volatiles in plants seem unwarranted because it is difficult, if not impossible, to mimic the way (site and process) insects damage a leaf, let alone to generate the damage as continually as the insect does. The only inference we can make

from our data is that repetitive picking of leaves from uninfested trees does not trigger enhanced production of volatiles found in the headspace of infested trees (Table 2). In fact, the levels of these volatiles diminished.

Earlier studies described volatiles from undamaged pear leaves (Miller et al., 1989) and fruits (Jennings et al., 1960). Miller et al. (1989) analyzed volatiles from pear leaves of two other pear cultivars: Bartlett and Bradford, cultivars considered to be susceptible and resistent to pear *Psylla* attack, respectively. They identified 14 compounds, eight of which were also present in one or more of our samples from infested trees: (*E*,*E*)-α-farnesene, (*Z*)-3-hexen-1-yl acetate, (*E*)-β-ocimene, linalool, α-copaene, β-caryophyllene, δ-cadinene, and limonene. The compound they tentatively identified as perillene is most probably (*E*)-4,8-dimethyl-1,3,7-nonatriene, a methylene terpenoid with the same molecular weight and almost the same mass spectrum as perillene and frequently present in volatile blends from arthropod-attacked plants [lima bean mites, apple mites, (Takabayashi et al., 1991); cotton caterpillars (McCall et al., 1994); corn caterpillars (Turlings et al., 1991); pear psyllids (present study)]. The reported Kovats index also corresponds well with the Kovats index on DB-1 for (*E*)-4,8-dimethyl-1,3,7-nonatriene (M. A. Posthumus, unpublished data; see also Dicke et al. 1990a). Some other volatiles, such as methyl salicylate were not found by Miller et al. (1989). These authors did not specify the infestation level of the trees they sampled, but in August, the time of sampling, it is rare to find trees devoid of psyllids in North American orchards (Watson and Wilde, 1963; Van de Baan and Croft, 1991). Furthermore the composition of the leaf volatiles suggests that the trees sampled by Miller et al. (1989) may have been infested at the time of leaf sampling.

Miller et al. (1989) found significant differences between the two cultivars: linalool was found only in Bradford, α-copaene only in Bartlett. We also have evidence for differences in volatile composition between two pear cultivars, i.e., Conference and Beurré Hardy, the latter failing to release methyl salicylate (Drukker and Posthumus, unpublished). Miller et al. (1989) argue that leaf volatiles are used by psyllids to locate their host plants. Thus, differences in volatile composition can be indicative of differential cultivar susceptibility. We suggest that the difference in volatile composition may, in addition, reflect cultivar-related differences in indirect defense by means of predator recruitment. The frequently reported heavy *Psylla* damage on Beurré Hardy compared to Conference (Drukker and Van der Blom, unpublished) may be explained by this difference.

Variability in Blend Composition. Several of the compounds we found associated with *Psylla* infestation were also found to be associated with damage in other, totally different plant–herbivore systems, such as apple spider mite (Takabayashi et al., 1991), lima bean spider mite (Dicke et al., 1990a), cucumber spider mite (Takabayashi et al., 1994), cotton caterpillar (McCall et al.,

1994), corn caterpillar (Turlings et al., 1991), Brussels sprout caterpillar (Mattiacci et al., 1994), broad bean aphid (Hardie et al., 1994), apple sawfly larvae (Boevé et al., 1996), and cabbage caterpillar (Blaakmeer et al., 1994). Five compounds found exclusively in our infested pear samples have not been mentioned before in association with herbivory: 2- and 3-methyl butanal, 5-ethyl-2(5H)-furanon, ethyl salicylate, and anisole.

Some of the variation in blend composition may be attributed to leaf age. We observed both qualitative and quantitative differences between young leaves in May and mature leaves in August, and not all of these differences could be attributed to the higher infestation level in August. For example, (*E*)-β-ocimene was found only in August in leaf samples that had a similar level of infestation to those in May. Alternatively, methyl salicylate was absent in highly infested and uninfested old leaves in August, whereas it was found in considerable quantities in infested young leaves in May. Age-related differences were also found by Takabayashi et al. (1994) in samples from spider mite-infested cucumber leaves.

Blend composition may also be affected by age, rootstock, and attack history of the trees. Our data leave these possible effects open, but we cannot assess their relative contributions. Further research is needed, especially because effects of tree age and attack history are known for direct defensive responses, as reviewed by Haukioja (1990; see also various chapters in Tallamy and Raupp, 1991). This author also makes a useful distinction between induced responses based on the time scale of the induction process; he distinguished between short-term and long-term (or delayed) induced responses. In our experiments, we used trees that originated from commercial nurseries. Hence, we cannot be absolutely sure that these trees had always been free of pests.

Another source of variation in blend composition stems from the accumulation of damage since herbivore colonization. We observed a steady increase over time for most compounds as the infestation proceeded. Some volatiles were observed even before the plants were infested [methyl salicylate in very low quantity, (*Z*)-3-hexen-1-yl acetate and (*Z*)-3-hexen-1-ol]; others were only found after the first appearance of *Psylla* nymphs and associated honeydew (1-hexyl acetate), five days later [(*E*,*E*)-α-farnesene, 5-ethyl-2(5H)-furanone] or 30 days later [(*E*)-2-hexenal].

Predator Response to Synomones. The responses of the two species of anthocorids to the three compounds tested are in line with findings by Dicke et al. (1990a) for phytoseiid predators. They too found a significant positive response to methyl salicylate and an indifferent response to (*Z*)-3-hexen-1-yl acetate. As the latter green-leaf compound was not indicative of spider mite damage, Dicke et al. (1990a) considered the indifferent response adaptive. However, in our pear trees this green-leaf compound is clearly more abundantly released by infested plants and, therefore, using the same line of reasoning

(Dicke et al., 1990a), we could have expected the bugs to respond to it, but they appeared not to. Also in cotton, (*Z*)-3-hexen-1-yl acetate was found to be associated with herbivory (McCall et al., 1994) and here parasitoids actually do respond to this and other green-leaf compounds (Whitman and Eller, 1990). Thus, it is not clear why anthocorids do not respond to the green-leaf compound, but do respond to the other two damage-related volatiles. However, synergistic effects among these (and any other) compounds in the headspace of the infested plants should not be ignored as a possibility. The other damage-related volatiles (Table 3) still remain to be tested, as does the attractiveness of different concentrations of all compounds to anthocorid predators found to be responding to the total blend in the field (Drukker et al., 1995).

The positive responses to the single compounds are surprising in that their information content can only be limited. The full blend, of course, contains much more information. Nevertheless, additional olfactometer tests showed that responses of wild-caught predators to full blends from infested pear leaves (*A. nemoralis*: 76%, $N = 73$; *A. nemorum*: 82%, $N = 61$; Drukker, unpublished data) were comparable to responses to (*E*,*E*)-α-farnesene and methyl salicylate (both species: 71–83%; see Table 4). Preliminary results showed that one of the damage-related compounds, methyl salicylate, elicits positive responses under field conditions as well; predatory bugs were found to be attracted to sticky traps with dispensers of methyl salicylate (on average 9.6 bugs per trap over a period of one to two months, compared to 0.8 in unbaited control traps; Drukker, unpublished data). A possible explanation for positive responses to a single compound may be that the compound is a common denominator of damage inflicted by a wide range of phytophages that include prey items on the menu. This may explain that *A. nemorum* responds to odor blends from French bean leaves infested by prey mites (*Tetranychus urticae* and *T. cinnabarinus*) (Dwumfour, 1992), because these blends are known to include methyl salicylate. Additional damage-related compounds may act in fine tuning the information content of the signal, thereby enabling preferential responses.

One wonders to what extent predatory arthropods can smell "the tree within the forest" among the wealth of chemical information contained in the blends released by plants upon herbivore attack. Whether their responses are plastic or fixed is a major question for future research. In some cases, natural enemies have been shown to change their behavior gradually over days of exposure to a stimulus (Dicke et al., 1990b; Dwumfour, 1992), in others, associative learning was demonstrated over short-term exposure to a stimulus (Lewis and Tumlinson, 1988). These possibilities are currently being investigated with respect to anthocorid predators (Drukker, in progress).

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EFFICIENCY OF SPRAYING MOUNTAIN PINE CONES WITH OLEORESIN OF SWISS STONE PINE CONES TO PREVENT INSECT ATTACK

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Abstract—The difference in cone colonization by specialized insects that had been observed between Swiss stone pine (*Pinus cembra* L.) and mountain pine (*P. uncinata* Ram.) prompted us to test whether volatiles and oleoresin of Swiss stone pine cones may serve as a barrier for insect attack. The volatiles emitted by cones of both pine species were sampled using headspace techniques in the French Alps. The cone volatiles of both species essentially consisted of seven similar monoterpenes, but the terpene profiles markedly differed between species. α-Pinene (67% of the total), β-pinene, and limonene + β-phellandrene were dominant in the cone volatiles of Swiss stone pine, while the cone emissions of mountain pine showed no dominant terpenoid. The cone oleoresin of *P. cembra* significantly differed from cone volatiles by a lower level of α-pinene and higher levels of β-pinene and limonene + β-phellandrene. Field sprays of mountain pine cones with oleoresin extracts of Swiss stone pine cones significantly reduced the overall damage of specialized cone insects. None of the cones sprayed with oleoresin were attacked, whereas 11% and 31% of the unsprayed control cones were damaged by insects. Specific cone damage due to a cone weevil, *Pissodes validirostris* Gyll. (Coleoptera: Curculionidae), and a cone pyralid, *Dioryctria mutatella* Fuchs (Lepidoptera: Pyralidae), were significantly decreased in one year. The volatile profile of the sprayed cones differed markedly from that of unsprayed mountain pine cones, especially in the higher level of myrcene. However, the volatile profile of the sprayed cones also differed from that of the oleoresin extract used for spraying. The role of monoterpenes in protecting the sprayed cones from insect attack is discussed.

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Key Words—*Pinus cembra*, *Pinus uncinata*, cone insect, volatile monoterpenes, host selection, repellent.

INTRODUCTION

Insects exploiting seed cones are considered important seed predators during the predispersal phase of seed development. The majority of these insects are host-specific, i.e., they can develop only in the seed cones of a few congeneric conifer species (Turgeon et al., 1994). In the Alps, the cone entomofauna of major conifers [e.g., European larch (*Larix decidua* Mill), Mountain pine (*Pinus uncinata* Ram.), and Norway spruce (*Picea abies* L.)] includes more than 10 species, of which about 70% are host-specific (Roques, 1993). In contrast, the cones of Swiss stone pine (*Pinus cembra* L.) are attacked by three nonspecialized species that can feed or develop in other habitats (foliage, buds, shoots, and twigs) (Dormont et al., 1996). The cone entomofauna of *Pinus cembra* was considered to have resulted from a secondary colonization following the Quaternary glaciations (Roques, 1991). It is assumed that insect species shifted from sympatric Alpine conifers that were taxonomically or ecologically close to Swiss stone pine, i.e., mountain pine and Norway spruce. However, the insects specialized to exploit cones of mountain pine or spruce [e.g., the pine cone weevil, *Pissodes validirostris* Gyll. (Coleoptera: Curculionidae), or the spruce seed moth, *Cydia strobilella* L. (Lepidoptera: Tortricidae)], have never been observed in cones of Swiss stone pine. The cone entomofauna of Asian stone pines (i.e., *Pinus pumila* Regel and *P. sibirica* Du Tour) similarly include a few species congeneric to those observed on cones of *P. cembra* (Florov, 1951; Khomentovsky and Efremova, 1991; Dormont et al., 1996). We therefore speculated that the limited insect colonization might have resulted from physical and/or chemical barriers depending on specific structures of cones of *P. cembra*.

Although the olfactory cues provided by plants have been shown to act as key factors in the process of host selection by many insect pests, especially those attacking conifers (Visser, 1986; Bernays and Chapman, 1994), volatiles emitted by cones have received only a little attention (Oshkaev, 1981; Mattson and Strauss, 1986; Roques, 1987; Marion-Poll and Thiéry, 1997). The proportion of cones attacked by insects has been correlated with the level of monoterpenes in some conifer species (Annila and Hiltunen, 1977; Rappaport et al., 1997). In other cases, a repellent action of monoterpenes is assumed (Rappaport et al., 1997).

Our objectives were to: (1) identify and compare the volatiles emitted by cones of Swiss stone pine and mountain pine, (2) analyze the composition of cone oleoresin in Swiss stone pine, and (3) test whether spraying mountain pine cones with resin extracts of Swiss stone pine cones can prevent colonization by insects.

METHODS AND MATERIALS

Headspace Sampling of Cone Volatiles. The study was carried out from 1994 to 1996 in the Briançonnais area of the southern French Alps. Swiss stone pine cones were collected in a pure natural stand of *Pinus cembra* including several thousands of mature trees, which was located at 1900 m elevation in the forest of Les Ayes (44°51′N, 6°36′E). Mountain pine cones were collected in a natural stand of *Pinus uncinata* mixed with *Larix decidua*, which was located at 1950 m elevation in the forest of Montgenèvre (44°55′N, 6°44′E). The mountain pine stand included about 200 mature trees. Two cone-bearing trees were randomly selected per site. On each of these trees, two branches bearing cones were sampled between June 5 and 20, 1995, i.e., during the attack period of cone insect pests (Roques et al., 1983). Immediately after cutting, the branches were brought to a field laboratory located at Briançon, about 15 km from both sites. Cones were removed from the branches and were processed immediately.

Cone volatiles were analyzed using a manifold effluvial headspace sampler designed by Rappaport et al. (1997), which enabled us to process eight samples simultaneously. Cones were individually sealed inside bags made of Tedlar, a nonreactive plastic. The bags were perforated with air inlet holes at both extremities. Teflon tubing with airtight brass couplings was used to connect the sample bags to glass cartridges filled with Amberlite XAD2 (Supelco), a substrate that is effective in capturing monoterpenes and sesquiterpenes. The glass cartridges were connected by Teflon tubes to a vacuum pump. Seven different cone samples and a control consisting of an empty Tedlar bag were connected to the pump during each experiment. Because we had to compare simultaneously the volatile emission of untreated cones of both pine species with that of mountain pine cones sprayed with a volatile solution (see below), each sampling experiment included both untreated and treated cones. However, the volatile sampling schedule was designed so that all cones were processed on the same day. Volatiles collections were carried out every 30 min between 11:00 and 16:00 hr in order to minimize possible differences in cone emission with time and to match the daily flight period of cone pests. In 1995, eight volatile samplings were organized. A total of 16 untreated cones of *P. cembra* were processed while volatile samplings of *P. uncinata* involved eight untreated cones and 32 cones that were submitted to different treatments (vs. 8 controls). In 1996, four sampling experiments were carried out, involving only *P. uncinata* (14 untreated cones and 14 treated cones vs. 4 controls). The effluvial sampling ran for 20 min. Once the effluvial sampling was completed, each glass cartridge was eluted with 3 ml of dichloromethane (CH_2Cl_2) for 2 hr. The resulting mixture was analyzed by gas chromatography (GC), and gas chromatography–mass spectrometry (GC-MS).

Gas Chromatography and Gas Chromatography–Mass Spectrometry of Cone Volatiles. Qualitative and quantitative analyses of the crude mixture were

done in a Varian Star 3400 CX chromatograph equipped with a SPI injector, a flame-ionization detector, and a fused silica capillary column WCOT CPSil-8CB, 0.32 mm × 25 m, (Chrompack). Carrier gas was helium with a flow rate of 3 ml/min. Injector and detector temperatures were 230°C and 240°C, respectively. The oven temperature program was 1 min isothermal at 35°C, then programmed at 15°C/min to 60°C, followed by 2 min isothermal at 60°C, and finally rising at 5°C/min to 240°C. Comparison of the chromatograms of the cone samples with that of a control from the same experiment allowed us to remove peaks corresponding either to adsorbent and Tedlar degradation or to volatiles present in the air surrounding the bags. Cone volatiles were identified based on retention time of external standards. In order to confirm the identification of the major components, GC-MS analyses were performed using an electronic impact ionization mode on a Nermag R10-10C quadrupole interfaced with a Varian 3300 apparatus. The Varian 3300 was equipped with a Ross injector (230°C) and a 25-m WCOT DB-5MS fused silica capillary column, 0.32 mm ID (J & W Scientifics), programmed at 1 min at 40°C and from 40°C to 260°C at 10°C/min, with helium as carrier gas (3 ml/min). Mass spectra were obtained by electron impact at 70 eV and identified by comparison with the EPA/NIH software library. Peaks were quantified using Star Chromatography Software. An internal standard, *p*-cymene, was injected in the sample just before the GC run in order to check retention times of terpenes present in the sample. The proportion of each compound was expressed with respect to total volatiles in order to compare volatile profiles of samples.

Extraction of Oleoresin from Cones of Swiss Stone Pine. The oleoresin of Swiss stone pine cones was collected at the forest of Les Ayes in June 1994 and June 1995. Each year, we selected at random two Swiss stone pine trees, from which we collected five cones per tree. Each sampled cone was cut, and 50 g of fresh cone was immediately extracted with 50 ml of *n*-pentane for 24 hr. Extracts were filtered and analyzed by GC and GC-MS as described above.

Field Tests of Spraying Oleoresin Solutions to Protect Mountain Pine Cones from Insect Damage. Field sprays of cones of *Pinus uncinata* with cone oleoresin of *P. cembra* were carried out at Montgenèvre in 1994 and 1995. We used the *n*-pentane extracts of cone oleoresin that had previously been collected the same year. The oleoresin solution consisted of 1ml extract, 0.5 ml dampening agent (Agral 90), 10ml paraffin oil, and 90 ml water. In order to check a possible influence of the chemicals added to the oleoresin solution, we used a control solution consisted of the same chemicals, except that 1ml of pure *n*-pentane replaced the oleoresin extract. Cones were sprayed using a pressurized-air sprayer Fungicrom.

The timing of the sprays was defined with respect to the attack period of cone insects. The cone entomofauna of *P. uncinata* previously observed in the area mostly consisted of two species, a pine cone weevil, *Pissodes validirostris*,

and a cone pyralid, *Diorcytria mutatella* Fuchs (Lepidoptera: Pyralida) (Roques et al., 1983). Female weevils first feed on the surface of pine cones before laying eggs in the cone scales during cone development, when the cone moisture content peaks. Cones with external feeding punctures but no larvae developing within tissues were tested by adult weevils and rejected for oviposition. At 1800–2000 m altitude, the cones usually reached the proper development stage around mid-June, but the period varied according to the year's weather conditions. The cone pyralid usually attacked cones during early July. Thus, 10 cones were randomly sampled each week to measure average water content. The spray was applied as soon as the moisture values were close to the peak values observed by Roques et al. (1983). The same cones were sprayed again 10 days after the first volatile application. Moreover, the cones to be sprayed were individually examined *in situ* before any application in order to ensure the absence of any visible insect damage.

The cone crop size was about three times higher in 1994 than in 1995. Most of the trees (80) bore cones in 1994, whereas only a few trees (ca. 20) were observed to bear cones in 1995. In 1994 as well as in 1995, 10 cone-bearing trees were randomly selected. Six branches were selected per tree, and all cones were tagged. On each tree, the cones of two branches were sprayed with the oleoresin solution, those of two other branches were sprayed with the control solution, and the cones of the last two branches were used as unsprayed controls. The treatment assignment was made at random. In 1994, a total of 135 cones were sprayed with the oleoresin solution, 121 cones with the control solution, and 133 cones left unsprayed. In 1995, these values were 155, 132, and 138, respectively. In order to analyze the change in volatile emissions of mountain pine cones following the spray, eight cones of *P. unicinata* were collected on two different trees two and five days after the first 1995 oleoresin applicaiton. Cone volatiles were sampled as above. The volatile sampling experiment carried out two days after treatment combined sprayed and unsprayed cones collected at the same time on the same tree.

In 1995, 16 additional cones of *P. uncinata* were sprayed with the control solution in order to test for the possible influence of both dampening agent and solvent on the release of monoterpenes by sprayed cones. Half the cones were collected two days after the spray, the remainder five days after, and the cone volatiles were analyzed as above. Finally, the action *per se* of oleoresin spraying was tested in 1996 by spraying cones of *P. uncinata* with an oleoresin solution of cones of the same species. The same techniques as described for *P. cembra* were used to extract and analyze oleoresin and to prepare the solution to be sprayed, except that 1 ml of oleoresin of *P. uncinata* was used. Sixteen cones of *P. uncinata* were randomly selected at Montgenèvre and sprayed with the oleoresin solution. Eight cones were collected two days after the resin application, and the remainder five days after the spray. An equivalent number of

unsprayed cones from the same tree were used as a control and collected at the same time. The volatiles of both sprayed and unsprayed cones were analyzed as above.

All the cones assigned for treatments (391 in 1994, 425 in 1995) were collected in late July 1994 and 1995, i.e., before any damaging insects had left the cones. The external surface of the cones was examined for weevil feeding punctures and other visible insect damage, e.g., frass of lepidopteran larvae. Cones were dissected in order to check for internal damage and to identify damaging larvae. Larvae were either preserved in 70% alcohol or reared to the adult stage.

Data Analysis. The volatile profiles of cones of the two pine species were compared using the relative amounts of compounds identified in the solutions. Volatile emissions and oleoresin of Swiss stone pine cones were compared in the same way. The transformation of the percent data, using the angular transformation (arcsin $\sqrt{x}$), did not allow us to normalize and homogenize variances. Therefore, the nonparametric test of Kruskal-Wallis was applied on untransformed data in order to test for differences between the profiles ($P < 0.05$) (Statistica 5.0 Microsoft).

The treatment effect was evaluated by comparing the proportion of cones without insect damage, the proportion of cones presenting *Pissodes* feeding punctures but no weevil larvae, the proportion of cones with *Pissodes* larvae developing within cone tissues, and the proportion of cones with *Dioryctria* larvae. As above, the angular transformation of the percent data did not allow us to normalize and homogenize treatment variances, and the untransformed data were submitted to the test of Kruskal-Wallis for treatment effect ($P < 0.05$).

RESULTS

Composition of Cone Volatiles and Cone Oleoresin. We identified seven major monoterpenes, as well as traces of one other monoterpene, bornyl acetate, and sesquiterpenes, in the cone volatiles of the two pine species (Table 1 and Figure 1). Although the major monoterpenes were common to cones of both *Pinus cembra* and *P. uncinata*, there were notable differences between the terpene profiles. α-Pinene was strongly prevalent in the cone volatiles of *Pinus cembra* while the levels of β-pinene and limonene + β-phellandrene (not separated in the quantitative analysis because of the GC temperature program we used) represented 15–20% of the volatile composition. Cone volatiles of *P. uncinata* did not show any dominant monoterpene. The respective levels of myrcene, α-pinene, limonene + β-phellandrene, and β-pinene accounted for

TABLE 1. COMPARISON OF PROFILE OF CONE OLEORESIN[a] AND VOLATILES[b] OF *Pinus cembra* WITH VOLATILE PROFILE OF CONES OF *Pinus uncinata* BEFORE AND AFTER SPRAYING WITH OLEORESIN EXTRACTS OF *P. cembra*[c]

Peak	Compound	*P. cembra* cones		*P. uncinata* cones	
		Oleoresin	Volatiles	Volatiles before spraying	Volatiles after spraying
1	Tricyclene	tr[d]	tr		
2	α-Pinene	52.2 (3.2)a[e]	67.1 (3.4)b	20.5 (1.9)c	17.6 (4.4)c
3	Camphene	0.9 (0.2)a	0.7 (0.2)a	3.5 (1.4)b	1.7 (0.6)b
4	Sabinene	0.2 (0.1)a	0.3 (0.1)a	6.1 (1.9)b	2.0 (1.1)c
5	β-Pinene	24.7 (3.3)a	18.2 (1.7)b	17.8 (3.4)b	3.4 (1.6)c
6	Myrcene	1.8 (0.4)a	1.4 (0.3)a	22.5 (2.1)b	45.4 (3.9)c
7	α-Phellandrene	tr			
8	Limonene/β-Phell.[f]	17.0 (1.4)a	11.1 (3.0)b	22.2 (3.1)c	29.3 (2.3)d
9	γ-Terpinene	tr			
10	Terpinolene	tr			
11	Azulene	tr			
12	Bornyl-acetate	tr	tr	tr	tr

[a] Plus traces of sesquiterpenes (see text).
[b] Plus traces of an unidentified sesquiterpene.
[c] Values are expressed as a mean percentage (±SE) relative to total components of the solution.
[d] Traces.
[e] For a compound, values in the same row followed by the same letter are not significantly different at $P = 0.05$ (Kruskal-Wallis test).
[f] Limonene and β-phellandrene were not separated in the GC analysis.

about 20% of the total volatiles. The two profiles differed by the relative level of most of the monoterpenes, except β-pinene.

A total of 12 monoterpenes were detected in the oleoresin extracts from fresh cones of *P. cembra* (Table 1 and Figure 1A). Bornyl acetate and many sesquiterpenes (e.g., caryophyllene, humulene) were also present as traces in oleoresin samples but most were not identified. The seven major components were the same monoterpenes observed in cone volatiles, but the oleresin profile differed by a lower level of α-pinene and higher levels of β-pinene and limonene + β-phellandrene.

The volatile emissions of mountain pine cones sprayed with oleoresin extracts of Swiss stone pine cones were similar two and five days after treatment. We therefore combined the samples in the data shown in Table 1. In contrast, the volatile profile differed greatly between sprayed and unsprayed cones of

TABLE 2. EFFECT OF SPRAYING CONE OLEORESIN[a] OF *Pinus cembra* ON INSECT DAMAGE TO CONES OF *Pinus uncinata*[a]

Year and treatment	Cones (*N*)	Insect damage			
		Pissodes feeding punctures	*Pissodes* larvae	*Dioryctria* larvae	Total insect damage
1994					
Oleoresin	135	0a[b]	0a	0a	0a
Control solution	121	9.4 (6.6)b	0a	10.4 (9.8)a	19.9 (15.8)b
Unsprayed	133	5.1 (5.0)ab	5.5 (5.1)a	0.4 (0.3)a	11.1 (11.0)b
1995					
Oleoresin	155	0a	0a	0a	0a
Control solution	132	6.1 (5.4)a	9.2 (8.3)b	14.1 (12.4)b	29.4 (15.2)b
Unsprayed	138	5.2 (4.9)a	13.2 (12.6)b	12.9 (12.7)b	31.4 (16.4)b

[a] Results of the 1994 and 1995 treatments (20 branches per treatment). Insect damage is expressed as the mean percentage (± standard error) per treated branch. The control solution consisted of replacing the oleoresin extract by pure *n*-pentane.

[b] In the same year, values of the same column followed by the same letter are not significantly different at $P = 0.05$ (Kruskal-Wallis test).

P. uncinata (Table 1, Figure 1D). Although the same compounds were noted, significant variations were observed in the level of most monoterpenes, except α-pinene and camphene. In particular, the level of myrcene greatly increased. However, the monoterpene profile of sprayed cones also differed from the profile of the cone oleoresin that was used for spraying, the respective proportions being significantly different for all of the volatile components (Table 1). The mountain pine cones sprayed with the control solution did not show a volatile emission different from that of unsprayed cones of the same tree. No difference was observed between the emission of mountain pine cones sprayed with oleoresin of the same species and those of unsprayed cones from the same tree.

Effect of Oleoresin Spraying on Cone Insect Damage. Damage to cones of *Pinus uncinata* was due only to the pine cone weevil and to the cone pyralid in 1994 and 1995 at Montgenèvre (Table 2). The overall insect damage was lower than 32% in both years. The larvae of the cone weevil were observed in 13.2% of the cones, at most, while the pyralids colonized up to 14.1%. None of the cones sprayed with oleoresin was attacked in either year. The overall insect damage was greater in the cones either sprayed with the control solution or

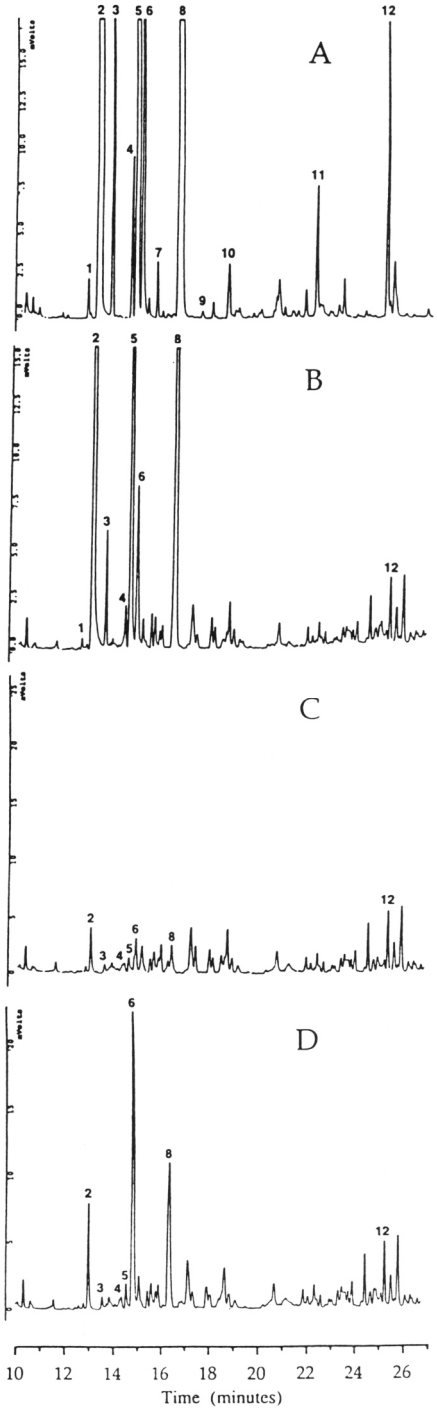


FIG. 1. Gas chromatograms of cone constituents of *Pinus cembra* (A: oleoresin; B: volatiles) and *Pinus uncinata* (C: volatiles emitted by untreated cones; D: volatiles emitted by cones sprayed with oleoresin extracts of *P. cembra*). Peak designations are given in Table 1.

unsprayed in 1994 and 1995. The spraying of cones reduced the specific occurrence of larvae of cone weevils and of pyralid larvae as well in 1995 but not in 1994. Although no feeding punctures of cone weevils were observed on sprayed cones in either year, the proportion of cones presenting only weevil feeding punctures but no larvae did not differ between the oleoresin treatment and the unsprayed controls.

DISCUSSION

Application of oleoresin extracts of Swiss stone pine prevented the colonization of mountain pine cones by insects. No larvae were observed within treated cones, and the 1995 treatment significantly decreased overall insect damage and specific damage by the two target pests. The absence of a significant difference between treatment and control for specific damage of weevil and pyralid in 1994 likely resulted from the patchy distribution of the pests, which attacked only a few trees that year. Most populations of cone insects are regulated primarily by larval food availability, and females tend to adjust oviposition by increasing or decreasing the number of attacked cones according to the size of the cone crop (Turgeon et al., 1994). Because the cone crop of *Pinus uncinata* was much heavier in 1994 than in 1995, overall damage was lower and had a more scattered distribution in 1994 than in the following year, where it increased and was concentrated on the few trees that bore cones (and were consequently used in the spraying experiment). The absence of feeding punctures also indicated that the cones sprayed with oleoresin extracts were not tested for host suitability by female weevils searching to lay eggs.

Since host selection by cone insects is probably mediated by olfactory cues (Roques, 1987; Turgeon et al., 1994; Marion-Poll and Thiéry, 1997), we suspect that the oleoresin treatment strongly disturbed the selection process, either during close-range orientation (due to changes in olfactory stimuli) or when the adult insect landed on the cone (due to changes in tactile stimuli). Such repellent or deterrent effects of pine oil and volatile terpenes have been observed in other insect species (Alfaro et al., 1984; Nordlander, 1991; Dubell, 1992; Nehlin et al., 1994). In our experiment, the treatment effect was attributed to Swiss stone pine oleoresin, and not to oleoresin alone, because the volatile profiles of mountain pine cones sprayed with mountain pine oleoresin did not differ from those of unsprayed mountain pine cones. However, we did not test *in situ* whether spraying mountain pine cones with their own oleoresin could modify insect attack.

Although volatile emissions from *P. cembra* and *P. uncinata* cones differed only by the relative level of a few monoterpenes, the monoterpene profile of cone volatiles of *P. uncinata* markedly changed following the spray by cone

oleoresin of *P. cembra*. The added compounds (dampening agent and solvent) used in the spraying mixture had probably no influence on cone volatile emissions since no difference was noted between cones sprayed with the control solution and unsprayed ones. A comparison of volatile emissions between cones of Swiss stone pine sprayed with the same control solution and unsprayed cones led to similar conclusions (Dormont and Roques, unpublished results). The resulting profile of mountain pine cones sprayed with Swiss stone pine oleoresin differed greatly, however, from that of the spraying solution and from that of cone emissions of both species as well. Levels of α-pinene and β-pinene, which were the most abundant of the monoterpenes found in cones of *P. cembra*, were lower than 18% of the total terpenes present in volatiles emitted by sprayed cones. These compounds probably evaporated to a great extent during spraying. The level of α-pinene was especially decreased in emissions of sprayed cones when compared to cone oleoresin of *P. cembra* and did not differ significantly from that observed in emissions of unsprayed cones of *P. uncinata*. In contrast, the most abundant monoterpenes found in cone volatiles of *P. uncinata* (i.e., myrcene and limonene + β-phellandrene but not β-pinene) dominated the volatiles emitted by the sprayed cones but the level of myrcene increased twofold in sprayed cones relative to unsprayed ones.

It was surprising that myrcene was the dominant terpene in the volatiles of sprayed cones because this compound represented less than 2% of the total terpenes in the solution used for spraying. However, the spray of oleoresin extracts of *P. cembra* probably resulted in increasing the amount of terpenes at the cone surface because resins usually contain a higher quantity of terpenes than volatile emissions (Knudson et al., 1993). We therefore speculate that some of the monoterpenes, not myrcene, evaporated rapidly after the spray application, thus modifying the relative ratios of monoterpenes that were observed in the volatile emissions two days after spray. Further experiments are needed to test the specific volatility of the monoterpenes present in the oleoresin solution.

Was the variation in the relative ratio of monoterpenes responsible for significant changes in the host selection process, thus, protecting the sprayed cones from insect damage? Although the monoterpenes considered are widely distributed in conifers, their relative levels in oleoresin have been shown to vary among genera and species, often under genetic control, and are thus used in chemosystematic studies (Von Rudloff, 1975). Similar variations in monoterpene content were reported for volatiles of needles (Schäfer et al., 1995) and for volatiles of seed cones of Norway spruce, larch species, and Douglas fir (Borg-Karlson et al., 1985; Rappaport et al., 1997).

Annila and Hiltunen (1977) reported that damage to Scots pine cones by *Pissodes validirostris* in Finland was correlated with the level of oleoresin monoterpenes. Weevil attack was observed to increase in relation to a high level of myrcene but was inversely correlated with the level of α-pinene. Unlike the

cones of *P. cembra*, which showed a high level of α-pinene, a low level of myrcene (in oleoresin as well as in volatiles), and little insect damage (Dormont et al., 1996), the sprayed cones of *P. uncinata* did not fit Annila and Hiltunen's observations. Annila and Hiltunnen (1977) used oil extracts from needles to test for resistance of Scots pine to cone weevil attack, however, and did not sample cone volatiles. Comparisons with our data are thus less relevant, since the proportions of monoterpenes within a profile are known to vary among organs of a tree (Hafizoglu and Reunanen, 1994) and may also differ between volatiles and related oleoresin, as our results showed for Swiss stone pine (see Table 1). In loblolly pine, Hanula et al. (1985) observed that a few volatiles from cones, i.e., α-pinene, myrcene, and limonene, were of great importance for host recognition by the southern pine coneworm, *Dioryctria amatella* Hulst. In both of the above studies, the level of myrcene was positively correlated with insect attack, whereas our results showed an inverse relationship. Whether the level of a specific monoterpene can substantially change the pattern of host selection and repel insects from sprayed cones remains to be determined.

Dethier et al. (1960) defined a repellent as "a chemical which causes an insect to make oriented movements away from its source." There was no evidence to suggest that the volatile emissions of sprayed cones acted as a true repellent for insects. Following Thiéry and Visser (1986), it could be suggested that the sprays rather resulted in masking the host olfactory cues with nonhost odor. Difference in the relative proportions of monoterpenes in volatile emissions of sprayed cones and the host chemical message, i.e., the natural volatile blend of mountain pine cones, might have disrupted insect searching behavior in the host recognition process (Bernays and Chapman, 1994). The importance of appropriate ratios among components present in host odors has been previously reported (see Visser, 1986). For example, Roseland et al. (1992) showed that a combination of five monoterpenes in specific relative proportions was the most attractive odor blend for the red sunflower seed weevil, *Smicronyx fulvus* Le Conte. In the pine weevil *Hylobius abietis* L., the highest attraction was obtained when the volatile mixture included the complete set of bark volatiles, but in "naturally occurring proportions" (Nordlander et al., 1986). Nordlander (1991) also observed that adding limonene could affect the response of *H. abietis* to host odors. Similarly, a large number of insect sex pheromones include several compounds in a specific ratio (Monti et al., 1995). Further studies are needed to test the olfactory responses of cone insects to the varying levels of terpenes found in host emissions. Although we did not test the influence of the enantiomeric composition of cone volatile monoterpenes on cone insect attack, enantiomers may induce different insect responses (Dickens, 1978). However, Nehlin et al. (1994) did not find any such difference in the effect of enantiomers on insect attack.

Considering these results, it is still unclear whether cone volatiles are

involved in the limited attack of *P. cembra* cones by insects. Although further experimentation is needed to test the efficiency of spraying cone oleoresin of *P. cembra* to protect cones from insect damage in other conifer species (e.g., European larch), the use of volatile terpenes may represent an additional tool for pest management, especially in seed orchards.

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ACQUISITION OF FEMALE-ATTRACTING FRAGRANCE BY MALES OF ORIENTAL FRUIT FLY FROM A HAWAIIAN LEI FLOWER, *Fagraea berteriana*

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Abstract—Males of the Oriental fruit fly, *Bactrocera dorsalis*, are strongly attracted to and compulsively feed on a fragrant lei flower, *Fagraea berteriana*. A series of phenylpropanoid components, *trans*-3,4-dimethoxycinnamyl alcohol, its acetate, and *trans*-3,4-dimethoxycinnamaldehyde were characterized as male attractants. The alcohol stimulated the same level of feeding activity as methyl eugenol. Males that fed on flowers selectively converted the attractant components into *trans*-coniferyl alcohol and stored it in rectal glands. Males scented with the phenylpropanoids were more successful in mating than unfed males, indicating the advantage of acquiring the fragrance in mating success.

Key Words—Oriental fruit fly, *Bactrocera dorsalis*, Tephritidae, Diptera, *Fagraea berteriana*, pheromone, attractant, 3,4-dimethoxycinnamyl alcohol, coniferyl alcohol, phenylpropanoid.

INTRODUCTION

A flower's fragrance often plays an important role in mutualistic relationships: the scent signals the location of nectar rewards to insect pollinators. However, in some cases, as in euglossine bee–orchid associations, males may seek the flower fragrance itself to obtain volatile components, although the biological function of the acquired fragrance has not been clearly elucidated in these

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instances (Dressler, 1982; Whitten et al., 1989). We have shown here the utilization by a male tephritid fly of a specific flower fragrance for synthesis of a sex pheromone to entice females during courtship.

Males of the Oriental fruit fly, *Bactrocera dorsalis* (Tephritidae), are strongly attracted to the blossoms of particular plants, such as the golden shower tree, *Cassia fistula* (Fabaceae) (Kawano et al., 1968). Methyl eugenol (**1**) has been identified as the major attractant in these cases (Fletcher et al., 1975; Shah and Patel, 1976; Lewis et al., 1988), and this chemical has been successfully used in eradication efforts via the technique of male annihilation against this very destructive fruit pest (Chambers, 1977). Recently, in Hawaii we observed dense aggregations of male *B. dorsalis* feeding on (licking) the fragrant flower, *Fagraea berteriana* (Loganiaceae), which is commonly used in lei (a necklace with flowers) making and is known locally as pua-kenikeni. The males fed compulsively on the petals (Figure 1A), even though flower extracts of *F. berteriana* were found to lack even a trace amount of **1** (Figure 2). In this study, we identify the chemical attractants in the flowers and demonstrate the selective accumulation of a derivative of these chemicals in the male rectal gland. In a series of competitive mating trials conducted in the laboratory, we then show that flower-fed males had a mating advantage over control, unfed males, further suggesting a pheromonal function for the acquired flower fragrance.

METHODS AND MATERIALS

Extraction and Purification of F. berteriana *Fragrance.* About 120 flowers of *F. berteriana* were extracted with ethanol (200 ml × 3). This extract (12.73 g) was partitioned between ether (100 ml) and water (50 ml), the water phase extracted again with ether (50 ml), and the combined ether layers dried over anhyd. Na_2SO_4. Removal of the solvent by evaporation under reduced pressure gave a yellow oil (0.94 g), which was chromatographed on a silica gel column (30 g, Wako gel C-200) into eight fractions eluting in sequence with a mixture of increasing concentrations of methyl acetate (%) in hexane (v/v, ml) as follows: Fractions 1 (0%, 100), 2 (5%, 100), 3 (10%, 100), 4 (15%, 100), 5 (20%, 100), 6 (30%, 100), 7 (40%, 50), 8 (50%, 50), 9 (60%, 50), 10 (100%, 100). Active compounds (**2, 3,** and **4**) were purified by a high-performance liquid chromatography (HPLC) using YMC-Pack A-024 S5 column (8 mm ID × 300 mm, eluted with mixtures of methyl acetate and hexane, 3 ml/min).

TLC Plate Bioassay. Small portions of the flower extract and fractions were subjected to thin-layer chromatography (TLC) on a precoated plate (HPTLC, silica gel 60 F_{254}, nano TLC, Merck) and developed with a mixture of benzene and ethyl acetate (2:1). The TLC plate, slightly moistened by misting with distilled water, was introduced into a small cage containing a number of male

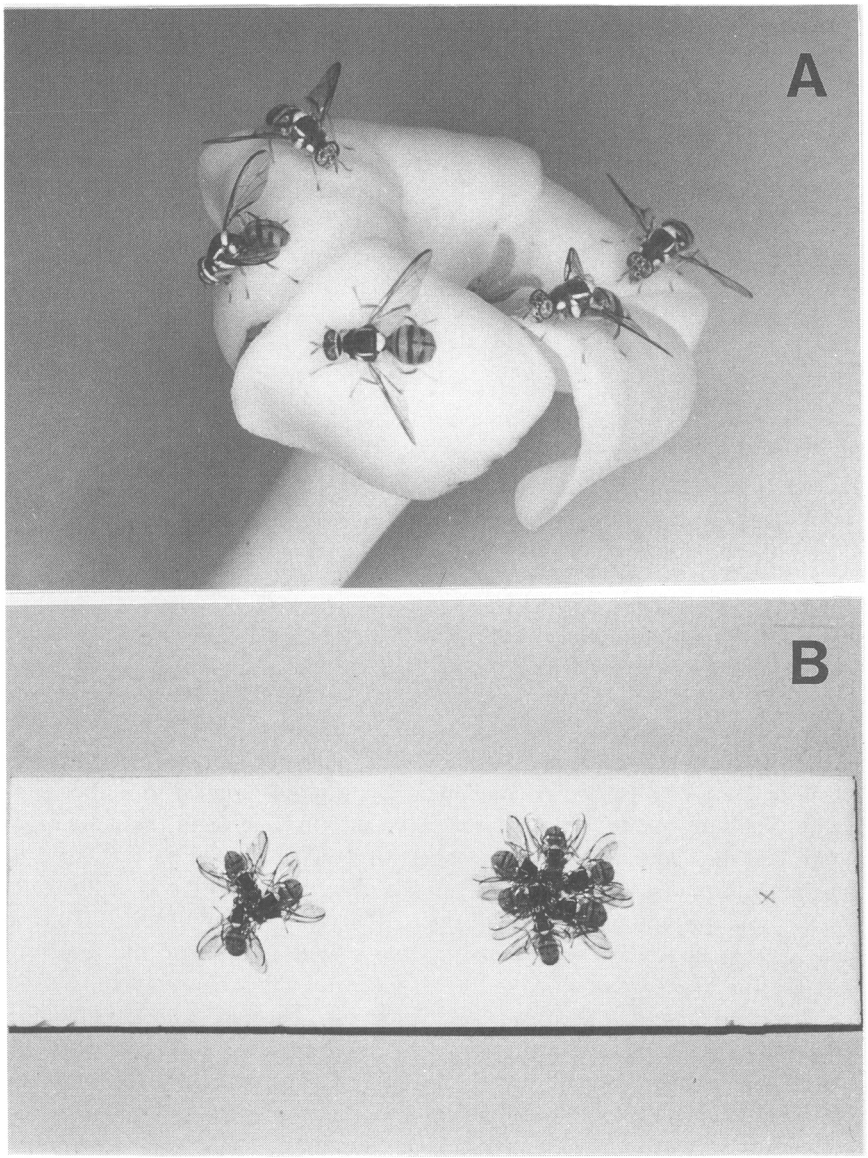


FIG. 1. A: Males of the Oriental fruit fly, *Bactrocera dorsalis*, feeding on the flower of *Fagraea berteriana*. B: Males of *B. dorsalis* feeding on attractive spots (R_f = 0.69 and 0.39) in the thin-layer plate bioassay after chromatography of a *Fagraea* flower extract (HPTLC silica gel 60 F_{254}, nano TLC Merck, developed with benzene–ethyl acetate 2:1, x-mark: original spot, left: solvent front).

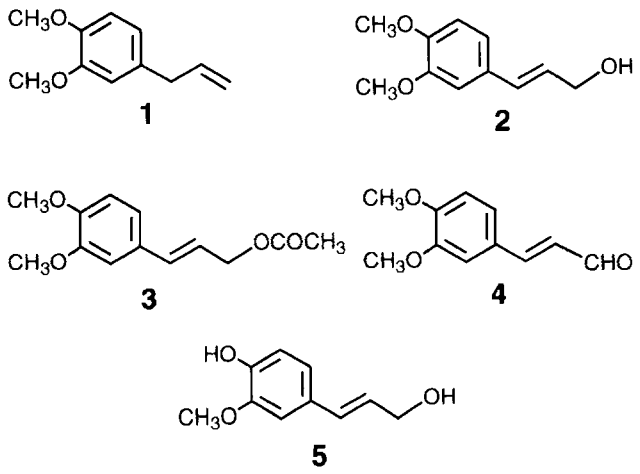


FIG. 2. Phenylpropanoids associated with the oriental fruit fly: methyl eugenol (**1**), *trans*-3,4-dimethoxycinnamyl alcohol (**2**), *trans*-3,4-dimethoxycinnamyl acetate (**3**), *trans*-3,4-dimethoxycinnamaldehyde (**4**), and *trans*-coniferyl alcohol (**5**).

flies (10–30 days old), and licking behavior was observed for about 10 min to determine the active zones (see Figure 1B).

Outdoor Cage Bioassay. B. dorsalis males (150–200 flies, 10–30 days old) were released in outdoor screen cages (2.2 m height × 3.0 m diameter) containing sticky traps suspended from the roof. Traps contained either a solvent blank or 1 mg of a particular compound (**2, 3,** and **4**) applied to a filter paper pellet. Each treatment was replicated in a cage (making eight traps per cage). Traps and flies were placed in the cages for 6 hr during the daytime, and the number of captured flies was recorded. The attractant activity was evaluated by Dunn's multiple comparison test (Daniel, 1990) (N = 2 for all tests). To compare the attractancy between **1** and **2,** males were exposed to the respective samples for 20 min period (N = 2 tests) following the same protocol.

Feeding Stimulant Bioassay. A strip of test filter paper, the tip (10 mm^2) of which was impregnated with each sample (compounds **1–4**) and moistened with distilled water immediately before the test, was brought to within 3 mm of the mouthparts of individual males (N = 50/compound) for 3 sec. The presence or absence of continuous licking on the test sample was scored.

Rectal Volatile Analysis. Males (20 days old) were exposed to *F. berteriana* flowers (3 flowers/male) and allowed to feed freely for one day. Rectal glands were dissected on the second day after feeding, and the gland and the remaining body tissues were extracted separately with ethanol for gas chromatographic quantification (cf. Nishida et al., 1988a, 1993). To test if synthetic **2** and **3** were incorporated into rectal glands, the males were exposed to each compound sup-

plied as a thin film (30 μg/male), allowed to feed for 1 hr, and then processed as above.

Flower Feeding and Mating Success Test. Males (20–25 days old) were allowed to feed on *F. berteriana* flowers for 6 hr and then held 2, 7, and 21 days before testing. Fed and unfed males (21–28 days old) were marked by placing enamel paint on the thorax. Although fed males held 21 days before testing were older than unfed males, Shelly and Dewire (1994) found age to have no effect on the mating success of mature males of *B. dorsalis*. In mating trials, three fed males, three unfed males, and three females (21–31 days old) were placed in glass cages (30-cm cubes) several hours before dusk (the period of sexual activity). Cages were checked 3–4 hr after dusk, and the identities of copulating males were scored (mating occurs at dusk, and pairs remain coupled until dawn). Males fed the synthetic mixture of **2** and **3** (1 : 1) were tested three or seven days after feeding. During exposure, 50 males were given access to the mixture (approximately 30 μg/male) for 6 hr. All flies were virgins and were used only once.

Behavioral Bioassay of B. dorsalis *Females Toward Chemically Treated Males and Dummies.* Males were individually fed on a mixture of **2** and **3** (30 μg each/male) at age 20 days and tested three days later. Males were anesthetized in a freezer and then slowly dragged (using a forceps) 3–4 mm in front of a virgin female (30–35 days old) for approximately 20 mm. A positive response was scored if the females chased and contacted the males and/or extruded their ovipositors toward the males within 10 sec ($N = 32$). Likewise, a male-sized piece of filter paper was impregnated with compound **5** (50 μg/dummy) and tested in the same manner as intact males ($N = 32$). The bioassay, both with the anesthetized fly and with the dummy, was carried out under dim light at 19:00–20:30 hr.

Instruments. Gas chromatography (GC)-mass spectra (MS) for the volatile components were obtained with a Hitachi M-80 mass spectrometer (electron impact, at 70 eV) connected to a GC column (24-m × 0.2-mm fused silica column coated with cross-linked methyl silicone HP-1, 0.33-μm film thickness, helium as carrier gas) programed from 80°C (approximately 2 min holding) to 240°C at a rate of 10°C/min. The ^{1}H NMR spectra were recorded with a Bruker AC300 spectrometer (300 MHz) with TMS as an internal standard (letters s, d, t representing singlet, doublet and triplet, J values in Hz).

RESULTS

Identification of Male Attractants in F. berteriana *Flower*

Males of *B. dorsalis* were exposed to a thin-layer plate which had been chromatographed with a crude extract of *F. berteriana* flowers. The flies were

attracted to and licked two restricted areas centered at the R_f values of 0.39 and 0.69 (Figure 1B). The pure active compounds responsible for stimulating feeding were isolated from fresh flowers of *F. berteriana*. Silica gel column chromatography separated two major active compounds (**2** and **3**) in fractions 8 and 5, which corresponded to the spots at the R_f values of 0.39 and 0.69, respectively. Fraction 8 was subjected to preparative HPLC, eluting with 60% methyl acetate in hexane. Active compound **2** was isolated at a retention time (R_t) of 15.0 min. Fraction 5 was separated by HPLC, eluting with 32% methyl acetate in hexane, and compound **3** was isolated at R_t = 13.0 min. A minor active component **4** was also recovered from R_t = 18.7 min from fraction 5. The quantities of compounds **2**, **3**, and **4** (mean content ± SD, N = 4) were 33.8 ± 15.1, 12.1 ± 5.6, 0.4 ± 0.1 μg/flower, respectively.

Compounds **2** and **3** were identified as *trans*-3,4-dimethoxycinnamyl alcohol and its acetate, respectively, from the following spectral data and synthesis: **2** was prepared from *trans*-3,4-dimethoxycinnamic acid methyl ester by reduction with $LiAlH_4$ (Nishida et al., 1988b); acetylation of **2** with acetic anhydride in pyridine yielded **3.**

Compound **2.** MS: *m/z*(%) 194 (98), 165 (22), 151 (100), 138 (67), 91 (41), 77 (33). ^{1}H NMR ($CDCl_3$): δ 3.88 (3H, s), 3.90 (3H, s), 4.31 (2H, double d, J = 1.4, 5.9), 6.23 (1H, double t, J = 5.9, 15.8), 6.56 (1H, broad d, J = 15.8), 6.83 (1H, d, J = 8.1), 6.93 (1H, double d, J = 1.8, 8.1), 6.95 (1H, broad s).

Compound **3.** MS: *m/z*(%) 236 (100), 193 (25), 177 (67), 165 (38), 146 (42), 91 (38), 77 (16), 43 (50). ^{1}H NMR ($CDCl_3$): δ2.09 (3H, s), 3.88 (3H, s), 3.90 (3H, s), 4.01 (2H, d, J = 6.6), 6.17 (1H, double t, J = 6.5, 16.1), 6.59 (1H, d, J = 16.1), 6.82 (1H, d, J = 7.9), 6.92 (1H, d, J = 7.9), 6.94 (1H, s).

The additional stimulant component (**4**) was identified as *trans*-3,4-dimethoxycinnamaldehyde from its diagnostic mass spectrum [*m/z*(%) 192 (100), 177 (22), 161 (71), 121 (24), 91 (31), 77 (49), 51 (43)]. Compound **4** was synthesized from **2** by oxidation with pyridinium chlorochromate in dichloromethane [^{1}H NMR ($CDCl_3$): δ 6.62(1H, double d, J = 7.7, 15.8), 6.91 (1H, d, J = 8.3), 7.08(1H, d, J = 2.0), 7.17(1H, double d, J = 2.0, 8.3), 7.41 (1H, d, J = 15.8), 9.67(1H, d, J = 7.7)].

Behavioral Effects of Phenylpropanoids **2**, **3**, *and* **4** *on Males*

Male response to *F. berteriana* volatiles consisted of two distinct behavioral steps: long-distance attraction followed by feeding. Attractant bioassays were conducted by releasing males into outdoor screen cages containing sticky traps with test chemicals. Compounds **2** and **3** were highly active, while the aldehyde **4** was not significantly different from the solvent blank (average number of male

flies captured ± SD/trap, $N = 2$ for all tests; compound **2:** 51.0 ± 9.7, **3:** 20.3 ± 8.6, **4:** 2.0 ± 2.4, blank: 1.5 ± 2.6; Dunn's multiple comparison test: **2** = **3** > **4** = blank; $\alpha = 0.15$). In tests comparing only **1** and **2**, **1** was found to be more attractive than **2** (**1:** 50.5 ± 16.8, **2:** 8.3 ± 6.7, blank: 0 ± 0 males/trap; $P < 0.05$, Mann-Whitney test).

Compounds **2**, **3**, and **4** evoked compulsive feeding responses from males when the chemicals were brought near the flies. Figure 3 shows the dose-response of phenylpropanoids (**1–4**), revealing that at low doses (both 1.0 and 3.0 ng) compound **2** stimulated a higher level of feeding than did **1** ($P < 0.001$; G test (2 × 2 contingency) with Yates correction). Compounds **3** and **4** also stimulated feeding activity but only at higher doses.

Accumulation of Compound 5 in Rectal Glands

B. dorsalis males used in the preceding experiment selectively converted both compounds **2** and **3** into *trans*-coniferyl alcohol (**5**), which was identified by GC–MS [*m/z*(%) 180 (69), 137 (100), 124 (56), 91 (34), 77 (20)] (cf. Nishida et al., 1988a).

When males were fed flowers of *F. berteriana*, they accumulated substantial quantities of **5** in the body tissues (2.7 ± 1.3 μg/male, $N = 8$), a large portion of which was concentrated in the rectal glands (1.4 ± 0.7 μg/gland, $N = 8$). Likewise, when males fed on a mixture of synthetic **2** and **3**, **5** accumulated in the rectal glands within a day after ingestion (rectal contents of **5** after ingestion of **2:** 17.4 ± 4.6; **3:** 15.1 ± 5.1 μg; unfed control: 0 μg/gland, $N = 5$).

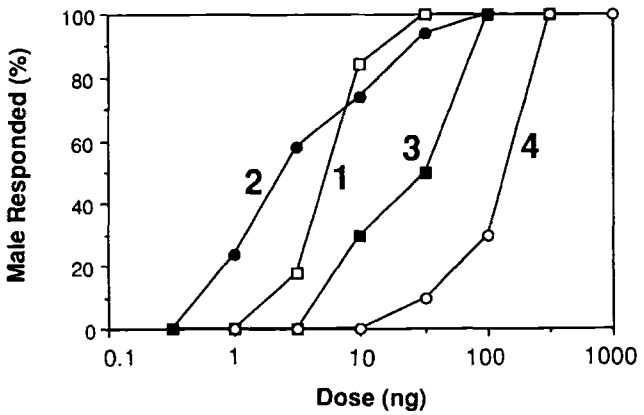


FIG. 3. Feeding stimulant activity of compounds **2–4** isolated from *Fagraea berteriana* flowers in the male feeding assay compared with methyl eugenol (**1**) ($N = 50$).

Effect of Flower and Phenylpropanoid Feeding on Mating Success

After feeding on pua-kenikeni flowers, males mated more successfully than unfed males. Males given access to flowers accounted for 67% (56/83), 80% (53/66), and 77% (78/101) of the total matings in tests conducted 2, 7, and 21 days after flower feeding ($P < 0.05$ all tests; binomial test) (Figure 4). Similarly, males fed a synthetic mixture of **2** and **3** had a mating advantage over unfed males both three days (67% = 51/76) and seven days (65% = 74/113) after feeding ($P < 0.05$ in both cases; binomial test). In these latter tests, many of the unmated females were observed chasing males that had been fed alcohol **2,** sometimes extruding their ovipositors directly toward the males. Such behavior was rarely observed towards the unfed males. Copulation often occurred immediately after ovipositor extrusion, suggesting this action to be a possible mate acceptance response of the female.

Behavioral Effect of Phenylpropanoids 2 and 3 on Females

To associate more rigorously female attraction with male feeding status, we monitored female response to anesthetized (and hence behaviorally equivalent) males fed or unfed the synthetic mixture of compounds **2** and **3.** Males treated with the chemicals attracted females more strongly than untreated males (15/32 vs. 5/32, respectively; $P < 0.05$; G test with Yates' correction). Similar results were obtained using the paper dummies treated with **5** (17/32 vs. 2/32, respectively; $P < 0.001$; G test with Yates' correction). In addition, among

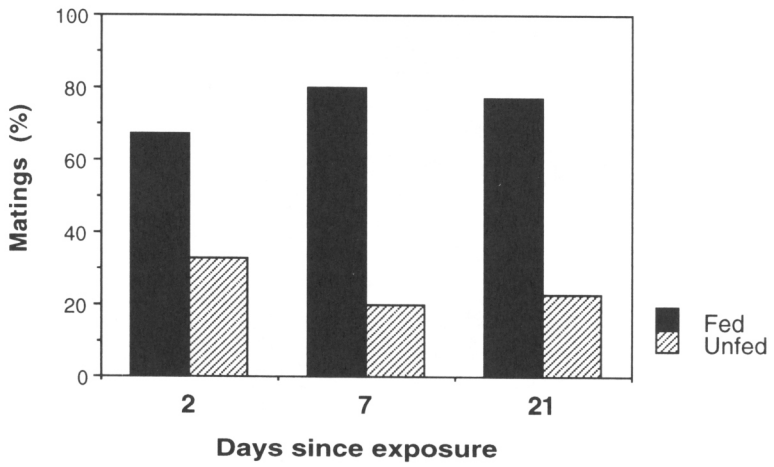


FIG. 4. Percent mating obtained by fed and unfed (control) males in trials conducted 2, 7, and 21 days after a 6-hr exposure of males to *Fagraea berteriana* flowers.

attracted females ovipositor extrusion was occasionally observed toward the fed males (2/15) or treated dummies (5/17) but was never observed toward unfed males or untreated dummies. Metabolite **5** thus appears to act as a short-range attractant and arrestant pheromone to elicit female acceptance of male courtship.

DISCUSSION

We have shown here that males of *B. dorsalis* are attracted to and compulsively feed on flowers of *F. berteriana* stimulated by three phenylpropanoid components **2, 3,** and **4.** Males selectively converted compounds **2** and **3** to coniferyl alcohol (**5**) for storage in the rectal glands and mated more frequently than unfed males, indicating the advantage of acquiring the fragrance in obtaining matings. Wild males of *B. dorsalis* and related species in the Oriental fruit fly complex incorporate methyl eugenol (**1**) metabolites in their body after foraging on methyl eugenol-containing plants (Nishida et al., 1988a,b; Nishida and Fukami, 1990; Tan and Nishida, 1996). Males of *B. dorsalis* fed on methyl eugenol were shown to have a mating advantage over the unfed males (Shelly and Dewire, 1994). Enhancement of mating success in *B. dorsalis* has also been shown by intact feeding on methyl eugenol-bearing flowers of *C. fistula* (Shelly, unpublished). Thus, acquisition of phenylpropanoid compounds by *B. dorsalis* and related species appears to have evolved within the context of sexual selection, particularly female preference for males scented with chemicals derived from flowers. However, the basis of this preference remains unknown. Females do not appear to derive any direct benefits (in terms of increased fecundity or offspring hatch rate) by selecting males fed compound **1** (Shelly, unpublished). Female preference could reflect a runaway process. If the ability to collect phenylpropanoid compounds has a heritable component, by mating with scented males, females may be increasing the likelihood that their sons will successfully locate and collect these compounds and thereby enjoy a mating advantage. Aditionally, phenylpropanoid **5** and its analogs have been shown to be repellent to avian predators (Nishida and Fukami, 1990; Jakubas et al., 1992). Since a large proportion of the wild population of *B. dorsalis* males stores these compounds in significant quantities, the acquired phenylpropanoids seem to play a role as defense substances (Tan, 1993; Tan and Nishida, 1996).

Although the rectal phenylpropanoid appears to play an important role as a sex pheromone under natural conditions, indoor males mate at a high frequency even without access to phenylpropanoids. Regardless of whether they had prior access to phenylpropanoid sources, males of *B. dorsalis* emit a smoke of a series of compounds that have been suspected to act as a sex pheromone (Ohinata et al., 1982). The rectal chemicals include fatty acids, trisodium phosphate, spiroketals, alkylamides, and other minor volatiles; the behavioral functions of

these in the courtship sequence have not, however, been clarified (Ohinata et al., 1982; Perkins et al., 1990). Further study is needed to understand the nature of the mating system of *B. dorsalis* in association with those endogenous rectal chemicals and pharmacophagously acquired phenylpropanoids (Nishida and Fukami, 1990).

The feeding location on *F. berteriana* flower (well away from the anthers) and the absence of pollen on examined individuals suggests that this *B. dorsalis*–flower association is not mutualistic. By contrast, floral spikes of *Spathiphyllum cannaefolium* (Araceae) attract males of *B. carambolae* and *B. papayae* with compounds **1, 2,** and **3** during the time when the spadix produces the characteristic fragrance and a large amount of pollen (Nishida and Tan, 1993; Chuah et al., 1996). Wild *B. carambolae* males bearing pollen clusters on the body surface were found to store substantial, although variable, quantities of metabolite **5** in the rectal glands, suggesting a possible mutualistic interaction through pollination (Nishida and Tan, 1993; Nishida and Tan, unpublished).

Regardless of the adaptive significance of lure attraction, the knowledge that feeding on lures enhances the mating performance of male tephritids may have tremendous practical value. The sterile insect technique (SIT) is a widely used, and environmentally safe control method involving the release of irradiated (sterile), mass-reared males to achieve sterile male–wild female matings and the production of infertile eggs. As the success of SIT hinges on the mating competitiveness of sterile males, prerelease exposure of sterile males to the lure may be a logistically simple way to enhance their mating frequency with wild females and consequently increase the effectiveness of the control program (Shelly, 1995; Shelly et al., 1996).

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AGGREGATION PHEROMONE OF PALM WEEVIL, *Dynamis borassi*

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Abstract—4-Methyl-5-nonanol (**1**) is the male-produced aggregation pheromone of the palm weevil, *Dynamis borassi* (F.) from Colombia. The pheromone was identified by coupled gas chromatographic-electroantennographic detection (GC-EAD) analysis of male- and female-produced volatiles, and comparative GC-mass spectrometry (MS) of weevil-produced **1**. In field experiments in Colombia, traps baited with a stereoisomeric mixture of synthetic **1** (3 mg/day) plus sugarcane captured more weevils than traps baited with **1** or sugarcane alone, suggesting that pheromone and plant volatiles are synergistically attractive. Use of a chiral, stereoisomer-separating Cyclodex-B column in GC-EAD and GC-MS analyses revealed that *D. borassi* males produce, and antennae of males and females respond to (4*S*,5*S*)-**1**. Previously identified palm weevil (*Rhynchophorus* spp.) aggregation phero-

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mones 5-methyl-4-octanol (cruentol) and 6-methyl-2-hepten-4-ol (rhynchophorol) also elicited antennal responses by *D. borassi*. In field experiments, *D. borassi* females were captured equally well in traps baited with sugarcane plus either **1**, cruentol or rhynchophorol. In contrast, *D. borassi* males were captured most often in traps baited with sugarcane plus **1**. Because *D. borassi* is a potential vector of the red ring nematode that causes the lethal red ring disease of palms, pheromone-based trapping of *D. borassi* could aid in monitoring or management of red ring disease in commercial palm plantations.

Key Words—Aggregation pheromone, *Bursaphelenchus cocophilus*, coconut, *Cocos nucifera*, Coleoptera, Curculionidae, *Dynamis borassi*, 4-methyl-5-nonanol, palm weevils, red ring disease, red ring nematode.

INTRODUCTION

The palm weevil, *Dynamis borassi* (F.) is widely distributed in South America (Wattanapongsiri, 1966). As a major pest of coconut (*Cocos nucifera* L.), it damages palm inflorescences and crown and vectors the lethal nematode [*Bursaphelenchus cocophilus* (Cobb)]-caused red ring disease (Gerber et al., 1990). *Dynamis borassi* by itself causes death of Arikury palm, *Syagrus schizophylla* (Martius), and *S. vagans* (Bondar) (Bondar, 1940; Landeiro, 1941), and is attracted to maturing fruit of star-nut palm, *Astrocaryum standleyanum* (Giblin-Davis et al., unpublished observation).

Males of many of the rhynchophorinae produce aggregation pheromones that mediate colonization of susceptible host plants (Giblin-Davis et al., 1996). Capture of *D. borassi* in traps baited with the synthetic aggregation pheromone (±)-6-methyl-2-hepten-4-ol (rhynchophorol) of the American palm weevil, *Rhynchophorus palmarum* (L.) and pesticide-treated sugarcane (Giblin-Davis et al., unpublished observation) suggest similarities in the chemical ecology of these weevils. We report the identification of the *D. borassi* aggregation pheromone and cross-attraction of *D. borassi* to pheromones of *Rhynchophorus* palm weevils.

METHODS AND MATERIALS

Volatile Collections. Dynamis borassi of mixed age and sex and of unknown mating status were field-collected in an African oil palm (*Elaeis guineensis* Jacquin) plantation at Palmas de Tumaco LTDA., Tumaco, Nariño, Colombia, provided with sugarcane, and shipped to Simon Fraser University, Canada, for capture of male- and female-produced volatiles in May 1994 and 1996. Groups of 5–10 weevils, separated by sex, were placed in modified 9-liter Naglene (Nalge Co., Rochester, New York) desiccators with 0–4-day-old apple slices.

A charcoal-filtered air stream (1.5 cm^3/min) was maintained through the desiccators for five days, collecting insect volatiles on 10 g of Porapak Q (50–80 mesh, Waters Associates, Inc., Milford, Massachusetts) packed in Pyrex glass tubing (Oehlschlager et al., 1988, 1992). Volatiles were eluted from the Porapak Q with distilled pentane and concentrated by distillation through a Dufton column (300 × 20 mm) (Quickfit & Quartz Ltd., Staffordshire, England).

Volatile Analyses. Porapak Q extracts were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), using a Hewlett Packard (HP) 5885B gas chromatograph equipped with a DB-5-coated, fused silica column (30 m × 0.25 mm ID) (J&W Scientific, Folsom, California). For GC-EAD recordings, an antenna was removed from a live weevil and suspended between two glass capillary electrodes with the scape inserted into one and the olfactory club impaled by the other. To obtain mass spectra of EAD-active compounds, a coupled HP 5985B GC–mass spectrometer (MS) equipped with an SP-1000-coated, fused silica column (30 m × 0.25 mm ID) (Supelco Inc., Bellefonte, Pennsylvania) was used in both electron impact (EI) (70 eV) and chemical ionization (CI) mode. To determine the weevil-produced stereoisomer(s) of 4-methyl-5-nonanol (**1**), weevil-produced **1** was isolated from the Porapak Q extract by high-performance liquid chromatography (HPLC), employing a Waters LC 626 high-performance liquid chromatograph equipped with a Waters 486 variable-wavelength UV–visible detector set at 210 nm, a Waters 746 data module integrator, and a Nova-Pak silica HPLC cartridge column (3.9 × 150 mm) with a 1 ml/min flow of isocratic hexane (96%)–ether (4%). HPLC-isolated **1** was then analyzed by GC-MS, employing a Varian Saturn GC-MS equipped with a fused silica Cyclodex-B-coated column (30 m × 0.25 mm ID) (J&W Scientific, Folsom, California). The temperature program was 80°C for 1 min, then 3°C/min to 95°C, then 95°C for 30 min. To determine the EAD-active stereoisomer of **1**, synthetic **1** was analyzed by GC-EAD with the Cyclodex-B column.

To study olfactory recognition of congeneric aggregation pheromones, a pentane solution was prepared for GC-EAD analyses containing the following *Rhynchophorus* pheromones at 5 ng each: (1) **1** (ferrugineol) (Hallett et al., 1993; Oehlschlager et al., 1995), (2) (±)-6-methyl-2-hepten-4-ol (rhynchophorol) (Oehlschlager et al., 1992; Rochat et al., 1991), (3) 5-methyl-4-octanol (cruentol) (Weissling et al., 1994), (4) 3-methyl-4-octanol (phoenicol) (Gries et al., 1993), and (5) 2-methyl-4-heptanol (Perez et al., 1997). All compounds were available in our laboratories from previous work (cited above) and were >95% chemically pure. Stereoisomers of **1** were synthesized using the methods described by Perez et al. (1996, 1997).

Field Experiments. Two field experiments were conducted near the borders of an African oil palm plantation at Palmas de Tumaco LTDA., Tumaco, Nariño, Colombia, in a completely randomized block design with traps at about 50-m

intervals and blocks >100 m apart. Lethal traps were constructed from closed 4-liter white plastic containers, similarly shaped to herbicide containers (30.0 high × 16.5 wide × 10.0 cm deep) with small holes in the bottom and two 6 × 13-cm entry slots cut into the sides. Each trap was suspended 1.5–1.8 m above ground from an African oil palm.

The first four-treatment experiment tested unbaited control traps and traps baited with **1** or fresh sugarcane alone (about 1 kg; ten 10-cm long pieces of freshly cut stem) and in combination. All traps contained a water-moistened rag. The sugarcane and/or rag was immersed in a suspension of liquid Sevin [21.3% carbaryl; 1-naphthyl *N*-methyl-carbamate (1.11 ml active ingredient/liter water)] to make each trap lethal to incoming weevils. About 90 mg of synthetic **1** and an indicator dye were hermetically sealed in a polymer membrane release device (ChemTica International, San Jose, Costa Rica) and hung inside the trap for pheromone dissemination at 3 mg/day at 25°C. The dye was used to indicate when the pheromone release device was empty.

Because of very low densities of *D. borassi*, we used the six replicate traps each week as subsamples that were pooled for each treatment. Each treatment was replicated in time (eight replicates) from February 7 to April 4, 1995. Captured weevils were removed from traps weekly, sexed, and counted. Clean traps were rebaited and rerandomized within each of the six blocks.

The second field experiment compared attraction of *D. borassi* to various *Rhynchophorus* pheromones. Traps were baited with sugarcane plus either synthetic **1**, rhynchophorol, cruentol, or phoenicol. Each pheromone was disseminated at 3 mg/day at 25°C. The experimental design was as in experiment 1 except that each treatment was replicated 12 times from April 4 to June 20, 1995.

Statistical Analyses. Data from field tests were square root-transformed ($x + 0.5$) to eliminate heteroscedasticity and analyzed by analysis of variance using PROC ANOVA (SAS Institute, 1985). Least significant difference tests (SAS Institute, 1985) were used for mean separation where significant ($P < 0.05$) statistical differences occurred.

RESULTS AND DISCUSSION

GC-EAD analysis of volatiles from male and female *D. borassi* revealed one male-specific compound that elicited strong antennal responses from male and female weevils (Figure 1). Mass spectral and retention characteristics of this compound were consistent with those of 4-methyl-5-nonanol (**1**), the main aggregation pheromone of *R. ferrugineus* (Oliv.) (Hallett et al., 1993), *R. bilineatus* (Montr.) (Oehlschlager et al., 1995), and *Metamasius hemipterus seri-*

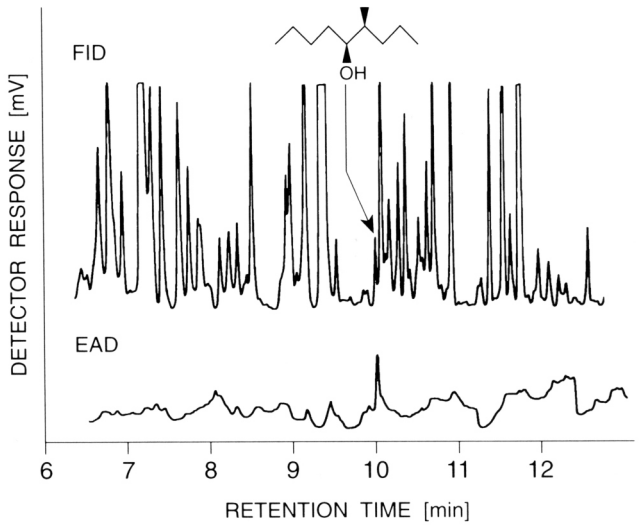


FIG 1. Flame ionization detector (FID) and electroantennographic detector (EAD: female *D. borassi* antenna) responses to volatiles collected for five days from *D. borassi* males feeding on apples. FID trace: 0.4 weevil hours of an extract containing 846 weevil hours of volatile production. Gas chromatograph (GC) conditions: linear flow velocity: 35 cm/sec, injector and detector temperatures: 240°C, temperature programming: 50°C 1 min, then 10°C/min to 240°C; DB-5 fused silica column (30 m × 0.25 mm ID). The EAD response is to 4*S*,5*S* compound **1**.

ceus (Oliv.) (Perez et al., 1997). In field experiment one, sugarcane plus **1** captured more male and female *D. borassi* than did **1** or sugarcane alone (Figure 2). This is consistent with reports of synergistic attraction of aggregation pheromones and food for at least seven species of the Rhynchophorinae (Giblin-Davis et al., 1996).

Identical retention times and highly comparable mass spectra of synthetic and *D. borassi*-produced **1** on a Cyclodex B column confirmed production of (4*S*,5*S*)-**1** by male weevils. GC-EAD analysis of synthetic **1** demonstrated that only (4*S*,5*S*)-**1** elicited responses by antennae of *D. borassi* (Figure 3). This result is consistent with findings that male *Rhynchophorus* weevils produce, and males and females respond to, one optical isomer (*S*, or *S*,*S*) of their chiral aggregation pheromone (Giblin-Davis et al., 1996). *Rhynchophorus ferrugineus* and *R. bilineatus* produce and respond to (4*S*,5*S*)-**1** (Oehlschlager et al., 1995; Perez et al., 1996). *Rhynchophorus cruentatus*, *R. phoenicis* (F.), and *R. palmarum* produce and respond to (5*S*,4*S*)-5-methyl-4-octanol (Perez et al., 1994),

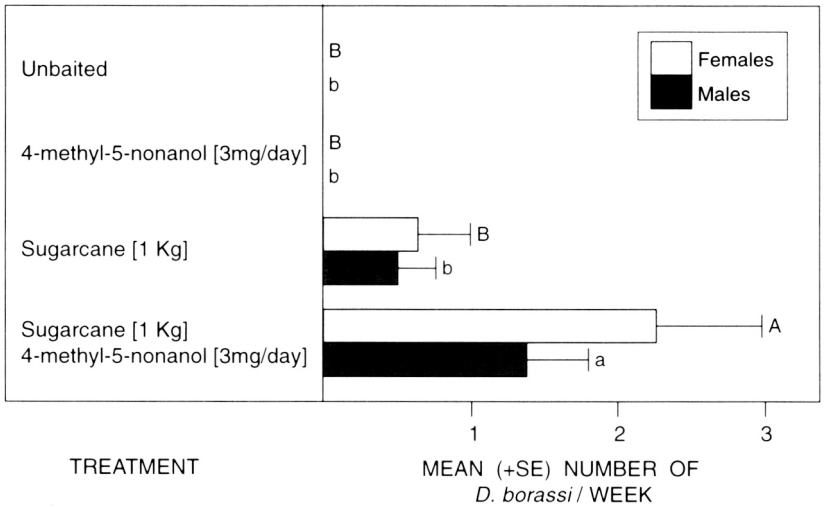


FIG. 2. Mean numbers (± SE) of *D. borassi* captured in lethal traps baited with sugarcane or 4-methyl-5-nonanol singly and in combination. Wet rags were used in traps without sugarcane. Rags or sugarcane were treated with pesticide to prevent weevil escape. The experiment (eight relicates) was conducted in an African oil palm plantation, Palmas de Tumaco LTDA., Tumaco, Colombia, from February 7 to April 4, 1995. Data transformed to approximate homogeneity are presented untransformed. Means within a sex with the same letter superscript are not significantly different ($P > 0.05$; least significant difference), ANOVA treatment effects—males: $F = 8.15$; $df = 3, 10$; $P < 0.001$; females: $F = 7.85$; $df = 3, 10$; $P < 0.002$.

(3*S*,4*S*)-3-methyl-4-octanol (Perez et al., 1994), and (4*S*,2*E*)-6-methyl-2-hepten-4-ol (Oehlschlager et al., 1992), respectively. In field trapping experiments with the preceding *Rhynchophorus* weevils, nonweevil produced stereoisomers in synthetic pheromones did not reduce attractiveness of the *S* or *S*,*S* isomers, indicating that stereoisomeric blends can be used for trapping weevils (Giblin-Davis et al., 1996). Low population densities of *D. borassi* did not allow for field tests of stereoisomers of **1**. Because chemical communication of *D. borassi* and *Rhynchophorus* weevils are similar, we hypothesize that nonnatural isomers of **1** in synthetic pheromone lures will not prevent optimal attraction of *D. borassi* to pheromone-baited traps.

D. borassi-produced **1**, and also cruentol, rhynchophorol, and 2-methyl-4-heptanol elicited responses by *D. borassi* antennae (Figure 4), suggesting olfac-

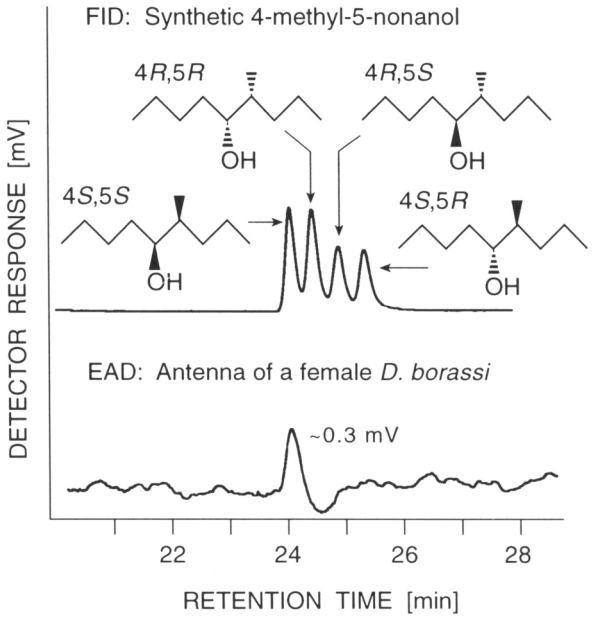


FIG. 3. Flame ionization detector (FID) and electroantennographic detector (EAD: female *D. borassi* antenna) responses to stereoisomers of 4-methyl-5-nonanol. Cyclodex-B column, isothermal 100°C, linear flow velocity of carrier gas 35 cm/sec; split injection; injector temperature 220°C.

tory recognition of aggregation pheromones of other weevils in the Rhynchophorinae (Giblin-Davis et al., 1996). This is similar to *M. hemipterus*, where antennal responses were elicited by pheromones of other weevils belonging to the same subfamily (Ramirez-Lucas et al., 1996). In field experiment 2, traps baited with sugarcane plus either cruentol, rhynchophorol, or **1** were equally attractive to *D. borassi* females and more attractive than traps baited with sugarcane plus phoenicol (Figure 5). More males were captured in traps baited with sugarcane plus **1** than in traps baited with sugarcane plus any of the other three pheromones (Figure 5). The attraction of *D. borassi* to cruentol produced by allopatric North American *R. cruentatus* is not understood. However, cross-attraction of *D. borassi* females to rhynchophorol, the aggregation pheromone of sympatric *R. palmarum*, may be adaptive in overcoming a palm's defense and in time-efficient use of a temporarily suitable resource. Sympatric West

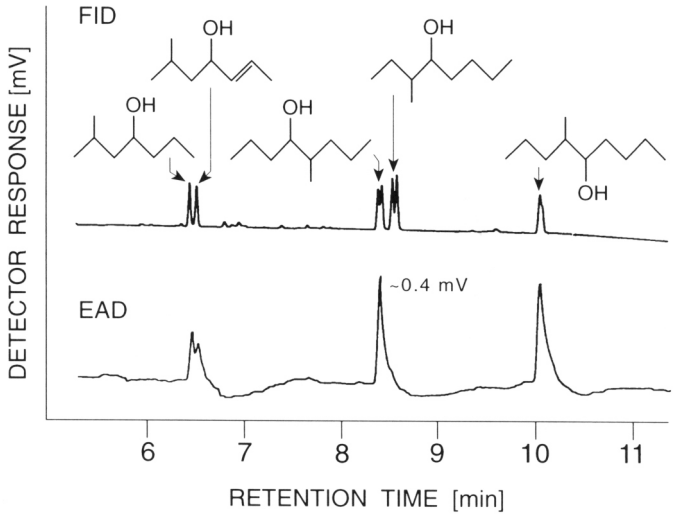


FIG. 4. Flame ionization detector (FID) and electroantennographic detector (EAD: female *D. borassi* antenna) responses to (left to right on figure) 2-methyl-4-heptanol, 6-methyl-2-hepten-4-ol (rhynchophorol), 5-methyl-4-octanol (cruentol), 3-methyl-4-octanol (phoenicol) and 4-methyl-5-nonanol (ferrugineol) (aggregation pheromones in the Rhynchophorinae). Column and chromatographic conditions as in Figure 1.

Indian sugarcane weevil, *M. h. sericeus*, which uses **1** as its major aggregation pheromone (Perez et al., 1997; Ramirez-Lucas et al., 1996) is also cross-attracted to rhynchophorol (Chinchilla et al., unpublished data). *Dynamis borassi* and *R. palmarum* are known vectors and *M. h. sericeus* is a suspected vector of the red ring nematode (*Bursaphelenchus cocophilus*) (Giblin-Davis et al., 1996). Because *D. borassi*, *R. palmarum*, and *M. h. sericeus* can coinhabit the crown of red ring diseased coconut palms (Gerber et al., 1990; Giblin-Davis et al., unpublished observation) these weevils together may overwhelm the palm's defense and become associated with red ring nematodes. As a result, they may increase their virulence, successfully occupying more hosts and enhancing their reproductive potential. Niche divergence and cannibalism of weevil larvae may serve to reduce interspecific competition in an occupied host (Giblin-Davis et al., 1996).

Knowledge of the chemical ecology of *D. borassi* can now help determine its population dynamics in palm plantations and in the wild and its relative importance in red ring nematode transmission.

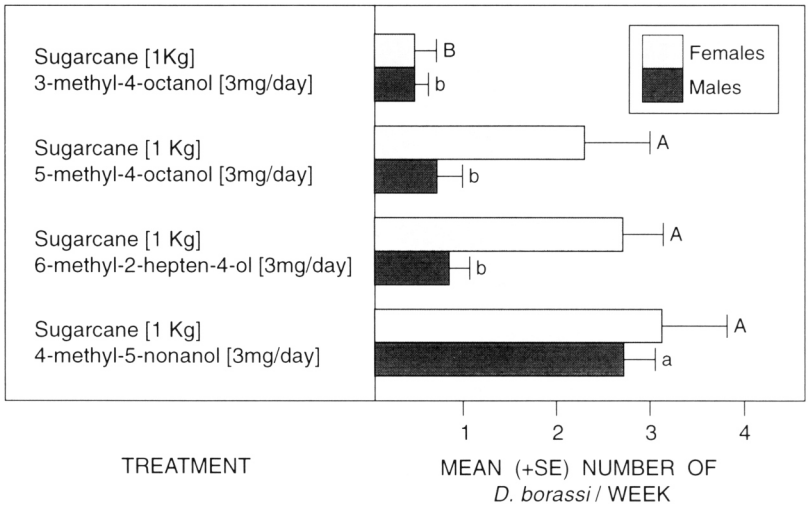


FIG. 5. Mean numbers (± SE) of *D. borassi* captured in lethal traps baited with sugarcane plus either 4-methyl-5-nonanol (ferrugineol), 6-methyl-2-hepten-4-ol (rhynchophorol), 5-methyl-4-octanol (cruentol), or 3-methyl-4-octanol (phoenicol). Sugarcane was treated with pesticide to prevent weevil escape. The experiment (12 replicates) was conducted in an African oil palm plantation, Palmas de Tumaco LTDA., Tumaco, Colombia, from April 4 to June 20, 1995. Data transformed to approximate homogeneity are presented untransformed. Bars for male and female weevils with the same letter superscript are not significantly different ($P > 0.05$; least significant difference), ANOVA treatment effects—males; $F = 22.22$; $df = 3, 14$; $P < 0.0001$; females: $F = 6.67$; $df = 3, 14$; $P = 0.002$.

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EICOSANOIDS AND EGG-HATCHING SYNCHRONY IN BARNACLES: EVIDENCE AGAINST A DIETARY PRECURSOR TO EGG-HATCHING PHEROMONE

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Abstract—The boreoarctic barnacle, *Semibalanus balanoides* (= *Balanus balanoides*) (L.), has the ability to synchronize the release of its nauplii with the spring phytoplankton bloom, thereby ensuring that the larvae can start their planktotrophic development successfully. Hatching is induced by an egg-hatching pheromone (an hydroxy fatty acid) released by the adult. Here, the possibility that the pheromone is an excretory metabolite of dietary eicosapentaenoic acid (EPA) is examined. Egg hatching could be induced by feeding gravid adult barnacles on *Skeletonema costatum*, but neither a concentrated culture of this diatom nor cell-free culture medium induced egg hatching in vitro. Following a 15-min incubation of EPA in seawater, a product with egg hatching activity was obtained, presumably by autooxidation. Egg hatching was not induced by feeding barnacles with lecithin liposomes containing EPA. Likewise, radiolabeled egg-hatching pheromone was not released by adult barnacles that had been fed with [^{14}C]EPA liposomes. Egg-hatching pheromone was not released by barnacles that were actively feeding on *S. costatum* prior to egg-hatching. The production of egg-hatching pheromone was inhibited in vitro and in vivo by lipoxygenase inhibitors. Taken together, the results suggest that egg-hatching pheromone is not an excretory metabolite but is derived from EPA released from membrane phospholipid and acted upon by a lipoxygenase. The nature of the stimulus to precursor fatty acid release has yet to be established, but a link with molting appears tenuous.

Key Words—Eicosanoids, pheromone, egg-hatcing, barnacle, phytoplankton, lipoxygenase.

INTRODUCTION

Egg-hatching and the liberation of planktotrophic nauplii are closely synchronized with the spring phytoplankton bloom in the boreoarctic barnacle, *Semi-*

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balanus balanoides (= *Balanus balanoides*) (e.g., Crisp, 1956; Barnes, 1957; Rzepishevsky, 1962; Clare et al., 1985; Starr et al., 1991; Clare, 1995). Over much of the species' range in the United Kingdom, the embryos, which are brooded in the adult's mantle cavity, are fully mature and capable of hatching approximately one to two months ahead of liberation in the field. Upon resuming feeding after the winter anecdysis, egg hatching is effected by the adult releasing a compound(s) into the mantle cavity (Crisp and Spencer, 1958; Barnes and Barnes, 1982). Because the mantle cavity is confluent with the seawater, the eggs are essentially brooded externally, and the active compound will hereafter be termed egg-hatching pheromone (EHP) sensu Karlson and Luscher (1959). It should be noted that the existence of an inhibitor to egg hatching has also been proposed (Barnes, 1989), but the critical experiments needed to test this hypothesis have yet to be performed.

The timing of ecdysis and egg hatching is closely linked (Patel and Crisp, 1961), a fact that led to the discovery that dopamine can elicit egg hatching in vitro (Clare et al., 1982; Clare and Walker, 1986) and in vivo (Clare, unpublished). However, based on physicochemical characteristics, dopamine is not the EHP per se (Clare et al., 1985), although a role for this neurotransmitter in the egg-hatching process cannot be dismissed. Instead, activity has been attributed to eicosanoids (Clare et al., 1985). The precise identity of the EHP is unclear (Gerhart et al., 1990), although trihydroxy fatty acids have been implicated (Hill et al., 1993). Much of the confusion regarding structure may have arisen from inappropriate assays of *S. balanoides* EHP. The original in vitro assay described by Crisp and Spencer (1958), and adopted in slightly modified form by Clare and coworkers (Clare et al., 1982, 1985; Clare and Walker, 1986), utilized conspecific egg masses of *S. balanoides*. While cross-specificity in activity of barnacle extracts has been noted (Crisp and Spencer, 1958; Clare and Walker, 1986), attempts to identify EHPs on the basis of allospecific bioassays are dubious, unless heterospecificity of pheromone action can also be demonstrated. Unfortunately, a prerequisite to the latter case requires the unambiguous identification of native pheromones. The possibility that EHP comprises a bouquet of compounds has also yet to be addressed (Clare, 1995).

The polyunsaturated fatty acid (PUFA), arachidonic acid, is central to eicosanoid biosynthesis in vertebrates (e.g., Shimizu and Wolfe, 1990) and might be utilized as a precursor to EHP (Clare, 1987). Eicosapentaenoic acid (EPA) is a more likely precursor, however, based on the putative structure(s) of the pheromone (Clare and Walker, 1986; Hill et al., 1993). This fatty acid may constitute as much as 25–30% of the free fatty acids in neutral and phospholipid fractions of *S. balanoides* (Clare and Walker, 1986) and is characteristically rich in the PUFAs of other marine organisms (e.g., Ackman, 1980). For example, EPA has been found to comprise up to 30% of the fatty acids in

the diatom *Skeletonema costatum* (Chuecas and Riley, 1969), although typically, lower values (~ 5–14%) have been reported (see Groth-Nard and Robert, 1993). In view of these figures, a simple mechanism to synchronize egg hatching with the spring phytoplankton bloom is suggested, namely, that coincident with the increase in feeding by the gravid, adult barnacle, there is an increase in excretion of dietary metabolites of EPA that are sensed by the mature embryos and signal an adequate food supply for their planktotrophic development (Clare, 1995). This hypothesis is reminiscent of, but distinct from, an earlier proposal by Barnes (1957) that the inductive cue is derived directly from diatoms. Indeed, Barnes (1957) reported that concentrated cultures of *S. costatum* increased hatching from egg masses in vitro. However, long periods of incubation (> 15 hr) were required so that, as pointed out by Barnes and Barnes (1963), EHP and the "diatom factor" are not directly related. Crisp and Spencer (1958) demonstrated that EHP, or to use their terminology "hatching substance," was the product of the barnacle's own metabolism and was produced whether the adult was fed on animal or plant food. Both these facts can be reconciled with the dietary metabolite hypothesis outlined above.

The conventional scheme of eicosanoid biosynthesis involves first the release of fatty acid precursor (e.g., EPA) from membrane lipid stores by the action of a phospholipase (Piomelli, 1993). The present study was carried out to examine which of the two hypothetical schemes—synthesis and secretion or metabolism and excretion—pertains to EHP production. Preliminary pharmacological evidence is presented to support lipoxygenase involvement in EHP synthesis as deduced from the putative structure of the pheromone.

METHODS AND MATERIALS

Organism Culture. Gravid *Semibalanus balanoides* attached to mussel, *Mytilus edulis*, shells, were supplied by the University Marine Biological Station, Millport, in February 1994 and January 1995. The adult barnacles were maintained in once weekly changes of aerated, UV-irradiated, filtered (0.2 μm) Eddystone seawater, hereafter abbreviated as FESW, at 9°C. The barnacles were not fed and have been so maintained, with viable egg masses, for several months (January through June). Gravid *Elminius modestus* were removed from the carapaces of *Carcinus maenas* that had been collected locally.

Seed cultures of the diatom *Skeletonema costatum* were obtained from the Plymouth Culture Collection. The cultures were grown in f/2 medium at 20 $\pm$ 1°C under continuous fluorescent lighting. Cell density was determined using an improved Neubauer hemacytometer.

Extraction of In Vitro Bioassay of Egg-Hatching Pheromone. For EHP

extraction, seawater (or seawater extracts of tissues) was acidified to pH 4.0 (activity is lost at or below pH 3.0) with 1 M citric acid and extracted with either chloroform or ethyl acetate (both HPLC grade). The *S. balanoides* in vitro assay described by Clare et al. (1985) was utilized, except for some of the assays of enzyme inhibitors which utilized *E. modestus* egg masses.

Preparation of Liposomes. Multilamellar liposomes of ca. 1 μm diameter were prepared from soybean lecithin (Sigma) according to the protocol of Deamer and Uster (1983), except that FESW was substituted for NaCl. Lecithin/EPA (Cayman) liposomes (4:1) were made by the same method. Radiolabeled liposomes were prepared by substituting 5 μCi [^{14}C]EPA (NEN) for cold EPA.

Analytical Techniques. High-performance thin-layer chromatography (HPTLC) was carried out in an Alltech linear HPTLC tank with Nano-sil-20 (UV254, 10 × 10 cm, 0.2 mm thick) HPTLC plates (Alltech). A solvent system of diethyl ether–petroleum ether–glacial acetic acid (50:50:1) (Hurst et al., 1987) was used. Racemic 8-HEPE (Cayman) was used as a marker. Typically, runs to a solvent front distance of 6 cm gave good separation of eicosanoid extracts. Spots were visualized under short- and long-wavelength UV light. For in vitro bioassay, spots were scraped off the plate, eluted with methanol, evaporated under nitrogen (oxygen free), and resuspended in FESW.

Prior to autoradiography, three coats of En^{3}Hance (NEN) were applied to HPTLC plates. Plates were exposed to Kodak X Omat AR autoradiography film at −70°C for 24 hr and the film developed according to the manufacturer's protocol.

Testing of Skeletonema costatum *Culture Medium.* A concentrated culture of *S. costatum* has previously been reported to induce egg hatching of *S. balanoides* in vitro (Barnes, 1957). As a prelude to comparing EHP with this diatom factor, *S. costatum* and cell-free culture medium were tested for activity in the in vitro bioassay: paired egg masses were incubated: in (i) 1.5 ml *S. costatum* at 1.86 × 10^5 cells/ml, (ii) 1.5 ml of centrifuged medium (to remove diatoms), and (iii) 1.5 ml FESW. Egg hatching was monitored after 15 min and 24 hr.

Release of Egg-Hatching Pheromone. If EHP is an excretory metabolite, it should be released by barnacles that are feeding. Accordingly, three treatments were set up using 600-ml beakers containing air-bubbled FESW: (i) barnacles fed on *S. costatum*, (ii) barnacles only, and (iii) *S. costatum* only. The experiment was performed on four occasions. Experiments 1–3 were each run for 24 hr in February, April, and May 1994, respectively, and employed gravid barnacles with mature egg masses. Experiment 4 was performed in January 1995 with barnacles containing immature (red eyespot stage) egg masses. The absence of the potential complication of eggs hatching permitted a longer feeding regime of six days in the latter instance. At the end of each experiment, the number of molts was counted and the barnacles were dissected to determine the number

that had liberated their nauplii. The seawater in each container was centrifuged (experiments 1–3), or filtered (Whatman No. 3) (experiment 4), to remove algae and fecal pellets and extracted for EHP. Experimental conditions were as follows: In experiment 1 there were 68 and 67 barnacles in treatments i and ii, respectively; 400 ml/treatment; 11.5°C; $\sim 2.8 \times 10^5$ cells/ml. In experiment 2 there were 24 and 25 barnacles in treatments i and ii, respectively; 200 ml; 12°C; $\sim 3.1 \times 10^5$ cells/ml; seawater not acidified prior to extraction. In experiment 3, there were 48 barnacles; 200 ml; 18.5°C; $\sim 2 \times 10^5$ cells/ml. In experiment 4 there were 36 and 37 barnacles in treatments i and ii, respectively; 200 ml; 9°C; $\sim 1.2 \times 10^5$ cells/ml; EHP extracted at 24 hr and six days. In each case, the solvent fractions were rotary evaporated to a small volume (~ 2 ml) and then evaporated to dryness under nitrogen (oxygen free) prior to resuspension in FESW for bioassay.

Effect of Liposome Diet on Egg-Hatching. If EHP is a dietary metabolite of EPA, it should be possible to induce egg hatching by feeding gravid barnacles on a diet that is enriched in EPA. Accordingly, four treatments were set up in 600-ml beakers containing 200 ml, air-bubbled, FESW: (i) $\sim 3 \times 10^5$ cells/ml *S. costatum* (positive control), (ii) $\sim 2 \times 10^5$ lecithin/EPA liposomes/ml, (iii) $\sim 2 \times 10^5$ lecithin liposomes/ml (negative control), and (iv) FESW only (negative control). After 48 hr, the number of molts was counted and the number of barnacles that had liberated their nauplii was determined by dissection. The experiment was performed on three occasions in March 1994.

Incorporation of [^{14}C]EPA into Excretory Metabolites. In order to determine if EHP is an excretory metabolite of EPA, barnacles were fed with radiolabeled liposomes and the metabolites examined by HPTLC with autoradiographic detection. Two treatments were used: (i) 300 ml FESW containing 48 *S. balanoides*, [^{14}C]EPA liposomes (5 μCi), and 4×10^4 cells/ml *S. costatum;* and (ii) 300 ml FESW containing [^{14}C]EPA liposomes (5 μCi), and 4×10^4 cells/ml *S. costatum.* Both beakers were air-bubbled and maintained at 17°C for 24 hr. The seawater was then acidified to pH 4.0 with 1 M citric acid and partitioned against an equal volume of chloroform. The chloroform extract was rotary evaporated to dryness and the residue taken up in a small volume of chloroform for HPTLC. Crude EHP (cold), 8-HEPE (cold), and [^{14}C]EPA were run together on the plate that was developed for 10–12 min. The plate was then viewed under UV light prior to autoradiography.

Incubation of EPA with Soybean Lipoxygenase. The ability of soybean lipoxygenase to convert EPA to a product with egg-hatching activity was tested with the in vitro assay. The following solutions were tested: (i) 1.5 mg EPA with 1.5 mg soybean lipoxygenase (type 1-B, Sigma) in 1.5 ml FESW, (ii) heated inactivated lipoxygenase (boiled for 15 min) with EPA, (iii) EPA in FESW, and (iv) 1% ethanol in FESW (ethanol was used as the carrier solvent for EPA).

Effect of Lipoxygenase Inhibitors on EHP Biosynthesis. Single, whole, adult barnacles were ground in FESW with or without the addition of a lipoxygenase inhibitor. The solutions were incubated at 20°C for 15 min, centrifuged at 16,000*g*, and the supernatant assayed for egg-hatching activity against the appropriate carrier solvent control. Two of the inhibitors, eicosatetraynoic acid (ETYA) and nordihydroguaiaretic acid (NDGA), were also tested in vivo, at a concentration of 10 μM, for their effect on feeding-induced egg-hatching. Three beakers were set up—ETYA, NDGA, and a 1% ethanol (carrier solvent) control—each containing 13 gravid barnacles and 200 ml, air-bubbled, seawater with 2×10^5 cells/ml *S. costatum* added. The solutions were changed after 24 hr and egg hatching and molting were recorded after 48 hr.

RESULTS

Contrary to the report of Barnes (1957), egg hatching was not elicited either by incubation with *S. costatum*, or in f/2 culture medium that remained after diatoms had been removed by centrifugation, even after 24 hr. EHP is, therefore, unlikely to be a factor that is associated with the barnacle's diet, concentrated by ingestion, and released to induce egg hatching. Rather, as stated previously (Crisp and Spencer, 1958), EHP is a product of the barnacle's own metabolism.

EHP can be extracted from seawater into which nauplii have been liberated by adult barnacles following feeding (Crisp and Spencer, 1958; Clare unpublished). Experiments that tested whether EHP release could be induced by feeding, other than at the time of egg hatching, failed to detect EHP in seawater containing either gravid barnacles with mature (experiments 1–3), or immature egg masses (experiment 4).

In order to examine whether EHP could be produced from dietary EPA, gravid barnacles were fed with an artificial diet of liposomes. A lecithin/EPA liposome diet increased the frequency of molting compared to barnacles that had been fed liposomes without EPA and the "no-additions" control, but only in the former instance was the result significant (*t* test, $t = 3.38$, $df = 2$, $P < 0.05$). Egg hatching was not induced by either of the liposome diets (Figure 1). One barnacle in the lecithin/EPA liposome treatment did not contain egg masses upon dissection. Since this was a small specimen and no nauplii were observed in the beaker, it is assumed that it did not originally contain egg masses. The diet of *S. costatum* increased molting compared to the seawater control ($t = 4.51$, $df = 3.18$, $P = 0.01$). However, even though no egg hatching was observed in the seawater controls, egg hatching was not induced significantly by the *S. costatum* diet, presumably due to the high degree of variability between replicates.

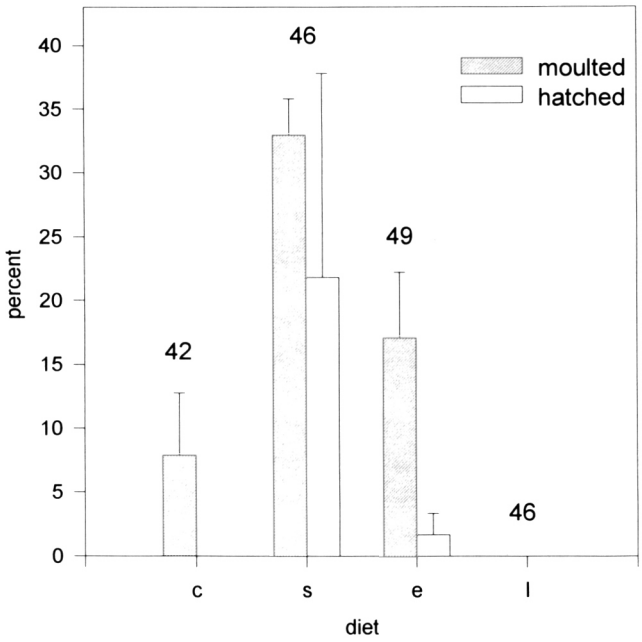


FIG. 1. *Semibalanus balanoides*. Incidence of molting and egg hatching in barnacles fed on various diets. Data are means of three replicate experiments (+ 1 SE). Numbers above error bars are total numbers of barnacles in each treatment. c, seawater control; s, *Skeletonema costatum;* e, lecithin/EPA liposomes; l, lecithin liposomes.

The failure to induce egg hatching in vivo by feeding an EPA diet could be attributed to an insufficient content of EPA in the liposomes. However, other evidence suggests that EHP is not an excretory metabolite of EPA: After feeding barnacles with [^{14}C]EPA, and separation of compounds extracted from seawater by HPTLC, autoradiography detected two bands: one more polar and the other less polar than EPA (Figure 2). Since the bands were detectable whether or not the beaker contained barnacles, it is possible that they represented autooxidation products of EPA and/or were produced by the algae. Neither band comigrated with cold egg-hatching pheromone (EHP zone in Figure 2A), nor the major autooxidation product of EPA determined in a previous experiment (data not shown).

If EHP is not an excretory metabolite of EPA, as the above results suggest, then perhaps EHP is synthesized de novo from EHP. The putative structure of *S. balanoides* EHP suggests lipoxygenase (LO) involvement (Hill et al., 1993). Previous studies with the cyclooxygenase inhibitors, acetylsalicylic acid and indomethacin (Clare et al., 1985) (see Table 2 below), support this inference.

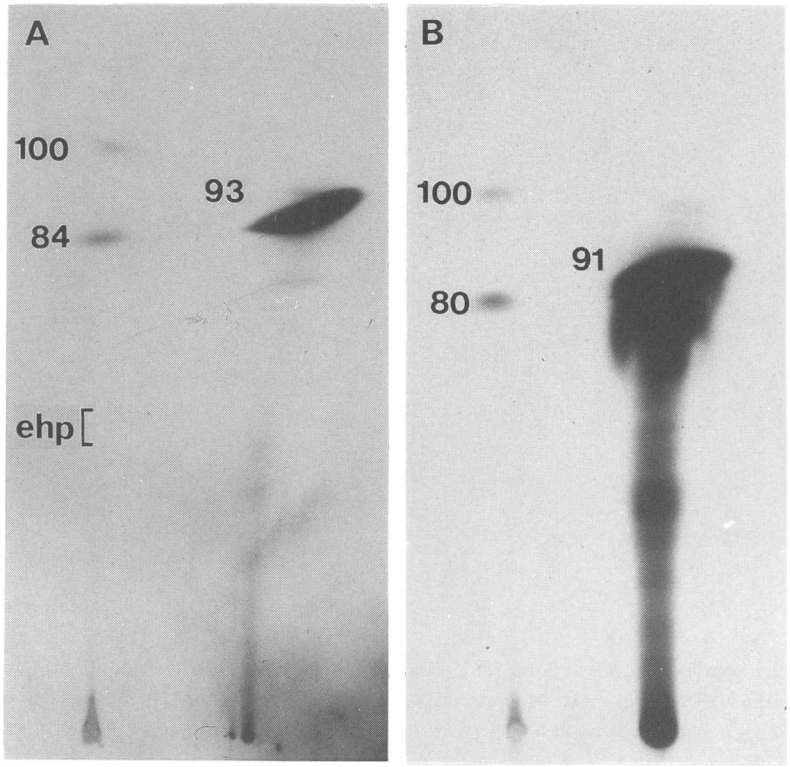


FIG. 2. *Semibalanus balanoides.* Autoradiogram of HPTLC plates. Right lanes in A and B show the migration of [^{14}C]EPA. Left lane in A shows the migration of radiolabeled compounds extracted from seawater containing barnacles that had been fed on [^{14}C]EPA liposomes. Left lane in B shows radiolabeled compounds extracted from the seawater control containing liposomes and *Skeletonema costatum* only. The band at $R_f(\times 100)$ = 80 may comprise two bands. ehp: region of the plate where cold egg-hatching pheromone migrated.

Nevertheless, as Table 1 shows, while incubating EPA in seawater resulted in a product(s) with egg-hatcing activity, the addition of soybean LO did not increased this activity. LO was toxic to hatched nauplii. The involvement of LO is, however, supported by the results of assaying LO inhibitors on EHP biosynthesis by adult tissue homogenates (Table 2). With the exception of ethyl 3,4-dihydrobenzylidene, all inhibitors of LO inhibited EHP biosynthesis, but to a variable degree. NDGA and baicalein were the most effective of the inhibitors (complete inhibition at 0.01 mM) tested.

TABLE 1. *Semibalanus balanoides:* EFFECT OF INCUBATION OF EICOSAPENTAENOIC ACID (EPA) WITH SOYBEAN LIPOXYGENASE ON EGG-HATCHING IN VITRO

Treatment	Hatch[a]	Comment
EPA (1.5 mg) + lipoxygenase (1.5 mg)	+	toxic
EPA (1.5 mg) + heat inactivated lipoxygenase (1.5 mg)	++	slightly toxic
EPA (1.5 mg)	++	slightly toxic
1% ethanol control	−	

[a] − no hatch; + small hatch; ++ large hatch (no complete hatches).

TABLE 2. EFFECT OF INHIBITORS OF EICOSANOID SYNTHESIS ON EGG-HATCHING PHEROMONE PRODUCTION IN TISSUE HOMOGENATES

Inhibitor	Specificity	Concentration (mM)	Effect on EHP synthesis
Acetylsalicylic acid[a]	CO[1,b,c]	10, 5, 1, 0.45	++, +, −, −[d]
Indomethacin[a]	CO[2]	10, 5, 1	−, −, −
Eicosatetraynoic acid	CO/LO[3]	0.1, 0.01	++, +
Nordihydroguaiaretic acid	5-LO[4]	0.01	++
Caffeic acid[e]	5-LO[5]	0.01	+
Baicalein[e]	12-LO[6]	0.01	++
Ethyl 3,4-dihydroxybenzylidene-cyanoacetate[e]	12/15-LO[7]	0.1, 0.01	+, +

[a] After Clare et al. (1985).
[b] [1]Vane and Botting (1987); [2]Laneuville et al. (1994); [3]Hamberg and Samuelson (1974); [4]Salari et al. (1984); [5]Koshihara et al. (1984); [6]Sekiya and Okuda (1982); [7]Cho et al. (1991).
[c] CO, cyclooxygenase; LO, lipoxygenase.
[d] ++, complete inhibition; +, partial inhibition; −, no effect.
[e] Assays done with *Elminius modestus.*

Both NDGA and ETYA partially inhibited feeding-induced liberation of nauplii from gravid adults (Table 3). Interestingly, the relative potency of these two inhibitors was the opposite of that observed in the in vitro experiments. This result may reflect the relative stabilities and/or uptake kinetics of the two compounds.

TABLE 3. *Semibalanus balanoides:* EFFECT OF ETYA AND NDGA (AT 10 μM) ON FEEDING-INDUCED EGG-HATCHING IN VIVO

Treatment	Released		Molted		Total (*N*)
	N	%	*N*	%	
ETYA	2	15.38	4	30.77	13
NDGA	5	38.46	0	0	13
Ethanol control	10	76.92	1	7.69	13

DISCUSSION

The results of this study do not support the hypothesis that EHP is an excretory metabolite of dietary EPA. The clearest evidence for this conclusion came from the inability to extract EHP from seawater that contained barnacles that had been feeding. It is well known that EHP is released into seawater at the time of egg hatching (Crisp and Spencer, 1958), and on the basis of the limited experiments reported here, it would appear that this is the only time the pheromone is released. A rejection of the hypothesis was lent further support by the failure to detect radiolabeled EHP in seawater when barnacles were fed with [^{14}C]EPA liposomes. Moreover, barnacles that were fed on lecithin/EPA liposomes did not liberate their nauplii. There was, nevertheless, an increase (>50%), albeit insignificant, in the molting of these barnacles compared to those that had not been fed, and a significant increase compared to those that had been fed on lecithin liposomes. Although it cannot be ruled out that the content of EPA was insufficient to induce egg hatching, the increase in molting suggests otherwise. It is also possible that excretory metabolites were not produced sufficiently rapidly following feeding to elicit hatching in the allotted experimental period. No previous data are available for barnacles, but in rats, eicosanoid excretion rapidly followed oral administration of arachidonic acid (Lands and Kulmacz, 1986).

The lack of any close correspondence between molting and egg hatching in the feeding experiments (Figure 1 and Table 3) was not surprising since it is well known that gravid *S. balanoides* can molt without liberating their nauplii (Crisp and Patel, 1960; Patel and Crisp, 1961). Thus, we can reject the suggestion made by Starr et al. (1991) that the arthropod molting hormone, 20-hydroxyecdysone, may be directly connected with the stimulus to egg hatching. On the other hand, the direct connection between feeding and release of EHP is not in dispute. Starr et al. (1991) argued that nutritional gain by the adult

barnacle is not required, as they observed hatching within 1 hr of feeding gravid adults on *S. costatum.*

Apparently, EHP is synthesized in accord with the conventional scheme of eicosanoid biosynthesis, i.e., from precursor fatty acid released from membrane lipid by phospholipase (Shimizu and Wolfe, 1990; Piomelli, 1993). The ability to induce egg hatching by a product(s) produced by incubating EPA in seawater supports the contention that EPA is able to serve as a precursor to EHP. Presumably, the active product was formed by autooxidation of EPA (Slater and Cheeseman, 1987). On this basis, it is likely that at least some of the activity formed by homogenization of adult barnacle tissues is the product of autooxidation and therefore an artifact of the preparation.

After release from membrane phospholipid, the results of the pharmacological experiments with lipoxygenase inhibitors suggest that such an enzyme acts on EPA as part of the EHP biosynthetic pathway. While several different classes of inhibitor were tested, with reported specificities for different lipoxygenases, the results of this study do not give a clear indication of which lipoxygenase is involved in EHP biosynthesis. The specificities of action reported in Table 2 are only meant as a guide, having been determined for mammals. Even this literature is very confused. Clearly more studies of the type reported by Miller and Stanley-Samuelson (1996) are needed for invertebrates.

Eicosanoids have diverse physiological functions (e.g., Sigal, 1991; Stanley-Samuelson, 1994), but are normally regarded as autocoids, or as second messengers in cell signaling. It is clear from studying barnacle egg hatching that eicosanoids can also function as pheromones in aquatic chemical communication. There have been two other reports of this phenomenon. First, there was the finding that spawning of the abalone, *Haliotis rufescens*, involves prostaglandins (Morse et al., 1977). More recently, $PGF_{2\alpha}$ metabolites have been found to function as pheromones in fish mating behavior (Sorenson, 1992; Stacey et al., 1994; Moore and Waring, 1996). Moreover, pheromones involved in male gamete attraction in brown seaweeds have been characterized, in the majority of cases, as C_{11} hydrocarbons. Interestingly, in the context of the present discussion, while not eicosanoids, the hydrocarbons are synthesized by an "offshoot of eicosanoid metabolism" (Gerwick, 1994). The metabolic pathway has been suggested to involve 9-lipoxygenase cleavage of hydroxy fatty acids derived from C_{20} PUFA precursors (e.g., arachidonic acid and EPA) (see Gerwick, 1994; Boland, 1995 for reviews). Given the phylogenetic diversity of these examples, the involvement of eicosanoids, and/or their metabolic products, in aquatic chemical signaling may be widespread.

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EFFECTS OF TERPENES AND PHENOLIC AND FLAVONOID GLYCOSIDES FROM DOUGLAS FIR ON WESTERN SPRUCE BUDWORM LARVAL GROWTH, PUPAL WEIGHT, AND ADULT WEIGHT

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Abstract—Monoterpenes, sesquiterpenes, and phenolic and flavonoid glycosides typical of the current year's foliage of Douglas fir (*Pseudotsuga menziesii*) were bioassayed using agar diets to determine the effect of these compounds on natural and colony populations of western spruce budworm (*Choristoneura occidentalis*). Several terpenes adversely affected budworm larval growth, supporting previous field and laboratory studies. However, larvae collected from a population in southeastern Idaho were significantly more tolerant of the monoterpenes than those from a Montana population. For the Montana population, agar diet studies showed that camphene, myrcene, terpinolene, bornyl acetate, and tricyclene adversely affected larval growth rate and pupal weight. For the Idaho population, agar diet studies showed that only terpinolene and bornyl acetate adversely affected larval growth and pupal weights. For both populations bornyl acetate was the most toxic, and this compound in addition to other monoterpenes represent defensive mechanisms in the current year's growth of Douglas fir. Sesquiterpene and phenolic-flavonoid glycoside fractions extracted from Douglas fir current year's growth also were bioassayed using agar diets. The sesquiterpene fraction showed a significant negative effect on budworm larval growth, but phenolics and flavonoid glycosides had no effect. Sesquiterpenes, in combination with tricyclene, camphene, myrcene, limonene, terpinolene, and the acetate fraction appear to represent an effective mixture of defensive compounds against the budworm.

Key Words—Monoterpenes, sesquiterpenes, phenolics, flavonoids, Douglas fir, *Pseudotsuga menziesii*, western spruce budworm, *Choristoneura occidentalis*.

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INTRODUCTION

Individual plant compounds as well as mixtures of compounds are known to play a significant role in the interactions between herbivorous insects and plants (Cates, 1996; Zou and Cates, 1994; Price et al., 1991; Cates and Redak, 1988; Berenbaum, 1983). Those roles include herbivore host selection (Raffa, 1995), tissue feeding preferences (Albert and Prisella, 1985), insect and plant population dynamics (Haukioja and Neuvonen, 1987; Wallner and Walton, 1979), and the degree of utilization of plant foliage (Macedo and Langenheim, 1989; Lindroth et al., 1988; Rosenthal and Janzen, 1979). The Douglas fir (*Pseudotsuga menziesii*)/western spruce budworm (*Choristoneura occidentalis*) system provides an ideal model for increasing our understanding of the roles that plant nutrients and other natural products play in the interactions between phytophagous insects and plants. Previous field studies have indicated that the performance of all life stages of the oligophagous western spruce budworm was adversely affected by various concentrations of specific terpenes in the foliage of Douglas fir (Cates and Zou, 1990; Cates and Redak, 1988; Cates et al., 1983). For example, a study conducted in Montana indicated that the level of budworm infestation (budworm density × amount of defoliation) was negatively correlated with myrcene, an unidentified terpene, and the total concentration of terpenoid acetates, and positively correlated with foliage concentrations of β-pinene (Cates et al., 1983).

In addition, compositional variations in the relative amounts of nutritional and defensive natural products of Douglas fir and other forest trees may contribute to reduced forest stand susceptibility (Cates, 1996; Macedo and Langenheim, 1989) and may be important selection pressures affecting budworm population dynamics (Zou and Cates, 1994; Cates and Zou, 1990; Wagner et al., 1989). Present evidence suggests that budworm populations have become locally adapted to tree population variation in foliage quality characteristics (Cates et al., 1991; Cates and Redak, 1988). The response of the budworm to among-population, among-trees-within-a-population, among-years, and other variations in foliage quality parameters of Douglas fir may have led to genetic divergence in budworm populations (Cates and Zou, 1990). This genetic divergence has been described by Willhite and Stock (1983) and is likely a major factor in the evolution of host races of budworm (Cates, 1996; Cates and Zou, 1990).

To date about 25 volatile compounds have been identified from Douglas fir foliage (Cates and Zou, 1990; Wagner et al., 1989). Some of these compounds are involved as mechanisms of resistance and have the potential to enhance the silvicultural management of forest pests (Wulf and Cates, 1987). Sesquiterpenes and phenolic and flavonoid glycosides are present in Douglas fir foliage, but their effect on the budworm is largely unknown. We have identified,

quantified, and purified major and minor components of the essential oil and the phenolic–flavonoid glycoside fractions in the current year's needles of Douglas fir. The availability of identified compounds and fractions containing specific groups of chemicals makes possible further study as to how these compounds may affect budworm larvae and pupae and whether some compounds are more important than others as inhibitors of budworm feeding.

We report the effects of low-molecular-weight tepenoids and of the sesquiterpene and phenolic and flavonoid glycoside fractions on budworm larval growth and pupal weight in a controlled environment. Individual terpene compounds were added to agar diets and fed to larvae reared from natural budworm populations in Montana and Idaho. In addition, sesquiterpene and phenolic and flavonoid glycoside fractions extracted from the current year's needles of Douglas fir were tested in feeding trials using a nondiapausing colony of budworm larvae.

METHODS AND MATERIALS

Budworm Rearing. Budworm pupae were collected from two natural diapausing populations near Greenough Creek, Montana, and Ashton, Idaho. Pupae were transported to the Chemical Ecology Laboratory at Brigham Young University and maintained in a growth chamber. Growth chamber conditions were 16L:8D photoperiod, 24°C constant temperature, and 45% relative humidity. Adults were allowed to emerge, mate, and lay eggs. Egg masses were disinfected before hatching and placed in Petri dishes containing sterilized gauze. Larvae emerged, grew to the second instar, and hibernated. Larvae in Petri dishes were subjected to a cold treatment of 4°C for 1 week and then stored for 200 days at 0°C. After the cold treatment, larvae were returned to a growth chamber in Petri dishes containing a control agar diet, allowed to grow to third instar, and sexed.

Diet Composition and Monoterpenes Used in Budworm Bioassay. Details concerning the composition of the diet and procedures for rearing budworms followed Robertson (1979) as modified by Zou and Cates (1994). Adjusting the nitrogen content of the diet to resemble more closely that of Douglas fir foliage was done by changing the concentration of vitamin-free casein, and then verified with an Auto Analyzer II. The total nitrogen content in all diets was 1.26%. The diets also contained 0.37% phosphorus and 0.33% potassium, verified with an atomic absorption spectrophotometer (Perkin Elmer 500). Once the diet was modified, 11 experimental diets were formulated; each of 10 included an individual terpene additive and one served as a control diet. Terpenes used in this study were (expressed as percentage of diet) tricyclene (0.110), camphene (0.355), myrcene (0.057), α-pinene (0.217), β-pinene (0.131), limonene (0.18),

terpinolene (0.076), bornyl acetate (0.35), geranyl acetate (0.095) and citronellyl acetate (0.095). Concentrations used were typical of those found in the current year's needles of Douglas fir on which budworm larvae were feeding. Samples analyzed from 125 trees from the Montana and Idaho populations were collected in early July 1986 and 1988 and were used to determine the terpene concentrations for the agar diets (Cates and Zou, 1990). From those samples, the 10 trees with the highest concentrations for each terpene were selected and averaged. Myrcene, terpinolene, geranyl acetate, and citronellyl acetate were found to be minor components of the Douglas fir foliage from these two populations. However, higher concentrations occur in other populations (Cates and Redak, 1988). Since a determination of their potential to inhibit larval growth and affect pupal weight was an objective of this study, their concentrations were increased to levels approaching the concentrations found in trees in other populations.

The agar diet was replaced every three days because previous study had shown that some monoterpenes in the agar diet decreased significantly in concentration by the end of the third day that the diet was in the Petri dish. Concentrations of the terpenes in the agar diets were routinely checked by capillary gas chromatography to verify constancy of concentration throughout the diet studies. Commercially available terpene compounds were purchased (Aldrich Chemical Company, Sigma Chemical Company, Wiley Organics) and their purity verified. Thirty-five third instars for each sex from each population were randomly assigned to each experimental diet. One larva was housed in each Petri dish, and each larva represented a single experimental unit. Therefore, the initial replication for each treatment was 35. Larvae were reared in 5-cm-diameter Petri dishes. The initial budworm larval weight in each treatment was determined by calculating the average of 10 larvae that were to be used in each diet. This was primarily done because our experience has indicated that increased handling of larvae resulted in higher larval mortality rates (R. G. Cates, unpublished data), and because we had 770 larvae to handle. Larval status was checked each day, and larvae and pupae were weighed upon death. After adults emerged, they were frozen, oven dried at 60°C for three days, and weighed. Data collection from these experiments were larval growth rate, pupal weight, and male and female adult dry weight [adult dry weight and mortality are reported in Cates et al. (1991)]. Growth rate was calculated as larval weight gain (wet wt)/larval living days to and including the date of pupation.

Terpenoid concentrations were determined by capillary gas chromatography using a 100% methyl polysiloxane column. Injector and detector temperature programs and detailed methods for determining the terpene concentrations are given in Cates and Zou (1990). Identification of compounds was accomplished by mass spectrometry (Hewlett Packard GC-MS, model 5995), and by cochromatography of standards using different columns. Response factors were determined in all cases.

Effect of Sesquiterpene and Phenolic Extracts on Budworm. Although sesquiterpenes and phenolics are produced in the current year's growth of Douglas fir, to our knowledge no test of their effectiveness against the western spruce budworm has been undertaken (Zou and Cates, 1995). Budworm larvae used to test the effects of these fractions were hatched from eggs obtained from a non-diapausing colony maintained by Robert McCron, Integrated Pest Management Division, Sault Ste. Marie, Canada. Upon emergence, larvae were placed on a control agar diet until they reached the third instar. Third instars were placed on diets containing either the sesquiterpene fraction, the phenolic fraction, or the control. A total of four experimental diets containing sesquiterpene and phenolic fractions were prepared with two different concentrations of each fraction (diet 1 = sesquiterpenes at 1% of the diet; diet 2 = sesquiterpenes at 0.5% of the diet; diet 3 = phenolics at 5% of the diet; diet 4 = phenolics at 2.5% of the diet). The low concentration of each diet was typical of resistant tree foliage, and the high concentration was arbitrarily set at twice the typical level. These experiments were run in the growth chamber for three weeks in the same manner as described for the other feeding trials. Fifteen budworm larvae were randomly assigned to each experimental diet, and at the end of the experiment budworm larval weight and growth rate were determined. There was an insufficient amount of the sesquiterpene fraction to run the experiment through to pupation, so experiments involving both fractions were terminated at the same time.

The sesquiterpenes and phenolic and flavonoid glycosides were extracted from Douglas fir foliage by grinding the needles in liquid nitrogen, filtering, and then exhaustively extracting with methylene chloride (3 × 300 ml). The methylene chloride extracts were combined and the water removed in a separatory funnel. The solvent was removed in a rotary evaporator, and the residue was absorbed onto silica gel and placed on a silica gel column. The column was washed with three column volumes of hexane, resulting in a colorless fraction containing only the sesquiterpenes as verified by capillary gas chromatography. These same needles were then extracted with methanol (3 × 300 ml). The methanol was removed, leaving a residue of phenolic and flavonoid glycosides as determined by thin-layer chromatography. This residue was redissolved in water and used in the agar diet study.

Statistical Procedures. Larval growth rate and pupal weight obtained from the agar diets containing terpenes were compared to the larval and pupal (wet wt) results from the control diets by ANOVA (Tables 1 and 2 below). Larval wet weight and growth rate obtained from the experimental diets containing sesquiterpenes and phenolic-flavonoid glycosides were compared to the control group by ANOVA (Table 3 below). Each budworm larva or pupa was treated as the experimental unit in all cases. SAS programs were used for all statistical analyses (SAS Institute, Inc., 1985).

RESULTS

Effects of Monoterpenes on Budworm from Two Natural Populations. Several terpenes were found to adversely affect budworm larval growth rates (Table 1). For the Montana population, camphene, myrcene, terpinolene, and bornyl acetate significantly reduced female larval growth, and tricyclene, camphene,

TABLE 1. MONTANA AND IDAHO BUDWORM LARVAL GROWTH RATES WHEN REARED ON AGAR DIETS CONTAINING TERPENES AND ACETATES[a]

	Budworm population			
	Montana		Idaho	
Sex/compound	Growth rate (mg/day wet wt, mean ± SD)	*P*	Growth rate (mg/day wet wt, mean, ± SD)	*P*
Female[b]				
Tricyclene	9 ± 2	0.80	7 ± 2	0.37
α-Pinene	11 ± 2	0.08	9 ± 2	0.28
β-Pinene	9 ± 1	0.92	9 ± 2	0.19
Camphene	5 ± 1	0.001	7 ± 1	0.50
Myrcene	8 ± 2	0.02	8 ± 2	0.63
Limonene	9 ± 2	0.27	7 ± 2	0.43
Terpinolene	8 ± 1	0.03	5 ± 1	0.02
Bornyl acetate	7 ± 2	0.001	6 ± 1	0.03
Geranyl acetate	8 ± 2	0.24	7 ± 1	0.43
Citronellyl acetate	10 ± 2	0.55	8 ± 2	0.71
Control[b]	9 ± 2		8 ± 1	
Male[b]				
Tricyclene	5 ± 2	0.02	6 ± 2	0.74
α-Pinene	6 ± 2	0.75	6 ± 2	0.88
β-Pinene	6 ± 2	0.94	5 ± 1	0.76
Camphene	5 ± 0	0.02	4 ± 1	0.09
Myrcene	6 ± 2	0.17	5 ± 1	0.25
Limonene	6 ± 2	0.94	6 ± 2	0.69
Terpinolene	5 ± 5	0.05	5 ± 1	0.34
Bornyl acetate	5 ± 1	0.01	4 ± 1	0.01
Geranyl acetate	6 ± 1	0.88	6 ± 2	0.87
Citronellyl acetate	7 ± 2	0.54	6 ± 1	0.88
Control[b]	6 ± 1		6 ± 1	

[a] ANOVA, significant differences $P \leq 0.05$ between the growth rate of larvae reared on the experimental diet vs. the control diet.
[b] For each diet $N = 35$ larvae/population.

terpinolene, and bornyl acetate adversely affected male larval growth when compared to the control. For the Idaho population, terpinolene and bornyl acetate significantly reduced female larval growth, while only bornyl acetate significantly reduced male growth. α-Pinene ($P = 0.08$) and camphene ($P = 0.09$) tended to reduce female growth rate in Montana larvae and male growth rate in Idaho larvae, respectively.

Female pupal weights of the Montana population were significantly reduced by camphene and bornyl acetate, with myrcene showing a strong trend, and male pupal weights were reduced by tricyclene and bornyl acetate, with camphene showing a strong trend towards a significant effect (Table 2). Female and male pupal weights from the Idaho population were significantly reduced by only bornyl acetate when experimental pupal weights were compared to the controls. For the Idaho female budworm, β-pinene and terpinolene showed trends towards a significant effect ($P = 0.10$) (Table 2).

Both concentrations of sesquiterpenes resulted in significant decreases in larval fresh weight production and growth rate of the Sault Ste. Marie budworm (Table 3). Alternatively, neither concentration of the phenolic and flavonoid glycoside fraction had a significant adverse effect on larval weight or growth rate.

DISCUSSION

This study indicates that different monoterpenes and the sesquiterpene fraction adversely affect budworm larval growth rate and pupal weight. The phenolic and flavonoid glycoside fraction was not effective in reducing larval fresh weight production or growth rate. This result is consistent with the observed decrease in phenolic compounds as the current year's needles develop throughout the growing season (Zou and Cates, 1995; Wagner et al., 1990). Although several terpenes, such as tricyclene, myrcene, and terpinolene, adversely affected growth rate and pupal weight, the most consistent terpenes to significantly reduce these parameters in both the Montana and Idaho populations were camphene and bornyl acetate. This study and those conducted by Cates et al. (1991) indicate that bornyl acetate is a significant anti-budworm defensive compound. Since the experiments involving bornyl acetate, as well as the other compounds such as the sesquiterpenes, were formulated around the natural concentrations found in the current year's growth of Douglas fir, these results are significant to understanding the mechanisms of defense by Douglas fir against the budworm. Noteworthy is the fact that male budworm were affected adversely by a greater array of terpenes than were females. Furthermore, combining terpenes into a single diet is known to exacerbate the performance of budworm (Zou and Cates, 1994).

The Idaho and Montana budworm populations used in this study respond

TABLE 2. MONTANA AND IDAHO BUDWORM PUPAL WEIGHTS FROM LARVAE REARED ON AGAR DIETS CONTAINING TERPENES AND ACETATES[a]

Sex/compound	Budworm population[b]			
	Montana		Idaho	
	Pupal weight (mg wet wt, mean ± SD)	*P*	Pupal weight (mg wet wt, mean ± SD)	*P*
Female				
Tricyclene	190 ± 40	0.73	160 ± 40	0.93
α-Pinene	200 ± 30	0.20	180 ± 40	0.28
β-Pinene	190 ± 20	0.91	190 ± 40	0.10
Camphene	120 ± 20	0.001	170 ± 20	0.85
Myrcene	170 ± 40	0.07	160 ± 30	0.66
Limonene	180 ± 50	0.28	160 ± 20	0.61
Terpinolene	170 ± 20	0.11	130 ± 40	0.10
Bornyl acetate	140 ± 0	0.001	180 ± 10	0.05
Geranyl acetate	180 ± 20	0.77	140 ± 20	0.14
Citronellyl acetate	200 ± 30	0.44	180 ± 16	0.28
Control	190 ± 30	—	190 ± 20	—
Male				
Tricyclene	100 ± 20	0.04	100 ± 30	0.86
α-Pinene	110 ± 20	0.83	100 ± 20	0.88
β-Pinene	110 ± 20	0.83	100 ± 20	0.88
Camphene	90 ± 10	0.06	90 ± 20	0.66
Myrcene	100 ± 20	0.23	90 ± 10	0.57
Limonene	120 ± 20	0.29	90 ± 30	0.63
Terpinolene	110 ± 10	0.25	90 ± 20	0.71
Bornyl acetate	90 ± 10	0.01	80 ± 10	0.03
Geranyl acetate	110 ± 10	0.95	100 ± 20	0.71
Citronellyl acetate	120 ± 30	0.37	100 ± 20	0.73
Control	110 ± 20	—	100 ± 10	—

[a] ANOVA, significant differences $P \leq 0.05$ between pupal weight of larvae reared on the experimental diet vs. the control diet.

[b] *N* for Montana females varied from 9 to 15 pupae, for Montana males from 11 to 14, for Idaho females from 6 to 10, and for Idaho males from 7 to 11 depending on the compound tested.

differently to the terpenes utilized in the agar diets. The Montana population, which was adversely affected by five different terpenes, appears to be less well adapted to foliage terpenes than the Idaho population, which was adversely affected by only two terpenes. These data suggest that genetic differences exist among these budworm populations, an aspect that has been reported previously (Cates and Zou, 1990; Willhite and Stock, 1983).

TABLE 3. LARVAL FRESH WEIGHT AND GROWTH RATE OF COLONY BUDWORM REARED ON AGAR DIETS CONTAINING SESQUITERPENE AND PHENOLIC-FLAVONOID GLYCOSIDE FRACTIONS[a]

Chemical fraction	Larval weight (mg)		Growth rate (mg/day)	
	$\bar{X} \pm$ SD	P	$\bar{X} \pm$ SD	P
Sesquiterpenes				
High	250 ± 30	0.006	2 ± 2	0.006
Low	360 ± 40	0.02	2 ± 3	0.024
Phenolic-flavonoid glycoside				
High	490 ± 40	0.23	3 ± 3	0.24
Low	590 ± 60	0.26	4 ± 4	0.26
Control	730 ± 50		5 ± 3	

[a]ANOVA, significant differences $P < 0.05$.

Reciprocal transplants of these two budworm populations among three Douglas fir populations further support the suggestion that genetic differences between the two budworm populations exist (Cates et al., 1991). When Montana budworm larvae were reared on foliage of trees at the Ashton, Idaho, site, male and female adults resulting from these rearings showed significantly less biomass production than did their cohorts reared on the current year's foliage of their "native" Montana Douglas fir population. However, budworm from the Ashton, Idaho, site, when reared on Montana trees, did not show significant reductions in biomass production on the foreign trees as compared to their biomass production on native trees. Finally, in this same study budworms from these two populations were reared on the current year's growth of Douglas fir at an Oquirrh Mountain site in Utah. Female budworm from these two populations when reared on Utah trees had dry biomass production reduced by 42–75% compared to their performance on native trees from which they were collected (Cates et al., 1991).

Noted in the study of Cates et al. (1991) were highly significant differences in the qualitative and quantitative production of terpenes, sesquiterpenes, and soluble carbohydrates among the Montana, Idaho, and Utah Douglas fir populations. This information on the terpene and carbohydrate differences when combined with the above laboratory feeding trials noted in this paper and in Cates et al. (1991) suggest that the two natural populations of budworm utilized in this study differ in their ability to cope with the metabolites found in the foliage of Douglas fir. The Idaho budworm population appears to have evolved a more efficient system of detoxification than the Montana population. However, neither

population was successful in utilizing the foliage of the Utah population. We propose that the adverse effect of the foliage from the Utah trees on the two populations of budworm was due to high concentrations of bornyl acetate, camphene, and especially the somewhat unique sesquiterpene fraction.

Little information is available concerning the mode of action by which terpenes adversely affect budworms or the ways in which budworms may detoxify terpenes. It is likely that terpenes, as well as other natural products, primarily affect the metabolic processes of herbivores (Berenbaum, 1983; Rhoades and Cates, 1976) and therefore represent strong selection pressures on the budworm. The most commonly suggested detoxification system against terpenes is the polysubstrate cytochrome P-450-dependent monooxygenases (PSMOs) in the microsomes of lepidopteran insects (Berry et al., 1980; Yu et al., 1979). For example, *Peridroma saucia* is known to detoxify monoterpenes utilizing the PMSO system, and presumably the western spruce budworm employs a similar system (Harwood et al., 1990; Moldenke et al., 1983). Further studies are needed on the mechanism of action of terpenes against budworms, particularly bornyl acetate, camphene, and the sesquiterpenes.

In summary, different life stages of the budworm may be negatively affected by the same as well as different compounds (Table 4) (Cates, 1996). Camphene and bornyl acetate have been shown to adversely affect every stage of the Montana budworm, and bornyl acetate is known to adversely affect every stage of the Idaho population (Table 4). In addition, overall mortality of the Montana population was increased when larvae were exposed to diets containing limonene, geranyl acetate, and citronellyl acetate (Cates et al., 1991). Limonene and terpinolene also were found to contribute to overall mortality in the Idaho population ($P = 0.08$) (Cates et al., 1991). Adult dry biomass production of males and females from the Montana and Idaho populations were adversely affected by six terpenoids (Cates et al., 1991). For the Idaho population, terpinolene, bornyl acetate, and geranyl acetate reduced female dry weight production, but male dry weights were reduced only by bornyl acetate. Finally, when these individual compounds are placed in mixtures with quantities of terpenes and carbohydrates typical of their concentration in Douglas fir foliage, they significantly increase larval mortality and decrease female and male larval growth rate and pupal weight (Zou and Cates, 1994). We suggest that these negative effects do reduce budworm fitness. For example, female budworm fecundity is significantly ($P = 0.01$) and positively correlated with female dry weight biomass production (Redak and Cates, 1984). We are assuming that the reduction of adult dry biomass production of 42–75% in budworm reared on resistant tree foliage compared to susceptible trees, and overall mortality based on agar diet studies of 55–89%, would adversely affect natural budworm fitness and population dynamics (Cates et al., 1991). Clearly these assumptions need to be tested.

TABLE 4. TERPENOIDS IN DOUGLAS FIR CURRENT YEAR GROWTH KNOWN TO ADVERSELY AFFECT MONTANA AND/OR IDAHO FEMALE AND MALE BUDWORM LIFE STAGES ($P \leq 0.05$)

Overall mortality[a]	P	Larval growth rate[a]	$P_{F,M}$[b]	Pupal weight[a]	$P_{F,M}$	Adult dry weight[a]	$P_{F,M}$
Bornyl acetate	0.00	Camphene	0.000, 0.02	Camphene	0.000, —	Camphene	0.000, 0.000
Camphene	0.00	Bornyl acetate	0.001, 0.01	Bornyl acetate	0.001, 0.01	Bornyl acetate	0.000, 0.000
Citronellyl acetate	0.00	Myrcene	0.02, —	Tricyclene	—, 0.04	Tricyclene	0.000, 0.000
Geranyl acetate	0.03	Tricyclene	—, 0.02			Terpinolene	0.000, 0.013
Limonene	0.01	Terpinolene	0.02, 0.05			Geranyl acetate	0.05, —
						Myrcene	—, 0.000

[a] Larval mortality and adult dry weight data for two populations of budworm modified from Cates et al. (1991); growth rate and pupal weight data from this paper (Tables 1 and 2).

[b] $P_{F,M}$ equals the P value for female followed by the P value for males. — indicates not significant for this sex.

Herbivory has been proposed as one factor that has led to the extensive qualitative and quantitative variation that is observed in the production of natural products within and among long-lived species. This variation is characteristic of vigorous, resistant host stands (Wulf and Cates, 1987; Carlson et al., 1985) and is important in the maintenance of herbivore populations at low numbers (Cates, 1996; Cates and Zou, 1990; Cates and Redak, 1988; Raffa and Berryman, 1983; Louda and Rodman, 1983; Whitman, 1983; Crankshaw and Langeheim, 1981). Interactions between the budworm and its host plants represent the most parsimonious explanation for the host-race formation observed among budworm and Douglas fir populations relative to foliage chemical variation and budworm metabolic characteristics.

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PATTERNS AND CONSEQUENCES OF BENZYL ACETONE FLORAL EMISSIONS FROM *Nicotiana attenuata* PLANTS

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Abstract—The emission of a single compound, benzyl acetone (BA, 4-phenyl-2-butanone), is barely detectable during the day in the headspace of flowers of the self-compatible disturbance species *Nicotiana attenuata*, but it increases dramatically (50×) in the evening, becoming the dominant component in the floral headspace. This striking temporal pattern of emission may be sculpted by its potential ecological roles (e.g., synomonal and kairomonal), which we examine here. We measured the nightly BA emissions from individual flowers at six different branch positions on plants receiving either self-pollen or pollen from another genotype and calculated the nightly whole-plant emission. The first flowers produced on a branch have a lower rate of emission than flowers produced later on the same branch; however, cross pollination did not influence the quantity of BA emitted from subsequently produced flowers. Informed by these measures of whole-plant emission, we constructed a device that released BA at a constant rate equivalent to that of a plant with 240 open flowers (an approximate 10× increase in emissions). This device and a control device were attached to 50 matched pairs of plants growing in a native population in Utah to estimate the fitness consequences of enhanced, constant BA emission. Plants with elevated BA emissions in the field were browsed more frequently than control plants and produced fewer capsules, so that lifetime seed production was reduced by 3.1%. However, both treatment and control plants were heavily attacked by negro bugs (*Cormelina* spp.) and produced light seeds with low viabilities, representing 47% and 23% of the mass per seed and viability, respectively, of unmanipulated plants, which flowered two weeks later in the same population. From glasshouse experiments, we estimated the consequences of out-crossing and attack by negro bugs on seed production. Out-crossing did not significantly affect seed production, seed

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mass or viability. In contrast, negro bug infestation dramatically decreased seed mass and viability. We conclude that while the phenological variation in attack rates might have obscured our ability to estimate the fitness consequences of enhanced BA emission, the effects are likely to be dominated by kairomonal rather than synomonal interactions for this self-compatible species.

Key Words—Floral volatiles, benzyl acetone, seed mass, seed number, seed germination, *Nicotiana attenuata*.

INTRODUCTION

Because floral odors play an important role in attracting pollinators (Dobson, 1994), they thereby function synomonally. However, these compounds may also function allomonally by repelling potential attackers or kairomonally by orienting herbivores (Pellmyr and Thien, 1986; Metcalf and Metcalf, 1991; Pellmyr et al., 1991). Indeed, a compound's functional role is as likely to be determined by its producer's ecological milieu as by any intrinsic characteristic of the chemical. For example, volatile compounds produced by flowers and functioning as feeding deterrents may have been coopted by animals and used initially as orientation cues to locate feeding, mating, and oviposition sites; only later may they have functioned in communication with pollination mutualists (Pellmyr et al., 1991).

By controlling the synthesis, emission, and release of allelochemicals in response to the environment, plants can limit the functional roles particular allelochemicals play. This plasticity can inform hypotheses about putative functional roles. For example, a synomonal role for floral volatiles is suggested by the high correlation between their emission and the activity of a plant's pollinators (Jakobsen and Olsen, 1994). However, the abrupt decrease in emission after pollination (Tollsten and Bergström, 1989; Euler and Baldwin, 1996) suggests that floral emissions also represent a liability and that the reduction in emission might minimize the costs associated with advertising for pollinators. These costs are likely to be ecological, associated with increases in a plant's apparency to predators, rather than biochemical, because the quantities of chemical emitted are small (see Dicke and Sabelis, 1989) and the details of the biochemical mechanisms responsible for the release suggest otherwise. For example, *Nicotiana sylvestris* and *N. suaveolens* flowers emit phenylpropanoid-derived volatiles in a diurnal pattern. When these flowers senesce, the corolla contains pools of glycosidically bound volatiles that are many times larger than the quantities emitted (Loughrin et al., 1990, 1992). Therefore, for these species, the plant has already invested the resources for the production of these metabolites, and this resource investment is not likely to be reduced by decreasing emission after pollination. We propose that the cost–benefit framework that forms the basis of the optimal defense and apparency theories of plant chemical

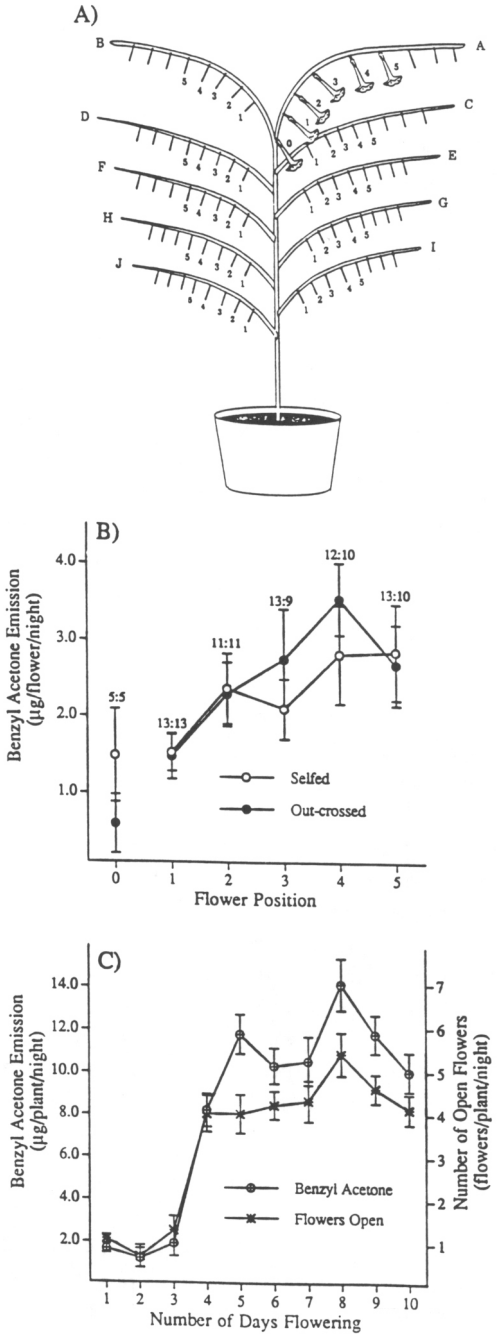
defense be applied to the volatile emissions that function in pollinator attraction. We test this framework by examining the fitness consequences of altering the pattern and magnitude of floral volatile emissions of plants growing in the field.

The flowers of *Nicotiana attenuata* emit greater quantities of volatiles at night. A single compound benzyl acetone (4-phenyl-2-butanone: BA) accounts for most of this change. Both the location and timing of BA emissions suggest a synomonal role in attracting pollinators: BA is released from the outer lip of the corolla; emissions increase dramatically shortly after dark, when moths are most active, and decrease after pollination (Euler and Baldwin, 1996). This species has many morphological characteristics in common with other hawkmoth-pollinated species (Knudsen and Tollsten, 1993), and BA is structurally similar to amyl salicylate and amyl benzoate—compounds reported to attract tobacco hornworm adults, *Manduca sexta* (Morgan and Lyon, 1928). *N. attenuata* can be found in recently disturbed or burned areas in the southwestern United States. Vegetative parts are attacked by mammalian browsers and hawkmoth (*Manduca* spp.) larvae, and the reproductive parts are commonly attacked by negro bugs (*Cormelina* spp.) that feed on the maturing seeds (Wells, 1959; Baldwin and Ohnmeiss, 1993). The flowers are self-compatible and are visited infrequently by hummingbirds, hawkmoths, and bees (Wells, 1959; Baldwin and Ohnmeiss, 1993; I. T. Baldwin, G. Y. Lynds and C. Preston, unpublished data).

To determine the consequences of successful pollinator attraction—evaluated by means of cross-pollination—on the rate of BA emission from subsequently produced flowers, we quantified the nightly BA emissions from individual flowers at six branch positions on plants whose flowers were hand-pollinated with pollen either from itself or from a plant of another genotype. With these measures of emission, we calculated whole-plant BA emissions and constructed a device that released BA at a constant rate, equivalent to that of a plant with 240 open flowers (approximately 10× more flowers than the average found on plants growing in 1-year-old burns). By placing this device on plants growing in native populations in Utah, we altered the pattern and magnitude of BA emission from plants and measured its effects on lifetime viable seed production. To help interpret the findings, we estimated the consequences of altered synomonal and kairomonal function on seed production by hand- and cross-pollinated plants and infesting plants with negro bugs in two glasshouse experiments.

METHODS AND MATERIALS

Reproductive Biology of N. attenuata. As this species matures, it produces a central racemose or narrowly paniculate indeterminate inflorescence with self-compatible perfect flowers. The flowers take three days to fully open their white,


A)
B
A
D
C
F
E
H
G
J
I
B)
Benzyl Acetone Emission (µg/flower/night)
5:5
13:13
11:11
13:9
12:10
13:10
Selfed
Out-crossed
Flower Position
C)
Benzyl Acetone Emission (µg/plant/night)
Number of Open Flowers (flowers/plant/night)
Benzyl Acetone
Flowers Open
Number of Days Flowering

tubular corollas; the corollas remain open for two to three nights, occasionally closing during the day. Flowers are produced sequentially on branches, and we depict panical development in Figure 1A. The fruit, a capsule, matures and dehisces 10–300 small (120–160 μg) seeds approximately 16 days after pollination. The seeds are largely dormant until they are exposed to aqueous extracts of wood smoke, a requirement that allows this species to synchronize its germination with the postfire environment (Baldwin et al., 1994; Baldwin and Morse, 1994).

Glasshouse Experiments: I. Plant Growth. Plants were grown from seeds collected in 1991 from a single plant at the DI Ranch (section T40S R19W: DI genotype) and from the Goldstrike Mine (section T39S R18W: GM genotype) area in southwestern Utah and germinated in flats of Cornell mix A (Boodley and Sheldrake, 1977). The two genotypes had slight morphological distinctions: the leaves and flowers of GM plants were approximately 5% larger than those of DI plants, and GM plants had a faint purple tint to their white flowers. After three weeks, 60 seedlings from the DI lineage and 10 from the GM lineage were transplanted into individual 4.0-liter pots and maintained in a glasshouse with supplemental lighting from 1500-W quartz halogen lamps for 16 hr/day (5 AM–9 PM), providing a minimum PAR of 320 μmol photons/m^2/sec. Twenty plants of the DI genotype were each used for the floral volatiles and negro bug infestation experiments, and five were used for the pollination experiment.

FIG. 1. (A) Diagram depicting the flower labeling scheme for *N. attenuata* plants. The first flower produced on each plant (which is formed before any branches) was labeled flower 0. Branches and flowers are produced sequentially from A to J and 1 to 5, respectively. The first flower to develop on each branch was designated as flower 1. Flowers 1–5 on branches A–C from all plants were individually enclosed in an open-ended trap for the collection of volatiles emitted during their first night open. (B) Effect of self- and cross-pollination on nightly benzyl acetone emissions from individual flowers at six flower positions on branches A–C of five plants in each pollination treatment. Plants had all flowers either hand-pollinated with their own pollen (selfed) or with pollen from another genotype (out-crossed). Values are mean ($\pm$SEM) quantities (μg/flower) of benzyl acetone emitted from intact flowers from 8 PM to 8 AM in headspace collections. The values above each symbol are the number of headspace samples from flowers that were to be pollinated either with their own pollen or with pollen from one GM plant, respectively. No differences were found between branches or pollination treatments; significant differences were found among flower positions. See Table 1 for statistical analysis of data. (C) Mean ($\pm$SEM) number of flowers open from the start of flowering for 10 days on each of 10 plants used in the experiment (right-hand axis) and the mean ($\pm$SEM) estimate of benzyl acetone emitted per plant per night (μg) for the first 10 days of flowering (left-hand axis). Since emission per flower and the number of flowers open per night were not affected by pollination treatment, values were averaged across pollination treatments.

Glasshouse Experiments: II. Effect of Flower Position and Pollen Source on BA Emissions. After panicle elongation and 5–10 days before the first flower was produced (at position 0: Figure 1A), 10 DI plants were paired by panical height and one member of each pair was randomly assigned to one of the following two treatments: (1) flowers hand-pollinated with pollen from one GM plant (out-crossed); and (2) flowers hand-pollinated with pollen collected from the same plant (selfed). The pollination technique is described below. Before hand pollination, volatiles from intact individual flowers were collected from the first five to seven consecutively opened flowers on branches A–C (Figure 1A) on all 10 plants. Flowers typically opened their corolla for the first time by 4 PM, and volatiles were collected from 8:00 PM to 8:00 AM the following morning. Flowers were then hand-pollinated with either their own pollen or pollen from a GM plant. Whole-plant emissions were calculated from the number of flowers open at a particular branch position on a night and the average nightly emission for a flower at that particular position.

Glasshouse Experiments: III. Effect of Pollen Source on Seed Production. To estimate the effect of out-crossing on seed production and viability, every flower produced on the first 10 branches of five plants of the DI genotype were alternately pollinated on their first night open (by 8 PM) with either their own pollen (selfed) or with pollen from one of five plants of the GM genotype (out-crossed). The choice of pollen for the first flower open on a branch alternated with each successively produced branch. Plants were inspected daily, and all mature flowers—expected to open for the first time that evening—were labeled with a code identifying the plant, treatment, branch, flower position, and date pollinated. No precautions were taken to prevent flowers from pollinating themselves; hence, it is possible that flowers in the GM-pollinated treatment received some of their own pollen. Plants in the self-pollinated group, however, were pollinated only with their own pollen. Plants were carefully bottom watered, and the glasshouse was kept free of insects to prevent unintentional pollen movement. In separate experiments, emasculated flowers never set seed unless they were hand-pollinated (Euler and Baldwin, unpublished data). While it is possible to emasculate flowers before the stigma becomes receptive, the damage caused to the corolla required for this manipulation increased the probability of fruit abortion.

Pollination was accomplished by removing pollen from two dehiscent anthers of the pollen-donor flower and applying it to the stigmatic surface with a glass pipet tip filled with glass-wool. A new pipet tip was used for each pollination. This procedure was estimated (from a microscopic examination of stigmas that were hand-pollinated with either self pollen or GM pollen) to deposit 1700 ± 200 ($N = 3$ for each pollen source) pollen grains on the stigma or more than five pollen grains per ovule for each flower. This procedure was repeated until the first five to seven consecutively opened flowers on each of the first

10 branches had been pollinated. In a preliminary experiment to determine if seed set or the mass of seed per capsule was influenced by the quantity of pollen reaching the stigmatic surface, we labeled 10 pairs of flowers (two on each of 5 DI plants), hand-pollinated one member of each pair with self pollen, and left the other member unmanipulated. No differences were found in either seed number ($t_9 = 0.003$) or seed mass per capsule ($t_9 = 1.211$) between pairs. We conclude that under our experimental conditions seed set and seed mass are not limited by the quantity of pollen reaching the stigma.

Dates of seed dehiscence or fruit abortion were recorded, and all seeds were individually collected in microcentrifuge tubes when each capsule opened. Seeds were air dried at 27–29°C and all seeds from each capsule were weighed to the nearest microgram on a Mettler MT5 microbalance. Ten seeds were haphazardly selected from each capsule and weighed to the nearest microgram to determine an average mass per seed. The number of seeds per capsule was calculated from the total seed mass per capsule and the average mass per seed data from each capsule. Seed viability was determined from a pooled sample of self- and cross-pollinated capsules from all six flower positions across the 10 branches from each plant.

Glasshouse Experiments: IV. Effect of Negro Bug Infestation on Seed Production. Twenty flowering DI plants were paired by panicle height and placed in individual 1-m^3 cages covered with fine "no-see-um" netting. Twenty adult negro bugs [*Cormelina* spp. (Heteroptera: Thyreocoridae), voucher specimens deposited at the Buffalo Museum of Science, Buffalo, New York] were introduced into half of the cages. After the plants had finished flowering (65 days), a pooled seed sample from all mature capsules was collected from each plant, and the average mass per seed and viability were determined for each plant.

Manipulation of BA Emission from Field-Grown Plants. A device was designed to release BA at a rate of 60 μg/hr, a rate equivalent to the nightly release rate of a plant with 240 emitting flowers, which represents approximately a 10× increase in whole-plant emission for large flowering plants, which average 23 flowers open each night. We used crimped-top 2-ml GC-MS vials with Teflon inner liners containing 200–400 μl of BA (Sigma Chemical Co., St. Louis, Missouri; lot 60H3446), and changed the number of puncture holes and the length of wick (single strand embroidery floss) protruding from the vial to achieve the desired rate of emission (Figure 2). Rates of release were determined by successively weighing three replicate vials from each treatment. The material remaining in the vial at the end of the field experiment was analyzed by GC-MS; the BA had not appreciably degraded.

The field manipulation was conducted during the 1996 growing season in an area (section 6 of T43S R17W) in southwestern Utah, which had burned on July 6, 1995 (BLM fire number 256) and had not been chained or seeded after the fire. *N. attenuata* and *Frasera albomarginata* (Gentianaceae) were the most

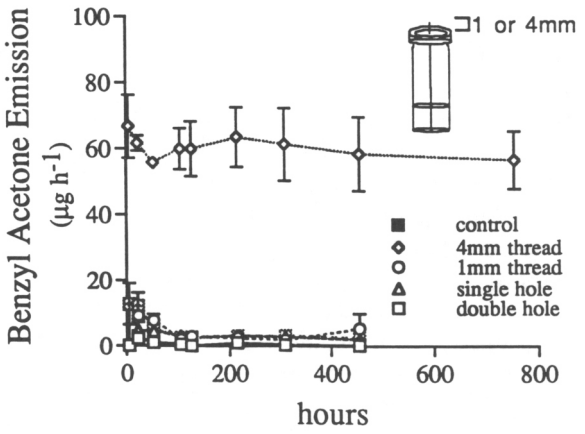


FIG. 2. Mean (±SEM) rate of benzyl acetone (BA) emitted from three sealed GC-MS vials containing 300 μl with the following manipulations to the septum: control, single hole, double hole, 1- or 4-mm wick protruding from the septum. The vials with the 4-mm wick were used to increase BA emissions from plants growing in the field by 240 open flower equivalents.

abundant plants growing among the burned juniper stumps. Fifty pairs of the largest *N. attenuata* plants were selected for the experiment on May 24 and 26, 1996. All plants had floral stalks at least 10 cm tall but had yet to produce their first open flower. All pairs had flowered by June 7. Selected plants grew no closer than 5 m to each other and had basal rosette diameters and stalk heights within 1 cm of each other. The BA-releasing device was positioned at the height of the developing panicle by attaching it with a twist-tie to a white wire surveyor's flag; control plants had the white marker without the BA-releasing device. Plants were followed until all had completed reproductive growth. At the end of the growing season, mature capsules were counted, mature seeds were collected, and mass per seed and viability were determined. The same type of data was collected from 12 other plants that had produced their first flowers a week later on June 21 and had noticeably fewer negro bugs attacking them. The percentage of each plant's leaf area removed by browsing mammals and orthopteran nymphs and adults (mostly *Trimerotropis pallidipennis*) and the number of hornworm larvae (*Manduca quinquemaculata* and *M. sexta*) on each plant were determined twice during the growing season. *Cormelina* bugs were censused, but their vagility and the lack of consistency between consecutive counts rendered the value of these counts doubtful.

Quantification of BA Emissions. Volatiles from individual flowers in the greenhouse were collected with a trap optimized for the quantification of BA emissions. Volatiles were trapped on 12.7 ± 0.2 mg of 100-mesh activated

charcoal secured in borosilicate glass Pasteur pipets with fine-pore polyester packing foam. Prior to collecting volatiles, 421 ± 4 ng of 1-phenyl-2-butanone was added to each trap under vacuum as an internal standard. 1-Phenyl-2-butanone, which is structurally similar to BA (4-phenyl-2-butanone), was not detected in headspace collections from *N. attenuata* (Euler and Baldwin, 1996). Volatiles were collected by enclosing each flower in a 30-ml open-ended test tube to which the trap was secured. Air was then drawn over the flower and through the trap with a vacuum pump at 928 ± 36 ml/min. The vacuum was produced by 2 Gast pumps (model DAA-V114-GB, Gast Mfg., Benton Harbor, Michigan) connected to a 15-port vacuum manifold, which allowed volatiles to be collected simultaneously from 14 individual flowers. One trap, the control, was used to collect ambient air from the glasshouse. This background sample was used to correct for the ambient air contribution to the volatile emission of each flower. Following collection, charcoal traps were stored at 5°C until extraction; traps were not reused. Volatiles were extracted from the activated charcoal by immersing the traps in 1 ml of CH_2Cl_2 and sonicating for 15 min. The eluent was transferred to a 2-ml crimp top vial and the samples stored at 5°C until analysis.

One microliter of each CH_2Cl_2 sample was injected into a 250°C injection port with an automatic sampler (Hewlett-Packard 7673, Kennett Square, Pennsylvania) with a 0.5-min splitless injection; volatiles were separated on a 25-m × 0.2-mm HP-1 column maintained at 50°C for 5 min and subsequently heated at 15°C/min to 200°C, with a continuous He flow of 0.6 ml/min, as determined by an electronic pressure control unit on the gas chromatograph (Hewlett-Packard 5890 series II). Compounds were detected as they eluted from the column with a mass spectrometer (Hewlett-Packard 5971) operated in the selective ion monitoring (SIMS) acquisition mode using 148 *m/z* as a target ion for both 1-phenyl-2-butanone and BA (4-phenyl-2-butanone), which had retention times of 10.4 and 10.6 min, respectively. A set of standard curve samples was prepared and analyzed with every 25 samples to determine relative response factors (RRFs) and to verify retention times. BA standard curves were prepared by adding 11.97 ± 0.11 mg of internal standard solutions containing 4.56 to 452.27 μg/ml BA to fresh charcoal traps. These spiked traps were processed and analyzed with the headspace samples. The coefficient of variability (CV) for the RRFs was 3.5%.

Seed Viability. We used a seed germination protocol that provided optimum conditions for germinating all viable seeds in 7–10 days (Baldwin et al., 1994; Baldwin and Morse, 1994). For each germination trial, three replicates of 20 seeds each were soaked for 1 hr in a 1:300 dilution (v/v) of Liquid Smoke (House of Herbs, Inc., Passaic, New Jersey) in 1 mM KNO_3 and transferred to soufflé cups (Solo, 1 oz., P100) containing approximately 5 g sterile sand. The sand was saturated with 1 mM KNO_3 so that seeds just touched the water layer.

Cups were sealed with transparent lids (Solo PL1 lids) and placed in a growth chamber (Percival, Boone, Iowa; model E-54U), which provided a 14L:10D photoperiod with 200 μmol photon/m^2/sec PAR and 32°C day:27°C night temperature cycle. Seeds were examined for germination (splitting of the seed coat and the emergence of the radicle) for up to 20 days from the initial transfer to the cups. Previous experiments had demonstrated that seeds not germinating within 20 days of being exposed to smoke extracts would not germinate over the next three months, during which time they tended to decompose.

Statistical Analysis. All data analysis was performed with the MGLH module of the SYSTAT statistical package (Evanston, Illinois).

RESULTS

Effect of Pollen Source on BA Emission. The nightly emissions of BA from individual flowers from all position 0 flowers (10 total) and of all flowers occupying positions 1–6 on branches B and C of all 10 plants were collected. On branch A, however, emissions were not collected from all flowers in positions 1–5 on all 10 plants. The number of collections for self- and cross-pollinated plants (of 15 potential collections per flower position), respectively, are given above each value in Figure 1B. We analyzed the complete data set from branches B and C with a three-way ANOVA and found no significant branch or pollination effects; but we did find a significant flower position effect (Table 1). Since branches did not differ, we collapsed the analysis into a two-way ANOVA by removing branch as a factor and included the data from branch A (Table 1; Figure 1B). Again, pollen source was not found to have any effect, but flower position remained highly significant. Flowers in position 4 on a branch emit approximately 2.1 μg of BA per night more than flowers in position 1, which represents a 1.4-fold increase in nightly BA emissions. We conclude that the consequences of cross-pollination and thus successfully attracting pollinators did not influence the quantity of BA emitted from subsequently produced flowers and that these plants have a fixed pattern of BA emission.

Since we recorded the number of flowers open each night on each plant and their location on the branch, and we know the amount of BA emitted from each flower at each position, we calculated nightly whole-plant BA emissions for the first 10 days of flowering for all 10 plants (Figure 1C). Since emission per flower and the number of flowers open per night were not affected by the pollination treatment, values were averaged across pollination treatments. Whole-plant BA emissions closely paralleled the number of flowers open for the first four days of flowering. However, on day 5 the whole-plant emissions were 50% higher on a per flower basis than during the previous four days. Plants retained this higher whole-plant emission rate through day 10 as a result of the higher per flower emission rate from flowers located at higher branch positions.

TABLE 1. DATA ANALYSIS OF BA EMISSION[a]

	df	MS (10^5)	*F*	*P*
I. Benzyl acetone emission (μg/flower/night)				
A. Branches B–C				
Branch (B)	1	2.93	0.10	0.751
Flower position (F)	6	82.15	2.84	0.014
Pollination treatment (P)	1	0.24	0.01	0.928
B*F	6	45.28	1.57	0.165
B*P	1	35.65	1.23	0.270
F*P	6	20.25	0.70	0.650
B*F*P	6	53.27	1.84	0.099
Error	101	28.93		
B. Branches A–C				
Pollination treatment (P)	1	112.01	4.37	0.001
Flower position (F)	5	0.23	0.01	0.925
P*F	5	14.39	0.56	0.729
Error	115	25.60		
II. Average Seed Mass (μg seed $^{-1}$)				
Branch (B)	9	208.03	1.70	0.094
Flower Position (F)	4	380.12	3.11	0.017
Pollination treatment (P)	1	2.06	0.02	0.897
B*F	36	159.99	1.31	0.137
B*P	9	262.76	2.15	0.029
F*P	4	166.58	1.36	0.250
B*F*P	36	204.41	1.67	0.018
Error	143			

[a] Degrees of freedom (*df*), mean squares (MS), *F* statistics, and *P* values of a three-way ANOVA on benzyl acetone emission from individual flowers at seven flower positions from branches B–C on all plants; two-way ANOVA on benzyl acetone emission from individual flowers (depicted in Figure 1) from the first five flowers on branches A–C; and a three-way ANOVA on the average seed mass (depicted in Figure 3) produced from the first five flowers from branches A–J on all plants.

Effect of Pollen Source on Seed Mass per Capsule, Average Mass per Seed, and Seed Viability. Since each plant had only one position 0 flower and all other flower positions were represented 10 times on each plant—one from each of the 10 branches (Figure 1A)—the seed mass data from capsules matured at position 0 were analyzed separately. All five position 0 flowers matured and produced seed. Neither the mass of seed per capsule nor the mass per seed at position 0 differed as a function of pollen source ($F_{1,4}$s = 0.701 and 0.561, respectively; Ps > 0.49; Figure 3). The probability of capsule maturation from positions 1–5 was also very high: of the 250 hand-pollinated flowers, 243 matured seed.

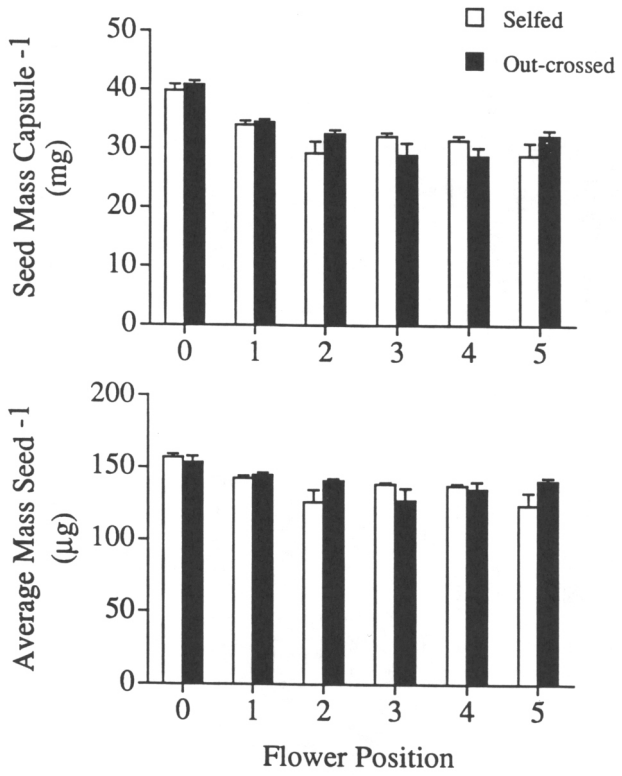


FIG. 3. Mean (±SEM) seed mass per capsule (mg) and average mass per seed (µg) (determined from the average mass of 10 seeds from each capsule) from self- and out-crossed flowers at positions 0–5 on branches A–J (see Figure 1) from five plants. Flowers sequentially produced on a branch were alternately hand-pollinated with either their own pollen (selfed) or with pollen from another genotype (out-crossed). For flower positions 1–5, each value is the mean of 23–25 capsules. Since there is only one position 0 flower on each plant, values at this position are the mean of five values. See Table 1 for statistical analysis of data.

Three-way ANOVAs revealed significant flower position effects on the average mass per seed and a significant branch × pollination treatment interaction (Table 1, Figure 3), but no significant effects were found for the number of seeds or the seed mass per capsule (all Ps > 0.30). For both measures, values declined from capsules matured at position 0 to those matured at positions 1–4 (by 10 µg/seed and 7 mg seed mass/capsule). If one estimates the number of seeds produced per capsule, by dividing the seed mass per capsule values by the average age mass per seed values, one finds that it declines significantly from lower to

higher branch positions. Clearly these declines across flower positions were much larger than any effects related to pollen source. Since the number of flowers pollinated each day reflects the number open on each plant each day, we analyzed these data to determine if pollen source affected the phenology of flowering. A one-way ANOVA on the number of flowers open from the start of flowering for each plant on each day revealed no effect of pollen source ($F_{1,240} = 0.034$).

Seeds were pooled by flower position and pollination treatment across branches and tested for viability. A two-way ANOVA on arcsine-transformed percentage viability revealed neither significant pollen source ($F_{1,53} = 0.23$) nor flower position ($F_{5,53} = 0.12$) effects. All seeds had viabilities approaching 100%.

Effect of Negro Bug Infestation. Infestation significantly ($F_{1,18}$s > 12; Ps < 0.001) reduced both the average mass per seed (from 147 $\pm$ 7 to 83 $\pm$ 9 μg/seed) and seed viability (from 98 $\pm$ 3% to 27 $\pm$ 3%).

Field Experiment with Enhanced BA. Of the 50 initial pairs of plants, 40 survived with the treatments intact to produce seed; 10 pairs had one or both members damaged by cows that rampaged through the site on June 1, or lost the BA device at some time during the growing season. Thirty-five (of the 80 plants that survived to produce seed) were browsed by rabbits and lost more than 20% of their leaf area after the BA-releasing device was attached; 26 of these plants included both members of a treatment pair, 7 were the BA-enhanced member of a pair, and 2 were the control member of the pair. One to seven eggs of *Manduca* sp. (Lepidoptera: Sphingidae) were found on 12 plants (seven BA-enhanced, five control plants), but these were parasitized before hatching. BA-enhanced plants produced significantly ($t_{39} = 2.119$; $P = 0.0405$) fewer capsules per plant than their matched controls (Figure 4; on average 5.6 fewer capsules, which represents a 3.1% reduction in lifetime seed production). The seed produced by these two groups of plants did not differ in either average seed mass or viability (t_{39}s < 0.4; Ps > 0.68; Figure 4); however, these values were only 47% and 23% of the mass per seed and viability, respectively, of smaller unmanipulated plants which flowered two weeks later in the growing season (Figure 4).

DISCUSSION

This study is the first to analyze the within-plant patterns of floral volatile emission and relate these to the within-plant patterns of seed production. Superimposed upon the diurnal rhythm of emissions is a fixed spatial pattern whereby the more recently produced flowers of each branch—those located at higher branch positions—consistently emitted more BA than the earlier-produced flow-

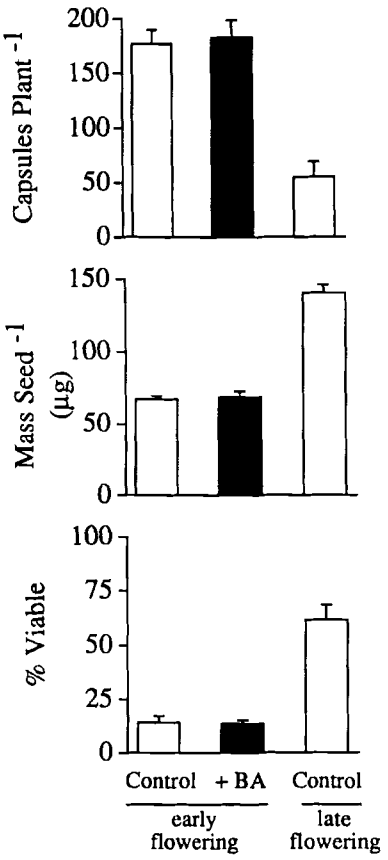


FIG. 4. Mean (±SEM) capsules per plant, mass per seed, and percentage of viable seed of native *N. attenuata* plants that were either the first to flower (early flowering) or flowered two weeks later (late flowering). One hundred early-flowering plants were paired by size and location and one member of each pair had a BA-releasing device (see Figure 2) attached to a white surveyor's flag at the height of the developing panicle, which increased the whole-plant emission of benzyl acetone by 60 µg/hr, a rate equivalent to 240 open flowers.

ers at lower branch positions (Figure 1). The patterns of seed production are the inverse of those in BA emission; flowers at higher branch positions matured fewer lighter seed than those at lower branch positions. That is, across positions on a branch, BA emission from flowers and the mass and number of seeds produced by these flowers are inversely correlated. Since reproduction meristems at higher branch positions are farther from the vegetative tissues that supply the resource requirements for seed filling and volatile emission, these results suggest that resource supply has opposing effects on these processes. Flowers at higher branch positions may function more to increase the plant's volatile plume and increase the plant's apparency to pollinators, and perhaps increase a plant's fitness through the male function by more efficiently attracting pollinators to carry pollen to other flowers.

This study also represents the first attempt to experimentally compare puta-

tive allomonal and synomonal functions of the emission of a floral volatile to understand the selective factors responsible for maintaining the pattern of emission. While the diurnal and spatial patterns of BA emissions suggest an important synomonal role in pollinator attraction, the fact that this pattern is unaffected by the consequences of successfully attracting pollinators—namely, out-crossing—suggests otherwise. Hand pollination of flowers with the pollen from another, morphologically distinct genotype did not alter the quantity of BA emitted from subsequently produced flowers (Figure 1). Moreover, since pollen source was not found to affect the timing or number of flowers produced, the time to seed maturation, or any of the seed production or viability measures (Figure 3), we were unable to detect any negative fitness consequences of inbreeding. While we have maintained this self-compatible species in the greenhouse for more than 12 generations of selfing without any apparent loss of fitness (I. T. Baldwin, unpublished results), these results may not adequately measure the potential fitness consequences of inbreeding and thereby underestimate the importance of the synomonal role played by BA for at least two reasons. First, our measures of offspring fitness (seed viability, mass) may not be sufficiently sensitive to detect ecologically relevant differences between selfed and out-crossed offspring. These seeds are thought to remain dormant in the seed bank for the intervals between fires in juniper forests (I. T. Baldwin, unpublished results)—which can be more than a century. Our measures of seed viability are unlikely to reflect differences in offspring quality important for survival during these intervals. Second, our experiments used only a single pollen source, in addition to self-pollen, and the results may have been different had mixtures of several genotypes been used. If pollinators deliver a diversity of pollen genotypes to the stigmatic surface, seed set or quality could increase as a result of increased gametophytic competition (Stephenson and Windsor, 1986) or increased opportunities for selective seed abortion (Wilson and Burley, 1983).

In contrast to the lack of significant correlations between fitness and putative synomonal function of BA emission, we found small (3.1%) but significant reductions in lifetime seed production in plants with experimentally enhanced BA emissions under field conditions. The reduction in capsule number was associated with a higher frequency of browsing, suggesting that the higher emission rate might function kairomonally to attract mammalian browsers. However, the large, early flowering plants used in this experiment were heavily infested by negro bugs, and both treatment and control plants produced light seeds with low viabilities that were only 47% and 23% of the mass per seed and viability, respectively, of unmanipulated plants which flowered two weeks later. Hence the large phenological variation in attack rate by negro bugs likely obliterated our ability to estimate the potentially large negative fitness consequences associated with the kairomonal function of BA emission. Hence, the dirunal patterns of scent production in *N. attenuata* may not only inform pollinators of the reward

status of the flower, but also help minimize the ecological costs of advertising for pollinators that result from increased apparency to herbivores. Clearly, several years of additional field experiments in several natural populations are needed to establish the importance of this kairomonal role. These results underscore the importance of the ecological milieu in determining the function of secondary metabolites and the importance of manipulative experiments to the evaluation of putative functional roles.

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A METHOD TO QUANTIFY THE ALLELOPATHIC COMPOUND BATATASIN-III IN EXTRACTS FROM *Empetrum hermaphroditum* USING GAS CHROMATOGRAPHY: APPLIED ON EXTRACTS FROM LEAVES OF DIFFERENT AGES

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Abstract—A gas chromatographic method was developed to quantify the allelopathic compound, batatasin-III, in organic and water extracts of leaves from the boreal dwarf shrub *Empetrum hermaphroditum*. The method provides a rapid and inexpensive quantification of small masses of batatasin-III in complex matrices. The compound was effectively separated from interfering compounds using solid-phase extraction techniques and chromatographed as a TMS-derivate on a nonpolar column. Batatasin-III was quantified in leaves and found to vary with the age of the shoot. The concentration of batatasin-III was highest in first- and second-year shoots, while third-year shoots contained about half that of younger shoots. Senescent leaves on older shoots still contained a considerable amount of batatasin-III. The method described provides an important tool for the demonstration of allelopathy.

Key Words—Allelopathy, batatasin-III, quantification, gas chromatographic method, SPE techniques, *Empetrum hermaphroditum*.

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INTRODUCTION

Allelopathy, which includes all types of chemical interactions among plants and microorganisms, is an interdisciplinary field where ecologists, physiologists, and chemists have to meet in order to achieve an understanding of the phenomenon (Rice, 1984). In the northern boreal forest, sites dominated by the dwarf shrub *Empetrum hermaphroditum* Hagerup. are usually associated with greater humus build-up, diminished tree productivity, and failure of regeneration after cutting (Sirén, 1955; Nilsson, 1992). Experiments conducted both in the field and greenhouse have shown that *E. hermaphroditum* exude leaf compounds that negatively affect seed germination, early plant establishment, growth, pine mycorrhizal symbiosis, and survival of both herbs and trees (Nilsson and Zackrisson, 1992; Zackrisson and Nilsson, 1992; Nilsson et al., 1993; Nilsson, 1994). Batatasin-III (3,3′-dihydroxy-5-methoxydihydrostilbene) has been identified as the compound that, at least in part, is responsible for the biological activity exhibited by *E. hermaphroditum* (Oden et al., 1992).

It has been postulated that in order to demonstrate allelopathy (the allelopathic significance of a plant), the first need is to distinguish allelopathic interference from competitive interference (Harper, 1975, 1977; Fuerst and Putnam, 1983; Williamson, 1990). Secondly, the toxin(s) should be isolated, characterized, and synthesized (or extracted), and, thirdly, the symptoms of allelopathy should be demonstrated by applying toxin(s) at the same concentrations and rates as those found under natural conditions in the absence of the allelopathic plants.

The aim of this work is to develop a method to quantify the total and water-soluble content of the putative allelopathic compound batatasin-III in *E. hermaphroditum* leaves. We know from previous work that the biological activity of *E. hermaphroditum* may change significantly during the season and that it differs among leaves of different ages (Zackrisson and Nilsson, 1992). The ultimate aim of the present work is to develop a standard analytical protocol for future physiological and field-based ecological research on the secondary metabolite produced by this plant.

METHODS AND MATERIALS

Chemicals and Glassware. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1,6-dihydroxynaphthalene (99% purity) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK) and pyridine (99.8% purity) from Fluka (Darmstadt, Germany). Batatasin-III was synthesized according to Hashimoto et al. (1974), at the Department of Organic Chemistry 2, Lund University, Sweden. Solvents were of HPLC grade (Fisons, Loughborough,

UK). All glassware was base washed and heated overnight at 400°C prior to use.

Plant Material. E. hermaphroditum plants with an intact upper mor humus soil profile, including the leached mineral soil (A_{00}–A_2), were collected on September 25, 1995, in 10 plastic trays (60 × 40 × 15 cm), from a sparsely regenerated forest site at Västra Månsträsk, northern Sweden (65°34′ N, 18°36′ E). The trays were kept outdoors under snow until they were brought to the greenhouse on December 2, 1995. The plants were grown at 22°C (day) and 15°C (night) under artificial illumination with a 20-hr photoperiod. On March 28, 1996, approximately five multibranched shoots from each tray were harvested.

To analyze the difference in batatasin-III content between shoots of different ages, each branch was physically divided into four age classes: (1) first-year shoots; (2) second-year shoots that had initiated in the spring of 1995; (3) third-year shoots initiated in the spring of 1994; and (4) brown senescent leaves still attached on the stem (i.e., older than three years). The first-year shoots were further separated according to the length of the shoots (< 10 mm long or > 10 mm long). The leaves were left to dry at 20°C for approximately two weeks, after which the leaves were removed from the branches and pooled according to age class. The leaves were stored frozen (−18°C) until analysis.

The extraction efficiency and the recovery of batatasin-III on solid-phase extraction (SPE) columns were evaluated using extracts of leaves from *E. hermaphroditum* collected in September 1993 at Rovågern outside Umeå (63°50′ N, 20°15′ E) and with leaves from *E. nigrum* L., a close relative to *E. hermaphroditum* that does not contain batatasin-III (Sterner et al., in preparation), collected in 1992 at Salmisjärvi (68°11′N, 21°50′E). The leaves were stored frozen (−18°C) prior to analysis.

Sample Treatment. Two batches (25 mg each) of leaves from each age class were extracted with 2 ml of ethyl acetate in Teflon-lined screw cap tubes and sonicated 2 × 10 min and left at 20°C for 24 hr. The solvent was transferred to another test tube and the leaf residues washed three times with 1.0 ml of ethyl acetate. The solvent was evaporated under nitrogen after which the extracts were lyophilized to remove possible water residues. The lyophilized samples were redissolved in 1200 μl of dry ethyl acetate; 125 μl was transferred to another test tube and mixed with 2375 μl of heptane, and 2000 μl of the solution was applied on an Isolute SPE SI column (100 mg/3 ml reservoir) (IST, Mid-Glamorgan, UK) previously conditioned with 2 ml of the same solvent composition. The impurities were absorbed on the silica while batatasin-III was eluted with 2 × 0.5 ml of ethyl acetate–acetic acid (98:2, v/v). The eluted fraction containing batatasin-III was lyophilized and then dissolved in 1000 μl of ethyl acetate.

Since we experienced problems with low repeatability (experiments not

shown here) when extracting small quantities of leaves with water, we chose to develop a method to enable quantification of batatasin-III in 5% water-extract, using the standard water extraction procedure developed by Zackrisson and Nilsson (1992). This meant extracting 50 g of dry leaves in 1000 ml of distilled water, continuously stirring at low revolution for 48 hr, and filtering through Munktell No. 3 filter paper. The water extracts (250 μl) were cleaned on Isolute SPE C-18 columns (100 mg/3 ml reservoir) (IST), previously activated with 2 ml of methanol and conditioned with 2 ml of water. More polar impurities were removed by rinsing the column with 3 ml of water and 2 ml of water–methanol (80:20, v/v). The column was dried by forcing air through the column before nonpolar impurities were removed with 3 ml of heptane. Batatasin-III was eluted with 2 × 0.5 ml of ethyl acetate–methanol–acetic acid (49.5:49.5:1, v/v/v). The fraction containing batatasin-III was lyophilized and redissolved in 1000 μl of methanol.

Subsamples of the cleaned ethyl acetate (20 μl) and water extracts (50 μl) were transferred to vial inserts (100 μl) inside vials equipped with rubber–Teflon screw caps and lyophilized before being derivatized at room temperature with 25 μl of BSTFA in 25 μl of pyridine containing 10 ng/μl of 1,6-dihydroxynaphthalene as an instrumental internal standard. The samples were injected within 24 hr after the derivatization. The concentration of batatasin-III calculated is the mean value of the duplicates.

Gas Chromatography. A Hewlett Packard 5890 GC equipped with a fused-silica DB-5 capillary column (30 m × 0.25 mm ID, film thickness 0.25 μm, J & W Scientific) was used for the quantification of batatasin-III. The column temperature was initially 150°C, with a linear increase of 7°C/min up to 290°C Hydrogen (99.998%) was used carried gas at a flow rate of 1.5 ml/min. Injections were made using a Hewlett Packard 6890 Series autosampler and the split was opened after 90 sec. The injection volume was 1 μl.

A standard calibration curve was drawn by plotting the area ratio between batatasin-III and 1,6-dihydroxynaphthalene for 14 different concentrations of batatasin-III (5–300 ng/μl). The correlation coefficient was determined to be 0.9994.

Gas Chromatography–Mass Spectrometry (GC-MS). A VG Trio-3 tandem quadropole mass spectrometer connected to a Hewlett Packard 5890 gas chromatograph was used to verify the presence of batatasin-III in the samples. Helium (99.995%) was used as a carrier gas at a flow rate of 1.0 ml/min. The injection mode, injector temperature, column, and temperature program were the same as for the GC analyses. Electron impact (EI) mass spectra were obtained using 70 eV ionizing energy and an ion source temperature of 250°C. The total ion current was recorded of a mass range of 50–450.

Extraction Efficiency. The extraction efficiency was evaluated and compared for three different extraction methods [Bligh and Dyer (1959), ethyl ace-

tate, and water extraction]. For each method, three batches of leaves (each 25 mg) were subsequently extracted three times. After each extraction, the yield of batatasin-III was analyzed by GC. The first method was modified after Bligh and Dyer (1959). The leaves were extracted with 2.0 ml of a single-phase methylene chloride–methanol–water (1:2:0.8, v/v/v) mixture in Teflon-lined screw cap tubes and sonicated 2 × 10 min and left at room temperature for 24 hr. Two phases were formed by adding 555 μl of methanol and 555 μl of methylene chloride, shaking vigorously, and centrifuging at 3000 rpm for 5 min. The organic phase containing batatasin-III was transferred to another test tube and the methanol–water phase was washed three times with 55 μl of methylene chloride before being removed. The second method involved extraction with 2.0 ml of ethyl acetate in Teflon-lined screw cap tubes. The leaves were sonicated 2 × 10 min and then left at room temperature for 24 hr. The solvent was transferred to another test tube and the left residues washed three times with 1.0 ml of ethyl acetate. In the third method, water was used instead of ethyl acetate. The difference in efficiency was tested statistically by a one-way ANOVA followed by a Tukey's test.

Recovery of Batatasin-III. The recovery of batatasin-III after cleaning the extracts on SPE columns was evaluated in two different ways. Extracts of *E. hermaphroditum* leaves were analyzed by GC before and after being cleaned on the columns, and the recoveries of batatasin-III were calculated in percentage ± SE. Three batches of leaves were extracted with ethyl acetate; each extract was cleaned on three different SI columns as described above. To evaluate how batatasin-III interfered with the matrix, leaves of *E. nigrum* were extracted as above and then spiked with synthesized batatasin-III to correspond to amounts found in extracts of leaves containing 10%, 5%, 1%, 0.5% and 0.05% of batatasin-III per leaf dry weight (LDW). The extracts were analyzed before and after being cleaned on SI columns (N = 3), and the recovery of batatasin-III was calculated. The recovery of water extracts after cleaning on C-18 columns was tested in the same manner, with 5% water extract of leaves from *E. hermaphroditum* and also with 5% water extract of leaves from *E. nigrum* spiked with synthesized batatasin-III to correspond to 1.0%, 0.5%, 0.1%, 0.05%, and 0.01% of batatasin-III per LDW.

RESULTS

Extraction Efficiency. The highest yield of batatasin-III in leaves was obtained when extracted with ethyl acetate, 0.032 ± 0.001 mg/mg LDW, of which 99.8% was obtained from the first extraction. The Bligh and Dyer method gave a significantly lower yield (P = 0.003), 0.025 ± 0.000 mg/mg LDW, of which 100% was obtained with the first extraction. The water extraction was

TABLE 1. RECOVERY OF BATATASIN-III IN LEAF EXTRACTS OF *Empetrum hermaphroditum* AND *E. Nigrum* AFTER CLEANING ON SPE COLUMNS

Plant material	Batatasin-III (% per leaf dry weight) in leaf extracts	Recovery of batatasin-III (%) from[a]	
		Ethyl acetate extracts cleaned on SI columns	Water extracts cleaned on C-18 columns
E. hermaphroditum	4	97 ± 1	94 ± 1
E. nigrum[b]	10	105 ± 3	ND
	5	103 ± 2	ND
	1	100 ± 2	86 ± 4
	0.5	99 ± 0	89 ± 1
	0.1	ND	84 ± 2
	0.05	100 ± 0	95 ± 1
	0.01	ND	83 ± 1

[a]The values represent mean ± SE, $N = 3$. ND = not determined.
[b]The leaf extracts of *E. nigrum* were spiked with different amounts of synthesized batatasin.

the least efficient. The total yield was 0.008 ± 0.001 mg/mg LDW, of which 16% was obtained with the first extraction, 36% with the second, and 48% with the last extraction.

Recovery. The cleaning of the ethyl acetate extracts of leaves from *E. hermaphroditum* on SI columns allowed a recovery of 97% while the recovery for the spiked extracts of *E. nigrum* varied between 99 and 105% (Table 1). Batatasin-III was detected in the extracts corresponding to 0.05% batatasin-III per LDW. However, an increased amount of batatasin-III needed to be injected onto the column to allow successful quantification. The cleaning of water extracts of leaves from *E. hermaphroditum* on C-18 columns allowed a recovery of 94% while the recovery for the spiked extracts of leaves from *E. nigrum* varied between 83 and 95% (Table 1). The integration of the peak corresponding to batatasin-III was complicated by the interference of a neighboring peak in the leaf extracts of *E. nigrum*.

Content of Batatasin-III in Leaves of Different Age Classes. The concentration of batatasin-III differed significantly[4] between the long and the short first-year shoots (Figure 1). The amount of batatasin-III in leaves was 0.062 mg/mg LDW in the long shoots, and 0.053 mg/mg LDW in the short shoots. However, there was no significant difference between the first-year long shoots and the second-year shoots (0.060 mg/mg LDW). The amount of batatasin-III was sig-

[4]Considering the variation within the method since pooled samples were used.

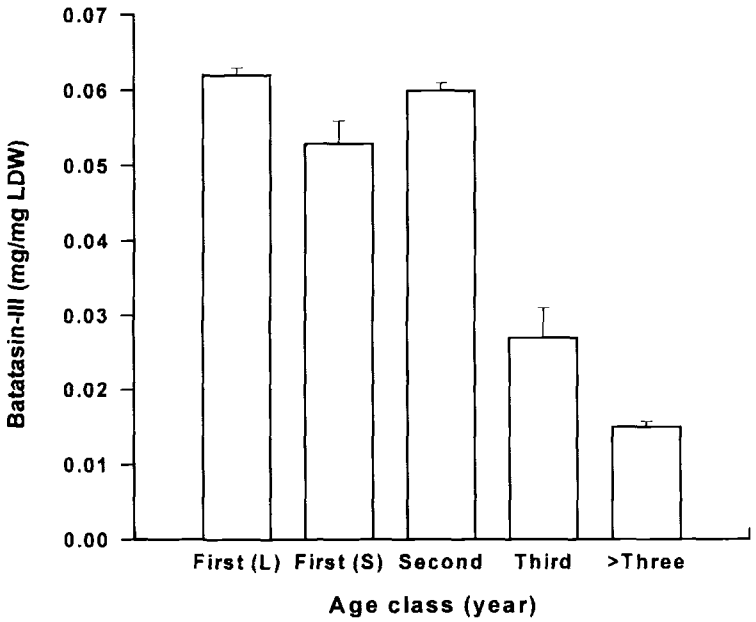


FIG. 1. The concentration of batatasin-III (mg/mg leaf dry weight) in leaves from *E. hermaphroditum*, sorted according to different age classes: first-year shoots (longer, L, or shorter, S, than 10 mm), second-year shoots, third-year shoots, and brown leaves still attached to the branches (i.e., older than three years). Error bars represent standard errors of mean ($N = 2$).

nificantly lower in the third-year shoots (0.027 mg/mg LDW), and in the brown leaves on the stem (0.014 mg/mg LDW). The 5% water-extract contained 0.138 mg/ml of batatasin-III. The retention time for batatasin-III was 13.5 min and for 1,6-dihydroxynaphthalene 7.9 min. The mass spectra confirmed the identity of the peak in the GC chromatograms (not shown here) used to quantify the amount of batatasin-III in the samples. The typical MS fragmentation pattern for silylated batatasin-III shows ions at *m/z* 388 (M^+), 209 (hydroxymethoxytropylium ion), and 179 (hydroxytropylium ion). The peak of *m/z* 73 represents $(CH_3)_3Si^+$.

DISCUSSION

The method developed to quantify the content of batatasin-III in leaves and also water extracts allows rapid and inexpensive quantification of small amounts of batatasin-III in complex matrices. Using SPE techniques, batatasin-III can be

effectively separated from interfering compounds in the matrix. The recovery was found to be above 97% for the ethyl acetate extracts. The lower values of recovery for the spiked water extracts of leaves from *E. nigrum* is most likely caused by an integration error since the peak corresponding to batatasin-III interfered with a neighboring peak in the matrix.

A method similar to ours has been used to quantify hydroxylated stilbenes in wine where six different stilbenols were extracted using C-18 extraction cartridges, the compounds silylated with BSTFA and quantified using GC-MS with the same kind of column and a similar temperature program (Lamikanra et al., 1996). Hydroxylated stilbenes have also been quantified using liquid chromatography (Kashiwada et al., 1989; Pezet et al., 1994; Sobolev et al., 1995). We have successfully isolated batatasin-III on a diol column using a HPLC system equipped with a light scattering detector (Wallstedt, 1994). However, we quantified batatasin-III using gas chromatography since the separation is faster (25 min between injections compared to 60 min) with a sensitivity in the nanogram rather than the microgram range. Our aim was to quantify batatasin-III extracted from as small a mass of leaves as possible without losing the repeatability; 25 mg of leaves (about 35–40 leaves) proved to be sufficient for the analysis. The samples were derivatized to increase the sensitivity and improve peak shape. Hydroxystilbenes have been successfully derivatized with methyl, acetyl, and trimethylsilyl (TMS) agents, of which TMS derivatives are the easiest to prepare (Gorham, 1989). We chose to silylate the samples with BSTFA in pyridine because the derivatization occurred instantaneously and quantitatively at room temperature. No peak corresponding to underivatized batatasin-III appeared. An excess of silylation reagent was used because it effectively consumed traces of water and helped to preserve the derivatives. The latter appeared to be stable; no change in peak area was observed when running derivatized samples previously stored in the refrigerator for more than two weeks. Derivatized batatasin-III showed sharper and less tailing peaks compared to underivatized batatasin-III, sensitivity increased approximately 30%, and derivatized batatasin-III could be detected down to 1 ng on the GC used.

Stilbenes have been reported to be unstable in light and sensitive to oxidation (Hart, 1981). Pezet et al. (1994) experienced denaturation of resveratrol and pterostilbene after concentration using rotary evaporation (in vacuo, 40°C, protected from light). Extracts of *E. hermaphroditum* have been found to withstand evaporation, long-term treatment with ultraviolet light, and high temperature without losing biological activity (Nilsson, 1992). During our work, we have seen no sign of oxidation or degradation of batatasin-III in extracts left in sunlight, heated, evaporated under nitrogen, concentrated using rotary evaporation or freeze-drying, or sonicated. Samples with batatasin-III have been stored for three years in a freezer (−18°C) without formation of degradation products.

Even though batatasin-III has quite hydrophobic properties, the compound is sufficiently water soluble to show biological activity in water extracts. The solubility of batatasin-III in distilled water is 0.35 mg/ml at 25°C (Olov Sterner, personal communication), but is markedly increased by a small addition of methanol. Water-soluble allelopathic compounds are considered to be washed off the leaf surface into the soil by rain, drizzle, and dew (Rice, 1984; Fischer et al., 1988, and 1989). Water extraction simulates this process and may allow a quantification of the allelopathic compounds released by the plant. The efficiency of different extraction methods partly depends on the properties of solvents to reach the inner tissue of the plant material during extraction. The lower yield of the water extraction is most likely because water fails to penetrate the waxy leaf surface and only washes the surface of the leaves, but when extracting with a solvent, like ethyl acetate or Bligh–Dyer solution, the internal tissues are more easily reached. Plant epidermal cells are not photosynthetic, except for stomata. Therefore if the extracts turn green with chlorophyll, it would indicate that internal tissues are being extracted (Waterman and Mole, 1994). Both the methylene chloride phase and the ethyl acetate extracts were bright green, indicating that internal tissues were being extracted. The extraction with ethyl acetate is to be preferred since it involves less work than the Bligh–Dyer extraction. However, results not presented here showed that extracting freeze-dried leaves with dry ethyl acetate lowered the yield of extracted batatasin-III. The yield was restored by saturating the ethyl acetate with water. Drying the leaves at room temperature seems to leave enough water in the leaves so as not to affect the yield regardless of the water content of the solvent. It is also preferable to dissolve the samples in dry ethyl acetate prior to purification on the SI columns, since we experienced problems with saturation of the silica with water when ethyl acetate, not stored over a drying agent, was used.

In agreement with our results, other studies have also found the concentration of allelochemicals to vary with the age of the plant organ (Rice, 1984). Batatasin-III has not been tested to see whether it has antiherbivore properties in *E. hermaphroditum*, but it is likely that the compound has a multipurpose function, both as allelopathic and antiherbivory agent, and it is known that the plant is rarely browsed by herbivores (Grime et al., 1988; Emanuelsson, 1984).

We observed that senescent leaves on the older shoots still contained a considerable amount of batatasin-III. This is compatible with results from earlier bioassays showing that senescent leaves exhibit inhibitory activity against seed germination of other species (Nilsson and Zackrisson, 1992). Senescent leaves remain attached to shoots for as long as 10 years before they fall off and form litter (Hagerup, 1922; Mork, 1946). It is tempting to speculate that the functional basis for this is to allow phytotoxic levels of batatasin-III to be released by rain to the soil surface while the leaves still are attached to the branches. A more

rapid decomposition of leaves and batatasin-III by soil microbes will occur when the leaves reach the soil surface. This process could be retarded to give the largest inhibitory effect on seed and seedling of other plants.

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EXAMINATION OF DIFFERENT TOBACCO (*Nicotiana* spp.) TYPES UNDER- AND OVERPRODUCING TOBACCO ANIONIC PEROXIDASE FOR THEIR LEAF RESISTANCE TO *Helicoverpa zea*[1]

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Abstract—First-instar larvae of the false tobacco budworm (corn earworm, *Helicoverpa zea*) that fed on either intact plants, leaf disks from undamaged plants, or leaf disks from insect-damaged plants of *Nicotiana sylvestris* and *N. tabacum* Coker plants overproducing a tobacco anionic peroxidase generally caused significantly less damage than those caged with corresponding material from wild-type plants. In some cases mortality was significantly higher and weights significantly less for caterpillars feeding on leaf material from overproducing vs. wild-type plants. First-instar *H. zea* fed on the same type of leaf material from *N. tabacum* Xanthi underproducing tobacco anionic peroxidase generally caused significantly more damage than those fed leaf material from wild-type plants. However, first-instar *H. zea* fed on underexpressing leaf material from *N. sylvestris* did not cause significantly greater damage compared to wild-type material. In cases where peroxidase enzyme activity was determined, significantly higher mean peroxidase activity was seen in leaves of plant types that also had significantly less mean feeding ratings. This information suggests that peroxidase activity can contribute to leaf resistance to chewing insects. However, the context of the peroxidase (cooccurring substrates, additional inducible factors) can mediate the degree of influence seen by changes in individual peroxidase isozyme levels.

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[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Key Words—Plant resistance, *Helicoverpa*, false tobacco budworm, *Nicotiana*, peroxidase, lignin, transgenic plants, corn earworm.

INTRODUCTION

Proteins are increasingly assigned a role in resistance to insects (Shah et al., 1995). Most of these act as direct toxins, such as Bt crystal proteins and lectins (Gatehouse et al., 1993). Others may act indirectly, such as protease inhibitors (Gatehouse et al., 1993, 1994). More recently, enzymes that interfere with insect processes, such as cholesterol regulation (Purcell et al., 1993; Corbin et al., 1994) or chitin formation (Cohen, 1993), have been described. Enzymes involved in host-plant responses to insects have also been investigated, and include polyphenol oxidases, peroxidases, and lipoxygenases (for review see Appel, 1993).

The role of plant peroxidases in insect resistance is unclear. Material oxidized by peroxidases can be more toxic and/or less nutritious to insects (Felton et al., 1989, 1992; Dowd, 1994; Dowd and Norton, 1995). However oxidation products that are thought to be produced by peroxidases can be less toxic than their precursors (Peng and Miles, 1988). Due to the complexity of interacting factors, predicting the role of peroxidase in a particular matrix is likely to be difficult (Appel, 1993; Miles and Oertli, 1993; Dowd and Lagrimini, 1997a). The production of plants (over- or under-) producing tobacco anionic peroxidase (Rothstein and Lagrimini, 1989; Lagrimini, 1992; Lagrimini et al., 1990, 1992, 1993) allows for the more direct examination of the potential for peroxidase to promote resistance to insects. Earlier studies have demonstrated enhanced resistance of some excised plant tissues from tobacco or tomato (over)producing a tobacco anionic peroxidase to different insects compared to wild-type plants (Dowd and Lagrimini, 1997b). In some cases production of enhanced levels of foreign peroxidase in nonsource plant species has increased susceptibility to some insects (Dowd and Lagrimini, 1997c). Thus, a better understanding may be had if the level of a naturally occurring peroxidase isozyme were genetically altered in the source plant species. Earlier studies demonstrated resistance of excised mature stems of *Nicotiana sylvestris* overproducing tobacco anionic peroxidase to feeding by first instars of the false tobacco budworm (corn earworm, *Helicoverpa zea*) compared to wild-type plants, but leaves of both plant types were equally susceptible to third instars (the only size tested) (Dowd and Lagrimini, 1997b). When seed became available for *Nicotiana sylvestris* over- and underproducing tobacco anionic peroxidase, and for two varieties of *N. tabacum*, one overproducing and the other underproducing tobacco anionic peroxidase (Lagrimini, 1992), it became possible to examine the activity in different, but closely related plants. Whole plant and leaf disk studies using caged first instars of the false tobacco budworm (corn earworm), *Helicoverpa*

zea, were used to examine relative resistance of the different tobacco variants to this insect.

METHODS AND MATERIALS

Insects. H. zea were reared on a pinto bean-based diet at 27 ± 1°C, 40 ± 10% relative humidity, and a 14L : 10D photoperiod (Dowd, 1988). Newly hatched instars (<24 hr old) were used in all assays.

Plants. At least twice-selfed seed from single lines of the transgenic tobacco anionic peroxidase overproducer (Lagrimini et al., 1990) and underproducer (Lagrimini, 1992) *N. sylvestris*, overproducer *N. tabacum* Coker (Lagrimini et al., 1990), and underproducer *N. tabacum* Xanthi (Lagrimini, 1992) was used. Seeds were planted in a previously described soil mix (Dowd and Lagrimini, 1997b). Plants were transplanted to 4-in.-diameter pots when two fully expanded true leaves were present (ca. 7–10 days). Plants were grown in an growth chamber (Environmental Growth Chambers G-10) containing 110-W fluorescent lights (Phillips) and 40-W fan crystal light bulbs (General Electric). The tops of the pots were 50 cm from the lights of the growth chamber. Plants were grown at 27 ± 1°C, 40 ± 10% relative humidity, and a 14L : 10D photoperiod. After transplanting, whenever plants were watered, liquid fertilizer with chelated iron was used (Dowd and Lagrimini, 1997b).

Intact Plant Bioassays. Cages were made to contain the small larvae on intact plants. Cages were made out of Solo clear plastic polyethylene cups with a 9-cm mouth diameter (10 oz.). The rolled lip of the cups was removed to facilitate pushing into the soil mix. For ventilation, ca. 2-cm squares were cut on opposite sides of the cup with the closest edge ca. 2 cm from the bottom of the cup. The holes were covered with organdy cloth secured with hot-melt glue. Plants were used for assays when they reached a size such that the largest leaf was ca. 3–5 cm. long (blade portion), which was approximately 20 days after germination. First-instars were added to the plant, the cup was inverted over the plant, and the top of the cup was pressed ca. 1 cm into the soil. Initially only two larvae per plant were used (with the first series of *N. sylvestris*). This number of larvae produced only limited damage, so 10 larvae per plant were used in subsequent assays. Plants remained in the growth chamber for one week and were then removed. The length of each leaf of a plant was measured, and the damage (in square millimeters) to each leaf was estimated. Initially the first four true leaves were not considered due to infrequent damage (with the first series of *N. sylvestris*), but with the 10 larvae per plant used in subsequent assays, these leaves were often damaged and so were also included. Larvae present were removed, counted, and weighed. Plants were returned to the growth chamber for recovery so that individual leaves could be used in subsequent leaf

disk assays (see below). As many plants as possible that were of an equivalent size of the different types being examined at a particular time were used in assays, and the number ranged from 13 to 22 plants of a particular type. All plant types of a particular cultivar were examined at the same time.

Leaf Disk Bioassays. For assays with disks from undamaged plants, leaf disks were removed from the newest leaf with a ca. 3 to 4-cm-long leaf blade. This size was reached approximately seven days after the leaf was initiated. In the first series of studies with *N. sylvestris*, both the younger leaves and leaves that were mature (second to third leaf from the bottom, approximately 14 days after the leaf initiation) were used. The maturity of the leaf influenced the amount of damage (see Results). Because age was initially determined to influence relative susceptibility of the leaf to caterpillar damage, it was important to select leaves of approximately the same maturity for assays. The younger leaves continued to be used due to their greater susceptibility to feeding. A ca. 2-cm disk was cut from the center of the leaf and used in bioassays. The disk was placed in a small Petri dish with a tight-fitting lid (Falcon 1006) along with 10 newly hatched *H. zea* larvae. The disk assay chambers were held under the same conditions used to rear the larvae except they were kept in the dark. Preliminary observations indicated cannibalism by the *H. zea* larvae was much lower if the leaves were kept in the dark. Assay chambers were examined for leaf feeding damage and caterpillar mortality after one and two days. Larvae were considered dead if they did not move when dishes were tapped. Mortalities were calculated as percentages of dead larvae of total larvae counted. Surviving larvae were weighed after two days using a Mettler AE163 analytical balance with a 10-μg accuracy. Damage to leaves was determined by two methods. When damage was generally less that 1/10 of the entire leaf for a particular group, a rating of the number of equivalent 0.25-mm^2 holes (approximating the head capsule size of the first instars) was used; 75 of these units were approximately equivalent to 1/10 of the leaf being consumed. When greater amounts of the leaf were consumed, a 1–10 scale was used. In cases where both degrees of damaged were seen in the same series, the conversion rate of 75 units = 1/10 of leaf consumed was used to convert values to those of the most prevalent rating unit. The number of leaves used depended on the number of plants available; typically 10–20 plants of a particular type were used. All assays with a particular plant cultivar were set up at the same time.

Insect feeding has been shown to differentially induce defensive agents, including peroxidases (e.g., Stout et al., 1994). Thus, it was of interest to test leaves from plants that had been damaged by caterpillars to see if the trend of resistance was the same as for leaves from plants that had not been damaged by the insects. The leaf used was the most terminal leaf from plants that had been damaged in the intact plant assays. After this leaf had expanded to usable

size (mean of ca. 5–8 cm of leaf blade length, depending on the cultivar) approximately seven days after initial damage, it was used in another series of leaf disk assays. Leaf tissue was removed, assays set up, and assays evaluated as described for the undamaged plant leaf disk assays.

Peroxidase Assays. Leaf disks 1 cm in diameter were removed from the same leaves of wild-type and underproducing *N. tabacum* Xanthi and wild-type and overproducing *N. tabacum* Coker as those used in leaf disk feeding assays prior to the start of the assay. They were assayed for peroxidase activity using guaiacol as a substrate by previously published procedures (Lagrimini et al., 1987).

Statistical Analyses. Mortality data was analyzed using chi-square analysis and leaf feeding ratings, weights, and enzyme activity were analyzed using analysis of variance (SAS Institute, 1985).

RESULTS

Differential Feeding Responses on Intact Plants. The total area of leaf consumed by caterpillars in intact plant assays was always less for plants overproducing peroxidase compared to wild-type plants and was significantly less for whole plants in some cases (Table 1). Intermediately aged leaves of the different plant types showed the greatest difference in feeding where obvious differences were present. For example, the sixth leaf from the bottom of the overproducing plants from the second series of *N. sylvestris* was damaged significantly less ($P < 0.05$) than the corresponding leaf from the wild-type plants. The fourth leaf from the bottom of the *N. tabacum* Coker overproducing plants was damaged significantly less ($P < 0.05$) than the corresponding leaf from the wild-type plants. More tissue was consumed by caterpillars caged with underproducing plants of *N. tabacum* Xanthi compared to wild-type plants, while the opposite was true for underproducing *N. sylvestris* (although differences for all leaves combined were not significantly different in any case). The sixth leaf from the bottom of the wild-type *N. tabacum* Xanthi was damaged significantly less ($P < 0.05$) compared to the corresponding leaf from underproducing plants. Because only small numbers of caterpillars were recovered from plants at the end of the seven-day assay period, weights are not reported.

Differential Feeding Responses in Leaf Disk Assays. In leaf disk assays, leaf disks from plants overproducing tobacco anionic peroxidase were always damaged less than those from wild-type plants to a significant degree in all cases except for mature *N. sylvestris* leaf disks (Table 2). There were few differences in mortality or weights of larvae feeding on leaf disks from these respective plants, although in some cases mortality was significantly greater, and weights significantly less, for caterpillars fed on disks from overproducing compared to

TABLE 1. EFFECTS OF FEEDING BY FIRST-INSTAR *H. zea* ON LEAVES OF INTACT PLANTS OF WILD-TYPE AND TRANSGENIC TOBACCOS OVER- AND UNDERPRODUCING TOBACCO ANIONIC PEROXIDASE[a]

Plant type	Leaf damage (mm^2) of entire plant	*N*
N. tabacum		
Xanthi		
Underproducer	123 ± 20a	22
Wild type	82 ± 11a	22
N. tabacum		
Coker		
Wild type	250 ± 56a	15
Overproducer	178 ± 15a	20
N. sylvestris set 1		
Underproducer	33 ± 9a	15
Wild type	54 ± 13a	22
Overproducer	38 ± 9a	13
N. sylvestris set 2		
Underproducer	123 ± 10a	20
Wild type	151 ± 13a	19
Overproducer	88 ± 8b	19

[a] Studies were initiated approximately 20 days after germination. *N. sylvestris* set 1 plants had two larvae per plant, while all other sets had 10 larvae per plant. Values are means ± standard errors. Values in columns of like studies followed by different letters are significantly different at $P < 0.05$ by analysis of variance.

wild-type plants. Leaf disks from underproducing plants were damaged to about the same degree as those from wild-type plants, and generally there were no differences in mortality and weights of larvae fed on these two different types of plant disks. Disks from old leaves of *N. sylvestris* were damaged much less that those from new leaves for each corresponding degree of expressing type of plant.

Leaf disks from insect-damaged plants (Table 3) were generally damaged less compared to disks from leaves of plants that had not been fed upon, especially for *N. tabacum* varieties (Table 2), but the length of leaves used were in some cases dissimilar. However, the same trends that were noted in Tables 1 and 2 were also noted with the leaf disks from the insect-damaged plants. Leaves from overproducing plants were generally damaged to a significantly lesser extent than wild-type plant leaf disks. Damage to underproducing *N. tabacum* Xanthi was significantly greater than to wild-type plants, while no significant differences in the degree of damage were seen for the underproducing vs. wild-type

TABLE 2. EFFECTS OF FEEDING BY FIRST-INSTAR *H. zea* ON LEAF DISKS FROM UNDAMAGED PLANTS OF WILD-TYPE AND TRANSGENIC TOBACCOS OVER- AND UNDERPPRODUCING TOBACCO ANIONIC PEROXIDASE[a]

Plant type	Damage rating		Mortality (%)		Weight (mg)
	Day 1	Day 2	Day 1	Day 2	
N. tabacum Xanthi					
Underproducer	74 ± 16a	3.6 ± 0.7a	5.6a	8.3a	0.17 ± 0.01a
Wild type	84 ± 12a	3.9 ± 0.4a	1.3b	9.0a	0.17 ± 0.01a
N. tabacum Coker					
Wild type	2.4 ± 0.4a	5.4 ± 0.5a	1.5a	2.2a	0.22 ± 0.01a
Overproducer	1.2 ± 0.1b	3.2 ± 0.5b	10.5b	6.4a	0.20 ± 0.01a
N. sylvestris set 1 (mature leaves)					
Underproducer	9 ± 1a	16 ± 2a	13.0a	11.8a	0.07 ± 0.01a
Wild type	8 ± 1a	15 ± 2a	10.5a	31.1b	0.07 ± 0.01a
Overproducer	7 ± 1a	15 ± 2a	6.3a	21.3ab	0.06 ± 0.01a
N. sylvestris set 1 (young leaves)					
Underproducer	46 ± 3a	79 ± 7a	10.2a	10.6a	0.11 ± 0.01a
Wild type	40 ± 4a	74 ± 6a	10.3a	14.3a	0.11 ± 0.01a
Overproducer	24 ± 2b	38 ± 4b	15.2a	28.3b	0.09 ± 0.01a
N. sylvestris set 2					
Underproducer	30 ± 3a	154 ± 37a	19.2a	27.9a	0.14 ± 0.01ab
Wild type	29 ± 3a	149 ± 30a	21.3a	20.3a	0.16 ± 0.01a
Overproducer	16 ± 1b	68 ± 16b	33.3b	23.0a	0.13 ± 0.01b

[a] Damage rating and weight values are means ± standard errors. Values in columns of like parameters and studies followed by different letters are significantly different at $P < 0.05$ by analysis of variance (damage ratings and weights) or chi-square (mortality) analysis. Ratings for day 2 of *N. tabacum* Xanthi and both days of *N. tabacum* Coker are on a 0–10 scale, other ratings are in 0.25 mm^2. Numbers of leaves for ecah treatment of all *N. tabcaum* studies are 20. For *N. sylvestris*, numbers of leaves used are 15 for underproducer, 22 for wild type, and 13 for overproducer for both leaf types of set 1; and 10 for underproducer, 14 for wild type, and 16 for overproducer for set 2. Mature leaf disks used for *N. sylvestris* were from leaves approximately 14 days after initiation; other leaf disks were from leaves approximately seven days after initiation.

N. sylvestris. As was seen for the other leaf disk assays, there were typically few differences in mortality or weights of caterpillars fed on the different leaf-disk types, although those fed on overproducing *N. sylvestris* were significantly smaller than those fed on wild-type plants. Caterpillars fed on leaf disks from wild-type *N. tabacum* Xanthi were significantly smaller that those fed on leaf disks from corresponding underproducing leaves. Overall, the mean of leaf sizes from the different expressing plants used in all of these concurrent assays was not significantly different ($P < 0.05$).

Peroxidase Activity of Leaf Tissues. Peroxidase activity was not signifi-

TABLE 3. EFFECTS OF FEEDING BY FIRST-INSTAR *H. zea* ON LEAF DISKS FROM DAMAGED PLANTS OF WILD-TYPE AND TRANSGENIC TOBACCOS OVER- AND UNDERPPRODUCING TOBACCO ANIONIC PEROXIDASE[a]

Plant type	Damage rating		Mortality (%)		Weight (mg)
	Day 1	Day 2	Day 1	Day 2	
N. tabacum Xanthi					
Underproducer	19 ± 2a	66 ± 9a	4.5a	22.1a	0.07 ± 0.01a
Wild type	11 ± 2b	29 ± 4b	1.1a	23.4a	0.04 ± 0.01b
N. tabacum Coker					
Wild type	17 ± 2a	47 ± 5a	ND	44.2a	0.10 ± 0.01a
Overproducer	9 ± 1b	23 ± 4b	ND	31.1a	0.11 ± 0.01a
N. sylvestris set 2					
Underproducer	21 ± 1a	59 ± 6a	8.9a	19.0a	0.13 ± 0.01ab
Wild type	17 ± 1ab	51 ± 6ab	9.8a	22.8a	0.15 ± 0.01a
Overproducer	16 ± 1b	39 ± 4b	8.9a	19.6a	0.12 ± 0.01b

[a] Damage rating and weight values are means ± standard errors. Values in columns of like parameters and studies followed by different letters are significantly different at $P < 0.05$ by analysis of variance (damage ratings and weights) or chi-square (mortality) analysis. Leaf ratings are all 0.25 mm^2. Numbers of leaves for each treatment are 17 for underproducer and 19 for wild type of *N. tabacum* Xanthi, 11 for both leaf types of *N. tabacum* Coker, and 20 for underproducer, 18 for wild type, and 19 for overproducer of *N. sylvestris*. Leaf disks were from leaves approximately seven days after initiation. ND = not determined.

TABLE 4. TOTAL PEROXIDASE ACTIVITY IN REPRESENTATIVE WILD-TYPE, OVER-, AND UNDERPRODUCING TOBACCO PLANTS[a]

Plant type	Peroxidase activity (Δ AU/min/g fresh weight)
N. tabacum Xanthi undamaged	
Underproducer	6.2 ± 1.0a
Wild type	8.2 ± 1.0a
N. tabacum Xanthi damaged	
Underproducer	24.1 ± 2.4a
Wild type	17.0 ± 1.3b
N. tabacum Coker undamaged	
Wild type	18.7 ± 6.1a
Overproducer	79.6 ± 15.5b
N. tabacum Coker damaged	
Wild type	20.9 ± 1.3a
Overproducer	89.1 ± 28.3b

[a] Values are means ± standard errors. Values in columns of like leaf types followed by the same letter are not significantly different at $P < 0.05$ by analysis of variance.

cantly different in underproducing vs. wild-type plants for undamaged leaves of *N. tabacum* Xanthi (Table 4). Peroxidase activity in underproducing leaves from damaged plants of *N. tabacum* Xanthi was unexpectedly significantly greater than that of the wild-type plants. In the cases of leaves from both damaged and undamaged plants of *N. tabacum* Coker, mean peroxidase activity in leaves from overproducing plants was approximately four to five times that of the wild-type plants.

DISCUSSION

Peroxidase-Mediated Resistance in Undamaged Plants. Peroxidases can promote insect resistance in plants by converting substrates to highly reactive chemical species that subsequently undergo a variety of reactions that can adversely affect insects. These highly reactive compounds can reduce nutritional quality by binding to nutrients and making them less digestible, inhibit digestive enzymes, act as direct cytotoxins, and polymerize and/or crosslink to increase tissue toughness (e.g., Appel, 1993; Felton et al., 1989, 1992; Dowd and Lagrimini, 1997a; Miles and Oertli, 1993). By examining the properties of the peroxidases in the different plants and differences in expression, substrate specificity, and substrate presence, it should be possible to gain some understanding of how changing peroxidase activity can alter resistance levels in transgenic plants compared to wild-type plants.

Chlorogenic acid and rutin are the more common allelochemicals in tobacco and are suitable substrates for peroxidases (Dowd and Vega, 1996). For *N. sylvestris* (Snook et al., 1986) and the different lines of *N. tabacum* (Schlotzhauer et al., 1981; Snook et al., 1986), the concentration of chlorogenic acid is about 10-fold that of rutin. Chlorogenic acid analogs are also common and are approximately equivalent in concentration (Snook et al., 1986). Chlorogenic acid oxidized by peroxidase binds to proteins and reduces their nutritional quality to insects (Felton et al., 1992). The products produced by peroxidase oxidation of chlorogenic acid and rutin are also more toxic to insects than the compounds themselves (Dowd and Vega, 1996). Thus, enhanced peroxidase activity in tobacco may result in more rapid generation of toxic metabolites, which additionally may bind to nutrients and reduce their digestibility. Reduced levels of peroxidase would tend to lower the production of these metabolites, making the tissues more acceptable to insects. Effects noted with leaf disks from undamaged plants were generally consistent with the idea of enhanced peroxidase activity being associated with enhanced insect resistance (overproducing plants), but reducing tobacco anionic peroxidase activity (underproducing plants) did not reduce insect resistance. The best explanation of these observations is that the total peroxidase activity is more important than the activity of individual peroxidase isozymes. Total peroxidase activity is enhanced by a significant level in the overproducing tobacco plants (present data, Dowd and

Lagrimini, 1997b), but in the present study the underproducing leaves examined had approximately equal levels of total peroxidase activity compared to the wild types. Isoforms other than the tobacco anionic peroxidase apparently provide enough net peroxidase activity so that feeding resistance to insects is approximately equal initially in underproducing and wild-type plants.

The fast (highly acidic) anionic peroxidases (equivalent to the tobacco anionic peroxidase studied in the present study) of different tobacco species can more rapidly oxidize the native substrates rutin and chlorogenic acid than slow anionic or cationic peroxidases (Sheen, 1974). However, the relative contribution of these peroxidase isozymes to total peroxidase activity varies in the different species (Sheen, 1974). For *N. sylvestris*, the rate of rutin oxidation by the fast anionic peroxidases is ca. 4×, and chlorogenic acid, ca. 2×, the combined rates for the other isozymes (Sheen, 1974). For *N. tabacum*, the contribution of the fast anionic peroxidases for rutin is ca. 10× the combined rate of the other peroxidases, and ca. 4× the rate of the other peroxidases for chlorogenic acid (Sheen, 1974). In our studies, the degree of insect damage in the underproducer was little affected compared to the wild-type in *N. sylvestris*. The higher proportion of isozymes other than the fast forms in *N. sylvestris* compared to *N. tabacum* may explain why the degree of insect damage of underproducing *N. sylvestris* was less different from the wild-type compared to corresponding plant types of *N. tabacum*. This difference in species response of the underproducing vs. wild-type plants was much more dramatic in damaged plants of *N. sylvestris* and *N. tabacum* compared to leaf disks from undamaged plants, suggesting other factors are interacting differentially with the changes in peroxidase activity in damaged vs. undamaged plants.

Peroxidase-Mediated Resistance in Damaged Plants. Damaging plants can significantly change the resistance level to insects. Leaf disks of the same plant type from insect-damaged plants of the tobacco varieties tested generally received much less damage than those from the corresponding undamaged plants. Both insect and mechanical damage to tomato leaves can increase resistance through the induction of both chemical and biochemical factors (Stout et al., 1994, 1996; Stout and Duffey, 1996). Induction of trypsin inhibitor in tobacco and tomato by insects occurs to a similar degree (Jongsma et al., 1994). Assuming additional factors are also induced by insect feeding on tobacco, this may explain the different effects seen with underproducing leaves from prefed upon vs. undamaged leaves of *N. tabacum* Xanthi. Enhanced peroxidase activity has been reported in soybean (*Glycine max*) (Bi and Felton, 1995) and tomato (*Lycopersicon esculentum*) (Stout et al., 1996) leaves in plants damaged by insects. However, in tomato, significantly enhanced peroxidase activity is only seen for the specific leaf that is damaged (Stout et al., 1996). Our results appear consistent with those with tomato in that leaves remote from the damaged leaf did not appear to have higher peroxidase activity compared to undamaged leaves in leaf disk assays.

The results seen with the leaf disks from insect-damaged plants compared to the disks from the undamaged plants, as well as the whole plant assays, suggest additional factors are interacting with the peroxidases that are mediating insect resistance. While the overproducing plants demonstrate similar levels of enhanced enzyme activity and insect resistance relative to the wild-type plants, leaf disks from damaged underproducing plants generally were more susceptible to feeding compared to the wild-type plants (significantly so in some cases). Net feeding was always less than for leaf disks from the undamaged plants for the same plant types. When mechanically damaged or insect damaged, the fast anionic peroxidases are only slightly induced, while the slow anionic and cathodic forms are highly induced in the damaged leaf (Lagrimini and Rothstein, 1987; Dowd and Lagrimini, 1997b). Insect damage can greatly enhance nonanionic peroxidase activity in *N. sylvestris*, but in overproducing plants the anionic peroxidases still contribute most of the total peroxidase activity (Dowd and Lagrimini, 1997b).

Wound-induced lignification occurs 24–48 hr sooner in the pith of plants overproducing the anionic peroxidase (Lagrimini, 1991). Properties of the overproducing tissue related to lignification include increased phenolic levels (Lagrimini, 1991) and smaller cells (Lagrimini, 1992). These may both increase the toughness of the tissue, which is a known deterrent to insect feeding (review Dowd and Lagrimini, 1997a). If a similar situation occurs in the leaves, then for the whole plant assays the advantage of the anionic peroxidase expression would be similar in *N. sylvestris* and *N. tabacum* if it is the more important defensive response, which was supported by data in the present study. By analogy, lignin production following damage in underproducing tissues should be significantly slower than in wild-type tissue with corresponding reductions in insect resistance; this was also seen with both plant species in the present study. Thus, the peroxidase reactions with rutin and chlorogenic acid may be more important in the short-term, immediate response (undamaged plants), while induction of lignification involving peroxidase would be more important in long-term, induced resistance. This trend is somewhat similar to the conclusion reached for investigations of mechanisms contributing to varietal resistance of corn. The allelochemical DIMBOA appeared to be more important in determining resistance to *Ostrinia nubilalis* in young leaves, while toughness of leaves (which can be mediated by peroxidase cross-linkings) was more important in determining resistance in older leaves (Bergvinson et al., 1995). However, our results suggest relative peroxidase activity can mediate both allelochemical and structural resistance in undamaged and damaged leaves, with the allelochemical interactions more important in the undamaged leaves and structural factors more important in the damaged leaves. The age of the leaves examined appears to be important in making conclusions about peroxidase activity, because after leaves catch up in terms of maturity, there is no difference in resistance in uninduced tissues (mature leaf assays with *N. sylvestris*). This same effect was noted with

wild-type and tobacco anionic peroxidase-producing leaves of sweetgum (*Liquidamber styraciflua*) of differing maturity when fed to some species of caterpillars (Dowd and Lagrimini, 1997a,c). Multiple mechanisms in addition to peroxidase may be involved that also are mediated by plant age, and damage type, as discussed previously.

CONCLUSIONS

Leaf material from two species of tobacco overproducing tobacco anionic peroxidase was often damaged significantly less than leaf material from wild-type plants by *H. zea* larvae. Leaf material underproducing tobacco anionic peroxidase was either damaged at the same rate or significantly more (depending on the plant species and leaf source) compared to wild-type leaf material. These results suggest expression of this particular peroxidase can mediate the degree of resistance of tobacco to feeding by chewing insects. This is likely to be one of several factors involved in resistance to insects, but nevertheless can be an important one. Because peroxidases are widely distributed in plants fed on by insect herbivores, peroxidase activity of appropriate isozymes is also likely to be important in determining relative insect resistance in other plants. However, the context of the peroxidase is important in determining its relative importance, due to the complexity of substrates and reactions involved.

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FEEDING STIMULATING SIGNAL IN LABIAL GLAND SECRETION OF THE SUBTERRANEAN TERMITE *Reticulitermes santonensis*

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Abstract—The paired labial glands of the French subterranean termite *Reticulitermes santonensis* Feytaud are located in the thorax. In the head, the glandular ducts join with those of the water sacs. In feeding choice tests with two semicircles of moist filter paper as food, workers of *R. santonensis* preferred the semicircle treated with labial gland extract compared to the semicircle treated with water (control). The labial gland secretion carries a signal that stimulates gnawing and feeding by termite workers during food exploitation. The extract of the labial gland even elicited feeding behavior when applied without food (on glass plates). The content of the water sacs was not effective as a feeding stimulus and neither were different body parts except for the fat-body. Water sacs are thus not a reservoir for the labial gland secretion, but their contents may serve as a solvent or carrier for the phagostimulant signal. The signal is highly polar, heat-resistant, nonvolatile, and thus very persistent. There is evidence that the signal from the labial gland is not species specific. Instead, it might be part of a general strategy by termites to exploit food sources.

Key Words—Termites, chemical communication, exocrine gland, pheromones, feeding behavior, feeding aggregation, phagostimulant.

INTRODUCTION

The social organization of termite colonies largely depends on chemical signals that are produced by various exocrine glands. These signals modulate a variety

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of behavioral patterns, mostly on a communal level involving a large number of nestmates. Chemical signals may be either released as airborne signals or deposited as low-volatility or nonvolatile signals on a solid substrate such as soil or food.

The paired labial glands are found in all termite species. The glands are located in the meso- and metathorax and their histology and fine structure have been described for several termite species (Pasteels, 1965; Noirot, 1969; Billen et al., 1989; Costa-Leonardo and Cruz-Landim, 1991; Kaib and Ziesmann, 1992). The glandular ducts open into the mouth cavity. Termites release the labial gland secretion onto the substrate. This secretion is reported to have various functions depending on the species and has been identified as a defensive substance in *Macrotermes* and *Odontotermes* soldiers (Maschwitz and Tho, 1974; Olagbemiro et al., 1990). Intraspecific functions, such as acting as a social nutrient or for regulating the nest microclimate and supporting fungal cultivation (Hewitt et al., 1971; Watson et al., 1971; Grassé, 1982; Brian, 1983), have been proposed. The labial glands are also supposed to excrete a cementing substance for nest construction or gallery building (Noirot, 1969). In addition, they have been suggested to be the source of digestive enzymes (Martin and Martin, 1978; Hogan et al., 1988; Veivers et al., 1991).

Despite the importance of chemical communication in social insects, the role of labial gland secretion in chemical communication has been the subject of few investigations. Only in the African termite, *Schedorhinotermes lamanianus*, has a pheromonal role of the labial gland secretion during communal food exploitation been demonstrated (Kaib and Ziesmann, 1992; Reinhard and Kaib, 1995). The secretions of the labial glands, released onto the food by gnawing and feeding termites, stimulate additional workers to gnaw at the same site, thereby forming aggregations of gnawing termites.

In this paper we investigate whether a pheromonal role of labial gland secretion during food exploitation might be a general phenomenon in termites. For comparison with the results on *S. lamanianus*, we have chosen the French termite *Reticulitermes santonensis* Feytaud (Isoptera: Rhinotermitidae) because of its different ecological habitat and nesting habits. While *S. lamanianus* is an arboreal termite that lives in humid forests of tropical Africa, *R. santonensis* occurs in a temperate zone of France in subterranean nests (Harris, 1968; Becker, 1970; Kaib and Brandl, 1992; Brandl et al., 1996). In addition, the foraging and recruitment processes of the two termite species are based on different strategies. In *S. lamanianus* foraging is initiated by soldiers. Termite workers follow the foraging trails of the soldiers only after reinforcement of the trails (Schedel and Kaib, 1987). During food exploitation, they form large aggregations of feeding workers protected by a surrounding ring of soldiers. In contrast, *R. santonensis* soldiers are not involved in foraging and recruitment (Reinhard et al., 1997). Foraging is initiated by workers only, which form rather small

feeding aggregations during food exploitation and are protected only by a shelter of gallery material. We examined the pheromonal function of the labial gland secretion in *R. santonensis* during food exploitation as well as the chemical properties of the secretion, concentrating on workers only.

METHODS AND MATERIALS

Collection and Culture of Termites. A colony of the subterranean termite, *Reticulitermes santonensis* Feytaud, was collected in August 1994 in Ile d'Oléron, France, and kept in the laboratory at constant conditions (25°C, 75% relative humidity). The colony was housed in a metal container with moist vermiculite serving both as soil substrate and water resource. From the container the termites had access to a foraging arena via a silicon tube (5 mm ID). The foraging arena of 10 × 10 cm (5 mm high) had a sand-blasted glass plate as floor and was covered by a glass plate. The termites were fed ab libitum with moist filter paper in the foraging arena.

Preparation of Extracts. For the labial gland extract, termite workers were killed by deep-freezing prior to dissection. Termites were fixed in a ventral position in distilled water and the paired labial glands were removed (Figure 1). Lots of 10 labial glands were simultaneously extracted in 100 μl distilled water for 12 hr at 4°C and then deep-frozen for 24 hr. Thereafter, the glands were removed from the extract and the extracts again deep-frozen until tested. The water sacs (Figure 1), crop, gut tissue, muscles, hemolymph, and fat-body of termite workers were extracted in the same way. For these extracts the volume of the body parts was three to four times higher compared to the glands, to

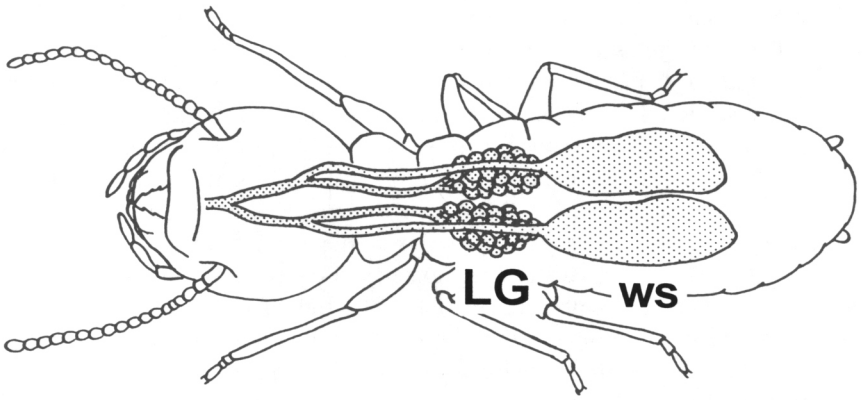


FIG. 1. Schematic illustration of the arrangement of the paired labial gland (LG) and the water sacs (ws) in *Reticulitermes santonensis* workers.

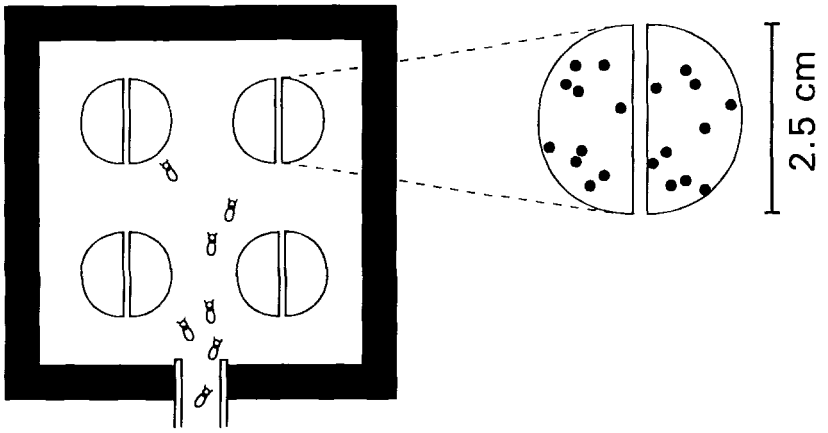


FIG. 2. Experimental set up for bioassaying the distribution of gnawing and feeding termites. The arena (10 × 10 cm) and the set of four simultaneous tests on semicircles of filter paper are shown on the left. Dots on the right represent the location of the first 20 gnawing events.

make sure to get a high enough concentration. For the hemolymph extract, ca. 50 μl of hemolymph were collected with a microcapillary and then dispersed into 100 μl of distilled water.

Bioassay. Tests were modified according to the experimental set up of Kaib and Ziesmann (1992) and Reinhard and Kaib (1995). They were carried out with workers on pieces of moist filter paper that were placed in the foraging arena. Four independent tests were run simultaneously. After each set of four tests the total foraging arena was replaced. Each test consisted of two semicircles (2.5 cm diameter) of filter paper placed close beside each other (Figure 2). One of the two semicircles was randomly chosen for application of 25 μl of labial gland extract or extracts of various body parts and then moistened with water. The other semicircle was just moistened. For the control experiments, both semicircles were only moistened with water. The bioassays were started immediately after applying the extracts. For investigating the chemical properties of the labial gland secretion, we first tested four extracts of labial glands made with solvents of decreasing polarity (water > methanol > dichloromethane > hexane). To test heat resistance, water extracts of the labial glands were exposed for 15 min each to temperatures of 40°C, 70°C, and 95°C in a waterbath before employing them in the bioassay. To test volatility, water extracts of the labial gland were applied on the semicircles of filter paper and were exposed to evaporation for 1 hr and 12 hr at 20°C and for 24 hr at 100°C in a heating oven. Afterwards, the semicircles were remoistened before testing. For the bioassay on

glass, a drop of water extract of the labial glands as well as a drop of water (25 μl each) were applied directly on the glass plate of the foraging arena without filter paper. Application sites were marked on the bottom side of the glass plate. The drops were allowed to dry up before employing the glass plate in the test.

Termites are light sensitive, so tests were made under red light illumination. Feeding in termite workers is expressed by gnawing behavior, which can be easily recognized by the hypognathous head position. Workers bore their mandibles into the food and wriggle their heads trying to tear off little pieces, which they then transport back to the nest. Frequencies and spatial distributions of gnawing workers were recorded by video techniques (IR-sensitive Kappa Camera) for 1 hr and subsequently analyzed. For each test the distribution of the first 20 gnawing termites on the two semicircles and the two drops, respectively, was determined.

Data Analysis. Statistical analysis of all data was carried out with Statistica by Statsoft Inc. The sign-test was used to analyze the data of the bioassays, and the significance level of acceptance was $P < 0.001$.

RESULTS

Signal Function of Labial Gland Secretion. When semicircles of filter paper were moistened with water only and were offered as food in the foraging arena, none of the two semicircles was significantly preferred, as the gnawing termites were equally distributed (Figure 3A). However, when one semicircle was treated with labial gland secretion, significantly more termites gnawed on it. When reaching a spot where labial gland secretion was applied, workers searching for food stopped and immediately started gnawing. Gradually aggregations of gnawing and feeding termites developed, and the treated semicircle of filter paper was eaten up much faster than the untreated one. This clearly demonstrates that the labial gland secretion of *R. santonensis* carries a signal that stimulates gnawing and feeding during food exploitation.

To test possible interactions between the labial gland secretion and the filter paper, we applied drops of labial gland secretion and drops of water as control directly on the glass plate of the foraging arena instead of on filter paper. The bioassay was started when the drops were dried up. Although there was no filter paper as food presented, termite workers aggregated on the spots with labial gland secretion and tried to gnaw on the glass (Figure 3B). On the spots with water, no aggregations of gnawing termites could be found. The response of the termite workers to the labial gland secretion was the same with and without filter paper.

As the ducts of the labial glands fuse in the head with those of the water sacs (Figure 1), we investigated a possible reservoir function of the water sacs

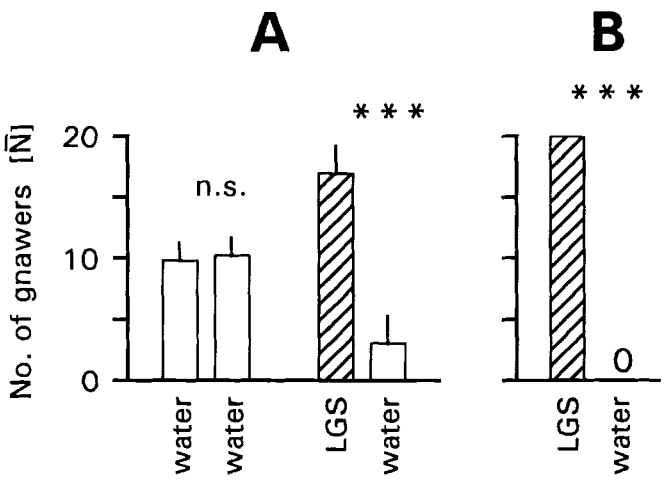


FIG. 3. Signal function of the labial gland secretion. Mean distribution of the first 20 gnawing termites (gnawers) (A) on two semicircles of filter paper and (B) on two dried up extract drops on the glass floor (±SD, $N = 20$). Choice test is represented by bars; empty bars: water, hatched bars: 25 μl labial gland secretion (LGS). (n.s.: no significant difference, ***: significant difference at $P < 0.001$, sign test.)

in a behavioral context. Water sac content had no effect on gnawing and feeding (Figure 4). Consequently, the water sacs do not contain a phagostimulant and do not function as reservoirs for the labial gland secretion. Extracts of crop, gut tissue, muscle, and hemolymph were not effective as feeding stimuli. Only the extract of the fat-body led to a preference of the treated semicircle. However, the effect was not as strong as after applying labial gland extract.

Chemical Properties. To examine the polarity of the phagostimulant signal, extracts of labial glands with solvents of decreasing polarity (water > methanol > dichloromethane > hexane) were tested. Only the water extract was effective as a feeding stimulus. None of the other solvents carried the signal (Figure 5A).

To test the heat resistance of the signal, water extracts of the labial glands were exposed to heat before being used in the bioassay. Heat-treatment did not affect the gnawing and feeding stimulus, and termites always preferred semicircles treated with secretion (Figure 5B).

To test the volatility of the signal, water extracts of the labial gland secretion were applied on the semicircles of filter paper and exposed to evaporation for 1 hr and 12 hr at 20°C as well as for 24 hr at 100°C. Regardless of the evaporation conditions, treated semicircles still carried the feeding stimulus (Figure 5C).

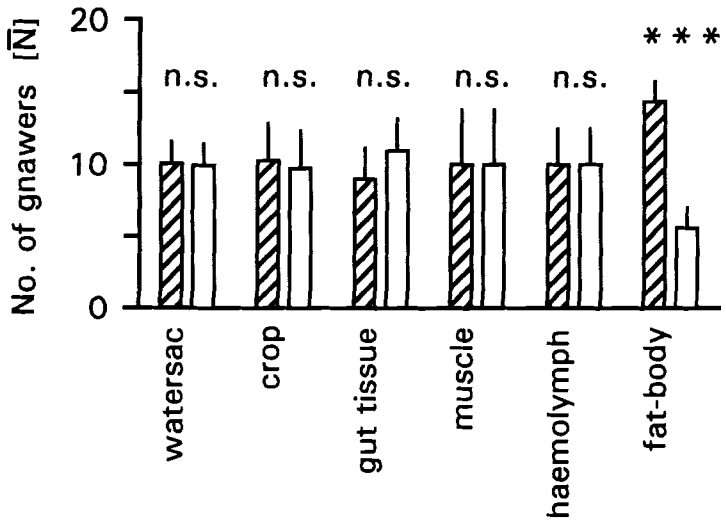


FIG. 4. Effect of body parts on feeding behavior. Mean distribution of the first 20 gnawing termites (gnawers) on two semicircles of filter paper (±SD, $N = 20$). Choice tests are represented by bars; hatched bars: 25 μl extract of body parts, empty bars: water. (n.s.: no significant difference, ***: significant difference at $P < 0.001$, sign-test.)

DISCUSSION

Although chemical communication plays a key role in the maintenance of insect societies (Noirot, 1969; Wilson, 1971; Hölldobler and Wilson, 1990), the role of the labial gland secretion in chemical communication has been examined only rarely (Kaib and Ziesmann, 1992; Reinhard and Kaib, 1995). The data we have presented document the function of the labial gland secretion during communal food exploitation by the subterranean termite *Reticulitermes santonensis*. The secretion is released onto the food by gnawing and feeding workers. The secretion carries a polar, heat-resistent, extremely persistent signal a polar, heat-resistant, extremely persistent signal that stimulates feeding and thus leads to aggregations of gnawing workers on the food source. The chemical identification of the phagostimulant signal requires further chemical analysis.

In *R. santonensis*, feeding stimulation could not be provoked by extracts of the water sacs, crop, gut tissue, muscle, and hemolymph. Consequently, feeding aggregations are not caused by general body substances. These results correspond to the observations of Kaib and Ziesmann (1992) for *S. lamanianus*, in which gnawing aggregations of workers are elicited by polar and persistent

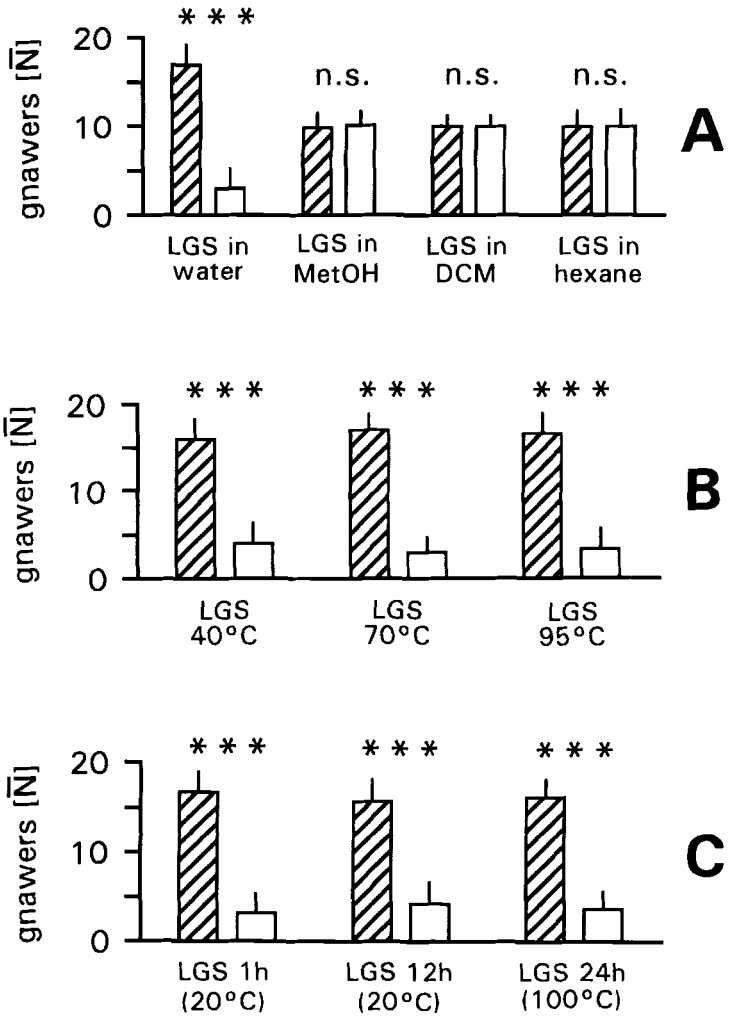


FIG. 5. Chemical properties of the labial gland secretion. Mean distribution of the first 20 gnawing termites (gnawers) on two semicircles of filter paper ($\pm$SD, $N = 20$). Choice test is represented by bars; hatched bars: 25 μl labial gland secretion (LGS), empty bars: water. (A) Polarity: LGS extracted in different solvents; (B) heat resistance: LGS exposed to heat before the test; (C) volatility: letting LGS on the semicircles evaporate before the test. (n.s.: no significant difference, ***: significant difference at $P < 0.001$, sign-test.)

labial gland secretion but not by samples taken from other body parts of termite workers.

In contrast to the results for *S. lamanianus*, fat-body extracts in addition to labial gland secretion of *R. santonensis* also stimulated feeding behavior. In the literature water sacs are quite often considered to be reservoirs of the labial gland secretion (Watson et al., 1971; Grassé, 1982; Alibert, 1983). Our results prove that, as in *S. lamanianus* (Kaib and Ziesmann, 1992), water sacs of *R. santonensis* do not carry a feeding stimulating signal. Nonetheless, the contents of the water sacs and the labial glands could be released simultaneously to form saliva. Therefore, water sac contents may serve as a carrier or solvent for the signal from the labial gland when applied on the food. In addition, water sac contents may modulate the signal concentration from the labial gland secretion.

Termites feed on cellulose, and the labial gland secretion of several termite species contains enzymes for the digestion of cellulose (Noirot, 1969; Zhuzhikov and Korovkina, 1972; Yamaoka and Nagatani, 1975; Martin and Martin, 1978; Hogan et al., 1988; Veivers et al., 1991). Therefore, when releasing the labial gland secretion on the food, products from an enzymatic breakdown of cellulose could elicit feeding behavior. To our knowledge, such a possible interaction between the secretion and the food has not been previously investigated. Our results from the secretion applied on a glass surface demonstrate that the labial gland secretion stimulates feeding behavior even in the absence of food. The heat resistance of the signal from the labial gland secretion provides further evidence that digestive enzymes do not play a role in the production of the feeding stimulus. Although not involved in the initial feeding stimulation, the enzymes may nevertheless enhance the mechanical break-up of solid food.

Functional and chemical properties of the phagostimulant signal in the labial gland secretion of the subterranean species *R. santonensis* agree to a great extent with the results obtained for the aboreal species *S. lamanianus* (Kaib and Ziesmann, 1992). The two species belong to different subfamilies of the Rhinotermitidae, and they differ in nesting habits as well as in their foraging and recruitment patterns (Harris, 1968; Noirot, 1969; Becker, 1970; Schedel and Kaib, 1987; Kaib and Brandl, 1992; Brandl et al., 1996; Reinhard et al., 1997). However, both species have the feeding stimulation system in common. This indicates that feeding stimulation by a chemical signal from the labial glands may be a general phenomenon.

R. santonensis and *S. lamanianus* are lower termites feeding on dead wood. To put our hypothesis of a general strategy on a broader phylogenetical and physiological scale, in a preliminary test we extracted labial glands of the grass-feeding termite *Trinervitermes trinervoides*, which belongs to the higher termites (Termitidae). These extracts were tested with both *R. santonensis* and *S. laman-*

ianus and proved to elicit a significant feeding preference in the two species. Therefore, we suggest that the signal function of the labial gland secretion for food exploitation is a phylogenetically old and non-species-specific principle in termites. However, termites do not employ only the general feeding stimulating signal from the labial gland during the exploitation of food sources. They also use highly species-specific foraging and recruitment signals during the search for food (e.g., Howard et al., 1976; Kaib et al., 1982; Hall and Traniello, 1985). Furthermore, these signals may interact with and modulate behavioral patterns (Reinhard and Kaib, 1995). Thus, by combining a general strategy during food exploitation with species-specific patterns in the search for food, several termite species may share common food sources without interspecific conflicts.

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MAMMALIAN EXOCRINE SECRETIONS XI. CONSTITUENTS OF THE PREORBITAL SECRETION OF KLIPSPRINGER, *Oreotragus oreotragus*

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Abstract—The ketones 3-pentanone, 4-methyl-2-pentanone, 5-methyl-3-hexanone, 4-methyl-3-hexanone, and the esters ethyl propanoate, 2-methylpropyl acetate, ethyl 3-methylbutanoate, and 2-methylpropyl propanoate were identified as the only volatile organic constituents of the preorbital secretion of the klipspringer, *Oreotragus oreotragus*. These compounds are considerably more volatile than those found in the preorbital secretions of other antelopes belonging to the tribe Antelopini. More than 50% of the preorbital secretion consists of proteinaceous material, which probably acts as a controlled-release substance in retarding the evaporation of the volatile constituents of the secretion from territorial marks.

Key Words—*Oreotragus oreotragus*, klipspringer, mammalian semiochemicals, mammalian pheromones, exocrine secretion, preorbital secretion, controlled release, proteins.

INTRODUCTION

The considerable damage done to vineyards, orchards, and pine plantations in various parts of South Africa by several small antelope species, the important

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role the preorbital secretions of these animals play in their territorial behavior, and the possibility of using synthetic preorbital constituents to keep these animals out of cultivated areas were the main reasons for the development of a research program aimed at the chemical characterization of their preorbital secretions. In the previous paper in the series on mammalian exocrine secretions (Mo et al., 1995) it was explained that a response-guided strategy in this research met with little success. We therefore decided to compare the chemical composition of the exocrine secretions of animals belonging to different species and genera to gain insight into the function of the various compounds and compound types present in the secretions of these animals. Two groups of small antelope, in which the structures of the preorbital glands differ, were chosen for future study. One of these was the duikers, of which there are 18 species in Africa, belonging to three genera. In the duikers the preorbital secretion is emitted from a very prominent preorbital gland through a row of tiny pores. The second group of antelope is the Raphicerini, in which the secretion accumulates in a preorbital cavity or pouch not much bigger than the size of a pea. Three members from the tribe Raphicerini, the grysbok, *Raphicerus melanotis* (Burger et al., 1996), the steenbok, *R. campestris* (Burger et al., unpublished), and the oribi, *Ourebia ourebi* (Mo et al., 1995), have already been studied. The klipspringer, *Oreotragus oreotragus*, in which marking with the preorbital secretion appears to play an extraordinarily important role in the animal's territorial behavior, was chosen as the next suitable candidate in this research program.

The klipspringer is a small antelope weighing 8–18 kg, with a mean height of 43–51 cm. The habitat of the klipspringer is typically rocky outcrops or the steep rocky slopes of mountains and gorges, where they may be found up to an altitude of 4000 m (Dorst and Dandelot, 1970). The name klipspringer comes from the Afrikaans language and derives from the animal's ability to jump from rock to rock and to bound up steep rock faces.

Klipspringers are territorial animals, an adult male establishing and defending an area that varies in size according to the mean annual rainfall (Norton, 1980). Klipspringers do not spend very much time chasing intruders off their territories; instead there is evidence that they use dunging and scent-marking to demarcate their territories. Dung heaps are scattered more or less randomly throughout their territories but there is a tendency to place dung heaps at prominent sites and near territorial boundaries (Norton, 1980). Scent-marking with secretion from the preorbital glands is possibly the most important method used by the klipspringer to define territorial boundaries. In both sexes the preorbital gland is a large, thin-walled pocket anterior to the forward corner of the eyes, in which cornified epidermal cellular material from the sac wall, secretions from the orbit of the eye, and various particles of environmental debris accumulate (Albone, 1984). The preorbital glands are prominent in both sexes and open to the surface in a naked, black, forwardly sloping slit or cavity in front of the

eyes. Scent-marking is carried out by either partner but, if the female scent marks, the male usually follows and overmarks. This suggests that scent-marking plays an important role in the maintenance of the pair-bond, as well as serving to demarcate a territory. Territorial marking is carried out by the careful choice of a low, bare twig of a small bush, which, by maneuvering the head, is carefully inserted into the open preorbital cavity. This leaves a 4- to 6-mm smear of black, tarry glandular exudate on the twig (Smithers, 1983). The territorial marks have a shiny black, resinous appearance. Most of the twigs, grass stems, and some of the rocks throughout the more intensively used areas of a home range have deposits of facial secretions if they are at face level. A male klipspringer has even been observed biting off grass tips and inserting the stalks into his preorbital glands (Kingdon, 1982). In the present investigation it was found that a male klipspringer invariably licked the black deposits before and/or after adding fresh exudate to a territorial mark.

A very interesting observation is that adults of the African tick, *Ixodes matopi*, are attracted to and aggregate on the preorbital secretion marks that the klipspringer leaves on vegetation (Spickett et al., 1981; Colborne et al., 1981). In this way, the tick stands an excellent chance of making contact with its host when the scent mark is revisited. Rechav et al. (1978) found that if the solid secretion is extracted with different solvents, only the water-soluble fraction is active in attracting another *Ixodes* species, *I. neitzi*. This phenomenon appears to be another example of the pheromone of one species being utilized as a kairomone by another species to find its prey or a host animal.

The secretion of the klipspringer is unique among the preorbital secretions of members of the tribe of the Antelopini in that the bulk of the secretion consists of solid material that probably acts as a controlled-release carrier material for the volatile constituents of the secretion. If a klipspringer is housed in a small pen having only a few objects that can be marked, preorbital material deposited on, for example, the corner of a feeding trough accumulates to form rodlike structures that can reach a length of 5 cm with a thickness of about 1 cm. The preorbital secretion of the grysbok, *Raphicerus melanotis*, in contrast, contains only minute quantities of nonvolatile material, which, after evaporation of all volatile material, has a black powdery or chalklike appearance.

In this paper the identification of the volatile organic constituents of the preorbital gland secretion of the klipspringer and the partial characterization of the nonvolatile constituents of the secretion are reported.

METHODS AND MATERIALS

General. All Pyrex glassware used in the handling of biological material and extracts, as well as in the preparation of reference compounds, was heated to 500°C in an annealing oven to remove any traces of organic material. Di-

chloromethane (Merck, Residue Analysis Grade) was used for extraction purposes. Syringes, stainless-steel needles, and similar equipment were cleaned with this solvent.

Gas Chromatography. Gas chromatographic (GC) analyses were carried out with Carlo Erba 4200 and 5300 gas chromatographs equipped with flame ionization detectors, Grob split-splitless injectors, and glass columns coated with a 0.25-μm film of the apolar stationary phase PS-089, a silanol-terminated 95%-dimethyl-5%-diphenylsiloxane copolymer. Helium was used as carrier gas at a linear velocity of 28.6 cm/sec at 40°C. The flame ionization detector was operated at 280°C and the injector was normally used at 220°C. Samples were injected in the split mode, the analytes thermally focused on the column at ca. 30°C and analyzed using a temperature program of 2°C/min from 40°C to 260°C (hold). In headspace gas analyses and in analyses in which solventless sample introduction was used, cryotrapping with Dry Ice was employed. Quantification was done with Barspec software and a 386 personal computer.

Mass Spectrometry. Electron impact (EI) mass spectra were recorded at 70 eV on a Carlo Erba QMD 1000 gas chromatograph–mass spectrometer (GC-MS system), using the columns and conditions described above. An interface temperature of 250°C was used. The ion source temperature was set at 200°C, and the pressure in the source housing was ca. 2×10^{-5} torr at a column temperature of 40°C, decreasing to ca. 1×10^{-5} torr towards the end of the temperature program.

An electrospray mass spectrum of the nonvolatile fraction of the preorbital secretion was obtained by dissolving 9 mg of the core of a territorial mark in 1 ml of deionized H_2O, centrifuging the solution to precipitate dust particles and other insoluble material, and diluting a few microliters of the supernatant solution with water containing 0.01% of TFA for injection into the electrospray source of a VG Quattro Triplequad mass spectrometer.

Sample Collection. Preorbital secretion was collected from a tame male klipspringer kept in a pen (10 m × 10 m) at the Tygerberg Zoo. The animal was allowed to mark a tubular Teflon scoop with an inside diameter of 5 mm and furnished with a Teflon plunger with which the collected material could be ejected into a 300-μl or 1-ml Reacti-Vial. About 20–30 μl of secretion were collected per marking event.

Extraction of Volatile Organic Constituents. The collected secretion was stirred with a thin glass rod, and dichloromethane was added to produce a homogeneous suspension. The suspension was centrifuged at 2000–3000 rpm for 15 min or longer to precipitate solid material such as dust particles and to separate the solvent from water and mucus. Only very small quantities of dichloromethane extract could be removed with a 100-μl syringe from underneath the supernatant water layer and transferred to a Reacti-Vial. This extract was stored at −30°C for subsequent GC and GC-MS analyses.

Solventless Sample Introduction. Two solventless sample introduction techniques were used. To obtain the strongest possible total ion chromatograms, about 20 μl of fresh, unprocessed preorbital secretion was introduced into the GC-MS system by inserting a glass injector liner containing the secretion supported on a few glass-wool fibers, into the injector. The glass-wool fibers were used in the liner to prevent the secretion from flowing down into the bottom of the injector. This technique is not suitable for retention time comparison, and the analytes could also undergo oxidation in the injector, as the injector liner has to be introduced into the injector at atmospheric pressure. These shortcomings were circumvented by introducing the sample-containing liner into the injector at 40°C, purging the injector with the carrier gas, and heating the injector at a reasonably slow rate to allow volatile compounds to be desorbed from the liner at as low a temperature as possible. The volatile material desorbed from the injector was cryotrapped on the column. As the secretion consists largely of water, a section of 1.0-mm fused silica tubing was used at the inlet end of the column to prevent the column being blocked by ice formation. The liner was cleaned by washing it with distilled water and drying it at 250°C in a GC oven after each analysis.

Analyses were also carried out using a solventless sample introduction technique in which the sample is introduced using a capillary tube with an open-ended sample cavity of about 15 μl (Burger et al., 1990a). The sample introduction capillary was weighed, the sample (2–3 μl) injected into the sample cavity, and the loaded capillary weighed again and introduced into the injector without delay, with the split and septum-purge valves closed. Various injector temperatures were used and the volatile material, desorbed from the capillary, was cryotrapped on the column.

Analysis of Headspace Gas of Secretion. Solid-phase microextraction (SPME) (Arthur et al., 1992) was used to extract the volatile organic compounds from the headspace of 50- to 100-μl samples of secretion in a 1-ml Reacti-Vial. The extracted volatiles were desorbed from the SPME fiber for 5 min at an injector temperature of 220°C and were cryotrapped on the column. After the volatile material had been desorbed, cryotrapping was terminated and the program started. In a few analyses the SPME fiber was left in the injector for varying lengths of time to allow desorption of any heavy material that could have been trapped. SPME also was carried out on territorial marks by placing a territorial mark in a 50-ml glass bottle fitted with a screw cap and Teflon-lined septum and sampling the headspace for 3 hr.

Determination of Affinity of Preorbital Protein for Volatile Preorbital Compounds. Two experiments were designed to find out whether the nonvolatile part of the secretion could act as a controlled-release substance for the volatile organic compounds present in the secretion. In the first of these experiments the headspace gas of a territorial mark was analyzed by the following procedure

once every 24 hr for as long as the target compounds could be detected. A territorial mark (2.6 g) was collected from the pen of the male klipspringer and suspended on a thin stainles-steel wire to facilitate the handling of the material. The solid material was placed into the 50-ml glass bottle described in the previous section, the headspace atmosphere of the mark sampled by SPME for 3 hr at 22°C, and the collected volatile compounds quantitatively analyzed by gas chromatography. The territorial mark was removed from the bottle after completion of each SPME sampling period and was exposed to the atmosphere in a shaded place until the next analysis was carried out 21 hr later. The mean maximum and minimum temperatures during the time it took to complete the experiment were 21° and 9°C, respectively. The humidity was relatively high during the first 24-hr period, and on the second day it started raining. The territorial mark remained soft for about the first two days of the experiment, but became hard and brittle towards the end of the experiment, which was continued for six days.

In a second experiment the capability of the preorbital protein to adsorb and retain the volatile constituents of the preorbital secretion was compared to that of other proteins. Glass-fiber wicks were individually coated with the proteins and exposed to the vapor of some of the volatile constituents of the preorbital secretion, and the amount of the volatile organic material adsorbed by each of the proteins was determined gas chromatograhically. The device employed in this experiment has been described in a paper on the pheromone-dispensing proteinaceous secretion of certain dung beetle species (Burger et al., 1990b) and is depicted in Figure 1. In the present investigation 2.0 mg each of the solid preorbital secretion, bovine albumin, bovine pancreas trypsin, and chicken egg white lysozyme were dissolved separately in 100 μl of distilled water or, in the case of the preorbital protein, in 0.1% TFA. The wicks were coated with the proteins by allowing the protein solutions to be drawn up into the wicks by capillary action, and the wet glass-fiber wicks were suspended on thin glass rods to dry. The coated wicks were further dried under carrier gas flow in a glass injection liner at an injector temperature of 60°C and were then slipped onto the four outer prongs of the glass framework in Figure 1. The uncoated pheromone-dispensing wick was slipped onto the central prong and was treated with 1 μl of a mixture of 3-pentanone, 3-pentanol, isobutyl acetate, and ethyl isovalerate.

The framework was placed in a glass vial (28 mm OD × 140 mm), which was closed with a ground-glass stopper without using stopcock grease. The vial with its contents was kept in a cupboard, away from radiant light or heat at 22°C for 24 hr, and the wicks were immediately sealed individually in soda glass tubes (5 mm ID) with a small flame. the wicks were left in the glass tubes for at least 6 hr to allow equilibration of the volatiles with the glass surfaces before the volatile material retained on each wick was analyzed. These analyses

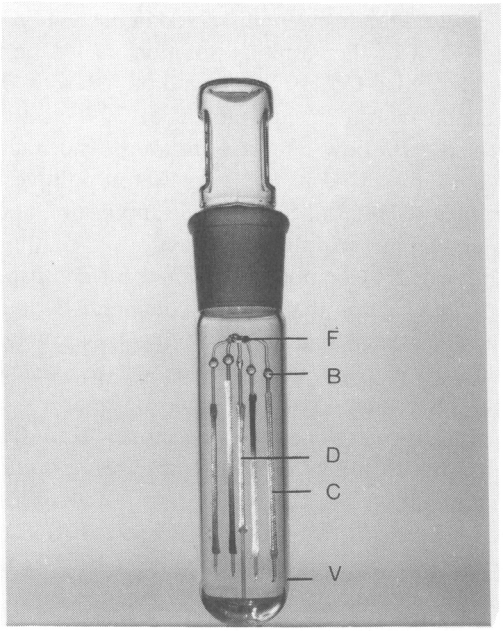


FIG. 1. Device for the determination of the affinity of various proteins for volatile organic compounds. V, glass vial (28 mm OD × 140 mm); F, five-pronged glass framework constructed from 1.0-mm glass rod and supported on the central prong; B, glass beads formed in the framework to keep the outer four prongs from touching the wall of the vial; D, dispensing glass-fiber wick treated with the volatile organic compound(s) under investigation; C, adsorbing wicks (collectors).

were done by inserting each wick into a wide-bore injector liner and desorbing the adsorbed compounds onto the capillary column at an injector temperature of, for example, 80°C for 30 min. After completion of an experiment, the wicks were stored in screw-capped glass tubes.

Electrophoresis. Polyacrylamide slab-gel electrophoresis in 20% acrylamide was performed in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970). Protein (100 μg/100 μl) was dissolved in the β-mercaptoethanol-containing application buffer of which 20 μl was applied per lane. Gels were stained with Coomassie brilliant blue R250.

Amino Acid Analysis. Proteins were hydrolyzed by gas-phase hydrolysis at 110°C for 24 hr in 5.7 M HCl–0.5% phenol (Bidlingmeyr et al., 1984). Amino acids were quantified by ion exchange chromatography on a Waters amino acid HPLC system using the OPA in a postcolumn derivative method as specified by the manufacturers.

HPLC Purification. Proteins were dissolved in 0.1% TFA at concentrations of 1 mg/ml, and 100 μl of the resulting solution loaded on a 30- × 0.4-cm column packed with Vydac-C4 [10 μm, equilibrated with 0.1% (w/v) TFA (solvent A) and eluted with a linear gradient of 70% acetonitrile containing 0.1% TFA (solvent B) at a flow rate of 1 ml/min]. The fractions were freeze dried and subsequently dissolved in 100 μl of water. Samples of 10 and 20 μl were dissolved in equal volumes of SDS sample application buffer and subjected to electrophoresis and amino acid determination.

Reference Compounds. Compounds required for comparison with constituents of the preorbital secretion that are not commercially available were synthesized from authentic starting materials. 5-Methyl-3-hexanone was synthesized by condensation of isobutyl magnesium bromide with propanal to give 5-methyl-3-hexanol (51 g, 44%). Oxidation of 5-methyl-3-hexanol with pyridinium chromate (Singh et al., 1979), gave 5-methyl-3-hexanone. ^{1}H: δ ($CDCl_3$) 0.914 [6H, d, (CH_3)$_2$-CH-], 1.046 (3H, t, CH_3-CH_2-), 2.151 [1H, m, -CH-(CH_3)$_2$], 2.398 (2H, q, -CH_2-CH_3), 2.280 (2H, d, -CH_2-CH-); ^{13}C: δ ($CDCl_3$) 7.787 (C1), 22.621 (C6, C7), 24.713 (C5), 36.461 (C2), 51.492 (C4), 211.520 (C3).

4-Methyl-3-hexanone was synthesized from *sec*-butyl magnesium bromide and propanal. Column chromatography of the final product on silica gel produced a sample of the pure reference compound, bp 134°C/760 mm. ^{1}H: δ ($CDCl_3$) 0.869 (3H, t, CH_3-CH_2-CO-), 1.047 (3H, t, CH_3-CH_2-CH-), 1.065 (3H, d, CH_3-CH-), 2.464 (2H, m, -CH_2-CO-), 2.440 (2H, m, -CH_2-CH_3), 6.479 (1H, m, -CH-CO-); ^{13}C: δ ($CDCl_3$) 7.083 (C1), 11.724 (C6), 16.051 (C7), 26.100 (C5), 34.294 (C2), 47.664 (C4), 215.38 (C3).

RESULTS AND DISCUSSION

The preorbital glands of the klipspringer are remarkably productive. The experimental male animal available for this research was tame enough to be coaxed into marking the Teflon scoop, which greatly simplified collection of material. In one experiment the animal marked the scoop 12 times and produced 102.1 mg of secretion in 22 min. The animal lost interest in the scoop after a while and started overmarking existing marks in his pen.

The secretion consisted of 38% nonvolatile material after water and other volatiles were evaporated at 100–110°C from freshly collected secretion. The high production of secretion and its relatively high content of nonvolatile material accounts for the accumulation of conspicuous territorial marks on twigs and other objects marked by the animal.

Characterization of Volatile Organic Constituents. The secretion is reasonably viscous, flowing quite slowly down the walls of vials in which it had been collected. When rubbed between the fingers, it is sticky and has a pleasant

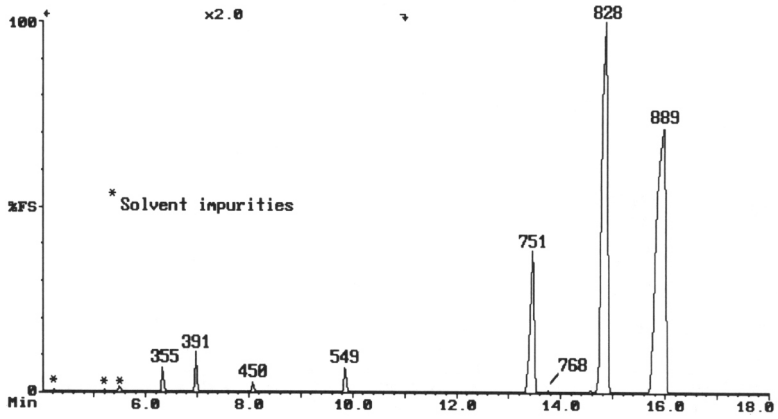


FIG. 2. Total ion chromatogram of volatile organic compounds extracted with dichloromethane from the preorbital secretion of a male klipspringer.

and distinctly fruity smell. In contrast to experience with other preorbital secretions, extraction of the volatile organic material from the secretion with dichloromethane was not very successful. An emulsion was formed and only a small percentage of the solvent was recovered when the emulsion was centrifuged. Nevertheless, using this extract, a satisfactory gas chromatogram was obtained (Figure 2). The extract contained only a few volatile constituents.

As the possibility existed that other, more volatile constituents could have been coeluted with the solvent, solventless sample introduction techniques were also employed. The neat secretion was, for example, injected onto glass wool in the injector liner and the volatile material desorbed from the glass wool by temperature programming the injector to allow the volatiles to be desorbed at the lowest possible temperature. Although the splitless introduction of large samples resulted in a total ion chromatogram with broad peaks and an unstable baseline, this method nevertheless produced results that confirmed the presence in the secretion of low concentrations of a few short-chain compounds.

The more elegant introduction of the neat secretion by a technique developed for the solventless introduction of biological material into the injectors of GC and GC-MS systems (Burger et al., 1990a) gave the total ion chromatogram shown in Figure 3. This method was used for qualitative as well as quantitative analyses.

Complications arising from the high water content of the secretion and the formation of ice in the column when applying cryotrapping were avoided by analyzing the headspace volatiles of the secretion after solid-phase microextraction (SPME) (Arthur et al., 1992). Total ion chromatograms exhibiting good

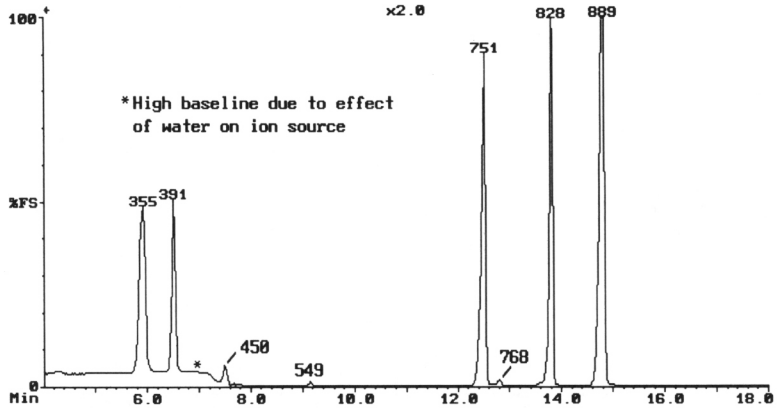


FIG. 3. Total ion chromatogram of volatile organic constituents of the preorbital secretion of a male klipspringer. The chromatogram was obtained by solventless introduction of the secretion into the injector of the GC-MS system.

capillary column resolution and an acceptable baseline were obtained. A typical total ion chromatogram obtained with this method is shown in Figure 4. Similar chromatograms were obtained when older material, deposited as territorial marks, was subjected to similar analyses (Figure 5). As expected, the more volatile constituents of the secretion were present in extremely low concentrations in the older territorial marks.

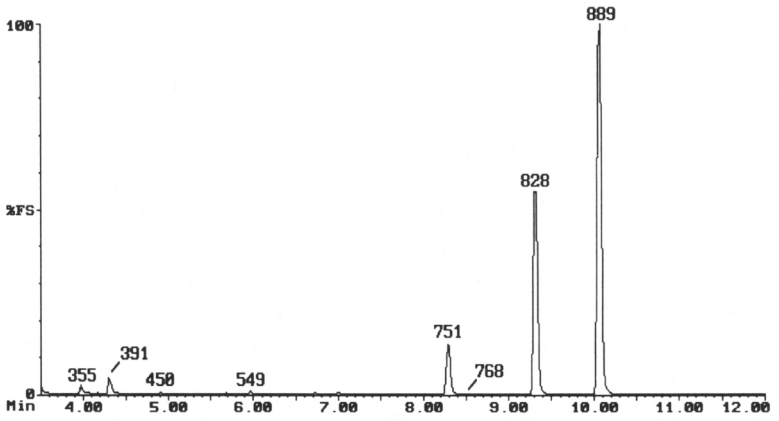


FIG. 4. Total ion chromatogram of the volatile organic constituents sampled by solid phase microextraction (SPME) from preorbital secretion, freshly collected from a male klipspringer.

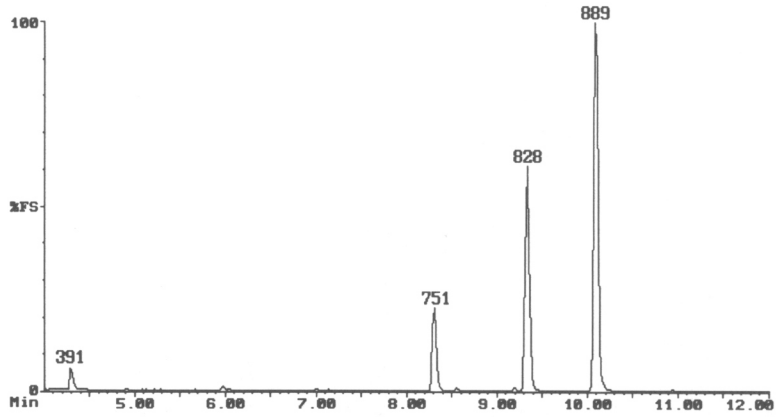


FIG. 5. Total ion chromatogram of volatile constituents sampled by SPME from a preorbital territorial mark deposited by a male klipspringer.

The volatile organic constituents identified in the preorbital secretion are listed in Table 1 together with the relevant mass spectral data and information on the quantitative composition of the secretion obtained in analyses with the synthetic substances as external standards.

Compared to the exocrine secretions from 12 other antelope species investigated in the Laboratory for Ecological Chemistry, the composition of the preorbital secretion of the klipspringer is extraordinarily simple. Whereas the other secretions typically contain at the very least about 80, and frequently more than 150 volatile organic constituents, only eight volatile organic compounds were consistently found in the fresh preorbital secretion of the klipspringer and in older territorial marks left in the field, regardless of the sample introduction technique employed. Five major constituents comprise about 98% of the total volatile organic fraction.

Characterization of Nonvolatile Fraction of Secretion. The fresh preorbital secretion has a tacky consistency when rubbed between the fingers. It was therefore assumed to contain a substantial amount of one or more polysaccharides or proteins.

Amino acid analysis after acid hydrolysis revealed that about 80% of the dry weight of the secretion consisted of protein. The results (Table 2) of the determination of the amino acid composition of the fresh (wet) secretion and old (dry) territorial mark gave practically identical results. Electrophoretic analysis (Figure 6) showed that both the fresh secretion and territorial marks consist of a relatively simple mixture of a few proteins ranging in molecular mass from 12 to 20 kDa. The similarity of fresh and old secretion is remarkable because

TABLE 1. COMPOUNDS IDENTIFIED IN PREORBITAL SECRETION OF KLIPSPRINGER

No. in Figure 2	Compound	Quantitative composition (μg/mg secretion)[a]	Mass spectra m/z (%)
355	3-Pentanone	0.020	86(30), 58(4), 57(100), 56(5), 55(4), 29(76), 27(40), 29(8)
391	Ethyl propanoate	0.032	102(14), 87(3), 75(14), 74(11), 73(7), 58(4), 57(100), 56(5), 45(12), 44(3), 43(6), 29(89), 27(39), 26(8)
450	4-Methyl-2-pentanone	0.0030	100(18), 85(15), 58(31), 57(20), 43(100), 42(14), 41(28), 39(26)
549	2-Methylpropyl acetate	0.0062	86(2), 73(12), 71(2), 61(2), 57(3), 56(20), 55(3), 44(3), 43(100), 42(11), 41(20), 39(17), 32(5), 29(11), 28(16), 27(14), 26(3)
751	5-Methyl-3-hexanone	0.083	114(20), 99(5), 85(40), 72(10), 58(7), 57(100), 56(5), 55(7), 43(31), 42(25), 41(56), 39(37), 32(19), 29(66), 28(49), 27(55)
768	4-Methyl-3-hexanone	0.0020	114(7), 86(5), 85(9), 58(4), 57(100), 56(8), 55(6), 53(3), 43(4), 42(10), 41(26), 40(4), 29(62), 28(39), 27(49), 26(9)
828	Ethyl 3-methylbutanoate	0.12	130(1), 115(10), 103(4), 88(100), 85(69), 70(19), 61(23), 60(50), 59(20), 57(84), 56(13), 55(12), 45(25), 43(58), 42(48), 41(94), 40(15), 39(79)
889	2-Methylpropyl propanoate	0.22	100(0.8), 86(7), 75(5), 57(100), 56(28), 55(6), 43(12), 41(27), 39(20), 32(6), 29(50), 28(27), 27(37), 26(7)

[a] A single collection from the two preorbital pouches of a klipspringer typically yielded 20–30 mg of secretion.

TABLE 2. AMINO ACID COMPOSITION OF PREORBITAL SECRETION OF KILPSPRINGER

Amino acid	Amount (mol %)[a]			
	Secretion		HPLC fractions	
	Dry	Fresh	F1	F2
Asp	9.3	9.4	2.1	13.8
Thr	11.2	11.7	14.5	5.7
Ser	14.5	15.7	24.6	6.0
Glu	11.6	11.6	1.6	19.1
Pro	nd	nd	16.8	nd
Gly	16.1	16.8	22.2	7.3
Ala	10.4	10.5	14.8	4.6
Val	3.3	3.4	0.1	5.9
Met	0.8	0.9	0.0	1.2
Ile	4.2	3.6	0.4	8.2
Leu	3.4	3.1	0.8	4.5
Tyr	3.1	2.9	0.3	4.7
Phe	3.6	2.1	0.8	7.7
Trp	nd	nd	0.0	nd
His	2.7	2.5	0.0	1.8
Lys	2.4	2.3	0.0	4.8
Arg	3.4	3.5	1.0	4.7

[a] Amounts are expressed in moles of amino acid per 100 moles of amino acid recovered. Dry and fresh refer to a dry territorial mark and freshly collected liquid sample, respectively. Amino acid analysis was done on 100 μg of each of these samples. F1 and F2 refer to fractions 1 and 2 in which the two major components were isolated from the freshly collected secretion by HPLC on a reverse-phase column.

territorial marks are subjected to harsh environmental conditions such as irradiation by sunlight and temperatures up to about 30°C. The frequent licking of the territorial marks, which brings the territorial marks into contact with certain enzymes, also does not seem to have any influence on the deposited material.

The molecular masses of three of the preorbital proteins were determined by electrospray mass spectrometry (ES-MS). The major protein has a molecular mass of 18,120.79 ± 15.05 Da and two of the minor proteins have molecular masses of 14,652.60 ± 19.02 and 17,631.34 ± 18.37 Da.

Further characterization of the proteins by reverse-phase chromatography on a Vydac C4 column revealed the presence of two fractions (Table 2, HPLC fractions 1 and 2) eluting at 20% and 40% acetonitrile concentration, respectively. Electrophoretic analysis of these fractions indicates that the more hydro-

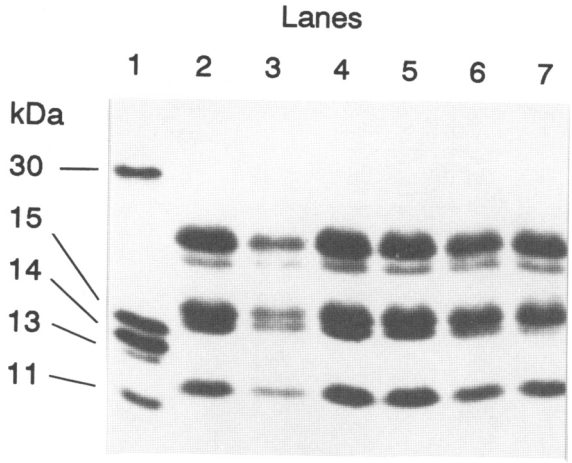


FIG. 6. SDS-PAGE of the proteinaceous fraction of the preorbital secretion of the klipspringer. Lane 1, chicken histones as mass marker proteins; lane 2, liquid secretion aged for several weeks at room temperature; lane 3, centrifuged liquid secretion (supernatant liquid); lane 4, fresh secretion; lane 5, centrifuged fresh secretion (supernatant liquid); lane 6, territorial mark dissolved/suspended in H_2O; lane 7, supernatant solution from a territorial mark dissolved in H_2O and centrifuged.

phobic fraction 2, comprising about 60% of the proteinaceous material, was heterogeneous and contained all the bands visible in the SDS-PAGE of the total secretion. Mass spectrometry (TOF-MALDI) revealed that fraction 2 contains proteins with molecular masses of about 18 kDa. The amino acid composition of this fraction is characteristic of a small globular albuminlike protein.

Fraction 1 has a very unusual composition in that it contained mainly five amino acids (Ser, Thr, Gly, Pro, and Ala). This, together with the fact that this fraction could not be stained by Coomassie blue protein stain, suggests the presence of a glycoprotein or mucoprotein (Hashimoto et al., 1964). TOF-MALDI mass spectrometry revealed that fraction 1 consisted of a broad peak at 25 kDa at various dilutions, indicating a fairly high degree of glycosylation. The anthrone test for carbohydrates showed that less than 3% of anthrone-positive sugars are present. The presence of other sugars cannot be excluded.

Preorbital Protein as Controlled-Release Medium. In order to find out whether the proteinaceous fraction of the klipspringer's preorbital secretion could possibly act as a controlled-release substance for the volatile organic constituents of the secretion, the desorption or evaporation of two of the major volatile constituents from the secretion was monitored using solid-phase microextraction (SPME). A large territorial mark that contained relatively old material was

selected for this experiment. The experiment was continued for six days, by which time the volatile components originally present in the mark were almost totally depleted and replaced by a large number of other compounds that were presumably extracted from the air. The results of this experiment are given in Figure 7. Arbitrary units are used, as absolute quantities have little significance in an experiment such as this, in which the territorial mark has been deposited over an unknown period and which is, furthermore, not homogeneous as far as the volatile content of its older parts and freshly deposited material are concerned. If it is taken into consideration that traces of the volatile constituents of the secretion will evaporate from a twig within a few hours if deposited in the absence of a controlled-release substance, it is clear that the proteinaceous material considerably prolongs the release of the volatile constituents of the secretion. However, it also has to be realized that this effect could be due merely to the bulk of material in which the volatiles are suspended and not necessarily to the protein being a particularly efficient controlled-release material.

An attempt was also made to find out whether the preorbital proteinaceous material has a higher affinity for the volatile organic constituents of the secretion than other proteins. The basic principle of an experiment devised for this purpose is the exposure of four glass-fiber wicks, coated with different proteins, to a source of the volatile material until equilibrium is reached. By determining the quantities of the volatile material retained by each of the proteins, a rough estimate of the affinity of the proteins for the volatiles can be obtained. Bovine albumin, bovine pancreas trypsin, and chicken egg white lysozyme were compared with the preorbital protein. Three of the volatile constituents of the preorbital secretion, 3-pentanone, isobutyl acetate, ethyl isovalerate, and another standard, 3-pentanol, were chosen as volatile organic compounds.

The experiment was repeated several times using different equilibration and

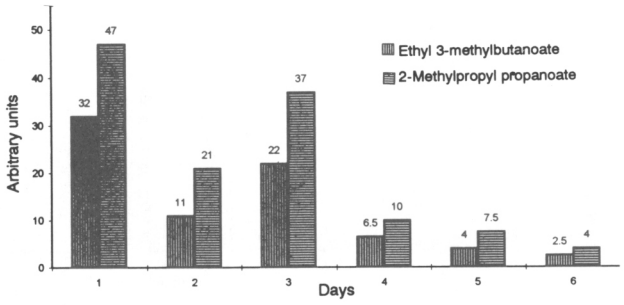


FIG. 7. Release of ethyl 3-methylbutanoate and 2-methylpropyl propanoate from a territorial mark. The headspace gas of the territorial mark (2.6 g) was sampled for 3 hr at 22°C once every 24 hr.

desorption parameters, starting with equilibration for 6 hr at 22°C and thermal desorption for 10 min at an injector temperature of 60°C. Although it was not attempted to determine the minimum time required to reach equilibrium, it was found that after 6 hr only very small quantities of the volatile compounds were left on the dispensing wick. With desorption of the volatiles from the protein-coated wicks for 15 min at an injector temperature of 60°C, the threshold conditions for complete desorption of the volatiles from the protein-coated wicks seemed to have been barely reached, as a faint smell of ethyl isovalerate could still be detected on removal of the wick from the injector before starting the analysis. Quantitative desorption was achieved in 15 min at an injector temperature of 80°C. Some of the results obtained in these experiments are summarized in Figure 8.

The affinity of the preorbital protein for organic volatiles of the secretion appears to be similar to that of albumin. Trypsin has a very low affinity for the volatiles, barely higher than the untreated glass fiber, whereas lysozyme appears to have a very high affinity, especially for the higher boiling esters. The contribution of the glass-fiber support to the retention of the volatiles is negligible. Apparently the klipspringer does not use a mixture of proteins having an excep-

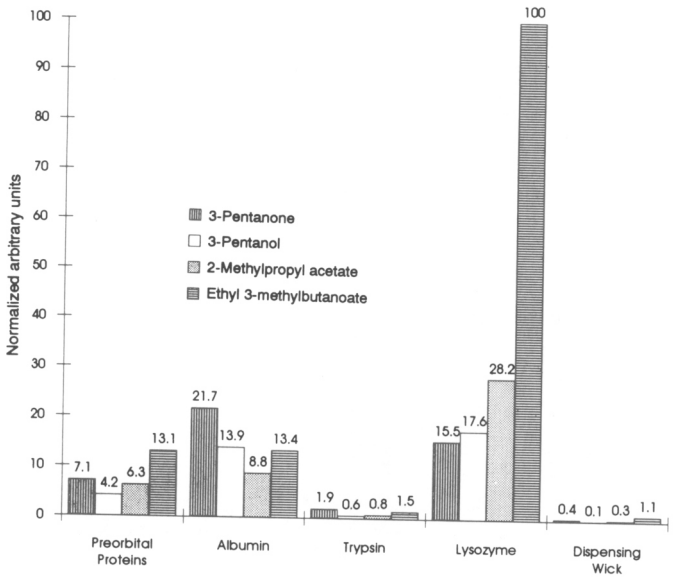


FIG. 8. Relative affinity of proteins for volatile organic compounds. Quantitative data were obtained by integrating the FID chromatograms of the volatiles desorbed (30 min at 80°C) from the dispensing wick and wicks coated with the proteins.

tionally high affinity for the volatiles in the secretion to control the release of these substances. However, the relatively large proportion of glycoprotein in the secretion results in an affinity for the hydrophobic volatile organic compounds approximately similar to the affinity of albumin. Lysozyme has an exceptionally high affinity for ethyl isovalerate. The large amounts of preorbital material deposited as territorial marks could possibly compensate for the lack of a highly specific affinity of the proteinaceous material for the volatile constituents of the secretion.

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PHENOLIC COMPOUNDS IN NATURAL SOLUTIONS OF A CONIFEROUS FOREST[1]

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Abstract—Phenolic compounds have been identified previously as potentially responsible for allelopathic interferences in spruce forest at high altitude. They have now been analyzed in canopy leachates, snow, and soil solutions collected from the three layers of the podsolic soil: OA, E, and B. Leachates were characterized by high tanning capacity and by *p*-hydroxyacetophenone (found at 10^{-6} M) also detected as the major (10^{-7} M) monomeric compound in snow. At least 10 phenolic monomers, including vanillic, *p*-hydroxybenzoic, and protocatechuic acids were identified in capillary waters extracted from the OA layer with their sum reaching 2×10^{-6} M. These monomers were only a small part (1%) of the total phenolics. In soil solutions, significant decreases in phenolic concentrations with depth were observed between the E and B layers, with qualitative modifications of the phenolic pattern. Spruce leachates and soil solutions exhibited high temporal variability, resulting in transitory allelopathic potential towards both aerial and subterranean parts of spruce seedlings. The occurrence of various and soluble phenolic coumpounds in the whole forest system, including *p*-hydroxyacetophenone, which persist from green needles to soil solutions, suggests that they could be involved in complex and interactive processes occurring during organic matter accumulation on the coniferous forest floor.

Key Words—Coniferous forest, leachates, snow, soil solutions, allelopathy, tanning activity, phenolic acids, *p*-hydroxyacetophenone, *Picea abies*.

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INTRODUCTION

There is growing evidence that phenolic compounds play a central role in controlling litter degradation rates and nutrient cycling processes in forest systems (Sugai and Schimel, 1993; Northrup et al., 1995). Direct toxicity of some of these molecules on tree seed germination (Odén et al., 1992; Pellissier, 1993), tree seedlings (Kil and Yim, 1983; Inderjit and Mallik, 1996), and microorganism growth (Field and Lettinga, 1992) is also now well documented, mostly from greenhouse experiments. The establishment of such interactions, also called allelopathy, has led to numerous identifications of phenolic compounds in plant materials (fresh or decaying leaves, roots, shoots, buds, flowers, etc.) and in the soil organic layers. Quantification procedures of these compounds have principally involved soil samples collected on the field and analysis by water leaching and extraction (Whitehead et al., 1983) or more drastic procedures using complexants such as sodium pyrophosphate (Vance et al., 1985) or organic solvents (Dalton et al., 1987). The major conclusion of these studies was that the quantitative results vary mainly with the method of analysis. Thus, controversy on the reality in the field of such chemical inhibitions has increased essentially because of the difficulties involved in measuring the amounts of allelochemicals really available to living organisms.

On the other hand, our knowledge of the transport and of the extracellular stability of such secondary metabolites in natural solutions is incomplete. However, leachates and throughfalls, especially in coniferous forests, are known to provide nonnegligible inputs of inorganic and organic compounds to the soil following interception of rainwater by canopy (or understorey) species (Parker, 1983; Arthur and Fahey, 1993). Furthermore, soil solutions contain various organic acids (including phenolic compounds) with chelating properties towards aluminum and iron, which play important roles in pedogenetic processes like podzolization (Vance et al., 1986, Pohlman and McColl, 1988). All these solutions could be suspected to be active in allelopathic processes, because of their strong ability to carry soluble molecules from the donor plant to the root system of the target plant.

The objective of this study was to verify if the potential allelochemicals (principally phenolic acids) previously isolated in plant, litter, and humus materials of high-altitude spruce (*Picea abies* L. Karsten) forest (Gallet and Lebreton, 1995) were recovered in detectable amounts in natural solutions in the vicinity of spruce seedlings, potentially sensitive to such metabolites. Among these compounds, *p*-hydroxyacetophenone represents an efficient marker of spruce metabolism, because of its abundance and specificity. The solutions studied included: throughfalls and snow, which could reach the aerial part of the target plant, and soil solutions in close contact with the root system, i.e., capillary waters (Zabowski and Ugolini, 1990).

METHODS AND MATERIALS

Study Site. Sampling in 1993 was at 1860 m in a bilberry–spruce forest (530 trees/ha) in Tarentaise (northern Alps, Savoie, France) on a humoferric podzol, characterized by poor spruce regeneration on decaying stumps (Bernier et al., 1993). The understorey was dominated by crowded bilberry (*Vaccinium myrtillus* L.) areas, with litterfalls of 360 kg dry wt/ha/yr. The mixed litter (3 cm thick) was spruce needles (litterfalls = 650 kg dry wt/ha/yr), bilberry leaves, and moss fragments on a mor humus OA (H_2O pH = 3.8, C-to-N ratio = 65). Thicknesses of the three layers of the humoferric podzol, OA, E, B (AFES, 1992), were 8, 20, and 25 cm, respectively, with weakly pronounced transitions.

Throughfalls and Snow Sampling. Throughfalls were collected during September and October with PVC gutters and glass funnels under three different adult trees, as previously described (Gallet, 1994). Throughfalls were protected from litter contact by a 2-mm PVC mesh. Incident rain (as control) was collected with the same apparatus at a single site in a natural clearing. The samples were brought back to the laboratory as soon as possible after rain, and immediately analyzed.

Triplicate snow samples were collected in under spruce during May, allowing potential leaching by melting snow of fallen stems and needles. Control snow was also collected at a single site in a clearing, where there were no influences of tree fallouts. The snow layer was divided into three 5-cm-high slices in spruce sites (e.g., surface, medium, and basic) and into two 10-cm-high slices in the control site (e.g., surface and basal). Volumes equivalent to 1 liter of water were stored in polyethylene bags and immediately analyzed after melting at room temperature and filtering.

Extraction of Soil Solutions. Many methods used to obtain soil solutions cause modifications of the chemical content. In this study, soil centrifugation was selected because of the negligible impact on the organic content of the capillary waters (Giesler and Lundstrom, 1993; Keller, 1995). Soil samples were collected from July through October after three significant rains had caused leaching down to the B layer. Once drainage was finished, soils were sampled with an auger. Because a minimum volume of 300 ml of soil solutions was found to be necessary for phenolic monomer extraction, the fresh weight of soil extracted was ca. 1 kg for the OA horizon, and ca. 2 kg for the E and B horizons. At least five different samples were bulked into a single sample for each horizon. After storage at −18°C and then thawing, soil samples were centrifuged at 3400*g* (Sorvall RC apparatus, HS4 rotor, and swinging buckets) at 4°C for 90 min (Keller, 1995). Centrifuged solutions were drawn from the collection cup with a pipet and bulked until 300 ml was obtained. The whole was then filtered.

Chemical Analysis. Dissolved organic carbon (DOC) was analyzed by IR detection, Shimadzu 500) and pH was measured on one aliquot of each solution.

Total phenols were determined with Folin-Ciocalteu reagent using gallic acid as a standard (Marigo, 1973). Tanning capacity was determined by the colorimetric method of Bate-Smith, modified by Schultz et al. (1981), with tannic acid as a standard. Monomeric compounds (phenolic acids sensu lato) were extracted from acidified solutions (by 2 N HCl) with ethyl ether and redissolved in ethanol before HPLC analysis as described earlier (Gallet, 1994).

Statistical Analysis. Matched data were compared using Wilcoxon signed-ranks test for dependant variables (for $N = 3$, P associated can not go below 0.125), and means were compared using the Mann-Whitney U test. Proportions were compared using the binomial test.

RESULTS

The brown-orange coloration of the spruce throughfalls, compared with the colorless incident rain sampled in the clearing gave evidence of great chemical changes, confirmed by the phenolics analysis (Table 1). The pH of the spruce leachates was significantly lower than that of the incident rain and the total phenolic content had increased by about 50-fold. Total phenolic compounds included mostly polymerized compounds, as revealed by the high tanning capacity compared with the low amounts of phenolic monomers detected by HPLC analysis. Amounts of *p*-hydroxyacetophenone reached a mean value close to 10^{-6} M (or 0.11 mg/liter, e.g., 0.4% of total phenols). Under the spruce canopy, comparisons between the two sampling dates gave relevant differences in total phenols and *p*-hydroxyacetophenone content.

Snow samples collected under spruce trees exhibited a few more total phenols than samples of the control site (Table 2). In spruce stands, surface slices (10–15 cm) contained significantly more total phenols compared to medium slices (5–10 cm). Differences between surface slices and basal slices (0–5 cm) were not significant, probably because of the very high variability of the latter. *p*-Hydroxyacetophenone was the only compound present in snow in detectable amounts, with higher concentrations in surface slices.

Organic analysis of capillary waters of the three layers of the podzolic soil (Table 3) revealed no differences in pH and total phenol amounts. More visible modifications occurred when reaching the B layer: the pH became less acidic, and the total phenolics were reduced to approximately half of those in the E layer. Phenolic carbon, estimated by Folin-Ciocalteu method, accounted only for about 10% of DOC, and without significant correlation between DOC and total phenols (in milligrams per liter) ($r = 0.33$). Temporal variability was significant, with far higher concentrations in total phenols observed for the first sampling date compared with the two others (Wilcoxon matched sign test, $N = 3$, $P = 0.125$). Monomeric analysis (Table 4) showed the same trend, i.e.,

TABLE 1. pH AND PHENOLIC COMPOSITION OF INCIDENT RAIN (SAMPLED AT ONE SITE IN A CLEARING) AND OF SPRUCE TTHROUGHFALLS (SAMPLED IN TRIPLICATE)[a]

	pH	Total phenols (mg eq/liter)	Tanning capacity (mg eq/liter)	Phenolic monomers (μmol/liter)	
				Pha	Others
Control (incident rain)					
September 25	4.9	0.9	0	0	0
October 2	5.3	0.4	0	0	0
Mean	5.3a	0.5a	0	0	0
Spruce[b]					
September 25	4.2 (0.2)	27.5 (5.5)	713 (40)	1.4 (0.7)	protocatechuic acid: tr *p*-hydroxybenzoic acid: tr
October 2	4.4 (0.3)	22.0 (2.1)	443 (343)	0.2 (0.1)	protocatechuic acid: tr *p*-hydroxybenzoic acid: tr
Mean	4.3b	24.7b	578	0.8	

[a]Different superscript letters for mean values indicate significant differences (Mann-Whitney test, $P < 0.05$).
[b]Mean (and SD) of three samples. Pha: *p*-hydroxyacetophenone. tr: trace.

TABLE 2. PHENOLIC COMPOSITION OF SNOW SAMPLED BY SLICES ON THE GROUND AT A CLEARING (ONE REPLICATE) AND OF SPRUCE FOREST (THREE REPLICATES) DURING MAY[a]

	pH	Total phenols (mg eq/liter)	Tanning capacity (mg eq/liter)	Pha (μmol/liter)
Control				
10–20 cm	5.6	0.68	0	0
0–10 cm	5.4	1.42	0	0
Spruce[b]				
10–15 cm	5.6 (0.2)	2.30 (1.43)	0	0.2
5–10 cm	5.5 (0.1)	1.01 (0.32)	0	0.05
0–5 cm	5.4 (0.1)	3.33 (2.51)	0	tr

[a]Thickness is given from the ground. Pha: *p*-hydroxyacetophenone.
[b]Mean (and SD) of three samples. For HPLC quantification, the three replicates were bulked before analysis.

TABLE 3. pH, DOC, AND TOTAL PHENOL CONTENTS OF CAPILLARY WATER COLLECTED IN THREE LAYERS OF PODZOLIC SOIL UNDER SPRUCE COVER[a]

	pH	DOC (mg/liter)	Total phenols	
			mg eq/liter	% of DOC
OA layer				
July 5	3.5	330	39.8	18
July 13	3.9	480	29.6	6
October 13	3.5	570	21.7	3
Mean	3.6a	460a	30.4a	8a
E layer				
July 5	3.6	450	65.9	16
July 13	3.4	360	18.1	7
October 13	3.7	318	16.4	8
Mean	3.6ab	376a	33.5a	11ab
B layer				
July 5	4	360	30.9	12
July 13	3.9	390	9.9	3
October 13	4.3	210	10.0	11
Mean	4.1ac	320a	16.9b	8ac

[a]Total phenols are expressed in milligrams of gallic acid equivalent per liter and as percentage of DOC (with 49.4% of C in gallic acid). Different superscript letters for mean values indicate significant differences (Wilcoxon signed-rank test, $P < 0.125$).

TABLE 4. PHENOLIC COMPOSITION OF CENTRIFUGE WATERS FROM THREE LAYERS OF PODZOLIC SOIL[a]

	Phenolic monomers (μmol/liter)							Sum	
	Phe[b]	Bal	Pro	Phb	Van	Cin	Pha	μmol/liter	‰ total phenols
OA layer									
5 july	0.37	0.03	0.32	0.55	0.98	0.14	0.14	2.52	11
13 july	0.09	0.02	0.06	0.17	0.30	0.15	0.02	0.80	5
13 october	0	0.01	0.12	0.18	0.30	0.25	0.09	0.94	7
Mean	0.15	0.02	0.17	0.30	0.53	0.18	0.08	1.43a	8a
Proportion	11%a	1%a	12%a	21%a	37%a	13%a	6%a		
E layer									
5 july	0.11	0.05	0.49	0.57	4.12	0.47	0.17	5.97	15
13 july	0	0	0.07	0.16	0.19	0.09	0.03	0.53	5
13 october	0	0	0.04	0.11	0.22	0.08	0.03	0.47	5
Mean	0.04	0.02	0.20	0.28	1.51	0.21	0.08	2.32ab	12ab
Proportion	2%b	1%a	9%a	12%b	65%b	9%a	3%ab		
B layer									
5 july	0	0	0.16	0.43	2.28	0.21	0.34	3.42	19
13 july	0	0	0.05	0.09	0.20	0.08	0.01	0.42	7
13 october	0	0	0.06	0.04	0.09	0.06	0.05	0.28	5
Mean	0	0	0.09	0.19	0.86	0.12	0.13	1.37ac	14ac
Proportion	0%b	0%a	7%a	14%b	63%b	9%a	9%ac		

[a]OA layer: 8–0 cm; E layer: 0–25 cm; B layer: 25–50.

[b]Phe: catechol + hydroquinone; Bal: *p*-hydroxybenzaldehyde; Pro: protocatechuic acid; Phb: *p*-hydroxybenzoic acid; Van: vanillic acid; Cin: cinnamic acids (caffeic + *p*-coumaric + ferulic acids); Pha: *p*-hydroxyacetophenone. Sum: sum of all the identified phenolic acids. Proportion indicates the percentage of each phenolic acid compared to the sum. Different superscript letters by column for mean values indicate significant differences (Wilcoxon signed-rank test, $P < 0.125$ for sum of phenolic acids; binomial test for percent, $P < 0.05$).

minor differences between OA and E solutions, but a significant decrease of the sum of all monomeric compounds between the E and B layers. The relative proportions of each phenolic acid identified in the soil solutions are similar for the three layers, with vanillic acid > *p*-hydroxybenzoic acid ~ ferulic + *p*-coumaric acids > protocatechuic acid > *p*-hydroxyacetophenone > catechol + hydroquinone > *p*-hydroxybenzaldehyde as the general pattern. The contributions of simple phenols and aldehydes to phenolic monomers decreased with depth, and these compounds were not present in B layer solutions. Protocatechuic acid and cinnamic acids were relatively stable in all solutions, while increasing proportions of *p*-hydroxyacetophenone and of vanillic acid occurred with depth. The contribution of phenolic acids to the total phenolic fraction was low and essentially constant with depth, with a significant correlation between their sum and the total phenols ($r = 0.93$, $N = 9$, $P = 0.001$). The phenolic acids content of the earliest samples was higher than the others for vanillic acid.

Total phenol and phenolic monomer amounts were of the same order in spruce throughfalls and in soil solutions collected below the OA layers, while snow samples appear much less concentrated. pH and total phenols were negatively correlated, when comparisons of throughfalls, snow, and soil solutions data are analyzed together ($r = -0.74$, $N = 22$, $P = 0.001$).

DISCUSSION

The phenolic patterns of the natural solutions sampled in the spruce stands show significant masses of numerous spruce metabolites and of some of their transformation products. For instance, spruce stems and needles release high quantities of tanning phenolics in throughfalls. Although no precise chemical identification has been attempted, it can be predicted that both proanthocyanidins found in needles (Gallet and Lebreton, 1995) and stilbenes present in bark (Hegnauer, 1966) will contribute to this fraction. The ecological impact of these polyphenols has been emphasized in living tissues through responses to pathogen attacks (Brignolas et al., 1995), but no studies on their extracellular and structural evolution (after tree death or after leaching outside) have been performed, especially in such acidic conditions. The great input of tannins close to the trunk could have major effects on the patterning of vegetation and microorganisms (Stöckli, 1991) in this area, through direct toxicity or by nitrogen (Schimel et al., 1996) and other elements, e.g., iron (Mila et al., 1996) immobilization. The concurrent slowing down of litter degradation would increase the organic matter accumulation, typical of such acidic coniferous system.

In the monomeric fraction, *p*-hydroxyacetophenone and vanillic acid appeared to be the most characteristic constituents of these natural solutions.

The ubiquitous occurrence (in snow, leachates, and soil) of *p*-hydroxyacetophenone is a proof of its persistence and its relative stability in the forest system. Indeed, unlike vanillic acid or other widespread phenolic acids identified in these solutions, *p*-hydroxyacetophenone is a specific metabolite, only synthesized by spruce, being neither a degradation product nor a microorganism metabolite. To our knowledge, this is the first report of the occurrence in snow of an allelochemical that appears to have been leached from the decaying needles visible in the snow. The greater concentration of *p*-hydroxyacetophenone in snow in close contact with soil (0–5 cm) compared to the upper layers could be through capillary action from the humus solutions below. In soil solutions, *p*-hydroxyacetophenone constitutes a higher proportion of the phenolic monomers in the deepest soil layer (B), suggesting a lack of specific microorganisms capable of degrading it. The extracellular stability of this compound is probably of great significance in spruce ecology, because of its fungistatic properties (Osswald et al., 1987) and as the aglycone moiety of the picein compound that has been identified as an indicator of spruce die-back (Hoque, 1985; Richter and Wild, 1992).

Following germination, the roots of spruce seedlings are in contact with macroporous water from the soil surface layer. Throughfalls and OA solutions exhibited qualitative as well as quantitative differences in their phenolic patterns, indicating inputs and/or transformation of materials as soon as the throughfalls reach the litter layers. For instance, the high tanning capacity observed in the spruce throughfalls will interact both with litter components and with microorganisms. The evolution of these phenolic patterns in the deeper layers gave some information on the biological activity in the soil. Biodegradation or adsorption and precipitation processes are generally low in the organomineral layers, although mainly active in the B layer, as previously shown by Guggenberger and Zech (1994). Qualitative differences in the patterns of phenolic acids in the different layers show biotransformations as, for example simplification of cinnamic acids (like ferulic acid) to benzoic acids (like vanillic acid). The disappearance of catechol, and to a lesser extent protocatechuic acid, with depth can be explained. These compounds are readily degraded or further polymerised (Barz and Weltring, 1985). The prevalence of vanillic acid in capillary waters was the result of its continuous release from spruce lignin, which is characterized by high vanillyl phenols content (Ziegler et al., 1985). Although *p*-hydroxyacetophenone, and to a lesser extent vanillic acid, can be seen as strict indicators of spruce metabolism, an important source of the other phenolic acids, such as the cinnamic acids, could be understorey species with dense cover like *Vaccinium myrtillus* (Gallet and Lebreton, 1995).

It is often difficult to demonstrate convincingly the allelopathic activity of phenolic compounds. The problem is that the concentrations of putative allelochemical compounds, when measured in field experiments are often lower

than the active concentrations displayed in greenhouse or in vitro experiments. For example, the inhibitory effect of *p*-hydroxyacetophenone on germination and primary growth of spruce seedlings has been established for concentrations of 10^{-4} M (Gallet, 1994), but the lower concentrations of the natural solutions found in this study (about 10^{-6} M in leachates, 10^{-7} M in snow and soil solutions) have been shown to be active against mycorrhizal fungi respiration (Boufalis and Pellissier, 1994). Comparable situations have been observed with other plant phenolic acids, often described as allelochemicals, despite their weak quantities in soils. In our study, the three types of solutions—leachates, snow, and OA solutions—were collected at different times over a four-month period. The results enabled a comparison of their respective concentrations, indicating increasing amounts (for total phenols, and sum of monomeric compounds) from snow to throughfalls and soil solutions, associated with decreasing pH. This is consistent with the conclusion that phenolic compounds could persist and accumulate in soil, but the polyphenols in leachates (e.g. tannins) probably have more of a complexing activity than the mixtures of polymerized molecules in the soil solutions. Indeed, phenolic monomers are only a minor fraction of the total phenolic fraction of forest soil solutions, determined as the "total phenols" using Folin-Ciocalteau reaction. Oligomers and soluble polymers (i.e., fulvic acids) of low molecular weight (< 1000) are known to be prevalent in soil solutions (Dawson et al., 1978), with as yet little detail available about their chemical structure, e.g., the number of aromatic rings. However, the influence of such compounds on plant growth has been shown (Dell'Agnola and Nardi, 1987; Vallini et al., 1993). Under field conditions, the aerial parts of the seedlings would be exposed from time to time to large amounts of *p*-hydroxyacetophenone and tannins, while the root system will be in permanent contact with a greater variety of phenolic monomers (at least 10 compounds) present in the macroporous and acidic waters of the organic layer.

Although certainly related to the interactive effects of climatic variations, soil status, and plant phenology, the observed temporal variability is difficult to explain because of the small number of replicates. Furthermore, it is possible that the variability is enhanced by the harsh conditions occurring at this altitude. Low-temperature, flooded conditions during the snowmelt period, and the brief growing period will all produce conditions unfavorable to degradation of the phenolic compounds (Dao, 1987), thereby producing peaks and troughs of phenolic fluxes throughout the spruce system.

The ubiquity, quantities, and activities of various spruce phenolic compounds seem compatible with their involvement in complex interactions occurring on forest floor, including direct allelopathic processes as well as nutrient immobilization. In all cases, a better knowledge of their field variability (spatial as well as temporal) might improve our understanding of their implications in forest dynamics.

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SEX PHEROMONE OF JAPANESE GIANT LOOPER, *Ascotis selenaria cretacea*: IDENTIFICATION AND FIELD TESTS

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Abstract—The Japanese giant looper, *Ascotis selenaria cretacea*, is a serious defoliator of tea gardens in Japan. GC-MS analysis of the virgin female extract confirmed the presence of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene. This compound had attracted male moths in a previous random screening test using C_{17}-C_{23} epoxydienes synthesized in a racemic form. Further GC and HPLC analyses with chiral columns showed that the natural pheromone was composed of 3*S*,4*R* and 3*R*,4*S* isomers in a ratio of 53:47, although the field evaluation revealed stronger activity of the pure 3*R*,4*S* epoxide than of other enantiomeric mixtures. This result indicates that the sexual communication system of this Japanese subspecies differs from that of the species in Israel, which is selectively attracted to an isomer with the opposite configuration. Interestingly, the 3*S*,4*R* epoxide attracted another geometric male, *Alcis angulifera*, in our field tests. (Z,Z,Z)-3,6-9-Nonadecatriene, a parent olefinic compound of the epoxy pheromone, was also identified in the gland extract of *A. s. cretacea*, but its effect on the attractive activity of the epoxide was not assured in field tests. The amount of the triene increased in the virgin female during photophase and also after decapitation. The epoxy component completely disappeared after decapitation, but could be increased by an injection of subesophageal ganglion extract. The data suggest that the triene is a biosynthetic precursor of the epoxide and that a neuropeptide hormone (PBAN) regulates the epoxydation.

Key Words—Sex pheromone, attractant, Japanese giant looper, *Ascotis selenaria cretacea*, (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene, (Z,Z,Z)-3,6,9-nonadecatriene, pheromone biosynthesis-activating neuropeptide.

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INTRODUCTION

The larva of the Japanese giant looper (mugwort looper), *Ascotis selenaria cretacea* Butler (Lepidoptera: Geometridae: Ennominae), is a polyphagous defoliator; it particularly favors tea, apple tree, and mugwort. Serious damage has been often observed in tea gardens in Mie and Shizuoka prefectures. By random screening tests of all the monoepoxy derivatives systematically synthesized from the (*Z*,*Z*,*Z*)-3,6,9-trienes and (*Z*,*Z*)-6,9-dienes with a C_{17}–C_{23} straight chain, it was found that the male Japanese giant looper moths are specifically attracted to (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene (epo3,*Z*6,*Z*9–19:H) (Ando et al., 1993, 1995). We now report here the identification of this epoxydiene in the extract of the pheromone glands of *A. s. cretacea* females by GC-MS analysis utilizing the synthetic standards. We also report the stereochemistry of the pheromone component and behavioral evaluation of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene (*Z*3,*Z*6,*Z*9-19:H), another component in the pheromone gland.

METHODS AND MATERIALS

Chromatography. Capillary GC was conducted with an HP 6890 Series gas chromatograph equipped with FID, a DB-23 column (50% cyanopropylmethylpolysiloxane, 0.25 mm ID × 30 m, J & W Scientific) or a Chiraldex A-PH column (0.25 mm ID × 20 m, ASTEC, Inc.), and helium as a carrier gas. The temperature program for the former column was 40°C for 1 min, 10°C/min to 160°C, and finally 4°C/min to 220°C, and samples were injected in a splitless mode. Analyses with the latter chiral column were conducted isothermally at 150°C, in a split mode with a 1:40 split ratio.

The HPLC was a JASCO PU-980 liquid chromatograph with a reversed-phase ODS column (8 mm ID × 25 cm, Senshukagaku, Tokyo, Japan) or Chiralpak AS column [4.6 mm ID × 50 cm (two 25-cm columns connected in series), Daicel Chemical Industry Co., Ltd., Tokyo, Japan]. The solvent used for the former reversed-phase column was 7% water in methanol (flow rate: 1.5 ml/min), and that for the latter chiral column was 0.1% 2-propanol in *n*-hexane (flow rate: 0.45 ml/min). Epo3,*Z*6,*Z*9–19:H was monitored by a UV detector operated at 215 nm.

GC-MS Analysis. Electron impact (EI) GC-MS was conducted with a Jeol JMS-SX 102A mass spectrometer with the previously listed DB-23 capillary column and the same temperature program as in GC analysis. The ionization voltage was 70 eV, and the ion source temperature was 240°C. Spectral ions (*m/z* 50–800) were measured twice per second in a cyclic scan mode lasting from 10 to 26 min after the sample injection.

Insects and Pheromone Extracts. Larvae of *A. s. cretacea* were reared on a semisynthetic diet for the silkworm [Insecta LF(S), Nippon-Nosan-Kogyo Co,

Yokohama, Japan] at 20°C in an uncovered container. They were kept in a dark room before the fourth instar to prevent their escape from the container, and on a 16L:8D cycle beginning with the fourth instar. Terminal abdominal segments (eighth to tenth), including the pheromone glands, were removed from about 400 female moths (2 or 3 days old) in the scotophase and soaked in *n*-hexane for 30 min to extract the sex pheromone. This extract was submitted to GC and GC-MS measurements without any purification. For the chiral HPLC analysis, this extract was partially purified by HPLC with an ODS column to obtain the fraction including epo3,Z6,Z9-19:H [retention time (R_t): 16.5 min]. To examine the quantitative change in the components in the pheromone gland in a day, some ovipositors of 2-day-old females were removed 2 or 6 hr after the beginning of the scotophase, and 4 or 12 hr after the beginning of the photophase. For each experiment, the pooled extracts from five ovipositors ($N = 6$) were analyzed by GC with a DB-23 capillary column.

Decapitation and Hormone Injection. One-day-old virgin females of *A. s. cretacea* were decapitated 2 hr after lights-off. One day after the decapitation, the females were injected with synthetic pheromone biosynthesis-activating neuropeptide (PBAN) of *Bombyx mori* (Bom-PBAN I, 20 ng/female) (Kitamura et al., 1989), which was purchased from Peninsula Laboratories, Inc. (Belmont, California), and dissolved in *B. mori* saline at a concentration of 1.0 μg/ml. Another group of the decapitated females was treated with an extract of brain–subesophageal ganglion (SG) complexes removed from the heads of *A. s. cretacea*. Fifty complexes of 1-day-old females in the scotophase were homogenized with 200 μl of saline, and the extract [20 μl, 5 female equivalent (FE)] was injected into each decapitated female after partial purification by centrifugation and heating in the same manner as for the brain-SG extract of *B. mori* (Ando et al., 1996). The pheromone glands of the females were removed 3 hr after the injection, and the extracts were analyzed by capillary GC and compared with those of the females just decapitated. For each experiment, six groups of five females were used.

Chemicals. Z3,Z6,Z9-19:H, epo3,Z6,Z9-19:H, and other authentic compounds, which were synthesized starting from linolenic acid (Ando et al., 1993), were purified by a Lobar column (Merck LiChroprep Si 60) (Ando et al., 1995), and chemicals with a purity >97% were used for the field experiments. Both enantiomers of epo3,Z6,Z9-19:H with >99% enantiomeric excess were obtained by chiral HPLC separation on a Chiralpak AS column (Qin et al., 1997).

Field Evaluation. One thousand micrograms of each of several mixtures (9:1, 5:5, 1:9) of Z3,Z6,Z9-19:H and epo3,Z6,Z9-19:H were applied to a gray rubber septum (8 mm OD), and field attraction was compared with that of the single component. Optically active isomers of epo3,Z6,Z9-19:H with 3*S*,4*R* or 3*R*,4*S* configuration and their mixtures (9:1, 8:2, 5:5, 2:8 and 1:9) were

tested at the level of 400 μg/septum. These lures were put in sticky traps (30 × 27 cm bottom plate with a roof, Takeda Chemical Ind., Ltd., Osaka, Japan) and placed at 1.0-m height from the ground in a tea garden (Kameyama-shi and Yokkaichi-shi in Mie prefecture) and at 1.5-m height in a mixed forest area in the suburbs of Tokyo (Rolling Land Laboratory, Tokyo University of Agriculture and Technology, Hachioji-shi, Tokyo).

RESULTS

Identification by GC-MS. An EI GC-MS analysis of 1 FE sex pheromone extract from *A. s. cretacea* produced the total ion chromatogram (TIC) shown in Figure 1A. Although there were many unknown compounds in the extract, the identity of two compounds eluting from the capillary column at 13.63 min (compound I) and 18.82 min (compound II) could be determined by comparing the R_ts and mass spectra with those of authentic compounds. The spectrum of compound I (Figure 1B), which presented M^+ at *m/z* 262 and fragment ions at *m/z* 206, 135, 108, and 79 (base peak), is identical with that of synthetic *Z*3,*Z*6,*Z*9–19:H. On the other hand, M^+ at *m/z* 278 and $[M\text{-}H_2O]^+$ at *m/z* 260 in Figure 1C indicated a possibility that compound II is a C_{19} epoxydiene. The spectrum of compound II including characteristic ions at *m/z* 220, 206, 108, and 79 (base peak) agrees well with that of synthetic epo3,*Z*6,*Z*9–19:H, but differs from (*Z*,*Z*)-3,9-*cis*-6,7-epoxynonadecadiene (*Z*3,epo6,*Z*9–19:H, R_t: 18.57 min) and (*Z*,*Z*)-3,6-*cis*-9,10-epoxynonadecadiene (*Z*3,*Z*6,epo9–19:H, R_t: 18.87 min), shown in Figures 1D and E. To confirm the position of the epoxy ring, mass chromatograms were recorded by monitoring the ions at *m/z* 220 and 206 for epo3,*Z*6,*Z*9–19:H, *m/z* 209, 195, and 111 for *Z*3,epo6,*Z*9–19:H; *m/z* 169 and 155 for *Z*3,*Z*6,epo9–19:H, and *m/z* 79 common to the three epoxides (Ando et al., 1993). Chromatograms of the pheromone extract unmixed and mixed with synthetic *Z*3,epo6,*Z*9–19:H and *Z*3,*Z*6,epo9–19:H are shown in Figure 2A and B, which reveal that the pheromone gland includes only epo3,*Z*6,*Z*9–19:H. Furthermore, the pheromone extract was examined in detail for traces of other C_{17}–C_{23} (*Z*,*Z*,*Z*)-3,6,9-trienes, (*Z*,*Z*)-6,9-dienes, and their monoepoxy derivatives. However, even with the injection of 10 FE, no other pheromonal candidates were detected on the mass chromatograms monitored for M^+ and characteristic ions (Ando et al., 1993, 1995).

Stereochemistry of Expoxydiene. Chiral capillary GC with an α-cyclodextrin-type column (Chiraldex A-PH) successfully separated two enantiomers of epo3,*Z*6,*Z*9–19:H, (3*S*,4*R*)-(+) isomer (R_t: 40.54 min) and (3*R*,4*S*)-(−) isomer (R_t: 39.87 min). Analysis of the crude pheromone extract (10 FE) showed both the 3*S*,4*R* and 3*R*,4*S* isomers in a ratio of 53:47 (Figure 2C). These enantiomers are also separable by chiral HPLC with an amylose-type column (Chiralpak AS)

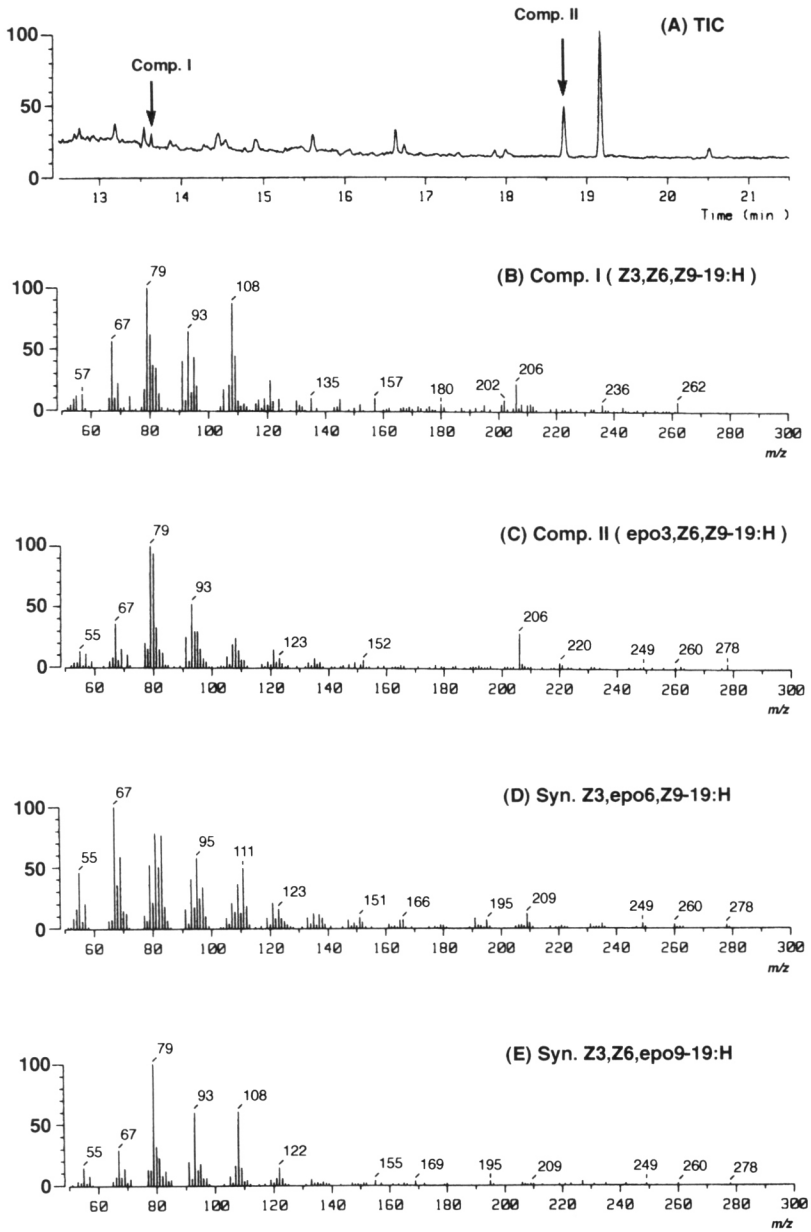


FIG. 1. GC-MS analysis of *A. s. cretacea* pheromone and synthetic standards. (A) Total ion chromatogram (TIC) of the pheromone gland extract (1FE), (B) mass spectrum of compound I (Z3,Z6,Z9-19:H), (C) mass spectrum of compound II (epo3,Z6,Z9-19:H), (D) mass spectrum of synthetic Z3,epo6,Z9-19:H, and (E) mass spectrum of synthetic Z3,Z6,epo9-19:H.

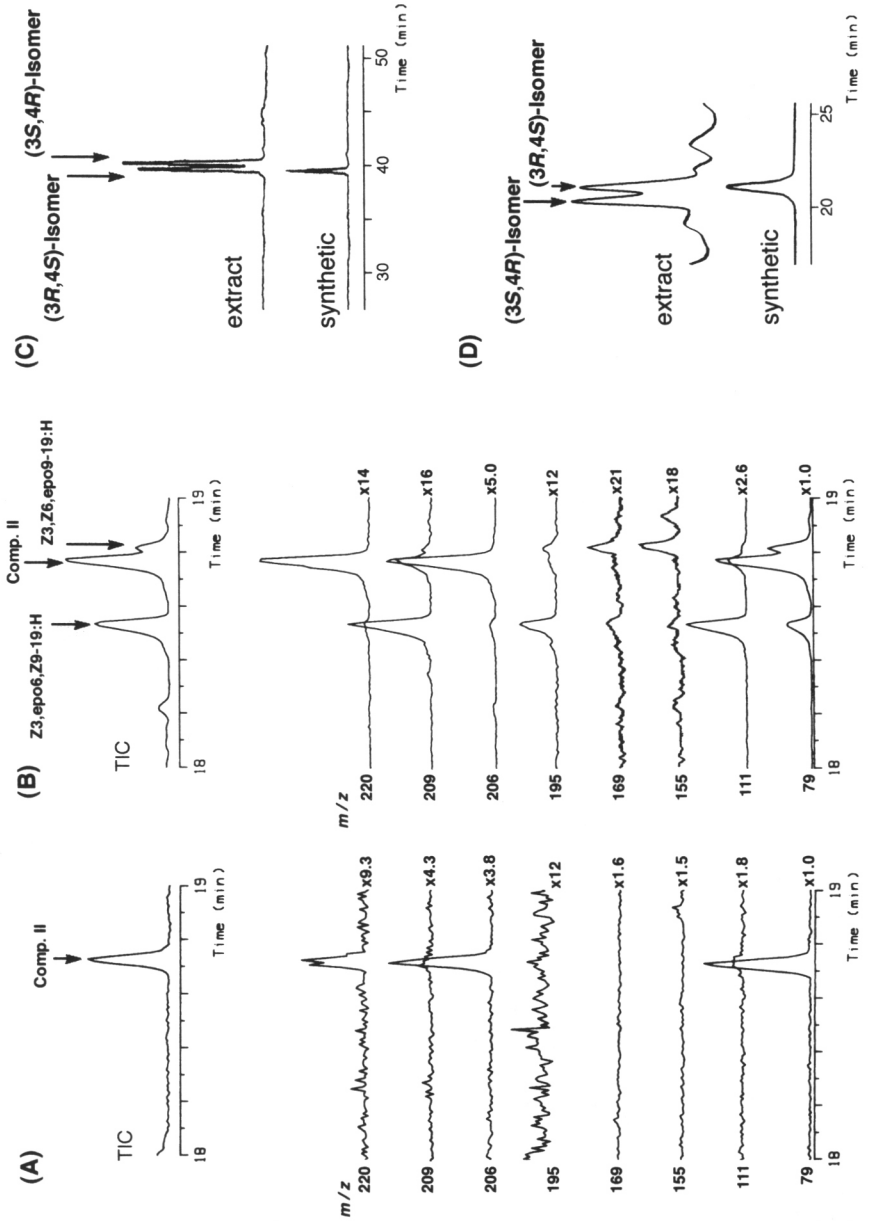

(A)
Comp. II
TIC
18
19
Time (min)
m/z
220
209
206
195
169
155
111
79
x9.3
x4.3
x3.8
x12
x1.6
x1.5
x1.8
x1.0
(B)
Comp. II
Z3,epo6,Z9-19:H
Z3,Z6,epo9-19:H
TIC
18
19
Time (min)
m/z
220
209
206
195
169
155
111
79
x14
x16
x5.0
x12
x21
x18
x2.6
x1.0
(C)
(3S,4R)-Isomer
(3R,4S)-Isomer
extract
synthetic
30
40
50
Time (min)
(D)
(3S,4R)-Isomer
(3R,4S)-Isomer
extract
synthetic
20
25
Time (min)

TABLE 1. QUANTITATIVE ANALYSIS OF TRIENE (Z3,Z6,Z9-19:H) AND EPOXIDE (epo3,Z6,Z9-19:H) IN PHEROMONE GLAND OF *Ascotis selenaria cretacea*[a]

Female[b]	Content (ng/female)		Ratio epoxide/triene
	Triene	Epoxide	
Normal			
2 hr after lights off	2.1 ± 0.6	71 ± 36	34
6 hr after lights off	2.6 ± 0.8	54 ± 21	21
4 hr after lights on	2.4 ± 0.8	63 ± 13	26
12 hr after lights on	16 ± 12	16 ± 11	1.0
Decapitated	6.5 ± 5.4	0.0 ± 0.0	0.0
Injected with Bom-PBAN[c]	2.6 ± 0.5	36 ± 13	14
Injected with SG extract[d]	1.9 ± 0.3	46 ± 19	24

[a]At least six groups of the extract from five females (2-day-old) were analyzed by GC.
[b]Insects were reared on a 16L:8D cycle from the fourth instar.
[c]Decapitated females were injected with synthetic Bom-PBAN I.
[d]Decapitated females were injected with an extract of brain-SG complexes of *A. s. cretacea*.

(Qin et al., 1997); 3*S*,4*R* isomer R_t = 20.78 min and 3*R*,4*S* isomer R_t = 21.39 min. After purification with reversed-phase HPLC, the natural epoxide (50 FE) was injected into the chiral column, and a ratio of the two isomers similar to that above was observed (Figure 2D).

Quantitative Analysis by GC. Quantities of Z3,Z6,Z9-19:H and epo3,Z6,Z9-19:H in the pheromone gland of 2-day-old virgin females were analyzed by GC. As shown in Table 1, the amount of each component varied somewhat in the scotophase and early photophase. The ratios of these two components were 1:21 and 1:34. In contrast, the triene increased remarkably at the end of the photophase, and the epoxide decreased to the level of the triene. After decapitation, the epoxide completely disappeared from the pheromone gland, while rather abundant triene was detected in it. The decapitated female produced the epoxide by an injection of synthetic Bom-PBAN I or an extract of brain-SG complexes removed from virgin females. The triene decreased again

FIG. 2. Mass chromatograms and chiral chromatograms of *A. s. cretacea* pheromone. (A and B) Mass chromatograms of the pheromone gland extract (1FE) without and with synthetic standards (Z3,epo6,Z9-19:H and Z3,Z6,epo9-19:H) monitoring characteristic fragment ions of monoepoxy derivatives of Z3,Z6,Z9-19:H, (C) chiral GC chromatograms of the pheromone extract (10FE) and authentic (3*R*,4*S*)-epo3,Z6,Z9-19:H on a Chiraldex A-PH column, and (D) chiral HPLC chromatograms of the pheromone (50FE) extract and authentic (3*R*,4*S*)-epo3,Z6,Z9-19:H on a Chiralpak AS column.

TABLE 2. FIELD TESTS OF MIXTURES OF SYNTHETIC TRIENE (*Z*3,*Z*6,*Z*9-19:H) AND EPOXIDE (epo3,*Z*6,*Z*9-19:H) FOR *Ascotis selenaria cretacea* IN TEA GARDENS IN MIE PREFECTURE (KAMEYAMA-SHI) IN 1996

Treatment (μg/septum)			Males captured (*N*)[a]	
Triene	Epoxide	Ratio	June 13-15[b]	September 7-17[c]
1000	0	10:0	0c	
900	100	9:1		19c
500	500	5:5	1c	51b
100	900	1:9	36b	120a
0	1000	0:10	64a	108a
0	0		0c	0d

[a]Numbers within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple range test.
[b]Captured by two traps.
[c]Captured by three traps.

in the injected females, and the ratios of the two components are almost the same as those of virgins in the scotophase.

Field Attraction. Table 2 shows the results of the field evaluation of synthetic *Z*3,*Z*6,*Z*9-19:H and epo3,*Z*6,*Z*9-19:H in the tea gardens in Mie prefecture. The lure baited only with the epoxide could efficiently attract males of *A. s. cretacea*. However, the triene was not attractive. The mixture of triene and epoxide in a 1:9 ratio seemed to have a synergistic effect on the activity of epo3,*Z*6,*Z*9-19:H in September, but attraction of the mixture was clearly weaker than that of the epoxide alone in June. The 5:5 ratio of the two components observed in the pheromone gland in the photophase is inadequate for attraction. The results from field tests with the two enantiomers of the epoxide are shown in Table 3. Although the natural pheromone is a 53:47 mixture of 3*S*,4*R* and 3*R*,4*S* isomers, traps baited with only the 3*R*,4*S* isomer caught the largest number of males in tea gardens in Mie prefecture and in a forest in Tokyo. In the forest, traps baited with the 3*S*,4*R* isomer captured another geometric species, *Alcis angulifera* Butler, which had been attracted to the racemic mixture of the epoxide in our previous random screening tests (Ando et al., 1993).

DISCUSSION

Female sex pheromones have been characterized from 29 species of Geometridae (Lepidoptera). Sex attractants of another 70 geometrid species have been found by field screening of synthetic compounds (Arn et al., 1992, personal

TABLE 3. FIELD TESTS WITH TWO ENANTIOMERS OF EPOXIDE (epo3,Z6,Z9-19:H) IN TEA GARDENS IN MIE PREFECTURE (KAMEYAMA-SHI AND YOKKAICHI-SHI) AND A FOREST IN TOKYO (HACHIOHOJI-SHI)

Treatment (μg/septum) epo3,Z6,Z9-19:H			Total captured males (N)[a]				
			Ascotis selenaria cretace			*Alcis angulifera*	
			Mie Prefecture		Tokyo	Tokyo	
(3S,4R)-(+) isomer	(3R,4S)-(−) isomer	Ratio	1995[b]	1996[c]	1995[d]	1995[e]	1996[f]
400	0	10:0	0c	0c	0c	78a	9a
360	40	9:1		1c			
320	80	8:2	3b		1c	8c	2b
200	200	5:5	5b	15b	21b	28b	1b
80	320	2:8	10b		17b	1c	0b
40	360	1:9		19a,b			
0	400	0:10	26a	29a	42a	0c	0b
0	0	0	0c	0c	0c	0c	0b

[a] Numbers within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple range test.
[b] Captured by two traps September 26–October 25 in Kameyama-shi.
[c] Captured by three traps September 9–21 in Yokkaichi-shi.
[d] Captured by two traps August 23–October 13.
[e] Captured by two traps October 6–November 2.
[f] Captured by two traps September 26–October 25.

data base of Ando). About 85% of these are composed of C_{17}–C_{22} hydrocarbons with two or three double bonds at the 3-, 6-, or 9-positions and their monoepoxy derivatives, which lack a terminal functional group, such as hydroxyl, acetoxyl, and aldehyde groups. Cossé et al. recently identified Z3,Z6,Z9-19:H (minor component) and epo3,Z6,Z9-19:H (major component) from a geometrid moth in Israel, the giant looper [*Ascotis* (= *Boarmia*) *selenaria* Denis & Schiffermüller] (Becker et al., 1983, 1990; Cossé et al., 1992). Our capillary GC-MS measurements have shown that the pheromone gland of the Japanese subspecies, *A. s. cretacea*, produces the same components in a similar quantity. In both species, the epoxide is essential for attracting males. The stereochemistry of the epoxy ring, however, is opposite. The males of the Israeli species are attracted to the 3*S*,4*R* isomer, and males of the Japanese subspecies are attracted to the 3*R*,4*S* isomer. Whereas the chirality of the natural pheromone component of the Israeli species is still unknown, chiral GC and HPLC analyses confirmed that the epoxide of the Japanese subspecies is almost in a racemate. Differences in the responses of males of the two subspecies suggest different pheromone receptors and different processing systems in their central nervous system. In our

field test, another geometrid species, *Alcis angulifera*, was attracted to the 3*S*,4*R* isomer.

Male attraction of *A. s. cretacea* to the epoxide was strongly inhibited when the parent triene Z3,Z6,Z9-19:H was mixed with it in a 1:1 ratio. However, the effect of the triene mixed in as a minor component was not clarified by our preliminary field tests in 1996. Further field evaluations are needed to find an optimum formulation of the synthetic pheromone for the Japanese subspecies. In the Israeli species, Z3,Z6,Z9-19:H seems to function as a precursor for the biologically active epoxide because the triene was found in the gland extract but not in the effluvia of the calling female, and it decreased behavioral responses of males to the epoxy lure in a wind tunnel (Cossé et al., 1992). Experiments with the Japanese subspecies led to a similar conclusion. Relatively high amounts of the triene were detected in a noncalling female in the photophase and also in decapitated females, which completely lost the epoxy pheromone component. PBAN, a neuropeptide, activates pheromone biosynthesis in *Helicoverpa zea* (Raina and Klun, 1984) and in several other lepidopterous species (Raina, 1993). The injection of synthetic Bom-PBAN or an SG extract stimulated production of epo3,Z6,Z9-19:H by decapitated females of *A. s. cretacea*, indicating that the epoxide production is controlled by the hormone. The triene decreased in the decapitated female after the injection. The triene may be an intermediate for synthesis of the epoxy pheromone.

The synthetic epo3,Z6,Z9-19:H may have possible application for predicting the emergence and population density of the looper. Because the homoconjugated epoxydiene is unstable, unlike ordinary pheromones with only one double bond, appropriate antioxidants and photostabilizers may play a significant role in offering a long life to the lure. There are other serious lepidopterous defoliators in a Japanese tea garden. Among those pests, sex pheromones of two tortricid species, the smaller tea tortrix (*Adoxophyes* sp.) and the Oriental tea tortrix (*Homona magnanima* Diakonoff), have been identified, and their synthetic pheromones have been used as a monitoring tool. The polyethylene tube formulation, which includes a common monoethyl component of their pheromones, is commercially available to directly control the population by disruption of their mating communication (Wakamura; 1992). We are eager to establish a similar disruption technique for *A. s. cretacea* in order to cultivate tea plants with a limited amount of insecticides.

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ORIENTATION DISRUPTION OF *Ctenopseustis herana*

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Abstract—Orientation disruption of *C. herana* was tested using pheromone traps baited with synthetic lures in small plot trials in apple orchards, using polyethylene rope dispensers (100/0.1-ha plot). Dispensers released either (a) 100% (*Z*)-5-tetradecenyl acetate (pheromone of *C. herana*), (b) a blend of 25% (*Z*)-5-tetradecenyl acetate and 75% (*Z*)-8-tetradecenyl acetate (multiple species disruptant blend), or (c) a blend of 50% (*Z*)-8-tetradecenyl acetate and 50% saturated tetradecenyl acetate (pheromone of *Planotortrix octo*). Blends (a) and (b) were effective at reducing trap catch by up to 100%, but disruption was not achieved with (c). Experiments with lures containing pheromone compared to pheromone plus (*Z*)-8-tetradecenyl acetate showed reduced attraction to (*Z*)-5-tetradecenyl acetate with the addition of (*Z*)-8 (1:3). These results indicate reduced attraction to lures but not disruption of orientation behavior from background release of this compound and in contrast to the reduced attraction and disruptive effect of the *C. herana* pheromone (a) on *P. octo*. Disruption of *C. herana* using the multiple species disruptant blend (b) deserves further investigation.

Key Words—Pheromone, disruption, polyethylene dispenser.

INTRODUCTION

Disruption of adult male moth behavior using synthetic sex pheromones is a possible control tactic for tortricid pests of orchards in New Zealand (Suckling, 1993). Successful disruption of the lightbrown apple moth (*Epiphyas postvittana* Walker) has been achieved in both the contexts of managing insecticide resistant populations (Suckling et al., 1990) and in the production of fruit with reduced organophosphate residues (Suckling and Shaw, 1995). However, up to four related endemic species of tortricids in the genera *Planotortrix* and *Ctenopseustis*

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attack horticultural crops in many areas of the country (Wearing et al., 1991). The distribution and importance of these species varies between regions (e.g., Foster, 1990; Suckling and Burnip, 1993; Shaw et al., 1994), and this geographical variation affects the potential for successful implementation of mating disruption. In Central Otago, *Planotortrix octo* (Dugdale) is the dominant pest leafroller (McLaren and Suckling, 1993; Wearing, 1995), while in northern North Island, all four native species may occur, together with *E. postvittana* (Suckling and Clearwater, unpublished data). The native species affecting apple orchards in the Canterbury Plains are *Planotortrix octo* and *Ctenopseustis herana* (Felder and Rogenhofer) (Suckling and Burnip, 1993), although *C. obliquana* is found nearby in parts of Banks Peninsula (Brown and Suckling, 1994).

Minks and Cardé (1988) reviewed the evidence for the assertion that the natural pheromone blend provides the most effective approach to disruption. However, there are several examples where disruption has been achieved without the natural blend. Tamaki et al. (1983) reported simultaneous mating disruption of two species of tea tortricids using a single pheromone component [(*Z*)-11-tetradecenyl acetate (Z11–14:OAc)] common to both. This blend has been in commercial use in Japan for over a decade (K. Ogawa, personal communication). However, other attempts to control multiple species complexes of related tortricids have been tried with somewhat erratic results using Z11–14:OAc in Europe (Minks and van Deventer, 1992; Boscheri et al., 1992) and North America (Knight, 1992). Geometric isomers can strongly affect the response of male moths to pheromone (Roelofs and Comeau, 1968), and some can be useful disruptants (Bengtsson et al., 1994; Suckling and Burnip, 1996). For multiple species complexes, the pheromone blend chosen for disruption against one species in a pest complex may contain an "off-ratio" or nonattractive blend for related species.

Simultaneous disruption of between one and four native leafroller species and *E. postvittana* will be necessary in most pipfruit regions of New Zealand. Multiple species disruption is therefore likely to use an off-ratio blend, which will not be the true pheromone for one or more species. The pheromone of *C. herana* is (*Z*)-5-tetradecenyl acetate (Z5–14:OAc) alone, while that of *C. obliquana* consists of a blend of 25% Z5–14:OAc and 75% (*Z*)-8-tetradecenyl acetate (Z8–14:OAc) (Clearwater et al., 1991). This blend has been proposed for use in New Zealand against the complex of four native species (Clearwater, personal communication).

Large-scale evaluation of mating disruption of *E. postvittana* has been undertaken in Nelson, where it is the dominant species (Suckling and Shaw, 1995). Disruption of *P. octo* using the off-ratio blend of partial pheromone and inhibitor ("b" below) (Suckling and Burnip, 1996) has also been successful at reducing fruit damage on several hectares of Central Otago stonefruit (McLaren

and Fraser, 1996), and useful against organophosphate-resistant populations on pipfruit orchards in Central Otago (Wearing, 1995) and Hawke's Bay (Lo et al., 1995).

The need for a better understanding of the potential for multiple species disruption led us to test disruption of *C. herana*, which has not been previously examined. We wanted to determine the extent of disruption from the pheromone (*Z*5–14:OAc) alone and in combination with *Z*8–14:OAc in the proposed multispecies blend. We also wanted to compare these results with disruption of *C. herana* from the pheromone of *P. octo* (*Z*8–14:OAc and saturated 14:OAc, 1:1), particularly as Foster and Roelofs (1987) identified the presence of saturated 14:OAc in the pheromone gland of female *C. herana*, although it was not cited a pheromone component.

METHODS AND MATERIALS

Dispensers. Three types of polyethylene tubing dispensers were obtained from Shin Etsu Chemical Co. Ltd. (Tokyo). Dispensers contained: (a) 80.1 mg of *Z*5–14:OAc (the pheromone for *C. herana*), (b) 19.6 mg of *Z*5–14:OAc (25%), with 58.7 mg of *Z*8–14:OAc (75%) (the multiple species blend, containing pheromone of *C. herana* and partial pheromone of *P. octo*), or (c) 40 mg of *Z*8–14:OAc (50%) and 41.0 mg of 14:OAc (50%) (pheromone of *P. octo*).

Blend Attractiveness. A trapping experiment was set up in order to determine the possible mechanisms involved in disruption through a better understanding of the impact of *Z*8–14:OAc on the attractiveness of the *C. herana* pheromone (*Z*5–14:OAc) released from rubber septa or polyethylene dispensers. Delta traps (Suckling and Burnip, 1996) were baited with a range of rubber septa with 1-, 3-, 10-, and 30-fold loadings of the standard lure (30 μg) of *Z*5–14:OAc for *C. herana* extracted from polyethylene tubing dispensers (below). Dilutions also were made and added to rubber septa baits from polyethylene tubing dispensers releasing the multiple species blend [*Z*5–14:OAc (25%) and *Z*8–14:OAc (75%)], also based on 1-, 3-, 10-, and 30-fold loadings of *Z*5–14:OAc, where 1 was the standard 30-μg lure. The dilutions were based on the manufacturer's technical information, supported by GC analysis (unpublished data). A volume of 100 μl of solution was added to each lure along with a further addition of the same volume of solvent alone after this had evaporated. Traps were baited with these eight lures, along with a single polyethylene dispenser of each type [(a) and (b) above], and untreated septa as blanks ($N = 5$). Traps were checked and rerandomized weekly from November 10, 1992 to February 17, 1993. Rubber septa lures were changed every six weeks, and sticky bases each month.

Disruption in Small Plots. Disruption of trap catch was tested using a similar experimental design in five trials at three Canterbury orchards over three years. The export orchards were 30–50 ha in total area. The details of the orchards and dispenser treatments are given in Table 1. A higher rate (2000 dispensers/ha) was used in the last year in an attempt to improve the level of disruption.

In each trial, trap catch (below) was compared among three treated and three control plots (0.1 ha, or ca. 33-m × 33-m plots). Dispenser treatments were the above formulations (a–c) implemented as described below. Treated and control plots were 50–100 m apart, and all three replicates were in the same orchard for each trial. The orchards were divided by poplar shelter belts (ca. 8 m high) into 1-ha blocks, with a single plot (treated or control) per sheltered 1-ha block. Plots, separated by two guard rows from the edge of the orchard, contained two traps operated internally and one trap externally (approx. 50 m away from the plots). Five of each type of dispenser were hung vertically in the orchard for study of release rates. The length of liquid in each dispenser was measured weekly, and the release rate of pheromone (milligrams per hectare per hour) was calculated. In trial 5, mean air temperature was recorded hourly on a data logger (model CR10, Campbell Scientific, Logan, Utah) using a temperature probe (model 107, Campbell Scientific), in order to model the pheromone release rate (Bradley et al., 1995). Trap catches in standard delta traps baited with 30 μg *Z*5–14:OAc were recorded. Traps were checked weekly from early October until the end of May. Trap bases and caps were replaced at

TABLE 1. DETAILS OF APPLE ORCHARDS AND TREATMENTS USED IN FIVE TRIALS OF ORIENTATION DISRUPTION OF *C. herana*

Parameters	Trials 1, 2, and 4	Trial 3	Trial 5
Orchard	A	B	C
Trees planted	1985	1986	1987
Cultivar tree spacing (m)	Braeburn, 2.5 × 4.5	Braeburn, 2.3 × 4.5	2.4 × 4.0
	Royal Gala and Fuji, 3.0 × 4.5	Fuji, 2.3 × 4.5	Royal Gala and Fuji, 3.0 × 4.0
Insecticides (pre/post pheromone application)	4/2	4/2	3/2
Dispensers applied (density/ha)	3/3/92 (1000) 10/2/93 (1000) 10/2/94 (2000)	12/2/93 (1000–4000)	11/2/94 (2000)

least once every six weeks. Trapping in the first generation provided a baseline for comparison between pheromone and control plots before the treatment with dispensers.

Statistical Analysis. Catch per trap per day was calculated from the total number of moths caught, divided by the number of traps in use and the trapping interval. For the trapping test, the significance of log (catch + 1) of trap catch was analyzed between treatments by analysis of variance. For the orientation disruption tests, the log (catch + 1) in the posttreatment period was subjected to analysis of covariance using trap catch before pheromone application in the same traps as a covariate. This was done to reduce the variability that existed in trap catches between different plots. The analysis of covariance (using the catch in the first generation as a covariate) was used to determine whether application of pheromone reduced trap catch in the treated plots below the expected level.

RESULTS AND DISCUSSION

Attractiveness of Blends. Catches were made across a range of loadings from 30 to 900 μg of Z5–14:OAc on rubber septa, but no dose–response was evident (Table 2). The trap baited with a polyethylene dispenser releasing Z5–14:OAc (80.1 mg loading) caught fewer males than those baited with this compound released from rubber septa. This was undoubtedly due to the higher release rate, which is likely to be disruptive to attraction at close range (Roelofs, 1978). Very few males of *C. herana* were caught in traps baited with polyethylene dispensers or the Z5/Z8–14:OAc blend contents diluted to within the range of attractant loadings of pheromone ($F_{1,36} = 19.68$, $P < 0.0001$, Table 2). This low catch indicates the effect of addition of the antagonist (Z8–14:OAc) on trapping of this species.

Orientation Disruption. Trap catches in the internal and external traps indicated two generations in each year, with a trough in late January–early February as shown in trial 5 (Figure 1, data for other years not shown for brevity). The timing of dispenser application was therefore appropriate for a successful catch of sufficient moths to permit analysis of covariance using the pretreatment catches to refine estimates of posttreatment effects. The timing for dispenser application in trial 1 was slightly later than the trough between generations, so subsequent trials used earlier application dates.

For all five trials, catches of *C. herana* were similar in external traps located near either treated or control plots before treatment and increased in the second generation near all treatments. Catches in the plots treated in February were similar to those in control plots prior to treatment (e.g., Figure 1), but traps present in all treatments in all trials releasing Z5–14:OAc caught significantly

TABLE 2. MEAN CATCH PER TRAP OF *C. herana* TO LURES CONTAINING PURE PHEROMONE OR PHEROMONE PLUS Z8–14:OAc

Lure	Mean ± SD	Significance[b]
Blank	0.0 ± 0.0	a
Z5–14:OAc × 1[a]	21.0 ± 17.4	b
Z5–14:OAc × 3	23.8 ± 16.0	b
Z5–14:OAc × 10	23.0 ± 11.1	b
Z5–14:OAc × 30	24.3 ± 25.3	b
Z5/Z8–14:OAc × 1[a]	1.3 ± 1.3	a
Z5/Z8–14:OAc × 3	1.5 ± 1.3	a
Z5/Z8–14:OAc × 10	0.8 ± 1.0	a
Z5/Z8–14:OAc × 30	0.0 ± 0.0	a
Z5/Z8–14:OAc dispenser	1.0 ± 1.2	a
Z5–14:OAc dispenser	0.5 ± 1.0	a

[a] 30 μg of Z5–14:OAc.
[b] Means designated by the same letter are not significantly different from each other.

fewer moths (Table 3), according to analysis of covariance. This amounted to 66–100% disruption of catch in all the trials (Table 4), with an average of 87% across the five trials, compared to 94% for the three trials with Z5–14:OAc alone.

In trials 2 and 3, the fourfold lower amount of Z5–14:OAc being released from Z5/Z8–14:OAc dispensers resulted in lower values of disruption in both cases, but the difference in disruption was not significant (Table 3). Catches in the treatment with fourfold more Z5/Z8–14:OAc dispensers (to make up for the 25% Z5–14:OAc contents) were as low as the catches with pure Z5–14:OAc, giving 97.8% disruption in both cases (Table 4).

There was no significant effect of the pheromone of *P. octo* (1:1 of Z8–14:OAc and saturated 14:OAc) on disruption of trap catch of *C. herana* in either trial 4 or 5 ($P < 0.05$), although 50% disruption was apparently achieved in trial 5 (compared to 0% in trial 4). This indicates that neither component was significantly disruptive despite the electroantennogram response to both compounds and the presence of specialist cells for Z8–14:OAc (Clearwater et al., 1991). Foster and Roelofs (1987) also reported no effect on attraction from the addition of saturated 14:OAc, despite it being present in pheromone glands.

There was a difference in behavioral effect of Z8–14:OAc between the two physically different situations reported here. It reduced trap catch when presented on the same lure (as would happen with calling females of the congeneric and

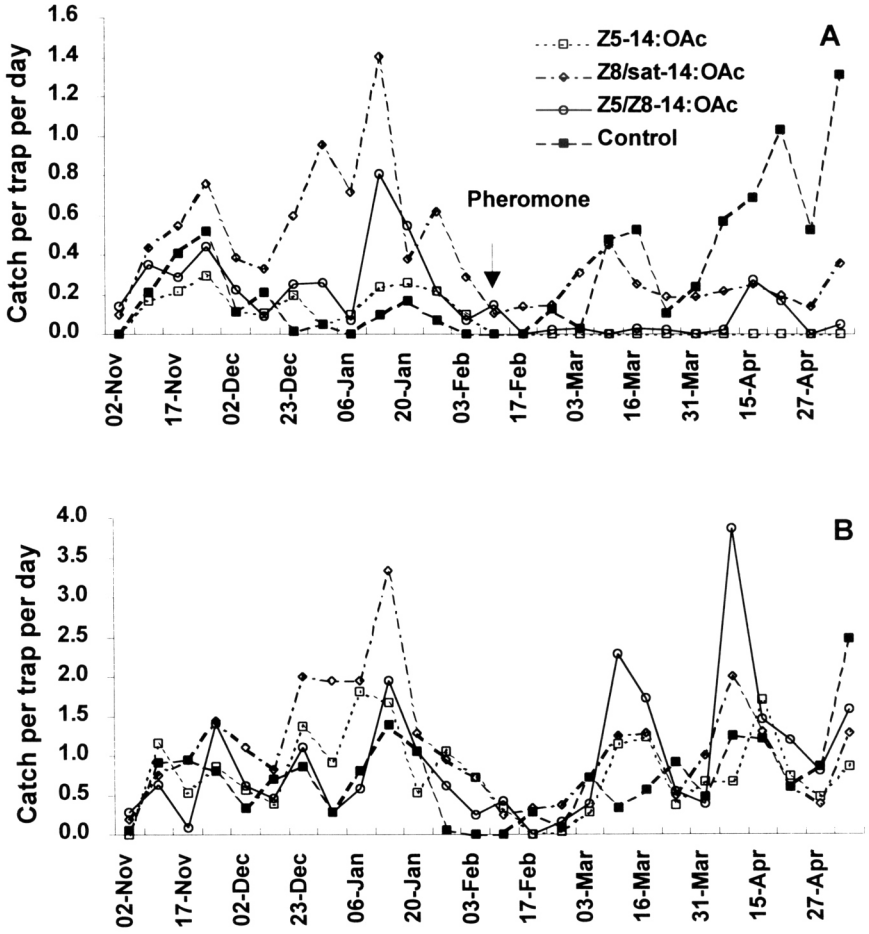


FIG. 1. Mean catch per trap per day of *C. herana* before and after treatment with dispensers (February 11, 1994) at internal (A) and external (B) traps in trial 5. Note higher *y* axis in B).

occasionally sympatric *C. obliquana*) but was not disruptive when released in orchard air by physically separated dispensers. This contrasts with the situation of *P. octo* reported by Suckling and Burnip (1996), where Z5–14:OAc both reduced trap catch and was a partial disruptant. However, changes in behavioral effect of trapping inhibitors or antagonists with physical separation from the lure have been recognized for some time (Rothschild, 1974; Kaae et al., 1974;

TABLE 3. ANALYSIS OF COVARIANCE OF TOTAL TRAP CATCH (LOG [X + 1]) OF *C. herana* FOLLOWING TREATMENT WITH POLYETHYLENE DISPENSERS RELEASING PHEROMONE AND RELATED COMPOUNDS, USING CATCH IN GENERATION BEFORE APPLICATIONS AS COVARIATE

Year	Trial	Z8-14:OAc sat 14:OAc	Z5-14:OAc Z8-14:OAc	4× Z5-14:OAc Z8-14:OAc[a]	Z5-14:OAc	Control	SEM
1991/92	1		0.05a[b]			1.13b	0.07
1992/93	2		0.56a		0.24a	0.85b	0.12
	3		0.32a	0.04a	0.05a	0.98b	0.10
1993/94	4	0.84b	0.27a			0.91b	0.09
	5	0.85b	0.12a		0.05a	1.53b	0.09

[a] Four times standard rate (i.e., 4000 dispensers/ha equivalent).
[b] Means with different letters within a row are significantly different ($P < 0.05$).

TABLE 4. PERCENTAGE DISRUPTION OF *C. herana* IN PLOTS TREATED WITH POLYETHYLENE DISPENSERS RELEASING PHEROMONE AND RELATED COMPOUNDS [100 − ((CATCH IN TREATMENT/CATCH IN CONTROL) × 100)]

Year	Trial	Orchard	Z8-14:OAc sat 14:OAc	Z5-14:OAc Z8-14:OAc	4× Z5-14:OAc Z8-14:OAc[a]	Z5-14:OAc
1991/92	1	A		99		
1992/93	2	A		66		85
	3	B		89	98	98
1993/94	4	A	0	91		
	5	C	50	89		100

Witzgall and Priesner, 1991; Rumbo et al., 1993), and *C. herana* may be similar to these cases.

The release rate of Z5-14:OAc per dispenser at dusk in trial 5 modeled from measurements of liquid length and air temperatures (Bradley et al., 1995) was in the range of 0.5–6 μg/hr for individual dispensers (Figure 2). The release

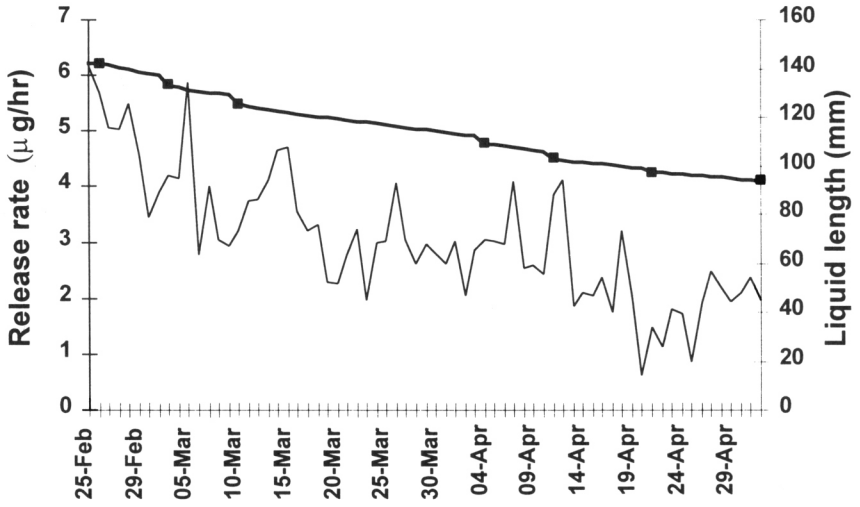


FIG. 2. Predicted release rate of Z5-14:OAc per dispenser at dusk, after treatment with dispensers containing Z5-14:OAc (25%) and Z8-14:OAc (75%) in trial 5 (1994), based on measured pheromone liquid length (data points), predicted changes in liquid length from accumulated air temperatures (thick line), and actual dusk air temperatures (not shown), using the model of Bradley et al. (1995).

rates in the plots (2000 dispensers/ha) were therefore above 1 mg/ha/hr, although the lowest values coincided with some trap catches towards the end of the season (Figure 1A). Release rates from the other types of dispensers present in trial 5 were similar (e.g., fourfold higher for dispensers with pure Z5–14:OAc), although these dispenser types did not warrant the development of a model.

Cardé (1990) reviewed evidence for habituation, which is more likely to be important in the case of behavioral antagonists, since "false trails" will not be possible where dispensers are not attractive. The Z5/Z8 dispensers appear to operate by mechanisms other than false trail following since they are not attractive. This is not surprising, given the use of this blend as the pheromone of the related species *C. obliquana* and the need for reproductive isolation (Clearwater et al., 1991). The Z5 dispensers could operate as false trails, although in untreated plots they were only weakly attractive. However, habituation to the higher background of pheromone in treated plots could raise the behavioral threshold, causing the individual dispensers to become attractive in an orchard.

There are few examples where potential disruption from mechanisms of false trail following and sensory overload have been compared directly (Minks and Cardé, 1988). In trial 3, this comparison was achieved with Z5 released at the same rate from either dispensers releasing only this compound (potential false trails) or from fourfold as many dispensers releasing a blend of Z5 and Z8–14:OAc (1:3) (nonattractive dispensers, possibly operating by sensory overload). The equivalent results from the two situations suggest that the natural blend of pure Z5–14:OAc is not necessary for disruption of trap catch, although release of the antagonist Z8–14:OAc in the environment appears not to impact on disruption to lures that contain only the pheromone. There is undoubtedly a minimum amount of Z5 needed, but the addition of Z8 appears not to adversely affect results. We can conclude that addition of a nonpheromone "antagonist" (Z8, needed for other species) did not reduce efficiency of the mixture over the natural blend against *C. herana*.

The lack of complete disruption of trapping in these small plot trials does not necessarily indicate a lack of potential for disruption of this species. It is of interest that larger quantities of Z5–14:OAc consistently achieved greater disruption (trials 2, 3, and 5), despite the lack of statistical significance in individual trials. Suckling and Burnip (1996) reported similar small plot trials for *P. octo*, which achieved less than complete disruption of trapping in some similar cases with young orchard plantings, while Wearing (1995) reported complete disruption of trapping and mating by tethered females of this species in Central Otago orchards, with larger areas under the same treatment. Factors such as pheromone rerelease from apple foliage (Karg et al., 1994) are likely to be important in maintaining a background level of pheromone in orchard air and impact positively on disruption in the case of mature trees (Suckling et al., 1996). Suckling and Angerilli (1996) showed that plots of 0.5 ha treated with pheromone of *E.*

postvittana had greater disruption of traps near the center, compared to the edges, which may be a function of the wind in the Canterbury region. A better understanding of these factors is needed. These results suggest that the potential for disruption of *C. herana* needs further testing with the multiple species blend over larger areas.

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RESPONSE OF EGG PARASITOID *Telenomus busseolae* TO SEX PHEROMONE OF *Sesamia nonagrioides*

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Abstract—The olfactory cues involved in the host-finding behavior of *Telenomus busseolae* Gahan, a solitary egg parasitoid of the corn stalk borer, *Sesamia nonagrioides* (Lefebvre), were studied. The female wasp was attracted to odors from calling virgin females of *S. nonagrioides* in a Y-tube olfactometer. No response was elicited by noncalling virgin female moths and/or mated female moths. A four-component commercially available sex pheromone of the corn borer was also tested, as were the individual components of the mixture. *T. busseolae* respond to the whole blend [(Z)-11-hexadecenyl acetate, (Z)-11-hexadecenol, (Z)-11-hexadecenal, and dodecyl acetate in the ratio of 8.5:1:1:2]. Z11-16:OH was the only compound that elicited no significant activity. These results suggest that the sex pheromone released by the host female *S. nonagrioides* is used by *T. busseolae* as an indirect cue to locate egg masses of this host.

Key Words—Parasitoid, Hymenoptera, Scelionidae, corn, Lepidoptera, Noctuidae, semiochemicals, Y-tube olfactometer, kairomone.

INTRODUCTION

The corn stalk borer, *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae), is one of the most serious pests of corn in Africa and many countries of the Mediterranean Basin (C.I.E., 1979). One of its main biological control agents is *Telenomus busseolae* Gahan (Hymenoptera: Scelionidae), a solitary egg parasitoid of various Lepidoptera belonging to the genus *Sesamia* (Alexandri

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and Tsitsipis, 1990; Sétamou and Schulthess, 1995). In the Mediterranean region of Turkey, *Telenomus* (=*Platytelenomus*) *busseolae* was reported as a common parasitoid of the corn stalk borer. *T. busseolae* is able to locate up to ≈60% of *S. nonagrioides* egg masses and parasitize ≈80% of the eggs in the total egg masses discovered (Kornosor et al., 1994). In Europe, this parasitoid has been found only in Greece (Polaszek et al., 1993), where it exhibits a similar level of parasitism (Alexandri and Tsitsipis, 1990).

Sesamia nonagrioides is also an important pest of maize and other cultivated crops in central and southern Italy (Nucifora, 1966; Laudonia et al., 1991). *T. busseolae* was introduced into Italy from Greece in 1991, but the parasitoid did not establish itself (Laudonia et al., 1991). In 1994, two new colonies of *T. busseolae*, from material provided by Dr. S. Kornosor (University of Çukurova, Adana, Turkey), were established at the Entomology Institutes in Perugia and Portici.

The efficiency of an egg parasitoid in a biological control program is highly dependent upon the semiochemical-mediated interrelationships of the environment, the plant, and the phytophagous insect (Vinson, 1985; Lewis and Martin, 1990; Vet and Dicke, 1992). Because of the importance of semiochemicals in many host–parasitoid interactions, we carried out a series of investigations to understand the possible role of the host sex pheromone in the host–parasitoid association *S. nonagrioides–T. busseolae*.

Sesamia nonagrioides virgin females produce a sex pheromone during the scotophase, with maximum calling occurring 4–6 hr after onset of scotophase. Females cease calling after mating (Babilis and Mazomenos, 1992a,b). The female-produced sex pheromone consists of a blend of five components (Rotundo et al., 1985, 1991; Mazomenos, 1989). Rotundo et al. (1991) obtained the highest attractiveness and specificity to *S. nonagrioides* males in field tests conducted in corn in southern Italy (Battipaglia, Salerno, Italy) by baiting traps with the following four components: (*Z*)-11-hexadecenyl acetate (Z11–16:Ac), (*Z*)-11-hexadecenol (Z11–16:OH), (*Z*)-11-hexadecenal (Z11–16:Ald), and dodecyl acetate (12:Ac) in the ratio 8.5:1:1:2. Hexadecyl acetate (16:Ac), the fifth compound detected in the gland extracts, was found to be inactive (Mazomenos, 1989). The average number of *S. nonagrioides* males captured was similar to that recorded using traps baited with two *S. nonagrioides* virgin females (Rotundo et al., 1991). Based on these results, a synthetic sex pheromone blend was commercialized (Traptest, by Istituto Guido Donegani, Agrimont, Italy) and is being used in traps to monitor infestation and establishment of *S. nonagrioides* in the field.

The aim of this work was to examine the response of *T. busseolae* to virgin and mated female *S. nonagrioides*, and the four-component synthetic sex pheromone blend.

METHODS AND MATERIALS

Insect Maintenance. T. busseolae adults were provided *S. nonagrioides* egg masses in 16-ml glass tubes with honey–water solution. *S. nonagrioides* was established from pupae collected in central Italy and reared on a meridic diet as described by Giacometti (1995). Colonies of both species were kept in the laboratory under a regime of 26 ± 1°C, 65 ± 5% relative humidity, and a photoperiod of 16L:8D. Freshly emerged *T. busseolae* females were held for 24 hr with males for mating, then they were individually isolated with a drop of honey solution and used in experiments when they were 2–3 days old.

Odor Sources. Female pupae of *S. nonagrioides* were kept separately at 26 ± 1°C, 65 ± 5% relative humidity, and 16L:8D photoperiod. After emergence, two groups of female moths were used in the tests: (1) virgin females during the first photophase following emergence, and (2) mated females during the third photophase following emergence. A second colony was held at reversed photoperiod (16L:8D) with scotophase beginning at 07:00 hr to allow bioassays to be conducted during the day between 09:00 and 14:00 hr. Two groups of female moths were used from this colony: (3) virgin females in calling activity during the first scotophase after emergence, and (4) mated females during the second scotophase after emergence.

The synthetic sex pheromone blend used in our experiments was formulated from commercially available products (Novapher, Milan, Italy), Z11-16:Ac/Z11-16:OH/Z11-16:Ald/12:Ac in a ratio of 8.5:1:1:2, loaded on rubber septa (5 × 9-mm rubber stoppers, sleeve type, Angelo Asceno, Milan, Italy) at a dosage of 1250 μg. Rubber septa contained an antioxidant, Tetrakis [methylene (3,5-di-*tert* butyl-4-hydroxydrocinate)] Irganox 1010 (0.5 mg), and an UV absorber, 2-hydroxy-4-*n*-octyloxy-benzophenone, Chimassorb 81 (0.5 mg). Single pheromone compounds, loaded on a rubber septum, were tested individually at the same dosage as used for the blend. Rubber septa containing only antioxidant and UV absorber were also tested. Rubber septa loaded with the synthetic pheromone or with the single compounds were obtained from Dr. E. Arsura, Novapher, Milan, Italy.

Behavioral Assays. The Y-olfactometer chamber was made of transparent Plexiglas (two arms 8 cm long and a common stem 8.5 cm long) sandwiched between two glass sheets (15 × 20 cm). Medical-grade compressed air flowed through both arms creating an airstream of 30 ml/min per arm. The flow was regulated by flowmeters, and the air was humidified by bubbling through a water jar before it passed into the olfactometer. Smoke tubes (Auer, Rauchröhrchen) were used to verify laminar airflow in the olfactometer. Both arms were connected to glass vials (350 ml) holding the odor source sealed with Teflon tape.

In each experiment, a single odor source was used in one arm of the

Y-olfactometer, while the other unbaited arm served as a control, i.e., with only humidified air. When *S. nonagrioides* adults were offered, three living *S. nonagrioides* females in glass vials were kept in a dark environment. Adults were observed under infrared light illumination (homogeneous emission of wavelengths at 950 nm provided by 108 LEDs) with a CCD camera (Sony SSC M370CE) equipped with a 16 mm/F1.6 lens to determine if they were calling. Bioassays were observed on a video monitor (Sony PVM 1371QM) connected with a monochrome CCD camera (Sony SSC M370CE) fitted with a 12.5–75 mm/F1.8 zoom lens. The Y-olfactometer was surrounded by a paper wall to minimize possible cues from the room and was illuminated by two 40-W TLD fluorescent tubes located above the device. After each experimental trial, the whole system was cleaned with hexane, chloroform, and acetone. The temperature in the bioassay room was $\approx 26°C$ at all times. A single wasp was introduced into the Y-olfactometer at the entrance of the common stem and observed for 10 min with the aid of a behavior recording program, The Observer release 3.0 Software Package (Noldus Information Technology, Wageningen, The Netherlands). Measurements were made of time spent by wasps in each arm of the olfactometer. Female wasps were tested singly, and all tests were replicated at least three times. Choices between test and control arm within a test were analyzed using Wilcoxon matched-pairs test (Software Package Statistica release 4.3) (Statistica, 1993).

RESULTS

With only clean air passing through both arms of the olfactometer, *T. busseolae* females spent an equal proportion of their time in each arm (26.22 $\pm$ 20.82 left vs. 22.96 $\pm$ 16.55 right, $N = 34$, $Z = 0.56$; $P = 0.58$). When exposed to *S. nonagrioides* females exhibiting calling behavior, female wasps spent significantly more time in the arm associated with the moths than in the control arm ($Z = 2.77$; $P = 0.00551$) (Table 1). In *S. nonagrioides*, the pheromone titer is synchronous with the calling rhythm and drops drastically after mating (Babilis and Mazomenos, 1992b). *T. busseolae* showed no significant preference from the moths over the control in the experiments with virgin non-calling moths ($Z = 0.94$, $P = 0.35$) or mated moths, both held during the photophase and the scotophase ($Z = 0.39$, $P = 0.69$; $Z = 1.736$, $P = 0.08$, respectively) (Table 1). These results suggest that the host sex pheromone, the maximal titer of which coincides with the calling activity, is essential to elicit a positive response by the wasps.

The responses of *T. busseolae* to the synthetic host pheromone blend and to each component are presented in Table 2. The wasps spent significantly more

TABLE 1. RESPONSE OF *T. busseolae* FEMALES IN Y-OLFACTOMETER TO FEMALES OF *S. nonagrioides*[a]

Odor source	Replicate	Average time spent in arm (% ± SD)		P^b
		Test	Control	
virg-no-act	24	15.023 ± 19.166	18.219 ± 20.370	NS
virg-act-pher	22	26.751 ± 21.395	15.211 ± 14.424	<0.01
mat-act	25	16.755 ± 12.908	16.173 ± 15.477	NS
mat-no-act	35	25.352 ± 22.414	16.554 ± 16.219	NS

[a]Conditions were: virgin females during the first photophase (virg-no-act); virgin females in calling activity during the first scotophase (virg-act-pher); mated females during the second scotophase (mat-act); and mated females during the third photophase (mat-no-act).
[b]Wilcoxon matched pair test.

TABLE 2. RESPONSE OF *T. busseolae* FEMALES IN Y-OLFACTOMETER TO SYNTHETIC SEX PHEROMONE BLEND OF *S. nonagrioides* FEMALES (SYNT-PHER) AND TO INDIVIDUAL COMPONENTS

Odor source	Replicate	Average time spent in arm (% ± SD)		P^a
		Test	Control	
Control	18	17.601 ± 15.424	19.422 ± 17.950	NS
synt-pher	47	37.055 ± 22.010	19.975 ± 14.851	<0.001
Z11-16:Ac	25	30.670 ± 20.249	13.631 ± 12.792	<0.01
Z11-16:OH	28	29.510 ± 13.660	23.008 ± 12.332	NS
Z11-16:Ald	24	32.586 ± 23.866	16.598 ± 18.069	≤0.05
12:Ac	26	36.534 ± 18.598	23.405 ± 21.092	≤0.05

[a]Wilcoxon matched pair test.

time in the arm containing the synthetic sex pheromone blend of *S. nonagrioides* than in the control arm ($Z = 3.89$, $P = 0.0001$). This response was not influenced by the presence of either the antioxidant or the UV absorber ($Z = 0.50$, $P = 0.62$). Among the compounds present in the synthetic sex pheromone blend, only (*Z*)-11-hexadecenol (Z11-16:OH) failed to elicit a significant response by *T. busseolae* females ($Z = 1.878$, $P = 0.062$).

DISCUSSION

Semiochemicals play a role in mediating the interactions between many hosts and their parasitoids. The volatile cues employed by parasitoids may originate from the plant–host complex (reviewed in Price et al., 1980), and their utility depends upon their reliability and detectability (Vet and Dicke, 1992). As for insect parasitoids attacking the egg stage, a major role seems to be played by the host. Numerous insect parasitoids locate suitable hosts by orienting themselves towards a range of chemical cues mainly associated with the host (reviewed in Vinson, 1985; Vet and Dicke, 1992; Godfray, 1994). In some cases, these cues are not directly associated with the attacked host stage, but help the parasitoids in restricting the search area. Such is the case with the host sex pheromones in several lepidopteran–egg parasitoid systems. Parasitoid searching behavior is directed toward an area where host mating is in progress and where an oviposition has probably taken place or is soon to occur (Vet and Dicke, 1992; Godfray, 1994). The sex pheromones of *Helicoverpa* (=*Heliothis*) *zea* (Boddie) and *Spodoptera frugiperda* (J. E. Smith) are exploited as important cues in locating host eggs and increasing rates of parasitization by their parasitoids, *Trichogramma* spp. and *Telenomus remus* Nixon (Lewis et al., 1982; Nordlund et al., 1983). *Telenomus euproctidis* Wilcox, the phoretic egg parasitoid of *Euproctis taiwana* (Shiraki), responds to the sex pheromone of its host, as well as to (*Z*)-16-methyl-9-heptadecenyl isobutyrate, a chemical in the abdominal tip of the host female (Arakaki et al., 1996). Other experiments have shown that indirect cues in addition to the host sex pheromone are involved in host location, e.g., *Trichogramma brassicae* Bedzenko (=*Trichogramma maidis* Pintureau and Voegelé) responds to sex pheromone from its host *Ostrinia nubilalis* Hbn. only when the pheromone is combined with the odor from host eggs and an extract from the host plant (Kaiser et al., 1989). Sétamou and Schulthess (1995) observed a positive correlation between the high population of adults of *Sesamia calamistis* Hampson and the efficacy of its parasitoid, *T. busseolae*, in maize fields, suggesting that the wasps were attracted to the sex pheromone emitted by host females.

The findings reported here show that *T. busseolae* is attracted to both virgin females of *S. nonagrioides* in calling activity and to a synthetic blend of the host's pheromone. The synthetic moth sex pheromone, formulated from commercially available products, is made of a blend of four components. *T. busseolae* was attracted to three of the four compounds, but not to Z11–16:OH. Field trapping experiments with synthetic pheromone indicate that Z11–16:OH plays an important role in reproductive isolation of the corn stalk borer from other related species, e.g., *Mythimna unipuncta* Hw. (Rotundo et al., 1985; Mazomenos and Bardas, 1988). However, the role of individual compounds under natural conditions has yet to be investigated. There are a few studies on

the adaptive value for an egg parasitoid in detecting the host sex pheromone (Godfray, 1994, and references therein), as well as the possibility of large-scale applications of host-seeking stimulants in pest control programs (Gross, 1981; Lewis et al., 1982). Pheromone application in the field to disrupt mating of the Douglas fir tussock moth, *Orgyia pseudotsufata* (McDunnough), did not result in reduced parasitism of two important egg parasitoids *Telenomus californicus* Ashmead and *Tetrastichus* sp. (Sower and Torgersen, 1979). The application of the host pheromone may be useful in future introductions of *T. busseolae* to aid its establishment.

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DO GERMINATION INDICES ADEQUATELY REFLECT ALLELOCHEMICAL EFFECTS ON THE GERMINATION PROCESS?

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Abstract—Allelopathy is often demonstrated by testing allelochemical effects on germination using many germination indices to assess them. The objective of this work was to compare four common germination indices (G_T, S, AS, and CRG) previously calculated with the same data in order to discuss their physiological meaning. Each index led to a different interpretation of allelochemical effect on germination. Considering all indices, the S index was the most sensitive, followed by the AS and CRG indices, which provided the same information. The G_T index, which gave only a global germination interpretation, was not precise enough in describing allelochemical actions during germination. Nevertheless, none of the indices exactly reflected the germination process. Thus, simple comparisons between the control and the test at each exposure time were more appropriate.

Key Words—Allelochemical, allelopathy, benzoxazolinone, germination, hydroxamic acid, indices, *Lactuca sativa*, phenol, *p*-hydroxybenzoic acid, protocatechuic acid.

INTRODUCTION

Allelopathy, a "direct or indirect effect of one plant upon another through the production of chemical compounds released into the environment" (Rice, 1984),

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is often demonstrated by testing allelochemical effects on germination. The germination process usually begins with water uptake by the seed (imbibition) and ends with the protrusion of the radicle through the testa. The seed germination test has been a widely used bioassay for the determination of allelopathy activity (Lovett and Ryuntyu, 1992).

This method has served to validate (or reject) allelopathy in ecosystems or agrosystems. For instance, effects of phenolic acids on seed germination and seedling growth in soils were contested by Krogmeier and Bremmer (1989) and by Kaminsky (1981). In contrast, the same method was used successfully by Pellissier (1993) to describe allelopathic inhibition of spruce germination and seedling growth by humic phenols.

Thus, the relevance of seed germination bioassays in allelopathy research must be given attention. First, the manner in which these bioassays are conducted has to be carefully considered. Indeed, optimal germination conditions are different for each species used (dormancy, temperature, photoperiod, volume of solution per Petri dish, etc.) and must be well identified (Leather and Einhellig, 1988). In addition, it is important to use suitable indices of germination. This is a major concern because the interpretation and the biological meaning of the bioassay results depend on it.

In many investigations different indices were used to show allelochemical effects on germination. The indices are usually of three types: maximum germination percentage (also termed germination capacity), germination progress (Bewley and Black, 1985), and shape of germination curve. The last approach consists of fitting the appropriate regression model to the data (Hedge and Miller, 1992). Several mathematical functions have been used without application of a general rule. Therefore, this approach has not been included in this study.

Objectives of the present study were to calculate four common germination indices from the same data in order to make comparisons between each of them and to discuss better ways of using those indices in order to improve the precision with which allelopathic physiological hypotheses were being tested.

METHODS AND MATERIALS

General Procedure. The experimental procedure involved testing the effect of two phenols and a hydroxamic acid on lettuce seed germination. The same data were then used to calculate and compare different indices.

Chemical Solutions. Three compounds (Sigma Chemical Co.) were selected for testing seed germination: protocatechuic acid (3,4-dihydroxybenzoic acid), *p*-hydroxybenzoic acid, and BOA (2-benzoxazolinone). Their selection was based on their allelopathic activity previously described by Sánchez et al. (1996), Christen and Lovett (1993), and Levi-Minzi et al. (1994). Aqueous solutions

were prepared at 10^{-3}, 10^{-4}, 10^{-6}, and 10^{-8} M with distilled water (buffered to pH 6).

Germination Tests. Fifty seeds of *Lactuca sativa* (var. Great Lakes) were placed in a Petri dish (9 cm diam.) on 1 Whatman 3 MM paper soaked in 4 ml chemical solution or distilled water (control). They were then placed in a thermostatically controlled light-box (total darkness at 28°C) to germinate. The number of germinated seeds was counted (rupture of seed coats and emergence of radicle) every 6 hr for two days, until no further seeds germinated. Treatments were replicated five times.

Germination Indices. Four germination indices were selected because of their common use in germination studies. They were calculated as described in Table 1.

Statistical Analysis. Germination tests and indices were compared to their respective controls using the Mann-Whitney U test ($P < 0.05$).

RESULTS

Effects of Allelochemicals on Germination at Each Exposure Time. The three allelochemicals used, protocatechuic acid, *p*-hydroxybenzoic acid, and BOA, acted differently. *p*-Hydroxybenzoic acid delayed germination at 12 hr of incubation (10^{-3} M and 10^{-4} M), whereas BOA induced a drastic inhibition of germination at 10^{-3} (Table 2). Two other concentrations of BOA, 10^{-4} M (from 12 to 24 hr) and 10^{-6} M (12 hr), only delayed germination. In contrast, protocatechuic acid had no effect on germination at any concentration at any stage.

Effects of Allelochemicals on Germination Based on Calculated Indices. The calculated indices are given in Table 3. Although the four indices were calculated with the same data, they gave different results with low variability. All indices were significant for BOA at 10^{-3} M, the only concentration causing inhibition of germination. For BOA at 10^{-4} M, three indices (S, AS, and CRG) were significant, but at 10^{-6} M only one (index S) was significant. *p*-Hydroxybenzoic acid effects on germination were detected for one concentration and by one index (S) only. For protocatechuic acid, none of the indices were significant, and it had no effect on germination.

DISCUSSION

The use of germination indices aids in the testing of allelochemical effects on the physiological germination process. One of the most common index values is the lethal concentration (LC_{50}), applied when inhibition of germination occurs (Fargasova, 1994). In the present study, inhibition of germination caused by

TABLE 1. FORMULAE TO CALCULATE GERMINATION INDICES

Germination index	Formula		References
Total germination (G_T)	$G_T = \left\lfloor \frac{N_T * 100}{N} \right\rfloor$	N_T: proportion of germinated seeds at each treatment for the last time measurement. N: Number of seeds used in the bioassay	Very common
Speed of germination (S)	$S = (N_1 * 1) + (N_2 - N_1) * \frac{1}{2} + (N_3 - N_2) * \frac{1}{3} + \cdots + (N_n - N_n - 1) * \frac{1}{n}$	$N_1, N_2, N_3, \ldots, N_{n-1}, N_n$: proportion of germinated seeds obtained the first (1), second (2), third (3), ..., $(n - 1)$, (n) days or hours.	Einhellig et al. (1982), Khandakar and Bradbeer (1983), Bradbeer (1988) in Wardle et al (1991)
Speed of Accumulated germination (AS)	$AS = \left\lfloor \frac{N_1}{1} + \frac{N_2}{2} + \frac{N_3}{3} + \cdots + \frac{N_n}{n} \right\rfloor$	$N_1, N_2, N_3, \cdots, N_n$: cumulative number of seeds which germinate on time 1, 2, 3, ..., N following set up of the experiment.	Adapted from Khandakar and Bradbeer (1983), Bradbeer (1988) in Wardle et al. (1991)
Coefficient of the rate of germination (CRG)	$CRG = \frac{[N_1 + N_2 + N_3 + \cdots + N_n]}{(N_1 * T_1) + (N_2 * T_2) + (N_3 * T_3) + \cdots + (N_n * T_n)} * 100$	N_1: number of germinated seeds on time T_1 N_2: number of germinated seeds on time T_2 N_n: number of germinated seeds on time T_n	Bewley and Black (1985)

TABLE 2. EFFECTS OF DIFFERENT CONCENTRATIONS OF PROTOCATECHUIC ACID, BOA AND *p*-HYDROXYBENZOIC ACID ON GERMINATION OF LETTUCE SEEDS EVERY 6 HOURS FOR TWO DAYS[a]

Compound and concentration (M)	Exposure time (hr)							
	6	12	18	24	30	36	42	48
Protocatechuic acid								
10^{-3}	0 ± 0	70.0 ± 20.9	109.3 ± 15.7	112.2 ± 12.4	103.9 ± 6.2	100.0 ± 5.3	96.5 ± 4.2	96.5 ± 4.2
10^{-4}	0 ± 0	60.0 ± 51.8	103.7 ± 47.4	109.9 ± 31.6	105.8 ± 16.3	104.1 ± 11.5	101.8 ± 7.5	100.9 ± 7.5
10^{-6}	0 ± 0	120.0 ± 54.2	113.7 ± 29.0	115.5 ± 17.3	105.8 ± 9.1	104.1 ± 6.2	102.6 ± 3.7	102.2 ± 4.2
10^{-8}	0 ± 0	185.0 ± 109.8	118.0 ± 17.7	111.0 ± 14.9	104.8 ± 9.3	102.8 ± 6.8	101.3 ± 5.4	101.3 ± 4.3
BOA								
10^{-3}	0 ± 0	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0 ± 0.0[b]	0.4 ± 1.0[b]	2.2 ± 2.2[b]	3.4 ± 3.6[b]	6.8 ± 7.0[b]
10^{-4}	0 ± 0	4.7 ± 7.0[b]	48.7 ± 21.7[b]	83.1 ± 15.2[b]	93.6 ± 5.1	94.6 ± 4.0	96.3 ± 3.7	96.7 ± 4.1
10^{-6}	0 ± 0	43.9 ± 26.4[b]	81.1 ± 24.3	89.4 ± 10.7	92.3 ± 6.4	93.3 ± 5.8	94.1 ± 5.0	95.4 ± 3.9
10^{-8}	0 ± 0	69.0 ± 29.0	86.7 ± 17.7	98.4 ± 3.3	99.3 ± 3.9	99.8 ± 3.6	100.1 ± 3.8	100.5 ± 3.4
p-Hydroxybenzoic acid								
10^{-3}	0 ± 0	12.5 ± 11.9[b]	100.1 ± 21.6	98.9 ± 3.6	98.9 ± 4.7	98.1 ± 3.9	98.4 ± 3.4	99.3 ± 2.9
10^{-4}	0 ± 0	36.1 ± 17.2[b]	90.6 ± 29.4	92.6 ± 10.9	96.7 ± 13.3	98.9 ± 11.1	99.7 ± 10.7	99.7 ± 10.7
10^{-6}	0 ± 0	64.3 ± 20.3	104.1 ± 17.5	104.3 ± 2.0	103.3 ± 2.2	102.8 ± 2.4	102.7 ± 3.0	102.7 ± 3.0
10^{-8}	0 ± 0	109.8 ± 24.2	88.4 ± 25.2	95.3 ± 6.1	98.5 ± 5.7	98.1 ± 5.8	97.5 ± 5.8	98.0 ± 5.3

[a]Results are expressed as percentage of the control.
[b]Significant differences, compared to the control for $P < 0.05$ according to Mann-Whitney U test.

TABLE 3. GERMINATION INDICES FOR EACH ALLELOCHEMICAL CONCENTRATION AND EXPRESSED AS PERCENTAGE OF CONTROL INDEX

Compound and concentration (M)	Index			
	G_T	S	AS	CRG
Protocatechuic acid				
10^{-3}	96.5 ± 4.2	100.7 ± 7.7	102.8 ± 8.5	108.2 ± 5.5
10^{-4}	100.9 ± 7.5	101.4 ± 20.1	102.9 ± 23.0	104.4 ± 16.4
10^{-6}	102.2 ± 4.2	107.4 ± 11.1	108.8 ± 12.9	107.3 ± 11.4
10^{-8}	101.3 ± 4.3	110.1 ± 4.5	110.9 ± 6.2	108.9 ± 7.8
BOA				
10^{-3}	6.8 ± 7.0[a]	2.8 ± 2.6[a]	1.4 ± 1.2[a]	35.5 ± 20.9[a]
10^{-4}	96.7 ± 4.1	74.1 ± 7.0[a]	73.6 ± 8.4[a]	79.0 ± 7.2[a]
10^{-6}	95.4 ± 3.9	85.0 ± 10.0[a]	84.9 ± 10.7	88.8 ± 8.1
10^{-8}	100.5 ± 3.4	93.6 ± 6.4	93.6 ± 6.2	92.9 ± 5.4
p-Hydroxybenzoic acid				
10^{-3}	99.3 ± 2.9	88.1 ± 6.3[a]	89.7 ± 7.1	92.3 ± 6.8
10^{-4}	99.7 ± 10.7	88.7 ± 11.8	89.0 ± 12.6	89.8 ± 7.1
10^{-6}	102.7 ± 3.0	98.3 ± 5.9	99.2 ± 5.8	97.7 ± 5.1
10^{-8}	98.0 ± 5.3	97.4 ± 8.9	96.8 ± 9.4	98.4 ± 9.4

[a] Significant difference, compared to control, for $P < 0.05$ according to Mann-Whitney U test.

allelochemicals was observed only with BOA at 10^{-3} M and the LC_{50} index has not been included.

It is clear from the results that each index leads to different interpretations of allelochemical effects on lettuce seed germination. Thus, two aspects could be discussed: germination capacity (G_T index) and germination progress or germination rate (S, CRG, and AS indices).

Germination capacity was calculated by means of the G_T index (maximum percentage germination), which is a widely used index (Hoffman et al. 1996; Baruah et al., 1994). In this study the G_T index revealed only germination inhibition by BOA at 10^{-3} M (Table 3). This does not mean that BOA affected the germination process only at this concentration. For instance, 10^{-4} and 10^{-6} M BOA delayed germination at 12, 18, and 24 hr (Table 2), but these effects were not reflected in the G_T index. The G_T index ignores slack times in germination. In this instance, a delay in germination was not involved even if the time of data collection had a significant impact on germination (Williams and Hoagland, 1982). Failure to consider the possibility of delayed germination leads to misunderstandings of allelochemical actions on germination. The G_T index

involved only final results and, hence, gave a global interpretation of germination (inhibition, stimulation, or no action). G_T could be useful to obtain ecological information in plant succession (Jäderlund et al., 1996), but it is not sensitive enough to study and validate allelochemical effects on a physiological process such as germination.

Unlike germination capacity, germination progress provided other interpretations through calculation of the three indices S, AS, and CRG. The S index was the most sensitive and closest to basic results (Table 2), since it was the only index that reflected the delayed germination with 10^{-6} M BOA. This confirmed its applicability and suggested that it would be useful for judging effects on germination, especially for delays (Einhellig et al., 1982). Furthermore, the S index has an important advantage over G_T because it is more sensitive as an indicator of allelopathic effects (Admed and Wardle, 1994), better revealing what occurred during the germination process.

Although S and AS have a similar formula (S considers the number of germinated seeds between two exposure times, whereas AS involves the cumulative number of germinated seeds at each exposure time), they did not give the same results. In contrast, AS and CRG had the same significant results and led to identical interpretations. They were less sensitive than S because they did not show the delayed germination by 10^{-3} M *p*-hydroxybenzoic acid and 10^{-6} M BOA. Only two results were significant (10^{-3} M and 10^{-4} M BOA). Care should be taken in interpretation of CRG or AS results.

Nevertheless, none of those four indices was able to reflect accurately all allelochemical effects on the germination process. Indeed, none revealed the delayed germination by 10^{-4} M *p*-hydroxybenzoic acid at 12 hr incubation time.

Bewley and Black (1985) proposed using more than one index to reflect the germination process accurately. They stated that "the behaviour of a seed population with respect to germination has several quantitative aspects that must be considered, and quantification obviously should not be limited to one parameter, say maximum germination percentage (germination capacity) or germination rate." Thus, several attempts have been made to include germination capacity and germination rate in a mathematical expression, but they were not entirely satisfactory. Developing a new index could be another possibility.

As the objective of the present study was to describe accurately allelochemical effects during germination, the indices were revealed as not precise enough. For these reasons, simple comparisons between the control and test at each exposure time appear more appropriate. Results could then be expressed as the cumulative number of germinated seeds or percentage germinated seeds and represented, for example, by time courses of germination. Important differences in behavior between seeds incubated with allelochemicals could be observed in such curves (Aliotta et al., 1994; Tarayre et al., 1995).

CONCLUSIONS

Results supported the hypothesis that data interpretations depended on the choice of germination index and also that the indices might not be suitable for precise description of allelopathic effects on the germination process. Using basic results for comparisons appeared more justified, otherwise interpretations could easily be criticized, and thus contribute to allelopathy being considered an imprecise science with regard to studies involving physiological processes.

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VOLATILE PLANT INFOCHEMICALS MEDIATE PLANT PREFERENCE OF *Trichogramma chilonis*

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Abstract—In India, eggs of the polyphagous noctuid moth *Helicoverpa armigera* on sorghum are parasitized to high levels by *Trichogramma* spp., but only rarely are parasitized eggs found on pigeonpea. This study was conducted to test whether volatile plant infochemicals contribute to the different parasitism levels observed on these two crops. In a four-armed airflow olfactometer, volatiles emitted by both sorghum and pigeonpea plants elicited a behavioral response form *Trichogramma chilonis* females. The parasitoids' response varied depending on the growth stage of the plant. Volatiles emitted by sorghum in the vegetative and reproductive stages arrested the parasitoids. *T. chilonis* females did not respond to volatiles from pigeonpea in the vegetative stage, but were repelled by volatiles from plants in the reproductive stage. Plants in the reproductive stage are preferred for oviposition by *H. armigera*. Thus, sorghum is attractive and pigeonpea repellent to *T. chilonis* females at the time when each plant is attractive to the host. This difference in the parasitoids' response may partly explain the different levels of egg parasitism reported from these two crops. The infochemicals involved in these plant–parasitoid interactions are discussed in the context of the current terminology.

Key Words—*Trichogramma chilonis*, Hymenoptera, Trichogrammatidae, pigeonpea, sorghum, *Helicoverpa armigera*, host location, airflow olfactometer, plant volatiles.

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INTRODUCTION

Parasitoids of herbivores use chemical stimuli (infochemicals) from both plants and hosts during the early steps in the host location process (Tumlinson et al., 1992; Vet and Dicke, 1992). These stimuli vary in their information content and in their detectability. Stimuli derived from the host are more reliable but generally difficult to detect, while stimuli from the plant are easier to detect but generally carry less host-specific information (Vet et al., 1991). Some parasitoids deal with this reliability–detectability problem by responding to herbivore-induced synomones, plant-produced infochemicals released after damage by the herbivore (Tumlinson et al., 1992; Vet and Dicke, 1992). Egg parasitoids such as *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) are not able to use stimuli derived from their hosts because insect eggs do not emit long-range volatiles (Kaiser et al., 1989; Noldus, 1989). Therefore, these parasitoids must rely on other information indicating the presence of host eggs on a plant, such as stimuli from another life stage of the host that may be easier to detect (infochemical detour; Vet et al., 1991). Two types of potential information are host sex pheromones and plant volatiles. Noldus (1989) listed several examples of *Trichogramma* spp. responding to host sex pheromones, and a number of studies have shown that *Trichogramma* spp. are attracted/arrested or repelled by plant volatiles (Bar et al., 1979; Cabello and Vargas, 1985; Nordlund et al., 1985; Kaiser et al., 1989).

The following study was conducted to test if *T. chilonis* Ishii females respond to volatiles emitted by pigeonpea [*Cajanus cajan* (L.) Millspaugh] and sorghum (*Sorghum bicolor* L.) plants. Pigeonpea is an important grain legume in the semiarid tropics (Nene and Sheila, 1990). Its most devastating pest in the Old World is the pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Lateef and Reed, 1990). One possible reason for the key pest status of *H. armigera* on pigeonpea is the low incidence and small impact of natural enemies, including *Trichogramma* spp., on this crop (Romeis and Shanower, 1996). In India, for example, reported parasitism levels by *Trichogramma* spp., mainly *T. chilonis*, can reach an average of 40% on eggs collected from sorghum, while less than 1% of eggs on pigeonpea are parasitized (Romeis and Shanower, 1996). Volatile plant infochemicals may partly explain the different parasitism levels of *H. armigera* eggs on these two crops.

METHODS AND MATERIALS

Parasitoids. T. chilonis was reared at 26 ± 2°C, 50 ± 10% relative humidity, and 16L:8D photoperiod on eggs of the factitious host *Corcyra*

cephalonica Stainton (Lepidoptera: Phyralidae). *C. cephalonica* was reared on sorghum grain. The parasitoid strain was collected in 1980 on *H. armigera* eggs from cotton and tomato plants near Hyderabad, India, and has been reared continuously on *C. cephalonica*. The female parasitoids used for the experiments were 1–2 days old, mated, inexperienced, and fed with honey agar.

Olfactometer Bioassay. The four-armed airflow olfactometer used in this study was slightly modified from the one used by Noldus (1988) and Noldus and van Lenteren (1985) for *Trichogramma* spp. The exposure chamber consisted of a single piece of white acetal (Delrin) covered with a glass plate. The setup was made airtight by a rubber ring fitted into a groove around the exposure chamber. Stainless-steel tubes were used for the connections. Each arm of the observation chamber was connected to a treatment chamber made of polycarbonate, a washing bottle filled with distilled water, and a flowmeter by which the airstream through each arm was separately regulated to 27 ml/min. The boundaries of the four flow fields were drawn on the cover glass plate. Thirty seconds after introducing one *T. chilonis* female in the entry tube, the tube was darkened and the parasitoid walked up into the exposure chamber (positive phototactic response). The airflow was established before the test insect entered the exposure chamber. Each wasp was observed for 10 min beginning when the parasitoid crossed a "first choice" line, 5 mm around the entry tube. This first choice, the total time spent by the parasitoid in each of the four flow fields, and whether or not a parasitoid entered the arms connected to the flow fields was recorded using the computer software package The Observer® (Noldus Information Technology, 1993). The first flow field chosen by the parasitoid was recorded to see if the wasp differentiated between the treatments before exiting the entry tube.

Plant material was offered in two opposite treatment chambers, leaving the other two as control. Air humidified with wet tissue paper (Kimwipes) was used as the control, as a preliminary experiment, also described in this paper, showed that *T. chilonis* females prefer humidified to unhumidified air. After every two to three observations, the exposure chamber was cleaned with 70% ethanol to remove traces of previous parasitoids (Salt, 1958). After every five observations, the apparatus was rotated to prevent bias due to environmental factors. A minimum of 40 wasps were observed for each test material. All experiments were conducted at ambient conditions of 26 $\pm$ 2°C and 50 $\pm$ 10% relative humidity.

When the distribution of the parasitoids over the four flow fields was significant after analysis of variance on angular transformed percentage data, the time spent in the two test fields was compared to the time spent in the two control fields based on the single degree of freedom contrast using the F test (Steel and Torrie, 1980). The number of parasitoids that spent more than 50%

of the observation time in the test fields as compared to the control fields was compared by a χ^2 test (Dicke et al., 1984). The number of test females that selected the flow fields containing the plant volatiles as their first choice was compared to the number selecting the control fields with a χ^2 test. The null hypothesis of equal distribution was used for both χ^2 tests.

Preliminary Experiment. As plant material offered in the treatment chambers increases the relative humidity of air, a preliminary experiment was conducted to test whether the parasitoids show any response to air humidified by wet tissue paper or plant material (20 pigeonpea pods, cv. ICPL 87; ~ 160 cm^2 pod surface). Pigeonpea pods plus wet tissue paper were tested against wet tissue paper alone to separate the effect of plant volatiles and relative humidity.

Sorghum Tests. Field collected sorghum (cv. CSH 9) leaves and panicles were tested. Panicles were checked for *H. armigera* eggs and larvae to ensure that they had no previous contact with the moth and were covered with paper bags one day prior to collection. The following quantities of each plant part were offered in a single treatment chamber: leaves, ~460 cm^2 leaf area; newly emerged panicle, one fresh exposed panicle of ~15 cm length, no flowers; flowering panicle, one panicle of ~15 cm length; soft dough panicle, one panicle with developing seeds of ~15 cm length, no flowers.

Pigeonpea Tests. Plant material was collected from undamaged greenhouse grown plants (cv. ICPL 87), and the following quantities of each plant part were offered in a single treatment chamber: vegetative tips, six tips of ~20 cm length, ~460 cm^2 leaf area; reproductive tips, three tips of ~20 cm length, with leaves, buds, flowers and pods; buds, flowers, three tips of ~20 cm length, with leaves, buds and flowers; and pods, 20 pods, ~ 160 cm^2 pod surface.

With the exception of pigeonpea pods, the plant material was placed in the treatment chamber with the cut end inserted in a glass vial filled with water and sealed with Parafilm.

RESULTS

The preliminary experiment showed that when offered a choice between air with a relatively high versus low humidity, the parasitoids spent a significantly longer time in the flow fields with the higher humidity (Table 1). This was true whether the source of the humidity was wet tissue paper [Contrast (Q) $\pm$ SE; $Q = 5.0 \pm 1.61$; $F_{1,244} = 9.6$] or plant material ($Q = 10.2 \pm 2.30$; $F_{1,156} = 19.7$). The parasitoids were repelled by volatiles from pigeonpea pods when offered together with wet tissue paper against humidified air ($Q = -8.0 \pm 1.59$; $F_{1,196} = 25.3$).

T. chilonis females were arrested by volatiles from flowering sorghum

TABLE 1. RESPONSE OF FEMALE *Trichogramma chilonis* TO HUMIDITY OR PLANT MATERIAL VERSUS CLEAN, UNHUMIDIFIED OR HUMIDIFIED AIR IN A FOUR-ARMED OLFACTOMETER

Test material	*N*	Time spent in flow fields (%)[a]	Longest time in flow fields *N*[b]	First choice *N*[c]
Wet tissue paper	62	57.0**[d]	40*	37
vs. unhumidified air		43.0	22	25
Pigeonpea pods	40	63.4***	27*	23
vs. unhumidified air		36.6	13	17
Pigeonpea pods + wet tissue paper	50	38.3***	11***	21
vs. wet tissue paper		61.7	39	29

[a] Percentage of observation time spent in the test fields as compared to the control fields. Means were compared based on the single degree of freedoom contrast.
[b] Number of parasitoids spending more than 50% of the observation time in the test fields as compared to the control fields. Numbers were analyzed using χ^2 test.
[c] Number of parasitoids crossing the first choice line in the test fields as compared to the control fields. Numbers were analyzed using χ^2 test.
[d] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

panicles ($Q = 8.3 \pm 1.95$; $F_{1,156} = 18.0$) or panicles in the soft dough stage ($Q = 7.6 \pm 1.79$; $F_{1,156} = 18.2$) (Table 2). Volatiles from newly emerged panicles without flowers, however, did not elicit a response from the parasitoids.

Volatiles emitted by pigeonpea plant material in the reproductive growth stage significantly repelled *T. chilonis* (Table 3). Repellency was observed by volatiles from tips containing buds, flowers, and pods ($Q = -8.4 \pm 1.74$; $F_{1,156} = 23.1$), tips containing buds and flowers ($Q = -3.9 \pm 1.89$; $F_{1,196} = 4.3$) and pods alone ($Q = -6.5 \pm 1.93$; $F_{1,284} = 11.5$). Prior to the reproductive growth stage, when only vegetative tissue was present, the wasps showed no response.

With the exception of the sorghum leaves (Table 2), the χ^2 test and the single degree of freedom comparisons produced similar results. For volatiles from sorghum leaves, a significant arrestment response was detected using the χ^2 test, while analysis based on the single degree of freedom comparison showed no significance.

No significant difference in the number of first choices was detected in

TABLE 2. RESPONSE OF FEMALE *Trichogramma chilonis* TO VOLATILES EMITTED BY SORGHUM PLANT PARTS VERSUS CLEAN, HUMIDIFIED AIR IN A FOUR-ARMED OLFACTOMETER

Plant material	*N*	Time spent in test fields (%)[a]	Longest time in test fields *N*[b]	First choice *N*[c]
Leaves	40	55.4	27*	18
Newly emerged panicle	40	47.9	15	22
Flowering panicle	40	61.3***[d]	31***	24
Soft dough panicle	40	60.6***	31***	24

[a]Percentage of observation time spent in the test fields as compared to the control fields. Means were compared based on the single degree of freedom contrast.
[b]Number of parasitoids spending more than 50% of the observation time in the test fields as compared to the control fields. Numbers were analyzed using χ^2 test.
[c]Number of parasitoids crossing the first choice line in the test fields as compared to the control fields. Numbers were analyzed using χ^2 test.
[d]* $P < 0.05$; *** $P < 0.001$.

TABLE 3. RESPONSE OF FEMALE *Trichogramma chilonis* TO VOLATILES EMITTED BY PIGEONPEA PLANT PARTS VERSUS CLEAN, HUMIDIFIED AIR IN A FOUR-ARMED OLFACTOMETER

Plant material	*N*	Time spent in test fields (%)[a]	Longest time in test fields *N*[b]	First choice *N*[c]
Vegetative tips	40	49.2	20	22
Reproductive tips	40	37.4***[d]	8***	20
Buds, flowers	50	43.7*	15**	23
Pods	72	41.0***	27*	31

[a]Percentage of observation time spent in the test fields as compared to the control fields. Means were compared based on the single degree of freedom contrast.
[b]Number of parasitoids spending more than 50% of the observation time in the test fields as compared to the control fields. Numbers were analyzed using χ^2 test.
[c]Number of parasitoids crossing the first choice line in the test fields as compared to the control fields. Numbers were analyzed using χ^2 test.
[d]* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

either test (Table 1–3). Most test females entered one or more arms connected to the exposure chamber, but they usually returned to the observation chamber. Thus, it was not possible to set a threshold time at which females made a final choice.

DISCUSSION

Sorghum plant material emitted volatiles that elicited an arrestment response in *T. chilonis*. Volatiles from sorghum panicles, the oviposition site for *H. armigera* (Parsons, 1940), arrested the parasitoids only after the flowers had opened. The infochemicals were not emitted by the flowers directly, however, as volatiles from panicles in the soft dough stage elicited the same response by the parasitoids. *T. chilonis* is known to parasitize insects ovipositing on sorghum leaves such as *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) (Sharma, 1985), and this may explain why the parasitoids were arrested by leaf volatiles. The sorghum volatiles that arrest *T. chilonis* are unknown. Whether infochemicals emitted by panicles in the flowering and soft dough stages and those emitted by the leaves are the same is also unknown. Sorghum volatiles have been shown to be attractive to hymenopteran and dipteran larval parasitoids of *Heliothis/Helicoverpa* spp. and stemborers (Schuster and Starks, 1974; Elzen et al., 1983; Martin et al., 1990; Ngi-Song et al., 1996).

The response of *T. chilonis* to volatiles from pigeonpea was strikingly different. Pigeonpea became repellent to *T. chilonis* when it had reached the reproductive growth stage, the most attractive stage for *H. armigera* oviposition. Volatiles emitted by plants in the vegetative growth stage did not mediate the behavior of searching parasitoids, and it is likely that the infochemicals responsible for the repellency of reproductive plants are emitted by the reproductive structures. This is supported by the finding that volatiles from pods alone were repellent. However, it is possible that leaves from plants in the reproductive growth stage may also emit the repellent infochemicals. Martin et al. (1990) reported that the response of *Eucelatoria bryani* Sabrosky (Diptera: Tachinidae), a tachinid parasitoid of *Heliothis/Helicoverpa* spp., to volatiles emitted by pigeonpea leaves varies with the growth stage of the plant. The pigeonpea infochemicals responsible for the repellency of *T. chilonis* are unknown and only a little information on pigeonpea volatiles exists. Six sesquiterpenes—α-bulnesene, α-humulene, α-guajene, β-caryophyllene, γ-muurolene and α-muurolene—are the major components in an aqueous steam distillate of pigeonpea leaves collected from plants in the flowering growth stage. A mixture of these compounds was attractive for female *H. armigera* in a flight tunnel and acted as an oviposition stimulant (Hartlieb and Rembold, 1996). These chemicals were emitted by pigeonpea leaves and are probably not responsible for the repellency of *T. chilonis*. Therefore it may be that different pigeonpea volatiles are involved in the attraction of *H. armigera* and the repellency of *T. chilonis*.

Our results show that *T. chilonis* is arrested by sorghum volatiles and repelled by volatiles from pigeonpea in the reproductive growth stage, the stage preferred for oviposition by the host *H. armigera*. Parasitoids were arrested by volatiles from pigeonpea pods when offered against unhumidified air; thus, the

attractiveness of humidity may overcome the repellency of pigeonpea. This may indicate that these infochemicals do not play a major role in the searching behavior of *T. chilonis* in the field and that other plant characters also contribute to the low parasitism levels on pigeonpea. A study to evaluate the impact of pigeonpea surface structures, i.e., trichomes and trichome exudates, on *T. chilonis* is currently in progress.

Using plant information is not expected to be adaptive for a generalist parasitoid such as *Trichogramma* spp. attacking a wide range of hosts (Vet and Dicke, 1992). However, *Trichogramma* spp. have been reported to respond to volatiles from tomato (Nordlund et al., 1985), maize (Kaiser et al., 1989), and cotton (Bar et al., 1979; Cabello and Vargas, 1985), in addition to sorghum and pigeonpea. Leaves from cabbage plants are the only example of plant material that did not elicit a response from *Trichogramma* spp. (Pak and van Lenteren, 1984). Therefore, the response of *Trichogramma* spp. to volatile plant infochemicals seems to be a common phenomenon. Wind plays an important role in the distribution of *Trichogramma* spp. (Hendricks, 1967; Keller et al., 1985), and because the parasitoids are unable to fly against strong winds (Steenburgh, 1934), it is likely that plant volatiles repel or arrest, rather than attract, trichogrammatids after entering a habitat. This may explain why in this study *T. chilonis* did not walk up the olfactometer arms connected to an attractive flow field as was reported for larval parasitoids (Dicke et al., 1984; Eller et al., 1988; Turlings et al., 1990). Arrestment rather than attraction of *Trichogramma* spp. to host sex pheromones has been reported by Noldus (1988) and Noldus and van Lenteren (1985).

According to the current infochemical terminology (Dicke and Sabelis, 1988), the sorghum volatiles responsible for the arrestment of *T. chilonis* are classified as synomones as both the plant (emitter) and the parasitoid (receiver) benefit from the interaction. The infochemicals involved in the *T. chilonis*–pigeonpea interaction are most difficult to classify but may be an example of an antimone, as neither the plant nor the parasitoid seem to benefit from the interaction. This term, first proposed by Whitman (1988), has been adapted to the current infochemical terminology by Takabayashi and Takahashi (1990) (see also Dicke and Sabelis, 1992).

Antimones have only been detected in a few systems. In two, the interaction was nonadaptive for the emitter and maladaptive for the parasitoid (receiver) (Takabayashi and Takahashi, 1990; Takabayashi et al., 1991). In contrast to these examples, *T. chilonis*–pigeonpea interactions seem to be maladaptive for both the emitter and the receiver of the infochemicals. A similar interaction has been reported for other *Trichogramma* species. Cotton is repellent to *T. semifumatum* Riley (Bar et al., 1979), and cotton leaves, but not flowers, are repellent to *T.* sp. p. *buesi* (Cabello and Vargas, 1985). *T. cordubensis* Vargas and Cabello is not repelled by cotton (Cabello and Vargas, 1985), and it is therefore

likely that the olfactory response varies between *Trichogramma* species. Differences in the olfactory response could be partly responsible for the reported habitat preference of *Trichogramma* spp. (Salt, 1935; Flanders, 1937; Lopez et al., 1982; Pinto and Oatman, 1988).

We believe that additional examples of volatile plant infochemicals that are repellent to parasitoids will be detected in the future. Two reasons may explain why they have only rarely been reported: (1) researchers focus on the attraction as opposed to the repellency of parasitoids or predators to specific host plants, and (2) some of the currently used methods, such as wind tunnels, are not designed to detect a repellent response to volatiles.

It is surprising that antimones exist as one would expect that communicative interactions have evolved by positive selection for transmission as well as reception (Otte, 1974). It remains unclear how such mutually disadvantageous interactions could evolve. One possible explanation is that production of or response to compounds acting as antimones in one context may be beneficial in another context. Most probably antimones will be found in systems where the interactants have not closely coevolved. Examples would be systems in which the interactants are of different origin or generalist parasitoids (such as *Trichogramma* spp.) attacking polyphagous hosts (such as *Heliothis/Helicoverpa* spp.). Antimones might therefore be a limiting factor for the use of 'new associations' in biological control.

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DISTRIBUTION AND DIFFERENTIAL EXPRESSION OF (*E*)-4,8-DIMETHYL-1,3,7-NONATRIENE IN LEAF AND FLORAL VOLATILES OF *Magnolia* AND *Liriodendron* TAXA

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Abstract—Analyses of volatiles emitted from artificially damaged leaves attached to branches of seven *Magnolia* taxa revealed the presence of (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol (the green odor compounds), and several mono- and sesquiterpenes, e.g., (*Z*)- and (*E*)-β-ocimene and caryophyllene. An herbivore-induced leaf volatile, (*E*)-4,8-dimethyl-1,3,7-nonatriene, known as a predator attractant in agricultural plants, was emitted 4–6 hr after leaves were damaged in *M. hypoleuca*. The damaged leaves of *M. grandiflora*, however, immediately released (*E*)-4,8-dimethyl-1,3,7-nonatriene. Undamaged leaves of *Magnolia* species examined did not emit volatile compounds. In addition, detached flowers of six *Magnolia* taxa and *Liriodendron tulipifera* also emit (*E*)-4,8-dimethyl-1,3,7-nonatriene as a floral volatile (up to 30% in some species); the chemical was also emitted from the intact flowers of *M. heptapeta* and *M. salicifolia*.

Key Words—Floral odor, green odor, monoterpene, sesquiterpene, 4,8-dimethyl-1,3,7-nonatriene, attractant, deterrent, herbivore, pollination biology, plant–insect interaction.

INTRODUCTION

Secondary compounds produced in plants are involved in many plant–insect relationships, e.g., protection against herbivores, pollination, and coevolution

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(Ehrlich and Raven, 1964; Rhoades, 1979; Wood, 1982; Williams and Whitten, 1983; Chew, 1988; Spencer, 1988; Harborne, 1993). Leaf injury of some agricultural plants by herbivores causes the delayed release of volatile secondary compounds that attract predators and parasitoids (Loughrin et al., 1994, 1995; Turlings et al., 1990, 1995; Turlings and Tumlinson, 1992; Takabayashi et al., 1994a,b). Moreover, feeding damage by herbivores not only affects the quality and quantity of released volatiles from the damaged leaves but also induces systemic emission of these volatiles from undamaged vegetative parts of the plant damaged (Röse et al., 1996; also see Farmer and Ryan, 1990; Shulaev et al., 1997). Exploitation of such chemical signals by predators and parasitoids to locate prey formed the basis for the hypothesis that plants may actively signal predators as a defensive mechanism (Price et al., 1980; Dicke and Sabelis, 1992; Turlings et al., 1990, 1995).

Volatiles emitted from flowers are also important insect attractants for pollination and may function as chemical cues for food, rest, mating, and/or larval development sites (Pellmyr and Thien, 1986). The fact that volatile compounds such as monoterpenes and fatty acid derivatives commonly occur in both (damaged) leaf and floral volatiles (Knudsen et al., 1993) suggests that floral volatiles may have originated from general leaf volatiles in the meshing of the life cycles of insects and plants (Pellmyr and Thien, 1986; Pellmyr et al., 1991).

In this paper we document the emissions of volatiles from artificially damaged leaves of seven *Magnolia* taxa and record the distributions of (*E*)-4,8-dimethyl-1,3,7-nonatriene in floral volatiles of *Magnolia* and *Liriodendron* taxa because it is known as one of the attractants of natural enemies emitted from feeding-damaged leaves (Turlings et al., 1990; Turlings and Tumlinson, 1992; Takabayashi et al., 1994a,b). This is the first documentation of the release of (*E*)-4,8-dimethyl-1,3,7-nonatriene from damaged leaves in non-agricultural plants.

METHODS AND MATERIALS

Collection of Volatiles. Seven species of *Magnolia*: *M. praecocissima* Koidz. (= *M. kobus*), *M. tomentosa* Thunb., *M. salicifolia* (Sieb. et Zucc.) Maxim., *M. hypoleuca* Sieb. et Zucc. (= *M. obovata*), *M. sieboldii* K. Koch ssp. *japonica* Ueda, *M. heptapeta* (Buchoz) Dandy (= *M. denudata*; Chinese taxon) and *M. grandiflora* L. (North American taxon) (Treseder, 1978; Ueda, 1980, 1985, 1986a,b), native to or cultivated in Japan were used for GC-MS analyses of volatiles emitted from leaves. Of the seven taxa, *M. grandiflora* is evergreen and the other species are deciduous trees or shrubs.

Leaves were damaged with a wire brush (while attached to the plant) and

then immediately enclosed in a polyethylene bag. Five to seven leaves on a branch were used at each sampling period except for *M. hypoleuca* (only one leaf, approximately 30 cm, was used). Leaf volatiles were collected from three different individuals in each taxon. Six successive samples were taken every 2 hr. Volatiles in the bag were pumped through a glass tube (7 mm ID × 5 cm) containing adsorbent (50 mg Tenax GC, mesh 60–80, GL Sciences, Tokyo, Japan) with a mini-pump (model MP-2N, Shibata Scientific Instrument Co., Ltd., Tokyo, Japan). The mini-pump was driven at approximately 1 liter/min. The glass tube containing the captured leaf volatiles was sealed with aluminum foil and stored at −20°C. Empty bags, adsorbent blanks, and bags containing undamaged leaves (controls) were analyzed for volatiles. Prior to use, the adsorbent was washed successively with two volumes of distilled methanol and ether and heated for 12 hr at 150°C.

Volatiles emitted from detached flowers of the 11 *Magnolia* and *Liriodendron tulipifera* species were analyzed by GC-MS. Intact flowers of five taxa, i.e., *M. sieboldii* ssp. *japonica*, *M. praecocissima*, *M. tomentosa*, *M. salicifolia*, and *M. heptapeta* were also analyzed. Throughout sampling, no floral organs were damaged. One to a few flowers were enclosed in a polyethylene bag in the field. The volatiles were collected in the same manner as described above, except sampling periods were for 2–3 hr (for detached flowers) and 9 hr (for intact flowers), respectively.

GC-MS Analysis. Leaf volatiles were eluted from the adsorbent with 350 μl of distilled diethyl ether. As an internal quantitative standard, 5 μl of *n*-octanol solution (10 μl *n*-octanol/1 ml diethylether) was added to the eluents. The eluents were carefully concentrated by N_2 flow to approximately 100 μl. Two microliters of the eluents were used for GC-MS analyses in all cases. GC-MS analysis was performed with a Hewlett-Packard 5971A mass selective detector, connected to a Hewlett-Packard 5890 Series II gas chromatograph. A fused silica capillary column (DB-17, 0.25 mm ID × 30 m, film thickness 0.25 μm) was used. The ion-source temperature was 185°C (the ionization energy was 70 eV). The injection temperature was 250°C and oven temperature was maintained at 50°C for the first 5 min, then programmed to 150°C at 5°C/min, finally raised to 250°C at 10°C/min, and then kept for 10 min. Helium was used as the carrier gas.

The chemical structures of the volatiles were identified by comparison of the GC retention times and mass spectra with those of authentic compounds, with computer data in the library, NIST PBM HP G1033A revision code A.00.00 (Hewlett-Packard), and with our own library of more than 100 mono- and sesquiterpenes. Most authentic compounds were obtained from commercial sources, but (*E*)-4,8-dimethyl-1,3,7-nonatriene was synthesized from citral by the Wittig reaction (Greenwald et al., 1963).

TABLE 1. VOLATILE COMPOUNDS EMITTED FROM ARTIFICIALLY DAMAGED LEAVES OF SEVEN *Magnolia* TAXA DURING 12 HOURS AFTER DAMAGE[a]

Compounds	Amounts of compounds collected (μg/12 hr/100 cm^2 leaf area)						
	M. pra. (N = 3)	M. tom. (N = 3)	M. sal. (N = 3)	M. hyp. (N = 3)	M. s.j. (N = 3)	M. hep. (N = 3)	M. gra. (N = 3)
Aliphatics							
(*Z*)-3-Hexenal					0.25 ± 0.40		
(*Z*)-3-Hexenol	3.09 ± 2.36	0.21 ± 0.12	0.06 ± 0.10	2.79 ± 0.80	8.17 ± 3.15		0.30 ± 0.28
(*Z*)-3-Hexenyl acetate	41.95 ± 19.48	38.43 ± 6.27	12.82 ± 1.57	21.7 ± 1.56	7.56 ± 6.09	0.13 ± 0.15	
Monoterpenes							
β-Myrcene	2.59 ± 1.34	2.28 ± 0.72				4.31 ± 1.11	0.24 ± 0.13
Limonene[b]	0.31 ± 0.19			0.22 ± 0.07			0.09 ± 0.08
(*Z*)-β-Ocimene	1.55 ± 0.74	3.71 ± 0.97	14.94 ± 5.94		0.04 ± 0.03		
(*E*)-β-Ocimene	6.59 ± 3.99	22.08 ± 1.29	3.52 ± 1.06	1.37 ± 0.60	0.02 ± 0.01		
p-Cymene						0.89 ± 0.34	
Linalool	0.15 ± 0.06			0.63 ± 0.62	0.59 ± 0.91		
Sesquiterpenes							
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	0.03 ± 0.06			1.74 ± 0.80			2.48 ± 0.78
Bicyclogermacrene[b]							4.18 ± 2.26
β-Elemene			0.93 ± 0.90		0.03 ± 0.03		7.65 ± 3.73
Caryophyllene[b]	0.30 ± 0.29	16.63 ± 6.17	0.02 ± 0.03	15.07 ± 5.59			2.73 ± 0.88
α-Humulene[b]		0.86 ± 0.51		1.90 ± 0.83			0.15 ± 0.08
γ-Cadinene[b]		1.25 ± 0.73					2.50 ± 0.75
α-Bisabolene[b]							6.23 ± 1.61
Unknowns							
43 56 61 69 84		0.38 ± 0.15					
91 93 121 136					0.19 ± 0.08		
79 91 105 121 136			3.32 ± 1.52				
43 91 119 134							0.10 ± 0.07
77 93 105 119 161							0.17 ± 0.09
67 82 93 107 121 136							0.23 ± 0.09
105 119 133 161 204							0.14 ± 0.10

[a] Data represent the average of three samples ± SD. Amount of each sample is the sum of six successive 2-hr samplings. Taxon abbreviations: M. pra.; *Magnolia praecocissima*, M. tom.; *M. tomentosa*, M. sal.; *M. salicifolia*, M. hyp.; *M. hypoleuca*, M. s.j.; *M. sieboldii* spp. *japonica*, M.hep.; *M. heptapeta*, M. gra.; *M. grandiflora*.

[b] Identification was made with only mass spectra of the original computer library.

RESULTS

Three aliphatics, six monoterpenes, and seven sesquiterpenes were identified in the volatiles from artificially damaged leaves of *Magnolia* (Table 1). The volatiles of *M. praecocissima*, *M. tomentosa*, *M. salicifolia*, *M. hypoleuca* and *M. sieboldii* ssp. *japonica*, were mainly composed of (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*Z*)-β-ocimene, (*E*)-β-ocimene and caryophyllene. In contrast, the volatiles of the evergreen *M. grandiflora* were dominated by sesquiterpenes, especially β-elemene, α-bisabolene, bicyclogermacrene, caryophyllene, γ-cadinene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene. β-Myrcene, limonene, linalool, β-elemene, and α-humulene were also found in the volatiles of some *Magnolia* taxa as minor components. (*E*)-4,8-Dimethyl-1,3,7-nonatriene occurred in the volatiles of *M. praecocissima*, *M. hypoleuca*, and *M. grandiflora*, although the amount in *M. praecocissima* was very low. These compounds were not found in volatiles from undamaged leaves (Table 2), in which no characteristic ion was detected in the GC-MS ion chromatograms, except a small amount of (*Z*)-3-hexenyl acetate was present in volatiles from *M. hypoleuca*.

The temporal pattern of the emissions of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, caryophyllene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene from artificially damaged leaves of selected taxa are shown in Figure 1. Generally, most volatiles, including (*Z*)-3-hexenyl acetate and (*E*)-β-ocimene, were emitted immediately after damage and then the amounts decreased rapidly, although (*E*)-β-ocimene from *M. hypoleuca* was emitted more abundantly at a later stage (Figure 1). In addition, caryophyllene was constantly emitted from the damaged leaves of *M. tomentosa*, *M. hypoleuca*, and *M. grandiflora* (Figure 1). The timing of emission of (*E*)-4,8-dimethyl-1,3,7-nonatriene from artificially damaged leaves

TABLE 2. VOLATILE COMPOUND EMITTED FROM UNDAMAGED LEAVES OF SEVEN *Magnolia* TAXA DURING 12 HOURS[a]

Compound	Amount of compound collected (μg/12 hr/100 cm² leaf area)						
	M. pra (*N* = 1)	M. tom. (*N* = 1)	M. sal. (*N* = 1)	M. hyp. (*N* = 1)	M. s.j. (*N* = 1)	M. hep. (*N* = 1)	M. gra. (*N* = 1)
Aliphatic							
(Z)-3-Hexenyl acetate	–[b]	–[b]	–[b]	0.68	–[b]	–[b]	–[b]

[a]Amount is the sum of six successive 2-hr samplings. See Table 1 for taxon abbreviations.
[b]No characteristic ion was detected in GC-MS ion chromatogram.

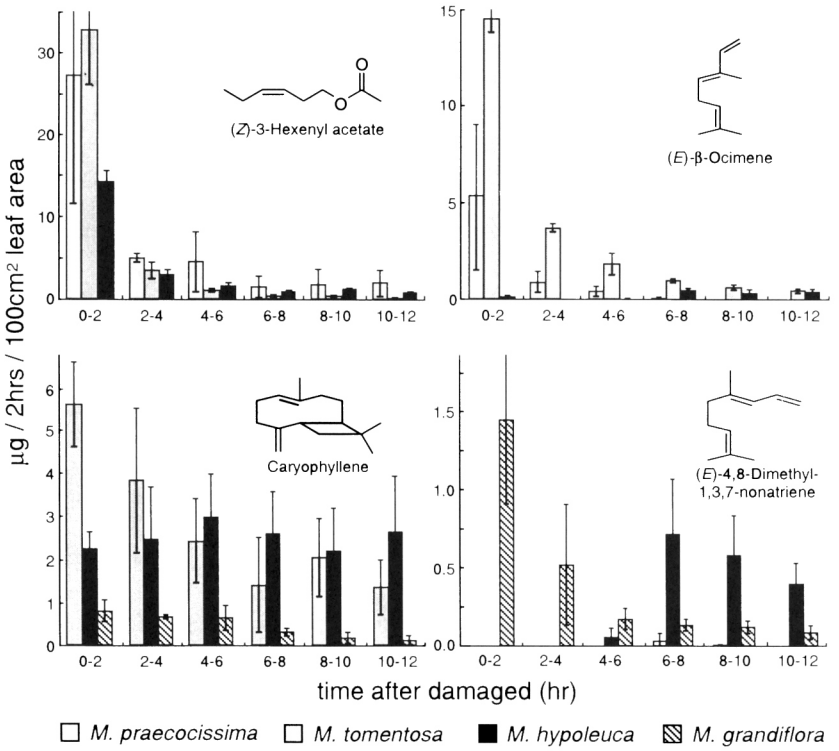


FIG. 1. Emissions of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, caryophyllene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene from artificially damaged leaves of four *Magnolia* taxa over time. Data represents the mean of three samples ± SD.

of *M. hypoleuca* was very different from those of (*Z*)-3-hexenyl acetate and other terpenes (Figure 1). In the early stages, it was not emitted from the damaged leaves at all. The emission began after 4–6 hr and peaked after 6–8 hr. Although (*E*)-4,8-dimethyl-1,3,7-nonatriene was emitted from the damaged leaves of *M. praecocissima* in a similar pattern as in *M. hypoleuca*, the quantity was very low. In contrast, the damaged evergreen leaves of *M. grandiflora* immediately released (*E*)-4,8-dimethyl-1,3,7-nonatriene after damage (Figure 1).

GC-MS analyses of floral volatiles of *Magnolia* and *Liriodendron* indicate the flowers emit various chemical compounds such as terpenoids, benzenoids, and fatty acid derivatives (H. Azuma, M. Toyota, Y. Asakawa, L. B. Thien, R. Yamaoka, and S. Kawano, unpublished data). (*E*)-4,8-Dimethyl-1,3,7-nonatriene also was detected in the volatiles of detached flowers of *M. virginiana*, *M. grandiflora*, *M. acuminata*, *M. praecocissima*, *M. tomentosa*, *M. heptapeta*,

and *Liriodendron tulipifera* ranging from $< 1\%$ to more than 50% of the floral volatiles (Table 3). Two Asiatic *Magnolia* taxa belonging to the subgenus *Yulania*, i.e., *M. tomentosa* and *M. heptapeta*, emitted especially high relative amounts of the compound. Intact flowers of *M. heptapeta* and *M. salicifolia* also emitted (*E*)-4,8-dimethyl-1,3,7-nonatriene (Table 3).

TABLE 3. DISTRIBUTION AND AMOUNT OF (*E*)-4,8-DIMETHYL-1,3,7-NONATRIENE IN ARTIFICIALLY DAMAGED AND UNDAMAGED LEAF VOLATILES AND RELATIVE AMOUNT IN FLORAL VOLATILES OF *Magnolia* AND *Liriodendron* TAXA[a]

			Flower[c]	
	Leaf[b] (μg/12 hr/100 cm^2 leaf area)		%/2 hr/1–3 flowers	% (μg)/9 hr/flower
Taxa	Damaged	Undamaged	Detached[b]	Intact
Magnolia				
Subgenus *Magnolia*				
M. virginiana			–[e]	
M. virginiana[f]			2.5	
M. grandiflora[s]	2.48	–[e]	0.5	
M. pyramidata			–[e]	
M. tripetala			–[e]	
M. hypoleuca	1.74	–[e]	–[e]	
M. sieboldii				
spp. *japonica*	–[e]	–[e]		–[e]
Subgenus *Yulania*				
M. acuminata			0.1	
M. praecocissima	0.03	–[e]	3.1	–[e]
M. tomentosa	–[e]	–[e]	37.9	–[e]
M. salicifolia	–[e]	–[e]	–[e]	8.3 (0.48)
M. heptapeta[s]	–[e]	–[e]	51.8	28.9 (39.37)
Liriodendron				
L. tulipifera			14.7	

[a] From H. Azuma, M. Toyota, Y. Asakawa, L. B. Thien, R. Yamaoka, and S. Kawano (unpublished data).
[b] Both damaged and undamaged leaves remained attached to branches during sampling.
[c] Floral organs were not damaged.
[d] Amounts of compounds compared with an internal standard were not examined.
[e] No characteristic ion indicating the compound was detected in GC-MS ion chromatogram.
[f] A cultivated plant in Tulane University was used.
[s] Cultivated plants in Kyoto University were used.

DISCUSSION

The artificially damaged leaves of *Magnolia* emitted the green odor compounds, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, and mono- and sesquiterpenes, particularly (*Z*)-β-ocimene, (*E*)-β-ocimene, and caryophyllene, in various combinations and quantities. Most of these volatiles are also emitted from the leaves of artificially damaged crop plants or plants attacked by herbivores (Takabayashi et al., 1994a,b; Loughrin et al., 1994, 1995; Röse et al., 1996). These volatiles also occur in floral fragrance of a wide range of angiosperms (Knudsen et al., 1993).

The green odor compounds, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, and other C_6 compounds, are synthesized from α-linolenic or linoleic acids from plastid membranes in green leaves when the leaves are damaged artificially or by herbivores (Hatanaka, 1993). Moreover, these compounds may elicit greater antennal olfactory responses than some monoterpenes in the parasitoid *Microplitis croceipes* (Hymenoptera) (Li et al., 1992). Corn, cotton, and other plants emit the green odors immediately after leaves are damaged, and then their release rapidly decreases. Large amounts of terpenoids such as (*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, and (*E*)-β-ocimene are induced in herbivore-damaged leaves after several hours of damage and attract natural enemies of the herbivores (Turlings and Tumlinson, 1992; Takabayashi et al., 1994a,b; Turlings et al., 1995; Röse et al., 1996). Emission of these chemicals by artificially damaged leaves of *Magnolia* species suggests similar signaling between first and third trophic levels may occur.

The composition of volatiles of damaged leaves of *M. grandiflora* differed considerably from the other *Magnolia* taxa, especially in the presence of relatively large amounts of several sesquiterpenes, the small amounts of green odors, and in the immediate emission of (*E*)-4,8-dimethyl-1,3,7-nonatriene. These differences suggest that the evergreen leaves of *M. grandiflora* store large amounts of volatiles, including a range of sesquiterpenes as (*E*)-4,8-dimethyl-1,3,7-nonatriene. The damaged leaves of *M. heptapeta* did not emit the green odors, which may be due to physiological conditions.

Analyses of the GC-MS data clearly show that the undamaged leaves of seven *Magnolia* taxa emit no volatiles, except for *M. hypoleuca*, which produced a small amount of (*Z*)-3-hexenyl acetate, probably caused by small scratches on the leaf surface. Many investigations, however, report that undamaged leaves emit similar quantities of volatile compounds as the damaged leaves (Andersen et al., 1988; Turlings and Tumlinson, 1992; Jakobsen et al., 1994; Loughrin et al., 1994, 1995; Mattiacci et al., 1994; Takabayashi et al., 1994a,b; Röse et al., 1996). In these investigations, undamaged leaf volatiles were collected from leaves cut from the plants or from potted plants, and very young plants were utilized as plant materials. The reasons for the absence of volatiles in the

undamaged leaves of *Magnolia* may simply be that leaves attached to the branches were used.

Induction of volatiles in agricultural plants is achieved by interactions of herbivore caterpillar regurgitant (Mattiacci et al., 1994) and/or application of β-glucosidase to mechanically damaged leaves that results in an emission of a blend of volatiles similar to those emitted by feeding herbivores (Mattiacci et al., 1995). Thus, in some taxa, enzymes such as β-glucosidase (an elicitor) probably activate biosynthesis of terpenoids (and other compounds), including two terpene alcohols, nerolidol and geranyl-linalool, that eventually lead to 4,8-dimethyl-1,3,7-nonatriene and 4,8,12-trimethyl-1,3,7,11-tridecatetraene (Boland et al., 1992). Jasmonic acid and its derivative, methyl jasmonate, are elicitors or signal transducers in some plants (Farmer and Ryan, 1990; Boland et al., 1995). Although the activities of β-glucosidase and jasmonic acid derivatives have not been analyzed in *Magnolia*, methyl jasmonate and jasmone were present as components of floral volatiles of some plants including *M. grandiflora* (Thien, et al., 1975; Kaiser, 1991; Yasukawa et al., 1992; Knudsen et al., 1993).

Emission of (*E*)-4,8-dimethyl-1,3,7-nonatriene by some taxa of *Magnolia* and *Liriodendron* comprised up to 30% of the floral volatiles (Table 3). Although more than 700 chemical compounds have been isolated in floral scents of angiosperms from 441 taxa (134 families) (Knudsen et al., 1993), the presence of (*E*)-4,8-dimethyl-1,3,7-nonatriene in floral scents is relatively rare (ca. 20 families) (Kaiser, 1991; Boland et al., 1992; Knudsen et al., 1993; Knudsen and Mori, 1996). The emission of (*E*)-4,8-dimethyl-1,3,7-nonatriene may be inductively generated in the detached flowers of some *Magnolia*, but the intact flowers of *M. heptapeta* constantly emit a high amount of the compound.

The family Magnoliaceae represents an ancient lineage of plants (Thorne, 1996), and the genus *Magnolia* is primarily composed of temperate and subtropical taxa that typically bear numerous, large, highly fragrant flowers over long periods of time and attract a wide range of insect visitors and pollinators (Thien, 1974; Yasukawa, et al., 1992). Despite the attraction of a large number of insects to the flowers, floral and leaf herbivory appears to be low on most *Magnolia* taxa, even though species of *Papilio* (Lepidoptera: Papilionidae) feed on members of the Magnoliaceae (Scriber, 1988).

Although the function of (*E*)-4,8-dimethyl-1,3,7-nonatriene in floral volatiles is still uncertain, the fact that this compound is emitted not only by the damaged leaves but also by the flowers of some *Magnolia* taxa and the common occurrence of several terpenoids such as β-ocimene, β-myrcene, linalool, and caryophyllene in floral volatiles of many flowering plants (Knudsen et al., 1993) suggest that the interaction between flower and pollinator and the chemical communication between the first and third trophic levels may be interrelated.

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RESPONSES TO GREEN LEAF VOLATILES IN TWO BIOGEOCLIMATIC ZONES BY STRIPED AMBROSIA BEETLE, *Trypodendron lineatum*

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Abstract—Experiments in the Coastal Western Hemlock (CWH) biogeoclimatic zone on the British Columbia (BC) coast and the Interior Douglas-fir (IDF) biogeoclimatic zone in the BC interior revealed pronounced differences in the effect of six-carbon green leaf volatiles on the response by striped ambrosia beetles, *Trypodendron lineatum* to multiple funnel traps baited with the aggregation pheromone lineatin. In the IDF zone, four green leaf alcohols [1-hexanol, (*E*)-2-hexen-1-ol, (Z)-2-hexen-1-ol, and (Z)-3-hexen-1-ol], released alone or in a quaternary blend at ca. 4 mg/24 hr/compound, caused a 63–78% reduction in trap catches. Two aldehydes, hexanal and (*E*)-2-hexenal, released together at ca. 13.0 mg/24 hr/compound were weakly disruptive in one of two experiments. Conversely, in the CWH zone, the two aldehydes together caused a slight, but significant, increase in the response over that to lineatin in one of two experiments, and the blend of all four alcohols caused only weak disruption of response in one of two experiments. None of the alcohols released alone was bioactive. These results may reflect adaptations that ensure accurate host location in the two ecological zones. In the CWH zone, *T. lineatum* attacks conifer logs almost exclusively, and surrenders the cut or broken stump habitat to *Gnathotrichus* spp. In the IDF zone, *T. lineatum* readily attacks the base of trees killed by bark beetles or fire and may utilize green leaf alcohols to detect and avoid the vertical silhouettes represented by nonhost angiosperms such as black cottonwood and trembling aspen.

Key Words—*Trypodendron lineatum*, ambrosia beetle, Coleoptera, Scolytidae, pheromone, green leaf volatiles, 1-hexanol, (*E*)-2-hexen-1-ol, (Z)-2-hexen-1-ol, (Z)-3-hexen-1-ol.

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INTRODUCTION

The striped ambrosia beetle, *Trypodendron lineatum* (Olivier), typically infests recently downed or dying coniferous trees, as well as stumps and slash resulting from logging (Borden, 1988). It is holarctic in distribution (Wood, 1957) and is a forest products pest throughout most of its range, degrading the value of manufactured products through its darkly stained galleries in the sapwood (McLean, 1985). Infested products are subject to export restrictions (Borden and McLean, 1981). On the British Columbia coast alone, the annual economic impact of ambrosia beetles, principally *T. lineatum*, is estimated to range from $95 to $189 million (Can.) (Lindgren and Fraser, 1994).

If *T. lineatum* is to attack successfully in the spring in coastal forests in the Pacific Northwest, trees must be felled by January, allowing time for the buildup of ethanol as a result of anaerobic metabolism (Kelsey, 1994). Ethanol acts as an attractant alone or in combination with α-pinene or the aggregation pheromone lineatin (Moeck, 1971; Nijholt and Schönherr, 1976; Shore and McLean, 1983; Shore and Lindgren, 1996), although its role as a synergist for lineatin has been questioned (Salom and McLean, 1988). It also acts as a boring stimulant (MacConnell et al., 1977). The primary attraction to host volatiles and lineatin can induce mass attack on sufficiently-aged forest products. These may be felled and bucked logs in forest settings, logs stored in piles on dry land or in log booms in the water (Richmond, 1969), or even freshly milled green lumber in sawmill sites (McLean and Borden, 1977a; Shore and McLean, 1985).

Because of the semiochemical-based nature of mass attack by *T. lineatum* and the high economic value of its hosts, several studies have sought to identify economically efficacious repellents that could deter attack by host-seeking beetles. Presumably, the aging process that results in the production of ethanol also allows for the dissipation of certain repellent monoterpenes (Moeck, 1970). Accordingly, Nijholt (1973) found that turpentine oil spray retarded attack by *T. lineatum* for up to five days. A commercial pine oil derived from pulp mill effluent and sprayed to the runoff point afforded a mean of 49.5 days of total protection of logs; a similar oleic acid treatment gave 41.2 days of protection (Nijholt, 1980). None of these materials has been developed as an operational repellent, probably due to the high cost of turpentine and oleic acid, the high volume of application, and the obnoxious odor of commercial pine oil.

In the process of host selection, *T. lineatum* rarely makes the mistake of attacking nonhost angiosperm logs. Such mistakes are so uncommon that they became the subject of anecdotal publications (Nijholt, 1981; Lindgren, 1986). Therefore, another possible source of repellents lies in green leaf volatiles (GLVs), six-carbon alcohols, aldehydes, and derivative esters commonly found in green plants (Visser, 1986). GLVs have been found to be repellent to bark beetles in the genera *Dendroctonus* and *Ips* (Dickens et al., 1991, 1992, 1993,

1995; Wilson et al., 1996), and may represent a key semiochemical signal in the discrimination between host and nonhost species by conifer-infesting bark beetles (Borden, 1996). We hypothesized that GLVs would also act as repellents for ambrosia beetles and tested this hypothesis against coastal and interior populations of *T. lineatum* in British Columbia (BC).

METHODS AND MATERIALS

Experiments in the BC interior were conducted at elevations of 1200–1500 m in the valleys of Willis and McNulty creeks near Princeton. These field sites are in the Interior Douglas-fir (IDF) biogeoclimatic zone (Hope et al., 1991), are dominated by mature lodgepole pine, *Pinus contorta* var. *latifolia* Engelm., with scattered veteran Douglas firs, *Pseudotsuga menziesii* (Mirb.) Franco; quaking aspens, *Populus tremuloides* Michx.; and black cottonwoods, *P. trichocarpa* Torr. & Gray. Coastal experiments were located at the University of British Columbia's Malcolm Knapp Research Forest, Maple Ridge, BC. The study site was at an elevation of 100–150 m in the Coastal Western Hemlock (CWH) biogeoclimatic zone (Pojar et al., 1991) in a mixed second growth stand that regenerated naturally following a fire in 1868. The stand is dominated by Douglas fir, western hemlock; *Tsuga heterophylla* (raf.) Sarg.; and western redcedar, *Thuja plicata* Donn ex D. Don.; and scattered vine maples, *Acer circinatum* Pursh; bigleaf maples, *Acer macrophyllum* Pursh: and paper birches, *Betula papyrifera* Marsh. The North Cascade Mountains lie between the two biogeoclimatic zones.

In both geographic areas, 12-unit multiple-funnel traps (Lindgren, 1983) were set up at least 15 m apart along the rights-of-way of forestry roads. Seven experiments, each with a randomized complete block design, were conducted with locations, numbers of replicates, and dates as in Table 1, and chemical stimuli, sources, purities, release devices, and release rates as in Table 2.

In 1994, experiment 1 tested a blend of two green leaf aldehydes [hexanal and (*E*)-2-hexanal] and three alcohols [1-hexanol, (*E*)-2-hexen-1-ol, and (*Z*)-3-hexen-1-ol], released from separate devices, for their ability to disrupt response to lineatin in the IDF zone. Hexanal and 1-hexanol were chosen because of their reported repellency to bark beetles in the southern United States (Dickens et al., 1992). The other compounds represented an additional array of inexpensive, common, and readily available GLVs; the three alcohols were individually repellent to the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, in BC (Wilson et al., 1996). In 1995, three sets of paired experiments with identical treatments were run in each of the biogeoclimatic zones (Table 1). Experiments 2 and 3 tested the potential disruptive capacity of a blend of GLVs [the same blend as in 1994, but with (*Z*)-2-hexen-1-ol added] compared to that of the

TABLE 1. LOCATION, REPLICATION, AND DATES FOR SEVEN RANDOMIZED BLOCK TRAPPING EXPERIMENTS ON EFFECT OF GLVS ON STRIPED AMBROSIA BEETLE. DIFFERENCES IN DATES ARE CONSISTENT WITH DIFFERENT FLIGHT PERIODS IN THE TWO BIOGEOCLIMATIC ZONES

Experiment	Biogeoclimatic zone	Location	Replicates (*N*)	Dates
1	IDF	Willis Creek	20	June 23–July 1, 1994 (10 reps) and July 1–11, 1994 (10 reps)
2	CWH	Malcolm Knapp Res. For.	20	Mar. 28–31, 1995 (10 reps) and Mar. 31–Apr. 19, 1995 (10 reps)
3	IDF	McNulty Creek	10	June 7–10, 1995
4	CWH	Malcolm Knapp Res. For.	10	Apr. 19–24, 1995
5	IDF	McNulty Creek	9	June 7–10, 1995
6	CWH	Malcolm Knapp Res. For.	14	Apr. 24–26, 1995 (7 reps) and Apr. 26–28, 1995 (7 reps)
7	IDF	McNulty Creek	10	June 7–10, 1995

aldehyde and alcohol blends alone. Experiments 4 and 5 compared the potential repellency of the two aldehydes tested alone and together, while experiments 6 and 7 tested the four alcohols alone and in a quaternary blend.

Captured beetles from all experiments were stored frozen in plastic bags until they were sexed and counted. Because of considerable variations in numbers of beetles between replicates and experiments, the counts were converted to the percentages of total captured beetles for each sex within each replicate. They were then transformed by arc sin $\sqrt{x}$ (Zar, 1984), and the means compared by ANOVA and the Ryan-Einot-Gabriel-Welsh (REGW) multiple Q test (Day and Quinn, 1989; SAS Institute, 1988). In all cases $\alpha = 0.05$.

RESULTS

The GLV blend tested in experiment 1 in the IDF zone reduced the responses of *T. lineatin* to its aggregation pheromone by 75% and 73% for males and females, respectively (Figure 1).

Experiment 2 in the CWH zone showed that the four green leaf alcohols

TABLE 2. DESCRIPTION OF CHEMICALS EMPLOYED IN SEVEN RANDOMIZED BLOCK TRAPPING EXPERIMENTS ON EFFECT OF GLVs ON STRIPED AMBROSIA BEETLE

Chemical[a]	Source[b]	Purity (%)[b]	Experiments	Release device	Release rate per 24 hr[c]
Lineatin	P	>99	1-7	Hercon strip	50.0 μg
Hexanal	A	98	1-3, 5, 6	bubble cap	13.0 mg
(*E*)-2-Hexanal	A	99	1-3, 5, 6	bubble cap	13.0 mg
1-Hexanol	A	98	1, 2, 4, 5, 7	bubble cap	3.8 mg
(*E*)-2-hexen-1-ol	A	95	1, 2, 4, 5, 7	bubble cap	3.8 mg
(*Z*)-2-hexen-1-ol	B	92	2, 4, 5, 7	bubble cap	3.8 mg
(*Z*)-3-Hexen-1-ol	A	98	1, 2, 4, 5, 7	bubble cap	3.8 mg

[a]All GLVs stabilized with 1-2% (wet weight) Ethanox 330 antioxidant (Ethyl Chemicals Group, Baton Rouge, Louisiana).
[b]Symbols as follows: P = Phero Tech Inc., Delta, BC; A = Aldrich Chemical Company, Milwaukee, Wisconsin; B = Bedoukian Research Inc., Danbury, Connecticut. Purities as determined by the manufacturer.
[c]Determined by Phero Tech (lineatin) or by the authors (GLVs) in the laboratory at 23-24°C.

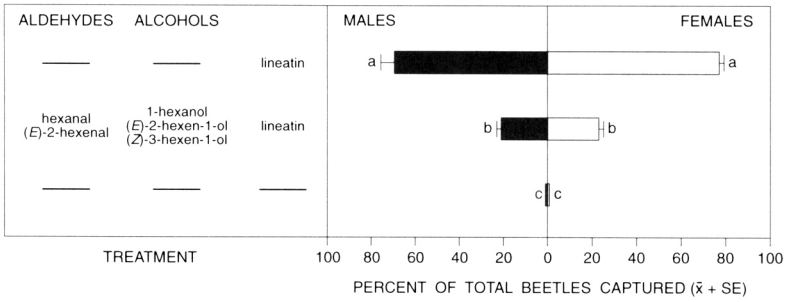


FIG. 1. Response of 31,009 male and 21,380 female *T. lineatum* in experiment 1 to multiple-funnel traps baited with lineatin alone or with a blend of two green leaf aldehydes and three alcohols in the IDF zone. Long dash indicates no treatment. Bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$.

tested together reduced the response to lineatin by 40% and 33% for males and females, respectively (Figure 2). The aldehydes had no significant effect, and the alcohol-aldehyde blend was no more disruptive than the alcohols alone. In contrast, experiment 3 in the IDF zone disclosed a very strong inhibitory effect of the alcohol blend, which reduced the response to lineatin by 85% and 79% for males and females, respectively (Figure 2). The two aldehydes together caused a slight, but significant, reduction in response to lineatin, but did not enhance the disruptive effect when added to the alcohols.

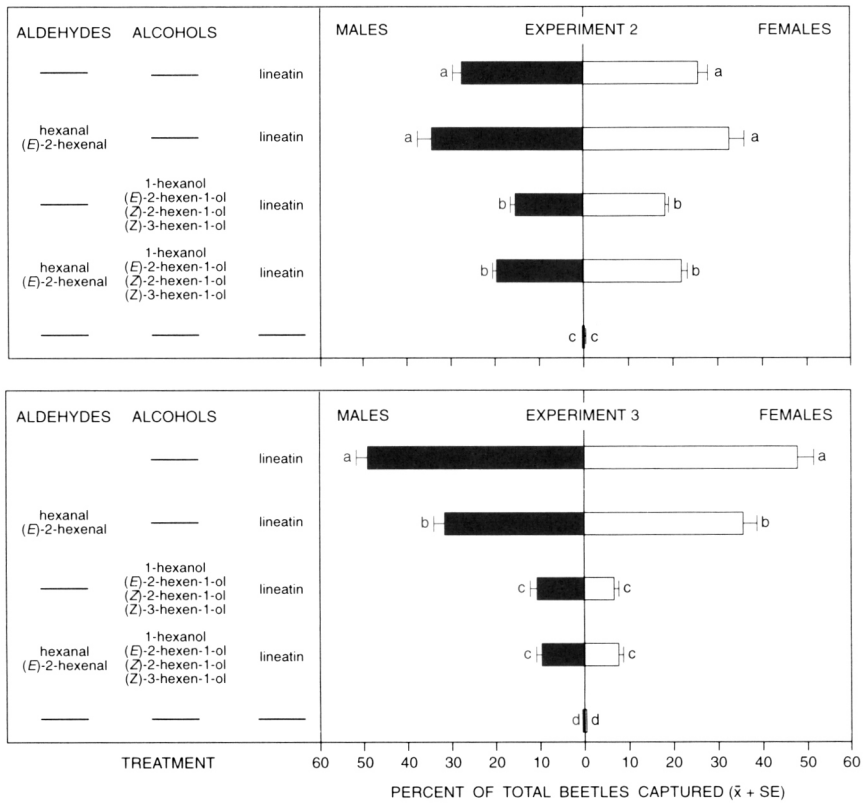


FIG. 2. Response of 15,552 male and 7350 female *T. lineatum* in experiment 2 in the CWH zone, and 2432 male and 1914 female *T. lineatum* in experiment 3 in the IDF zone to multiple-funnel traps baited with lineatin alone or with a blend of two green leaf aldehydes, four green leaf alcohols, or all GLVs together. Long dash indicates no treatment. Bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$.

In experiment 4 in the CWH zone, neither of the aldehydes tested alone had a significant effect on the response to lineatin, but when tested together they caused a weakly significant increase in the response by males (Figure 3). The slight inhibitory effect of the aldehydes seen in experiment 3 in the IDF zone (Figure 2) was not repeated in experiment 5, in which neither aldehyde alone nor in combination was bioactive (Figure 3).

In the CWH zone, experiment 6 showed that no green leaf alcohol alone or in a quaternary blend disrupted the response of either sex to lineatin (Figure 4). The slight disruptive effect of the alcohol blend in experiment 2 (Figure 2) was thus not supported by the results of experiment 6, a result similar to that

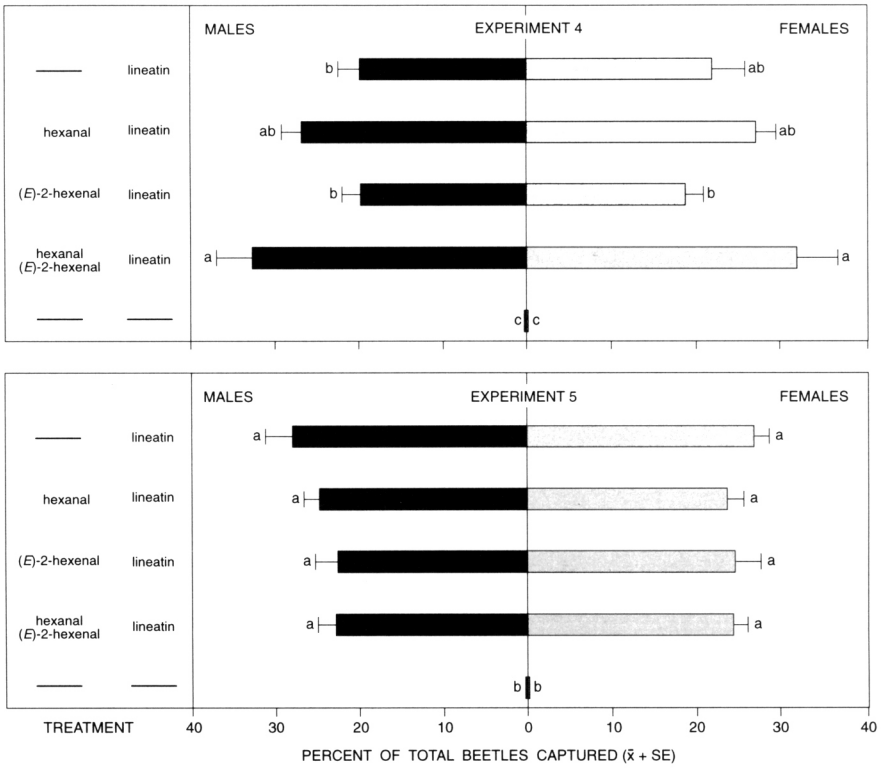


FIG. 3. Response of 19,568 male and 8202 female *T. lineatum* in experiment 4 in the CWH zone, and 2221 male and 1486 female *T. lineatum* in experiment 5 in the IDF zone to multiple-funnel traps baited with lineatin alone or with one of two green leaf aldehydes or both together. Long dash indicates no treatment. Bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$.

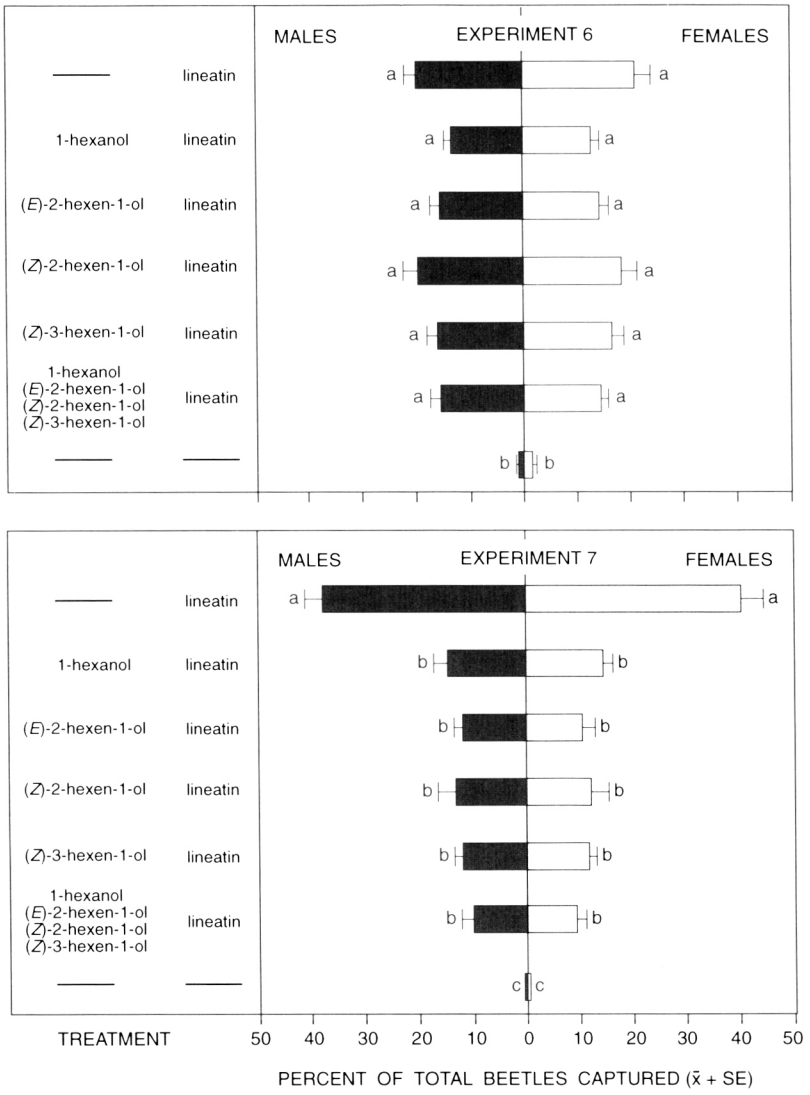


FIG 4. Response of 12,632 male and 6854 female *T. lineatum* in experiment 6 in the CWH zone, and 3554 male and 2738 female *T. lineatum* in experiment 7 in the IDF zone to multiple-funnel traps baited with lineatin alone, with one of four green leaf alcohols, or all four together. Long dash indicates no treatment. Bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$.

found for the aldehydes in the IDF zone (Figures 2 and 3). In contrast to the results of experiment 6 in the CWH zone, experiment 7 in the IDF zone revealed that the four green leaf alcohols tested alone were all strongly bioactive, causing the responses to lineatin to decline by 63–78% (Figure 4). The disruptive effect of the four alcohols tested together was no greater than that to any alcohol alone, indicating that there was neither an additive nor a synergistic interaction between them. Although 56% of the beetles captured were males, the effect of the alcohols was almost equal between sexes.

DISCUSSION

Our results support the findings that GLVs can be disruptive semiochemicals for bark beetles (Dickens et al., 1992; Wilson et al., 1996) and extend the known range of bioactivity of GLVs within the Scolytidae to sapwood-dwelling ambrosia beetles. We hypothesize that conifer-infesting scolytids utilize GLVs primarily as semiochemicals that indicate the presence of nonhost species (Borden, 1996), e.g., angiosperm trees, shrubs, and herbaceous plants in which GLVs are common (Visser, 1986). However, it is unwise to generalize about the behavioral effects of GLVs. GLVs were patented as "inhibitors" of pine beetle aggregation pheromones on the basis of experiments demonstrating that hexanal and 1-hexanol were repellents to the southern pine beetle, *Dendroctonus frontalis* Zimmerman; the small southern pine engraver, *Ips avulsus* (Eickhoff); and the eastern fivespined ips, *I. grandicollis* (Eickhoff) (Dickens et al., 1993, 1995). However, subsequent experimentation demonstrated that hexanal was inactive for *D. ponderosae*, and 1-hexanol was the weakest of four green leaf alcohols tested against this species (Wilson et al., 1996). Moreover, 1-hexanol has recently been demonstrated to be a multifunctional pheromone produced by male *Pityogenes knechteli* Swaine, another pine bark beetle (Savoie et al., 1997). At very low release rates it was attractive in combination with the aggregation pheromone, ipsdienol, and at high release rates it was repellent.

The variability in response by *T. lineatum* to GLVs in two BC biogeoclimatic zones approximately 150 km apart (Figures 1–4) indicates that without experimental data it may also be unwise to use the result of an experiment in one location to generalize about the effect of GLVs throughout the variable habitats of a species with a wide geographic range. Geographic variation in production of and response to pheromones has been demonstrated before in scolytids. Spruce beetles, *Dendroctonus rufipennis* (Kirby), responded to the aggregation pheromone 1-methyl-2-cyclohexen-1-ol (MCOL) of different chirality, in different parts of their range, and responded to another pheromone, verbenene, in only one of five test locations (Borden et al., 1996). Pine engravers, *Ips pini* (Say), both produce and respond to the pheromone ipsdienol of

variable chirality in broad east–west and north–south gradients, but two populations within 100 km of each other in southeastern British Columbia differed markedly in both respects (Miller et al., 1989, 1996; Seybold et al., 1995). Such variation may reflect variable selection pressure to maintain species specificity in pheromone communication channels driven by a mosaic of partially sympatric competing species (Miller et al., 1996).

A comparative study on two continents revealed pronounced variability in the responses of *T. lineatum* to semiochemicals (Borden et al., 1982). In the CWH zone of British Columbia, *T. lineatum* responded to drainpipe traps baited with lineatin alone at much higher levels than when they were baited with ethanol and α-pinene, whereas in England the responses to the two sitmuli in the same type of trap were approximately equal. In England the responses to lineatin were synergized by ethanol, α-pinene, or both together, but in coastal British Columbia not even an additive effect was observed. This variation is suggestive of being driven by minor, but important, differences in host habitat. For example, *T. lineatum* in both coastal British Columbia and England attack felled trees, but in England they also attack the stumps of recently cut trees (J.H.B., personal observation). Thus, it may be adaptive for *T. lineatum* in England to respond to vertical silhouettes (drainpipe traps that simulate high stumps caused by natural wind breakage) that are redolent with the odor of host kairomones. Such a response would not be adaptive for *T. lineatum* in the CWH zone because within this zone stumps are the least preferred habitat (Dyer, 1963) and for the most part are surrendered to *Gnathotrichus* spp. (McLean and Borden, 1977b; J.H.B., personal observation). In contrast, *T. lineatum* in the IDF zone characteristically attacks around the base of trees killed by bark beetles or fire (J.H.B., personal observation). It may thus be adapted to respond negatively to GLVs emanating from the vertical silhouettes represented by nonhost angiosperm trees, e.g., trembling aspen and black cottonwood. This hypothesis is consistent with the report that *T. lineatum* attacking a nonhost angiosperm (alder) in Europe produced lineatin, but attracted mainly congeneric heterospecifics (Klimetzek et al., 1981). It is also consistent with Schroeder's (1992) finding that the odors of bolts cut from nonhost poplar and brich trees, *Populus tremula* L. and *Betula pendula* Roth, disrupted the response to ethanol by the European bark beetles *Tomicus piniperda* (L.) and *Hylurgops palliatus* (Gyll.). Finally, this hypothesis is supported by our recent detection (unpublished results) of certain GLVs among the bark volatiles of several angiosperm trees in British Columbia, although there are no data yet available on any differences in green leaf volatiles produced by nonhost trees in different biogeoclimatic zones.

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RESPONSES TO ODORS OF DOMINANT AND SUBORDINATE HOUSE MICE (*Mus domesticus*) IN LIVE TRAPS AND RESPONSES TO ODORS IN LIVE TRAPS BY DOMINANT AND SUBORDINATE MALES

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Abstract—Data from 16 0.1-ha field enclosures containing populations of wild house mice (*Mus domesticus*) were used to test two sets of questions. Do mice respond differentially to odors from dominant and subordinate males in live traps? Do dominant and subordinate males respond differentially to odors in live traps from other mice? A trap was considered odorized if it had captured a mouse during the previous week, during which there were always two trapping nights. Dominant and subordinate males did not differ with respect to the time they were alive in the enclosures, the frequency of capture, or home range size. Overall, mice were more attracted to the odor of dominant males compared to subordinate males. This difference was due primarily to the attraction of estrous and juvenile females to the traps odorized by dominant males. Dominant males were more likely to be captured in traps odorized by estrous females than in traps odorized by juvenile males or females, nonestrous females, or pregnant/lactating females. Subordinate males were caught more frequently in traps odorized by dominant males than were other dominant males. Conversely, dominant males were captured more frequently in traps odorized by subordinate males than were other subordinate males. Rates of capture of self-odor were higher for subordinate than for dominant males. The results may be explained by a combination of factors related to the social and reproduction systems of house mice.

Key Words—*Mus domesticus*, social status, dominance, chemosignals, odors, house mice.

INTRODUCTION

Many rodents from a variety of genera use chemical cues for communication and social interaction, including *Microtus* (Wolff and Johnson, 1979; Ferguson

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et al., 1986; Ferkin and Johnston, 1995), *Peromyscus* (Bronson and Marsden, 1964; Doty, 1972; Drickamer, 1984) *Rattus* (Galef and Clark, 1971; Galef and Stein, 1985), and *Mus* (Vandenbergh, 1983; Hurst, 1987, 1990a). In house mice (*Mus domesticus*), olfactory communication is important with respect to recognition of sex, age, and reproductive condition (Bowers and Alexander, 1967; Hurst, 1990b–d), and olfactory cues can influence reproductive physiology and age of puberty (Drickamer, 1986; Vandenbergh and Coppola, 1986). Female house mice in estrus discriminate dominant from subordinate males based on odor cues (Drickamer, 1992; Mossman and Drickamer, 1996).

Spatial and group relations in house mice are mediated, in part, by chemical cues, primarily from urine (Rowe and Redfern, 1969; Hurst 1990b–d). Dominant males mark to announce their home areas, and marks by all mice living in an area serve to maintain group ties, to intimidate intruders, and for orientation within their range. Some aspects of dominance relations in males appear to be mediated via the olfactory channel (Kimelman and Lubow, 1974; Desjardins et al., 1973; Hurst, 1990b). Dominant males mark at higher frequencies than subdominant males living in the same area and more than nonresident males. Dominant males countermark urine deposits from subdominants and any intruders (Hurst, 1990b).

Studying chemical cue preferences in house mice in a laboratory setting generally involves some type of olfactometer or similar discrimination apparatus (Bowers and Alexander, 1967; Lenington, 1988; Drickamer, 1989a) or populations housed in small indoor enclosures or arenas (Lenington, 1983, 1991; Drickamer, 1989b; Hurst 1990b–d). Under field conditions, the problem becomes more difficult since house mice are small, nocturnal rodents. One means of examining cue preferences in house mice as well as other rodents has been the use of traps containing odors from conspecifics (Mazdzer et al., 1976; Daly et al., 1980; Heske, 1987). Soiled traps were entered more frequently than clean traps by individuals of several rodent species, including *Apodemus sylvaticus*, *A. flavicollis*, *Clethrionomys glareolus*, *Microtus agrestis*, and *M. townsendii* (Boosntra and Krebs, 1976; Stoddart, 1982; Gurnell and Little, 1992). Drickamer (1995) reported similar findings for *Mus domesticus*. In the latter study, I found that adult males preferred odors from juvenile and estrous females and avoided odors of other males significantly more than expected and that estrous females selected traps containing odors from adult males significantly more than expected.

Based on the foregoing information, I conducted the experiments presented here to determine whether we can learn additional information about the roles of olfactory cues in social and reproductive biology of house mice in a semi-natural field setting by knowing the social dominance status of males. Two types of questions were addressed using the odorized trap technique (Drickamer, 1995). First, do the mice respond differentially to odors in live traps that most recently

contained dominant versus subordinate males? Second, do dominant and subordinate males respond differentially to odors in traps that have most recently captured other conspecifics?

METHODS AND MATERIALS

Enclosures. Eight 0.1-ha enclosures located in an abandoned horse pasture were used for these experiments during 1992 and 1993. Drickamer and Mikesic (1990) contains a full description of the enclosures. Each enclosure was 25 × 40 m, had wire mesh and aluminum flashing side walls, and contained four symmetrically placed 2-m-diameter structures (called mouse cities; King, 1983) constructed of 2-m-high aluminum flashing affixed to wooden posts. Mouse cities had a plywood roof, two entrances on opposite sides just above ground level, nesting cavities inside cinder blocks located in the interior, and were supplied weekly with food and water. A 1.5-m strip was mowed around the interior perimeter of each enclosure approximately once a month; in all other areas of each enclosure the vegetation was allowed to grow uncut, attaining heights of 1–1.5 m. The mowed strips served as a pathway for human travel and also prevented vegetation near the walls from attaining heights that could enable mice to escape by climbing. Each enclosure contained a 6- × 9-m trapping grid with 5-m intervals between trap stations. One Sherman live-trap was placed at each station. The trapping grid thus had 26 trap stations located along the walls of each enclosure and 28 in the interior.

Enclosure Procedures. Data were collected for all eight enclosures in each of two years, 1992 and 1993. In late March of each year populations were introduced into the enclosures. Two different stocks were used, with four enclosures started from each stock each year. One stock consisted of second-generation mice bred from animals captured at the Southern Illinois University Swine Center. Eight females and two males of this stock were introduced into each city (total of 32 females and 8 males per enclosure). The other stock was bred from mice originally captured near Fort Collins, Colorado, and cross-bred in the laboratory of Dr. Sarah Lenington at Rutgers University, Newark, New Jersey, with an inbred stock (LT.MA). Four females and two males were placed in each city (total of 16 females and 8 males per enclosure). There were no differences in the trap response behavior of mice of these two stocks (Drickamer et al., 1992); data were combined from all enclosures for the analyses reported in this paper.

Starter mice were all 110–160 days of age at the time enclosures were stocked. They had all been held in same-sex sibling grops of three to five mice per cage since weaning at 25–30 days of age. No same-sex siblings were placed into the same enclosure. One week prior to stocking the enclosures, each mouse

was weighed to the nearest 0.5 g with a Pesola scale, reproductive condition was checked, and each mouse was given a unique toe-clip ear-punch number. Mice were defined as prepubertal until they reached 15 g body mass with the testes descended (males) or 13 g body mass with the vagina open (females). After those criteria were attained, mice were defined as adults. Estrous condition was determined by an examination of the external vagina (Drickamer, 1995). Pregnancy and lactation were assessed by a combination of body mass and conformation and by the condition of the nipples.

Trapping Procedures. Each live trap was placed inside a 1.75-liter wax-coated milk or juice carton to provide extra insulation and protection from the elements. Each trap contained one cotton square (2.54 × 2.54 × 0.3 cm thick) called a Nestlet (Ancare Corp., Belmont, New York). Traps were baited with a mixture of peanut butter and rolled oats. Live traps were operated for two nonconsecutive nights per week from early May until mid-October (25 weeks each year). Traps were opened and baited between 16:00 and 18:00 hr and were checked the next morning between 06:00 and 09:00 hr. Captured mice were checked for identification, body mass, and reproductive condition and were released at the site of capture. Previously uncaptured individuals (recruits) were given a toe-clip ear-punch number. Traps were not cleaned between captures and cotton squares were replaced on an as-needed basis, when they disintegrated, or, as happened in a few instances, when they became soaked after it rained. All traps were examined every other week to ensure that they were functioning properly. Approximately 10 traps, of 432 in use, were replaced each summer due to malfunctions. No traps were moved between stations within or between any of the enclosures. While such manipulations could prove quite interesting, moving traps would likely result in disturbance of the general contextual cues surrounding each trap and thus could have resulted in problems similar to those we encountered when we cleaned traps and conducted odor manipulations in a previous study (Drickamer et al., 1992). All mice were removed from the enclosures at the conclusion of the field season in late October of each year using a combination of live and kill traps.

For purposes of experimental manipulation, a trap was defined as being odorized if it had caught a mouse during either of the two trap nights previous to the current trap night (Drickamer, 1995). If a trap had not caught anything for that period, then it was not again considered odorized until another mouse was caught in it. Thus, a trap had all of the contextual cues of previous captures and the surroundings, but the experimental odor was the one that predominated from the most recent capture.

Dominance Testing. For male mice used to initiate enclosure populations, round-robin tests using all eight males from an enclosure were conducted prior to placing mice in the field. Follow-up tests on these mice as well as tests on

recruits born in the enclosures were conducted in the field during the course of the field season. All field testing of dominance involved opportunistic captures of mice. When two males that had not previously encountered one another or had not met for more than one month were captured on the same morning, they were tested. Only adult males (≥ 15 g and testes descended) were used in these encounters. When males weighed 19 g or less they were paired only with other males that weighed within ± 2 g of their body weight. Mice were tested in a standard opaque polypropylene shoe-box rodent cage (15 × 28 × 14 cm deep) with a wire screen lid. To avoid possible confounding effects from residual odor cues, cages were cleaned after each test.

Pairs of mice were placed in the cage simultaneously and observed for 10 min. There were three possible outcomes. When five consecutive attacks occurred by one mouse, the encounter was terminated and that mouse was recorded as an aggressive winner (score = 3), and the other was recorded as a defeated loser (score = 0). When one mouse consistently displaced the other or engaged in a few mild attacks, that mouse was recorded as a passive winner (score = 2) and the other as a passive loser (score = 0). When neither mouse consistently displaced the other and there were no attacks during the test period, the encounter was recorded as a draw (both score = 1). No mice received any observable wounds during any encounter.

The scores for each male were averaged to obtained a measure of his dominance status. To be used as a possible source of odor or as a responder to trap odors, a male must have had at least two encounters to provide a baseline estimate of the dominance status; males with a single encounter or no encounters during the field season were not used. Over the two field seasons 87% of all adult males met this criterion those that did not were all younger adult males of ≤ 17 g. Dominant (high social status) males were defined as those with average scores of ≥ 2.0, and all males with lower scores were subordinate (low social status).

Position Effects. One potential problem with a study of this type is that mice were caught in particular traps simply because the trap was within their home range (Montgomery, 1987; Gurnell and Little, 1992). This effect would be true only if the number of potential traps that a mouse could enter on a particular night were severely limited. As in a previously published report (Drickamer, 1995), I believe that the following sequence of logic ensures that a position effect is unlikely in this study. Average home ranges ($\pm$SE) for females were 250 ± 21 m^2 and 400 ± 54 m^2 for males in the enclosures (Mikesic and Drickamer, 1992). Based on the 5-m trap interval in the grid within each enclosure, there were 10–18 traps within the average female home range and 16–24 traps within the average male home range. From the trapping data, it is possible to calculate that, on average, for a particular trap night, there were

18–23 traps (of the 54 present) in each enclosure that had caught a mouse on one of the two previous trap nights and thus served as the predominant odor. Combining the previous two points results in a determination that for females there were three to seven odorized traps in her home range on the average trap night and there were five to 10 traps in the home range of each male. Using all the data from both years, I also calculated the average number of traps odorized by the different age and sex classes that were present in each enclosure for an average trap night. There were 3.2 ± 0.1 traps containing odor from juvenile females, 6.5 ± 0.2 with odor from adult females, 2.8 ± 0.1 with odor from juvenile males, and 7.7 ± 0.2 with odor from adult males. Of the traps with odor from adult males, there were, on average 3.7 ± 0.2 that were odorized by dominant males and 4.0 ± 0.2 odorized by subordinate males.

Analyses. A data base was maintained with information for each mouse caught. The dominant and subordinate males were analyzed in terms of the time they were present in the enclosures and the proportion of available trap nights on which they were caught. These analyses were designed to test for possible biases due to differential presence of one dominance class of males and to make certain that both dominant and subordinate males were equally trappable. To test for a possible bias due to different home range sizes for dominant and subordinate males, a home range area was claculated for each male using the minimum convex polygon method (Mohr, 1947). Parametric unpaired *t* tests were used in all three of these analyses (Zar, 1996).

To test for possible differential responses of various classes of mice to odors from dominant versus subordinate males, I first calculated the number of odorized traps available for each trapping date for the two male social status types within each enclosure. These values were summed to provide an estimate of the total availability of that odor across the 25 weeks of trapping. The ratio of mice caught in these odorized traps was calculated relative to availability within each of five classes: juvenile males, estrous females, nonestrous females, pregnant and/or lactating females, and juvenile females. These ratios were computed for each pen across the entire year for each of the two years of the study (total $N = 16$). Analyses of these ratios were carried out with parametric unpaired *t* tests (Zar, 1996).

To test for possible differential responses by dominant versus subordinate males to odors of all types in the traps, the numbers of traps were calculated in each enclosure for each trapping date that were available as having been odorized by juvenile males, estrous females, nonestrous females, pregnant and/or lactating females, and juvenile females. For each dominant or subordinate male the total available odorized traps of these five types were calculated for the weeks that each specific male was present in the enclosure. Then, a series of ratios was computed for each male using the number of times it was captured in traps

odorized by mice of a particular class as the numerator and the total odorized traps available of that type for the weeks that the male was present (total N = 118; 49 dominant and 69 subordinate males). Analyses of these ratios were carried out with unpaired parametric t tests (Zar, 1996).

I also tested the responses by dominant and subordinate males to their own odor, after being captured in a trap that they had odorized. For this analysis, the number of times that a male was caught was counted, as was the times that he was caught in his own odorized trap. A ratio was computed using the self-capture frequency in the numerator and the availability of his own odorized traps as the denominator. These ratios were analyzed using parametric paired t tests (Zar, 1996) with 49 dominant and 69 subordinate males.

Finally, the responses of dominant and subordinate males were tested to traps that had been odorized by dominant and subordinate males. Self-captures were excluded from these analyses. The availability of traps odorized by dominant and subordinate males within each enclosure across the field season was counted and then the number of captures in traps odorized by dominant and subordinate males was tallied for each male (N = 18). With the data corrected for the number of weeks a given male was present, two ratios were calculated for each mouse. The first ratio was for its capture rate in traps odorized by dominant males divided by the number available for traps odorized by dominant males. The second ratio was for its capture rate in traps odorized by subordinate males divided by the number available for traps odorized by subordinate males. Two separate parametric t tests were run on these data, one for traps odorized by dominant males and one for traps odorized by subordinate males.

RESULTS

Of the adult male mice in the 16 test enclosures over the two years, 49 were dominant and 69 were subordinate, based on the previously stated criteria. Initial analyses revealed that dominant (X = 15.2 $\pm$ 0.9 weeks; mean $\pm$ 1 SE) and subordinate (14.3 $\pm$ 0.6 weeks) males did not differ in terms of the mean number of weeks they were present in an enclosure (t = 0.891; df = 116; P > 0.30). Dominant (X = 45.1 $\pm$ 2.6%) and subordinate (47.6 $\pm$ 2.5%) males were not different in terms of the proportion of the available trap nights on which they were caught (t = 0.207; df = 116; P > 0.50). Dominant (X = 231.5 $\pm$ 24.2 m^2) and subordinate (X = 201.2 $\pm$ 14.6 m^2) males did not differ in terms of home range area (t = 1.134; df = 116; P > 0.25).

Responses to Odors from Dominant and Subordinate Males. Overall, mice from the five age and sex classes tested were significantly more likely to enter traps odorized by dominant males than subordinate males. Examination of the

TABLE 1. RATES OF CAPTURE FOR HOUSE MICE OF DIFFERENT AGES AND SEXES IN LIVE TRAPS THAT HAD CAUGHT DOMINANT OR SUBORDINATE MALE MOUSE IN THE PREVIOUS WEEK[a]

Class of responder	% Capture rate (mean ± SE)	
	Dominant odor	Subordinate odor
Juvenile males ($t = 0.729$; $P > 0.50$)	9.5 ± 2.4	8.5 ± 1.5
Estrous females ($t = 2.860$; $P < 0.005$)	5.5 ± 1.1	2.1 ± 0.6
Nonestrous females ($t = 0.282$; $P > 0.25$)	2.9 ± 0.5	2.3 ± 0.3
Pregnant/lactating females ($t = 0.552$; $P > 0.50$)	2.7 ± 0.4	2.4 ± 0.4
Juvenile females ($t = 2.194$; $P < 0.05$)	2.1 ± 0.2	1.7 ± 0.1
Total captures in traps odorized by dominant and subordinate males ($t = 2.194$; $P < 0.05$)	21.7 ± 2.1	16.9 ± 0.9

[a] All t tests are unpaired with $df = 116$.

responses within individual classes revealed that estrous females and juvenile females were significantly more likely to enter traps that had been odorized by dominant males than those odorized by subordinate males (Table 1). None of the remaining three t tests indicated any significant differential responses to the odors of males of different social status.

Responses by Dominant and Subordinate Males. Overall dominant males were more likely than subordinate males to enter odorized traps (Table 2). This difference is due primarily to the fact that dominant males were significantly more likely to enter traps that had been odorized by estrous females (Table 2). None of the other t tests provided evidence for significant differential responses by dominant and subordinate male mice to odors from conspecifics.

Self-Captures and Responses to Dominance Status of Males. The mean rate of self-captures for dominant males (15.7 ± 2.7) was significantly lower than the mean rate of self-captures for subordinate males (26.1 ± 2.8) ($t = 2.535$; $df = 116$; $P < 0.02$). Subordinate males (7.3 ± 2.1) were significantly more likely than dominant males (3.0 ± 1.0) to enter traps odorized by dominant males ($t = 2.006$; $df = 116$; $P < 0.05$). Conversely, dominant males (14.8 ± 2.1) were significantly more likely than subordinate males (8.0 ± 1.0) to enter

TABLE 2. RATES OF CAPTURE OF DOMINANT AND SUBORDINATE HOUSE MICE IN LIVE TRAPS THAT HAD CAUGHT A MOUSE IN THE PREVIOUS WEEK[a]

Class of odor source	% Capture rate (mean ± SE)	
	Dominant social status	Subordinate social status
Juvenile males ($t = 0.322$; $P > 0.50$)	5.3 ± 2.2	6.1 ± 2.9
Estrous females ($t = 2.727$; $P < 0.001$)	8.2 ± 2.3	2.0 ± 1.9
Nonestrous females ($t = 0.232$; $P > 0.50$)	14.3 ± 3.1	13.2 ± 3.0
Pregnant/lactating females ($t = 1.212$; $0.30 < P < 0.30$)	13.2 ± 3.3	9.1 ± 1.8
Juvenile females ($t = 0.001$; $P > 0.50$)	3.3 ± 1.1	3.2 ± 0.9
Total captures in odorized traps ($t = 3.168$; $P < 0.001$)	44.3 ± 1.4	33.6 ± 1.2

[a]All t tests are unpaired with $df = 116$.

traps that had been odorized by subordinate males ($t = 3.145$; $df = 116$; $P < 0.001$).

DISCUSSION

The control tests indicate that males did not differ in their average time in the enclosures or the overall rate of capture. For home range size, there was no difference between males of the two social status categories. These results mean then that the other findings are not biased by basic differences in the habits of dominant and subordinate males.

The fact that in the overall test, more mice entered traps odorized by dominant males than those odorized by subordinate males is somewhat unexpected. However, it may be that dominant males are less of a threat to these other mice than subordinate males. It may be that mice living in a particular area are familiar with the odor of the dominant male and thus they do not respond by avoiding that odor. The overall effect was due primarily to the attraction of estrous and juvenile females to the dominant male odor.

Dominant male odor is more attractive to females in estrus than subordinate male odor. Furthermore, dominant males are more likely to enter traps that recently contained an estrous female than are subordinate males. Taken together, these results support the conclusion that dominance status may confer some advantages in terms of mating and reproductive success (Oakeshott, 1974; D'Amato, 1988). These findings support a recent report in which mice were tested for odor preferences in 10-min field trials (Mossman and Drickamer, 1996). It is also clear from these findings that the attraction works both ways; females are attracted to the odor of dominant males, and dominant males are attracted to estrus females. A corollary of the latter finding is that, for some reason, subordinate males are not captured as often as dominant males in traps that recently contained an estrus female. If this is a behavioral avoidance of estrus females by subordinate males, it would not be very adaptive. On the other hand, the observed effect could be the result of either direct behavioral interference of dominant males with subordinate males near traps odorized by estrus females. Alternatively, the effect could result from a tendency of subordinate males to expect the presence of dominant males near the odor from an estrus female and, therefore, to avoid overt conflict subordinate males tend to be more cautious about approaching the odor from an estrous female.

Juvenile females are differentially attracted to traps with the odor of a dominant male. They may be seeking potential mates and are biased toward males of high rank as part of a mate-selection process. Since very young juvenile females of about 7 g or less do not enter the traps or do not set them off, the sample of juveniles is biased toward those that are slightly heavier and thus a bit older, approaching puberty. Females approaching puberty might be expected to seek potential mates. Male odor can accelerate sexual development in female house mice (Vandenbergh, 1969). In the laboratory, young female house mice avoid male odors until they are approaching puberty (Drickamer, 1989a). At this time they exhibit a preference for male odor. From the data analyzed here it appears that they also can selectively approach odors from dominant males.

Dominant males enter traps odorized by subordinate males more frequently than do subordinate males. There are at least two possible explanations for this finding. Dominant male mice may be familiar with the odors of neighboring dominant males or dominant males whose ranges they overlap, in which case, they may avoid such odors, choosing, instead, to investigate odors from subordinate males, particularly those with which they are not familiar, attempting to countermark locations where such males have been (Hurst, 1987). Dominant males seemingly avoid other high-ranking males, but they readily explore the odors of lower-ranking males. If their range is generally nonoverlapping with the ranges of other dominant males, there could be less opportunity for entry of traps odorized by dominant males. Secondly, avoiding odors from other high-

ranking males could be adaptive for the dominant males, as encounters with those males could occur and might result in overt aggression, with potential negative consequences. Since the overall rate of capture of dominant and subordinate males was not different, yet there was a difference in the rate of entry of odorized traps by these males, the subordinate males entered more nonodorized traps than dominant males.

Rates of capture to self odor in a trap odorized by that same male were higher for subordinate males than for dominant males. Dominant males may be spending more of their time checking out the odors of other mice, such as females in estrus and subordinate males. They may be less interested in checking out their own odor. Second, subordinate males may be attracted to the peanut butter and oats bait in a trap. The safest location to find such food would be in a place that had their own scent as the predominant mouse odor cue.

That subordinate males enter traps odorized by dominant males more frequently than do dominant males is curious. Home ranges of dominant males may have only a small degree of overlap. Thus, dominant males might have fewer opportunities to encounter traps odorized by other dominant males. It might be expected that subordinates would avoid more dominant males to preclude the possibility of overt aggression, resulting in potentially serious injury. However, if they are familiar with the odors of the dominant males whose ranges their area overlaps, they may not avoid such odors.

House mice apparently use odor cues pertaining to social dominance status in at least two major contexts. Firstly, odor cues convey information about social status and spatial relations (Hurst, 1990b,d). Differential responses by other adult males indicate that they can discriminate between odors from subordinate versus dominant individuals. They may use this information in terms of their spatial activity. Secondly, odor cues play a role in finding mates. Estrous females are capable of discriminating dominant male odor from subordinate male odor in the field, as previously reported based on laboratory studies (Drickamer, 1992). Whether odor cues play a pivotal role in actual mate selection remains to be tested.

It may seem curious that home range areas for dominant and subordinate males are not different. This could be an artifact of the enclosure situation with the presence of the mouse cities. However, it may also be a result of the fact that in feral situations, house mice exhibit a pattern of overlapping home ranges; they are not territorial. Thus, without active defense of an exclusive area, there would be nothing to prevent subordinate males from occupying areas nearly as large as those used by dominant males.

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6-BROMOINDOLE-3-CARBALDEHYDE, FROM AN *Acinetobacter* SP. BACTERIUM ASSOCIATED WITH THE ASCIDIAN *Stomozoa murrayi*

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Abstract—The ascidian *Stomozoa murrayi* from Caribbean coral reefs is not overgrown by macroepibionts. Chemical extraction of this organism gave 6-bromoindole-3-carbaldehyde (**1**) and its debromo analog (**2**). These two compounds, previously obtained from several marine organisms, were also extracted from an *Acinetobacter* sp. bacterium isolated from the surface of *S. murrayi*. Compound **1** exhibits in vitro settlement inhibition of barnacle larvae, moderate antibacterial properties, but not antialgal or fish-feeding deterrent activities. Its potential role in contributing to the reduced fouling in *S. murrayi* is proposed.

Key Words—6-Bromoindole-3-carbaldehyde, *Stomozoa murrayi*, marine bacteria, *Acinetobacter*, antifouling activity, chemical defense.

INTRODUCTION

In recent years it has been repeatedly suggested that associated microorganisms could be the true producers of biologically active secondary metabolites obtained from marine benthic invertebrates (e.g., Ireland et al., 1988; Banaigs et al., 1993). Yet there are few data supporting this hypothesis and the fact is still largely a matter of speculation (Fenical, 1993; Kang et al., 1996).

Recent work has provided convincing evidence for the production by microorganisms of natural products originally attributed to their hosts. In a very elegant work, Unson et al. (1994) demonstrated that a brominated diphenyl

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[1]In memory of Hélène Débard, who died during the preparation of this manuscript.

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ether is produced by *Oscillatoria spongeliae*, a cyanobacterial symbiont of the sponge *Dysidea herbacea*.

Oclarit et al. (1994) isolated an anti-bacillus peptide from the sponge *Hyatella* sp. and showed that this product is in fact synthesized by an associated *Vibrio* sp. Bewley et al. (1996) provided another good example of bioactive metabolites from the sponge, *Theonella swinhoei*, produced by microbial symbionts.

The ecological role of microbial metabolites in the marine environment is not yet well understood. Some marine bacteria inhibit marine yeasts (Buck et al., 1963; Burkholder, 1973), while other marine bacteria protect crustacean embryos against fungal infections (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992). Microbial films are known to promote the settlement of many benthic organisms (Pawlik, 1993) or, in contrast, to inhibit larval settlement of barnacles (Maki et al., 1988; Holmström et al., 1992; Rittschof et al., 1992). Kon-Ya et al. (1995) showed that a bacterium of the genus *Alteromonas*, associated with the sponge *Halichondria okadai*, produces ubiquinones that inhibit the settlement of *Balanus amphitrite* larvae.

Among bromoindole derivatives, the metabolite 2,5,6-tribromo-1-methylgramine, isolated by Kon-Ya et al. (1994a) from the bryozoan *Zoobotryon pellucidum*, and other compounds of the same family (Kon-Ya et al., 1994b) have shown potent anti-fouling activity against barnacle larvae.

The present work was undertaken to determine if secondary metabolites present in the Caribbean ascidian *Stomozoa murrayi* (Kott, 1957) could be found in associated bacteria isolated from the surface of the ascidian. If such a hypothesis could be confirmed, then comments about bioactivity of these compounds would be an important part of profiling the compounds, allowing speculation on their ecological role as antifouling agents.

METHODS AND MATERIALS

Collection and Extraction of the Ascidian. In the summer of 1992, 250 g (fresh weight) of *S. murrayi* were collected by scuba diving (3–5 m) near the marine research station of the Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México (ICML-UNAM), at Puerto Morelos, 86°52′W, 20°50′N (Q. Roo, Mexico), on the east coast of the Yucatan Peninsula. This black massive colonial ascidian was identified by Claude Monniot and Françoise Monniot, from the Muséum National d'Histoire Naturelle (Paris), as *Stomozoa murrayi*, a species originally described by Kott (1957) from the Red Sea, and later found in Brazil (Millar 1977) and in New Caledonia (Monniot, 1988). This is the first observation of the species in the Caribbean Sea.

Fresh ascidians were preserved in ethanol for two weeks. The ascidians were mixed in a Waring blender, filtered, and the ethanol evaporated. The remaining aqueous extract (700 ml) was partitioned with heptane (3 × 500 ml), ether (3 × 500 ml), dichloromethane (3 × 500 ml) and butanol (3 × 350 ml). The weight of all combined extracts was 3.9 g, and the dry weight of the ascidian residue was 40 g. The extracts were frozen and transported to France. Work-up was done immediately.

Isolation and Extraction of Bacteria from the Ascidian. Four bacterial isolates were obtained by cutting very thin layers from different parts of an ascidian colony (freshly collected) with a sterile razor blade. These layers were gently rinsed with sterile seawater and then added to 10 ml sterile seawater and shaken vigorously. Serial log dilutions were prepared (two replicates). From the third dilution, 200 μl was inoculated in sterile Petri dishes containing Bacto Marine Agar 2216 (Difco). Dishes were incubated for five days at 28°C in darkness. From the two replicates, we obtained two strains from the surface layer of the ascidian, namely SM-S1 and SM-S2. These strains were transferred in separate sterile Petri dishes and subcultured to ensure the purity of the strains. Two other bacterial strains were obtained from the same specimen, one from the inner layer of the tunic (strain SM-INT), and another one from an individual zooid (strain SM-Z). The pure strains were stored in tubes containing Bacto Marine Agar 2216 (Difco) and kept at 4°C. They were transported to France at environmental temperature, placed in a refrigerator and subcultured monthly.

The culture medium was prepared with natural filtered seawater and diluted with distilled water (75:25, v/v). Then, 5 g bactopeptone, 0.5 g NH_4Cl, and 0.1 g $FePO_4$ were dissolved in 1 liter of the diluted seawater. The four strains were grown separately in 0.5 liter of this medium (pH 6.8) for 10 days in darkness at 28°C. No significant pH variation was observed when the growth process was stopped.

Culture broths were extracted with ethyl acetate. The extracts were evaporated to dryness and partitioned between water (500 ml) and heptane (2 × 500 ml), followed by ether (2 × 500 ml). Ether fractions were submitted to GC-MS analysis. Sterile medium (500 ml) was extracted as a reference.

The strain SM-S1 was grown again for two days at 28°C in 50 ml of culture medium (see above), after which 5 ml of the 2-day-old culture was transferred to a 2-liter fermentor containing 1 liter of culture medium and growth continued at 28°C in darkness for two days with 350 rpm agitation and 2 liter/min aeration. The ether extract (5 mg) from 1 liter of bacterial broth of the strain SM-S1 (*Acinetobacter* sp.), was submitted to GC-MS analysis under the conditions described below.

Identification of Metabolites. Gas chromatographic separation was performed with an Unicam 610 gas chromatograph coupled to an Unicam System 2 Benchtop quadrupole mass spectrometer. A 30-m × 0.25-mm SPB 1701

(Supelco) column was used. The oven was programmed at 3°C/min from 160°C to 250°C (He carrier gas, 1 bar). Ether extract from bacterial culture of the strain SM-S1 was evaporated to dryness, and the resulting residue was dissolved in CH_2Cl_2 at 50 mg/ml. One microliter of the dichloromethane solution was injected in splitless mode at 150°C. For the MS determinations the ionization voltage was 70 eV, the pressure of the detector was 10^{-4} torr, and the mass spectral scan range was set to 50–500 *m/z*.

The ether-soluble material (520 mg) from 40 g (evaluated dry weight) of the ascidian was submitted to silica gel chromatography (Si 60). The 9:1 CH_2Cl_2–MeOH fraction (60 mg) was purified by HPLC on a C_8 reverse-phase column, with 8:2 MeOH–H_2O as solvent mixture, and it yielded 3 mg (0.007% of the ascidian dry weight, i.e., 75 μg/g dry tissue) of pure 6-bromoindole-3-carbaldehyde (**1**) and 1.5 mg (0.0035% = 37.5 μg/g dry tissue) of pure indole-3-carbaldehyde (**2**). The structures were determined by NMR spectral data (Wratten et al., 1977; Dellar et al., 1981; Rasmussen et al., 1993).

Synthesis of **1**. Bromination of **2** in acetic acid (Da Settimo et al., 1967) gave a mixture of the starting product, with the 5- and 6-brominated homologs. Pure **1** was obtained by repeated HPLC separation on a Lichrosorb Si 60 250-10 column (Merck) with 60:70:70:1 $CHCl_3$–EtOAc–heptane–EtOH.

GC/MS Control. To avoid false positive results, as recently reported for tetrodotoxin production by bacteria (Matsumura, 1995), the sterile culture medium (500 ml) was also examined as background with the same extractive and analytical conditions. Compounds **1** and **2** were not detected even as traces in the culture medium extracts, attesting to the bacterial production of both metabolites.

Biological Activities. Antimicrobial activities of **1** and **2** were assayed by the disc diffusion method against eight marine bacteria: *Deleya marina* (CIP: 748-T), *Alteromonas haloplanktis* (CIP: 103197-T), *Vibrio campbellii* (CIP: 751-T), *Bacillus marinus* (CIP: 103308-T), and SM-S1, SM-S2, SM-INT, and SM-Z (Table 2). Except for *B. marinus*, which is Gram-positive, and the strain SM-S2 (Gram variable), all strains are Gram-negative.

It was necessary to apply 60 μg of each compound to a sterile paper disc (6 mm, Filtres Durieux) to obtain reliable growth inhibition; after solvent evaporation, the discs were placed on a Marine Agar 2216 (Difco) test dish (three discs per dish) containing a bacterial culture. Growth inhibition was measured after 48 hr of incubation at 30°C. Two replicates containing three discs each were performed for each strain, and the results were presented as a simple average of the data.

The antialgal activity of **1** and **2** was tested against the diatom *Nitzschia acicularis* with a similar method of bacterial plating assay. Sterile paper discs were impregnated with 60 μg of each compound. In our experimental conditions,

10 days at 18°C with a 12-hr photoperiod were necessary before measuring zones of inhibition.

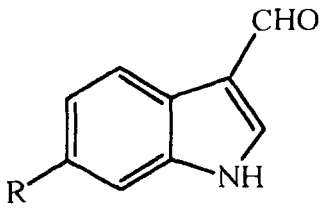
Settlement inhibition was tested in vitro on *Balanus amphitrite* larvae, following the techniques of Rittschof et al. (1984, 1985, 1986, 1992). Compounds **1** and **2** were tested at concentrations ranging from 100 μg to 0.01 μg/ml. Test solutions were prepared by dissolving 1 mg of compound in 30 μl dimethyl sulfoxide (DMSO) and diluting to appropriate concentrations in filtered seawater. Maximal concentration of DMSO in the tests was 0.3%. The tests were carried out in four replicates: 20–30 larvae (4-day-old cyprid larvae) were pipetted into polystyrene cell wells (Corning, 35 mm) containing 2 ml of test situation, filtered seawater control, or 0.3% DMSO in seawater. Test dishes were incubated for 24 hr at 26–28°C with a 15L:9D photoperiod. Tests were stopped by the addition of two drops of 10% formaldehyde, and the numbers of attached and free larvae were counted under a dissecting microscope. The frequency of permanent attachment (expressed as percentage settlements) in treatments was compared to attachment in the control by William's adjusted G test for independence. Results were rejected if the replicates differed significantly or if the settlement in the controls was less than 30%.

Fishes used in the feed-deterrent assay were *Serranus cabrilla*. Compounds **1** and **2** were dissolved in ethanol at 1 mg/ml and 10 mg/ml. For both concentrations, 1 ml of the solution was added to 24 ml of nonsolidified agar (conserved at 40°C) and poured in Petri dishes containing small pieces (0.5 g) of thawed shrimp. After solidification, pieces of agar were cut in pellets of 1 g containing 0.5 g of agar and0.5 g of shrimp. From one extreme to the other, final test concentrations per pellet for both compounds were 250 μg for the highest one and 25 μg for the lowest one. Fishes were fed with four pellets per day. They were conditioned to feeding on control pellets (containing EtOH only) for seven days prior to experimentation.

RESULTS

The main chemical result was the identification of compounds **1** and **2** (Figure 1) from the ether extract of the ascidian *Stomozoa murrayi*. This was accomplished by means of MS analysis and extensive ^{1}H and ^{13}C NMR studies, particularly normal (DQF-COSY) and reverse (HMQC and HMBC) 2D NMR.

Identification of Bacteria. Identification of the four strains isolated from the ascidian *S. murrayi* was not possible with the usual biochemical tests. Strain SM-S1 is a rod-shaped, nonmotile bacterium, which stains Gram-negative and produces white, spreading colonies. It cannot utilize arabinose, lactose, rhamnose, inositol, sorbitol, *N*-acetylglycosamin, acetate, phenylaccetate, pyruvate,



1 R = Br
2 R = H

FIG. 1. 6-bromoindole-3-carbaldehyde **1** and indole-3-carbaldehyde **2**.

γ-hydroxybutyrate, or malonate. It utilizes glucose, galactose, mannose, fructose, saccharose, maltose, fucose, glycerol, mannitol, lactate, succinate, L-alanine, L-histidine, and L-proline. Preliminary studies based on 16S ribosomal DNA sequences of this strain indicate that it belongs to the genus *Acinetobacter*.

Compounds **1** *and* **2** *from* Acinetobacter *sp.* An unidentified polyhalogenated compound (isotopic cluster at *m/z* 327, 329, 331, 333) was detected at R_t 7:43 min. A second compound was detected at R_t 17:42 min (Figure 2B) and identified as the aldehyde **2** (M^+ 145, $[M-H]^+$ 144, $[M-COH]^+$ 116). A third compound was detected at R_t 28:46 min (Figure 3B) and identified as the monobrominated aldehyde **1** based on molecular ion cluster (M^+ 223–225, $[M-H]^+$ 222–224) and the fragmentation pattern ($[M-COH]^+$ 194–196, $[M-COH-HCN]^+$ 167–169, $[M-H-Br]^+$ 143, $[M-COH-Br]^+$ 115). Figure 2 presents the quite similar mass spectra observed at R_t 17 min for two GC-MS independent analyses of bacterial extract and coinjected indole-3-carbaldehyde **2** from ascidian and commercial origin (90:10 w/w).

The mass spectra of **1** from bacterial and ascidian origin (Figure 3) show the same fragmentation pattern with some variation in the relative abundances (Table 1). Table 1 also presents the relative abundance of **1** isolated from the sponge *Pseudosuberites hyalinus* (Rasmussen et al., 1993), for which the Danish group "cannot exclude the possibility that it originates with some associated microorganisms." Synthetic compound **1** (authenticated by NMR spectra: $^{\delta}CDCl_3$ = 8.20, 7.82, 7.61, 7.44) has an R_t of 28:30 min in the same analytical conditions.

Antimicrobial Activity. The bromo compound **1** inhibited the growth of four of eight strains tested, while the debromo analog **2** does not (Table 2). No autotoxicity was observed for strain SM-S1. Compound **2** had no effect on the growth of the diatom *Nitzschia acicularis*. Compound **1** displayed a very low inhibitory activity (300 times less active than Cu^{2+} salts) on this diatom with a zone of inhibition measured as excess radius of 2.5 mm.

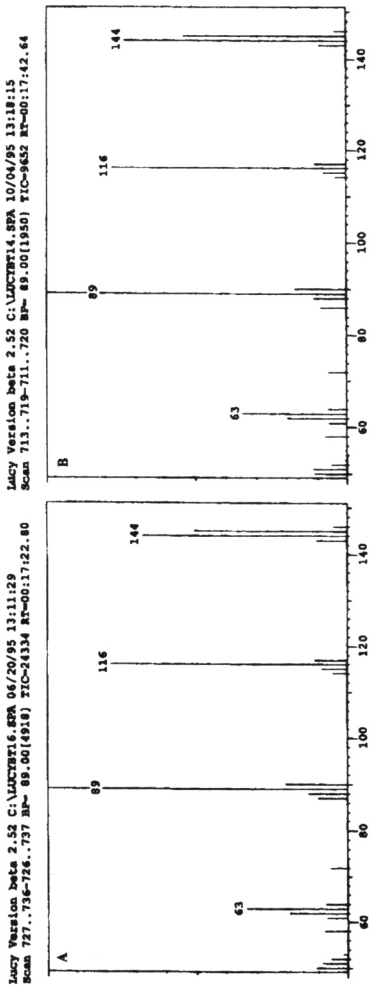


FIG. 2. Mass spectra of indole-3-carbaldehyde **2** from ascidian origin (A) and from bacterial origin (B).

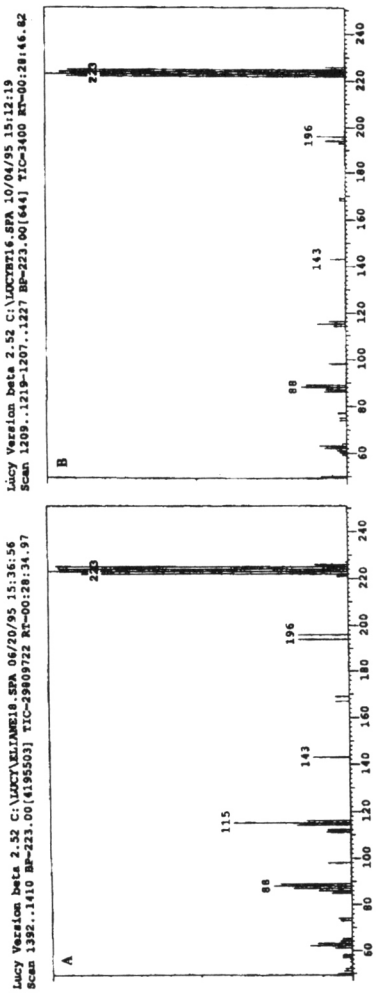


FIG. 3. Mass spectra of 6-bromoindole-3-carbaldehyde **1** from ascidian origin (A) and from bacterial origin (B).

TABLE 1. FRAGMENTATION PATTERN OF 6-BROMOINDOLE-3-CARBALDEHYDE **1** FROM NATURAL AND SYNTHETIC ORIGIN

	$[M]^+$ $[M-H]^+$				$[M-COH]^+$		$[M-COH-HCN]^+$		$[M-H-Br]^+$	$[M-COH-Br]^+$
Origin	225	224	223	222	196	194	169	167	143	115
Stomozoa murrayi	98	97	100	89	17	17	5	6	12	39
Acinetobacter sp.	93	96	100	93	9	7	2	2	2	10
Synthetic **1**	97	100	95	83	17	18	not observed		6	22
Pseudosuberites hyalinus[a]	100	100	100	100	28	28	12	12	24	37

[a]Rasmussen et al. (1993).

TABLE 2. ANTIBACTERIAL ACTIVITY OF **1** AND **2** ON EIGHT MARINE STRAINS[a]

	Strain SM-S1	Strain SM-S2	Strain SM-INT	Strain SM-Z	*Alteromonas haloplanktis*	*Abcillus marinus*	*Deleya marina*	*Vibrio campbellii*
1 (60)	−	++	−	+	−	++	−	++
2 (60)	−	−	−	−	−	−	−	−
C (30)	+++	+++	+++	+++	nt	nt	nt	nt
S (10)	+	++	++	+	nt	nt	nt	nt
P (10)	−	+++	−	−	nt	nt	nt	nt
E (15)	++	+++	++	++	nt	nt	nt	nt

[a]Values are indicated as excess radius. Concentration is indicated in parentheses in μg. +, 4–5 mm radius; ++, 5–10 mm radius, +++, 10–15 mm radius. C = Chloramphenicol, S = Streptomycin, P = Penicillin, and E = Erythromycin. nt = non tested.

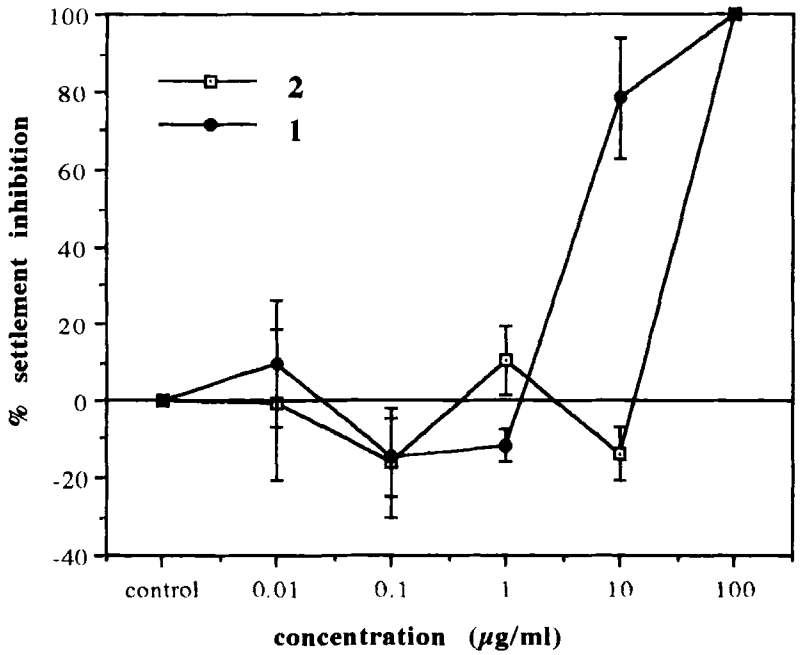


FIG. 4. Inhibition of settlement of *Balanus amphitrite* larvae by 6-bromoindole-3-carbaldehyde **1** and indole-3-carbaldehyde **2**. Control includes seawater and DMSO controls. Each point is the mean of four replicates ± SD.

Inhibition of Settlement. Both compounds completely inhibited settlement of cyprid larvae of *Balanus amphitrite* at the highest concentration tested (100 μg/ml) (Figure 4). The most active metabolite **1** inhibited settlement of larvae by 80% at 10 μg/ml. Settlement inhibition by compound **2** showed a sharp increase at 100 μg/ml, with no significant effect at lower concentrations. EC_{50} values of compounds **1** and **2** were calculated to be 5 μg/ml and 28 μg/ml, respectively. In similar experiments, the EC_{50} value of $CuSO_4$ was found to be 0.85 μg/ml.

Feeding Deterrency Activity. We have not observed any natural predation on *Stomozoa murrayi* by fishes, but when we cut the tunic, damselfishes greedily ate the exposed zooids and spat out tunic fragments. Although tested at concentrations corresponding to between 2 and 20 times stronger than the natural concentrations in the ascidian, no repellent effect of **1** and **2** has been observed. It is worth noting that fishes in this experiment are Mediterranean *Serranus cabrilla* and do not belong to the environment of the Caribbean ascidian.

DISCUSSION

As in the case of other marine benthic invertebrates, the ascidian *S. murrayi* is subjected to a continuous pressure of settlement by larvae and spores, but neither our field observations nor earlier descriptions give any evidence of fouling on this species. Thus, the existence of an antifouling defense is expected. The biological activity of compound **1** isolated from the ascidian supports this hypothesis. The present of compounds **1** and **2** in the ascidian and in a bacterium from its surface may indicate a symbiotic relationship. Compound **1** together with the debromo analog **2** previously was described from at least one bacterium and different taxonomic groups of invertebrates (Table 3).

Four bacterial strains from the ascidian *S. murrayi* have been isolated and cultured. Only one strain produced compounds **1** and **2**. Compound **1** displays low antibacterial activity (Table 2). Moreover, this compound is not present in large quantity in the ascidian (75 μg/g dry tissue, and 12 μg/g wet tissue). For the first time, these indolic compounds are shown to be produced by a bacterium associated with an ascidian.

The activity of **1** on strain SM-S2 is surprising because this bacterium grows at the ascidian surface along with strain SM-S1 that produces **1**. When grown together, the two bacteria are present in Petri dishes after seven days and in a fermentor after three days. Possibilities that could explain this observation are that strains SM-S1 and SM-S2 in the ascidian are separated by many layers of cell-tissue; the production of **1** in culture by SM-S1 is low; and the concentration of **1** in the test is higher than in the environment.

Only four bacterial strains have been isolated, but marine bacteria are dif-

TABLE 3. SUMMARY OF NATURALLY OCCURRING 6-BROMOINDOLE-3-CARBALDEHYDE **1** TOGETHER WITH INDOLE-3-CARBALDEHYDE **2**

Species	Taxonomic group	Site	Reference
Pseudomonas sp.	Bacterium	California	Wratten et al. (1977)
Acinetobacter sp.	Bacterium	Southeastern Mexico	This paper
Iotrochota sp.	Sponge	Western Australia	Dellar et al. (1981)
Pleroma menoui	Sponge	Southeastern Noumea	Guella et al. (1989)
Oceanapia bartschi	Sponge	Bahamas	Cafieri et al. (1993)
Pseudosuberites hyalinus	Sponge	Faroe Islands	Rasmussen et al. (1993)
Tubastrea coccinea	Coral	Hawaii	Okuda et al. (1982)
Tubastrea faulkneri	Coral	Northeastern Australia	Rashid et al. (1995)
Pyura sacciformis	Tunicate	Japan	Niwa et al. (1988)
Stomozoa murrayi	Tunicate	Southeastern Mexico	This paper

ficult to isolate and cultivate, because only a small percentage of the viable bacterial cells in marine samples ultimately grow under standard culture conditions (Fenical, 1993). The exact nature of the association between the *Acinetobacter* sp. strain (SM-S1) and the ascidian *S. murrayi* is not yet established, and we do not know if this bacterium is typical to *S. murrayi*.

Marine life is the largest source of naturally occurring organohalogen compounds, and in many marine organisms these compounds may serve in chemical defense, as either feeding deterrents, irritants, or poisons (Gribble, 1992). The question of the true origin of these metabolites has not been resolved. Bromoindole alkaloids isolated from several phyla have been shown, at least in one case, to originate from a dietary source (Fahy et al., 1991). A symbiotic origin is often suggested but rarely proved (Rasmussen et al., 1993). Very few microorganisms express the biochemical potential for introducing a bromo atom in the indole nucleus (Wratten et al., 1977; Norton and Wells, 1982).

When a metabolite is widely distributed, bacteria or cyanobacteria either as true symbionts or as nonobligate associates have been suspected to be responsible for production (Fenical, 1993). We have no information on whether compound **1** obtained from other marine invertebrates is of bacterial origin or whether those organisms are fouled or not. The production of both compounds **1** and **2** by a marine bacterium isolated from the surface of a benthic invertebrate could explain the presence of the same products in phylogenetically unrelated benthic invertebrates. Pietra (1995) hypothesized that the presence of similar natural products in different phyla could stem from endosymbiotic bacterial contribution of genetic information to the host, but this hypothesis remains to be proved.

The information about bacterial epibiosis on ascidians is quite limited (Wahl et al., 1994; Wahl, 1995). Bacterial associations with ascidians have been described (Holmström et al., 1992; Holmström and Kjelleberg, 1993), and reveal that bacteria produce inhibitory substances in the marine environment, even if they are not specifically antibiotic producers.

Marine organisms, such as ascidians, can prevent fouling by the production of secondary metabolites (Davis and Wright, 1990). For the ascidian *S. murrayi*, bromo compound **1** inhibits larval settlement, but compound **2** does not. Compound **1**, although not present at a high level, was present in sufficient quantity to inhibit settlement of barnacles (EC_{50} = 5 μg/ml). This could explain why the ascidian *S. murrayi* is not overgrown by macroepibionts such as *Balanus amphitrite*. We cannot estimate an equivalence between the concentration of the products on the ascidian, in dry weight, to the test in solution, and we think that it is necessary to incorporate the compound in a matrix to evaluate its antifouling properties. Compound **1** displays an activity against barnacle larvae settlement close to the activities of 2,5,6-tribromo-1-methylgramine (EC_{50} = 1 μg/ml) studied by Kon-Ya et al. (1994a) or $CuSO_4$. The fact that compound **1** is also produced by strain SM-S1, probably the true producer, agrees with the concept that larval settlement can be affected in a negative way by bioactive secondary metabolites released by associated bacteria (Avelin et al., 1993).

Wratten et al. (1977) report that products **1** and **2** are from extracellular origin, and our results are in agreement with the suggestion of Maki et al. (1988) that bacterial extracellular products may be the causal agents of larval settlement inhibition. We propose that compound **1** reduces fouling on the surface of the ascidian *S. murrayi*, but it should be tested against naturally occurring epibionts to ascribe to it a defensive role.

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DIFFERENTIAL DISPLAY OF INVESTIGATIVE BEHAVIOR PERMITS DISCRIMINATION OF SCENT SIGNATURES FROM FAMILIAR AND UNFAMILIAR SOCIALLY DOMINANT FEMALE MARMOSET MONKEYS (*Callithrix jacchus*)

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Abstract—The hypothesis was tested that marmosets have differential displays of investigatory behavior towards circumgenital scent marks from socially dominant females based on degree of familiarity. In a series of two-choice behavioral discrimination bioassays, dominant males together with subordinate and dominant females were presented concurrently with scent secretions from familiar versus unfamiliar dominant females, and their investigative behaviors towards the scent were recorded. Test animals directed significantly different amounts of investigative behaviors towards familiar versus unfamiliar scents when the scent stimuli involved complete circumgenital scent marks, glandular secretion only, or urine only. When animals discriminated between the two scent samples, they did so by directing significantly increased amounts of behavior to scent from unfamiliar females. Chemical cues promoting differential displays of behavior to familiar versus unfamiliar scents appeared to be volatile in nature and were effective in the scent mark for at least one day following deposition. The reliable ability of marmosets to consistently discriminate between odors from familiar versus unfamiliar dominant females provides strong circumstantial evidence that individual female marmosets have distinct chemical signatures. The ability of marmosets to discriminate the odor

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from familiar versus unfamiliar dominant females may be important in the context of intergroup relations.

Key Words--Odor communication, callitrichid, *Callithrix jacchus*, scent-marking, individual differences, two-choice discrimination, volatile chemicals, familiarity, marmoset.

INTRODUCTION

Complex odor communication systems have evolved in a large number of both solitary and social species, across most animal taxa (for reviews see Albone, 1984; Epple, 1986; Ralls, 1971; Macdonald et al., 1990; Doty and Muller-Schwarze, 1992). Odors signal a multitude of characteristics about an individual donor such as their sex (Smith, 1994), hormonal status (Ziegler et al., 1993), social rank (Mossman and Drickamer, 1997), individual identity (Johnston and Jerigan, 1994), and even major histocompatability (MHC) type (Brown et al., 1990). This chemical information subsequently plays an important role in regulating behavior (Signoret, 1990) and, in many cases, reproductive physiology (Novotny et al., 1990) of conspecifics.

Marmosets and tamarins, small, arboreal, group-living New World primates, have a well-developed odor communication system. These taxa provide particularly useful models for the investigation of the role of odor in regulating social interactions (see review in Epple, 1986; Epple et al., 1993). In the wild, common marmosets (*Callithrix jacchus*) live in social groups containing 3–15 individuals (Scanlon et al., 1988; Digby, 1995; Pontes Mendes and Monteiro da Cruz, 1995). Within these social groups, both in captivity and in the field, reproduction is typically limited to one (behaviorally dominant) female (Abbott, 1984; Hubrecht, 1984; Stevenson and Rylands, 1988; also see Digby and Ferrari, 1994; Digby, 1995). Common marmosets have morphological and behavioral adaptations that result in the effective formation, deposition, and reception of scent material (Sutcliffe and Poole, 1978; Coimbra-Filho and Mittermeier, 1978; Hunter et al., 1984; Stevenson and Rylands, 1988; Epple et al., 1993; Taniguchi et al., 1992). Both males and females possess specialized circumgenital glands in the anogenital region. Only males possess sternal glands, the development of which is dependent on male dominance status (Sutcliffe and Poole, 1978). Scent marking frequency in the common marmoset is significantly modified by social and physiological contexts and by environmental factors (for a review see Epple et al., 1993). These findings suggested that scent marking in common marmosets, as in other callitrichid species, plays an important role in communication (Epple, 1986; Epple et al., 1993; Cebul et al., 1978; Barrett et al., 1990, 1993; Smith, 1994).

Common marmosets are cooperative breeders, demonstrating a strong reli-

ance on intragroup cooperation for infant care (Tardif, 1996), foraging (Garber, 1993), territory defense (Goldizen, 1987), and antipredator vigilance (Koenig, 1992; Caine, 1993). Hand in hand with this cooperation is a complex social organization characterized by frequent, affiliative social interactions between group members (Schaffner et al., 1995). Marmoset social interactions are not limited, however, to members of the same social group. In the wild, neighboring groups of marmosets encounter each other from once a week to several times a day (Garber et al., 1993; Hubrecht, 1984, 1985; Rylands, 1986; Savage, 1990; Buchanan-Smith, 1991). Behavioral interactions during these intergroup encounters are often very different from behavioral interactions occurring between members of the same social group. Intergroup behavioral interactions are characterized by visual, vocal, and acoustic agonistic displays between adult group members (Buchanan-Smith, 1991), but can include physical contact, biting, and aggression (Rylands, 1986). It has been suggested that odor cues in marmoset scent marks play an important role in regulating group movements and therefore intergroup spacing, and by doing so the marks help minimize disputes between neighboring groups (Lacher et al., 1981). For example, marmosets are exudate eaters, gouging holes in branches and feeding on the sap that flows into the holes. Observations on wild populations of common marmosets, black-and-white tassel ear marmosets (*C. humeralifer*), and black-tufted-ear marmosets (*C. p. penicillata*), report animals depositing the majority of their scent marks (i.e., up to 99%) at feeding sites. Lacher et al. (1981) proposed that animals use information in scent marks at these feeding sites to determine the time that has elapsed since another group fed from the hole and to regulate their movement in relation to the position of neighboring groups, thereby avoiding encounters. In addition, results from several studies of captive and wild marmosets have shown that scent marking is not randomly distributed over the home range, and is instead concentrated at specific locations such as feeding areas (Coimbra-Filho and Mittermeier, 1978; Rylands, 1985), sleeping sites (Sutcliffe and Poole, 1978), and areas where home ranges of neighboring groups overlap (Rylands, 1990). These observations have suggested that scent marking plays an important role in defending the home territory and resources against neighboring groups (Epple, 1970, 1986; Epple et al., 1993).

In order for scent marks to play important roles in the context of regulating intergroup spacing and resource defense, marmosets need to be able to distinguish between scents deposited by members of their own group versus scents deposited by nongroup members. The aim of the present study was to determine whether male and female marmosets display differential investigative behavior that would indicate discrimination between scents deposited by members of their own social group (familiar animals) versus scents deposited by members of another social group (unfamiliar animals). Consistent differential displays of behavior towards the two presented odors would provide evidence that scent

marks from common marmosets could be distinguished by conspecifics on the basis of familiarity. To investigate the nature of the chemical cues providing discrimination between familiar versus unfamiliar scents, the behavioral responses of test animals were assessed when the presented scents involved: (1) unmanipulated circumgenital scent marks; (2) unmanipulated scent marks presented 3 mm below a perforated sheath, allowing the test animal contact with only volatile cues; (3) secretions only from the circumgenital gland; (4) urine alone; and (5) 24-hr-old unmanipulated circumgenital scent marks.

METHODS AND MATERIALS

Ten intact females exhibiting regular ovarian cyclicity (defined as dominant females), served as both scent donors and test subjects in two-choice behavioral discrimination tests. Seven females held dominant status in social groups and three females were pair-housed with an intact male. In addition, six subordinate females (anovulatory) and 11 dominant males living in social groups were used as test subjects (but not scent donors).

The animals used in the study were captive born and housed at the Institute of Zoology, London, as previously described by Hearn (1983). All marmosets were postpubertal. Social groups were formed by placing two to four unrelated males and two to four unrelated females in an observation room (2.9 × 2.2 × 1.7 m) fitted with natural branches, a feeding platform (75 × 75 cm) and two nest boxes raised 1.2 m off floor level. Once a group was established, it was transferred to a home cage (100 × 50 × 75 cm) and given access to an exercise cage (2 × 1 × 2 m) every five to seven days. Behavioral observations were made on each group for 1.5 hr daily for the first 10 days following group formation. All submissive and dominant behavioral interactions were recorded, excluding interactions involving feeding and drinking (Abbott, 1984; Abbott et al., 1988). A dominance hierarchy was established within each sex approximately three days following group formation. Among the females, the hierarchy typically comprised a dominant female (rank 1) and one to two subordinate females (rank 2 and below). The most dominant individual (rank 1) received least aggression and most submission and the most subordinate individual received most aggression and least submission (Abbott and Hearn, 1978; Abbott, 1984). All social groups of animals used in the study were stable and had been established for at least six months.

In this study, familiar dominant females were defined as the resident dominant female in the social group of the respective subject. Unfamiliar dominant females were defined as females with whom the subject had neither been housed nor had had physical contact. All marmosets involved in the study shared a single animal room and were in unobstructed visual, auditory, and olfactory

contact. Animals were fed a varied diet once daily that consisted of Mazuri pellets (Mazuri New World Diet) soaked in either condensed milk (Nestles) or a black currant fruit drink (Robinson); Farex (Farley Baby Products); fruit (apple, orange, banana, grapes), and mealworms. Animal room temperature was maintained at 20–25°C and humidity at 50–70%. The animals experienced an unvarying photoperiod regime of 12 hr light (08:00–20:00 hr) and 12 hr dark (20:00–08:00 hr). All the experiments were performed at the Institute of Zoology, London.

Determination of Ovulatory Function

Scent was always collected from females in the luteal phase of the ovarian cycle to control for ovarian hormonal influences on scent secretion or deposition. Dominant female common marmosets exhibit a 28-day ovarian cycle with a luteal phase characterized by levels of plasma progesterone elevated to concentrations greater than 10 ng/ml for approximately 20 days (Harlow et al., 1983). The hormonal status of all females was monitored throughout the experiment by determining concentrations of plasma progesterone in twice weekly blood samples with an enzyme-linked immunoabsorbent technique (Hodges et al., 1988). The day of ovulation was determined as the day prior (± 1 day) (Abbott et al., 1988) to an increase in levels of progesterone to luteal phase concentrations (i.e., >10 ng/ml). Early pregnancy and/or an extended luteal phase was terminated by administering 0.5 mg of cloprostenol, a prostaglandin F_{2a} analog (Estrumate; ICI, Macclesfield; Summers et al., 1985) by intramuscular injection, approximately 15–25 days following ovulation. Cloprostenol administration induced luteolysis, and the female returned to a follicular phase (plasma progesterone <10 ng/ml) for approximately nine days, prior to subsequent normal ovulation.

Collection and Manipulation of Scent Material

Scent Mark Collection Apparatus. Frosted Pyrex collection tubes (7.5 × 1 cm; Figure 1) were placed in a foil-lined groove running the length of a wooden perch (48 × 4 cm). The tube-fitted wooden perch was placed into the home cage of the scent donor in place of the usual wooden perch. A well, approximately 0.5 cm deep, ran along half the length of each collection tube. The well was intended to mimic the feeding holes that marmosets typically scent mark in the wild and to encourage scent marking (Coimbra-Filho and Mittermeier, 1976, 1978; Rylands, 1985; Stevenson and Rylands, 1988). All glass apparatus was cleaned by rinsing in tap water 5 min, boiling in disinfectant (Pyroneg, Diversity Ltd., Northampton, UK) for 45 min, again rinsing for 5 min in tap water, rinsing three times in distilled water, boiling twice in distilled

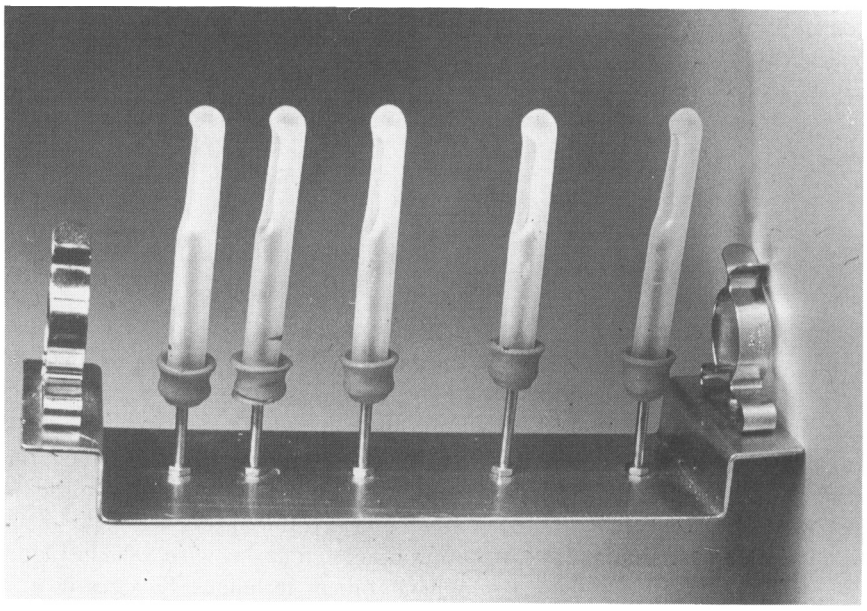


FIG. 1. Presentation frame fitted with five collection tubes, used to present a single scent stimulus in a two-choice behavior discrimination test. In any behavior discrimination test, two such presentation frames were presented to the test animal.

water for 45 min each time, and finally by baking at 200°C for 8 hr. Aluminum foil and Teflon-lined lids were baked at 150°C for 8 and 2 hr, respectively.

Marmosets were trained to deposit circumgenital scent marks on the specialized scent mark collection apparatus by training with a positive reinforcement technique: each deposition of a scent mark was rewarded by 0.5 ml black currant fruit drink. One of us (T.E.S.) was responsible for the collection of all scent material and conducting all the training procedures. There were four consecutive stages to the training procedure. An animal experienced only one training period per day. Each animal had to become competent at each stage (Table 1) before it proceeded to the next stage of training. Animals varied in the length of time taken to reach competency in each stage of training, as shown in Table 1. An animal was defined as trained when it consistently deposited a scent mark within 5 sec of the researcher approaching the cage with a syringe of fruit juice. In all experiments, 15 scent marks from an individual donor constituted a single scent stimulus. All scent material was collected at 07:00–09:00 hr, prior to the morning feed.

TABLE 1. DETAILS OF FOUR CONSECUTIVE STAGES OF TRAINING MARMOSETS TO DEPOSIT CIRCUMGENITAL SCENT MARKS ON SCENT MARK COLLECTION APPARATUS[a]

Stage of training	Variation in time taken for animals to reach competency	Competency requirement	Summary of procedure for each stage of training
1	2–10 days	Habituate animal to scent collection apparatus	A wooden perch fitted with Pyrex collection tubes was placed into the cage with the animal for 4 hr daily. Habituation to the apparatus was signified by the animal freely contacting the equipment.
2	5–10 days	Habituate animal to prolonged presence of observer	The observer was positioned quietly, for 15 min daily, 30 cm in front of the animals' cage. Habituation was indicated by the absence of prolonged visual attention towards the observer (i.e., when the animal stared at the observer for no longer than 1 min per 15-min session) and the absence of behaviors indicating distress, such as rapid movement and twitter vocalizations.
3a	2–6 days	Condition animal to scent mark in its cage	The animal was observed for 20–40 min daily and was rewarded with 0.5 ml fruit juice drink when it scent marked anywhere in the cage.
3b	1–2 days	Condition animal to scent mark collection apparatus	The animal was observed for 20–40 min daily and rewarded with 0.5 ml fruit juice drink when it scent marked the collection apparatus. Following initial conditioning to scent marking the apparatus, the procedure was repeated for three days to reinforce the marking response.
4	2–4 days	Habituate animal to observer periodically replacing scent marked collection tubes	As for stage 3b, except that when a collection tube had been scent marked once it was removed from the wooden perch using clean forceps and replaced with a clean tube.

[a] Animals had to be competent at each stage before proceeding to the next stage. Time (days) taken for animals to attain competency at each stage is presented.

Collection of Glandular Material. Scent donors were removed from the cage and their circumgenital scent gland was thoroughly cleaned. First, the gland and surrounding area were gently wiped with excess distilled water to remove scent material, feces, and urine adhering to the gland. Second, the gland was cleaned using an 80% ethanol solution, after which it was rinsed with excess distilled water followed by a further cleansing with 80% ethanol solution. Finally, the gland was thoroughly rinsed with excess distilled water. To collect a gland wipe, the clean circumgenital gland was swabbed with a frosted Pyrex collection tube while applying pressure to the gland to express secretion. A single swab was defined as one gland wipe, and 15 gland wipes served as a single scent stimulus in a choice discrimination test. Glandular material was used in discrimination tests within 20 min of collection.

Urine Collection. At 08:00 hr, the animals were wakened, removed from the cage, and the circumgenital gland cleaned thoroughly (as described above) to prevent contamination of the urine sample with glandular material. Animals were gently held for a maximum of 10 min, during which period they urinated on approximately 80% of occasions. Urine was collected on aluminum foil, transferred to sealed glass vials and stored on ice until presented in behavior tests. If subjects had not urinated within 10 min they were returned to their home cage.

Preparation of Organic Solvent Scent Mark Extracts. Following the deposition of one, or at the most two, circumgenital scent marks on a collection tube, the tube was removed from the wooden perch with clean aluminum forceps and placed in a glass tube (16 × 2 cm) containing a 2-ml mixture of methanol–methylene chloride (1:3 v/v). The tube containing the solvent mixture and scent marked collection tube was vortexed gently for 1 min. The collection tube was removed from the extraction solvent with a glass rod and the process repeated until 15 single scent marks from the donor had been extracted into the 2-ml volume. Organic solvent extracts were stored at −20°C for a maximum of 1.5 hr before presentation in a behavior discrimination test.

Two-Choice Behavioral Discrimination Test

Apparatus. A single scent stimulus was presented on five collection tubes attached at 2-cm intervals to a metal presentation frame (20 × 6 cm) (Figure 1). The second scent stimulus was carried by a second set of five collection tubes attached to a second, identical presentation frame. Each scent stimulus comprised a pool of 15 scent marks (treated as described below for each experiment) from the respective familiar and unfamiliar dominant female. For a discrimination test, two presentation frames (each complete with five collection tubes bearing scent from a single animal) were fitted longitudinally onto the front of the home cage of the test animal, approximately 28 cm apart. The two

sets of collection tubes carrying the scent protruded through the front bars of the cage and into the home cage of the test animal, to a depth of approximately 6 cm. In tests presenting solvent extracts of scent marks, a 2-ml extract containing 15 scent marks was defined as one scent stimulus. Each extract was evenly divided between the wells on one set of five collection tubes. Extracts were only presented in discrimination tests immediately following their evaporation to dryness. Evaporation to dryness took approximately 20 min.

Procedure. Five minutes prior to testing, animals residing with the test animal were removed from the home cage and placed in an adjacent cage. The test animal remained in auditory (and possibly olfactory) but not visual contact with its cage mates. Five minutes later, the test animal was shut in its nest box at the side of the cage for approximately 30 sec while the two presentation frames, each complete with five fitted tubes carrying the respective scent stimuli, were fitted onto the front of the cage. When the animal was released from the nest box, a 5-min test period commenced. The following behaviors directed by the test animal at the scent stimuli were recorded by an observer (T.E.S.) familiar to the animals, sitting at a distance of 2 m from the cage and visible to the animal at all times:

(1) Sniff: the marmoset's muzzle was moved to within 0.5 cm of the collection tube and the animal sniffed the equipment. (2) Close contact nuzzling: a characteristic facial expression resembling the flehmen posture reported in ungulates, lemurs, and closely related tamarin species (the saddle-back tamarin) was observed (Estes, 1972; Hunter et al., 1984). The upper lip was drawn up close to the nostrils exposing the teeth. The lower and upper jaw met, however, leaving no space between the teeth. Maintaining this expression and constant contact with the tube, an animal would move its muzzle along the longitudinal axis of the collection tubes. A rapid rate of inhalation was observed. (3) Lick: the animal protruded the tongue and licked the scent stimuli at a rapid rate. (4) Scent marking: an animal circumgenital scent marked or ventral rubbed the scent stimuli (Sutcliffe and Poole, 1978).

Behaviors were expressed as frequency of sniffs and scent marks and total time spent nuzzling and licking per 5 min. Animals were tested no more than once a day, with the order of testing randomized. The side of stimulus presentation was counterbalanced to control for side preference. Six series of two-choice behavioral discrimination tests were conducted to test the hypothesis that dominant and subordinate females and dominant males displayed differential amounts of investigative behavior towards the presented stimuli, so as to suggest spontaneous discrimination between circumgenital scent marks deposited by their resident (familiar) dominant female versus circumgenital scent marks deposited by nonresident (unfamiliar) dominant females. When dominant females were used as test animals, the two concurrently presented scent stimuli were donated by themselves (familiar scent) and a nonresident dominant female (unfamiliar

scent). Test animals were not presented with the scent from a single unfamiliar donor more than once in any series of discrimination tests. For each series of experiments, the scent source, testing conditions, number and gender of test animals, and number of trials per test animal are summarized in Table 2.

Control. Two control procedures were conducted. The first assessed the behavioral displays of marmosets (nine males and nine females) towards naturally deposited unmanipulated circumgenital scent marks from two individual, unfamiliar dominant females. The results were analyzed to investigate whether test animals exhibited differential displays of behavior to the scent presented on

TABLE 2. SUMMARY OF TWO-CHOICE BEHAVIORAL DISCRIMINATION TESTS[a]

Experiment	Scent source	Scent donors	Test conditions	No. and gender of test animals	Test per subject (*N*)
Unmanipulated scent marks					
1a	SM	FDF vs UDF	Unman contact	8 M	2
1b	SM	FDF vs UDF	Unman contact	6 SF	4
1c	SM	SELF vs UDF	Unman contact	10 DF	2
Volatile cues only					
2a	SM	FDF vs UDF	No contact	8 M	2
2b	SM	SELF vs UDF	No contact	10 DF	2
Solvent extracted marks					
3a	SM	FDF vs UDF	Solvent Extract	8 M	2
3b	SM	FDF vs UDF	Solvent Extract	6 SF	4
3c	SM	SELF vs UDF	Solvent Extract	10 DF	2
Gland secretion					
4a	GW	FDF vs UDF	Unman contact	10 M	2
4b	GW	SELF vs FD	Unman contact	10 DF	2
Urine					
5a	U	FDF vs UDF	Unman contact	10 M	2
5b	U	SELF vs FD	Unman contact	10 DF	2
Scent marks aged 1 day					
6a	SM	FDF vs UDF	24 hour old	11 M	1
6b	SM	SELF vs FD	24 hour old	10 DF	2

[a]Abbreviations used: SM = circumgenital scent mark; GW = glandular secretion; U = urine; UDF = unfamiliar dominant female; FDF = familiar dominant female; Unman contact = unmanipulated marks that animals could freely contact; No contact = unmanipulated marks in the noncontact condition; Solvent extract = organic solvent extracts of scent marks; Aged 1 day = scent marks aged 1 day; M = male; DF = dominant female; SF = subordinate females.

the right side of the cage versus the left side of the cage. Furthermore, the extent to which these test animals consistently discriminated between scent from unfamiliar female A and unfamiliar female B was assessed in six pairs of females.

The second control procedure assessed the effect of the extraction mixture on the behavioral responses of marmosets. Eleven males and 10 dominant females were each presented once with a presentation frame fitted with five clean collection tubes (bearing no scent) and a presentation frame fitted with five tubes over which 2 ml of the organic solvent mix (methanol–methylene chloride, 1 : 3 v/v) had been distributed.

Unmanipulated, Naturally Deposited Scent Marks (Experiment 1). In the first series of two-choice behavior discrimination tests, the scent stimuli were unmanipulated, naturally deposited scent marks from the two donor females. The collection tubes protruding through the front bars of the home cage were not covered in any way, so the test animals could freely contact and manipulate the scent.

Volatile Cues (Experiment 2). In experiment 2 we investigated whether or not test animals were able to discriminate between naturally deposited scent marks from the resident (familiar) dominant female versus a nonresident (unfamiliar) dominant female on the basis of volatile cues only. Scent stimuli were presented to test animals, as described above, but a perforated plastic sheath (holes 2 mm in diameter, spaced 3 mm apart) was placed at a height of 3 mm around the collection tube bearing the scent mark. Animals could not directly contact the scent mark and were only exposed to volatile cues that permeated through the perforated plastic sheath.

Solvent Extracted Marks (Experiment 3). This experiment challenged subjects to discriminate between organic solvent extracts (methanol–methylene chloride, 1 : 3 v/v) of scent marks from familiar versus unfamiliar dominant females.

Glandular Secretion (Experiment 4). The circumgenital scent mark of the common marmoset is a cocktail of many scents including: (1) secretions from a specialized glandular region in the anogenital area and from nonspecialized circumanal glands, (2) urine, and (3) a combination of genital discharge, feces, and old scent material adhering to the scent glands (Sutcliffe and Poole, 1978). This series of discrimination tests was conducted to determine whether secretions from the circumgenital gland alone contained chemical cues distinguishing between scent marks from familiar versus unfamiliar females. Test subjects were therefore presented with wipes collected from cleaned circumgenital glands of familiar and unfamiliar dominant females.

Urine (Experiment 5). Urine is a major component of the circumgenital scent mark of common marmosets (Sutcliffe and Poole, 1978). We investigated whether marmosets could discriminate between void urine (1 ml samples that

were not contaminated with glandular material) from familiar versus unfamiliar dominant females.

One-Day-Old Scent Marks (Experiment 6). An advantage of odor communication is that the communicatory message can be released over a period of time. We determined whether the chemical cues permitting discrimination of scent marks from different females were still present one day after the scent mark had been deposited. To investigate the hypothesis, subjects were presented with 1-day-old scent marks from familiar versus unfamiliar dominant females. Scent marks that were 1 day old had remained unmanipulated and suspended in the air (attached to the presentation frame) in an animal room (temperature: 20–25°C; humidity: 50–70%).

Statistical Analysis of Behavioral Data

For each experiment the mean frequency of sniffing and scent marking together with the mean duration of nuzzling and licking was computed for each test animal. The behavioral responses of the test animals to the two scent stimuli concurrently presented in each experiment were analyzed using Wilcoxon matched-pairs signed-rank test (Siegel, 1956). An alpha level of 0.05 was adopted. Data for the two-choice behavioral discrimination tests are expressed as median ± interquartile range.

RESULTS

Behavioral Discrimination Tests (Table 1)

Control Procedures. When test males and test females were presented with circumgenital scent marks collected from two unfamiliar dominant females, they showed no discrimination between the scent stimuli presented on the right side of the cage versus scent presented on the left side of the cage (male test animals: sniff, 3.9 ± 0.6 vs. 3.5 ± 0.7, NS; nuzzle, 7.25 ± 2.0 vs. 6.1 ± 1.5, NS; three males deposited scent marks over the test stimuli and only two males licked the scent marks; female test animals: sniff, 2.0 ± 4.5 vs. 1.8 ± 1, NS; nuzzle, 3.7 ± 0.7 vs. 4.2 ± 1.5, NS; scent mark 0.8 ± 1 vs. 1.2 ± 0.9, NS). Control tests consistently demonstrated that animals did not discriminate between the scent marks from two unfamiliar animals.

There was no significant difference in the quality and quantity of investigative behavior directed by either males or females at clean collection tubes versus collection tubes carrying solvent extract control blanks (male test animals: sniff, 2.5 ± 1.1 vs. 3.5 ± 2.0, NS; nuzzle 3.0 ± 2.1 vs. 1.8 ± 2.2, NS; female test animals: sniff, 2.1 ± 0.5, NS; nuzzle, 4.1 ± 2.5, NS). A single

male subject deposited two scent marks over the solvent extract control blanks, and one male deposited a single scent mark over both a clean collection tube and a collection tube carrying the solvent extract control blanks. Two females deposited one and two scent marks, respectively, over the solvent extract control blanks, and one female subject deposited two scent marks over each of the two scent stimuli. No test subjects licked the scent stimuli. The results indicated that evaporated organic solvent residue caused no significant bias in the investigative behavior of test animals.

Unmanipulated Scent Marks (Experiment 1). When test males and test subordinate females were presented with unmanipulated scent marks from familiar versus unfamiliar dominant females, test animals spent significantly more time nuzzling marks deposited by unfamiliar females [male test animals: UDF (unfamiliar dominant female) = 8.3 ± 1.3, FDF (familiar dominant female) = 3.2 ± 1.2, $z = 2$, $P < 0.05$; subordinate female test animals: UDF = 8.5 ± 0.5, FDF = 2.5 ± 1, $z = 2.2$, $P < 0.05$; Figure 2a and b]. Neither males nor females discriminated with sniffing behavior (these test animals also did not lick the scent stimuli or deposit any scent marks over the stimuli from dominant females). Dominant females similarly displayed differential investigatory behaviors between their own scent marks and those of an unfamiliar dominant. Dom-

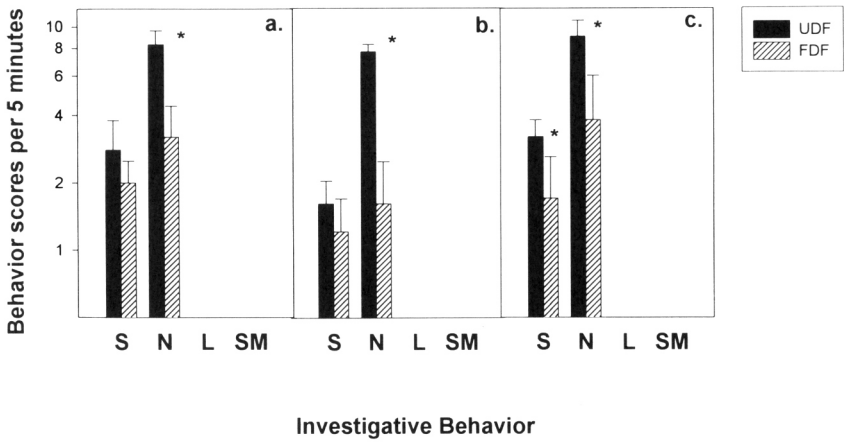


FIG. 2. Levels of investigative behaviors directed towards freshly deposited unmanipulated marks by (a) males, (b) subordinate females, and (c) dominant females. Behavior scores shown are for the investigatory behaviors sniff (S), close contact nuzzling (*N*), lick (L) and scent mark (SM) per 5 min (log scale; median ± interquartile range). In all experiments test animals were presented with scent from an unfamiliar dominant female (UDF) and a familiar dominant female (FDF).

inant females spent significantly more time nuzzling, and directed significantly more sniffs to, the scent marks of unfamiliar dominant females in comparison to their own marks (dominant female test animals: nuzzle, self = 3.8 ± 2.2; UDF = 9 ± 1.6, $z = 2.2$, $P < 0.05$; sniff, self = 3.25 ± 0.6, UDF = 4.35 ± 0.5, $z = 2.1$, $P < 0.05$; Figure 2c). Dominant females scent marked both scents with similar frequency and did not lick the presented samples.

Volatile Cues (Experiment 2). Males and dominant females similarly displayed differential investigatory behavior between scent marks from familiar versus unfamiliar dominant females when the scent stimuli were presented 3 mm below a perforated plastic sheath. Males directed significantly more sniffs to scent stimuli from unfamiliar dominant females (male test animals: UDF = 3.0 ± 0.5, FDF = 1.5 ± 0.4, $z = 2.5$, $P < 0.01$; Figure 3a). They showed no discrimination in terms of nuzzling or licking behavior and did not deposit any scent marks over the scent stimuli. Dominant females spent more time nuzzling marks from an unfamiliar dominant female than their own marks, but showed no discrimination in terms of sniffing or scent marking behavior (dominant female test animals: UDF = 7.8 ± 2.6, self = 4.0 ± 2.3, $z = 2$, $P < 0.05$; Figure 3b). Females did not lick the presented scent stimuli.

Solvent Extracted Scent Marks (Experiment 3). Both dominant and subordinate females spent significantly more time nuzzling solvent extracted scent mark pools donated by unfamiliar as opposed to familiar dominant females

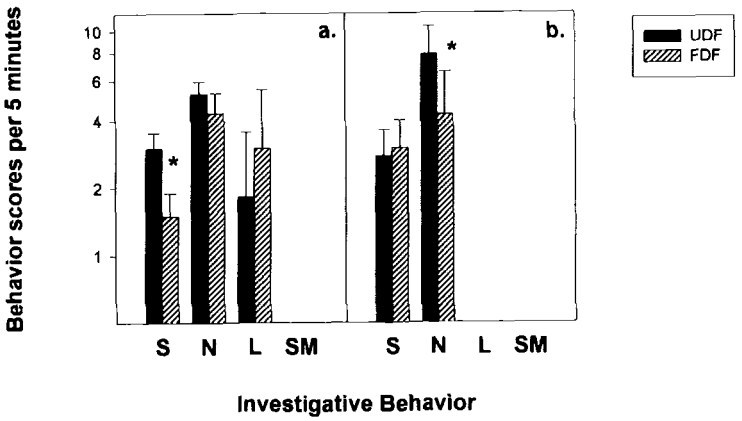


FIG. 3. Levels of investigative behavior directed towards freshly deposited scent marks presented under a perforated sheath so test animals were only exposed to volatile airborne cues from the scent mark. Behavior scores are shown for (a) males and (b) dominant females. Further details are given in Figure 2 legend.

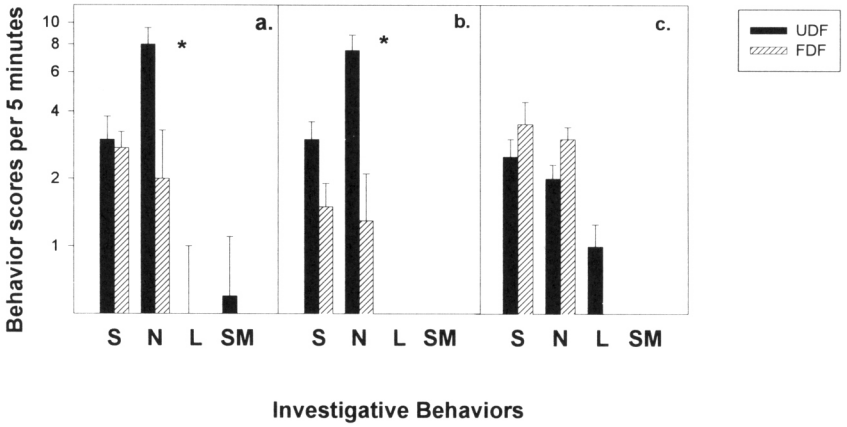


FIG. 4. Levels of investigative behavior directed by (a) dominant females, (b) subordinate females, and (c) males towards organic solvent extracts of freshly deposited scent marks. Further details are given in Figure 2 legend.

(dominant female test animals: UDF = 8.0 ± 1.5, self = 2.0 ± 1.3, $z = 2$, $P < 0.05$; subordinate female test animals: UDF = 7.5 ± 1.3, FDF = 1.3 ± 0.8, $z = 2$, $P < 0.05$; Figure 4a and b). Females in neither test group demonstrated discrimination between marks based on their sniffing or licking behavior or by depositing scent marks on the presented scents. Male test animals showed no difference in investigative behavior between solvent extracts of scent marks deposited by familiar versus unfamiliar dominant females (Figure 4c).

Glandular Material (Experiment 4). Dominant females, when presented with scent from their own circumgenital gland or that of an unfamiliar dominant female, spent significantly more time nuzzling the glandular scent from the unfamiliar dominant female (dominant female test animals: UDF = 9.4 ± 1.0, self = 5.2 ± 0.8, $z = 2.0$, $P < 0.01$; Figure 5a). Dominant females did not exhibit significant bias in terms of sniffing behaviors and did not lick or deposit any scent marks over the scent stimuli. Males did not display differential investigative behavior towards glandular material alone from familiar versus unfamiliar dominant females (Figure 5b).

Urine (Experiment 5). Urine alone contained chemical cues that caused only males to exhibit differential investigative behavior between urine samples collected from different individuals (Figure 6a and b). Test males directed significantly more sniffs to urine samples deposited by unfamiliar versus familiar dominant females (male test animals: UDF = 4.2 ± 1.2, FDF = 2.2 ± 0.8, $z = 2.7$, $P < 0.01$). Males showed no behavioral bias in terms of nuzzling or

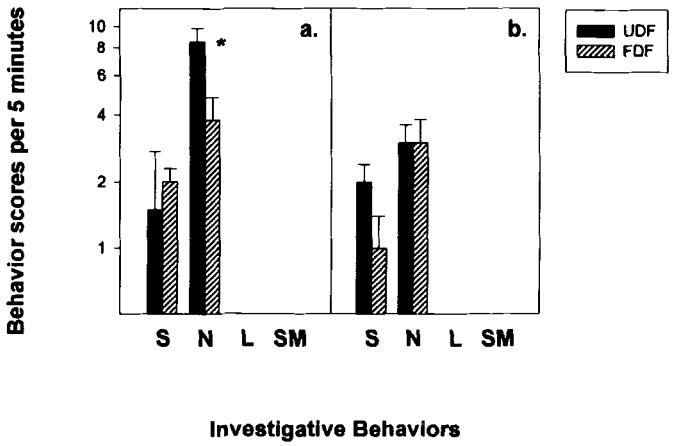


FIG. 5. Levels of investigative behavior directed by (a) dominant females and (b) males towards glandular secretion only. Further details are given in Figure 2 legend.

scent marking behavior, and only two males licked the urine samples. There was no significant difference between the concentration of urine samples from the two donor groups used in the bioassays (i.e., mean creatinine concentration $\pm$ SEM for urine donated by familiar dominant females was 1.13 $\pm$ 0.2 mg/ml versus 1.07 $\pm$ 0.1 mg/ml for samples from unfamiliar dominant females). Differential urine concentration, therefore, could not be implicated in any behavioral bias exhibited by the test animals.

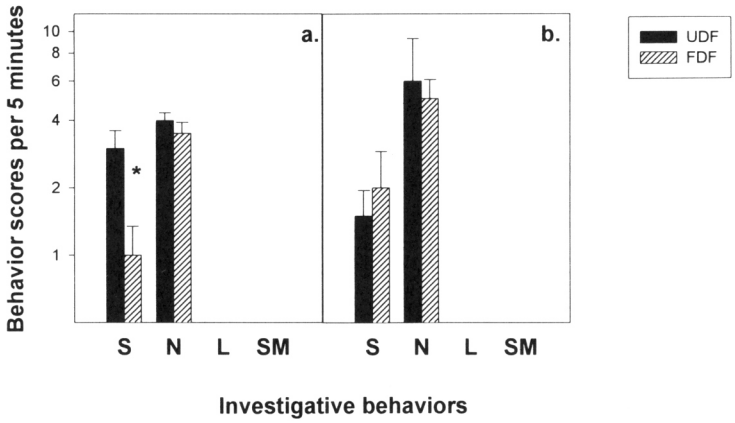


FIG. 6. Levels of investigative behavior directed by (a) males and (b) dominant females towards urine samples only. Further details are given in Figure 2 legend.

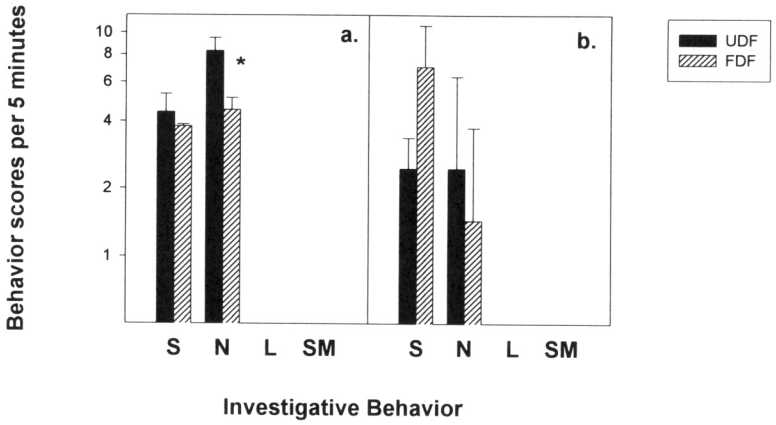


FIG. 7. Levels of investigative behavior directed by (a) dominant females and (b) males towards 1-day-old scent marks. Further details are given in Figure 2 legend.

One-Day-Old Scent Marks (Experiment 6). One day after deposition, scent marks still retained chemical cues permitting discrimination of familiar and unfamiliar females. Dominant females spent significantly longer nuzzling 1-day-old scent marks from unfamiliar females than scent marks of equivalent age deposited by themselves (dominant female test animals: UDF = 8.3 $\pm$ 1.2, self = 4.5 $\pm$ 0.6, $z = 2.4$, $P < 0.05$; Figure 7b). Test females did not exhibit similar bias in terms of sniffing, licking or scent marking behavior. Males, however, did not behaviorally differentiate between 1-day-old scent marks deposited by familiar versus unfamiliar dominant females.

DISCUSSION

The present study demonstrated that male marmosets, together with dominant and subordinate female marmosets, directed differential displays of behavior towards scent deposited by the dominant female of their own social group (familiar odor) versus a dominant female from another social group (unfamiliar odor). When animals behaviorally differentiated between scents from familiar versus unfamiliar animals, they did so by preferentially directing higher levels of investigative behaviors to scent from unfamiliar conspecifics. Chemical cues eliciting these differential displays of behavior were contained in freshly deposited, unmanipulated circumgenital scent marks, 1-day-old circumgenital scent marks, urine alone, and glandular secretion alone. Furthermore, these differential displays of behavior were still observed when animals were unable to directly contact the scent mark but were instead only presented with the volatile,

airborne chemicals in the complete scent mark. Cues enabling discrimination between familiar versus unfamiliar scent marks could, in addition, be extracted into organic solvent.

The clear discriminatory behavior exhibited by test marmosets towards scent from familiar versus unfamiliar dominant females suggests that the scent stimuli presented from the individual scent donors were olfactorally distinct, that test animals were generally motivated to investigate the scent marks presented, and that the odor communicated important differences (Johnston et al., 1993). On the other hand, when subjects failed to direct significantly greater amounts of investigatory behaviors to one particular scent, this did not imply that the two scent stimuli had indistinguishable odors. For example, female but not male marmosets demonstrated discriminatory behavior towards scent marks deposited by familiar versus unfamiliar dominant females when the scent marks were 1 day old. The lack of discriminatory behavior displayed by males could not be the result of the two scent stimuli having indistinguishable odors since the discriminatory behavior demonstrated by test females towards these two scents clearly implied that the odors were distinct. In addition, the total lack of discriminatory behavior in the current study certainly did not imply that the marmosets were unable to perceive differences between such odors or were unable to discriminate between them (e.g., see Taylor and Dewsbury, 1990). A lack of discrimination between two scents may have occurred because animals were not motivated to discriminate or because the test animals might not have perceived the odor differences as being important enough to warrant differential displays of investigative behavior. The controlled experimental context under which the animals encountered the scents may also have rendered irrelevant odor differences that would be important under more natural conditions.

Two-choice behavioral discrimination tests were used in this study as experimental tools to determine whether marmosets were able to discriminate between scents deposited by familiar versus unfamiliar conspecifics. This methodology resembles that of habituation testing paradigms, since comparisons are being made between the amounts of investigative behavior directed to familiar versus novel scents (Johnston, 1993). In habituation tests, consistent differential displays of behavior towards the familiar scent or the novel scent is taken as evidence that the two scents are olfactorally distinct (e.g., Mertle, 1975; Epple et al., 1988; Johnston, 1993; Johnston and Jerigan, 1994). Similarly, the present experiment compared the behavioral responses of test animals to familiar versus novel scent. In the current experiment, however, the familiar scent was previously known to the test animals since it was the scent of a fellow group member with whom the test animal had frequent physical contact and social experience. Test animals might, therefore, have associated the familiar scent with significant experiential and social referents. In habituation paradigms, test animals would

typically not have had previous exposure to either of the scents or the scent donors prior to the experiment, and therefore neither scent should be associated with social or experiential referents.

By habituating animals to the scent from one individual (= familiar scent), and then observing increased levels of investigatory behavior to a scent from a second individual (= novel scent), several studies have utilized habituation paradigms to demonstrate the existence of individual odors (Epple et al., 1988; Johnston, 1993; Tang-Martinez et al., 1993; Johnston and Jerigan, 1994, but see Halpin, 1986). In the same way that the ability of animals to discriminate between a novel and familiar scent from two individuals in habituation tests provides evidence that the two odors are distinct, the demonstrated ability of test animals in the present experiment to spontaneously discriminate between odors from a familiar versus a novel individual would suggest that the odors from the two individual scent donors were olfactorally distinct. We suggest, therefore, that the present experiment provides indirect evidence for the existence of individual odors in common marmosets.

Epple (1973) utilized yet another variation on the habituation paradigm to demonstrate the existence of individual odors in saddleback tamarins, a closely related species to the common marmoset. In the latter study, pairs of tamarins encountered an unfamiliar animal for 10-min periods, during which aggressive interactions occurred between the trio. Following the encounter, the resident pairs were presented concurrently with the odor from the recent opponent and a familiar animal with whom the test animals had had a previous (aggressive) encounter a longer time ago. Test animals directed significantly increased amounts of investigatory behavior to the scent of the recent opponent. On the basis of these results, it was proposed that saddleback tamarins have individual odors and that animals are able to discriminate between these odor signatures. The familiar scent presented to test animals in the experimental paradigm used by Epple (1973) would, as in the present study, have had significant social meaning attached to it.

In the present study test animals directed significantly increased amounts of investigative behaviors to scent deposited by unfamiliar dominant females, compared to familiar dominant females. Similarly, we have shown previously that male and female common marmosets exhibit differential displays of behavior towards scent from familiar versus unfamiliar dominant male marmosets, in all cases directing increased levels of investigative behavior towards scent from the unfamiliar male (Smith, 1994). In a series of single-scent presentation tests, tufted capuchin monkeys (*Cebus apella*) directed increased levels of investigation towards a bar marked with scent from conspecifics of a different social group compared to a bar marked by members of their own social group (Ueno, 1994). Increased amounts of investigative behavior towards scent from nongroup

members versus scent from resident group members has also been demonstrated for two species of lemur, *Lemur fulvus* and *L. macaco* (Fornasieri and Roeder, 1992). In addition, male and female ring-tailed lemurs, *L. catta*, directed increased amounts of investigatory and marking behavior towards labial marks (females) and antebrachial secretion (males) from unfamiliar same-sex conspecifics compared to their own marks (Dugmore and Evans, 1990; Ramsay and Giller, 1996). Heightened investigation of unfamiliar scents might enable the animal investigating the scent to obtain important information on the unfamiliar scent donor, such as their sex, hormonal condition, and social status. If the investigating animal has the ability to associate the unfamiliar scent with the donor of the unfamiliar scent, then prior knowledge of the scent donor's sex, hormonal, and social status might facilitate appropriate behavioral interactions when the animal investigating the scent and the unfamiliar scent donor encounter each other.

It was not surprising that marmosets were able to discriminate familiar versus unfamiliar animals on the basis of volatile, airborne cues alone, since chemical cues of high volatility communicate important information in scent mixtures from a large number of species (e.g., reviews by Albone, 1984; Novotny et al., 1990; Epple et al., 1993). Furthermore, cotton-top tamarins and saddleback tamarins, closely related species to common marmosets, were able to discriminate odors from conspecifics versus other callitrichid species on the basis of volatile airborne cues alone. In the case of cotton-top tamarins, however, only four subjects from a group of 12 subjects displayed discriminatory behaviors to volatile cues (Belcher et al., 1988; Epple et al., 1993).

While the present study has demonstrated that marmosets discriminate between odors from familiar versus unfamiliar dominant females, the results do not imply that marmosets recognize that the scent was deposited by an unfamiliar or familiar dominant female. The demonstrated ability of marmosets to discriminate between familiar versus unfamiliar dominant female odors might, however, serve important functions for this species. First, discrimination of familiar from unfamiliar dominant female odors may facilitate an animal's spatial orientation in the environment. Marmosets deposit high concentrations of scent marks at group resources such as sleeping sites, feeding trees, and travel routes (e.g., Sutcliffe and Poole, 1978; Lacher et al., 1981; Rylands, 1985, 1990), which, if animals are able to discriminate familiar odors, may help animals orientate to these important foci. Furthermore, marmosets often travel up to several kilometers a day in search of food (Stevenson and Rylands, 1988) and have been observed to deposit significantly high numbers of scent marks along the routes traversed (Rylands, 1990). Familiar scent marks on frequently traveled routes may facilitate group cohesion (Milton, 1975; Epple, 1986).

An additional function for the discrimination of familiar versus unfamiliar

dominant female odors might be to regulate group movements and therefore social interactions between neighboring groups. Marmosets scent mark predominantly within or at the periphery of the home territory as opposed to the territory of other groups (Rylands, 1990). By orientating towards the familiar scent from the resident dominant female, marmosets may remain in their home territory as opposed to that of another marmoset group and may regulate the frequency of intergroup encounters and disputes.

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SEX PHEROMONE STEREOCHEMISTRY AND PURITY AFFECT FIELD CATCHES OF MALE APHIDS

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Abstract—Male blackberry-cereal aphids, *Sitobion fragariae* (Walker), were caught in the field by water traps releasing synthetic (4a*S*, 7*S*, 7a*R*)-nepetalactone, the major sex pheromone component. The presence of the enantiomer (4a*R*,7*R*,7a*S*)-nepetalactone reduced catches and a plant-extracted (4a*S*,7*S*,7a*R*)-nepetalactone was less effective than the 99% (7*S*)-lactone. A more extensive trial with the bird cherry-oat aphid, *Rhopalosiphum padi*, involving the pheromone comprising the stereochemically related (4a*S*,7*S*,7a*R*)-nepetalactol showed a similar trend. The (7*R*)-isomer caught fewer males than the (7*S*)-isomer, but in this case the addition of high-purity (7*S*)-lactol to make a 50% blend caught as many males as pure (7*S*)-lactol. With plant-derived lactol, further purification did not significantly increase the catch. It is suggested that trace compounds associated with reduced enantiomeric purity in terms of the (7*S*)-configuration or from plant sources reduce activity of the sex pheromone components. Male damson-hop aphids, *Phorodon humuli* (Schrank), were also caught in the synthetic lactol traps, and it is suggested that this is due to traces of the (4a*R*,7*S*,7a*S*)-nepetalactols, which comprise the sex pheromone for this species. The significance for aphid chemical ecology studies and pest control strategies is discussed.

Key Words—Aphid, sex pheromone, stereochemistry, (4a*S*,7*S*,7a*R*)-nepetalactone, (4a*S*,7*S*,7a*R*)-nepetalactols.

INTRODUCTION

The existence of aphid sex pheromones has been known for many years, and in the early 1970s, Pettersson (1970, 1971) demonstrated that sexual female

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aphids (oviparae) of the genus *Schizaphis* Börner released a volatile pheromone capable of effecting behavioral changes in walking aphid males. Later studies by Marsh also demonstrated a sex pheromone for the vetch aphid, *Megoura viciae* Buckton (Marsh, 1972, 1975). It was shown that the pheromone was released from scent plaques on the hind tibiae of the females and was detected by the sensory cells of specific olfactory organs, the secondary rhinaria, on the third segment of male antennae. Preliminary behavioral studies suggested that males would respond only within a few centimeters of the calling females.

The sex pheromones for many aphid species, principally the pests in the subfamily Aphidinae, have been identified as one or both of the cyclopentanoid monoterpenoids (4a*S*,7*S*,7a*R*)-nepetalactone (**1**) and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**2**) (Dawson et al., 1987, 1996) (Figure 1). The ratio of the two components can differ between aphids species and between samples collected either from leg washings or by air entrainment (Dawson et al., 1990). The differences between the ratios and quantities observed (Dawson et al., 1990; Hardie et al., 1990) from tibial extracts and air entrainment suggests that the nepetalactol **2** may be continually produced from a glycoside precursor and that a proportion of this is sequentially oxidised to the nepetalactone **1** (Dawson et al., 1990). Male aphids can certainly discriminate blends in the laboratory and the field (Hardie et al., 1990, 1994).

In contrast to the other aphid species studied, oviparous females of the damson-hop aphid, *Phorodon humuli* (Schrank), produce neither **1** nor **2**, but a mixture of the two diastereoisomeric (4a*R*,7*S*,7a*S*)-nepetalactols, **3a** and **3b**. Although the exact ratio of components with regard to the stereochemistry at C-1 has not been fully determined for the sex pheromone, males are highly responsive to a 7:3 synthetic mixture of the 1*S*- and 1*R*-diastereoisomers (**3a**) and (**3b**), respectively (Campbell et al., 1990). Early attempts to attract flying aphid males to calling females were unsuccessful, both in field trials (Tamaki et al., 1970) and in the laboratory (Steffan, 1987). After identification, however, field studies using water traps releasing the synthetic **3a** and **3b** caught thousands of male *P. humuli* against only tens in the control traps. This was far more than conventional suction traps, which process large volumes of air (Campbell et al., 1990). Thus, by using synthetic material, it was demonstrated for the first time that aphid sex pheromones could be used to catch males in the field. Since then, field trials have confirmed this finding with other aphid species with other sex pheromones. For example, traps releasing synthetic nepetalactone **1** caught significantly more *S. fragariae* than control traps (Hardie et al., 1992) while nepetalactol **2** caught *Rhopalosiphum padi* (L.) (Hardie et al., 1994). These results indicate that the sex pheromones are capable of influencing aphid behavior and could perhaps be used for disrupting the mating process. Although it is unlikely that sex pheromones alone could be used to control aphid populations, they

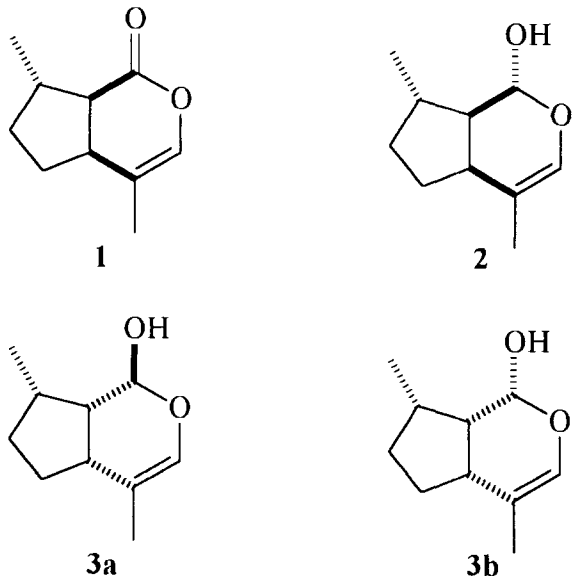


FIG. 1.

could be used as part of an integrated control strategy employing aphid pathogens (Pickett et al., 1994).

Materials employed for field studies were obtained both by synthesis and extraction from a plant source. Purity was determined by gas chromatography and by spectroscopic analysis. The optical purity can have a marked effect on the efficacy of these materials, as can the presence of minor impurities. The purpose of this study was to investigate the impact of such aspects on the trap catches of male aphids in the field.

METHODS AND MATERIALS

Chemicals. Starting from citronellol with varying enantiomeric purities with respect to the *S* isomer of 99%, 95%, racemic citronellol (i.e., comprising 50% each of (*S*)- and (*R*)-citronellol), and 50%, and also 98% (*R*)-citronellol, compounds **1** and **2** were synthesized by a route modified from Schreiber et al., (1986) and described fully elsewhere (Dawson et al., 1996). The nepetalactone **1** was also obtained from *Nepeta cataria* (Lamiaceae = Labiatae), with DIBAL-H reduction giving the corresponding nepetalactol **2** (Dawson et al., 1987).

Field Trials. Four sites were chosen in parkland at Imperial College, Silwood Park, Berkshire, England. The experimental design was a randomized

block, with each site treated as a block, and each treatment was represented twice within each block. Water traps were constructed from clear plastic Petri dishes (14 cm diameter × 2 cm deep), and mounted 1.1 m above the ground and 2 m apart. Pheromones in Et_2O (50 μl) were placed into glass vials (08-CPV Chromacol) from which the pheromone was released through a 1-mm hole drilled in the plastic cap of the vial. Control vials were prepared using only Et_2O. Dilute nonionic detergent solution was placed in the trap, and the pheromone vial was suspended centrally from a wire loop, so that the plastic cap of the lure was 6 cm above the Petri dish. Traps were emptied three times per week, after which the trap position was randomly reallocated. Pheromone vials were replaced every two weeks.

Two field trials were carried out: one running for 29 days in 1994, for *S. fragariae*, and one running over 42 days in 1995, for *R. padi*. These aphid species were chosen for trapping because the sex pheromones for each essentially comprise single components, thereby avoiding the complication of releasing specific blends.

Data Analysis. The data were analyzed using GLIM and, as the response variable consisted of count data, Poisson errors were declared. Overdispersion in the data was corrected by declaring an empirical scale parameter (Crawley, 1993). Where there was a significant treatment effect *a posteriori*, comparisons were used to compare individual treatment means. With the larger 1995 trial, not only was it possible to compare between the treatments, but the treatment structure also enabled the interaction between the (7*R*)- and (7*S*)-lactols for *R. padi* catches to be assessed. Each isomer was considered as a factor at two levels, present and absent, and for this the 99% (7*S*)-lactol, 98% (7*R*)-lactol, 1:1 (7*S*)/(7*R*)-lactol, and the blank data were analyzed.

RESULTS AND DISCUSSION

Field Trial with S. fragariae, *1994.* Analysis of the *S. fragariae* catch showed a significant effect of treatment ($P < 0.001$, deviance 77.23, 4 *df*; Table 1). The lactone prepared from 99% (*S*)-citronellol gave the highest trap catch, while the plant-derived lactone and the 95% (7*S*)-lactone had statistically similar activity and substantially lower than from that of the 99% (7*S*)-lactone. The racemic (7*S*)-lactone showed about one third the activity of the 99% (7*S*)-lactone, but caught significantly more than the control. It was surprising that the plant-derived lactone did not result in similar or even higher catches than the 99% (7*S*)-lactone, since the enantiomeric purity at C-7 is high from the plant (Eisenbraun et al., 1980). However, the plant-derived lactone, from the essential oil of the catmint plant, *N. cataria*, was purified by column chromatography using a steep elution gradient to preserve yield. This may have allowed

TABLE 1. TOTAL NUMBERS OF *S. fragariae* ALATE MALES CAUGHT IN WATER TRAPS RELEASING SYNTHETIC AND PLANT-DERIVED (4*aS*,7*S*,7*aR*)-NEPETALACTONE (**1**) IN 1994

Source of nepetalactone	Males (*N*)[a]
Blank	12d
Racemic Citronellol	32c
95% (*S*)-Citronellol	59b
99% (*S*)-Citronellol	104a
Plant extract	72b

[a]The letter following the number indicates statistical significance with $P < 0.05$.

retention of traces of some other volatile materials. Aphids may not respond to sex pheromone components blended with plant volatiles, thereby allowing them to avoid inappropriate interactions with plants coincidentally containing such pheromone components. Such a mechanism has been encountered before in aphid chemical ecology, where other plant sesquiterpenes interfere with the activity of coincidental production of the alarm pheromone by plants (Dawson et al., 1984). Thus, these other volatile materials may have had some masking or repellency effect on the attractiveness of the sex pheromone, thereby explaining the diminished response to the plant-derived lactone in comparison to synthetically pure materials. Further studies are planned to identify these antagonistic components.

Field Trial with R. padi, *1995.* This field trial was on a larger scale than for 1994 so as to incorporate several new lines of investigation. The (7*R*)-lactone was not available for testing with *S. fragariae*, and it was difficult to assess whether treatments that contained increasing quantities of the (7*R*)-lactone [i.e., from 99% (*S*) to the racemate] showed diminution in activity because of an effect arising from the (7*R*)-stereochemistry, or because there was simply less (7*S*)-lactone [i.e., for the racemic lactone sample, only 5 mg of total 10 mg in the lure has the (7*S*)-configuration]. Therefore, to assess the effect on *R. padi*, the (7*R*)-lactol was tested alone, as a blend of 50% (7*S*)- and (7*R*)-lactols (5 mg of each of the pure (7*S*)- and (7*R*)-enantiomers mixed in a single vial to afford a racemic mixture), and in paired vials, each containing either 10 mg of the (7*S*)- or (7*R*)-lactols. The results of these treatments could then be compared with that of the racemic lactol prepared from racemic citronellol. Furthermore, to investigate the suggested masking or repellency of other volatile materials contaminating the plant-derived pheromone sample, the nepetalactone **1** was purified by column chromatography, using a gradual elution gradient, to obtain

a highly purified lactone extract. This purified lactone and the standard plant lactone were treated with DIBAL-H to produce the required lactols. These were tested to determine whether the cleaner plant lactol caught more *R. padi* males.

Initial analysis of the separate treatments on the catch of *R. padi* was highly significant ($P < 0.001$, deviance 85.74, 8 *df*; Table 2). In trap catches of *R. padi*, all treatments that contained some synthetic (7*S*)-lactol caught significantly more males than the other treatments, but were not significantly different from one another. This was in contrast to that observed for *S. fragariae*, where significant reduction in trap catch occurred with decreasing relative quantities of the (7*S*)-lactone. Although there was a general downward trend in trap catches from 95/99% (7*S*) to 50% (7*S*) with *R. padi*, the differences were not significant. The purified plant-derived lactol caught significantly fewer males than 95/99% (7*S*)-lactols, but did not catch more males than the 50% (7*S*)-lactols or the standard plant-derived lactol. The standard plant-derived lactol caught more males than the control vials but fewer males than the synthetic lactols, again suggesting other volatile materials might be lowering the attractiveness. The (7*R*)-lactol caught more males than the plant products but fewer than the 95/99% (7*S*)-lactols.

The interaction between the (7*R*)- and (7*S*)-lactols was significant ($P < 0.01$, deviance 25.86, 1 *df*). Thus, the response to either the (7*R*)- or the (7*S*)-

TABLE 2. TOTAL NUMBERS OF *R. padi* ALATE MALES CAUGHT IN WATER TRAPS RELEASING SYNTHETIC AND PLANT-DERIVED (1*RS*,4*aS*,7*S*,7*aR*)-NEPETALACTOL (**2**) IN 1995

Source of nepetalactol	Males (*N*)[a]
Blank	6e
Racemic Citronellol	75abc
50% (*S*) + 50% (*R*) racemic blend[b]	84abc
95% (*S*)-Citronellol	115a
99% (*S*)-Citronellol	106a
98% (*R*)-Citronellol	60bc
1:1 99% (*S*)/98% (*R*) citronellols[c]	94ab
Standard plant extract	38d
Purified plant extract	50cd

[a]The letter following the number indicates statistical significance with $P < 0.05$.
[b]A racemic blend was prepared by mixing 5 mg of pure 99% (7*S*)- and 5 mg of 98% (7*R*)-lactols to obtain 10 mg of a racemic mixture, to compare with racemic (7*S*)-lactol obtained from racemic citronellol.
[c]One vial with 10 mg of 99% (7*S*)-lactol and one vial with 10 mg of 98% (7*R*)-lactol.

TABLE 3. TOTAL NUMBERS OF *P. humuli* ALATE MALES CAUGHT IN WATER TRAPS RELEASING SYNTHETIC AND PLANT-DERIVED (1*RS*,4*aS*,7*S*,7*aR*)-NEPETALACTOL (**2**) IN 1995

Source of nepetalactol	Males (*N*)[a]
Blank	10c
Racemic-citronellol	30c
50% (*S*) + 50% (*R*) racemic blend[b]	82ab
95% (*S*)-Citronellol	97a
99% (*S*)-Citronellol	103a
98% (*R*)-Citronellol	27c
1:1 99% (*S*)-98% (*R*)-citronellols[c]	59b
Standard plant extract	8c
Purified plant extract	14c

[a]The letter following the number indicates statistical significance with $P < 0.05$.
[b]A racemic blend was prepared by mixing 5 mg of pure 99% (7*S*)- and 5 mg of pure 98% (7*R*)-lactols to obtain 10 mg of a racemic mixture, to compare with racemic (7*S*)-lactol obtained from racemic citronellol.
[c]One vial with 10 mg of 99% (7*S*)-lactol and one vial with 10 mg of 98% (7*R*)-lactol.

lactol was influenced by the presence or absence of the other, and the response to the (7*S*)-lactol was much reduced in the presence of the (7*R*)-lactol.

The field traps releasing lactol also yielded significant numbers of male *P. humuli* [see Table 3, treatments as per Table 2]. This was unexpected since the pheromone for *P. humuli* comprises only the lactols **3a** and **3b**. Statistical analysis showed that the plant-derived lactols caught no more males than the control, nor did the (7*R*)-lactol. When citronellol used for the synthesis of **2** contains any (*S*)-citronellol, small quantities [6–8% of the total (7*S*)-lactol produced] of the diastereoisomers **3a** and **3b** are also produced, and the presence of these by-products appears to be sufficient to attract significantly more *P. humuli* males than the other treatments.

This extensive field study provides valuable information for chemical synthesis of aphid sex pheromone components appropriate for maximizing the aphid catch. However, this study also indicates the need for further investigation of plant-derived products if these are to be developed as the basis for aphid control in the longer term (Hick et al., 1996).

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FIELD DISCRIMINATION OF OILSEED RAPE, *Brassica napus* VOLATILES BY CABBAGE SEED WEEVIL, *Ceutorhynchus assimilis*

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Abstract—The response of the cabbage seed weevil, *Ceutorhynchus assimilis* to yellow water traps baited with some components of oilseed rape (*Brassica napus*) odor was studied in a series of field experiments. Four isothiocyanates (NCS), five other amino acid derivatives (aromatic compounds), and two fatty acid derivatives are detected by peripheral olfactory receptors of *C. assimilis*. 3-Butenyl, 4-pentenyl, and 2-phenylethyl NCS were attractive when released individually during the period when weevils were immigrating to the host crop, but allyl NCS was not. A mixture of these four isothiocyanates was attractive during the periods of weevil immigration and dispersal. However, during the host colonization phase, 2-phenylethyl NCS, like the mixture of the four isothiocyanates, elicited no response or was repellent. Phenylacetonitrile and benzyl alcohol were attractive during the colonization phase. The fatty acid derivative (*Z*)-3-hexen-1-ol did not affect weevil catch in an experiment conducted when the insects were already established in the crop, but it depressed the catch in another conducted when weevils were dispersing from the crop. These results suggest that yellow traps baited with 2-phenylethyl NCS can be used to monitor immigration into crops in the spring and that phenylacetonitrile may be useful for assessing numbers of weevils colonizing the crop throughout the summer.

Key Words—*Ceutorhynchus assimilis*, cabbage seed weevil, Coleoptera, Curculionidae, plant kairomones, isothiocyanates, phenylacetonitrile, volatiles, insect-plant, trap, semiochemicals.

INTRODUCTION

The cabbage seed weevil, *Ceutorhynchus assimilis* (Paykull) (Syn: *C. obstrictus*, Marsham) (Coleoptera: Curculionidae) is a pest of oilseed rape, *Brassica*

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napus L., in Europe (Bonnemaison, 1965; Winfield, 1992) and the Pacific Northwest, USA (Hill, 1991). After overwintering in soil or leaf litter, adult weevils migrate in the spring to brassicaceous (= cruciferous) host plants, which they colonize throughout the summer. The female lays her eggs, usually singly, in young pods, and the larvae feed on the developing seeds. Mature larvae leave the pods and pupate in the soil. The new adults emerge in the late summer and feed for a short period before dispersing to overwintering sites (Bonnemaison, 1957).

Most phytophagous insects respond to a combination of chemical (olfactory and gustatory) and visual stimuli during the host-finding and acceptance process (Miller and Strickler, 1984; Prokopy, 1986; Bernays and Chapman, 1994). Olfactory stimuli from plants are particularly important as orientation cues (Visser, 1986) and may operate alone at various distances from the plant or act additively or synergistically with visual cues (Judd and Borden, 1991; Aluja and Prokopy, 1993). Thus *C. assimilis*, which is oligophagus on Brassicaceae (Doucette, 1947; Dmoch, 1965), has been shown to be attracted by the odor and color of brassicaceous plants (Görnitz, 1953; Evans and Allen-Williams, 1989, 1993). In field cage experiments weevils flew upwind within the odor plume of a host crop (Kjaer-Pedersen, 1992). In further field experiments, traps baited with extracts of *B. napus* were located by *C. assimilis* from distances of at least 20 m (Evans and Allen-Williams, 1993). Walking weevils also oriented to the odors of oilseed rape flowers and their extracts in olfactometer studies (Evans and Allen-Williams, 1989; Bartlet et al., 1993).

Plant odors contain complex mixtures of volatile compounds, some of which, e.g., the green leaf volatiles (Visser et al., 1979; Buttery and Ling, 1984; Flath et al., 1985), are common to many families and species of plant. Others are associated with a particular plant family or groups of families, e.g., isothiocyanates, which are characteristic of the Brassicaceae and a few species in some other families (Fenwick et al., 1983). It appears that most insects do not use the total volatile chemical signal from a host plant as an orientation cue, but instead use a subset of the volatiles (Hopkins and Young, 1990; Roseland et al., 1992). Some phytophagous insects, e.g., the onion fly, *Delia antiqua* (Vernon et al., 1981), corn rootworms, *Diabrotica* spp. (Andersen and Metcalf, 1986; Lampman et al., 1987), *C. assimilis* (see below), and *Meligethes* spp. (Smart and Blight, in preparation) are attracted by individual components of the odor of a plant.

Electroantennogram (EAG) (Kozlowski, 1984; Evans and Allen-Williams, 1992; Blight et al., 1992, 1995) and single-cell recording (SCR) techniques (Blight et al., 1989, 1995) have been used to detect compounds in the odors of brassicaceous plants that stimulate the peripheral olfactory sensilla of *C. assimilis* and thus could affect the insect's behavior. At a physiologically discrim-

inating level, the antenna of the seed weevil detects at least 25 of the more than 50 volatile compounds released by flowering oilseed rape (Blight et al., 1992, 1995). Most of the compounds, which comprise isothiocyanates (NCS), other amino acid derivatives (aromatic compounds), fatty acid derivatives and isoprenoids, are perceived by specialist antennal cells. A high proportion of the cells recorded in one study (Blight et al., 1989) responded specifically to the isothiocyanates, suggesting that these compounds may be particularly important as cues for host-plant location. This premise was supported by the results of field and laboratory olfactometer experiments. Migrating seed weevils were attracted to yellow sticky or water traps baited with a mixture of 3-butenyl, 4-pentenyl, 2-phenylethyl, and allyl NCS (Blight et al., 1992; Smart et al., 1993, 1997), and walking weevils responded in a linear track olfactometer to a mixture of 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS (Bartlet et al., 1993). However, it is very likely that many of the other compounds detected by the antenna are also involved in orientation to *B. napus*, particularly since modern cultivars of oilseed rape contain only trace amounts of glucosinolates, the thioglucosides from which isothiocyanates are derived by catabolism.

In this paper we describe the results of five field experiments in which we examined the response of *C. assimilis* to yellow water traps baited individually with 11 synthetic compounds found in oilseed rape odor. The objective was to assess the effects of lures of single compounds on the seed weevil during the migratory or host-plant colonization phases. Traps baited with attractive compounds might be useful for detecting and monitoring populations of *C. assimilis*, whereas repellent compounds could be used in push–pull pest management strategies (Smart et al., 1994).

METHODS AND MATERIALS

Experimental Design and Data Analysis. The response of *C. assimilis* to 11 compounds was studied in five field experiments (A–E), conducted on Rothamsted Farm, Harpenden, during 1993–1995.

Each experiment comprised a randomized block (Latin square) design as used in previous experiments (Smart et al., 1997). Traps representing one replicate (i.e., row) of a block were set out in a straight line at 10 m spacing. Where feasible, blocks were rerandomized when a mean of at least five weevils per treatment had been captured in a replicate.

An unbaited (control) trap and a multi-isothiocyanate-baited trap were included as two of the six treatments in each experiment (see Results).

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. Where appropriate, trans-

formed treatment means were compared at $P = 0.05$, using Duncan's multiple-range test (Duncan, 1955). Means were then transformed back, and these are shown in the Results (Tables 1–5 below).

Traps. All experiments used water bowl traps that were brush painted canary yellow (ICI autocolour BS381/cP383, BS0409), and two-thirds filled with detergent solution (0.5%). They have been described previously (Smart et al., 1997). Lures were wired to a clear plastic "cross bar" secured across the top of the bowl, and the traps were placed on wooden platforms that were mounted on poles, ca. 1 m above ground level.

Chemicals. All chemicals were obtained from commercial sources (Aldrich Chemical Co., Fluka Chemie AG, Lancaster Synthesis, Avocado Research Chemicals), except for 3-butenyl isothiocyanate (NCS) and 4-pentenyl NCS, which were synthesized (Dawson et al., 1993). All the compounds were ≥98% pure, except for 4-pentenyl NCS (97%) and allyl NCS (94%).

Lures. Each compound was released individually, by diffusion, from a polyethylene bag. The chemical was applied to a piece of cellulose sponge (3 mm thick, code 0032 6865, or 10 mm thick, code 0013 4811, J. Sainsbury plc), which was then heat-sealed into a bag made from polyethylene tubing (A1 Packagings Ltd., London). Different release rates were obtained by altering the type and surface area of the sponge and the gauge of the polyethylene.

RESULTS

Experiment A (Table 1). This experiment, conducted in the spring, consisted of four blocks. Three blocks were on overwintering sites where oilseed rape had been grown in the previous year, and the fourth was adjacent to flowering oilseed rape. The capture of *C. assimilis* in traps baited individually with 2-phenylethyl, 3-butenyl, 4-pentenyl, and allyl NCS, and the mixture of all four isothiocyanates, was studied. There were no site differences, so the data from all four blocks were combined in one ANOVA (Table 1).

All the isothiocyanate treatments, apart from allyl NCS, caught significantly more weevils than the unbaited trap. There were no significant differences between them (Duncan's multiple range test, $P = 0.05$). The allyl NCS treatment caught significantly fewer weevils than the combined isothiocyanate treatment but was not significantly different from the other single isothiocyanate treatments.

Experiment B (Table 2). This was done in the summer adjacent to flowering oilseed rape that was already colonized by weevils. The response to 2-phenylethyl NCS was compared to the responses to 2-phenylethanol, phenylacetonitrile, methyl salicylate, and the mixture of all four substances.

Phenylacetonitrile attracted significant numbers of weevils, but the other

TABLE 1. EXPERIMENT A: RESPONSE OF *Ceutorhynchus assimilis* TO YELLOW WATER BOWL TRAPS BAITED WITH ISOTHIOCYANATES (NCS), APRIL 12–MAY 11, 1993[a]

Compound	Release rate (mg/day)	*C. assimilis* caught per replicate [*N* (range)]
Unbaited		0.7 c (0–15)
2-Phenylethyl NCS	5	1.6 ab (0–29)
3-Butenyl NCS	5	2.0 ab (0–35)
4-Pentenyl NCS	5	1.6 ab (0–16)
Allyl NCS	30	1.3 bc (0–15)
NCS multibait[b]	(see below)	2.2 a (0–34)

[a]In all, 568 weevils were caught in four blocks (24 replicates) on four different sites. ANOVA showed there were no site differences. Combined ANOVA gave $F = 5.27$; $df = 5,80$; $P < 0.001$. Means followed by different letters are significantly different (Duncan's multiple-range test, $P = 0.05$). SE of a treatment mean was 0.04.
[b]Combination of the four NCS. Each NCS was released separately at the rate shown above.

TABLE 2. EXPERIMENT B: RESPONSE OF *Ceutorhynchus assimilis* TO YELLOW WATER BOWL TRAPS BAITED WITH AROMATIC COMPOUNDS, JUNE 1–27, 1994[a]

Compound	Release rate (mg/day)	*C. assimilis* caught per replicate [*N* (range)]
Unbaited		20.0 b (9–41)
2-Phenylethanol	6	20.9 b (9–63)
2-Phenylethyl NCS	5	13.8 b (6–35)
Phenylacetonitrile	7	40.3 a (20–74)
Methyl salicylate	35	12.6 b (6–40)
Multibait[b]	(see below)	15.9 b (4–28)

[a]In all, 870 weevils were caught in six replicates. ANOVA gave $F = 5.66$; $df = 5,20$; $P = 0.002$. Means followed by different letters are significantly different (Duncan's multiple-range test, $P = 0.05$). SE of a treatment mean was 0.07.
[b]Combination of the compounds used in the other treatments. Compounds were released separately at the rates used in the other treatments.

treatments, including the mixture, were not significantly different from the unbaited trap.

Experiment C (Table 3). This experiment was carried out in the late summer on a site where oilseed rape had recently been harvested, and from which newly emerged weevils were dispersing. The response to a mixture of 3-butenyl,

TABLE 3. EXPERIMENT C: RESPONSE OF *Ceutorhynchus assimilis* TO YELLOW WATER BOWL TRAPS BAITED WITH (Z)-3-HEXEN-1-OL, BENZALDEHYDE, OR A MIXTURE OF ISOTHIOCYANATES (NCS), AUGUST 9–26, 1994[a]

Compound	Release rate (mg/day)	*C. assimilis* caught per replicate [*N* (range)]
Unbaited		3.1 b (0–11)
(Z)-3-Hexen-1-ol	4	3.2 b (0–9)
(Z)-3-Hexen-1-ol	13	1.6 c (0–5)
Benzaldehyde	2	2.5 bc (0–9)
Benzaldehyde	30	3.7 b (0–8)
NCS[b]	(see below)	12.5 a (2–28)

[a]In all, 210 weevils were caught in six replicates. ANOVA gave $F = 17.0$; $df = 5,19$; $P < 0.001$. Means followed by different letters are significantly different (Duncan's multiple-range test, $P = 0.05$). SE of a treatment mean was 0.06.

[b]The lure released allyl NCS (30 mg/day), 3-butenyl NCS (5 mg/day), 4-pentenyl NCS (5 mg/day), and 2-phenylethyl NCS (5 mg/day).

4-pentenyl, 2-phenylethyl, and allyl NCS was compared to responses to (Z)-3-hexen-1-ol and benzaldehyde. Each of the latter two compounds was released at two different rates.

Only the trap baited with the mixture of isothiocyanates attracted significantly more weevils than the unbaited trap. Neither of the benzaldehyde treatments nor the lower release of (Z)-3-hexen-1-ol (4 mg per day) had an effect on weevil capture. However, (Z)-3-hexen-1-ol released at 13 mg/day reduced the catch significantly, compared to the unbaited treatment.

Experiment D (Table 4). This experiment was done in the spring on a site similar to those used for experiments A and B. The response to 2-phenylethyl NCS was compared to the responses to (Z)-3-hexen-1-ol and phenylacetonitrile. Each of the latter compounds was released at two different rates. Only the higher release of phenylacetonitrile (26 mg/day) caught significantly more weevils than the unbaited trap. None of the other treatments differed significantly from the unbaited.

Experiment E (Table 5). This experiment was conducted in the summer adjacent to oilseed rape already colonized by weevils. The response to 2-phenylethyl NCS was compared to the responses to two release rates of benzyl alcohol and of hexan-1-ol. Benzyl alcohol, released at 2 mg/day, attracted significantly more weevils than the unbaited treatment, but the higher release (12 mg/day) of this compound did not affect trap capture. Neither hexan-1-ol treatment was significantly different from the unbaited treatment. The 2-phenylethyl NCS treatment depressed the catch compared to the unbaited.

TABLE 4. EXPERIMENT D: RESPONSE OF *Ceutorhynchus assimilis* TO YELLOW WATER BOWL TRAPS BAITED WITH (Z)-3-HEXEN-1-OL, PHENYLACETONITRILE, OR 2-PHENYLETHYL ISOTHIOCYANATE (NCS), APRIL 10–MAY 3, 1995[a]

Compound	Release rate (mg/day)	*C. assimilis* caught per replicate [*N* (range)]
Unbaited		1.8 bc (0–23)
(Z)-3-Hexen-1-ol	4	2.3 abc (0–61)
(Z)-3-Hexen-1-ol	13	1.4 c (0–51)
Phenylacetonitrile	7	3.0 ab (0–91)
Phenylacetonitrile	26	4.0 a (0–228)
2-Phenylethyl NCS	5	2.1 bc (0–20)

[a]In all, 748 weevils were caught in two blocks (12 replicates). ANOVA gave $F = 3.26$; $df = 5,40$; $P = 0.02$. Means followed by different letters are significantly different (Duncan's multiple-range test, $P = 0.05$). SE of a treatment mean was 0.06.

TABLE 5. EXPERIMENT E: RESPONSE OF *Ceutorhynchus assimilis* TO YELLOW WATER BOWL TRAPS BAITED WITH BENZYL ALCOHOL, HEXAN-1-OL, OR 2-PHENYLETHYL ISOTHIOCYANATE (NCS), MAY 24–JUNE 30, 1995[a]

Compound	Release rate (mg/day)	*C. assimilis* caught per replicate [*N* (range)]
Unbaited		8.2 bc (0–39)
Benzyl alcohol	2	12.4 a (1–55)
Benzyl alcohol	12	9.8 ab (1–36)
Hexan-1-ol	3	8.9 abc (0–50)
Hexan-1-ol	13	6.4 cd (0–25)
2-Phenylethyl NCS	5	5.0 d (0–22)

[a]In all, 932 weevils were caught in two blocks (12 replicates). ANOVA gave $F = 5.84$; $df = 5,39$; $P < 0.001$. Means followed by different letters are significantly different (Duncan's multiple-range test, $P = 0.05$). SE of a treatment mean was 0.07.

DISCUSSION

The experiments reported here were part of a series carried out from 1991 to 1996 to examine the response of *C. assimilis* in the field to approximately 20 plant volatile compounds known to be perceived by the antenna. However, during this period, populations of *C. assimilis* in the UK dropped from a peak

in 1990 to a very low level (Lane and Walters, 1994), and further results are dependent on population resurgence. Nevertheless, these results are relevant to the development of a baited trap for monitoring the seed weevil, which, in Europe, is an unpredictable pest.

Previous experiments (Smart et al., 1997) showed that *C. assimilis* is attracted to a mixture of 3-butenyl, 4-pentenyl, 2-phenylethyl, and allyl NCS during its period of dispersal to overwintering sites, but not when it is established in the crop in the summer. In the present work, response to the isothiocyanate mixture during the dispersive phase was confirmed (experiment C) and it was shown (experiment A) that the mixture also attracted weevils that were migrating to host plants in the spring.

3-Butenyl, 4-pentenyl, and 2-phenylethyl NCS, released individually at 5 mg/day, were also attractive during the spring migration phase (experiment A). Although not significantly different from these three, allyl NCS released at 30 mg/day, i.e., six times the rate used for the others, was not significantly attractive compared to an unbaited yellow trap, and it was significantly less attractive than the mixture of the four isothiocyanates. Therefore it probably does not contribute much to the activity of the mixture (this study; Smart et al., 1997) and is only very weakly attractive to seed weevils compared to the other isothiocyanates investigated. This was to be expected because antennal cells of *C. assimilis* only respond to allyl NCS at unnaturally high stimulus concentrations (Kozlowski, 1984, Blight et al., 1989, 1995). It is also in agreement with Finch's observations (Finch, 1977, and personal communication) that a very high release of allyl NCS (ca. 3 g/day) was attractive to *C. assimilis* during the spring migration. Many other insects that specialize on the Brassicaceae have been reported to respond to unspecified release rates of allyl NCS. Among those known to be attracted to smaller releases are *Phyllotreta* spp. flea beetles (ca. 4 mg/day; Pivnick et al., 1992), the diamond back moth, *Plutella xylostella* (ca. 55 mg/day; Pivnick et al., 1994), and the oilseed rape pest, the male pod midge, *Dasineura brassicae* (12–13 mg/day; Murchie et al., 1997). Perhaps surprisingly, the parasitoid of *D. brassicae*, *Platygaster subuliformis*, is not attracted to allyl NCS (Murchie et al., 1997).

Previous results (Smart et al., 1993, 1997) indicated that a mixture of 3-butenyl, 4-pentenyl, 2-phenylethyl, and allyl NCS could be used for monitoring movement of *C. assimilis*, but the data presented here suggest that there is no disadvantage to using only one of these compounds. Since 2-phenylethyl NCS is readily available commercially and is more stable than the alkenyl isothiocyanates, it is now being used in experimental monitoring trials and has successfully captured migrating weevils in spring (Smart et al., 1996).

Like the mixture of all four isothiocyanates (Smart et al., 1997), 2-phenylethyl NCS was unattractive (experiments C and E) when the weevils were colonizing host plants. Unexpectedly, it was also inactive in experiment D,

which was carried out from April 10 to May 3, 1995. In most years weevils would still have been immigrating to host plants at this time, but because of mild weather in the first part of 1995, they moved from overwintering sites unusually early. Thus most of the insects captured in experiment D were already colonizing plants.

There are differences in the olfactory perception of the alkenyl (3-butenyl and 4-pentenyl) isothiocyanates and 2-phenyl ethyl NCS (Blight et al., 1989), suggesting that they might have different behavioral effects. This is true of walking weevils, which were not attracted in a linear track olfactometer (Bartlet et al., 1993) to a range of concentrations of 2-phenylethyl NCS, but oriented to mixtures of 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS and to 3-butenyl and 4-pentenyl NCS, tested individually. The negative response to 2-phenylethyl NCS is in agreement with the field experiment results because the weevils used in the olfactometer had been kept in culture on oilseed rape. The field response of weevils already colonizing the crop to the individual release of 3-butenyl and 4-pentenyl NCS is being tested.

In contrast to the isothiocyanates, all the other seven volatile compounds tested are associated with many plant families. The amino acid derivatives, benzyl alcohol, methyl salicylate, benzaldehyde, and 2-phenylethanol, are common floral volatile compounds produced by more than 30 plant species (Knudsen et al., 1993), and they have also been identified as leaf volatiles. Phenylacetonitrile is less widely distributed but has been identified as a floral volatile from at least nine families (Tatsuka et al., 1990; Knudsen et al., 1993, Leal et al., 1994). It is a fruit volatile (Flath et al., 1990; Boevé et al., 1996) and has also been identified from leaf tissue (MacLeod et al., 1981; Loughrin et al., 1995). The two fatty acid derivatives (green leaf volatiles), (*Z*)-3-hexen-1-ol and hexan-1-ol, are very widely distributed (Visser et al., 1979).

Except for methyl salicylate and 2-phenylethanol, the compounds were tested at two different release rates because this factor can influence the type and magnitude of insect response (Lampman et al., 1987; Pivnick, 1993; Smart and Blight, unpublished). (Additional experiments were attempted with methyl salicylate and 2-phenylethanol, but too few weevils were captured to enable conclusions to be drawn). Three of the seven compounds, phenylacetonitrile, benzyl alcohol, and (*Z*)-3-hexen-1-ol, showed biological activity. In experiments conducted during the colonization phase, both phenylacetonitrile (experiments B and D) and benzyl alcohol (experiment E) attracted significant numbers of weevils compared to an unbaited trap. During the dispersive phase (*Z*)-3-hexen-1-ol lowered the catch compared to the unbaited trap (experiment C), but in experiment D, conducted during the host colonization period, it did not affect the trap catch. In a linear track olfactometer, it attracted walking weevils (Bartlet et al., 1997).

Of the other compounds tested, benzaldehyde had no effect on trap catch

when tested during the dispersive phase. Similarly 2-phenylethanol, methyl salicylate, and hexan-1-ol elicited no responses during the colonization phase. However, methyl salicylate attracted walking weevils in the linear track olfactometer (Bartlet et al., 1997) and hexan-1-ol has been reported previously to have a repellent effect on walking male and female *C. assimilis* in a four-way olfactometer (Evans and Allen-Williams, 1989). These compounds (see above) and (Z)-3-hexen-1-ol are therefore being field-tested at other release rates. Compounds exhibiting repellency could be useful in push–pull strategies for the management of *C. assimilis* populations (Smart et al., 1994).

To our knowledge, this is the first time that field attraction of the seed weevil to phenylacetonitrile and benzyl alcohol has been demonstrated, although walking weevils orient to phenylacetonitrile in the linear track olfactometer (Bartlet et al., 1997). Both phenylacetonitrile and benzyl alcohol have been identified previously as components of plant-produced insect kairomones. They are both constituents of the scarab beetle (*Anomala octiescostata*) attractant produced by dandelion (*Taraxacum officinale*) flowers (Leal et al., 1994). Phenylacetonitrile is a component of an aggregation kairomone for the Japanese beetle, *Popillia japonica* (Loughrin et al., 1995) and benzyl alcohol is implicated in the attraction of the blueberry maggot fruit fly, *Rhagoletis mendax*, to wild blueberry, *Vaccinium angustifolium* (Lugemwa et al., 1989).

In general we have found that when phytophagous insects are established on host plants, they cannot be attracted to semiochemical-baited traps, and we therefore did not expect phenylacetonitrile and benzyl alcohol to attract *C. assimilis* during the colonization phase. Further investigation of the activities of these substances is in progress. They may be useful for monitoring in-field weevil populations.

Although four of the seven compounds did not affect trap capture when tested alone, it remains likely that they contribute to the attraction of *C. assimilis* to oilseed rape odor. It has been suggested (see Loughrin et al., 1996, and references therein) that specialized phytophagous insects, like *C. assimilis*, respond to specific ratios of host-plant volatiles while generalists, e.g., the Japanese beetle, *Popillia japonica*, respond to a range of individual compounds and nonspecific blends. Therefore it is surprising that *C. assimilis* responds to individual host volatile components that are widely distributed across a variety of plant species. However, relatively large quantities of single compounds must be released in order to affect *C. assimilis* behavior, and one would expect that the weevil will be more responsive to a mixture of volatiles released in the proportions in which they are present in oilseed rape. When a mixture of 23 compounds known to stimulate the *C. assimilis* antenna was tested in the linear track olfactometer (Bartlet et al., 1997), a high proportion (60%) of the weevils oriented to it, but it was not significantly attractive in a conservative statistical

analysis. Fieldwork with further combinations of oilseed rape volatiles is in progress.

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SPECIES-SPECIFIC RESPONSE OF GLUCOSINOLATE CONTENT TO ELEVATED ATMOSPHERIC CO_2

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Abstract—The carbon/nutrient balance hypothesis has recently been interpreted to predict that plants grown under elevated CO_2 environments will allocate excess carbon to defense, resulting in an increase in carbon-based secondary compounds. A related prediction is that, because plant growth will be increasingly nitrogen-limited under elevated CO_2 environments, plants will allocate less nitrogen to defense, resulting in decreased levels of nitrogen-containing secondary compounds. We present the first evidence of decreased investment in nitrogen-containing secondary compounds for a plant grown under elevated CO_2. We also present evidence that plant response is species-specific and is not correlated with changes in leaf nitrogen content or leaf carbon–nitrogen ratio. When three crucifers were grown at 724 ± 8 ppm CO_2, total foliar glucosinolate content decreased significantly for mustard, but not for radish or turnip. Glucosinolate content of the second and fourth youngest mustard leaves decreased by 45% and 31%, respectively. In contrast, no significant change in total glucosinolate content was observed in turnip or radish leaves, despite significant decreases in leaf nitrogen content. Total glucosinolate content differed significantly among leaves of different age; however, the trend differed among species. For both mustard and turnip, glucosinolate content was significantly higher in older leaves, while the opposite was true for radish. No significant CO_2 × leaf age interaction was observed, suggesting that intraplant patterns of allocation to defense will not change for these species. Changes in nitrogen allocation strategy are likely to be species-specific as plants experience increasing atmospheric CO_2 levels.

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The ecological consequences of CO_2-induced changes in plant defensive investment remain to be investigated.

Key Words—Elevated CO_2, glucosinolates, crucifers, plant defense, carbon/nutrient balance hypothesis.

INTRODUCTION

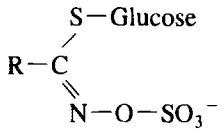
It is now clear that atmospheric carbon dioxide levels are increasing rapidly. The current average concentration of approximately 360 ppm is expected to double by the end of the twenty-first century (Houghton et al., 1990; Graedel and Crutzen, 1993; Gates, 1993). Independent of its potential effects on global climate, increasing atmospheric CO_2 may profoundly impact natural ecosystems by directly influencing plant growth and chemistry. Although plant growth is often increased under elevated CO_2 (Strain and Cure, 1985; Bazzaz, 1990; Mooney et al., 1991), leaves generally contain decreased levels of nitrogen and water and increased leaf carbon–nitrogen ratios (Lincoln et al., 1984; Bentley and Johnson, 1991; Baxter et al., 1994; Epron et al., 1996; Karowe, unpublished data). Despite increased consumption of elevated-CO_2-grown plants, insect herbivores generally exhibit reduced survivorship and/or growth (Lincoln et al., 1984; Fajer et al., 1989, 1991; Johnson and Lincoln, 1990; Bentley and Johnson, 1991; Lindroth et al., 1993; Karowe, unpublished data).

Despite the long-recognized importance of plant secondary chemistry in influencing plant–herbivore interactions (Rosenthal and Janzen, 1979; Spencer, 1988; Rosenthal and Berenbaum, 1991; Bernays, 1992; Romero et al., 1997), little information exists about the response of plant secondary chemistry to elevated atmospheric CO_2 (Fajer et al., 1992; Lindroth et al., 1993; Julkunen-Tiitto et al., 1993; Lavola and Julkunen-Tiitto, 1994). Moreover, all secondary compounds investigated thus far are carbon-based, i.e., do not contain nitrogen (Penuelas et al., 1996). Since the primary direct effect of elevated CO_2 appears to be dilution of plant nitrogen, it is plausible that elevated CO_2 may exert a particularly strong effect on nitrogen-containing secondary compounds.

The carbon/nutrient balance hypothesis (Bryant et al., 1983) predicts that the increased C:N ratio of plants grown under elevated CO_2 will result in increased production of carbon-based secondary compounds (Fajer et al., 1992; Ayres, 1993; Lindroth et al., 1993). A logical extension of this hypothesis is that under elevated CO_2, leaves will contain lower concentrations of nitrogen-based secondary compounds (Baldwin and Ohnmeiss, 1994) because increased vegetative growth will require a larger proportion of available nitrogen. To our knowledge, the response of nitrogen-based secondary compounds to elevated CO_2 has not yet been investigated.

Glucosinolates and their derivative mustard oils are ubiquitous nitrogen-

containing secondary compounds of the order Capparales, most notably of the family Brassicaceae (Cruciferae). Nearly 100 glucosinolates have been identified among the Brassicaceae (Louda and Mole, 1991). All known glucosinolates are relatively small secondary compounds with the basic structure:



where the R group may be aromatic or aliphatic (Larsen, 1981; Chew, 1988a; Van Etten and Tookey, 1991). Glucosinolates are hydrolyzed by myrosinases to glucose, sulfate, and a mustard oil. In general, glucosinolates and myrosinases are stored separately within the plant; in commercially important crucifers, glucosinolates apparently are stored in vacuoles while myrosinases appear to be associated with cytosolic membranes (Chew, 1988a).

Glucosinolates have played a major role in the development of theories about chemically mediated plant–herbivore coevolution (Rhoades and Cates, 1976; Feeny, 1976, 1977) and are often cited as a classic "qualitative" secondary defense (sensu Feeny, 1976). Several recent reviews have concluded that, while they are not the sole determinant of crucifer–herbivore interactions, glucosinolates nonetheless have played and continue to play an integral part in the evolution of crucifer–herbivore relationships (Chew, 1988b; Louda and Mole, 1991; Renwick 1997). As nitrogen-containing secondary compounds of demonstrated ecological and evolutionary importance, glucosinolates provide an excellent system for investigating the effect of elevated atmospheric CO_2 on investment in nitrogen-containing allelochemicals. Therefore, in this study we ask: (1) Does the total glucosinolate content of crucifer species change under elevated CO_2? (2) If so, are plant responses to elevated CO_2 species-specific? (3) Are changes in within-plant nitrogen allocation consistent with those expected under the carbon/nutrient balance hypothesis?

METHODS AND MATERIALS

Seeds of mustard, *Brassica juncea* (var. Florida Broadleaf), turnip, *B. rapa* (var. Purple-top), and radish, *Raphanus sativus* (var. French Breakfast), all obtained from W. Atlee Burpee & Co. (Warminster, Pennsylvania), were sown in Hyponex potting soil and allowed to germinate in the greenhouse at the University of Michigan Biological Station in Pellston, Michigan. Seven days after sowing, plants were transplanted into 6-in. pots containing potting soil. For each species, 32 pots were planted, each containing two plants. Four pots of each species were placed into each of eight 0.5-m^3 open-topped field cham-

bers similar to those described by Drake et al. (1989). Four chambers were maintained at ambient CO_2 levels (363 $\pm$ 6 ppm) and four were maintained at elevated CO_2 levels (724 $\pm$ 8 ppm.). CO_2 content was elevated by dispensing 100% CO_2 into the inlet port of an outlet blower connected by dryer hose to the base of each elevated chamber. CO_2 levels were monitored continuously by pumping air from one ambient and all elevated chambers to a microcomputer-controlled valve manifold that directed the gas stream past an infrared gas analyzer. Levels detected by the gas analyzer were recorded to disc approximately every 5 min starting June 27. CO_2 flow to each elevated chamber was adjusted via a manual flowmeter.

Plants were watered twice daily, inspected periodically for insect herbivores and, when necessary, sprayed with Safer Soap insecticide. Plants were arranged into four blocks, each containing plants sufficient to fill one ambient and one elevated chamber. In order to minimize block effects, plant blocks were rotated among chambers such that each elevated CO_2 block spent approximately equal time within each elevated CO_2 chamber. On August 18, both plants from two pots per species per chamber were sampled for glucosinolate content. Leaf discs 0.5-in.-diam. were cut from the distal tip of the second and fourth youngest leaves from one plant per pot and from the second youngest leaf from the other. Leaf discs were weighed, immediately placed in a 1.5-ml microfuge tube containing 0.5 ml boiling distilled water, and boiled for 5 min to denature hydrolytic enzymes. After boiling, 0.5 ml of 60 mM barium lead acetate solution was added to remove myrosinase by precipitation, and the tube was placed immediately on ice. Tubes were stored at $-80°C$ for one week before shipment on dry ice to the University of Montana.

Total glucosinolate content was determined by a protocol originally developed by Heaney and Fenwick (1981) and modified by MacGregor (unpublished) and more recently by Mitchell-Olds et al. (unpublished data) to handle larger sample sizes. Plant tissue in each tube was homogenized using a variable-speed hand drill, vortexed, and centrifuged. One milliliter of supernatant was added to a 0.3-ml DEAE Sephadex column, washed twice with 500 μl 4 M acetic acid and twice with 1 ml H_2O. One hundred microliters of 3.0 mg/ml myrosinase was added, left overnight, and eluted with 1 ml H_2O. One tenth of 1 ml eluate was assayed at 490 nm in a Biorad microplate reader, after addition of 0.1 ml glucose oxidase–peroxidase color reagent. The glucose oxidase–peroxidase color reagent is a mixture of equal volumes of color reagents 1 and 2, which are both made in imidazole buffer (0.98% imidizole, 0.03% sodium azide, and 0.42 ml glacial acetic acid brought to volume in 100 ml H_2O). Color reagent 1 contains 0.058% glucose oxidase type X-S from *Aspergillus niger* and 0.115% 4-aminoantipyrene. Color reagent 2 contains 0.009% horseradish peroxidase and 0.58% phenol. Samples were quantified by comparison to a glucose dilution series. Reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri).

To determine the water and nitrogen contents of leaves, 0.5-in.-diam. leaf discs were cut from second and fourth youngest leaves of eight additional plants of each species at each CO_2 level (two from each chamber) on August 19. Each disc was weighed, dried to constant weight at 60°C, reweighed to determine water content, ground under liquid nitrogen, dried again to constant weight at 60°C, and analyzed for nitrogen, carbon, and hydrogen content with a Perkin Elmer CHN Elemental Analyzer. Within each species–CO_2–leaf age–block combination, average leaf water content was used to estimate the dry weight of leaf discs used in the glucosinolate analysis described above.

To determine the extent to which exposure to elevated CO_2 affected plant growth rates (Coleman et al., 1993), on August 19 all plants were inspected for initiation of flowering (bolting). To determine whether plant response to elevated CO_2 may have been constrained by root restriction (McConnaughay et al., 1993), all plants were subsequently removed from their pots and inspected for root crowding.

For each species, glucosinolate content, expressed as millimolar glucosinolate per gram dry leaf weight, nitrogen content, and carbon–nitrogen ratio were analyzed by three-way analysis of variance with CO_2 level, leaf age, and block as main effects.

RESULTS

Total glucosinolate content of both young and older mustard leaves was significantly lower for plants grown at elevated atmospheric CO_2 levels (Tables 1 and 2). Glucosinolate content of the second youngest mustard leaf decreased

TABLE 1 LEAF GLUCOSINOLATE CONTENT OF MUSTARD, RADISH, AND TURNIP GROWN AT AMBIENT (3 63 ± PPM) AND ELEVATED (724 ± 8 PPM) CO_2[a]

	Glucosinolate (mM/g dry wt)			
	Second youngest leaf		Fourth youngest leaf	
Species	Ambient	Elevated	Ambient	Elevated
Mustard	0 584	0 297	1 833	1 034
	(0 280)	(0 150)	(0 756)	(0 537)
Radish	0 528	0 813	1 134	1 080
	(0 358)	(0 502)	(0 296)	(0 807)
Turnip	1 628	1 496	0 833	0 593
	(0 537)	(0 893)	(0 352)	(0 425)

[a] Means are given, with standard deviations in parentheses Two way analysis of variance is presented in Table 2

TABLE 2. TWO-WAY ANALYSIS OF VARIANCE FOR TOTAL LEAF GLUCOSINOLATE CONTENT OF MUSTARD, RADISH, AND TURNIP[a]

Source of variation	SS	*df*	MS	*F*	*P*
Mustard					
Main effects					
CO_2 level	3.15	1	3.15	18.37	<0.001
Leaf age	10.51	1	10.51	61.37	<0.001
Interaction					
CO_2 × leaf age	0.70	1	0.70	4.10	0.0490
Error	7.54	44	0.17		
Total	21.26	47			
Radish					
Main effects					
CO_2 level	0.14	1	0.14	0.57	0.4622
Leaf age	2.03	1	2.03	8.20	0.0064
Interaction					
CO_2 × leaf age	0.31	1	0.31	1.24	0.2710
Error	10.88	44	0.25		
Total	13.57	47			
Turnip					
Main effects					
CO_2 level	0.43	1	0.43	1.14	0.2921
Leaf age	9.00	1	9.00	23.47	<0.001
Interaction					
CO_2 × leaf age	0.04	1	0.06	0.10	0.7618
Error	18.40	48	0.38		
Total	28.02	51			

[a]Corresponding means are presented in Table 1.

by 45% and that of the fourth youngest leaf decreased by 31%. In contrast, elevated CO_2 did not significantly affect the total glucosinolate content of young or old radish or turnip leaves (Tables 1 and 2).

For each plant species, glucosinolate content differed significantly between leaves of different age (Tables 1 and 2); however, the trend differed between species. For both mustard and radish, older leaves contained more glucosinolates than did younger leaves; this was true under both ambient and elevated CO_2. In contrast, older turnip leaves contained less glucosinolates than did younger leaves. For mustard only, the effect of CO_2 level depended on leaf age (significant CO_2 × leaf age interaction in Table 2). Under ambient CO_2, glucosinolate

TABLE 3. LEAF NITROGEN CONTENT AND CARBON–NITROGEN RATIO FOR MUSTARD, RADISH, AND TURNIP GROWN AT AMBIENT (363 ± 6 PPM) AND ELEVATED (724 ± 8 PPM) CO_2[a]

Species	Second youngest leaf		Fourth youngest leaf	
	Ambient	Elevated	Ambient	Elevated
Nitrogen content (% dry wt)				
Mustard	2.79	2.46	2.18	1.71
	(0.54)	(0.50)	(0.30)	(0.39)
Radish	2.94	2.83	2.49	1.83
	(0.41)	(0.44)	(0.35)	(0.23)
Turnip	3.49	2.98	2.54	2.12
	(0.83)	(0.55)	(0.26)	(0.34)
Carbon-nitrogen ratio				
Mustard	15.35	16.91	19.36	25.16
	(3.37)	(3.92)	(2.96)	(6.56)
Radish	13.67	14.08	16.40	22.15
	(1.95)	(1.92)	(2.03)	(2.59)
Turnip	12.16	13.86	15.82	19.60
	(2.91)	(2.34)	(1.53)	(3.33)

[a]Two-way analysis of variance is presented in Table 4.

content of older mustard leaves was 2.8-fold greater than for younger leaves; under elevated CO_2, the difference increased to 3.8-fold.

For each species, growth under elevated CO_2 resulted in significantly lower leaf nitrogen content and significantly higher leaf carbon-nitrogen ratio (Tables 3 and 4). When grown under elevated CO_2, nitrogen content of young leaves decreased by 3.7–14.6% and carbon–nitrogen ratios increased by 3.0–14.0%. Changes were more dramatic for old leaves. When grown under elevated CO_2, nitrogen content of old leaves decreased by 16.5–26.5%, and carbon–nitrogen ratios increased by 23.9–35.1% (Table 3). Only radish exhibited a significant CO_2 × leaf age interaction for nitrogen content and carbon–nitrogen ratio (Table 4).

Inspection of mustard plants on August 19 revealed no evidence of phenological advancement under elevated CO_2. The proportion of mustard plants that had bolted did not differ significantly between ambient (9.3%) and elevated (7.8%) CO_2 treatments ($\chi^2 = 0.03$, $P > 0.9$). No radish or turnip plants had bolted by this time in either CO_2 treatment. Inspection of all three species revealed no evidence that plants suffered from root restriction.

TABLE 4. TWO-WAY ANALYSIS OF VARIANCE FOR LEAF NITROGEN CONTENT AND CARBON–NITROGEN RATIO FOR MUSTARD, RADISH, AND TURNIP[a]

Source of variation	SS	*df*	MS	*F*	*P*
Mustard					
Nitrogen content					
Main effects					
CO_2 level	1.28	1	1.28	6.53	0.0163
Leaf age	3.71	1	3.71	18.90	<0.001
Interactions					
CO_2 × leaf age	0.04	1	0.04	0.19	0.6689
Error	5.50	28	0.20		
Total	10.54	31			
Carbon–nitrogen ratio					
Main effects					
CO_2 level	108.6	1	108.6	5.53	0.0259
Leaf age	300.5	1	300.5	15.32	<0.001
Interactions					
CO_2 × leaf age	36.1	1	36.1	1.84	0.1861
Error	549.9	28	19.6		
Total	995.1	31			
Radish					
Nitrogen content					
Main effects					
CO_2 level	1.21	1	1.21	8.93	0.0058
Leaf age	4.17	1	4.17	30.72	<0.001
Interactions					
CO_2 × leaf age	0.59	1	0.59	4.33	0.0467
Error	3.80	28	0.14		
Total	9.76	31			
Carbon–nitrogen ratio					
Main effects					
CO_2 level	75.9	1	75.9	16.61	<0.001
Leaf age	233.3	1	233.3	51.05	<0.001
Interactions					
CO_2 × leaf age	57.5	1	57.5	12.57	0.0014
Error	128.0	28	4.6		
Total	494.7	31			
Turnip					
Nitrogen content					
Main effects					
CO_2 level	1.71	1	1.71	5.78	0.0231
Leaf age	6.53	1	6.53	22.09	<0.001
Interactions					
CO_2 × leaf age	0.02	1	0.02	0.06	0.8166
Error	8.27	28	0.30		
Total	16.52	31			

TABLE 4. CONTINUED

Source of variation	SS	*df*	MS	*F*	*P*
Carbon-nitrogen ratio					
Main effects					
CO_2 level	60.1	1	60.1	8.76	0.0062
Leaf age	176.9	1	176.9	25.80	<0.001
Interactions					
CO_2 × leaf age	8.7	1	8.7	1.26	0.2705
Error	192.0	28	6.9		
Total	437.6	31			

[a] Corresponding means are presented in Table 3.

DISCUSSION

The results of this study suggest that, for some plants, investment in nitrogen-containing secondary compounds may change with increasing atmospheric CO_2 concentration, but that the magnitude and even direction of change are likely to be species-specific. The decrease in total glucosinolate content of mustard grown under elevated CO_2 is consistent with the prediction of the carbon/nutrient balance hypothesis (Bryant et al., 1983) that increased nitrogen limitation results in reduced allocation of nitrogen to defense. However, glucosinolate content of turnip and radish did not change significantly under elevated CO_2 despite significantly reduced leaf nitrogen contents and significantly increased leaf carbon–nitrogen ratios. Clearly, for these three crucifers, change in leaf nitrogen content is not a reliable predictor of change in leaf secondary chemistry.

Species-specific responses have also been observed with carbon-based defenses. For instance, under elevated CO_2, total volatile leaf mono- and sesquiterpenes in peppermint did not change (Lincoln and Couvet, 1989), but concentrations of the iridoid glycosides aucubin and catalpol in plantain decreased significantly (Fajer et al., 1992). Elevated CO_2 resulted in an increase in total phenolic concentration of wheat, no change in orange, and a decrease in total phenolic concentration of pine (Penuelas et al., 1996). In the most comprehensive study to date, Lindroth et al. (1993) showed that the effects of elevated CO_2 on carbon-based allelochemicals differed among three tree species and, moreover, varied among types of phenolics within species. Elevated CO_2 resulted in significant increases in foliar concentrations of salicortin (a phenolic glycoside) in trembling aspen and of ellagitannin and condensed tannin in sugar

maple. However, elevated CO_2 also resulted in a significant decrease in foliar ellagitannin in red oak, but no significant change in gallotannin levels in maple or oak or in condensed tannin levels in aspen or oak. Clearly, broad generalizations about responses of carbon- and nitrogen-based allelochemicals to elevated CO_2 are not warranted at this time.

Independent of CO_2-induced changes, total glucosinolate content also differed significantly between young and old leaves for all three crucifer species; again, the magnitude and direction of differences were species-specific. Moreover, the significant $CO_2 \times$ leaf age interaction for mustard, but not for radish or turnip, suggests that elevated atmospheric CO_2 will affect patterns of defensive allocation among leaves in some species but not in others.

Because glucosinolates affect a wide variety of herbivores both adversely and positively, the ecological consequences of changes in glucosinolate content are hard to predict. Although they do not defend crucifers against all nonadapted insect herbivores (Chew, 1988b; Louda and Mole, 1991), glucosinolates constitute an effective chemical barrier to most noncrucifer feeders (reviewed by Schoonhoven, 1972; Chew, 1988a; Louda and Mole, 1991). For instance, growth and survivorship were markedly reduced for larvae of the umbellifer specialist black swallowtail fed celery (Erickson and Feeny, 1974) or wild carrot (Blau et al., 1978) containing less than 1% allylglucosinolate. Similarly, incorporation of 1% allyl isothiocyanate into artificial diets strongly decreased larval growth and survivorship of the generalist Bertha armyworm (McCloskey and Isman, 1993). In addition, glucosinolates and their breakdown products inhibit growth of some fungi (Holley and Jones, 1985) and feeding by some mammals (Van Etten and Tookey, 1991).

In contrast, many crucifer-adapted herbivores are not adversely affected by glucosinolates and may use glucosinolates or mustard oils as attractants (Feeny et al., 1970; Schoonhoven, 1972; Pivnick et al., 1994), oviposition stimulants (David and Gardiner, 1962; Rothschild, 1987; Huang et al., 1994; Chew and Renwick, 1995), and/or larval feeding stimulants (Ma and Schoonhoven, 1973; Schoonhoven and Blom, 1988; Reed et al., 1989). It is therefore plausible that decreased glucosinolate content may result in reduced herbivory by adapted herbivores (e.g., the cabbage butterfly) that use mustard oils as attractants and/or feeding stimulants. Concomitantly, decreased glucosinolate content may result in increased herbivory by nonadapted generalists that can tolerate low glucosinolate levels (e.g., the southern armyworm; Blau et al., 1978) and may also result in increased herbivory by species that, despite feeding on crucifers, appear to be deterred by specific glucosinolates (e.g., flea beetles; Feeny, 1977; Louda and Rodman, 1983). Finally, Siemens et al. (1996) recently demonstrated that herbivory by two crucifer-adapted herbivores, flea beetles and the diamondback moth, is highest at intermediate glucosinolate levels. Clearly, since glucosino-

lates have various effects on adapted, unadapted, and potential herbivores, CO_2-induced changes in glucosinolate content are likely to have multiple, complicated effects.

Inferences about the ecological consequences of changes in glucosinolate content of crucifers are complicated by our use of a method that does not distinguish among specific glucosinolates. However, we believe this constraint would tend to obscure, rather than accentuate, the effect of elevated CO_2 on glucosinolate content. Among crucifers, glucosinolate profiles (identities and proportions of individual glucosinolates) are species- or genus-specific (Rodman and Chew, 1980; Rodman, 1981). It is possible that although elevated CO_2 did not result in different total glucosinolate content of radish or turnip, the proportions of individual glucosinolates may have changed significantly. Since individual glucosinolates differ dramatically in their biological effects (reviewed in Renwick, 1997), CO_2-induced changes in glucosinolate profiles may strongly impact herbivores, despite unaltered total glucosinolate content.

The results of this study suggest that investment of nitrogen in defensive compounds may decrease for some plants as they experience increasing atmospheric CO_2 levels. However, plant responses to elevated CO_2 are likely to be species-specific and do not appear to be predictable from changes in leaf nitrogen content or carbon-nitrogen ratio. The ecological consequences of changes in plant defensive strategy remain to be elucidated, but may be profound.

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INHIBITION OF *Nitrosomonas europaea* BY MONOTERPENES FROM COASTAL REDWOOD (*Sequoia sempervirens*) IN WHOLE-CELL STUDIES

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Abstract—Inhibition by allelochemicals, including monoterpenes, has been suggested as a factor in the extremely low nitrification rates observed in coastal redwood forests. Similarities between the molecular structure of known nitrification inhibitors and some conifer monoterpenes have been suggested as one reason for the inhibition of autotrophic nitrifiers by conifer monoterpenes. The effect of monoterpenes on nitrification rate and growth of *Nitrosomonas europaea* was examined in whole-cell pure culture experiments using the five most abundant monoterpenes in coastal redwood needles. These are (in order of decreasing concentration in the needles) limonene, α-pinene, sabinene, myrcene, and γ-terpinene. Four of the five compounds significantly inhibited growth of *N. europaea* in batch culture experiments. Short-term kinetic studies of the two most inhibitory monoterpenes, limonene and α-pinene, were performed on whole cells to evaluate the mode of interaction between these chemicals and nitrification rates. Inhibition constants (K_i) of limonene (38 μM) and α-pinene (95 μM) were determined. Lineweaver-Burk plots of nitrification in the presence of monoterpenes appear to fit a noncompetitive inhibition model; however, the mechanisms of inhibition may be more complex.

Key Words—*Nitrosomonas europaea*, nitrification, inhibition, kinetics, monoterpenes, *Sequoia sempervirens*, conifers, nitrogen cycling.

INTRODUCTION

The forest floor (the combined fresh litter, fermentation, and humic horizons) has a profound effect on nitrogen cycling in forest ecosystems (Hart and Fire-

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stone, 1991; Killham, 1990; White, 1986, 1991, 1994). The effects of secondary chemicals in leaf litter on nutrient cycling have not been thoroughly investigated, but have been suggested as a factor that might control nutrient cycling in forests (Horner et al., 1988; Lohdi and Killingbeck, 1980). Monoterpenes have been shown to inhibit nitrification in a variety of ecological and laboratory settings. Foliar monoterpenes inhibit nitrification in soil nitrification potential bioassays, and field studies suggest that monoterpenes inhibit nitrification beneath California bay [*Umbellularia californica* (Hook & Arn) Nutt.] in coastal woodlands (Wood, 1996). Monoterpenes from needle resins are implicated in the inhibition of nitrification in a ponderosa pine (*Pinus ponderosa* Laws) ecosystem on the basis of soil bioassay experiments (White, 1988, 1990, 1991).

White (1988) proposed that monoterpenes inhibit ammonia monooxygenase (AMO, the first enzyme in the ammonia oxidation pathway in autotrophic nitrifying bacteria) based on chemical similarities between some conifer monoterpenes and known nitrification inhibitors that act on AMO. AMO is inhibited by a number of *n*-alkanes, *n*-terminal alkenes and alkynes, and by some cyclic compounds (Bedard and Knowles, 1989; Hooper and Terry, 1973; Hyman et al., 1985; Lees, 1952; McCarty and Bremner, 1991; Ward, 1990).

Oxidation products, which are produced by ammonia-oxidizing bacteria, of some of the cyclic inhibitory compounds, such as benzene, phenol, and Nitrapyrin, suggest that these compounds are alternative substrates for AMO (Hyman et al., 1985; Vannelli and Hooper, 1992). The hypothesis that conifer monoterpenes are competitive inhibitors for AMO is consistent with observations from White's (1991) study in which nitrification inhibition by ponderosa pine monoterpenes was inversely proportional to initial concentrations of ammonium in soil bioassays. A noncompetitive mode of inhibition for monoterpenes would be consistent with the study by Keener and Arp (1993), in which the mode of inhibition for a series of hydrocarbons and halogenated hydrocarbons was investigated. In these whole cell experiments, it was inferred that inactivation of AMO was caused by binding of large hydrocarbons at a site other than the active site of the enzyme (Keener and Arp, 1993). Regardless of the mode of inhibition, it is evident that structure and relative concentration of inhibitor and substrate are primary factors controlling the magnitude of the inhibition (White, 1994).

The multiple effects of monoterpenes on edaphic microbes have not been thoroughly investigated. Monoterpenes have antimicrobial properties, disrupt electron transport, and uncouple oxidative phosphorylation (Bedard and Knowles, 1989; Knobloch et al., 1986; Rice, 1984; Uribe et al., 1985). They may also serve as a carbon source for some heterotrophic bacteria (Shukla et al., 1968; Harder and Probian, 1995). Many factors could influence the fate of monoterpenes in the soil solution, thus influencing their efficacy as inhibitors of nitrification. For example, at high concentrations, nonspecific toxicity to microbes

by monoterpenes could prevent decomposition and mineralization. A reduced pool size of nitrate in soil bioassays would appear to result from inhibition of nitrification.

Soil bioassays do not precisely determine the roles of monoterpenes in the forest floor. Therefore, the present study was designed to determine whether monoterpenes, and in particular coastal redwood monoterpenes, are specific inhibitors of the autotrophic soil nitrifier, *Nitrosomonas europaea*. This investigation focused on the low concentration range for both the primary substrate, ammonium, and for the inhibitors, in an attempt to test substrate concentrations likely to be encountered on the forest floor. Nitrapyrin, a commercial nitrification inhibitor, was used as a positive control for inhibition. Although its exact mechanism of inhibition is unknown, Nitrapyrin is oxidized by ammonia monooxygenase and is a good model with which to compare the monoterpenes (Vanelli and Hooper, 1992). Growth rates in batch culture were used to determine the degree of inhibition caused by each monoterpene. Short-term kinetic experiments were also performed to investigate the mode of action by the two most inhibitory monoterpenes, limonene, and α-pinene. AMO has proven difficult to purify in active form, so these experiments were performed on whole cells. An additional advantage of using whole cells is that they may be more applicable than purified enzymes for predicting the behavior of whole cells in the environment. A disadvantage is the difficulty in distinguishing multiple possible interactions between potential inhibitors and multiple sites of action in the cell.

The needle resin of coastal redwood is composed of 15 monoterpenes, several sesquiterpenes, and other minor oxygenated terpenoids (Okomoto et al., 1981; Hall and Langenheim, 1986a). For this study, we chose the five most abundant monoterpenes whose combined concentration comprises over 90% of the total yield of coastal redwood needle resin. These five, limonene, sabinene, α-pinene, myrcene, and γ-terpinene, are all hydrocarbon monoterpenes. One additional (structurally similar) monoterpene, β-pinene, was also included.

METHODS AND MATERIALS

Strains and Culture Conditions. Nitrosomonas europaea (ATCC No. 19718) was maintained in liquid culture at room temperature (approximately 25°C) in the dark in a freshwater medium derived from Soriano and Walker (1968). Phenol red was used as a pH indicator, and pH was maintained near neutral by the addition of sterile K_2CO_3 (5% solution in distilled water) as required. The culture was ascertained to be pure at the beginning of the set of experiments by spreading on rich organic medium (5 g peptone per liter) and by periodic inoculations into liquid rich medium.

Growth Inhibition Experiments. Experiments were carried out in the above

medium (Soriano and Walker, 1968) with 100 ml of culture in 250-ml flasks and an initial ammonium concentration of 50 mM. The flasks were inoculated with 1 ml of exponentially growing stock cultures and incubated statically in the dark at room temperature.

Monoterpenes (technical grade, >90% purity) were obtained from Aldrich Chemical Company, Inc., and Nitrapyrin (2-chlor-6-trichloromethyl-pyridine) was obtained from Dow Chemical Corp. Their chemical structures are shown in Figure 1. Potential inhibitors, including Nitrapyrin, were emulsified in 0.5% ethanol in distilled water and added to the cultures at the time of inoculation. Nitrapyrin served as a positive control for inhibition and was used at the prescribed field concentration of 1 μg/ml. Monoterpenes were used at final concentrations of 1 and 10 μg/ml, and each treatment was performed in duplicate or triplicate. One set of flasks received only 0.05% ethanol as a control for solvent effects. Growth was monitored by approximately daily measurement of nitrite concentration with a colorimetric method (Strickland and Parsons, 1972) in 1-ml aliquots that were removed from the growth flask with sterile technique.

Growth rate (approximated by nitrification rate) was measured during the initial portion of the exponential phase of the batch cultures. Growth rates were compared statistically by regression analysis of the log-transformed data in a two-tailed *t* test (Systat).

Kinetics of Monoterpene Inhibition of Ammonia Oxidation. A semicontinuous batch culture was initiated by inoculating 500 ml of a 1-week-old batch culture of *N. europaea* into 2 liters of medium containing 50 mM ammonium. The 4-liter culture bottle was equipped with sterile sampling and medium-addition ports but was not stirred. It was incubated in the dark at room temperature and growth was monitored by periodic determination of nitrite concentration. The culture was maintained in exponential phase by periodic dilution (removal of 2 liters of culture for experimentation and replacement with 2 litres fresh medium).

For kinetic experiments, cells from 2 liters of semibatch culture were harvested on 0.3-μm-pore size 47-mm-diameter polycarbonate filters by filtration under gentle vacuum. Cells were resuspended (at a concentration of approximately 10^5 cells/ml, see below) in ammonium-free medium and transferred into experimental flasks (50 ml experimental volume in 125-ml flasks). Experimental medium was identical to culture medium except that ammonium concentrations varied according to experimental design (0.1–100 μg/ml, equivalent to 0.0056–5.6 mM as NH_4^+). Monoterpenes (1 μg/ml for α-pinene and 10 μg/ml for limonene) were added as described above. Samples were removed from each experimental flask at the time of inoculation for measurement of initial ammonium concentration (Grasshoff et al., 1983) and cell number (epifluorescence enumeration using DAPI) (Porter and Feig, 1980). Each experimental treatment was performed in triplicate. Each flask was sampled using sterile pipets five to

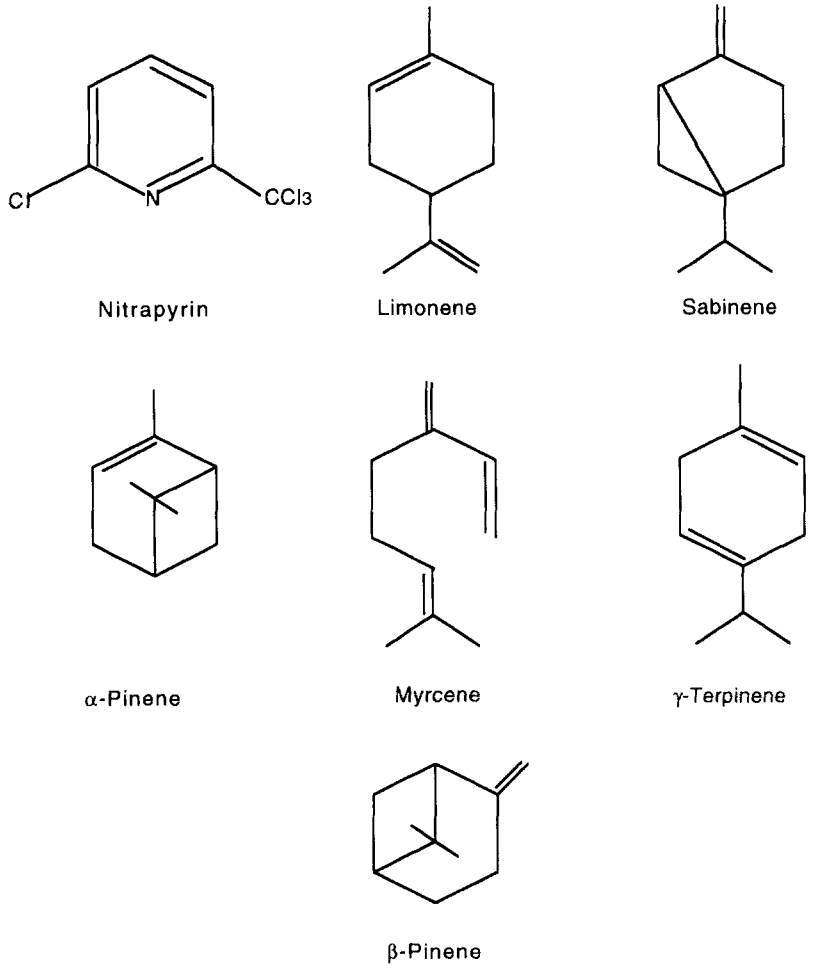


FIG. 1. Molecular structures of the potential inhibitory compounds tested in batch culture experiments.

seven times over the course of 4–5 hr for measurement of nitrite concentration (Strickland and Parsons, 1972).

Initial velocities of nitrite production (μmol NO_2^-/cell/hr) were calculated by linear regression from the increase in nitrite concentration with time. Double-reciprocal plots (Lineweaver-Burk) of initial rate and substrate concentration were used to calculate kinetic parameters. Regressions and probabilities were determined using Systat.

RESULTS

Growth Inhibition. Growth of *N. europaea* in batch culture was estimated as net nitrite production rate and was measured by daily changes of the NO_2^- concentration. Growth rates were calculated from the semi-log plot of nitrite concentration versus time during exponential phase and then normalized to the control. In the absence of inhibitors, *N. europaea* grew in a normal exponential fashion following a short lag phase after inoculation (Figure 2). Ethanol is an inhibitor of ammonia oxidation at 0.09 M concentration (Hooper and Terry 1973), but the 0.05% (0.011 M) ethanol treatments (used here as an addition control) were not significantly different from the controls (Table 1). The monoterpene treatments also exhibited logarithmic growth curves (i.e., with slopes significantly different from zero; Figure 2).

The growth rate of *N. europaea* in batch culture was significantly reduced by 1 μg/ml of the commercial nitrification inhibitor, Nitrapyrin ($P < 0.05$). Most of the monoterpenes significantly inhibited the growth of *N. europaea*, at least at the 10 μg/ml level (Table 1). Myrcene and limonene were inhibitory at the 1 μg/ml level and limonene at the 10 μg/ml level; the large variability for the 10 μg/ml myrcene treatments precluded detection of a significant effect. α-Pinene and γ-terpinene had a significant effect only at 10 μg/ml. Sabinene and β-pinene did not produce significant inhibition at either concentration, and in fact, β-pinene appeared to be stimulatory at the higher concentration.

Kinetics of Monoterpene Inhibition of Ammonia Oxidation. Ammonium oxidation rate (NO_2^- μmol/cell/hr) in the absence of inhibitors increased with increasing substrate concentration up to at least 5.6 mM (as NH_4^+) and exhibited apparently normal first-order kinetics as described by the Michaelis-Menten equation:

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]}$$

Double reciprocal plots of initial rates of nitrite production versus substrate concentration were linear and consistent with Michaelis-Menten kinetics (Figure 3). Kinetic parameters were calculated from the Lineweaver-Burk rearrangement of the above equation:

$$\frac{1}{v} = \frac{K_m}{V_{\max} \cdot [S]} + \frac{1}{V_{\max}}$$

Ammonia, rather than ammonium, is the substrate of ammonia monooxygenase, the enzyme that catalyzes the first step in the oxidation of ammonia to nitrite. Therefore, K_m is expressed as the combined concentration of $NH_4^+ + NH_3$. In the absence of inhibitors at pH 7.6, the K_m for $NH_4^+ + NH_3$ was 532

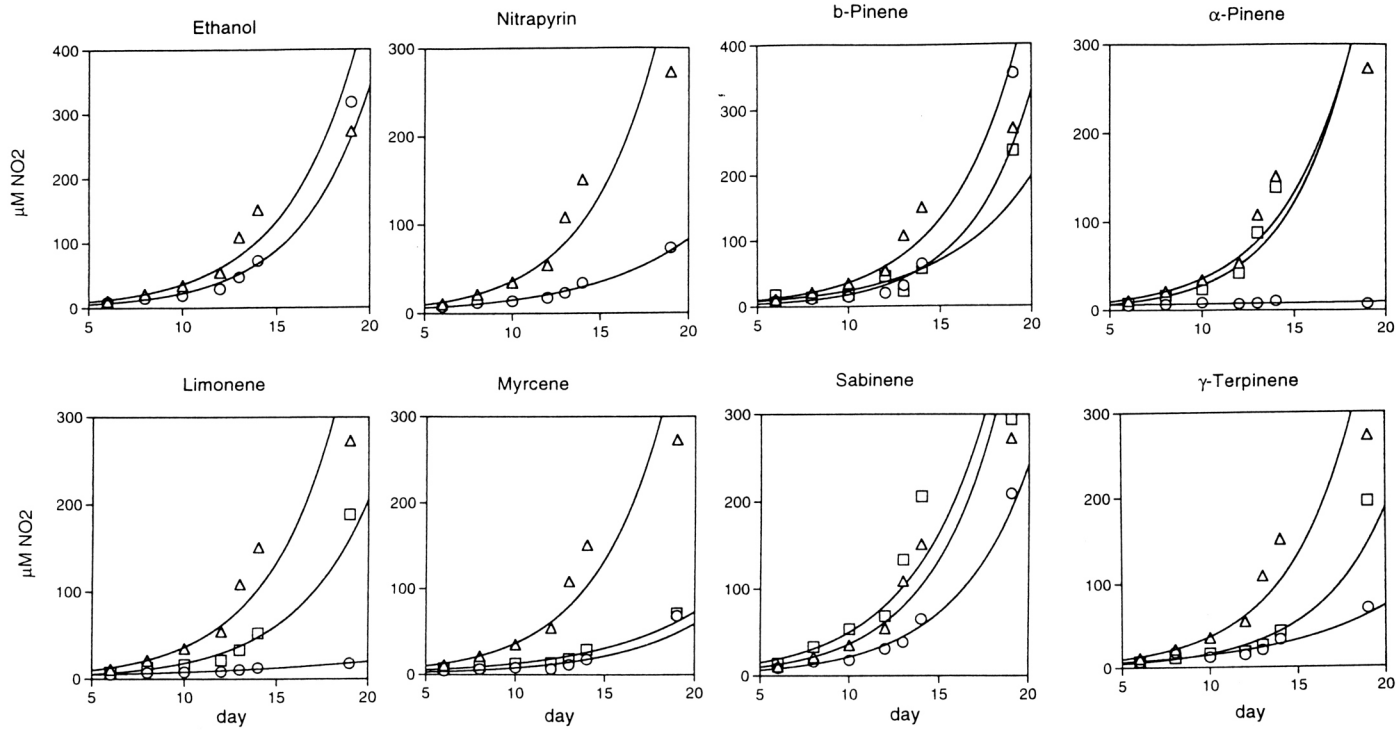


FIG. 2. Growth curves from batch cultures of *N. europaea*. Growth was monitored by nitrite accumulation in the medium over a period of 19 days; Δ, nitrite concentration in the absence of inhibitor. □, nitrite concentration in the presence of 1 μg/ml potential inhibitor; ○, nitrite concentration in the presence of 10 μg/ml of potential inhibitor.

TABLE 1. EFFECT OF POTENTIAL ON GROWTH (NITRITE PRODUCTION) OF *N. europaea* IN BATCH CULTURE[a]

Treatment	Relative nitrite production rate (mean ± SD)	*P*
control	1.00 ± 0.011	
ethanol (0.05%)	1.06 ± 0.009	0.424
nitrapyrin (1 μg ml^{-1})	0.43 ± 0.024	0.041*
γ-terpinene (1 μg ml^{-1})	0.87 ± 0.032	0.486
γ-terpinene (10 μg ml^{-1})	0.62 ± 0.004	0.018*
β-pinene (1 μg ml^{-1})	0.93 ± 0.043	0.782
β-pinene (10 μg ml^{-1})	1.15 ± 0.003	0.062
limonene (1 μg ml^{-1})	0.74 ± 0.007	0.047*
limonene (10 μg ml^{-1})	0.35 ± 0.013	0.014*
sabinene (1 μg ml^{-1})	0.92 ± 0.018	0.449
sabinene (10 μg ml^{-1})	0.96 ± 0.001	0.645
α-pinene (1 μg ml^{-1})	1.07 ± 0.022	0.645
α-pinene (10 μg ml^{-1})	0.11 ± 0.009	0.001**
myrcene (1 μg ml^{-1})	0.56 ± 0.010	0.023*
myrcene (10 μg ml^{-1})	0.45 ± 0.030	0.062

[a]Results are reported as the rate of nitrite production (mean of two or three replicates) in the experimental flasks as a fraction of the rate in the uninhibited control flasks. The standard deviation is that associated with the mean nitrite production rate in the replicate flasks. *$\alpha < 0.05$ and **$\alpha < 0.001$ denote rates significantly different from rates in the control flasks.

μM (the average of two control experiments; see Figure 3A and B). These results compare well to those of Suzuki et al. (1974) who reported a K_m for $NH_4^+ + NH_3$ of 480 μM at pH 8. The V_{max} we observed averaged 1.32×10^{-8} μmol/cell/hr.

In batch cultures, α-pinene inhibited growth in *N. europaea* more strongly than did limonene at the 10 μg/ml level (Figure 2, Table 1). Both α-pinene and limonene inhibited ammonia oxidation in short-term kinetic experiments. The lower concentration of α-pinene, which was not inhibitory in the growth experiment, exhibited significant inhibition in the kinetic experiments. Double-reciprocal plots again were linear, but differed in both slope and *y* intercept between the control and monoterpene treatments. In the presence of 10 μg/ml limonene, the apparent K_m for $NH_4^+ + NH_3$ was 1220 μM and in the presence of 1 μg α-pinene, the apparent K_m was 344 μM. The double-reciprocal plots for limonene are consistent with a noncompetitive mode for the interaction between limonene and ammonia; both the slope and the *y* intercept of the double-reciprocal plots (Figure 3A) are significantly different ($P < 0.001$). In the case of

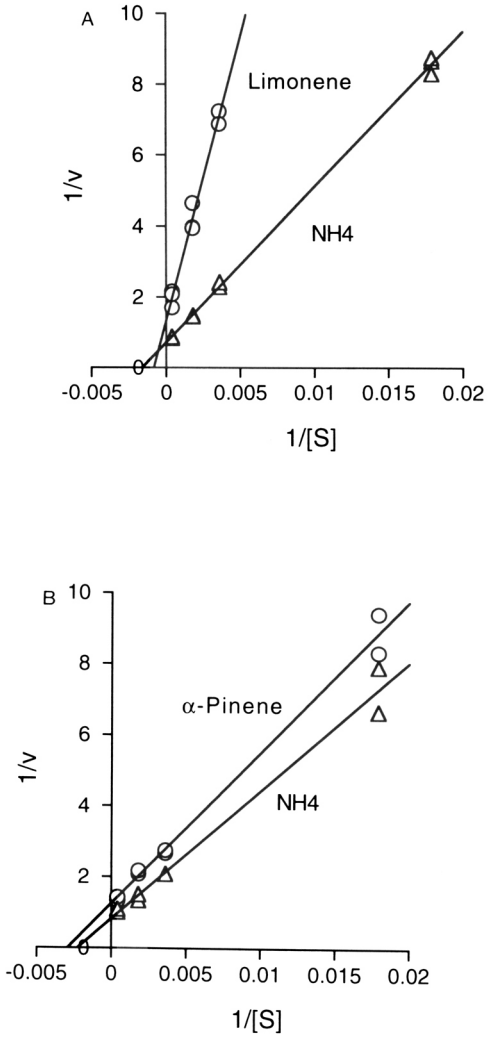


FIG. 3. Double-reciprocal plots ($1/v = 1/(\text{mol/cell/hr} \times 10^{-8})$; $1/[S] = 1/\mu M$) of the effect of limonene (A) and α-pinene (B) on ammonia oxidation by *N. europaea*. Δ, nitrite production rate in the absence of inhibitor; ○, nitrite production rate in the presence of inhibitor. Data derived from the regression equations are shown in Figure 2. Curve fits: (A) limonene: $R^2 = 0.987$, $N = 9$; ammonium: $R^2 = 0.999$, $N = 12$; (B) α-pinene: $R^2 = 0.991$, $N = 8$; ammonium: $R^2 = 0.984$, $N = 8$.

α-pinene, the slopes of the double-reciprocal plots (Figure 3B) are different ($P < 0.025$) but the y intercepts are not significantly different ($P > 0.05$). Therefore, these data do not clearly distinguish between noncompetitive and competitive modes of inhibition.

In both the competitive and noncompetitive inhibition model, the apparent half saturation constant for inhibition can be computed from:

$$K_i = \frac{K_m(1 + [I])}{K'_m}$$

where K'_m is the apparent half saturation constant of the reaction rate in the presence of inhibitor and [I] is the concentration of the inhibitor. The inhibition constant, K_i, corresponds to the concentration of inhibitor that reduces the V_{max} of the primary substrate reaction rate by 50%. K_i's computed from the data in Figure 3 were 38 μM for limonene and 95 μM for α-pinene. These values imply strong inhibition by both monoterpenes.

DISCUSSION

Growth of *N. europaea* in batch culture exhibited conventional logarithmic growth. The lag phase (not shown in Figure 2), was short, if present, probably because the cultures were all inoculated from rapidly growing cultures from the same medium and temperature conditions. The effects of the putative inhibitors were easily discernible during the early exponential phase of growth in batch culture, and clear differences in the strength of inhibition were demonstrated among the monoterpenes.

The inhibition of growth and nitrification by *N. europaea* in batch culture by redwood monoterpenes is consistent with the hypothesis that monoterpenes specifically inhibit ammonia oxidation and may be responsible for the slow rates of nitrification in coniferous forests (White, 1990, 1991, 1994). The different degrees of inhibition indicating that each compound's inhibitory capability is specific to its molecular structure demonstrates that these are specific inhibitors of *N. europaea*. In contrast, general toxicity by monoterpenes requires high concentrations (5 mM) to disrupt electron transport and uncouple oxidative phosphorylation in cell free extracts of bacterial membranes (Uribe et al., 1985; Knobloch et al., 1986).

The most inhibitory monoterpenes in batch cultures and kinetic experiments with *N. europaea*, limonene, and α-pinene were also the most active monoterpenes in soil bioassays (White, 1991). The degree of inhibition differed with molecular structure but not in consistent ways related to degree of terminal bond saturation or cyclicity. Thus, although inhibition by these terpenes appears to

be compound-specific, the variation in inhibition depending on structure of the inhibitor may imply that more than one mode of inhibition is occurring.

Direct measurements of monoterpene concentrations in the water layer, which is the actual site of bacterial colonization, of natural litter under redwood canopies have not been reported, so it is not possible to compare the concentrations found to be inhibitory in this study with those occurring naturally. Monoterpene yields in needles and litter, however, can provide a basis for predicting whether natural levels could be inhibitory. White (1986, 1991, 1994), in studies on ponderosa pine stands, reported total monoterpene levels in the upper, relatively fresh litter layer of 1.4 mg/g, decreasing to 0.15 mg/g in year-old litter. Wilt et al. (1993) reported a similar pattern for pinyon pine (*Pinus monophylla* Torf. & Frem.), with total monoterpenes decreasing from 3.6 mg/g in the fresh litter to less than 40% of that level in senescent needles. Total yield of monoterpenes in redwood litter ranged from over 6 mg/g dry wt in the fall to 1 mg/g dry wt in the late spring months following the wet season (Wood et al., 1995), indicating that most of the monoterpenes were lost during the winter (wet) months. As in redwood needles (Hall and Langenheim, 1986a, b), α-pinene and limonene were major constituents of the total monoterpene yield in the pinyon pine needles and litter (Wilt et al., 1993). Limonene and α-pinene each contribute more than 20% of the total monoterpene yield in both redwood and pinyon pine needles and litter. The observed decrease in monoterpene content upon senescence of the needles and decay of the litter implies major loss by volatilization or leaching. Field studies showed that *Umbellularia* litter lost between 65% and 85% of the monoterpene total yield in one rainy season (Wood et al., 1995). Laboratory studies corroborated the importance of water leaching in the loss of monoterpenes from both *Umbellularia* and *Sequoia* litter and further substantiated the importance of monoterpene water solubility (Wood, 1996).

Even though monoterpenes are volatile hydrocarbons, they are more water soluble than previously recognized (Weidenhamer et al., 1993) and are found at detectable levels in fjords and river water bordering forested areas (Button, 1984; Bertsch and Anderson, 1975). The exposure levels used in the present study (1 and 10 μg/ml) are less than the solubilities reported by Weidenhamer et al. (1993) for all monoterpenes tested except myrcene (whose solubility is less than 10 μg/ml). Fischer (1991) and Fischer et al. (1994) also showed that monoterpenes not only are water soluble, but in many cases the compounds are biologically active at concentrations well below the limits of their aqueous water solubilities. The monoterpene content of redwood litter (see above) suggests that significant levels of monoterpenes could be present in the soil solution during the rainy season. With a K_i of 38 μM for limonene (equivalent to 5.2 μg/ml), and 2.6% inhibition of ammonia oxidation by 1 μg/ml limonene (Table

TABLE 2. KINETIC PARAMETERS FOR NITRITE PRODUCTION IN SHORT-TERM KINETIC STUDIES OF *N. europaea* WITH AND WITHOUT MONOTERPENE INHIBITORS

Substrate or inhibitor	V_{max} (μmol/cell/hr)	K_m (μM)	K_i (μM)
$NH_4^+ + NH_3$	$1.32 \times 10^{-8} \pm 0.33 \times 10^{-8}$	532 ± 127	
NH_3		2.65 ± 0.63	
Limonene	7.58×10^{-9}		38
α-Pinene	8.08×10^{-9}		95

2), it seems likely that limonene may affect nitrification in situ as the monoterpenes in litter percolate through the soil. The K_i of 95 μM for α-pinene (equivalent to 13 μg/ml) suggests that α-pinene at naturally occurring concentrations could also be a factor influencing litter nitrification rates.

Molecular aggregation may be a mechanism that increases monoterpene activity when combined with other terpenoids or unknown additional soil components (Tanrisever et al., 1981). Monoterpenes are found aggregated with soil colloids into droplets (Muller and Del Moral, 1996). Aggregation may explain the large standard deviations observed in some of the treatments (e.g., 10 μg/ml limonene and 1 μg/ml sabinene), due to droplets formed in solution. The effects of synergistic compounds should also be considered as potentially important factors regulating the activity and availability of monoterpenes in nature.

Mechanisms of Monoterpene Inhibition. Ammonia monooxygenase (AMO) is a mixed function oxidase, and its mechanism is hypothesized to be similar to that of tyrosinase, including the role of copper in the active site (Lerch, 1981; Shears and Wood, 1985). Some inhibitors of AMO are effective because they are substrate analogs, and many can be partially oxidized by the enzyme (Hyman and Wood 1985; Bedard and Knowles, 1989; Hyman et al., 1988; Hooper and Terry, 1973; Bremner and McCarty, 1991; Ward, 1987). Metal chelators constitute a second set of inhibitors for AMO, probably because they bind the copper ions in AMO (Lees, 1946, 1952; Hooper and Terry, 1973). Based on studies of the kinetics of ammonium and hydrocarbon oxidation, hydrocarbon inhibitors have been described as competitive inhibitors of AMO (benzene: Hyman et al., 1985; methane: Hyman and Wood, 1983; Ward, 1987; ethylene: Hyman and Wood, 1985; Vanelli and Hooper, 1992). Chelating agents, such as allylthiourea, guanidine, and L-histidine, which inhibit by binding to the copper ions, exhibit noncompetitive inhibition (Lees, 1946). Copper binding inhibitors, which fit a classical noncompetitive model of inhibition, are thought to inhibit

by binding copper at the active site (Lees, 1952). It is counterintuitive that this chelation results in noncompetitive inhibition, since chelation of the copper ion would seem likely to affect binding by the substrate at that site.

Larger hydrocarbons, such as monoterpenes, may exhibit a third mechanism of inhibition. Although Keener and Arp (1993) did not investigate monoterpenes directly, a logical extension of their argument is that monoterpenes should inhibit noncompetitively; they hypothesized that nonpolar compounds would bind with AMO at a hydrophobic region separate from the active site of the enzyme. Like the copper-binding inhibitors (above), monoterpenes acting as noncompetitive inhibitors should have no effect on substrate binding and should not affect the K_m because the enzyme and enzyme-inhibitor complex have the same affinity for the substrate (Segel, 1976).

Any organic molecule with unshared or π electrons may chelate metal ions. Therefore, inhibitors with unsaturated bonds (i.e., C=C) may bind copper at the active site of AMO. The apparent similarity in kinetic behavior between monoterpenes and metal chelators could imply that a common mechanism is at work and that the monoterpenes inhibit by chelating copper at the active site. An equally viable interpretation is that the monoterpenes bind or obstruct at a site removed from the active site, in such a manner as to inhibit substrate oxidation but not substrate binding. This is the mechanism proposed by Keener and Arp (1993).

In the experiments reported here, inhibition by both limonene and α-pinene was consistent with a noncompetitive inhibition model, although competitive inhibition, in which part of the α-pinene molecule competes for substrate binding site with the primary substrate, may also be occurring to some extent.

Ammonia oxidizers are also sensitive to the usual cell poisons and uncouplers of phosphorylation, although these effects are not directly related to AMO. We therefore expected that monoterpenes might exhibit two kinds of inhibitory effects: a specific inhibition of AMO by competitive or noncompetitive inhibition at low concentrations, and a general toxicity at high concentrations. Benzene and phenol are also inhibitors of autotrophic nitrifiers and are oxidized by ammonium monooyxgenase to phenol and hydroxyquinone, respectively (Hyman et al., 1985). However, kinetic studies of benzene at low concentrations do not fit either competitive or noncompetitive inhibition models, indicating a more complex behavior (Hyman et al., 1985). In the same study, higher concentrations of benzene (4 mM) added to cell-free extracts with 500 μM hydroxylamine and 10 μM allylthiourea inhibited oxygen uptake. These results suggest that at high concentrations, benzene is inhibitory at a site other than AMO. Results from kinetic studies may not have entirely illustrated the true mechanism of benzene inhibition because of these two potential modes of inhibition. Because the consumption of monoterpenes and the increase of possible oxidation products were

not monitored in these experiments, it is possible that the inhibitory monoterpenes were also oxidized by AMO and thus fail to fit a simple competitive inhibition model for the same reasons.

This investigation focused on the low concentration range for both the primary substrate, ammonium, and for the inhibitors, choosing substrate concentrations likely to be encountered on the forest floor. The results imply that levels of redwood monoterpenes that occur in forest litter are capable of direct specific inhibition of *N. europaea*-like ammonia oxidizers in forest soils. Several of the compounds were very inhibitory, both in batch culture and in short-term kinetic experiments. These observations are consistent with the hypothesis that direct inhibition by monoterpenes could be a factor in regulating nitrification rates in some forest soils. The whole-cell experiments reported here clearly identify a physiological basis for direct inhibition of nitrification by monoterpenes. The net effect of monoterpenes on soil nitrogen cycling may not be easy to detect or quantify in natural systems, however, due to the multitude of chemicals and chemical–microbial interactions possible in such a complex system.

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SEMIOCHEMICALS MEDIATING SPACING BEHAVIOR OF BIRD CHERRY-OAT APHID, *Rhopalosiphum padi* FEEDING ON CEREALS

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Abstract—Olfactometry using an apterous individual of *Rhopalosiphum padi* (L.) showed an arresting effect by volatiles from a wheat seedling and a repellent effect by volatiles from a wheat seedling infested with aphids at a high population density (ca. 9 aphids/cm^2). Four compounds, 6-methyl-5-hepten-2-one, (−)- and (+)-6-methyl-5-hepten-2-ol, and 2-tridecanone, were identified by GC-MS in air entrainments from the wheat seedlings with high aphid density but not from the wheat seedlings alone. The mixture of the four compounds in the natural proportion counteracted the attractivity of the volatiles from the intact uninfested wheat seedling. The likely role of these compounds in the spacing behavior of this aphid species, when present in high densities on wheat, is discussed.

Key Words—Spacing pheromones, olfactometry, aphids, wheat.

INTRODUCTION

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Homoptera: Aphididae), behaves as a holocyclic species with alternating winter and summer hosts in the most temperate areas of its distribution. The winter primary host is *Prunus padus* L. (Rosaceae), and several Gramineae (= Poaceae) may serve as secondary

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summer hosts. Volatile semiochemicals have been demonstrated as mediating several key processes in the behavioral ecology of *R. padi*. Thus, during the autumn migration from Gramineae, the gynoparae react positively to primary host-plant semiochemicals (Pettersson, 1970a), and a pheromone associated with the gynoparae is possibly involved in the settling of this morph in the canopy of *P. padus* (Pettersson, 1993). The sex pheromone isolated from several aphid species (Pickett et al., 1992) is attractive towards males of *R. padi* and is synergized by benzaldehyde, a major volatile in *P. padus*. Furthermore, the volatile primary host-plant component, methyl salicylate, switches off during the spring, with the aggregation odor stimulus keeping the colonies of fundatrices together on *P. padus* (Pettersson, 1994).

Laboratory experiments have shown that the attraction of *R. padi* to oat, one of its graminaceous summer hosts, is mediated by plant volatiles (Quiroz and Niemeyer, 1998a). Mobility of apterae of *R. padi* within the secondary summer hosts has been examined, and a density dependent deaggregation behavior was attributed to volatiles emitted by the aphid–plant complex (Pettersson et al., 1994, 1995). This work extends the preliminary study on the relationship between density and mobility of *R. padi* (Pettersson et al., 1995), with the aim of identifying compounds involved in the mutual repellence of aphids present at high densities on wheat seedlings.

METHODS AND MATERIALS

Aphids. Aphids for starting the cultures were collected in grass fields near the Laboratorio de Química Ecológica in Santiago, Chile, and maintained on wheat or oats for at least five generations in a greenhouse at 18–22°C, a 18L:6D photoperiod, and 50–70% relative humidity. All individuals used in the experiments had recently molted to adults and were in an active food site-searching mood. Additionally, experiments were performed in the middle of the day since a diurnal rhythm in the readiness of an aphid to respond to olfactory stimuli may also give rise to variation among individuals.

Olfactometry. Behavioral assays were performed in an olfactometer originally described by Pettersson (1970b). One aphid was enclosed in an arena permeated by air coming from each of its four outstretched arms and drawn out through a hole above the center of the arena. The test stimulus was applied to a filter paper that was placed in the stimulus arm of the olfactometer. Equivalent amounts of solvent were placed on filter papers in the control arms. The aphid was constantly under observation for 15 min, and the time spent in each arm was noted. The olfactometer rotated every minute. Each experiment was replicated 10 times, and results were analyzed using nonparametric statistics (Wil-

coxon one-tailed rank-sum test for two groups), the total time spent in the treatment arm being compared with the mean time spent in the control arms.

Entrainment of Volatiles. Volatiles were entrained from two different sources: wheat (*Triticum aestivum* L. cv. Ciko) seedlings at growth stage 12 (Zadoks et al., 1974), and wheat seedlings with apterae of *R. padi* at high density (9 aphids/cm^2). Air, dried and purified by passage through activated 5 Å molecular sieves and charcoal, was drawn at 1 liter/min for 48 hr through two bell jars containing the odor sources. Volatiles were adsorbed onto Porapak Q and were desorbed by elution with freshly distilled diethyl ether (Pettersson et al., 1994). The resulting extract was concentrated under a stream of nitrogen and stored in sealed glass ampoules at −20°C prior to analysis. Aliquots of extract used in olfactometry contained between 10 and 60 ng of the repellent semiochemicals identified.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). Volatile extracts obtained as above were injected into a capillary GC column (CBP20, 25 m × 0.25 mm ID) directly coupled to a mass detector with an integrated data system (GC model HP5890, MD model HP5972). Ionization was by electron impact at 70 eV and 280°C. The GC oven was maintained at 35°C for 5 min and then programmed to increase at 5°C/min to 200°C. Tentative identifications by GC-MS were confirmed using Kovats indices and coinjection of authentic samples when available. The pure chemicals (>99% purity) were obtained from Aldrich and diluted in hexane for behavioral assays. Chromatograms were recorded as total ion current (TIC) in time (minutes).

Determination of Enantiomeric Composition of 6-Methyl-5-hepten-2-ol. One of the compounds identified in entrainments from wheat seedlings infested with a high density of aphids was 6-methyl-5-hepten-2-ol. The proportion of enantiomers present in the extract was determined by esterification of the commercial racemate of the alcohol with (1*S*)-(−)-camphanic chloride (Aldrich Chem. Co.) to obtain the diastereomeric esters, microesterification of the extract with (1*S*)-(−)-camphanic chloride, and quantification of the peaks generated by calibration lines produced with the pure diastereomeric esters. Pure enantiomeric alcohols were generated by hydrolysis of the corresponding diastereomeric esters (Quiroz and Niemeyer, 1998b).

RESULTS

Summer generation apterae of *R. padi* were attracted in the olfactometer towards volatiles from an undamaged wheat seedling and were repelled by a wheat seedling with a high aphid density (Table 1). Entrainment volatiles produced by these systems were attractive and repellent towards apterae of *R. padi*, respectively (Table 1).

TABLE 1. RESPONSE OF APTERAE OF *R. padi* INTRODUCED SINGLY INTO AN OLFACTOMETER TO UNINFESTED WHEAT SEEDLINGS, WHEAT SEEDLINGS INFESTED WITH APHIDS, AND ENTRAINMENTS OF AIR ABOVE WHEAT SEEDLING AND WHEAT SEEDLING INFESTED WITH APHIDS

Stimulus applied	Average time spent in each arm (min)	*P*
Wheat seedling	5.11 ± 0.16	0.0014
Blank	2.78 ± 0.13	
Wheat seedling with a high density of apterous *R. padi* (9 aphid/cm^2)	2.13 ± 0.20	0.0018
Wheat seedling	4.24 ± 0.06	
Volatiles from wheat seedling (10 μl)	5.35 ± 0.15	0.0015
Solvent (10 μl)	2.78 ± 0.05	
Volatiles (10 μl) from wheat seedling with high density of apterous *R. padi* (9 aphids/cm^2)	2.17 ± 0.21	
Volatiles from wheat seedling (10 μl)	4.17 ± 0.07	0.0018

Figure 1 shows the GC-MS analysis of an entrainment sample from a wheat seedling with high aphid density and the identity of compounds present. Peaks labeled A, B, and C, were present only in entrainments from wheat seedlings with aphids at high population density. These compounds were identified by

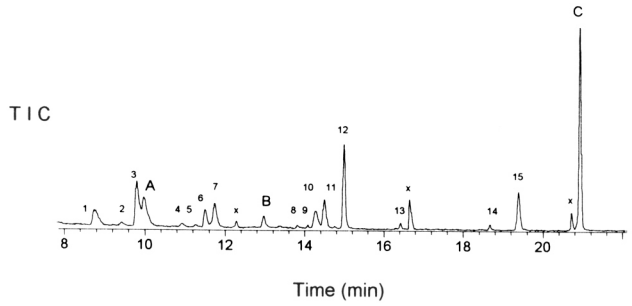


FIG. 1. Trace from the gas chromatographic run of air entrainments from a wheat seedling infested with a high density of aphids. Identification of peaks is a follows: 1, octanal; 2, (*Z*)-3-hexenyl acetate; 3, (*E*)-2-hexenyl acetate; A, 6-methyl-5-hepten-2-one; 4, (*Z*)-3-hexenol; 5, nonanal; 6, (*E*)-2-hexenol; 7, (Z)-2-hexenol; B, 6-methyl-5-hepten-2-ol; 8, decanal; 9, pentadecane; 10, camphor; 11, benzaldehyde; 12, linalool; 13, hexadecane; 14, heptadecane; 15, naphthalene; 16, octadecane; C, 2-tridecanone.

coupled GC-MS as 6-methyl-5-hepten-2-one (peak A, MHO), 6-methyl-5-hepten-2-ol (peak B, MHOH), and 2-tridecanone (peak C, 2-T). MHOH consisted of the (+) and the (−) enantiomers in a 1:3 ratio.

Identifications were confirmed with Kovats indices and peak enhancement on coinjection with authentic commercial samples. The absence of these compounds in entrainments from uninfested wheat seedlings was confirmed by single ion monitoring (SIM) in the corresponding regions of the chromatogram using characteristic or abundant ions from MHO (111, 83, 69, 55), MHOH (110, 69, 55), and 2-T (183, 85, 71). These ions were absent from the SIM runs.

In the olfactometer, MHO, MHOH [1:3 mixture of (+) and (−) enantiomers] and 2-T were repellent to apterae of *R. padi* either as pure compounds or as a mixture in the proportions occurring in the natural samples (Table 2) or when combined as the stimulus with an undamaged wheat seedling (Table 3). The compounds also exhibited dose-dependent repellent activity with respect to a blank (Figure 2).

DISCUSSION

Previous investigations have demonstrated that walking apterae of *R. padi* avoid oat plants with aphid colonies above a certain density (Pettersson et al., 1995). Olfactometer experiments were designed to confirm these observations using wheat as the aphid host plant. Odors released from a wheat seedling

TABLE 2. RESPONSE OF APTERAE OF *R. padi* INTRODUCED SINGLY INTO AN OLFACTOMETER AND EXPOSED TO CHEMICALS PRESENT IN AIR ENTRAINED FROM WHEAT SEEDLINGS WITH APHIDS IN HIGH DENSITY

Stimulus applied*	Average time spent in each arm (min)	*P*
0.01 μg of MHO	2.48 ± 0.77	
Hexane	3.73 ± 0.60	0.0375
0.01 μg of MHOH [1:3 mixture of (+) and (−) enantiomers]	2.71 ± 0.16	0.008
Hexane	3.52 ± 0.26	
0.01 μg of 2-hexane	2.61 ± 0.07	0.045
Hexane	3.85 ± 0.21	
0.01 μg of MHO, MHOH [1:3 mixture of (+) and (−)-enantiomers], and 2-T in ratio 3:1:10	2.11 ± 0.44	0.005
Hexane	3.97 ± 0.48	

*MHO = 6-methyl-5-hepten-2-one; MHOH = 6-methyl-5-hepten-2-ol; 2-T = 2-tridecanone.

TABLE 3. RESPONSE OF APTERAE OF *R. padi* INTRODUCED SINGLY INTO AN OLFACTOMETER TO WHEAT SEEDLING AND WHEAT SEEDLING PLUS DIFFERENT CHEMICALS CONTAINED IN A GLASS CAPILLARY TUBE PLACED INSIDE BELL JAR CONTAINING WHEAT SEEDLING

Stimulus applied[a]	Average time spent in each arm (min)	*P*
Undamaged wheat	5.22 ± 0.23	0.0013
Blank	2.49 ± 0.11	
Undamaged wheat + 10 μg of MHO	2.17 ± 0.09	0.0051
Undamaged wheat	4.46 ± 0.14	
Undamaged wheat + 10 μg of MHOH [1:3 mixture of (+) and (−) enantiomers]	2.36 ± 0.08	
Undamaged wheat	5.01 ± 0.85	0.015
Undamaged wheat + 10 μg 2-T	2.30 ± 0.20	0.045
Undamaged wheat	4.11 ± 0.18	
Undamaged wheat + 10 μg of MHO, MHOH [1:3 mixture of (+) and (−) enantiomers], and 2-T in ratio 3:1:10	2.16 ± 0.09	
Hexane	5.15 ± 0.85	0.0015

[a]MHO = 6-methyl-5-hepten-2-one; MHOH = 6-methyl-5-hepten-2-ol; 2-T = 2-tridecanone.

without aphids attracted apterae of *R. padi*, and the odors released by the plant infested with a high density (9 aphids/cm^2) of apterous *R. padi* repelled apterae of *R. padi*. Similar effects were obtained with air entrained from the systems MHO, MHOH [1:3 mixture of (+) and (−) enantiomers], and 2-T were found in entrainments from infested wheat but not from wheat alone. These compounds, either alone or in the natural mixture, elicited a repellent response similar to that shown by the volatiles released from wheat with a high aphid density, suggesting that they are the main active compounds in the volatile extract.

The present work does not establish the origin of the active compounds, i.e., whether they are released by the plant or by the aphid. Neither are the causes of the release addressed, i.e., a defense of the plant against high aphid infestation or a reaction of aphids to overcrowding and hence to overexploitation of its food source. The presence of any of these three compounds is rather frequent both in plants as well as in some animals, although seldom have their roles as semiochemicals been demonstrated. For example, MHO has been

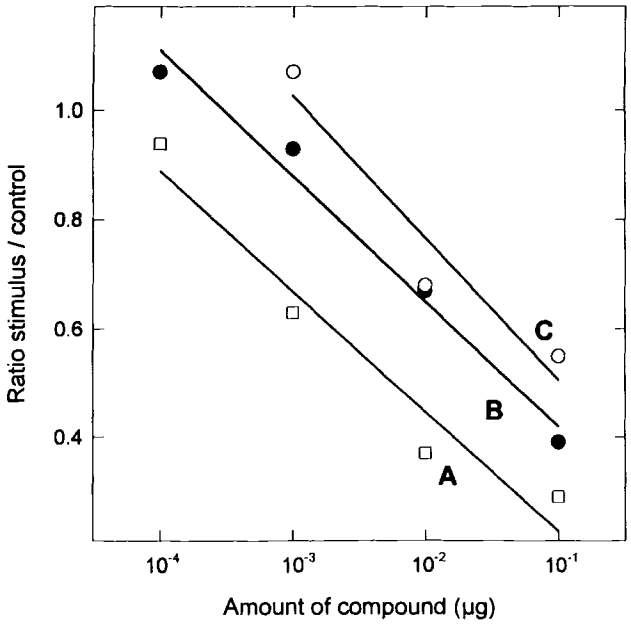


FIG. 2. Dose–response curves for the olfactometric activity of 6-methyl-5-hepten-2-one (A), 6-methyl-5-hepten-2-ol [1:3 mixture of (+) and (−) enantiomers] (B), and 2-tridecanone (C). Ratios smaller than 1 indicate a repellent effect of the stimulus.

reported as a defense compound released by bees (Davies and Madden, 1985; Hefetz et al., 1982; Tomalski et al., 1987; Wittmann et al., 1990; Voelkl et al., 1994), as an ant alarm pheromone that can be used by spiders as a kairomone (Allan et al., 1996), and as a sexual and spacing pheromone in the aphid hyperparasitoid *Alloxysta victrix* (Micha et al., 1993). MHOH has been reported as a population aggregation pheromone released by the beetle *Gnathotrichus sulcatus* (Byrne et al., 1974). The third substance, 2-T, an insect repellent of wide activity (Eisner et al., 1972), is present in secretions of several species of ants (do Nascimento et al., 1993) and bees (Fisher et al., 1993) but is also secreted by the glandular hairs of the wild tomato, *Lycopersicon hirsutum* f. *glabratum* (Solanaceae), and is toxic towards the aphid *Aphis gossypii* (Williams et al., 1980). It seems likely that in the present study the heptenone and the heptenol are emitted by the aphid and the 2-tridecanone is released by the plant. However, further studies are necessary to determine the origin of these substances.

The ecological effect of this spacing semiochemical complex is likely to be important as a colony density regulation mechanism in *R. padi*. The behavioral expression of this complex is defined in the present laboratory system as an increase in mobility (walking) by aphid individuals. In a natural environment,

other related consequences may be a higher sensitivity to disturbance (colony mates, wind, etc.) and prolonged time between probing impulses.

Application of the semiochemicals identified here in crop fields may have various effects. The first might be to repel aphids towards places where the semiochemicals have not been sprayed, which in turn would reduce the aphid population in the crop. A second is that increased mobility could increase exposure of affected aphids to natural enemies and the risks of disorientation and accidents. Another is that time spent on walking as an adult could lead to a reduction in the time available for producing offspring on a plant. Finally, the aphid population could be distributed more evenly between plants in the stand, leading to a dilution of the stress on individual plants.

It is interesting to note that MHO has been described in the aphid hyperparasitoid *Alloxysta victrix* (Westwood) (Hymenoptera: Alloxystidae) as a sex and spacing pheromone (Micha et al., 1993) and as a semiochemical that disperses females of the aphid parasitoid *Aphidius uzbekistanicus* (Luzhetski) (Hymenoptera: Aphidiidae) (Höller et al., 1994) and also in the related species *Alloxysta brevis* as a semiochemical repelling attacks by the honeydew-collecting ant *Lasius niger* (Voelkl et al., 1994). Our finding that this compound also constitutes a spacing semiochemical in aphids points to it as a key element in the regulation of the predator–prey relationship in this multitrophic chain. The different sensitivity to this compound of the members of this chain may provide a unique mechanism for pest control in the sustaining crop.

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A SURVEY OF BIRDS ODOROUS OR UNPALATABLE TO HUMANS: POSSIBLE INDICATIONS OF CHEMICAL DEFENSE

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Abstract—We solicited information from field and museum ornithologists on the birds that they consider odorous and/or unpalatable in order to identify species that may use chemicals to deter predators. Ninety-two ornithologists from the Americas and Eastern Europe responded to our survey. Eighty genera and 10 families representing 17 orders, primarily Procellariiformes, Falconiformes, Psittaciformes, Cuculiformes, Piciformes, and Passeriformes, were cited as containing malodorous or uniquely odorous birds. Two orders (Opisthocomiformes and Trogoniformes), five families (Procellariidae, Cuculidae, Bucconidae, Picidae, and Furnariidae), and one subfamily (Drepanidinae) were reported to us as either containing many odorous species or consisting primarily of them. Thirty genera and three families representing 13 orders, primarily Passeriformes, were reported to us as unpalatable. The birds cited in our survey and those previously reported as odorous and/or unpalatable are tabulated. Our survey and review point to a number of taxa that may use chemicals to deter predators, although we acknowledge that compounds imparting aversive or unique odors or flavors may arise for a variety of reasons, e.g., as dietary by-products.

Key Words—Aves, birds, odorous, unpalatable, chemical defense.

INTRODUCTION

Interest in the possible use by birds of chemicals to deter predators has been stimulated by the discovery of homobatrachotoxin, a neurotoxic alkaloid first isolated from Colombian poison-dart frogs, in the feathers, skin, and other

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tissues of pitohuis (*Pitohui* spp., Passeriformes) from New Guinea (Dumbacher et al., 1992). The reputed inedibility of the hooded pitohui (*P. dichrous*), referred to by native New Guineans as a "rubbish bird" not to be eaten unless specially prepared, and the numbness, burning sensation, and sneezing experienced by the field biologists collecting it, prompted the chemical investigation of this and two related species, the variable pitohui (*P. kirhocephalus*) and the rusty pitohui (*P. ferrugineus*). *P. dichrous* and *P. kirhocephalus* also are reported to emit a strong "sour" odor (Dumbacher et al., 1992). This scent is not attributed to homobatrachotoxin, but it may constitute an aposematic cue to potential predators denoting the toxicity of these birds (see Diamond, 1992, 1994).

As Glendining (1993) remarks, appropriate tests of relevant predators are needed to document avian chemical defenses. Nonetheless, the olfactory and gustatory perceptions of humans often have suggested chemically protected organisms. Indeed, humans are natural predators for some species and may be appropriate subjects in studies of chemical defense.

We conducted a survey of ornithologists for their opinions of the birds that they consider odorous and/or unpalatable in order to identify species that may use chemicals to deter predators. Our survey was similar to one conducted more than 50 years ago by Cott (1945a,b), who solicited opinions on the edibility of nongame birds in order to assess the possible correlation between unpalatability and conspicuous plumage (cf., Götmark, 1994). In addition to summarizing our survey results and original observations, we tabulate here other bird species reported to be odorous and/or unpalatable.

METHODS AND MATERIALS

We solicited information from field and museum ornithologists on the birds that they consider odorous (intact or skinned) or unpalatable (cooked). We referred to the membership list of the American Ornithologists' Union for most of our contacts. Opinions relating only to free-ranging birds were sought to avoid instances in which odors or flavors might arise from unnatural diets or as other artifacts of captivity. We do not consider here suggestions on the unpalatability of eggs or the toxicity of birds; neither do we consider the possible distastefulness of feathers, a potentially important aspect of avian chemical defense. We adopt the nomenclature of the American Ornithologists' Union (1983 and supplements) for most American species and that of Peters (1931–1986) for most others.

RESULTS AND DISCUSSION

Ninety-two ornithologists from Argentina, Bahamas, Belize, Brazil, Canada, Chile, Colombia, Costa Rica, Denmark, France, Germany, Jamaica, Mex-

ico, Netherlands, Panama, Peru, Trinidad, United Kingdom, and United States, including Puerto Rico, responded to our survey (see Acknowledgments). Most respondents reported on birds that are malodorous or uniquely odorous; in this respect, 80 genera and 10 families representing 17 orders, primarily Procellariiformes, Falconiformes, Psittaciformes, Cuculiformes, Piciformes, and Passeriformes, were cited (Table 1). Two orders (Opisthocomiformes and Trogoniformes), five families (Procellariidae, Cuculidae, Bucconidae, Picidae, and Furnariidae), and one subfamily (Drepanidinae) were reported to us as either containing many odorous species or consisting primarily of them.

Procellariiforms are widely acknowledged to be malodorous (e.g., Murphy, 1936; Thomson, 1964; Warham, 1990; Sick, 1993), thus it is not surprising that they were reported in our survey. The odors of some procellariids, at least, may be derived from the secreted stomach oils that they spray at offenders, in some cases, from distances of more than 2 m (Godman, 1907–1910; Warham, 1977; Jacob, 1982). Surface-nesting fulmars, including species of *Daption*, *Fulmarus*, and *Macronectes*, routinely spray these secretions in defense (Armstrong, 1951; Duffey, 1951; Warham et al., 1976); *F. glacialis* may do so even before hatching by protruding its beak through a hole in the shell (Lees, 1950). Predatory birds contacted by these stomach oils may be disabled and die as a result of damage to their plumage that impairs their abilities to fly and repel water (Swennen, 1974; Warham, 1977).

The turkey vulture (*Cathartes aura*) and the black vulture (*Coragyps atratus*) were the most frequently reported falconiforms in our survey. These birds, which are specialized scavengers, were described by several respondents as emitting a carrion-like scent. Hocking (personal communication) and Inigo-Elias (personal communication) state that the carcasses of vultures themselves are ignored by scavenging animals (see Cott, 1947), an impression that should be tested because it may reflect the unacceptability of these birds to predators.

A number of cuculiforms were reported to us as malodorous, primarily anis (*Crotophaga* spp.). We also have detected a strong odor emitted by the groove-billed ani (*C. sulcirostris*) from Veracruz, Mexico. Sick (1993) reported that the odor of *Crotophaga* spp. can be detected several meters away when these birds gather in groups. Davis (1940) stated that the entire Crotophaginae possesses a disagreeable scent.

The hoatzin (*Opisthocomus hoazin*, Opisthocomiformes) was cited frequently in our survey. This species, which is well known for its foul odor, is dubbed "stink bird," "stinking pheasant," and similar folk-names throughout its range in northern South America (e.g., Young, 1888; Kirke, 1898; Cott, 1947; Austin, 1961). Cutright (1940, p. 190) states that because of its strong odor "the natives never eat the hoatzin, and it may well be that other animals are similarly repelled by its flesh." *O. hoazin* is the only known avian folivore to engage in pregastric fermentation (Grajal et al., 1989), a process by which it presumably generates its stench (e.g., Sick, 1993). High concentrations of

TABLE 1. BIRDS REPORTED IN OUR SURVEY AND THOSE PREVIOUSLY DESCRIBED AS ODOROUS AND/OR UNPALATABLE TO HUMANS[a]

Order Family/Subfamily Genus Species	Respondents (*N*) and references	
	Odorous	Unpalatable
Gaviiformes		
Gaviidae		
Gavia arctica	0	0 Vesey-Fitzgerald in Cott, 1947
G. immer	0	0 Selby, 1825–1833; Wilson and Bonaparte, 1871; Vesey-Fitzgerald in Cott, 1947
G. stellata	0	0 Fisher in Cott, 1947; Vesey-Fitzgerald in Cott, 1947
Podicipediformes		
Podicipedidae		
Podiceps cristatus	0	0 Andersson, 1872
Procellariiformes	1 Murphy, 1936; Thomson, 1964; Warham, 1990; Sick, 1993	0
Procellariidae	1 Welty and Baptista, 1988	0
Bulweria bulwerii	0 Thibault and Holyoak, 1978	0
Daption capense	1 Andersson, 1872; Godman, 1907–1910	0
Fulmarus glacialis	0	0 Fisher in Cott, 1947
F. g. rodgersi	0	0 Godman, 1907–1910
F. glacialoides	1	0
Macronectes gigantea	1 Matthews in Cott, 1947	0
Pachyptila belcheri	1	0
P. desolata	1	0
Procellaria aequinoctialis	1	0
Pterodroma rostrata	0 Thibault and Holyoak, 1978	0
Puffinis spp.	0 Thibault and Holyoak, 1978	0

P. griseus	1	0
P. lherminieri	1	0
Hydrobatidae	0 Thibault and Holyoak, 1978	0
Oceanites gracilis	1	0
O. oceanicus	1	0
Oceanodroma hornbyi	1	0
Spheniscifformes		
Spheniscidae		
Spheniscus humboldti	1	0
Pelecaniformes		
Sulidae		
Morus bassanus	*0 Cott, 1947	0
M. capensis	*0 Jarvis in Cott and Benson, 1970	0
Pelecanidae		
Pelecanus thagus	1	0
Phalacrocoracidae		
Phalacrocorax aristotelis	0	0 Macpherson, 1897
P. brasilianus	0	0 Chubb, 1916
P. capensis	*0 Layard and Sharpe, 1875–1884	0
P. carbo	0 Bolam, 1913	0 Simon, 1981
Anhingidae		
Anhinga anhinga	0 Bartram in Wilson and Bonaparte, 1871	0 Bartram in Wilson and Bonaparte, 1871
Fregatidae		
Fregata andrewsi	*0 Cott and Benson, 1970	0
Ciconiiformes		
Ardeidae		
Ardea cinerea	0	0 Vesey-Fitzgerald in Cott, 1947; Goodman and Hobbs, 1990
Bubulcus ibis ibis	0	0 Messel in Cott, 1947
Casmerodius albus	0	1
Scopidae		
Scopus umbretta	0	0 Cott and Benson, 1970

TABLE 1. CONTINUED

Order Family/Subfamily Genus Species	Respondents (N) and references	
	Odorous	Unpalatable
Threskiornithidae		
Endocimus ruber	0	0 Cott, 1947
Geronticus eremita	*0 Meinertzhagen, 1940	0
Anseriformes		
Anatidae		
Aix galericulata	0	0 Simon, 1981
Alopochen aegyptiaca	0	0 Andersson, 1872; Horsbrugh, 1912; Meinertzhagen, 1930
Amazonetta brasiliensis	0	1
Anas spp.	1	1
A. acuta	0	0 Phillips, 1922–1926; Simon, 1981
A. clypeata	0	0 Hume and Marshall, 1879; Baker, 1908; Gibson, 1920
A. georgica	0	0 Steinen, 1890 cited in Phillips, 1922–1926
A. penelope	0	0 Selby, 1825–1833; Hume and Marshall, 1879; Millais, 1902
A. platyrhynchos	1	0 Simon, 1981
A. querquedula	0	0 Mallais, 1902; Simon, 1981
A. strepera	0	0 Belding in Grinnell et al., 1918
Anseranas semipalmata	0 Thomson, 1964	0
Aythya spp.	1	0
A. affinis	0	0 Phillips, 1922–1926
A. ferina	0	0 Baker, 1921–1930; Phillips, 1922–1926
A. fuligula	0	0 Hume and Marshall, 1879; Dixon, 1900

A. marila	0	0 Hume and Marshall, 1879; Phillips, 1922–1926
A. novaeseelandiae	0	0 Simon, 1981
A. nyroca	0	0 Baker, 1908, 1921–1930
Biziura lobata	0 Mellor in Mathews, 1914–1915; Thomson, 1964; Simon, 1981; Welty and Baptista, 1988	0 Phillips, 1922–1926; Simon, 1981
Bucephala albeola	0	0 Simon, 1981
Cairina spp.	1	0
C. moschata	0 Thomson, 1964; Simon, 1981	0 Heinroth, 1911 cited in Phillips, 1922–1926
Chloephaga hybrida	0 Hamilton in Cott, 1947	0 Murphy, 1936; Cott, 1964
Clangula hyemalis	0	0 Phillips, 1922–1926
Cygnus olor	0	0 Duke of Bedford in Cott, 1947
Dendrocygna bicolor	0	0 Horsbrugh, 1912
D. javanica	0	0 Hume and Marshall, 1879
Marmaronetta angustirostris	0	0 Chapman and Buck, 1978
Melanitta fusca	0	0 Simon, 1981
M. nigra	0	0 Yarrell, 1884–1885; Dixon, 1900; Phillips, 1922–1926; Simon, 1981
M. perspicillata	0	0 Dixon, 1900; Simon, 1981
Mergellus albellus	0	0 Dixon, 1900; Baker, 1908; Cott, 1964
Mergus merganser	*1	2 Hume and Marshall, 1879; Dixon, 1900; Baker, 1908; Davidson in Cott, 1947
M. serrator	0	0 Phillips, 1922–1926
Netta rufina	0	0 Hume and Marshall, 1879
Oxyura spp.	1	0
O. leucocephala	0	0 Radde, 1884 cited in Phillips, 1922–1926
Plectropterus gambensis	0	0 Phillips, 1922–1926
Rhodonessa caryophyllacea	0	0 Simson, 1884
Somateria mollissima	0	0 Phillips, 1922–1926
Tachyeres brachypterus	0 Hamilton in Cott, 1947	0 Cunningham, 1868; Hamilton in Cott, 1947

TABLE 1. CONTINUED

Order Family/Subfamily Genus Species	Respondents (N) and references	
	Odorous	Unpalatable
Tadorna cana	0	0 Horsbrugh, 1912
T. ferruginea	0	0 Hume and Marshall, 1879; Dixon, 1990
T. radjah	0	0 Hubbard, 1907
T. tadorna	0 Yarrell, 1884–1885	0 Hume and Marshall, 1879; Yarrell, 1884–1885; Phillips, 1922–1926; Matthews, Meinertzhagen, Vesey-Fitzgerald in Cott, 1947, 1964; Simon, 1981
Thalassornis leuconotus	0	0 Stark and Sclater, 1906; Simon, 1981
Anhimidae		
Anhima cornuta	0	1
Falconiformes		
Cathartidae		
Cathartes spp.	4	1
C. aura	7 Wilson and Bonaparte, 1871; Cott, 1947	3
C. burrovianus	1	0
C. melambrotus	1	0
Coragyps atratus	5	1
Sarcoramphus papa	2 Sick, 1993	0
Accipitridae		
Accipiter nisus	0	0 Vesey-Fitzgerald in Cott, 1947
Gyps rueppellii	0	0 Meinertzhagen in Cott, 1947
Pandion haliaetus	1	0
Falconidae		
Caracara spp.	1	0

Daptrius americana	1	0
D. ater	1	0
Galliformes		
Megapodiidae		
Megapodius freycinet	‡0 Diamond, 1994	0
Phasianidae		
Centrocercus urophasianus	0	1 Simon, 1981
Francolinus africanus gutturalis	0	0 Blanford, 1870
Ithaginis cruentus	0	0 Meinertzhagen in Cott, 1947
Lophura leucomelanos	0	0 Gammie cited in Baker, 1921–1930; Simon, 1981
Tetrao urogallus	0	0 Lloyd, 1867; Morris, 1897; Dixon, 1900
Tragopan satyra	0	0 Meinertzhagen in Cott, 1947
Gruiformes		
Gruidae		
Grus antigone	0	0 Meinertzhagen in Cott, 1947
Rallidae		
Fulica spp.	1	0
Limnocorax flavirostra	0	0 Cott and Benson, 1970
Otidae		
Afrotis afra	0	0 Meinertzhagen, 1951
Choriotis kori	0	0 Jackson, 1938
Neotis cafra	0	0 Horsbrugh, 1912
N. cafra denhami	0	0 Uys in Cott and Benson, 1970
Charadriiformes		
Chionididae		
Chionis alba	0 Wood, 1862	0
Haematopodidae		
Haematopus ostralegus	0 Naumann, 1905	0 Naumann, 1905, Cott, 1964
Charadriidae		
Vanellus armatus	0	0 Cott and Benson, 1970
V. crassirostris	0	0 Cott and Benson, 1970
V. vanellus	0	0 Dixon, 1900

TABLE 1. CONTINUED

Order Family/Subfamily Genus Species	Respondents (N) and references	
	Odorous	Unpalatable
Recurvirostridae		
Recurvirostra avosetta	0	0 Andersson, 1872
Jacanidae		
Microparra capensis	0	0 Cott and Benson, 1970
Scolopacidae		
Calidris testaceus	0	0 Andersson, 1872
Tringa melanoleuca	0	0 Herbert, 1873
T. stagnatilis	0	0 Cott and Benson, 1970
Thinocoridae		
Attagis gayi	1	1
Thinocorus orbignyianus	1	1
Glareolidae		
Pluvianus aegyptius	0	0 Cott, 1964
Laridae		
Larus atricilla	0	0 Irvine, Fisher in Cott, 1947
L. hyperboreus	0	0 Irvine, Fisher in Cott, 1947
L. scoresbii	*1	0
Phaetusa simplex	1	0
Rhynchops niger	1	0
Sterna superciliaris	1	0
Alcidae		
Aethia cristatella	1 Thomson, 1964	0
Fratercula arctica	0	0 Simon, 1981
Uria aalge	0	0 Simon, 1981
U. lomvia	0	0 Fisher in Cott, 1947

Columbiformes		
Columbidae		
Columba spp.	0	1
C. arquatrix	0	0 Jackson, 1938
C. cayennensis	0	1
C. palumbus	0	0 Bolam, 1913; Sick, 1993
C. plumbea	0	1
C. rufina	0	0 Chubb, 1916
C. subvinacea	0	1
Hemiphaga novaeseelandiae	0	0 Buller, 1873
Nesoenas mayeri	0	0 Temple, 1994
Streptopelia turtur	0	0 Meinertzhagen in Cott, 1947
Treron pompadora phayrei	0	0 Baker, 1913
T. waalia	0	0 Meinertzhagen in Cott, 1947
Psittaciformes		
Psittacidae		
Amazona spp.	2	0
A. albifrons	1	0
Anodorhynchus spp.	1	0
Ara spp.	2	0
Aratinga spp.	2	0
Bolborhynchus spp.	1	0
Brotogeris spp.	1	0
Forpus spp.	1	0
Myiopsitta spp.	1	0
Pionites spp.	1	0
Pionopsitta spp.	1	0
Pionus spp.	1	0
Pyrrhura spp.	1	0
Touit spp.	1	0
Cuculiformes		
Cuculidae	1	0
Centropus menbeki	‡0 Diamond, 1994	0

TABLE 1. CONTINUED

Order Family/Subfamily Genus Species	Respondents (*N*) and references	
	Odorous	Unpalatable
Chrysococcyx caprius	0	0 Cott and Benson, 1970
C. cupreus intermedius	0	0 Roberts in Cott, 1947
C. klaas klaas	0	0 Roberts in Cott, 1947
Coccyzus americanus	1	0
Cuculus clamosus	0	0 Cott and Benson, 1970
C. solitarius	0	0 Roberts in Cott, 1947
Dromococcyx phasianellus	1	0
Hyetornis pluvialis	3	0
Cuculidae/Crotophaginae	0 Davis, 1940	0
Crotophaga spp.	3	0
C. ani	7	0 Quelch in Chubb, 1916
C. major	3	1
C. sulcirostris	5	0
Guira guira	1	0
Musophagidae		
Musophaga violacea rossae	0	0 Meinertzhagen in Cott, 1947
Tauraco fischeri	0	0 Meinertzhagen in Cott, 1947
Opisthocomiformes		
Opisthocomidae		
Opisthocomus hoazin	6 Young, 1888; Kirke, 1898; Brown, Schomburgk in Chubb, 1916; Cutright, 1940; Cott, 1947; Austin, 1961; Thomson, 1964; Sick, 1993; Thomas, 1996	4 Cott, 1964
Strigiformes		
Strigidae		
Otus spp.	*1	0

Caprimulgiformes		
Steatornithidae		
Steatornis caripensis	0 Snow, 1961	0
Caprimulgidae (some species)	*2	1
Nyctibiidae (some species)	*1	0
Apodiformes		
Apodidae		
Apus melba	0	0 Meinertzhagen in Cott, 1947
Trochilidae (some species)	1	2
Trogoniformes	*1 Sick, 1993	0
Coraciiformes		
Upupidae		
Rhinopomastus cyanomelas	0 Marshall, 1902	0 Vesey-Fitzgerald in Cott, 1947
Upupa epops	0 Wood, 1862; Gillard, 1958; Austin, 1961; Thomson, 1964; Vaurie, 1973; Welty and Baptista, 1988	0
Phoeniculidae		
Phoeniculus bellei jacksoni	0 Granvik in Jackson, 1938	0
P. purpureus	1 Marshall, 1902; Bannerman, 1930–1951; Cott and Benson, 1970; McLachlan and Liversidge, 1971	1 Roberts in Cott, 1947
P. p. marwitzi	0 Belcher, 1930; Jackson, 1938	0
P. p. erythrorhyncus	0 Andersson, 1872	0
Momotidae		
Baryphthengus martii	1	0
Momotus momota	2	0
Alcedinidae	0	0 Cott, 1964
Alcedo atthis	0 Naumann, 1905	0
A. cristata	0	0 Roberts in Cott, 1947
Ceryle rudis	0	0 Cott and Benson, 1970
Halcyon albiventris	0	0 Roberts in Cott, 1947
H. chelicuti damarensis	0	0 Roberts in Cott, 1947
Ispidina picta natalensis	0	0 Roberts in Cott, 1947

TABLE 1. CONTINUED

Order Family/Subfamily Genus Species	Respondents (N) and references	
	Odorous	Unpalatable
Piciformes		
Galbulidae	*0 Sick, 1993	0
Bucconidae	3	0
Bucco spp.	1	0
B. macrorhynchos	1	0
Chelidoptera tenebrosa	1	0
Malacoptila spp.	1	0
M. panamensis	4	0
M. fusca semicincta	1	0
Micromonarcha spp.	1	0
Monasa spp.	1	0
M. nigrifrons	1	0
Nonnula spp.	1	0
M. brunnea	1	0
Notharchus spp.	1	0
Nystalus spp.	1	0
Capitonidae		
Eubucco richardsoni	1	0
Indicatoridae		
Indicator indicator	0	0 Cott and Benson, 1970
Picidae	1	0
Campephilus spp.	*1	0
Celeus spp.	*3	1
C. brachyurus	1	0
C. castaneus	1	0
C. elegans	1	0

Dendrocopos hyperythrus	1	0
D. major	0 Naumann, 1905	0
D. syriacus	*0 Cott, 1947	0
Dryocopus spp.	1	0
Hemicircus canente	0 Bock and Short, 1971	0
H. concretus	0 Bock and Short, 1971	0
Melanerpes spp.	1	0
M. carolinus	1	0
Picus viridis	0 Naumann, 1905	0
Veniliornis spp.	*2	0
Rhamphastidae (some species)	1	0
Bucerotiformes		
Bucerotidae	0	0 Cott, 1964
Ceratogymna brevis	0 Swynnerton, 1916b	0 Meinertzhagen in Cott, 1947
Passeriformes		
Furnariidae	2	0
Synallaxis albescens	1	0
S. brachyura	1	0
S. gujanensis	1	0
S. unirufa	1	0
Dendrocolaptidae (some species)	1 Sick, 1933	0
Rhinocryptidae		
Psilorhamphus guttatus	0 Sick, 1993	0
Scytalopus spp.	1	0
Tyrannidae		
Mionectes oleagineus	0	1
M. olivaceaus	0	1
M. striaticollis	0	1
Onychorhyncus coronatus	0 Sick, 1993	0
Pachyramphus spp.	0	1
Pyrocephalus rubinus	0	1
Tityra spp.	0	1
Tyrannus spp.	0	1
T. tyrannus	0	1

TABLE 1. CONTINUED

Order Family/Subfamily Genus Species	Respondents (N) and references	
	Odorous	Unpalatable
Cotingidae		
Ampelion spp.	0	1
Conioptilon mcilhennyi	0	1
Lipaugus spp.	0	1
Pipreola spp.	0	1
P. riefferii	0	1
Porphyrolaema porphyrolaema	0	1
Pipridae	0	1
Alaudidae		
Calandrella cinerea	0	0 Cott and Benson, 1970
Mirafra nigricans	0	0 Cott and Benson, 1970
Campephagidae		
Campephaga phoenicea flava	0	0 Roberts in Cott, 1947
Coracina caesia	0	0 Roberts in Cott, 1947
Pachycephalidae		
Pitohui dichrous	0 Dumbacher et al., 1992	0
P. kirhocephalus	0 Dumbacher et al., 1992	0
Monarchidae		
Terpsiphone viridis	0	0 Roberts in Cott, 1947; Cott and Benson, 1970
Paradisaeidae		
Epimachus albertisii	0	0 Pratt, 1906
Corvidae		
Corvus corax	1	0
Pica pica	0	0 Vesey-Fitzgerald in Cott, 1947
Dicruridae	0	0 Cott, 1964

Paridae		
Parus leucomelas	0	0 Cott and Benson, 1970
P. niger	0	0 Cott and Benson, 1970
Certhiidae		
Salpornis spilonotus	0	0 Cott and Benson, 1970
Sylviidae		
Eremomela icteropygialis	0	0 Cott and Benson, 1970
Muscicapidae		
Cossypha heuglini	0	0 Cott and Benson, 1970
Entomodestes spp.	0	1
Ifrita kowaldi	0	0 Majnep and Bulmer, 1977
Myadestes spp.	0	1
Myrmecocichla arnoti	0	0 Cott and Benson, 1970
M. nigra	0	0 Cott and Benson, 1970
Neocossyphus rufus	*0 Ziegler, 1971	0
Platycichla spp.	0	1
Turdus spp.	0	1
T. viscivorus	0	0 Meinertzhagen in Cott, 1947
Laniidae		
Lanius excubitor	0	0 Meinertzhagen in Cott, 1947
Tchagra senegala	0	0 Cott and Benson, 1970
Sturnidae		
Sturnus vulgaris	2	1 Brown in Cott, 1947; Macpherson, 1897
Emberizidae		
Ergaticus ruber	0	0 Escalante et al., 1994
Hemispingus atropileus	1	0
H. a. calophrys	1	0
Sporophila aurita	1	0
Emberizidae/Icterinae	0 Sick, 1993	0
Cacicus spp.	2	0
C. cela	1	0
C. melanicterus	1	0
C. solitarius	1	0

TABLE 1. CONTINUED

Order Family/Subfamily Genus Species	Respondents (N) and references	
	Odorous	Unpalatable
C. uropygialis	2	0
Gnorimopsar spp.	1	0
Gymnostinops spp.	1	0
G. montezuma	1	0
Ocyalus latirostris	1	0
Psarocolius spp.	2	0
Quiscalus mexicanus	1	0
Q. niger	1	0
Q. quiscula	1	0
Catamblyrhynchidae		
Catamblyrhynchus diadema	1	0
Fringillidae		
Coccothraustes coccothraustes	0	0 Meinertzhagen in Cott. 1947
Pyrrhula pyrrhula	0	0 Naumann. 1905
Fringillidae/Drepanidinae	2 Perkins, 1901, 1903; Thomson, 1964; Pratt, 1992a,b	0
Chloridops kona	0 Perkins, 1893, 1901, 1903	0
Drepanis funerea	0 Perkins, 1895, 1901, 1903	0
Hemignathus spp.	0 Perkins, 1893, 1901, 1903	0
H. munroi	0 Perkins, 1893, 1901, 1903	0
H. obscurus	0 Perkins, 1893	0
H. parvus	0 Pratt, 1992a	0
H. sagittirostris	0 Pratt, 1992a	0
H. virens	0 Pratt, 1992a	0
Himatione sanguinea	0 Pratt, 1992a	0

Loxiodes bailleui	0 Perkins, 1893	0
Loxops coccineus	0 Pratt, 1992a	0
Oreomystis bairdi	0 Pratt, 1992a	0
O. mana	0 Engilis in Pratt, 1992a	0
Palmeria dolei	0 Perkins, 1895, 1903	0
Pseudonestor xanthophrys	0 Perkins, 1895; Henshaw, 1902	0
Psittirostra psittacea	0 Perkins, 1893, 1901; Henshaw, 1902	0
Rhodacanthis palmeri	0 Perkins, 1893	0
Vestiaria coccinea	0 Pratt, 1992a	0
Ploceidae		
Ploceus st. thomae	0 Bannerman, 1930–1950	0
Estrildidae		
Uraeginthus angolensis	0	0 Cott and Benson, 1970

[a]The number of survey respondents citing each taxon as odorous or unpalatable is given; our personal observations (described in text) are included. Birds that are malodorous after skinning (*) or immediately after death (‡) are indicated. Species cited in Cott and Benson (1970) include both those for which palatability scores were 4.9 ("distasteful") or lower on a scale of 2.0 ("inedible") to 9.0 ("excellent") and those described as unpalatable by members of the tasting panel.

volatile fatty acids typical of those produced by microbial fermentation have been detected in the crop and esophagus of this species (Grajal et al., 1989).

Among the coraciiforms cited as odorous in our survey was the green woodhoopoe (*Phoeniculus purpureus*). Short (personal communication) noted that the greater honey-guide (*Indicator indicator*), an obligate nest parasite, when raised by *P. purpureus*, retains the odor of its foster parents for a month or more after fledging. Another coraciiform, the hoopoe (*Upupa epops*), was not cited in our survey, but it is widely considered malodorous (e.g., Wood, 1862; Welty and Baptista, 1988). Austin (1961, p. 182) remarks that "hoopoes are notorious for their foul, smelly nests, from which they never remove droppings or food debris. In addition, the female has a strong repulsive musty odor that emanates from her preen gland, and is believed to have a protective function like attar of skunk." Female *U. epops* defend their nest by spraying encroaching predators with their odorous uropygial gland fluids (Gilliard, 1958). The young also defensively discharge these secretions, but adult males apparently do not (Vaurie, 1973).

The piciforms cited as odorous in our survey include primarily bucconids and picids. We have detected a pungent odor from the chestnut-colored woodpecker (*Celeus castaneus*, Picidae) in southern Veracruz, Mexico, a feature reported in congeners by several respondents in our survey. Woodpeckers of the genus *Hemicircus* are known to discharge a resin-scented secretion from a tuft of bristly feathers in the middle of their back (Bock and Short, 1971). This viscid exudate (function unknown) is not produced by a discrete gland but by "fat quills," specialized feathers that excrete lipids.

Numerous passeriforms, primarily icterines such as oropendolas (*Cacicus* spp.) and grackles (*Quiscalus* spp.), were described to us as odorous. We also have noted that the great-tailed grackle (*Q. mexicanus*) from Texas produces a disagreeable scent that clings to the skin and clothing after handling. Sick (1993) states that all icterines, especially colonial species, emit a strong odor from their feathers, not from their skin. Perez-Rivera (personal communication), on the other hand, suggests that the odor of the Greater Antillean grackle (*Q. niger*) emanates from its legs. These suggestions contrast with the common assumption that bird scents arise principally from the uropygial gland (e.g., Thomson, 1964; Welty and Baptista, 1988).

Among the odorous passeriforms reported to us are the Hawaiian honeycreepers (Drepanidinae), many of which emit a scent reminiscent of that of "old canvas" (Perkins, 1893, 1895, 1901, 1903; Pratt, 1992a, b). This so-called "drepanidine odor," which persists for years in museum specimens, is thought to be diagnostic of the subfamily, although it may be less noticeable in species from arid habitats (Pratt, 1992a) or during particular seasons (Perkins cited in Henshaw, 1902).

Thirty genera and three families representing 13 orders, primarily Passeriformes, were reported to us as unpalatable (Table 1). We received fewer reports of unpalatable taxa than of odorous ones, possibly because the consumption of birds other than domestic fowl is uncommon among modern field ornithologists. A number of unpalatable birds are cited in Cott (1947) and Cott and Benson (1970). Species indicated as unpalatable (primarily from first-hand experience) by these or other authors are included in Table 1.

Among the birds cited as unpalatable in our survey were several taxa occasionally consumed by humans, e.g., anatids. As Cott (1947) notes, opinions vary on the palatability of some species, which may be distasteful only in certain regions or during particular seasons (see also Chubb, 1916). Palatability also may vary according to the anatomical region of the bird as well as the diet, age, and sex of the individual (Swynnerton, 1916a; Cott, 1947; Simon, 1981). The possible influence of diet on the taste of bird flesh has been mentioned by many authors (e.g., Blanford, 1870; Buller, 1873; Hume and Marshall, 1879; Chubb, 1916; Jackson, 1938; Sick, 1993). Divergent opinions on the edibility of birds also may reflect human cultural differences in taste preference or experience (Goodman and Hobbs, 1990).

Our survey results and other anecdotal reports point to a number of odorous and/or unpalatable birds. Although our survey was undertaken to identify species that may use chemicals to deter predators, we acknowledge that compounds imparting unique or aversive odors or flavors may arise for a variety of reasons, including ectoparasite control (Poulsen, 1994; Mouritsen and Madsen, 1994), antimicrobial defense (Jacob and Ziswiler, 1982), pheromonal communication (Bohnet et al., 1991), orientation to nests (Thibault and Holyoak, 1978), or as mere dietary by-products.

Adventitious contact with foods, feces, or substrates also may account for the odors of some birds. The stench of cathartid vultures, for example, may result from contact with the carcasses on which these birds feed or with regurgitates discharged in response to threats (Rea, 1983). Some ducks may acquire the odors of the foul-smelling feces that they spray on their eggs before fleeing the nest when alarmed by predators (Kear, 1963; Swennen, 1968; Clark and Wobeser, 1997). Teixiera (personal communication) notes that a pungent odor often emanating from woodpeckers of the genera *Campephilus*, *Celeus*, and *Veniliornis* is similar to the scent of the termite (*Nasutitermes* spp.) colonies on which these birds feed. In addition, many passerines are known to smear ants and other odorous materials onto their plumage (e.g., Simmons, 1966; Erhlich et al., 1986; Clayton and Vernon, 1993), or they incorporate aromatic plants into their nest (e.g., Clark and Mason, 1985, 1988); these activities may serve to appropriate chemicals to control ectoparasites. Studies of the responses of predatory vertebrates, insects (Cott, 1947; Thiollay, 1991), and other consumers are needed to test the possible defensive properties of avian chemicals.

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Sean S. Duffey

Sean Duffey died suddenly in Davis, California, on May 21, 1997, from an embolism precipitated by unsuspected, aggressive, and difficult to diagnose lung cancer. To the last he was unaware that he was ill and was vigorous and active up to the moment of his death. Sean is survived by his wife, Anne, and his sons, Brendan and Seth, of Davis and his parents, Betty and Laurance Duffey of Calgary, Alberta. He was born November 28, 1943, in Toronto and received his Bachelor's and Master's degrees in Zoology and his Ph.D. in Botany from the University of British Columbia in Vancouver, the latter in 1974. After receiving his doctorate he spent two years on a NATO/National Research Council of Canada Postdoctoral Fellowship at the University of Georgia. He joined the faculty of the Entomology Department at the University of California, Davis in 1976.

Sean's research program focused on Chemical Ecology and his efforts ranged widely over the interactions involving chemicals, plants, and insects. His first studies were of the cardiac glycosides produced by milkweeds (Asclepiadaceae) and their sequestration by the insects that fed on them. His primary efforts concentrated on the milkweed bugs, *Oncopeltus fasciatus* and *Lygaeus kalmii*, but he also worked with monarch butterflies and milkweed beetles. Among his important discoveries was the fact that a biophysical system was operating in the sequestering of cardiac glycosides. While continuing his research on cardiac glycosides, Sean began an analysis of the remarkable cyanogenic defensive secretions of polydesmid millipedes. There followed several papers on the biochemistry of HCN production and the production of other defensive compounds in these interesting animals. After arriving at UC Davis, Sean began a long series of brilliant studies on the chemical mechanisms used by plants to fend off attack by insects and various pathogens. This work centered on resistance in tomatoes, and over the years he collaborated with numerous students and colleagues. Studies analyzed the role of numerous chemicals produced by plants including tomatine, proteinase inhibitors and various plant oxidative enzymes. Recent studies had included analyses of iduced defenses and the interactions of chemicals with the biological agents such as parasitoids and baculoviruses used in various IPM and biological control programs.

A constant theme and frequently emphasized message in Sean's work was the fact that chemical-biological interactions were rarely simple and straight-

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forward. He stressed that in order to understand plant-insect interactions, for example, it was necessary to understand the interactions among plant chemicals, the overall characteristics of the insect's diet, the physiological state of the insect, and the modifiable characteristics of plant and insect. Chemical and biological context and chemical mixture were seen as critical determinants of biological activity; a simple view that natural products functioned merely as "toxins" or isolated defensive factors was often misleading. Sean worked closely with colleagues in ecology and plant pathology to help him expand on this thesis.

Sean was a member of several societies including the Entomological Society of America and was a member of the editorial boards of *Physiological Entomology*, *Journal of Chemical Ecology*, and *Entomologia Experimentalis et Applicata*. Other professional activities included invited papers at the International Congress of Entomology and the Gordon Conference and service on the Entomology/Nematology Competitive Grants panel at the USDA. He was extremely active in departmental and university affairs, serving on and chairing numerous committees. He was Vice-Chairperson of the Department of Entomology at the time of his death and was the Acting Chairperson in 1994-1995.

Teaching was always a priority and a passion for Sean, and he participated at one time or another in some 20 different courses. His course in insect-host plant interactions was legendary, with voluminous hand-outs and study guides to lead the students through the intricacies and arcana of the diverse chemical and biological factors. He regarded good teaching of undergraduates early on in their careers as an important duty and devoted several years to teaching a course in insect natural history to nonmajors in biology or entomology and to participating in the large general biology course for all majors in the biological sciences. He actively engaged in curricular revision at both graduate and undergraduate levels.

Sean was always extremely popular with students and his passion for good mentoring matched that of his teaching. He served as Master Graduate Adviser for the department and chaired its Graduate Policy Committee. His lab was a busy place with undergraduates, graduate students, and postdocs from both his and other laboratories carrying out projects. Always approachable, Sean not only advised and directed his own students, but was an inspiration and help to others from fields as diverse as toxicology and anthropology.

Never one to let grass grow, Sean actively pursued outside interests in his "spare time." He was a dedicated runner, putting in several miles most noontimes. He had an intense interest in good music, and the strains of Bach, Mozart, and other masters nearly always emanated from his office. He read widely, often startling colleagues with his depth of understanding of seemingly arcane subjects. When his sons started playing soccer, he immersed himself enthusiastically in the local program, refereeing games and serving as head referee for several

years. His garden was a riot of blooming plants, several carefully chosen to attract butterflies and other insects. The number of lives that Sean touched was remarkably revealed at his memorial service where hundreds of mourners overwhelmed the capacity of the church and flowed around the alter, clogged the aisles, and spilled out onto the lawn outside.

Sean wrote at the top of his resumé "I place a strong emphasis on balancing multiple activities in the academic community." In that endeavor he succeeded incredibly well, and there can be no better epitaph. In memory of an exceptional colleague, a scholarship fund has been established to support students working in the area of chemical ecology in the UC Davis Department of Entomology. Contributions can be sent to the Sean Duffey Scholarship Fund, Department of Entomology, University of California, Davis, CA 95616. Checks should be made payable to the Regents, University of California.

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PLANT VOLATILES MEDIATE HOST-FINDING BEHAVIOR OF THE APPLE LEAFCURLING MIDGE

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Abstract—The behavioral responses of apple leafcurling midge, *Dasineura mali* Kieffer, mated females to foliage from host and nonhost trees were investigated in a wind tunnel. When released downwind of apple or pear foliage, females exposed to apple were more likely to exhibit upwind flight and to approach and land on foliage. On apple foliage, landings were concentrated on buds and immature leaves. Probability of taking flight and latency to flight did not differ for females exposed to apple and pear. When foliage was placed behind screens to obscure plant visual stimuli, females again distinguished between apple and pear, with more of the females exposed to apple odors flying upwind and landing. Females exposed to pear odors were more likely to fly upwind, approach, and land than females exposed to clean air. Odors from immature apple foliage triggered orientation responses in a larger percentage of females than odors from mature apple foliage. A dichloromethane extract of immature apple foliage also triggered orientation responses.

Key Words—Diptera, Cecidomyiidae, *Dasineura mali*, apple, flight, wind tunnel, oviposition.

INTRODUCTION

Despite the economic importance of many plant-feeding species in the family Cecidomyiidae, little is known about their host-finding behavior. Plant-feeding cecidomyiids are typically highly specialized in their host-plant associations, being found only on a single plant species or a limited number of plant species

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within a single plant genus (Gagné, 1989). As the immature states of cecidomyiids usually have limited mobility, suitable host selection by the ovipositing female is critical. The small number of studies that have investigated the behavior of cecidomyiid females indicate that plant chemicals play an important role in both the finding of plant hosts (Pettersson, 1976; Foster and Harris, 1992) and the identification of hosts after the female lands on a plant (Foster and Harris, 1992).

The apple leafcurling midge, *Dasineura mali* Kieffer (Diptera: Cecidomyiidae), is a specialist herbivore that attacks apple trees in Europe, Great Britain, North America, and New Zealand (Gagné, 1989). In New Zealand, it was formerly of little concern to orchardists but has recently become a serious pest because of large increases in its numbers (Smith and Chapman, 1995). Adult *D. mali* emerge from pupation sites in the soil beneath apple trees (Todd, 1959). Virgin females produce a sex pheromone that triggers upwind flight in males (Harris et al., 1996) and, after mating, lay their eggs on young apple leaves. These eggs give rise to larvae that feed and thereby cause distortions in leaf growth (curled leaves) and early senescence of leaves. In addition to reducing the productivity of apple trees (Allison et al., 1995), *D. mali* larvae and pupae found on harvested fruit can lead to the rejection of fruit for export (Lowe, 1994).

We have initiated research to determine how mated female *D. mali* find young apple leaves. For this study we observed the behavior of individual mated females introduced into a wind tunnel containing various combinations of odor and visual stimuli. The following questions were addressed: Do females exhibit flight responses to host foliage, and immature apple foliage in particular? Are flight responses mediated by plant volatile chemicals? Do visual stimuli from plants play an important role in flight orientation?

METHODS AND MATERIALS

Insects. Curled leaves of apple (various cultivars, e.g., Fuji, Braeburn) infested with late-instar midge larvae were collected from orchards in Auckland from December 1995 to March 1997. In the laboratory, the curled part of infested leaves was broken open and placed on the surface of a 1-cm layer of horticultural bark medium that lined the bottom of plastic boxes (15 × 10 × 5 cm). Mature larvae exited leaves and pupated in the bark medium. Boxes were covered, placed in a controlled environment chamber (21 ± 1°C, 60–80% relative humidity, 14L:10D with lights turned on at 7:00 AM), and watered when the medium dried out. Twelve to 30 days later, adults emerged between 6:00 and 10:00 AM and were collected daily.

General Experimental Methods. Behavioral responses of mated *D. mali* females were tested in a wind tunnel based on the design of Miller and Roelofs

(1978). The main body of the wind tunnel measured 0.95 m wide × 1 m high × 2 m long (Plexiglas walls and an aluminum frame), had two white cotton mesh screens at the upwind end (these screens were replaced by colored screens in one experiment), and had a 5-cm-deep layer of moist soil lining the floor of the tunnel. Upwind of the main body of the wind tunnel was a chamber made of wood (0.95 cm wide × 1 m high × 15 cm long) that was used to hold odor sources and other treatments. A 0.7 m diameters fan connected to a variable motor speed controller (Woods Air Movement, G.E.C., Wellington, NZ) also was housed in this chamber. This fan pushed air through the wind tunnel at speeds of 0.2–0.3 m/sec (Turbometer, Davis Instruments, Hayward, California). At the downwind end of the wind tunnel, an exhaust tube evacuated air to the outside of the building. Fourteen full-spectrum composition fluorescent tubes with high-frequency control circuits (Biolux tubes in Quicktronic Deluxe ballasts, Biolux, Hamburg, Germany) provided light within the tunnel (310 μW/cm^2). Temperature in the wind tunnel was 24 ± 3°C.

Mated females used in experiments were obtained as follows. Females of similar body size that eclosed during the hour of peak emergence (6:30–7:30 AM) (Galanihe, 1996) were collected daily and introduced at 7:30 AM into a plastic container (13 height × 13 cm diam.) along with a similar number of males. At 8:30 AM females were removed by aspiration and placed individually in glass vials (10 long × 2.5 cm diam), each vial having a plastic lid with a mesh window. For the next 30 min, females were observed to determine physiological status: females that were observed extending the terminal segments of the ovipositor (the calling posture of virgin *D. mali* females) (Harris et al., 1996) were discarded. In experiments on foliar extracts in which females were introduced into the wind tunnel in groups of 10 or 20, individual females were transferred by aspirator from glass vials to a cylindrical plastic container (6 cm long × 4 cm diam.) that had a removable plastic lid at one end and a mesh window at the other end.

All tests with mated females were carried out between 1:00 and 6:00 PM, the period when flight behavior is exhibited by mated females (Galanihe, 1996). In the majority of experiments, females were introduced individually into the wind tunnel (method given below). For these experiments, a single female was considered to be a replicate. This testing procedure was changed for two experiments testing foliar extracts of apple. Here, groups of either 10 or 20 females were introduced simultaneously into the wind tunnel (see experiments for method), and each group was considered to be a replicate. This change in testing procedure was necessary because experiments on foliar extracts were carried out in the latter part of the apple growing season, a time when the immature apple leaves needed to make extract were scarce. By increasing the number of females released per unit of foliar extract, we were able to test the behavioral responses of a larger number with the limited amount of foliar extract available to us.

The most commonly used method of introducing females into the wind

tunnel, i.e., introduction of individuals, was achieved by orienting a glass vial holding a single female in an upright position, so that the female moved to the lid of the vial, and removing the lid from the vial. The lid was then moved to the middle of the wind tunnel, where it was turned over and attached to a wooden pole (30 cm long) by means of an adhesive material. This wooden pole was located midway between the side walls of the tunnel, 50 cm downwind of the screen at the upwind end of the tunnel, and 30 cm downwind of the foliage or visual target that, in some experiments, was placed at the upwind end of the wind tunnel. We chose these relatively short distances between release points and targets/screens because female *D. mali* are small (body length ca. 2 mm) and hard to track visually as they fly in the wind tunnel. Females that flew away from the vial cap before it was placed on the rod or that were lost from sight immediately after take-off from the vial cap were not included in the data set for analysis. Females that remained in the vial cap for the 5-min observation period were recorded as nonresponders.

For females that flew away from the vial cap, the following were recorded: (1) the time the vial cap with the female was placed on top of the wooden rod; (2) the time that the female flew away from the vial cap; (3) the direction of flight after take-off (the possibilities being upwind, downwind, crosswind, with upwind flight scored only if upwind flight was sustained over distances of at least 20 cm); (4) directional changes during flight, especially a change from downwind flight to upwind flight; (5) arrival in a 5-cm-radius zone surrounding the target or a 5-cm zone downwind of the screen at the upwind end of the wind tunnel; (6) landing on the target or the screen; and (7) location of landing site on the target or screen. All observations were recorded manually using three stop watches and a check sheet.

Responses to Apple and Pear Foliage. Three fresh shoots (30 cm high, ca. 30 leaves) of either apple (cultivar Dunn's Favorite) or pear (cultivar Beurre Bosc) held vertically in a flask of water were placed 20 cm downwind of the upwind screened end of the wind tunnel. Individual females were released (as described previously) from a platform placed at a height that was level with the midpoint of the apple or pear shoots and 30 cm downwind of the foliage. Nine blocks were run, each block consisting of five responsive females (i.e., females taking flight) tested for each of the two treatments (total of 45 females tested for each of the two plant types).

In a second test, visual cues in the wind tunnel were held constant by placing one of two identical models of shoots (ca. 10 green artificial cloth leaves attached to a central plastic rod) at the upwind end of the main body of the wind tunnel (models placed as apple shoots were placed in the previous experiment, i.e., 20 cm downwind of the upwind screened end of the wind tunnel), while odor sources were varied by placing apple or pear shoots (same cultivars as above) behind two screens in a chamber upwind of the main body of the wind

tunnel. Model shoots placed in the upwind chamber plus a model at the upwind end of the main body of the wind tunnel constituted the third treatment (i.e., no foliar odor) and served as a control for the turbulence caused by real shoots lying in an airstream. Fifteen blocks were run, each block consisting of five responsive females tested for each of the three treatments (total of 75 females tested per treatment).

Responses to Odors From Mature and Immature Apple Foliage. Odors from either immature or mature apple foliage were introduced into the wind tunnel by placing the foliage behind two screens in a chamber upwind of the main body of the wind tunnel. Immature apple foliage consisted of shoots (ca. 15) with a single bud and three small newly opened apple leaves (mature leaves were removed from the shoot). Mature apple foliage consisted of shoots (ca. 15) with only mature leaves (i.e., buds and immature leaves removed). All shoots were positioned upright in a flask of water and at mid-height stood ca. 30 cm above the floor of the wind tunnel. In this experiment, because there was no actual plant or plant model within the main body of the wind tunnel, the behavior of individual female midges was scored relative to the screen at the upwind end of the tunnel (i.e., approaching the screen, landing on the screen). All females that landed on the screen did so in the region of the screen that was immediately downwind of odor sources. Fifteen blocks were run, each block consisting of five responsive females tested for each of the two treatments (total of 75 females tested per treatment).

Responses to Extracts of Apple Foliage. To make extracts, 50 immature apple leaves (cultivar Gravenstein) collected from unsprayed apple trees were dipped for 50 sec in dichloromethane (AR Grade, Mallinckrodt Speciality Chemical Co., Paris, Kentucky). This method probably extracted only chemicals present on the surface of apple leaves as leaves were not damaged and the cut end of the petiole did not contact the solvent. The extract was concentrated down from 1 leaf equivalent to 2.5 leaf equivalents/ml by passive evaporation in an extractor hood and stored at $-15°C$.

For the test, a total of 50 leaf equivalents of apple foliar extract were released from filter papers placed at the upwind end of the main body of the wind tunnel. To do this, 12.5 leaf equivalents (5 ml) of the extract was applied to each of four filter paper discs (Whatman No. 1, 7.0 cm diam.). Four discs treated with the same volume of dichloromethane served as controls. Five minutes later, after the solvent had evaporated, the four discs were hung in a 2×2 formation from the middle of two parallel strings (5 cm apart) that were suspended across the upwind end of the wind tunnel (strings 35–40 cm above the tunnel floor). Immediately after filter papers were positioned, a release cage containing 10 females was introduced into the wind tunnel and placed on a 30-cm-tall wooden rod, 30 cm downwind of the filter paper discs. The cage was positioned so that the long axis of the cylinder was parallel to the floor of the

wind tunnel and the removable lid faced upwind. The lid of the cage was then removed. For each group of females that was released, the numbers that performed the following behaviors were recorded: flight out of the container, flight upwind, approach within 5 cm of the filter paper disks, and landing on filter paper disks. Ten blocks were run, each block consisting of 10 females tested for each of the two treatments (total of 100 females tested per treatment).

In a second experiment on foliar extracts, we tested behavioral responses of *D. mali* to apple foliar extracts that, relative to the original extract, had reduced concentrations of highly volatile chemicals. Eight hundred leaf equivalents of apple foliar extract were made using 400 ml dichloromethane, as above. This extract was divided into two parts, one of which was allowed to evaporate passively from 200 to 100 ml. The other part was actively evaporated to dryness using a stream of nitrogen. Using the latter method, a solid wax residue remained after evaporation. This wax residue was dissolved in 100 ml of dichloromethane. Both parts of the extract were stored at −15°C. These extracts were tested at 50 leaf equivalents using the same procedure as above. Seven blocks were run, each block consisting of 20 females tested for each of the two treatments (total of 140 females tested per treatment).

Responses to Visual Stimuli in Presence and Absence of Apple Odor. To test whether female *D. mali* orient to plant visual stimuli, we made models of apple shoots and presented these models in the wind tunnel with and without apple odors. There were five treatments in this experiment: (1) a foliar model in the main body of the wind tunnel and apple foliage placed behind a screen in a chamber upwind of the main body of the wind tunnel, (2) a foliar model in the wind tunnel and a foliar model placed in the upwind chamber, (3) no model in the wind tunnel and apple foliage placed in the upwind chamber, (4) no model in the wind tunnel and a model placed in the upwind chamber, and (5) apple foliage in the main body of the wind tunnel and a model placed in the upwind chamber. Foliar-models consisted of 10 model shoots, each shoot consisting of a wooden rod (6 mm diam. × 30 cm long) with five "leaves" made from bright green construction paper. "Leaves" were the shape and size of apple leaves (ca. 7 cm long and 5 cm wide, each with a surface area of ca. 49 cm^2) and were fixed to the wooden rod at 45° angles. No attempt was made to match the color of real apple leaves. Foliar models placed in the chamber upwind of the main body of the wind tunnel served as a control for the turbulence created by real apple shoots. Model shoots ($N = 10$) as well as apple shoots ($N = 10$) were held in an upright position in flasks of water. Ten blocks were run, each block consisting of five responsive females tested for each of the five treatments (total of 50 females tested per treatment).

In a second experiment on visual responses, we explored the hypothesis that *D. mali* females would be more likely to fly towards apple odors if those odors were presented along with visual stimuli characteristic of foliage (yellow)

rather than visual stimuli characteristic of sky (blue). Yellow and blue also correspond to regions of the light spectrum that are important for the oviposition behavior of a related cecidomyiid, the Hessian fly (Harris et al., 1993). The two color fields placed at the upwind end of the wind tunnel were created by dying nylon mesh with fabric dye (Dylon International Ltd., London; turquoise No. 07 and yellow No. 08). When dry, the dyed fabric was stretched over two separate frames and these frames were used to separate the main body of the wind tunnel from the upwind chamber that held the odor source (ca. 50 apple leaves) or a foliar model (ca. 50 model leaves). There were four treatments; (1) a yellow screen and model foliage placed upwind of the screen, (2) a blue screen and model foliage placed upwind, (3) a yellow screen and apple foliage placed upwind, and (4) a blue screen and apple foliage placed upwind. Ten blocks were run, each block consisting of five responsive females tested for each of the four treatments (total of 50 females tested per treatment).

Experimental Design and Statistical Analyses. All experiments used a complete block design, with the order of presentation of treatments randomized within each block. Most commonly, a single stimulus treatment was set up in the wind tunnel, and individual females were tested sequentially until five had taken flight from the release platform. After these five were tested, the treatment was changed, with this process continuing until each treatment had been tested with five responsive females. For these experiments, the females tested for the full set of treatments comprised a block (i.e., 5 females/treatment/block). For analysis, data were analyzed as the number of females that took flight from the test platform out of the total number of females introduced into the wind tunnel and as the number of females that flew upwind, approached, or landed on the target out of the five females that took flight. Latencies to flight were also analyzed by two-way ANOVA, (i.e., latencies were not averaged within a block).

For the two experiments testing foliar extracts, the experiment was again run as a randomized complete block design. However, because females were placed in the wind tunnel in groups of 10 or 20 rather than individually, all behaviors were scored as the number of females out of the group that took flight, flew upwind, and approached and/or landed on the filter paper target. Here, a block of treatments consisted of the two groups of females tested for the two treatments. Order of presentation of the two treatments within blocks was alternated.

Data were analyzed using JMP version 3.1 (SAS Institute, 1995) and were first subjected to Bartlett's test for homogeneity of variance. When variances were shown by this test to be heterogeneous, data were transformed to achieve homogeneity (arcsin transformation for all percentage data). Transformed data that did achieve homogeneity were analyzed by two-way ANOVA tests or ANOVAs for factorial designs. In a number of cases in which transformed data did

not achieve homogeneity, data were subjected to a Welch ANOVA (SAS Institute, 1995), a test that is valid when group sample variances are heterogeneous. When ANOVAs indicated significant treatment differences, treatment means were separated at $P < 0.05$ by the Tukey-Kramer HSD (honestly significant difference) (SAS Institute, 1995). When sample sizes were small because only small numbers of females exhibited a behavior, data were pooled over all blocks. These distributions were subjected to Log Likelihood tests (SAS Institute, 1995). When no treatment or block differences were found at $P < 0.05$, this is indicated in the text as not significant (NS).

RESULTS

Responses to Apple and Pear Foliage. When given 5 min to respond after being placed on a platform in the middle of the wind tunnel, similar percentages (Figure 1) of the *D. mali* females tested with apple or pear foliage initiated flight (treatment effects, NS; block effects, $P < 0.001$). Latencies from placement on the platform to initiation of flight were 56 ± 10 sec and 34 ± 10 sec ($X \pm$ SE) in the presence of apple and pear foliage, respectively (treatment and

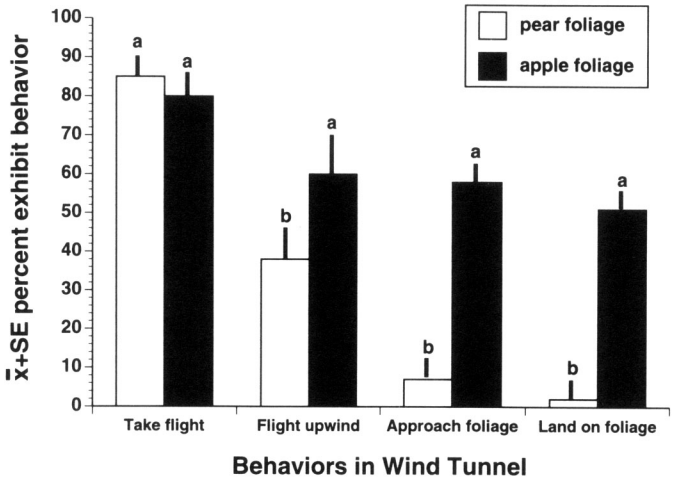


FIG. 1. Behavioral responses of mated female *D. mali* released downwind of pear or apple foliage in a wind tunnel. "Flight upwind" was scored when upwind flight occurred over distances of at least 20 cm. "Approach foliage" was scored when females entered a 5-cm-radius zone around foliage. Within each behavioral category, treatment means accompanied by different letters were significantly different at $P < 0.05$ (two-way ANOVA on arcsin transformed data).

block effects, NS). Upon initiating flight, females exhibited one of three types of flight: (1) crosswind or downwind flight, (2) direct upwind flight towards the foliage, or (3) an initial crosswind or downwind flight followed by upwind flight towards the foliage. The latter two types of flight, classed together as upwind flight (Figure 1), were more commonly exhibited by females exposed to apple foliage than females exposed to pear (treatment effect, $P < 0.05$; block effect, $P < 0.02$). Relative to females flying towards pear foliage, females flying towards apple foliage (Figure 1) also were more likely to progress upwind, the result being that more of these females approached (treatment effect, $P < 0.001$; block effect, NS) and landed on foliage (treatment effect, $P < 0.001$; block effect, NS). Of the total number of females that oriented to and landed on apple foliage, 74% landed on buds or immature leaves, 9% landed on the shoot, and 17% landed on the mature leaves. Thus, landing responses were not distributed independently of foliage type (chi-square test, $P < 0.001$). As buds and immature leaves on apple shoots represented a much smaller proportion of the total leaf area than the mature apple leaves, differences in landings on the two leaf types would have been even greater if expressed as landings per leaf type per unit leaf area. As well as leaf area, immature and mature apple foliage differed in color, with immature foliage being brighter green (i.e., reflecting more yellow/green light).

In this experiment, odor stimuli were varied by placing apple, pear, or model foliage behind two white mesh screens at the upwind end of the wind tunnel, and visual stimuli were held constant by placing one of three identical foliar models 20 cm downwind of the mesh screens. While percentages of females taking flight and latencies to flight were similar across treatments (treatment and block effects, NS), females exposed to apple odors were more likely to exhibit upwind flight, and approach and land, than were females exposed to pear odors or no foliar odors (Figure 2; for upwind flight treatment effects, $P < 0.001$ and block effects, NS; for approach treatment effects, $P < 0.001$ and block effects, NS; for landing treatment effects, $P < 0.007$ and block effects, NS). Of the females that landed after flying upwind to apple or pear odors, the majority (86%) flew past the foliar model and landed on the white screen at the upwind end of the wind tunnel, with the rest (14%) landing on the foliar model. Relative to females flying in the wind tunnel without any foliar odors, females exposed to pear odors were more likely to fly upwind, approach, and land (Figure 2) (Log Likelihood test for distribution of upwind flight, $P < 0.001$; approach, $P < 0.001$; land, $P < 0.003$). When no plant odors were present in the wind tunnel, only one of the 75 females that took flight exhibited sustained upwind flight. This female did not approach or land on the model or screen.

Responses to Odors from Mature and Immature Apple Foliage. Females given odor of immature or mature apple foliage did not differ in percentages of

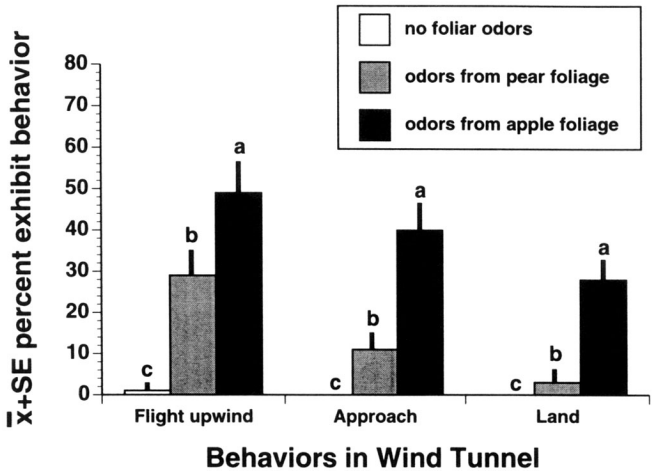


FIG. 2. Behavioral responses of mated female *D. mali* released downwind of odor sources held behind screens in a chamber upwind of the main body of the wind tunnel. For the control treatment (no foliar odors), a foliar model resembling pear or apple foliage in leaf number and size was placed behind the screens. Behaviors were scored as described in Figure 1 except that "Approach" or "Landing" were scored, respectively, when females entered the 5-cm-radius zone or landed on the foliar model or screen. Within each behavioral category, treatment means accompanied by different letters were significantly different at $P < 0.05$ (two-way ANOVA on arcsin transformed data followed by Tukey multiple comparison or LogLikelihood ratio tests).

individuals that took flight (mean ± SE: 90 ± 1.5% and 91 ± 1.5%, respectively; treatment effects, NS; block effects, $P < 0.001$) or latencies to take-off (110 ± 13.9 and 104 ± 13.9 secs, respectively; treatment and block effects, NS). However, females exposed to odors from immature apple foliage (Figure 3) were more likely to fly upwind (treatment and block effects, $P < 0.001$), approach (treatment and block effects, $P < 0.001$), and land (treatment and block effects, $P < 0.001$) on the screen at the upwind end of the wind tunnel than females exposed to odors from mature apple foliage.

Responses to Extracts of Apple Foliage. When filter papers treated with 50 leaf equivalents of a dichloromethane extract of apple foliage or dichloromethane alone (control) were placed at the upwind end of the wind tunnel, similar percentages of females flew out of the release chamber (Figure 4; treatment and block effects, NS). However, for the foliar extract, greater percentages of females flew upwind (treatment effect, $P < 0.01$; block effect, NS) and entered a 5-cm zone downwind of the filter paper discs (treatment effect, $P < 0.001$; block effect, NS). Of the females tested with the foliar extract, 29/100 landed on the

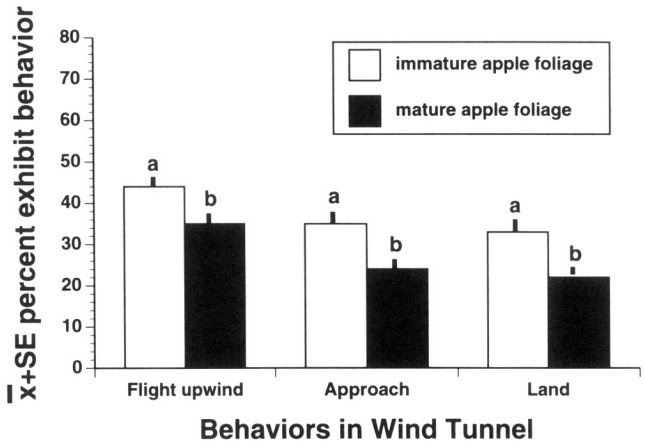


FIG. 3. Behavioral responses of mated female *D. mali* released downwind of immature or mature apple foliage held behind screens at the upwind end of the wind tunnel. Behaviors were scored as in Figure 2. Within each behavioral category, treatment means accompanied by different letters were significantly different at $P < 0.05$ (two-way ANOVA on arcsin transformed data).

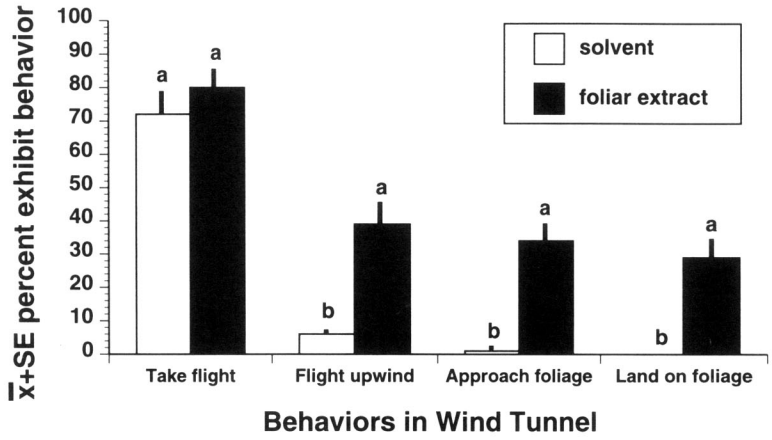


FIG. 4. Behavioral responses of mated female *D. mali* released downwind of filter papers treated with either a solvent (dichloromethane control) or a dichloromethane extract of apple foliage. Behaviors were scored relative to treated filter papers as in Figure 1. Within each behavioral category, treatment means accompanied by different letters were significantly different at $P < 0.05$ (two-way ANOVA on arcsin transformed data).

filter paper discs versus 0/100 for the solvent-treated papers (treatment effect, $P < 0.003$; block effect, NS). Four of the 29 females landing on the apple extract-treated filter papers exhibited examining behaviors (antennation and probing with the terminal segments of the abdomen); however, none of these females laid eggs.

In a second test using apple foliar extract, females were less likely to approach and land on filter papers treated with foliar extract that had been evaporated to dryness under a stream of nitrogen than on filter papers treated with extract that had not been evaporated to dryness (for approach, treatment effect, $P < 0.004$ and block effect, NS; for land, treatment effect, $P < 0.001$ and block effect, $P < 0.057$). Of the females that took flight, 47 $\pm$ 7.2% and 27 $\pm$ 3.2% approached and landed, respectively, on the filter papers treated with the extract that had not been evaporated to dryness. With extract that had been evaporated to dryness, only 10 $\pm$ 7.1% and 2.9 $\pm$ 3.2%, respectively, of the females approached and landed on treated filter papers.

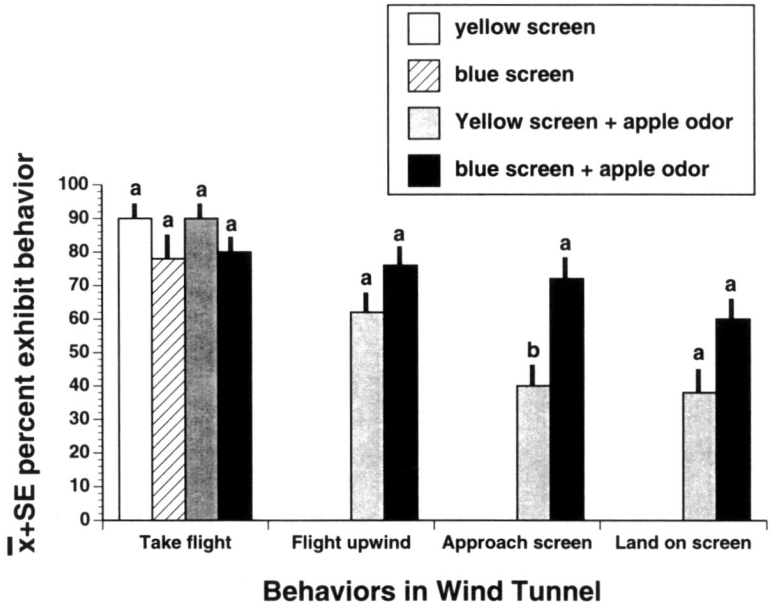


FIG. 5. Behavioral responses of mated female *D. mali* released downwind of an odor source (apple foliage) held upwind of either yellow or blue screens. Behaviors were scored as in Figure 2. Within each behavioral cateogry, treatment means accompanied by different letters were significantly different at $P < 0.05$ (two-way ANOVA on arcsin transformed data followed by a Tukey multiple comparison test).

Responses to Visual Stimuli in the Presence and Absence of Apple Odor. Rather than increasing behavioral responses to apple volatiles, foliar models placed at the upwind end of the wind tunnel significantly reduced the percentage of females that took flight from the test platform, both when apples odors were present in the tunnel and when they were absent (take-off reduced from 84–94% to 58–62%; treatment and block effects, $P < 0.001$; Tukey test, $P < 0.05$). The addition of a foliar model to an airstream containing apple odor also reduced the proportion of females exhibiting upwind flight (reduced from 45–66% to 38%) and landing (reduced from 48–56% to 34%; treatment and block effects, $P < 0.001$; Tukey test, $P < 0.05$).

In a second experiment we explored the hypothesis that *D. mali* females would be more likely to fly towards apple odors if those odors were presented along with visual stimuli characteristic of foliage (yellow) rather than visual stimuli characteristic of sky (blue). To do this, we presented one of two large colored screens (yellow or blue) at the upwind end of the wind tunnel, both in the presence and absence of apple odors (Figure 5). Color and odor had no effect on percentages of female that took flight or on latencies to flight (treatment and block effects, all NS, except block effect for take-off, $P < 0.01$). When apple odors were not present in the wind tunnel, females did not fly upwind (Figure 5). Females responding to the two remaining treatments, yellow screen + apple odor and blue screen + apple odor, exhibited no significant difference in upwind flight responses (Figure 5; odor effects, $P < 0.004$; block, color and color × odor effects, NS). Of the females that exhibited upwind flight, a greater proportion of females flying towards the blue screen approached and entered the 0 to 5-cm zone at the upwind end of the wind tunnel (Figure 5; color and color × odor effects, $P < 0.004$ and $P < 0.02$, respectively; block and odor effects, NS). Of the females that entered this zone, similar percentages of females landed on the yellow and blue screens (Figure 5; all treatment and block effects, NS).

DISCUSSION

When introduced into a wind tunnel, mated female *D. mali* released downwind of apple foliage were more likely to fly upwind, approach foliage, and land on foliage than females released downwind of pear foliage (Figure 1). This greater responsiveness to apple foliage would appear to function as a behavioral mechanism for concentrating eggs on the only known host plant of *D. mali*, apple, *Malus domestica* Borkh. (Rosaceae). In reality, little is known about the host range of *D. mali*. Like many other *Dasineura* species, *D. mali* has been defined as a *Dasineura* midge that causes a particular type of damage on a particular host species (R. J. Gagné, Systematics Entomology Laboratory, USDA, personal communication). Similarly, the midge that curls the leaves of

pear, *Pyrus communis* L. (Rosaceae) is referred to as *D. pyri* Bouche, the pear leafcurling midge. Leafcurling midges found on apples and pears do not show obvious differences in traits commonly used to distinguish cedidomyiid species (i.e., genitalia) and therefore have not been regarded as well-defined species (R. J. Gagné, Systematics Entomology Laboratory, USDA, personal communication). However, *D. mali* males oriented positively in a wind tunnel to virgin midge females reared from apple but not to virgin midge females reared from pear (Harris et al., 1996). Moreover, *D. pyri* larvae placed on apple did not survive (Barnes, 1948) and apple blocks heavily infested with midges have been found next to pear blocks that have no midges, and vice versa (Whitcomb, 1934). Thus, it appears that *D. mali* and *D. pyri* are two distinct species, and that *D. mali* attacks apple but not closely related species such as pear.

Odors originating from apple foliage appear to be important orientation stimuli for female *D. mali*. When foliar visual stimuli were obscured by placing apple and pear foliage behind screens at the upwind end of the wind tunnel, females given odors from apple foliage were more likely to fly upwind, approach, and land (Figure 2). The flight behavior of female *D. mali* exposed to apple odors was similar to that of male *D. mali* exposed to female sex pheromone (Harris et al., 1996), i.e., narrow zigzagging upwind flights, occasionally interrupted by lateral casting across the wind axis. Another *Dasineura* species that uses plant odors to locate host plants is *D. brassicae*. Females of this species have been observed flying in a predominantly upwind direction within fields of host plants (Sylven, 1970). In an olfactometer, female *D. brassicae* accumulated in greater numbers in olfactometer arms with host-plant odors than in arms with clean air (Petterson, 1976).

That greater numbers of *D. mali* females oriented to immature versus mature apple foliage (Figure 3) implies that behaviorally active odors released from apple foliage change in blend or concentration as the leaf matures. Little is known about the volatile emissions of apple leaves (except see Ebel et al., 1995), how they change as the leaf develops, or how they differ from closely related species, such as pear. The low but significant level of responsiveness of *D. mali* to pear odors (Figures 1 and 2) indicates that apple and pear (and perhaps other plant species) may share one or more behaviorally active volatile components, with immature apple leaves either releasing larger quantities of these chemicals and/or a different blend of chemicals. The greater responsiveness of *D. mali* to immature apple foliage in the wind tunnel corresponds with observations of egg-laying sites in the orchard (Whitcomb, 1934; Barnes, 1948; Todd, 1959). Young leaves that are less than two thirds unfolded are preferred for oviposition, with older leaves only chosen when young leaves become scarce over the four to five generations of *D. mali* that occur during a single growing season.

Plant chemicals eliciting behavioral responses in apple midges were removed from apple leaves by brief (50 sec) extractions in dichloromethane. This extrac-

tion method generally removes lipids (epicuticular waxes) from the leaf surface (Eigenbrode and Espelie, 1995). Extractable lipids vary among plant species, among genotypes within a species, and among parts within plants, and are thought to have a number of ecological functions, including protection of the leaf from dehydration and mediation of interactions between the plant and its parasites, e.g., insects and microorganisms (Eigenbrode and Espelie, 1995). Extractable lipids of wheat and other cereals have been shown to mediate the behavior of another cecidomyiid, the Hessian fly, *Mayetiola destructor* (Harris and Rose, 1990; Foster and Harris, 1992). However, unlike the Hessian fly, which was influenced by extractable lipids not only during flight and landing but also during on-plant examining and egg-laying behavior (Foster and Harris, 1992), *D. mali* rarely exhibited examining behaviors after landing on extract-treated filter papers (only 4/29 females) and never laid eggs on these papers. Thus, it appears that additional plant stimuli are required before *D. mali* females will oviposit on a surface. Such stimuli could be chemical compounds that were not extracted by dichloromethane extraction or tactile stimuli associated with the dense hairs that cover the immature apple leaves preferred by *D. mali* (Whitcomb, 1934).

It is clear that female apple midges respond to volatile chemical(s) in dichloromethane extracts of apple leaves. Our preparation of extracts, which included some passive evaporation of extracts, would be expected to leave both involatile and reasonably volatile compounds in the extract. When we evaporated the extract to dryness and reconstituted it in dichloromethane, the extract elicited a much lower response in females. This suggests that one or several of the chemicals eliciting behavioral responses is of reasonable volatility (i.e., is lost fairly rapidly from the matrix of the extract, either during or immediately after evaporation of the dichloromethane).

In *D. mali*, plant odors did not influence either the probability of take-off from the release platform (Figure 2) or latency to take-off. In another experiment (Galanihe, 1996), *D. mali* that mated between 7:30 and 8:30 AM were introduced into a wind tunnel containing apple foliage at hourly intervals from 9:00 AM to 5:00 PM. As the day progressed, females were more likely to take off from the release platform and had shorter latencies to take-off. In a second experiment (Galanihe, 1996), mated female *D. mali* held in glass vials with little or no air movement exhibited walking and short flights with increasing frequency as the day progressed. Taken together, these results indicate that flight initiation in female *D. mali* is more influenced by internal physiological factors (Barton Brown, 1993) than by plant volatiles.

We used models of plants to explore the influence of visual stimuli on *D. mali*, but, using these models, found little evidence to support a major role for plant visual stimuli in take-off, flight, or approach behaviors. For example, in one experiment testing for visual responses, model apple branches added to

an airstream containing apple odors decreased rather than increased percentages of females taking off, flying upwind, and landing. This result indicates that, rather than sensing models through the visual sense, female *D. mali* perceived foliar models via disruptive effects of the models on airflow and/or the odor plume itself (e.g., increased turbulence) (Wyatt et al., 1993). Of the females that did fly upwind and land in this experiment, only a few landed on the models (14%). Most females did not reduce their rate of upwind flight near the model and eventually landed on a white screen at the upwind end of the wind tunnel, a screen that was positioned ca. 10 cm downwind of the apple branches that served as odor sources. As the chamber that held the apple branches was dark, and the two screens that separated the apple branches from the main body of the wind tunnel were made of a close mesh, it is not likely that female *D. mali* oriented to visual stimuli from these branches.

Our inability to identify behavioral responses of *D. mali* to plant visual stimuli may have been related to deficiencies in our plant models: the color of our models did not precisely match the colors of immature apple foliage, and host-plant odor stimuli originated from an odor source placed upwind of the model rather than from the model itself. However, similar models have proven adequate for studying the visual behavior of a related cecidomyiid, the Hessian fly (Harris et al., 1993), and in the Hessian fly elicited strong visual responses to color and spatial stimuli characteristic of host plants (i.e., grasses). Moreover, mated Hessian fly females oriented to and landed on foliar models throughout their egg-laying phase, regardless of whether these models emitted host-plant volatiles (Harris et al., 1993). It is interesting to note that, unlike the apple leafcurling midge, Hessian fly females do not exhibit upwind flight responses to host-plant odors in a wind tunnel or olfactometers (Harris, unpublished results).

In summary, mated female *D. mali* oriented to odors from plant foliage in a wind tunnel and, during such orientation, distinguished between host and nonhost plants as well as immature and mature foliage of their only known host plant, apple. A dichloromethane extract of immature apple foliage also triggered orientation responses. The next step of our research will be to work with chemists to isolate and identify the behaviorally active volatile chemicals released from immature apple foliage. Such chemicals could prove useful in monitoring programs for *D. mali*. At present, monitoring of *D. mali* is achieved by counting eggs, a time-consuming method that requires expertise and close examination of small leaves under high magnification.

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DODECANOIC ACID IS THE OVIPOSITION PHEROMONE OF *Lutzomyia longipalpis*

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Abstract—The sandfly, *Lutzomyia longipalpis* (Lutz and Neiva), produces an oviposition pheromone in the accessory glands that is secreted onto the eggs during oviposition. This compound attracts and/or stimulates gravid females for egg-laying. The compound was identified as dodecanoic acid, using gas chromatography–mass spectrometry, gas chromatography, and chemical derivatizations. The synthetic analog induced the same behavioral response in gravid sandflies as the whole egg extract when present in biologically relevant quantities. When single sensillum recordings were taken from the ascoid on the antennae of female sandflies, the response to egg extract and dodecanoic acid was similar. There was a dose dependent response to dodecanoic acid and preferential sensitivity compared to other fatty acids. There was a strong additive interaction upon the behavior of *L. longipalpis* when dodecanoic acid was tested in a bioassay with the known oviposition attractant apneumones from rabbit feces, hexanal, and 2-methyl-2-butanol. The results suggested that sandflies acquired hexadecanoic acid (palmitic acid) from the blood meal and over a period of four days this was converted to dodecanoic acid. The role of these "token" semiochemicals in sandfly oviposition is discussed with respect to additive interactions and the significance of their origin from larval food resources.

Key Words—*Lutzomyia longipalpis*, oviposition, pheromone, apneumone, additive interaction, single sensillum recording, gas chromatography–mass spectrometry, oviposition bioassay, dodecanoic acid.

INTRODUCTION

Choice of a suitable oviposition site by a gravid insect is of great importance for the survival of her progeny (Chapman, 1985). One way in which she locates

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an appropriate site is by orienting towards semiochemical oviposition attractants (McCall and Cameron, 1995). This allows the female to provide an optimal food resource for her progeny at an energetically low maternal cost (Dicke and Sabelis, 1988). Depending on the physiological state of the insect, the same semiochemicals may induce different behavioral responses, reducing the genetic and energetic burden on the olfactory system. For example, mosquito oviposition attractants include volatile compounds such as fatty acid esters, indoles, cresols, and alcohols (Bentley and Day, 1989; Davis and Bowen, 1994). Some of these compounds are also used when mosquitoes search for a host (Cork and Park, 1996; Knolls et al., 1996).

The sandfly, *Lutzomyia longipalpis* Lutz and Neiva (Diptera: Psychodidae), is currently being studied to determine the stimuli that mediate oviposition for this vector of visceral leishmaniasis in Latin America. In the wild, female sandflies are believed to lay their eggs in microhabitats, such as the cracks and crevices of chicken coops and cow stables, sites that are rich in organic nutrients (Lewis and Kirk, 1954; Ward, 1974). Here, females may lay up to 100 eggs, whereas in laboratory colonies, the usual number is 20–30 eggs. A range of organically rich substrates, such as rabbit food, rabbit feces, and colony frass are attractive to gravid sandflies (ElNaiem and Ward, 1992). In the laboratory, larvae are reared on diet that contains 25% rabbit feces. 2-Methyl-2-butanol and hexanal isolated from the feces attracted ovipositing females (Dougherty et al., 1995), apparently serving as apneumones from the organic material indicative of the presence of an adequate food resource for the progeny. This is one of the requirements for a good choice of oviposition site (Chapman, 1985). In addition, an oviposition pheromone from the accessory glands of gravid females (Dougherty et al., 1992) was found on oviposited eggs (ElNaiem and Ward, 1991). Greatly enhanced oviposition aggregation was achieved when both attractant sources were combined (Dougherty et al., 1993).

The oviposition pheromone of *L. longipalpis* has been isolated and shown to be a single compound (Dougherty et al., 1994); however, it has not yet been characterized. The present investigation set out to identify this pheromone. Single sensillum electrophysiology was undertaken to measure the sensitivity of antennal receptors to the pheromone, compared to related synthetic analogs. Synthetic oviposition pheromone was used with synthetic hexanal and 2-methyl-2-butanol to investigate additive interactions. Once identified, the accumulation of the oviposition pheromone in the female accessory gland over a period of time was examined.

METHODS AND MATERIALS

Sandflies. Sandflies were originally collected from Jacobina, Bahia State, Brazil, and Curarigua, Lara State, Venezuela, and reared for more than 73 and

12 generations, respectively, according to the method of Modi and Tesh (1983). Female flies used in the oviposition bioassays were blood-fed three days after emerging on Syrian hamsters anesthetized with a 12 mg/liter solution of sodium pentobarbitone. Only females that had obviously fed on blood were then isolated with an equal number of males at 27 ± 1°C with a photoperiod of 12L:12D for four days to allow copulation, defecation, and oogenesis. Flies for electrophysiological investigation were used two days after taking a blood meal.

Preparation of Oviposition Pheromone and Apneumone Extracts. Extracts were prepared from 1200 eggs by placing them in a 2-ml microvial with 100 μl of hexane (all solvents were from BDH, UK, and of pesticide residue analysis grade). The sealed vials were left for 48 hr at −20°C to allow complete extraction and minimize loss of volatiles. Extracts were reduced in volume on ice under N_2 to 30 μl. Two microliters of egg extract, containing 500 egg equivalents (EE), were pipetted on a 4-cm × 2-cm filter paper strip and placed into the syringe of the odor delivery system for electrophysiological stimulation (Dougherty et al., 1995). To determine when the oviposition pheromone was accumulated, five female flies were isolated at three points during their life cycle: one day before a blood meal (BM; five days after emergence), one day after a BM, and four days after a BM, the normal time of oviposition. Each fly was extracted individually in 20 μl of dichloromethane for 24 hr in a flame-sealed glass ampoule. The extract was reduced in volume, as described above, to 1 μl for chemical analysis. Air-entrained volatiles of rabbit feces were obtained with the method of Dougherty et al. (1995). Hexane extracts of rabbit feces volatiles were reduced to 5 μl on ice under N_2.

Gas Chromatography–Mass Spectrometry (GC-MS). Analysis was carried out on a Hewlett Packard 5890 II+ gas chromatograph with an HP-5MS column (Hewlett Packard UK; 30 m × 0.25 mm ID, 0.25 μm film thickness) or a DB-Wax column (J & W Scientific, UK; 30 m × 0.25 mm ID, 0.25 μm film thickness) coupled directly to a Hewlett Packard 5972A bench top mass spectrometer (EI, 70 eV, 165°C). Samples were introduced via a split/splitless injector (splitless mode, sampling time 0.7 min, 180°C). Two temperature programs were used. For the HP-5MS column, the temperature was initially 50°C for 4 min, then increased at 5°C/min up to 310°C. The DB-Wax column was initially at 50°C for 4 min, then increased at 10°C/min to 220°C. Helium was the carrier gas under constant pressure with an initial linear velocity of 1 m/sec at 50°C.

Two microliters each of Jacobina or Curarigua egg extracts (80 EE) were injected. The pheromone peak was initially located in the GC-MS total ion chromatogram by its retention relative to a hydrocarbon series and by comparison of chromatogram profiles obtained by gas chromatography (GC) (Dougherty et al., 1994). The spectra of the putative pheromone in each egg extract were obtained and compared to those in the computer-based spectral library. Dodec-

anoic acid gave the closest match to the pheromone peak. Synthetic dodecanoic acid (200 ng) in 2 μl of hexane was analyzed on both columns under the same conditions and temperature programs as above, and the spectra were compared with those of the oviposition pheromone.

To confirm the molecular weight of the pheromone, the two egg extracts were analyzed by GC-MS in chemical ionization (CI) mode. The injector and ion source temperatures were both 180°C, and the ionizing gas was methane. A DB-Wax column was used with the parameters as stated above. The spectrum of the pheromone peak was obtained and compared to those in the computer-based spectral library. Synthetic dodecanoic acid was analyzed (200 ng) and the spectrum compared to the pheromone peak. To confirm the carboxylic acid functional group, both egg extract and dodecanoic acid were chemically modified by esterification with diazomethane in ether. The reaction products were analyzed by GC-MS in EI mode on a DB-Wax column and with the corresponding operating conditions. A spectrum was obtained for each of the reaction products and compared to those in the computer-based spectral library and to synthetic dodecanoic acid methyl ester (200 ng).

Preparation of Synthetics. Hexanal, 2-methyl-2-butanol (Aldrich, UK, >99% purity) and dodecanoic acid (Sigma, UK, 99% purity) were prepared in hexane so that 1 μl contained 100 ng. For electrophysiological stimulation, straight-chain, saturated carboxylic acids, C_8–C_{14} (Aldrich, UK, 99% purity), were prepared at four concentrations; 1 μg, 10 μg, 100 μg, and 1 mg/10 μl of hexane. For chemical analysis, synthetic C_{12}, C_{14}, and C_{16} carboxylic acids (Aldrich, UK, 99% purity) were prepared in hexane at concentrations of 1 ng, 10 ng, 100 ng, and 1 μg/1 μl of hexane. A solution containing 100 ng/μl dodecanoic acid *trans*-methyl ester (Aldrich, UK; 99% purity) was prepared in hexane.

Oviposition Pheromone Bioassay. The bioassay was an adaptation of the method used by Dougherty et al. (1995). Cored GC septa (Dyson Instruments, UK), heat sealed at one end, were placed into holes opposite each other (10 cm apart) in the plaster of Paris base of a polymethylpentane (PMP) larval rearing pot. Between each experiment, the PMP pots were cleaned by first removing the plaster, then washing with alcohol and distilled water, and finally were autoclaved before being replastered. Both test and control septa were covered with a pad of cotton wool to prevent access by flies. A solution of the chemical to be tested was placed dropwise into the test septum. The control septum had an equal volume of solvent alone added to the core. The following samples were tested: 40 ng of dodecanoic acid; egg extract so that 100 EE were present; and a control of solvents only. The concentration of synthetic compound found in 100 sandfly eggs was approximately 40 ng. Twenty females were left in the dark for 24 hr, and the number of eggs on the test and control sites were counted. Ten replicates were carried out for the above treatments.

Interaction Between Pheromone and Apneumones. The bioassay used was the same as above. Solutions to be tested were added dropwise to the test septa while an equal volume of solvent was added to the control septa. The following samples were tested: combined synthetics [dodecanoic acid (40 ng/100 EE), hexanal (200 ng), and 2-methyl-2-butanol (200 ng)]; entrained rabbit feces volatiles (2 μl); hexanal and 2-methyl-2-butanol (200 ng of each); and a control of solvent only. The synthetic compounds tested were present in quantities at which they would naturally occur in attractant samples of entrained rabbit feces volatiles or sandfly eggs. Eight replicates were undertaken for every sample with 20 females in each.

Data Analysis. The data were not normally distributed when tested with the Anderson-Darling normality test (Minitab for Windows, Version 9). Therefore, differences in the number of eggs laid at test and control sites were statistically analyzed with the Wilcoxon matched-pair signed-rank test. To test for differences between treatments used in the bioassay, the number of eggs laid at the test site for each material was log-transformed and an analysis of variance (ANOVA) performed. This treatment was used rather than a nonparametric ANOVA because the variability of each test treatment was not the same. Finally, a Tukey's multiple comparison test was undertaken to reveal any statistical difference between treatments.

Accumulation of Oviposition Pheromone Within Sandflies. One microliter of female extract from each of the three age groups was analyzed by GC-MS on a HP-5MS column, using the operating parameters described previously. Spectra were obtained for the oviposition pheromone, possible precursors, and breakdown products at each of the sample times in the life cycle of the adult fly and compared to those in the computer-based spectral library. Once the compounds were identified, a quantitative analysis was carried out on a GC 17A (Dyson Instruments, UK) with a DB-Wax column (temperature program: 45°C, then increased at 10°C to 240°C, and held for 10 min). Dichloromethane extracts were introduced via an on-column injector (40°C) with helium as the carrier gas under constant pressure (linear flow of 1 m/sec at 40°C). Detection was via a flame ionization detector (320°C). Three injections of C_{12}–C_{14} carboxylic acids at the four concentrations were made, and a calibration curve plotted. The quantity of carboxylic acids in the biological extracts was then obtained using the equation of the calibration curves.

Electrophysiological Responses to Oviposition Pheromone. Single sensillum recordings were taken from the ascoid sensillum on the antennae of female sandflies (Dougherty et al., 1995). Neurons in the ascoid were stimulated with egg extract, dodecanoic acid, and some of its analogs (carboxylic acids C_8–C_{14}) at four concentrations. In all experiments, the stimuli were tested on a minimum of 8 flies. The neuron sensitive to the oviposition pheromone was characterized, and the dose–response plotted for each of the synthetic compounds. Double

consecutive stimulations were undertaken with egg extract and dodecanoic acid to ensure the same neuron was responding to both.

RESULTS

Structural Characterization of Oviposition Pheromone. Total ion chromatograms for GC-EI-MS analyses of egg extracts were characterized by several peaks common to both Jacobina and Curarigua populations. The pheromone peak eluted at 26.31 min (Jacobina population) and 26.26 min (Curarigua population) on the HP-5MS column and at 23.20 min and 23.17 min, respectively, on the DB-Wax column. Mass spectra of the pheromone peak showed ions at *m/z*(abundance) 28(43), 41(70), 43(78), 60(98), 73(100), 85(26), 115(13), 129(27), 157(18), and 200(M^+, 15), corresponding to dodecanoic acid.

Dodecanoic acid eluted at 26.25 min on the HP-5MS column and at 23.17 min on the DB-Wax column. The mass spectrum of authentic dodecanoic acid contained ions at *m/z*(abundance) 28(43), 41(70), 43(78), 60(98), 73(100), 85(26), 115(13), 129(27), 157(18), and 200(M^+, 15). GC-CI-MS confirmed the molecular weight of dodecanoic acid as 200 amu ($M+H^+ = 201$) with a base peak at 73 amu. The retention times and spectra of synthetic dodecanoic acid matched that of the pheromone peak in both the Jacobina and Curarigua *L. longipalpis* samples. When the pheromone peak and dodecanoic acid were methyl-esterified, their molecular weights increased to 214 amu, with similar spectra that matched synthetic dodecanoic acid methyl ester.

Oviposition Pheromone Bioassay. The results of the bioassay are shown in Figure 1. Both whole egg extract ($P = 0.036$) and dodecanoic acid ($P = 0.036$) produced a targeted oviposition response from gravid *L. longipalpis* with significantly more eggs being laid at the test site than control. There was no significant difference between the numbers of eggs laid at test sites treated with egg extract or dodecanoic acid. None of the solvent controls induced an oviposition response, and no positional bias of the eggs was observed between the test and control sites.

Interaction Between Pheromone and Apneumones. Extract of rabbit feces volatiles ($P = 0.031$), hexanal with 2-methyl-2-butanol ($P = 0.034$), and the combined synthetic oviposition attractants (hexanal, 2-methyl-2-butanol, and dodecanoic acid) ($P = 0.03$), all produced a positive oviposition response (Figure 2). Furthermore, significantly more eggs were laid at sites containing the combined synthetic oviposition attractants compared to sites treated with either extract of rabbit feces volatiles or hexanal with 2-methyl-2-butanol.

Accumulation of Oviposition Pheromone Within Sandflies. Before the blood meal, dichloromethane extracts of female sandflies contained no fatty acid compounds, except trace quantities of dodecanoic acid (Table 1). During the period

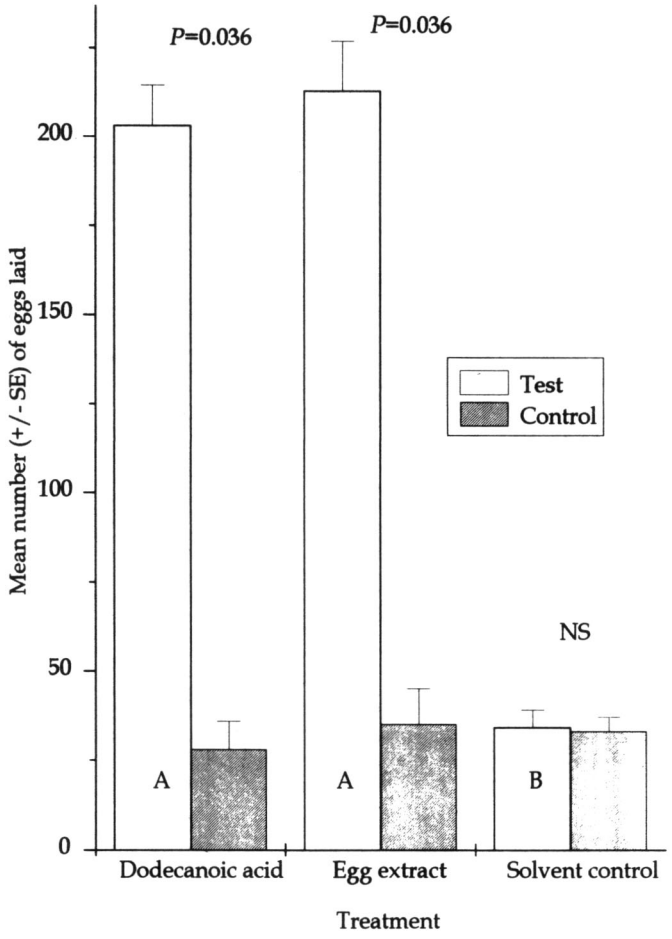


FIG. 1. Oviposition response of *L. longipalpis* to test sites treated with dodecanoic acid (40 ng), egg extract (100 EE), and a control of hexane solvent (4 μl). Numbers of eggs laid at the test and control sites were compared using Wilcoxon matched-pair signed-rank test. A comparison between treatments was carried out after a logarithmic transformation of the data by ANOVA; treatments with the same letter are not significantly different. Open bars = test site, hatched bars = control site.

of the experiment, several compounds that could be precursors or breakdown products of dodecanoic acid were found. These included: hexadecanoic acid *m/z*(abundance) [43(100), 55(57), 58(73), 60(84), 68(36), 73(93), 83(22), 85(21), 129(22), and 257(M^+, 0.13)]; hexadecanoic acid acetate *m/z*(abundance)[41(34), 43(40), 69(16), 74(100), 75(21), 87(72), 97(11),

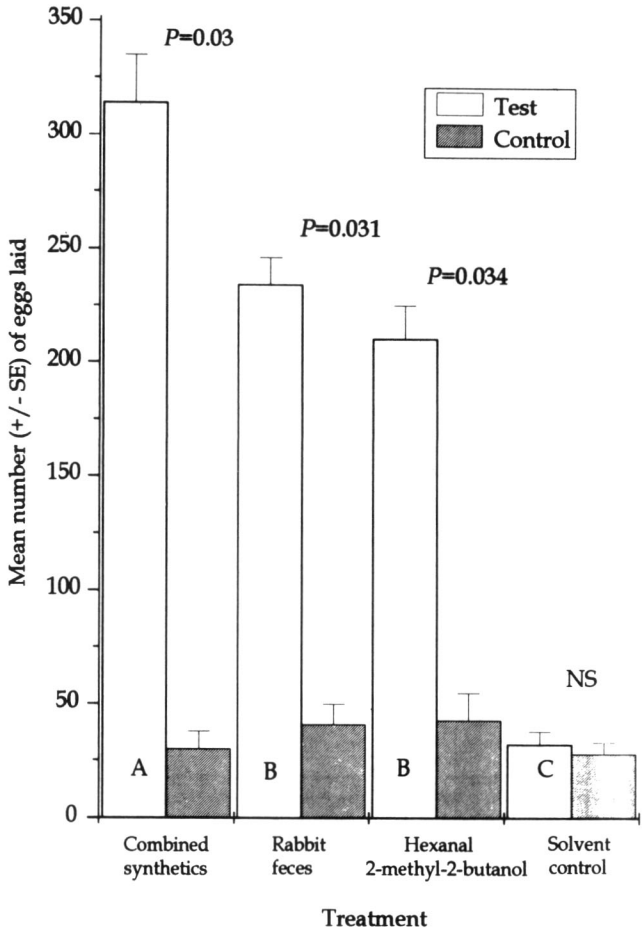


FIG. 2. Oviposition response of *L. longipalpis* to test sites treated with combined synthetics (dodecanoic acid 40 ng, hexanal 200 ng, and 2-methyl-2-butanol 200 ng), entrained volatiles of rabbit feces (2 μl of 100× concentrated extracted solution), hexanal (200 ng) with 2-methyl-2-butanol (200 ng), and a control of hexane solvent (4 μl). Numbers of eggs laid at the test and control sites were compared using Wilcoxon matched-pair signed-rank test. A comparison between treatments was carried out after a logarithmic transformation of the data by ANOVA; treatments with the same letter are not significantly different. Open bars = test site, hatched bars = control site.

TABLE 1. CARBOXYLIC ACID PRESENT IN FEMALE *L. longipalpis* ONE DAY BEFORE AND ONE AND FOUR DAYS AFTER A BLOOD MEAL (BM)

Age of fly	Carboxylic acid (ng/fly ± SE)[a]		
	C_{12}	C_{14}	C_{16}
1 day before BM	0.15 ± 0.08		
1 day after BM	0.83 ± 0.05	0.46 ± 0.20	9.25 ± 0.68
3 days after BM	8.79 ± 0.45	0.83 ± 0.09	1.44 ± 0.07

[a] C_{12}: dodecanoic, C_{14}: tetradecanoic, and C_{16}: hexadecanoic acid.

143(16), 270(15), and 272(M^+, 0.5)]; tetradecanoic acid *m/z*(abundance) [43(67), 55(49), 57(57), 60(83), 71(31), 73(100), 85(21), 97(11), 129(32), 149(19), and 230(M^+, 0.1)]; and tetradecanoic acid acetate *m/z*(abundance) [41(26), 43(33), 55(22), 69(11), 74(100), 75(14), 87(59), 143(84), 199(6), and 244(M^+, 0.3)]. One day after the blood meal, hexadecanoic acid was predominantly found, along with its acetate. Tetradecanoic acid and its acetate were also found in smaller quantities. Four days after the blood meal, both hexadecanoic acid and tetradecanoic acid were greatly reduced in female bodies, whereas, dodecanoic was the predominant fatty acid (Table 1).

Electrophysiological Responses to Oviposition Pheromone. A neuron sensitive to the hexane egg extract was located in the ascoid sensillum. The neuron response had a relatively large amplitude with equal positive and negative components (Figure 3). Double consecutive stimulations showed that the same neuron response increased in frequency when exposed to either hexane egg extract or synthetic dodecanoic acid (Figure 3). All synthetic carboxylic acid analogs caused an increase in frequency response of this cell in a dose-dependent manner, although there was preferential sensitivity to dodecanoic acid (Figure 4).

DISCUSSION

The compound identified as the biologically active pheromone component of hexane extract of *L. longipalpis* eggs was dodecanoic acid. It was isolated from egg extracts of flies from Jacobina (Brazil) and Curarigua (Venezuela). The presence of dodecanoic acid, 2-methyl-2-butanol, and hexanal in biologically relevant quantities mimicked the presence in extracts of eggs and rabbit feces in the oviposition bioassay (Dougherty et al., 1993). There was no significant difference between these two test preparations. The perception of several

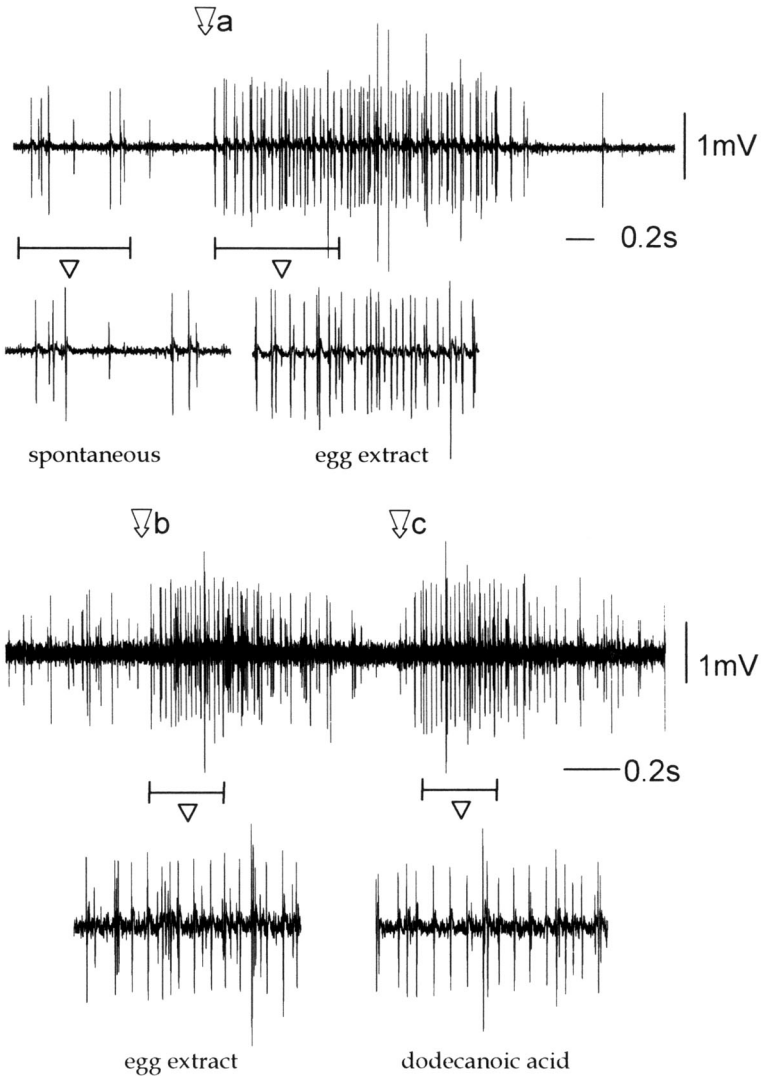


FIG. 3. Electrophysiological responses of olfactory cells within the ascoid sensillum of a female *L. longipalpis* to stimulation with egg extract (500 EE) (a), and a double consecutive stimulation with egg extract (500 EE) (b) and dodecanoic acid (1 μg) (c). Open arrows on the upper traces indicate the start of stimulation, which lasted 1 sec; the lower traces are expanded sections of the upper traces and show the response in greater detail.

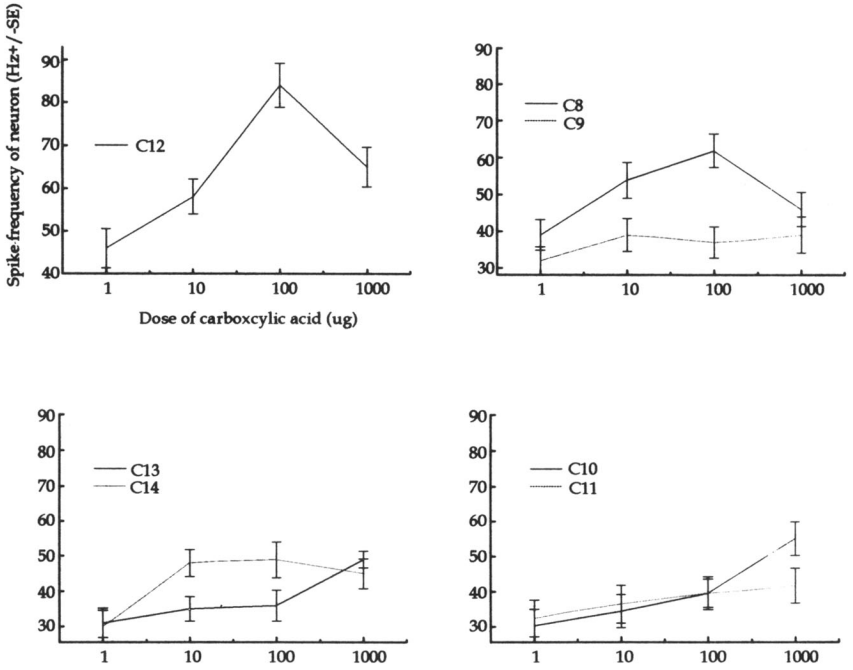


FIG. 4. The frequency of the individual neuron response in the ascoid sensillum on the antennae of female *L. longipalpis* to seven straight-chain, saturated carboxylic acids with carbon numbers of C_8–C_{14}, over a range of four log concentrations.

semiochemicals that have a greater effect on insect behavior than the individual components is well studied. *Tomicus minor* (pine shoot beetle) uses a pheromone and host odors to target more accurately its host and mate (Lanne et al., 1987). The oviposition pheromone of *Culex quinquefasciatus* has an additive effect on stagnant water when combined as an oviposition attractant (Blackwell et al., 1992; Mordue et al., 1993), becoming both electrophysiologically (by electroantenogram) and biologically more active than the individual components.

Fatty acids are obtained from the blood meal by hematophagus insects, and phytophagous insects synthesize them in the fat body. The most common one used as a food reserve is palmitic acid or hexadecanoic acid (Chapman, 1985). In times of nonfeeding, this compound is mobilized by hydrolysis with a lipase enzyme. It then forms lipoprotein complexes, which enter the hemolymph. Upon entering muscle, they are hydrolyzed, and the fatty acids are progressively oxidized to produce acetyl-coenzyme A (Chapman, 1985). From the results, it

would appear that sandflies obtain hexadecanoic acid from their blood meal, and this compound is then metabolized by chain shortening to dodecanoic acid over a period of four days. It is interesting to note that the oviposition pheromone of *C. quinquefasciatus*, erythro-6-acetoxy-5-hexadecanolide (Laurence and Pickett, 1985), and of *C. tarsalis*, 1,3-diglycerides of acetylated hydroxy fatty acids (Staratt and Osgood, 1973), may have synthesis starting points from hexadecanoic acid. The hydroxylation of this compound is normal for mobilization of the food source. The product is then a methanolysis step away from the active lactone compound (Bentley and Day, 1989).

A neuron sensitive to dodecanoic acid was located in the ascoid sensillum on the antennae of female *L. longipalpis*. The dose-dependent response and the preferential sensitivity to dodecanoic acid support this compound as the oviposition pheromone. Neurons sensitive to 2-methyl-2-butanol and hexanal were identified by Dougherty et al. (1995) and were also present in the ascoid sensillum. The response generated when the ascoid was exposed to a combination of these two compounds was complex, with hexanal inhibiting one neuron and stimulating another and 2-methyl-2-butanol stimulating a third cell. Dougherty et al. (1996) also found that hexanal and benzaldehyde present in fox gland extract activated one neuron and inhibited another. The latter neuron increased its frequency of firing when stimulated with carboxylic acids and is the same cell found in the present investigation. At a time when females would be locating a blood meal host, carboxylic acids did not attract sandflies as well as aldehydes in a dual-odor plume olfactometer (Dougherty et al., 1996). Why should the perception of compounds, such as hexanal, from the oviposition site inhibit the perception of carboxylic acids including the oviposition pheromone? One hypothesis is that inhibition of the carboxylic acid neuron is part of a coding mechanism that is required for the interpretation of the complex firing patterns of neurons stimulated by the mix of compounds from the oviposition site (Isman, 1992). Alternatively, Bowen et al. (1995) showed that developmental changes in the sensory system of *Aedes bahamensis* altered the relative responsiveness of sensilla that perceived odors from plant and vertebrate hosts. The authors gave a model for the sensory physiological basis of nutritional preference in this mosquito, with attraction to the host being switched from plants to vertebrates, depending on the age and physiological state of the insect. It is also known that endogenous factors can alter the sensitivity of receptors (Abisgold and Simpson, 1988; Davis et al., 1987). It would be interesting to investigate the possibility of interactions between the three neurons identified regarding the perception of the two different semiochemicals sources involved in the orientation of *L. longipalpis* to the oviposition site.

The role of dodecanoic acid on sandfly eggs may be more complex than for the sole purpose of communication. The compound could form part of the

water proof coating that covers the chorion of the egg (Chapman, 1985). Furthermore, first instars often ingest their egg case, and dodecanoic acid would give nourishment to newly emerged larvae. It is interesting to note that 2-methyl-2-butanol and hexanal are derived from substrates that also give the larvae nourishment. Both sets of semiochemicals are derived from food resources and, through evolution, have been targeted as good markers for an adequate food supply. No resource-unique compounds (host or oviposition site) that cause attraction of insects have yet been identified (Isman, 1992). Such compounds would give the insect a specific signal similar to the perception of a sex pheromone. Mitchell et al. (1990) found that *Leptinotarsa decemlineata* shows a characteristic electrophysiological response to compounds that represent nutritional resources (Mitchell, 1988) in the leaf sap of the potato plant. Sap from nonhost plants produced highly variable responses. The wide range of organically rich substrates (ElNaiem and Ward, 1992) and the interaction of several organic compounds that attract *L. longipalpis* for oviposition (Dougherty et al., 1995) imply that some of the unique individual chemical tokens that attract sandflies for oviposition have been identified, although they are not resource-specific.

McCall and Cameron (1995) reviewed oviposition pheromones in insect vectors. These authors stated that "Whether or not an insect species aggregates (for oviposition) is probably determined by the selection pressures on its eggs and larval environment." Aggregation for oviposition could take place where ample food resources exist for the progeny, and, if food resources are limited, insects may produce aggregation deterrents. *L. longipalpis* uses the presence of volatile compounds from organically rich substrates as an indicator of a suitable oviposition site. This suitability is further enhanced when the gravid female perceives the oviposition pheromone and egg-laying follows.

Otieno et al. (1988) produced a successful trap for *C. quinquefasciatus* using the oviposition pheromone placed in contaminated pools. Dougherty et al. (1993) were able to trap 63% of *L. longipalpis* females flies over a 72-hr period in a laboratory oviposition trap using a combined extract of oviposition pheromone and rabbit food. It is hoped that with further insight into the behavioral and neurophysiological interactions between the oviposition pheromone of *L. longipalpis* and the semiochemicals from organic larval food sources, an oviposition monitoring/control trap can be developed.

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NIGHTTIME VARIATION IN VOLATILE CONTENT OF FLOWERS OF THE NIGHT BLOOMING PLANT *Gaura drummondii*[1]

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Abstract—Nighttime amounts were determined for five volatile chemicals identified in extracts of *Gaura drummondii* flowers. The major component (2-methylbutanal oxime, isomers *1* and *2*) occurred at the highest level during the earliest sampling period (20:00–23:50 hr) in greenhouse and field-grown plants. Oxime levels decreased at subsequent sampling periods. Benzyl alcohol levels were greater in the latter part of the night (03:00–06:00 hr). 2-Phenylethanol or phenylacetaldehyde amounts did not change with respect to time. Average amounts (ηg/bloom) of each chemical summed for all time periods were greater for greenhouse plants than for field grown plants. Three compounds that had not been identified previously in *Gaura drummondii* were identified in methylene chloride extracts. These were benzyl alcohol, (*Z*)-jasmone, and eugenol.

Key Words—*Gaura drummondii*, Onagraceae, flower, odor, volatiles, 2-methylbutanal oxime, benzyl alcohol, phenylacetaldehyde, 2-phenylethanol.

INTRODUCTION

Plant-derived chemicals that attract adult insects for feeding and/or oviposition have been proposed as an integral part of an adult control strategy whereby insect pest populations can be managed in the mobile, more susceptible adult stage instead of the larval stage (Lingren et al., 1988, 1989, 1990). Several plant species have been hypothesized to be attractive to *Helicoverpa zea* (Bod-

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die) (Lepidoptera: Noctuidae) because of direct observations of nocturnal feeding (Raulston et al., 1998) or indirect observations of pollen found on the proboscis or other body parts of captured insects (Bryant et al., 1991; Lingren et al., 1993, 1994). Several species of *Gaura* are under study as possibly attractive plants to *H. zea* and other noctuids. *G. drummondii* (Spach) Torr. & Gray is a rhizomatous perennial herb that blooms at night, with initiation of blooming about dusk. Flowers remain on the plant throughout the night, but fall off the following day. A total of 12 volatile chemicals were identified from hexane extracts of *G. drummondii* (Teranishi et al., 1991), and 21 and 25 volatile chemicals were identified from hexane extracts of *G. suffulta* Gray and *G. longiflora* Spach, respectively (Kint et al., 1993). In other plant species, odor from flowers is variable, with an endogenous rhythm (Overland, 1960; Altenburger and Matile, 1988; Matile and Altenburger, 1988) during which volatiles are produced in a rhythmical, diurnal manner. One of the first activities of some moths 1 day old or older is to feed (Adler, 1987; Lingren et al., 1979). Nocturnal insects have major feeding periods within the night, and it is important to know the relationship that exists between levels of flower volatile chemicals that may attract insects and time of night. It is also important to determine possible rhythmic fluctuations in volatile concentration for planning collections of the plant material for chemical analyses and testing for activity in bioassays. Tests reported herein were conducted to determine the relationship between time of night and volatile concentration in blooms collected from *G. drummondii* from field grown and greenhouse plants.

METHODS AND MATERIALS

Gas Chromatography. A Hewlett-Packard 5890 Series II gas chromatograph (GC) was used for gas chromatographic analyses. The instrument was equipped with a split/splitless injector, a Hewlett-Packard 7673 autosampler, and a DB-I fused silica capillary column (60 m × 0.32 mm ID, film thickness of 0.25 μm; J&B Scientific).

A 5.0-μl aliquot of sample was analyzed unless otherwise stated. The temperature gradient program utilized for the separations was as follows: initial temperature, 40°C, hold 5 min; increase to 100°C at 3.5°C/min, hold 5.0 min; increase to 150°C at 2.0°C/min, hold 15 min. Total programmed run time was 67.14 min. Data analysis used the Hewlett-Packard 3365 Series II Chemstation, version A.03.01.

Mass Spectrometry. Compounds were identified with a Hewlett-Packard 5971A GC–mass spectrometer. The instrument was equipped with a split/splitless injector and a DB-I column identical to that used for the GC analyses. The linear velocity of the carrier gas was adjusted to 30 cm/sec at 150°C.

For the mass spectrometric analyses, a 10-μl sample was injected using the following temperature gradient program: initial temperature, 40°C, hold 5 min; increase to 100°C at 3.5°C/min, hold 5 min; increase to 150°C at 2.0°C/min, hold 5 min; increase to 250°C at 5.0°C/min, hold 15 min, for a total run time of 110.47 min. Compounds were identified by comparing retention times and mass spectra of unknowns to retention times and mass spectra of known standards run under identical conditions.

Chemical Standards. The following chemicals were used as received from the suppliers: (*E*)-2-hexenal, (*Z*)-3-hexenol, phenylacetaldehyde, 2-phenylethanol, methyl salicylate, and methyl 2-methoxybenzoate (Aldrich Chemical Co., Milwaukee, Wisconsin); (*Z*)-3-hexenyl acetate (Sigma Chemical Co., St. Louis, Missouri); (*Z*)-jasmone (Pflatz & Bauer, Waterbury, Connecticut); and 3,4-dihydro-8-hydroxy-3-methyl-1H-2-benzopyran-1-one (mellein) (Fluka Chemika-Biochemika, Ronkonkoma, New York).

2-Methylbutanal oxime (a mixture of isomers 1 and 2) was prepared in the laboratory according to the procedure of Roberts et al. (1985). The oxime purity was ascertained to be >90% by gas chromatography, and there was an approximately 70:30 ratio of isomers 1 and 2, respectively.

Methylene Chloride Extracts—Volatile Chemical Levels, Time Study. Gaura drummondii blooms were collected from plants grown in test plots maintained in Burleson County, Texas. Three bloom samples (500 blooms/sample) were picked for each of three time periods, starting at 22:00 (T1), 02:00 (T2), and 06:00 hr (T4) for the night of August 9, 1993. On subsequent dates in April–June, 1994, sampling occurred at four time periods with either two or three samples collected per time period (500 blooms/sample) starting at 20:30 (T1), 24:00 (T2), 03:00 (T3), and 05:30 hr (T4). Additional samples of *G. drummondii* flowers were obtained at similar time periods from the greenhouse for comparison.

Methylene chloride was immediately added to collected blooms, and the mixture was allowed to steep for four days at 4°C. The approximate total volume of blooms plus methylene chloride was 200 ml. Extracts were filtered by vacuum using 9-cm Whatman No. 1 filter paper, a 9-cm Buchner funnel, and a 500-ml Buchner filtering flask. The bloom residue was washed with two 50-ml portions of methylene chloride. Each filtered extract was combined with its respective washes and stored at −10°C until concentrated. Water contained in the extracts was removed with a separatory funnel.

The volatiles were enriched in the extracts by removing excess solvent via fractional distillation in a 30-cm Vigreux column. Distillate temperature was not allowed to rise above 40°C. Samples were concentrated to an approximate volume of 2–5 ml. The enriched samples were placed in 100-ml pear-shaped flasks and polished (removal of nonvolatile material) by transferring under vacuum (2 mTorr) to clean, empty, pear-shaped collection flasks cooled with liquid

N_2. The connection between flasks was made with a U-shaped connecting tube with a side arm (at the collection end) connected to a vacuum manifold. All connections were ground-glass fittings, with a 14/20 standard taper fitting between the flasks and the connecting tube, and a 18/9 ball-and-socket fitting between the side arm and manifold. Vacuum was obtained with a mechanical vacuum pump and liquid cooled diffusion pump. After transfer, each sample was brought to a final volume of 10.0 ml and dried with anhydrous sodium sulfate. A 5.0-μl aliquot of each extract was analyzed for individual volatile compounds via gas chromatography with external standards for quantitation. The five volatile compounds analyzed were 2-methylbutanal oxime (Isomers 1 and 2), benzyl alcohol, phenylacetaldehyde, and 2-phenylethanol.

Extract Preparation for Mass Spectral Analysis. Replicate methylene chloride extracts for each time period were combined and further concentrated via a microfractional distillation apparatus with the use of a 10-cm Vigreux column. The combined extract was concentrated to 300–500 μl. A 10-μl aliquot was analyzed with the mass spectrometer.

Hexane Extracts. Bulk hexane extracts of *G. drummondii* were prepared for comparison of volatile content to that determined for the methylene chloride extracts. A total of 2500 blooms was picked between 21:00 and 24:00 hr and placed in a 4-liter bottle. Sufficient hexane (usually 2 liters) was added to cover the blooms. The extract was then processed as previously described for concentration and polishing of methylene chloride extracts. Further concentration via the microfractional distillation apparatus was performed as the final step for mass spectral analysis.

Statistical Analyses. Student's *t* test was used (Steele and Torrie, 1980) to compare replicate analyses for differences between greenhouse and field blooms. Significance was determined at $P \geq 0.05$.

RESULTS

Methylene Chloride Extracts—Volatile Chemical Levels, Time Study. Gaura drummondii blooms collected from plants grown in field test plots generally had a smaller bloom size [$\bar{X} = 34.6 \pm 0.5$ (SE) mg/bloom] than greenhouse plants [$\bar{X} = 49.9 \pm 1.3$ mg/bloom].

The first collection period (20:00–23:30 hr) was begun following the initiation of bloom opening. For both the field and greenhouse blooms, isomers 1 and 2 of 2-methylbutanal oxime were detected at their highest level during this period (Figures (1 and 2). Mean levels of isomer 1 were 1362.4 $\pm$ 121.1 (SE) ηg/bloom for the field plants and 2102.1 $\pm$ 348.3 ηg/bloom for greenhouse plants. After 03:00 hr, the mean amount of 2-methylbutanal oxime, isomer 1, had decreased to 117.8 $\pm$ 30.4 ηg/bloom for the field plants and 174.0 $\pm$ 4.9

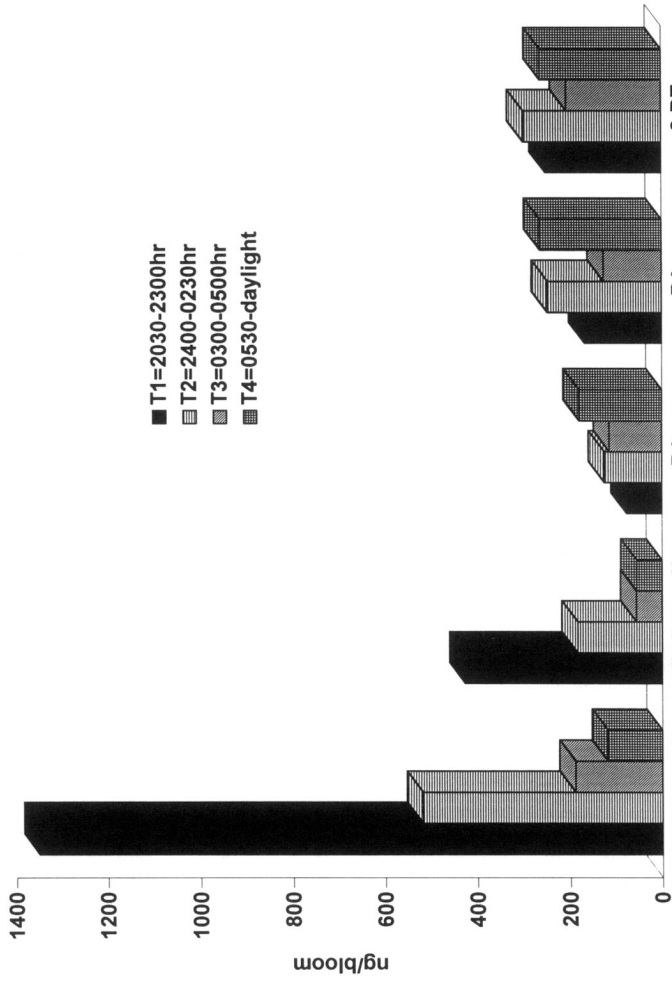


FIG. 1. Field bloom ($N = 500$) mean volatile quantity per bloom for four time periods. MBO (Iso 1 & 2): 2-methylbutanal oxime isomers 1 and 2; BA: benzyl alcohol; PA: phenylacetaldehyde; 2-PE:2-phenylethanol. Dates: August 1993 and April–June 1994.

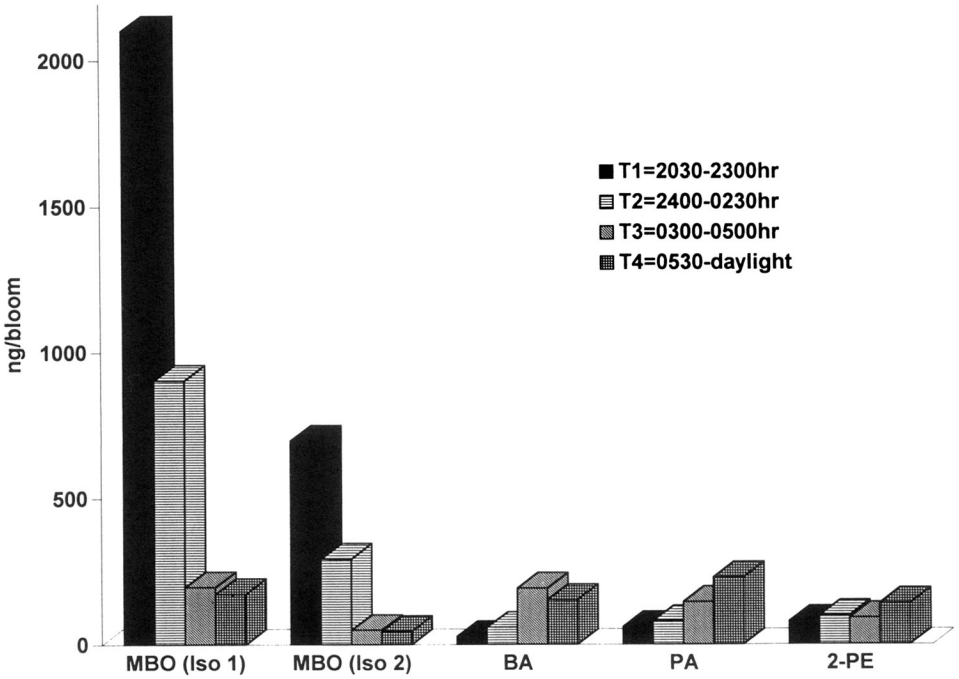


FIG. 2. Greenhouse (N = 500) mean volatile quantity per bloom for four time periods. Abbreviations as in Figure 1 legend. Dates: December 1993 and April–June 1994.

ηg/bloom for the greenhouse plants. Isomer 2 levels of the 2-methylbutanal oxime were considerably lower than isomer 1, especially at T1 and T2; however, the amount–time profiles were similar when compared to isomer 1. Even though amounts of isomers 1 and 2 of 2-methylbutanal oxime were different for both field and greenhouse blooms, the same response pattern, T1 > T2 > T3 = T4 ($P \geq 0.05$), was exhibited.

Benzyl alcohol amount–time variation for field and greenhouse blooms was T4 > T3 = T2 > T1 ($P \geq 0.05$) and T4 = T3 > T2 = T1 ($P \geq 0.05$), respectively. 2-Phenylethanol amounts were not different with respect to time (T1 = T2 = T3 = T4, $P > 0.05$) for either field and greenhouse plants. Mean phenylacetaldehyde levels for field and greenhouse blooms were quite variable for the time periods, studied; however, there were no significant differences (T1 = T2 = T3 = T4; $P > 0.05$).

The mean amount of each chemical over all time periods (T1 + T2 + T3 + T4)/4 shows that greenhouse blooms have significantly greater quantities ($P \geq 0.05$) per bloom for the five chemicals analyzed as compared with field blooms. When the overall averages are converted to volatile concentration per

unit weight, these differences are eliminated except for 2-phenylethanol and phenylacetaldehyde, which are significantly higher in the blooms collected from field plants (Figure 3).

The volatile makeup of methylene chloride and hexane extracts of *G. drummondii* determined in our laboratory was compared to a hexane extract of *G. drummondii* reported by Teranishi et al. (1991) (Table 1). Our mass spectral analysis of the methylene chloride extracts was in agreement with respect to content except that we did not detect limonene or (*E*)-cinnamaldehyde. Furthermore, the concentration of 2-phenylethanol was approximately four times that previously reported (Teranishi et al., 1991). A match in the retention time in the total ion chromatogram and mass spectra between commercial standards and unknowns in the methylene chloride extract confirmed the presence of benzyl alcohol, (*Z*)-jasmone, and eugenol in *G. drummondii* (Table 1). These three compounds have not been reported previously in *G. drummondii*.

Hexane extracts of *G. drummondii* are identical in content to methylene chloride extracts.

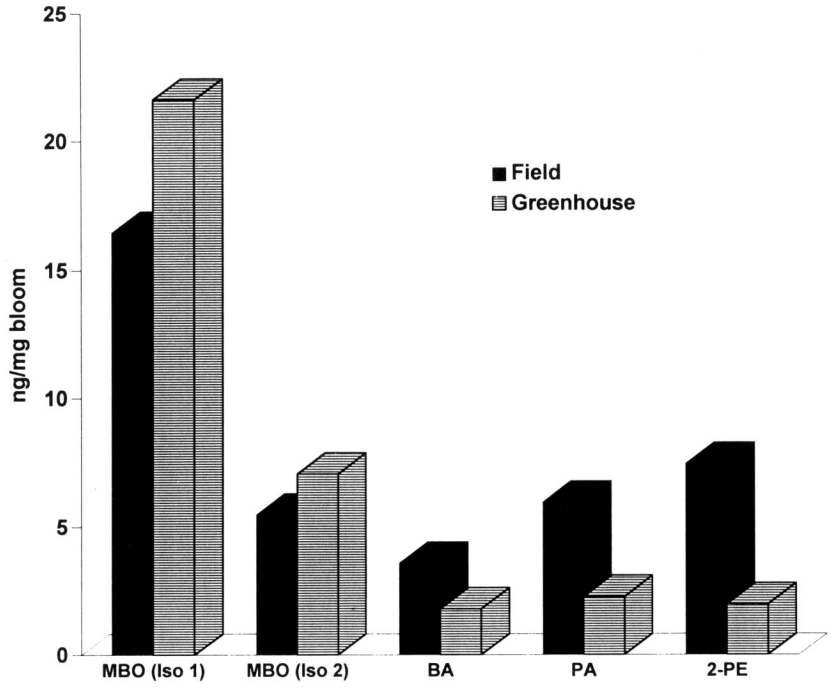


FIG. 3. Mean quantity of chemicals over all time periods, adjusted for bloom weight, field vs. greenhouse.

TABLE 1. CHEMICAL COMPOSITION OF *Gaura drummondii* BLOOMS FROM THE FIELD IN METHYLENE CHLORIDE AND HEXANE EXTRACTS

Compound	Percent of total		
	Methylene chloride extract	Hexane extract	Hexane extract (Teranishi et al., 1991)
(*E*)-2-Hexenal	0.1	2.5	0.8
(*Z*)-Hexenol	0.7	2.1	1.9
2-Methylbutanal oxime			
Isomer 1	41.0	28.3	47.0
Isomer 2	9.8	13.7	22.0
(*Z*)-3-Hexenyl acetate	0.4	1.2	1.5
Benzyl alcohol	8.9	0.8	[a]
Phenylacetaldehyde	1.3	3.1	5.7
Limonene	[a]	[a]	5.9
2-Phenylethanol	23.4	20.6	5.5
Methyl salicylate	1.0	3.8	2.2
(*E*)-Cinnamaldehyde	[a]	[a]	0.7
Methyl-2-methoxybenzoate	5.1	3.2	4.8
Eugenol	3.1	2.9	[a]
(*Z*)-Jasmone	0.7	0.9	[a]
3,4-dihydro-8-hydroxy-3-methyl-1H-2-benzopyran-1-one	4.9	17.4	2.1

[a] Chemicals not detected.

DISCUSSION

G. drummondii initiate blooming shortly after sunset and reach full bloom capacity within 1–2 hr. *Helicoverpa zea*, the corn earworm moth, begins its feeding activity shortly after sunset and continues for several hours afterward. Feeding activity resumes again in the early morning hours. Therefore, the quantities of attractive volatile chemicals during the time periods that insects are responsive to feeding attractants could be a very important factor in determining the attractiveness of the plant. Quantities of the two isomers of 2-methylbutanal oxime decreased significantly with time, indicating that the oximes were either being emitted by the plant or metabolically altered to other compounds at a greater rate than synthesis during the night. Amounts of phenylacetaldehyde and 2-phenylethanol remained relatively constant throughout the night while benzyl alcohol increased slightly with time. For a quantitative time study of a solvent extract of plant material, a constant, decreasing, or increasing level of volatile chemicals will not necessarily relate to the levels of volatile chemicals released

into the atmosphere (headspace). This knowledge can, however, give important information as to the chemicals present at a specific time period and the amounts available. The volatility of the chemicals and the specific combination of chemicals in the headspace will influence the overall odor signature of the plant material. The composite odor signature of the volatile blend released may be the trigger for an insect's detection and subsequent response or nonresponse. The response or lack of it can then be utilized to evaluate the attractancy potential of volatile chemicals. We are presently evaluating headspace volatile composition from *G. drummondii* and determining the quantities of volatile chemicals released.

Teranishi et al. (1991) reported that the two isomers of 2-methylbutanal oxime are the major constituents in a hexane extract of *G. drummondii*, comprising 47.0 and 22.0% of the total volatile mix. Results of our tests indicated a similar pattern during the first few hours after bloom initiation each night, but the relative quantity of the two oxime isomers decreases to approximately equal to or less than the other three components during the last three hours of the night, or approximately 6–9 hr after bloom initiation. It is possible that the blooms obtained for Teranishi's extract were picked within a few hours after initiation of blooming of *G. drummondii* for the night.

Mass spectral data and retention time confirm the presence of benzyl alcohol, (*Z*)-jasmone, and eugenol in methylene chloride and hexane extracts prepared in our laboratory. These chemicals were not reported by Teranishi et al. (1991). We did not detect limonene or (*E*)-cinnamaldehyde. However, the plant material used in identification by Teranishi et al. (1991) was obtained from a wild population of *G. drummondii* in Hidalgo County, Texas. Plant material used in the current study was obtained from field plots and greenhouse plants in Burleson County and Brazos County, Texas, and were therefore grown under very different conditions regarding temperature, soil, and moisture. All of these factors could contribute to differences in chemical makeup of plant material.

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ISOLATES OF SOIL ACTINOMYCETES WITH POTENTIAL FOR PHYTOTOXIN PRODUCTION

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Abstract—Agar plate screening of 220 isolates of soil actinomycetes for phytotoxin production resulted in selection of six isolates that inhibited ($\geq 70\%$) one or both indicator plants for further studies. Cell-free broth of the two isolates, diluted in a 1:1 ratio, significantly reduced seed germination and radicle growth of six plants species on filter paper bioassay. Both isolates were inhibitory to velvetleaf (*Abutilon theophrasti*), a difficult weed to control. Phytotoxin from cell-free fermentation broth of one of the isolates (no. 48), sequestered by solvent extraction, was bioassayed on filter paper. Seed germination and growth of cress (*Lepidium sativum*) and barnyardgrass (*Echinochloa crusgalli*) were severely reduced at 1.5 and 3.0 mg/ml concentration, respectively. Solvent extract of mycelia was marginally inhibitory indicating that the phytotoxin was largely extracellular.

Key Words—Actinomycetes, agar plate screening, bioassay, fermentation, microbial isolates, phytotoxin, weed control.

INTRODUCTION

Increased awareness of the environmental damage caused by continued use or misuse of conventional synthetic agrochemicals has aroused great interest in biodegradable and more selective herbicides (Hoagland, 1990). Herbicide application is likely to increase as a consequence of increased emphasis on reduced-tillage agriculture (Duke, 1986). The increasing incidence of herbicide resistance of weeds, reviewed by Altman (1993), has stimulated research for herbicidal compounds with new or novel sites and modes of action. Microbial metabolites have become the focus of attention of researchers searching for natural product alternatives to conventional pesticides.

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Lynch (1976) extensively reviewed microbial products in relation to plant growth. Soil microorganisms produce a wide range of bioactive compounds that are potentially phytotoxic to higher plants. Research on isolation and screening of microbial metabolites for new herbicidal compounds has witnessed an upsurge as indicated by the number of reviews published during the last two decades (Duke and Abbas, 1995; Heisey et al., 1988; Hoagland, 1990). Several microbial metabolites with herbicidal potential have been isolated (Howell and Stipanovic, 1984; Misato, 1983; Takahashi et al., 1995). Anisomycin, a product of *Streptomyces toyocaensis*, provided the chemical basis for the development of a new synthetic commercial herbicide (Yamada et al., 1972, 1974). Bialaphos, presently marketed in Japan as a broad spectrum herbicide, was isolated from *Streptomyces viridochromogenes* (Sekizawa and Takematsu, 1983). This article reports results of screening of actinomycetes for metabolites with herbicidal potential.

METHODS AND MATERIALS

Isolation of Actinomycetes. Soil samples collected aseptically from horse and cow barns were air-dried, sieved, and the pH determined. Following the "soil enrichment" method for proliferation of actinomycetes, soil samples with added $CaCO_3$ to raise pH to 7.0 were incubated for two weeks under humid conditions (Tsao et al., 1960). Soil samples thus prepared were used for isolation of actinomycetes by the soil dilution method on glucose–asparagine–dipotassium phosphate (GAP) agar plates (Waksman, 1967). Cycloheximide (an antifungal antibiotic) at 100 μg/ml was added to the medium after autoclaving. One-milliliter soil suspensions of 10^{-3}–10^{-5} dilutions were added to 9.0 ml of 0.8% water agar, mixed thoroughly, and overlaid on GAP agar plates poured a day before. This procedure allowed microbial colonies to appear on the surface of the agar plates. The agar plates were incubated at 26–28°C for up to three weeks to allow sufficient time for growth of slow-growing actinomycetes. All isolates that appeared morphologically different were removed and streaked on fresh GAP agar plates. Isolates free of contamination were transferred onto slants of GAP agar and oatmeal agar for maintenance and preservation (Shirling and Gottlieb, 1966).

Screening of Isolates. The growth of an isolate from a GAP agar plate was scraped and transferred aseptically into a tube containing about 2–3 ml of sterile distilled water. Tissues were fragmented by a glass rod and mixed thoroughly with a Pasteur pipet. A few drops of this suspension were placed in the center of a GAP agar plate. The inoculum was spread by a bent glass rod over about a 2.0-cm-wide band area in the center of the plate (9.0 cm diam.) The plates were incubated at 27–29°C under humid conditions for 15–18 days. This incubation period was adequate for growth of the microbial isolate and diffusion of

metabolites in the agar medium. Uninoculated GAP agar plates similarly incubated served as controls.

At the end of the incubation period, 10 seeds of garden cress (*Lepidium sativum* L.) or annual ryegrass (*Lolium multiforum* Lam.) were implanted on two sides of the inoculum band and also on uninoculated control plates. Ryegrass seeds were surface sterilized with 25% Clorox, which was thoroughly washed off with sterile distilled water. It was not necessary to surface sterilize cress seeds. Plates were incubated under humid conditions at 27–29°C in the dark for 90 hr. Three replicate plates of each indicator species were prepared. Cress and ryegrass were selected for bioassay, to represent di- and monocotyledonous plants, respectively.

The lengths of radicles and hypocotyl/coleoptiles were measured, and percent germination was determined for each species. Any isolate indicating bioactivity was retested. Germination and radicle growth were assigned higher ratings than hypocotyl growth in selecting bioactive isolates because of the greater sensitivity of germination and radicle growth to stress.

Broth Culture of Selected Isolates. Selected isolates indicating inhibition of germination and/or reduction of radicle length were grown in modified A-9 broth (Warren et al., 1955). The modified broth (PMC) contained 5.0 g glucose, 2.5 g Bacto peptone (Difco Lab.) 5.0 g molasses and 1.0 g corn steep solid (Sigma) in 1 liter distilled water, adjusted to pH 7.0 with 1 N NaOH. Cornsteep solid was separately boiled in about 100 ml distilled water, filtered, and the filtrate added to the broth.

To prepare primary inoculum for broth culture, the growth of an isolate on GAP agar plate was scraped into a tube containing about 2.0 ml sterile distilled water and was fragmented by a glass rod. This inoculum was used to inoculate a flask containing 25 ml PMC. The culture was grown for five days on a rotary shaker at 28°C. The inoculum thus prepared was used to inoculate a larger volume of PMC broth; the ratio of the inoculum to broth was 1:20. The culture was grown on a rotary shaker in batches at 28°C for 5, 7 or 10 days. We noted that the broth from the 7-day-old culture produced adequate bioactive metabolite. Therefore, growth of the culture was terminated seven days after inoculation. The broth was vacuum filtered through Whatman No. 4 filter paper and the cell-free broth bioassayed for bioactivity.

Bioassay. The following five dicot and two monocot seeds were used: cress, lambsquarters (*Chenopodium album* L.), redroot pigweed (*Amaranthus retroflexus* L.), velvetleaf (*Abutilon theophrasti* Medic.), yellow sweetclover [*Melilotus indicus* (L.) All.], barnyardgrass (*Echinochloa crusgalli* [L.] Beauv.), and annual ryegrass. Based on agar plate screening, six highly phytotoxic isolates were selected for testing in broth culture.

The cell-free broth was diluted 1:1 with sterile distilled water. A sterile Whatman No. 4 filter paper in a 9-cm Petri dish was moistened with diluted

cell-free broth. Filter paper similarly moistened with uninoculated broth served as a control. Ten seeds of a species were placed on the filter paper. Three replicate plates were prepared for each treatment. After 96 hr of incubation in the dark at 28–30°C under humid conditions, percent germination was calculated, and lengths of radicle and hypocotyl were measured.

The presence of phytotoxin in the fermentation broth was also tested in sand culture. Ten seeds of cress, barnyardgrass, or velvetleaf were planted in styrofoam cups containing sand moistened with diluted cell-free broth or uninoculated broth. Sand cultures moistened with distilled water and seeded similarly served as controls. Cups were arranged in a randomized complete block design with three replicates in a phytotron (14-hr day length, day/night temperature 25/30°C, 80% relative humidity, photosynthetic photon flux density 490 μmol m^{-2} sec^{-1} at top of plants). Seed germination count was taken at seven days after planting; plant height and dry weight were determined at 15 days after planting.

Extraction of Phytotoxin. After seven days' growth of isolate 48 on a shaker, cell-free broth was extracted with dichloromethane (3× volume of the broth). The solvent was evaporated to near dryness by rotary evaporation at <30°C. The residue in the evaporation flask was dried on a jet stream of N_2 gas and the dry weight determined. The residue (insoluble in water, only partially soluble in ether and methyl alcohol) was dissolved in a small volume of dichloromethane and diluted with ether. The ether solutions of the residue containing different concentrations were bioassayed on filter paper with cress and barnyardgrass seeds. The ether solution applied on filter paper was evaporated and moistened with water before implanting seeds. Uninoculated broth, similarly extracted with dichloromethane, served as a control.

The cells collected from the fermentation broth after vacuum filtration were extracted with 80% acetone. After evaporation of acetone by rotary evaporation, the aqueous phase was extracted with dichloromethane in the same way as the cell-free broth. Uninoculated broth extracted with the same solvent served as a control. Ether solutions of the residue from cell extraction were bioassayed on filter paper with cress seeds.

Data were analyzed by analysis of variance using Statistical Analysis System (SAS Institute, 1989). Differences between treatment means were evaluated by a t test at the 1% level.

RESULTS AND DISCUSSION

Isolation of Phytotoxin-Producing Actinomycetes. Of 220 isolates of microorganisms screened on agar plates, six significantly reduced germination and growth of cress; isolates 119 and CB21.

Although they severely reduced radicle and hypocotyl growth, they did not

TABLE 1. RESPONSE OF SEEDS OF GARDEN CRESS AND RYEGRASS TO SELECTED ISOLATES OF ACTINOMYCETES ON AGAR PLATE[a]

Isolate	Cress			Ryegrass		
	Germination (%)	Radicle (mm)	Hypocotyl (mm)	Germination (%)	Radicle (mm)	Coleoptile (mm)
Control	97.0(100)	26.1(100)	34.8(100)	83.3(100)	20.8(100)	24.4(100)
#14	23.3(−76.0)*	3.7(−85.8)*	6.8(−80.5)*	70.0(−16.0)	6.9(−66.8)*	11.3(−53.7)*
#48	16.6(−82.9)*	7.6(−70.9)*	6.6(−81.0)*	85.0(+2.0)	5.0(−76.0)*	14.9(−38.9)
#119	96.7(−0.3)	2.1(−92.0)*	3.9(−88.8)*	90.0(+8.0)	5.0(−76.0)*	19.5(−20.1)
#HB21	56.7(−41.6)*	5.2(−80.1)*	2.3(−93.4)*	40.0(−52.0)	3.6(−82.7)*	14.1(−42.2)*
CB6	10.0(−89.7)*	15.5(−40.6)*	19.0(−45.4)*	70.0(−16.0)	7.5(−63.9)*	16.0(−34.4)
CB21	90.0(−7.2)	3.3(−87.4)*	6.8(−80.5)*	85.0(+2.0)	12.1(−41.8)*	15.9(−34.8)
CB31	16.7(−82.8)*	2.8(−89.3)*	3.3(−90.5)*	73.0(−12.4)	1.9(−90.9)*	10.8(−55.7)*
CB36	13.3(−86.3)*	2.0(−92.3)*	2.3(−93.4)*	66.7(−19.9)	2.9(−86.1)*	11.7(−52.0)*
S.E.	1.37	0.64	0.86	4.28	0.44	0.89

[a]Values in parenthesis indicate percent increase (+) or decrease (−) over control. Asterisks indicate significant differences from control within each variable (*t* test at the 1% level).

inhibit germination (Table 1). Eight isolates were inhibitory to radicle growth but not germination of ryegrass. Isolates 14, HB21, CB31, and CB36 were also inhibitory to coleoptile growth of ryegrass. These four isolates also inhibited germination and growth of cress. Three isolates stimulated radicle growth of ryegrass but not of cress (data not shown). DeFrank and Putnam (1985) reported that 7% of the isolates of actinomycetes they screened inhibited 60% germination and growth of cucumber (*Cucumis sativus* L.) and barnyardgrass. Heisey et al. (1985) found that 10–12% of 347 isolates they tested on solid medium were highly inhibitory to three indicator seedlings.

Unfermented broth diluted 50% showed a reduction in seed germination and seedling growth of almost all species tested. Therefore, unfermented broth instead of water was used as a control in the bioassay. Six isolates (48, 119, HB21, CB21, CB31, CB36) were grown in broth. Growth of isolate 119 was profuse; dry weight of cells produced per 100 ml broth was 0.30 g, and that of the remaining isolates ranged between 0.020 and 0.22 g/100 ml broth. Data are presented (Figure 1) for isolates 48, 119, and CB31 only, since phytotoxicity of isolates HB21, CB21, and CB36 was similar to that of CB31.

The cell-free broth of 119 was highly toxic to four of the five dicot. species tested; yellow clover remained unaffected (Figure 1). Both germination and seedling growth of barnyardgrass, but only germination of ryegrass, were significantly reduced. The phytotoxin produced in the broth of isolate 48 was highly potent; it inhibited both dicot. and monocot. species. Suppression of radicle growth was associated with discoloration of the tip. This phenomenon suggests rapid absorption of the phytotoxin from the fermented broths of 119 and 48.

Isolate CB31 in broth culture was not inhibitory to any species, although the same isolate significantly inhibited cress and ryegrass on agar medium. This indicates that phytotoxin production on agar medium does not always correspond to a similar production in submerged culture. An antibiotic, fumaramidmycin, was discovered from *Streptomyces kurrsanovii*, which produced antibiosis on agar medium but not in submerged culture (Maruyama et al., 1975). Shomura et al. (1979) described a similar experience where they identified 25 strains of actinomycetales that produced antibiotics only on agar medium.

In the sand culture experiment, fermented broth of isolate 48 at 25% concentration significantly inhibited cress and velvetleaf, but not barnyardgrass (Table 2). Growth was generally stunted in all three species tested; this was reflected in reduced dry weights of shoots. Germination was not affected except in cress. It was noted that velvetleaf was very sensitive to the phytotoxin of isolate 48. These results generally corroborate those of the bioassays on filter paper. It appears that the phytotoxin from isolate 48 is more effective against dicot. than monocot. plants.

Stunted growth of a weed may be a useful strategy in weed management. Cell-free fermentation broth of *Pseudomonas fluorescens* strain D7 isolated from

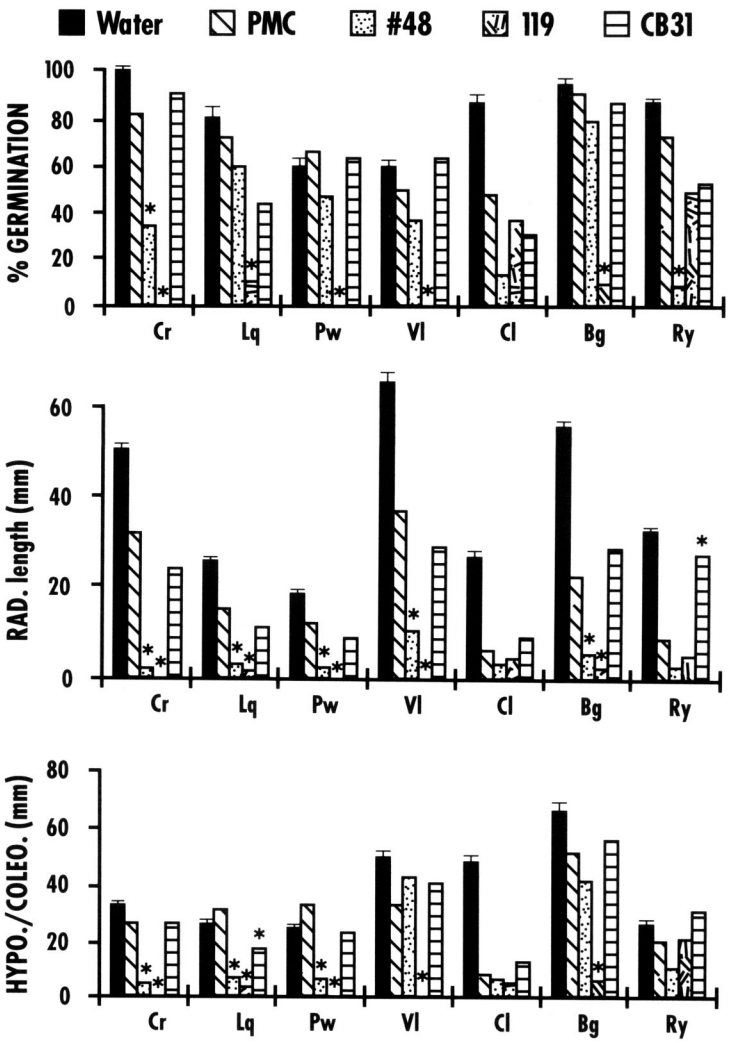


FIG. 1. Response of seeds from five dicotyledons and two monocotyledons on filter paper moistened with diluted (1:1) fresh and fermented broths of selected isolates. Differences between isolates were evaluated against controls for each species. The bars represent pooled standard errors of the means for a species. Cr = cress, Lq = lambsquarters, Pw = pigweed, V1 = velvetleaf, Cl = yellow clover, Bg = barnyardgrass, Ry = ryegrass.

*Significantly different from fresh (PMC) broth by *t* test at the 1% level.

TABLE 2. RESPONSE OF SEEDS OF TEST PLANTS IN SAND MOISTENED WITH DILUTED FRESH (UNINOCULATED) AND CELL-FREE BROTH OF ISOLATE 48

Broth (%)	Germination (%)		Shoot height (mm)		Shoot dry weight (mm)	
	Uninoc.	Fermen.	Uninoc.	Fermen.	Uninoc.	Fermen.
Cress						
25	104.2	93.8	81.5	65.1[a]	118.7	83.6
50	104.2	41.7[a]	71.9	32.6[a]	101.4	29.4[a]
75	86.5	24.0[a]	52.2	25.7[a]	75.7	22.9[a]
Control (water)	100		100		100	
SE	0.38		1.63		4.40	
Barnyardgrass						
25	118.6	114.3	115.7	91.0	32.6	13.5
50	104.3	108.6	174.7	71.9	75.6	6.7[a]
75	118.6	108.6	163.1	60.3[a]	54.9	18.6[a]
Control (water)	100		100		100	
SE	0.12		1.63		5.58	
Velvetleaf						
25	106.6	106.6	125.1	62.0[a]	92.3	21.9[a]
50	95.5	92.2	85.1	40.2[a]	33.5	6.8[a]
Control (water)	100		100		100	
SE	0.28		5.27		3.30	

[a]Significantly different from corresponding dilution of uninoculated broth by t test at the 1% level.

wheat rhizosphere reduced root growth of downy brome grass (*Bromus tectorum* L.) in hydroponic culture; field application of the bacterium selectively suppressed downy brome in winter wheat test plots (Tranel et al., 1993).

Sequestration of Phytotoxin. Methylene chloride extract of fermentation broth from isolate 48 at 0.5 mg/ml concentration severely inhibited germination and growth of cress and completely suppressed germination at 3.0 mg/ml concentration (Table 3). Germination of barnyardgrass was also negatively affected at the two higher concentrations. Cell extracts were also inhibitory, but to a lesser degree, indicating that the phytotoxin of isolate 48 is extracellular (Table 4). These results agree in general with those of crude broth.

It is noteworthy that the metabolites of isolates 119 and 48 completely suppressed germination and significantly reduced growth, respectively, of velvetleaf, a major weed that is difficult to control with currently available herbi-

TABLE 3. RESPONSE OF SEEDS OF TEST PLANTS ON FILTER PAPER MOISTENED WITH SOLVENT EXTRACTS OF FRESH (UNINOCULATED) AND CELL-FREE BROTH OF ISOLATE 48

Conc. of extract (mg/ml)	Germination (%)		Hypo./Coleop. length (mm)		Radicle length (mm)	
	Uninoc.	Fermen.	Uninoc.	Fermen.	Uninoc.	Fermen.
Cress						
0.5	106.7	66.6[a]	118.3	71.8[a]	114.0	68.2[a]
1.5	103.3	3.3[a]	111.4	54.4[a]	101.6	46.5[a]
3.0	103.3	0.0[a]	116.8	0.0[a]	110.8	0.0[a]
5.0	103.3	0.0[a]	92.2	0.0[a]	98.1	0.0[a]
Control (water)	100		100		100	
SE	0.19		0.32		0.57	
Barnyardgrass						
0.5	107.1	57.1	122.3	110.2	119.7	66.2[a]
1.5	114.3	57.1	121.0	115.3	108.4	50.7[a]
3.0	114.3	21.4[a]	113.4	52.9[a]	103.3	46.9[a]
5.0	121.3	0.0[a]	107.0	0.0[a]	118.3	0.0[a]
Control (water)	100		100		100	
SE	0.30		0.55		0.94	

[a]Significantly different from corresponding concentration of uninoculated broth by t test at the 1% level.

TABLE 4. RESPONSE OF CRESS SEEDS ON FILTER PAPER MOISTENED WITH SOLVENT EXTRACTS OF UNINOCULATED BROTH AND CELLS OF ISOLATE 48

Conc. of extract (mg/ml)	Germination (%)		Hypocotyl length (mm)		Radicle length (mm)	
	Uninoc.	Cells	Uninoc.	Cells	Uninoc.	Cells
0.5	103.3	55.5[a]	100.6	55.4	95.1	58.6
1.5	103.3	70.0	91.5	42.4[a]	81.9	33.9[a]
3.0	100.0	55.5[a]	92.1	59.9[a]	95.1	53.0[a]
5.0	106.7	22.2[a]	87.6	42.9[a]	88.5	40.5[a]
Control (water)	100		100		100	
SE	0.33		0.45		0.65	

[a]Significantly different from corresponding concentration of uninoculated broth by t test at 1% level.

cides. Investigations into the mode and site of action of the phytotoxins from these isolates may prove worthwhile. Phytotoxic compounds from both may serve as templates for new synthetic herbicide design.

Mishra et al. (1987) reported that crude extract of fermentation broth of *Streptomyces hygroscopicus* strain 30001 sprayed on potted cucumber caused reduced growth and development of necrosis. A similar result was obtained with another strain of the same species. A herbicidal compound, phosphonothrixin, was isolated from fermentation broth of *Saccharothrix* sp. The compound was reported to inhibit germination and develop chlorosis by foliar application on several graminaceous and broad-leaf weeds tested (Takahashi et al., 1995). Gealy et al. (1996) reported that cell-free fermentation broth and ethyl acetate extract of the broth of *Pseudomonas syringae* strain 3366 suppressed germination of seeds and reduced root and shoot growth of downy brome, winter wheat Hill 81, and 10 other crop and weed species.

In conclusion, the agar plate screening method is a rapid and inexpensive procedure for recognizing bioactive microbial strains. The results indicate that the potential to discover new phytotoxins for herbicidal purposes is unlimited. The two isolates mentioned here may serve as leads in the search for compounds with desirable herbicidal activity against velvetleaf.

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CHARACTERISTICS OF HYDROXAMIC ACID INDUCTION IN WHEAT TRIGGERED BY APHID INFESTATION

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Abstract—Hydroxamic acids (Hx) are natural products of Gramineae that are associated with cereal resistance to pests. We aimed at characterizing the induction of Hx accumulation in seedlings of wheat, *Triticum aestivum*, by short-term infestation of the cereal aphid, *Rhopalosiphum padi*. A load of 25 aphids increased significantly the Hx levels in the infested primary leaf in comparison with control levels. Lower loads did not increase Hx concentration. Aphid infestation lasting 16 hr did not elicit induction of Hx, even after a time-lag of 32 hr to allow the expression of any induced response. Forty-eight hours was the minimum duration of aphid infestation required to trigger Hx induction. The age of the infested tissue (the primary leaf) did not affect induction. Similar increases of Hx were found in unfolding, expanding, and totally expanded primary leaves. It was determined that the regime of nutrient supply (N-intensive nutritive solutions at low and high concentration) to wheat seedlings had no effect on the magnitude of the aphid-induced Hx (N-based secondary metabolites). Results obtained are discussed in the framework of general theories of plant defense allocation.

Key Words—Defense, herbivory, aphids, wheat, Gramineae, hydroxamic acids, Defense theory, Carbon/Nutrient theory.

INTRODUCTION

The last decade has seen a considerable increase in the number of reports on herbivory-induced phytochemical responses. However, seldom have these induced responses (IR) been subjected to a comprehensive characterization in

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terms of mechanisms, spatial and temporal dynamics, biotic and abiotic constraints to their expression, and associated costs imposed to the plant. Outstanding exceptions to this generalization come from the works on IR of wild tobacco alkaloids (Baldwin, 1989; Baldwin et al., 1990) and wild parsnip furanocoumarins (Zangerl, 1990; Zangerl and Berenbaum, 1995). Although work on IR has mainly addressed questions regarding theoretical issues such as optimal patterns of defense allocation (Ohnmeiss and Baldwin, 1994), patterns of insect grazing (Gibberd et al., 1988), and plant competition (Edwards et al., 1992), it recently has included applied approaches stressing the usefulness of induced resistance as a tool in pest management programs (English-Loeb et al., 1993). In order to achieve their goals, both major foci of IR studies require a thorough knowledge of the already mentioned characteristics of IR.

This paper aims at characterizing the phenomenon of induced phytochemical responses in wheat, *Triticum aestivum* L., by examining the induction of its main natural products, the hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones, Figure 1) (Niemeyer, 1988), following a short-term infestation by the bird cherry-oat aphid, *Rhopalosiphum padi* (L.). Hydroxamic acids (Hx) are defensive metabolites typical of Gramineae (Niemeyer and Pérez, 1995). Hx are induced in wheat by aphid feeding, and this induction is affected by plant genotype (Niemeyer et al., 1989), aphid genotype (Gianoli et al., 1997), and environmental conditions (Gianoli and Niemeyer, 1996). In addition, constitutive accumulation of Hx reduced aphid infestation in the field but did not impose a cost in grain yield to wheat cultivars (Gianoli et al., 1996). In a similar vein, IR of Hx by aphid infestation on the wild wheat, *T. uniaristatum*, were shown to be not costly to the plant in terms of growth and survival (Gianoli and Niemeyer, 1997) and were characterized as localized, short-term IR (Gianoli and Niemeyer, 1998). In the present work, we attempt to determine the threshold of aphid load and the minimum time of aphid infestation required to elicit the

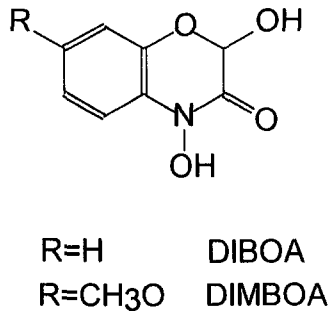


FIG. 1. Chemical structures of DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one), the main hydroxamic acid aglucones present in wheat extracts.

induction of Hx in wheat seedlings. In addition, we gain insights into the modulation of such IR by biotic and abiotic factors by examining the effect of leaf age and nutrient supply on Hx induction. Results are discussed in the framework of the optimal defense theory (McKey, 1974; Rhoades 1979) and, in the experiment on the effect of nutrients, they are also tested against predictions of the carbon/nutrient theory (Bryant et al., 1983).

METHODS AND MATERIALS

Basic Experiment on Induction of Hx. 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, and then grown in a growth chamber at 15°C and a photoperiod of 12L:12D. When seedlings attained growth stage (gs) 11 (primary leaf fully unfolded, secondary leaf visible, Zadoks et al. 1974), each one was infested with 20 apterae of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (second or third instar), confined in a clip cage attached to the primary leaf. Aphids came from a laboratory colony reared on oat seedlings at 20°C and a photoperiod of 12L:12D. Empty clip cages were placed on control plants. After 48 hr of infestation, aphids were removed from the infested seedlings and primary leaves of both control and treated plants were analyzed for Hx. No difference in fresh weight between control and infested leaves was found by the time of analysis of Hx in all the experiments performed (data not shown), thus allowing comparisons of concentrations of Hx.

Threshold of Aphid Load. This experiment assessed the minimum load of aphids required to elicit the induction of Hx in the infested leaf (primary leaf). Densities of 2, 10, and 25 aphids per clip cage were used in replicated basic experiments of induction. A LSD test following a one-way ANOVA was performed to examine differences of means among treatments ($N = 8$). This experiment was performed with late gs 12 seedlings (see below).

Duration of Infestation. Given that previous work showed 48 hr of continuous aphid infestation as sufficient to generate IR of Hx in wheat (Niemeyer et al., 1989; Gianoli and Niemeyer, 1996), we evaluated whether 16 hr of infestation could trigger IR as well. In order to determine whether a hypothetical lack of IR at the end of the 16-hr infestation could be due to either an insufficient stimulus or to a temporal constraint to the expression of the response, a treatment was included consisting of 16 hr of infestation and Hx analysis 32 hr later (to complete 48 hr from the onset of infestation until chemical analysis). Comparisons of control and infested seedlings were performed for each treatment by one-way ANOVAs. Treatments were defined as 48–48, 16–16, and 16–48, where the first number refers to duration (hours) of aphid infestation and the

second to the time elapsed from the onset of infestation to the analysis of Hx ($N = 8$).

Leaf Age. The basic experiment of induction was replicated at three different ontogenetic stages of the primary leaf related to three phenological stages of the seedling (Zadoks et al., 1974): gs 10 (primary leaf through coleoptile, seedling height 8–10 cm), gs 11 (primary leaf unfolded and secondary leaf visible, 12–14 cm) and gs 12 (primary leaf totally expanded, secondary leaf unfolded and tertiary leaf visible, 16–18 cm). A two-way ANOVA for Hx concentration was performed, with leaf age and aphid infestation as fixed effects ($N = 8$ for each treatment).

Nutrient Supply. The basic experiment of induction was replicated under two regimes of nutrient supply (high and low). Seeds were sown (October 15, 1996) in individual pots (25 ml) filled with vermiculite and watered exclusively with H_2O for five days; thereafter seedlings were subjected to either regimen of nutrient supply. Nutrient solutions were obtained from a master N : P : K aqueous solution (30, 20, and 15 g/liter, respectively) diluted 1/100 and 1/1000 for the high and low nutrient treatment, respectively. Seedlings received 10 ml of nutrient solution on October 21, 24, 28, and 30. On October 30, the protocol of infestation was implemented, and 48 hr later primary leaves were analyzed for Hx content. A two-way ANOVA for Hx concentration was performed, with nutrient regime and aphids as fixed effects ($N = 8$ for each treatment).

Chemical Analysis. Plant material was macerated with 1 ml H_2O, using a mortar and pestle. The aqueous extract was left at room temperature for 15 min and then taken 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 constant 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 1 liter H_2O): 0–7 min, 30% A; 7–9 min, 100% A; 7–13 min, 30% A. Detection of DIMBOA, the main Hx aglucone present, was performed at 263 nm.

RESULTS

Twenty five was the minimum number of aphids per clip cage able to trigger an Hx accumulation in the infested leaf that was significantly different ($P < 0.05$, LSD-test following a one-way ANOVA) from the control treatment (Figure 2). The lower levels of Hx observed in control seedlings in comparison with those of other experiments (Figure 3) are due to the use of late gs 12 seedlings, which are in the decreasing phase of Hx accumulation (see Discussion).

It was found that 16 hr of aphid infestation did not elicit an induction of Hx ($P < 0.05$, one-way ANOVA) even after a time-lag of 32 hr prior to Hx

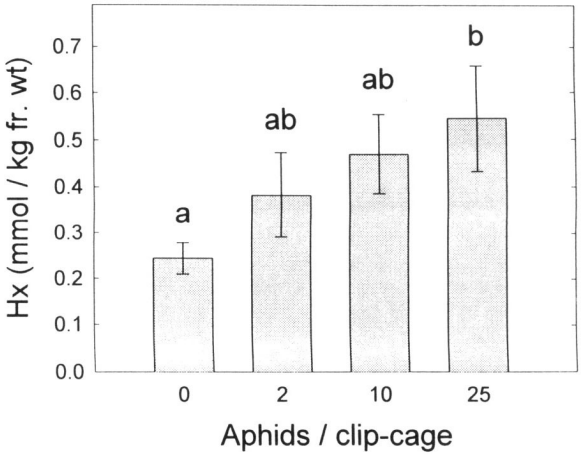


FIG. 2. Hydroxamic acid (Hx) content (mean ± SE, eight replicates) of the primary leaf of wheat seedlings after 48 hr of infestation by different levels of aphid infestation. Means in columns sharing lowercase letters are not significantly different (LSD test following one-way ANOVA).

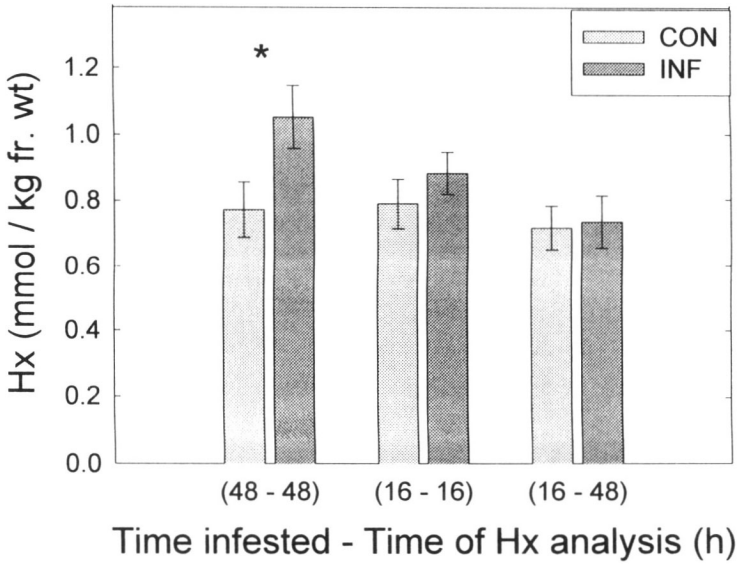


FIG. 3. Hydroxamic acid (Hx) content (mean ± SE, eight replicates) of the primary leaf of aphid-infested (20 individuals) (INF) and noninfested control (CON) seedlings of wheat after different duration of infestation and time elapsed to Hx analysis since the onset of infestation. *Significant differences between control and infested plants at $P < 0.05$ (one-way ANOVA).

TABLE 1. EFFECTS OF APHID INFESTATION (20 APHIDS FOR 48 HR) AND LEAF AGE (AS RELATED TO SEEDLING GROWTH STAGE, gs) ON ACCUMULATION OF HYDROXAMIC ACIDS IN PRIMARY LEAF OF WHEAT SEEDLINGS

Mean ± SE hydroxamic acids content (mmol/kg fresh weight)		
Leaf age	Control	Infested
gs 10	0.629 ± 0.092	0.843 ± 0.048
gs 11	0.772 ± 0.084	1.050 ± 0.059
gs 12	0.588 ± 0.025	0.694 ± 0.045

	Analysis of variance			
Source	*df*	Mean square	*F* ratio	*P*
Aphid infestation (A)	1	0.126	4.875	0.034
Leaf age (L)	2	0.153	5.942	0.006
A × L	2	0.013	<1.0	NS[a]
Error	42	0.026		

[a]NS = not significant.

analysis. As expected, 48 hr of infestation was needed to produce IR (Figure 3).

There was no effect of leaf age on Hx induction ($P > 0.61$ for the interaction of effects: aphid × leaf age, two-way ANOVA) (Table 1). However, both aphid infestation and leaf age had significant effects on Hx levels of the primary leaf ($P < 0.04$ and $P < 0.01$, respectively; Table 1). Higher Hx levels were found on infested plants, and an increase followed by a decrease of Hx was the pattern with leaf age (Table 1).

Nutrient supply regime did not affect Hx induction since the interaction of effects (aphid × nutrients) was not significant ($P > 0.85$, two-way ANOVA) (Table 2). Aphid infestation did affect Hx accumulation on the primary leaf ($P < 0.025$), infested tissues containing higher Hx levels (Table 2). Higher nutrient input produced no increase in Hx levels, the effect being marginally nonsignificant ($P = 0.061$) (Table 2).

DISCUSSION

The intensity of herbivory has been considered to affect the magnitude of IR (Karban, 1991). In the present system, significant induction of Hx in wheat

TABLE 2. EFFECTS OF APHID INFESTATION (20 APHIDS FOR 48 HR) AND NUTRIENT SUPPLY REGIME ON ACCUMULATION OF HYDROXAMIC ACIDS IN PRIMARY LEAF OF WHEAT SEEDLINGS

Mean ± SE hydroxamic acids content (mmol/kg fresh weight)		
Nutrients	Control	Infested
Low	0.499 ± 0.043	0.595 ± 0.020
High	0.574 ± 0.052	0.686 ± 0.042

	Analysis of variance			
Source	*df*	Mean square	*F* ratio	*P*
Aphid infestation (A)	1	0.090	**5.922**	0.021
Nutrients (N)	1	0.058	**3.800**	0.061
A × N	1	0.001	**<1.0**	NS[a]
Error	28	0.015		

[a]NS = not significant.

seedlings was restricted to the higher aphid load tested (25 individuals per clip cage) and did not appear following 16-hr infestations. Lower aphid densities did not increase Hx levels in infested tissues. Thus, an infestation of 25 aphids (or 20, as this and previous work reported) for 48 hr seems to be the threshold for eliciting IR of Hx in wheat seedlings. Interestingly, a similar work on aphid-induced Hx in a wild wheat showed that a load of 10 aphids (for 48 hr) was enough to obtain IR and, furthermore, induced Hx levels remained constant following greater densities of infestation (Gianoli and Niemeyer, 1998). It may be that cultivated wheat, subjected to artificial selection oriented towards agronomic features, has suffered a loss in its capacity to react readily to cues of herbivore attack by increasing the concentration of secondary metabolites. Alternatively, different "sensitivities" to aphid infestation may be a consequence of differences in tolerance, i.e., the capacity to recover after herbivory. Thus, plants with greater tolerance levels could delay the allocation of defenses to attacked tissues until the degree of damage threatened its fitness, hence saving resources to devote to growth or reproduction (see Zangerl and Bazzaz, 1992).

Leaf age is a factor usually overlooked in IR studies (Coleman and Jones, 1991). Contrary to previous reports on indole alkaloids (Frischknecht et al., 1987) and phenols (Hartley and Firn, 1989), and in accordance with recent work on furanocoumarins (Zangerl and Berenbaum, 1995), we found that age of the

infested leaf did not affect induction of Hx. Both aphid infestation and leaf age, however, had singly a significant effect on Hx levels of the primary leaf. The aphid effect was expected as Hx are inducible; likewise, an effect of leaf age was also expected since the dynamics of Hx accumulation in wheat seedlings is highly sensitive to age (Argandoña et al., 1981). Thus, the pattern of Hx accumulation for the whole plant as well as for any single leaf consists of an initial increase until reaching an early peak and a steady decrease thereafter. As can be seen (Table 1), the seedling growth stages (gs) utilized in this experiment fall in the upward-maximum-downward part of the curve of Hx accumulation.

The optimal defense (OD) theory (McKey, 1974; Rhoades, 1979) would predict that younger—and hence more valuable—plant parts receive better protection by enhanced defense allocation (see Bryant and Julkunen-Tiitto, 1995). To some extent this was the case for the primary leaf of wheat seedlings if constitutive Hx levels are considered (Table 1). However, as shown by the statistical analysis performed, this leaf showed comparable degrees of Hx induction for the three growth stages evaluated. This lack of effect of leaf age may be an artifact of the limited range of seedling phenological stages studied. Older phenological stages and hence lower Hx constitutive levels (in accordance with the dynamics of accumulation) could constitute a scenario in which greater levels of induced Hx could be produced in order to achieve a better protection.

Results obtained showed that nutrient availability did not affect induction of Hx by aphid infestation. Similar results were obtained with lupine alkaloids (Johnson et al., 1989), tobacco alkaloids (Ohnmeiss and Baldwin, 1994), and furanocoumarins (Zangerl and Berenbaum, 1995). As expected, aphid infestation affected Hx accumulation. As was reported for corn seedlings (Manuwoto and Scriber, 1985), nutrient supply regime affected overall Hx accumulation in wheat seedlings, but its effect was not significant. Regarding the close relationship between available nitrogen in the substrate and concentration of N-based compounds in wheat (Kowalski and Visser, 1987) and given that Hx may be classified as N-based secondary metabolites (Figure 1) and that nutrient solution was predominantly nitrogen (55%), this effect was not surprising. Nonetheless, in view of this fact, it is reasonable to expect a higher induction in the high-nutrient regime due to the greater availability of nitrogen; however, this did not occur. A rationalization for these results may be produced by examining general theories of plant defense allocation.

OD theory predicts enhanced allocation of (induced) defenses to tissues with high fitness value, costly to replace, and/or with high probability of attack. This allocation should reflect the costs and benefits of investing those resources in defense instead of in growth or reproduction. On the other hand, carbon/nutrient (C/N) theory (Bryant et al., 1983) would consider production of induced defenses limited by plant requirements of either C-based or N-based compounds

for growth and in relation to environmental conditions. Thus, occurrence of IR (according to whether the defenses are C-based or N-based) would be conditioned to internal imbalances in either type of compound and to the effect of herbivory on the C/N ratio in the tissue concerned. Given that both theories address the relationship of defense to growth, it is important to point out that growth of the infested primary leaf did not differ from that of the control in either nutrient treatment (data not shown).

Our system consisted of: (1) plants treated with different levels of N in the substrate; (2) a phloem-feeder that depletes proportionally more C-based compounds than N-based ones (see Hayashi and Chino, 1986), i.e., relatively increases available N; and (3) a N-based natural product. Therefore, C/N theory would predict a higher level of induction of Hx in the high-nutrient treatment. This prediction was not supported by the results obtained. Alternatively, from the standpoint of OD theory, a more complex scenario may be conceived. On the one hand, a greater N budget would allow an enhanced allocation to N-based induced defenses without major penalties in growth for such resource investment. Then a higher level of induction of Hx in the high-nutrient treatment may be predicted. On the other hand, tissue of plants in richer habitats is of lesser value since its replacement is less costly to the plant and hence should receive less chemical protection than a comparable tissue in a plant growing in a poor habitat. Although the expression of such an advantage in growth and tissue replacement was not evident in our experiment, it should appear with longer periods of evaluation. This view would lead one to expect a lower level of induction of Hx in the high-nutrient treatment. We speculate that under the experimental conditions of this study both trends occurred, and hence the outcome was a lack of effect of nutrients on Hx induction. Further studies involving longer evaluations and including whole-plant allocation patterns will confirm or reject this preliminary observation.

Finally, another point relevant to the lack of effect of nutrients on Hx induction concerns the mechanism of induction. Whether Hx are synthesized in situ or transported to the attacked tissue would determine the relative importance of a surplus of nitrogen for Hx induction, since in situ synthesis is expected to be more sensitive to resource availability than translocation. Since aphid-induced Hx in the primary leaf of a wild wheat was shown to be a consequence of translocation from the stem (Gianoli and Niemeyer, 1997), it is likely that a similar process is operating in the present system, hence supporting the pattern found.

The characteristics of the induction of Hx by aphid infestation in wheat seedlings were determined in terms of the minimal stimuli able to generate it and the biotic as well as abiotic constraints to its expression. Although evaluations were restricted to a limited set of conditions, they represent a first approach

to the knowledge of such a complex trait as inducible plant defenses and thereby provide valuable information to both theoretical and applied research projects. The phenomenon of Hx induction in wheat seedlings appeared to be dependent on initial stimulus conditions (duration and magnitude of the infestation) but insensitive to biotic (leaf age) as well as abiotic (nutrient supply regime) factors. Results obtained were shown to be partially in accordance with OD theory predictions.

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BEHAVIORAL EFFECTS OF CAROTENOID SEQUESTRATION BY THE PARSNIP WEBWORM, *Depressaria pastinacella*

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Abstract—The parsnip webworm, *Depressaria pastinacella*, acquires a distinct yellow stripe when it consumes the yellow flowers of its principal host plant, *Pastinaca sativa*, the wild parsnip. Caterpillars raised on artificial diet lacking host-plant material lack this yellow coloration. By chemical characterization and comparison of caterpillars raised on parsnip flowers and on artificial diet, we were able to determine that lutein, along with smaller amounts of other xanthophylls from the host plant, is selectively sequestered in the fat body. In bioassays designed to measure avoidance of ultraviolet light, caterpillars raised on parsnip flowers or on artificial diet supplemented with lutein were less likely to avoid exposure to ultraviolet light than caterpillars raised on unaugmented artificial diet and thus lacking sequestered carotenoids. The ability to sequester xanthophylls, which are highly effective antioxidants, may confer a selective advantage on these caterpillars, whose apiaceous host plants produce large quantities of furanocoumarins, natural products that are photoactivated by light wavelengths in the ultraviolet region; such sequestered pigments may reduce not only the oxidative stress associated with ultraviolet light and diurnal foraging but also the photooxidative stress associated with ingestion of photoactive furanocoumarins.

Key Words—*Depressaria pastinacella*, parsnip webworm, *Pastinaca sativa*, wild parsnip, lutein, UV avoidance, phototoxicity, antioxidant, carotenoid, Lepidoptera.

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INTRODUCTION

Carotenoids are tetraterpenes that collectively constitute the most widely distributed plant pigments. Due to their ability to absorb wavelengths of light in the range of 450–500 nm, these compounds serve as important accessory pigments for photosynthesis. Moreover, as singlet oxygen quenchers and free radical scavengers, carotenoids aid plants in coping with oxidative stress associated with photosynthesis (Larson, 1988). Ecologically, these highly colored—generally bright yellow, orange, or red—compounds play an important role as signal substances and are frequently involved in mediating interactions between plants and their animal mutualists, particularly pollinators and dispersers of seeds and fruits. In insects, carotenoid pigments are generally derived from precursors obtained in foods, although in some exceptional instances they are produced by symbiotic yeasts (Kayser, 1985). As in plants, carotenoids in insects are important in mediating both inter- and intraspecific communication and play a role in species recognition and aposematic signalling (Rothschild et al., 1986).

The physiological role of carotenoids in insects is less clear than is their behavioral importance. The metabolic significance of carotenoids in most animals is related to the fact that they can be converted into vitamin A, although only a small number of plant carotenoids function as provitamins. There is currently no evidence of an absolute requirement for vitamin A in any insect species (Skinner et al., 1995), although inclusion of vitamin A in diets can have growth-promoting effects (Shimizu et al., 1981). Vitamin A in insects is involved in light perception (Goldsmith and Warner, 1964), and dietary carotenoid deficiencies are associated with loss of phototactic responses over the course of development (Shimizu et al., 1981). In at least one instance, inclusion in diets of lutein, a nonprovitamin, as well as β-carotene restored visual sensitivity in house flies deprived of a carotenoid source during development (Goldsmith, 1964). Lutein may also play a role in detection of light cues associated with homochromy, or background-matching, in *Pieris brassicae* (Rothschild et al., 1975).

Recent evidence also suggests that, due to their ability to quench singlet oxygen and act as free radical scavengers, carotenoids sequestered from food may act to reduce photooxidative stress experienced by herbivorous insects. Aucoin et al. (1990) demonstrated that elevated dietary intake of β-carotene reduced the phototoxicity of the oxygen-dependent photosensitizer α-terthienyl in *Manduca sexta*, and Green and Berenbaum (1994) showed that vitamin A ameliorates the phototoxicity of citral to the cabbage looper, *Trichoplusia ni*. A function for nonprovitamin carotenoids has not yet been demonstrated, despite the fact that they are commonly sequestered by insects.

The parsnip webworm, *Depressaria pastinacella*, is a herbivorous lepidopteran likely to experience high levels of photooxidative stress as a conse-

quence of its diet. This oligophagous caterpillar feeds exclusively on species in two genera of Apiaceae (Berenbaum and Zangerl, 1991). Both genera—*Pastinaca* and *Heracleum*—are characterized by the production of furanocoumarins, benz-2-pyrone compounds that are highly phototoxic (Murray et al., 1982). In the presence of ultraviolet light, furanocoumarins can form an excited triplet state that can interact directly with DNA, protein, or lipids to cause toxicity; triplet-state furanocoumarins can also react with ground-state oxygen to generate singlet oxygen, superoxide anion, and hydroxyl radicals (Berenbaum, 1991). These toxic oxygen species can in turn cause damage by denaturing proteins, disrupting lipids, and damaging other biomolecules.

D. pastinacella possesses a number of biochemical mechanisms for reducing its exposure to oxidative stress. Although these caterpillars excrete a small amount of furanocoumarins unmetabolized in silk (Nitao, 1990), the principal mechanism by which furanocoumarins are processed is by cytochrome P-450-mediated metabolism (Nitao, 1989; Berenbaum and Zangerl, 1992). In addition, constitutive levels of antioxidant enzymes in this species are high relative to insects not normally associated with phototoxic plants (Lee and Berenbaum, 1990); this suite of enzymes may be involved in neutralizing hydrogen peroxide, superoxide anion, and other toxic oxyradicals generated as a result of photoactivation of unmetabolized furanocoumarins.

Some evidence exists that webworms may also rely to a certain extent on behavioral mechanisms of protection against phototoxicity. Normally, they are found ensconced in a silken web, into which parts of the developing umbel are woven. Caterpillars are rarely found moving about on the plant during daylight hours, although whether or not this behavior is mediated by detection and avoidance of sunlight has not previously been determined. Berenbaum (1978) has suggested that concealed feeding is disproportionately common in oecophorids (and other insects) associated with phototoxic plants, serving as a means of behavioral resistance. Guillet et al. (1995) documented induction of shelter-building behavior in the tortricid larva *Sparganothis reticulatana* on the phototoxic daisy *Chrysanthemum leucanthemum* and preferential oviposition by the adults of this species in shade. The diurnal burrowing habits of the chrysomelid beetles *Chrysolina quadrigemina* and *C. hyperici* on their host plant *Hypericum perforatum* may represent another form of behavior induced by light exposure that serves to reduce photooxidative stress caused by ingestion of the phototoxic anthraquinone hypericin (Fields et al., 1990).

In addition to biochemical and behavioral defenses, the possibility exists that parsnip webworms also possess physical defenses against the phototoxins in their host plants. In the field, caterpillars in the last instar characteristically possess a bright yellow stripe visible along the underside of the abdomen (Riley, 1889; Hodges, 1974). This yellow pigmentation is absent in caterpillars raised in the laboratory on artificial diets, indicating that the yellow color results from

sequestration of a host-plant-derived pigment. Aucoin et al. (1990) suggested that the bright orange–red fat body of *Chrysolina hyperici*, associated with *Hypericum perforatum*, contains β-carotene sequestered from the host plant, which protects the beetle larva from hypericin-associated photooxidative damage. In webworms, the sequestered pigment may serve a similar function.

In this study, we set out to characterize the behavioral responses of the parsnip webworm to visible and ultraviolet light and to determine whether these responses are influenced by sequestration of pigments from the plant. We had two objectives: to characterize and identify the pigment(s) sequestered from the host plant by the parsnip webworm and to determine whether sequestration of pigments from the host plant influences webworm response to visible and ultraviolet light.

METHODS AND MATERIALS

Plants and Insects. *Pastinaca sativa* (wild parsnip) and *Depressaria pastinacella* (parsnip webworms) were collected from roadside populations in Savoy, Illinois (Champaign County) in mid-June 1995, unless otherwise indicated. Preliminary bioassay experiments were conducted with webworms from this population but, due to timing and availability, webworm collections were made in Minnesota for additional experiments. These webworms were collected as eggs from an overgrown field in late May 1996 on the east side of Minnesota State Highway 61, located 1.6 km north of Lock and Dam #5 in Goodhue County, Minnesota. The field was located between the highway and a railroad. Both the highway and the railroad ran parallel to the Mississippi River (located about 100 m east of the highway).

Larvae were maintained in 1-oz plastic creamer cups either on artificial diet (Nitao and Berenbaum, 1987), on artificial diet supplemented with lutein (Sigma, St. Louis, Missouri), or on excised flowering umbels of parsnip collected in the field and brought to the laboratory on a regular basis, depending upon the purpose for which they were being reared. Rearing conditions were maintained at approximately 20–23°C with a photoperiod of 16L:8D.

Pigment Identification. Leaves, buds, flowers, and fruits were examined for pigment content, as were both pigmented and unpigmented larvae. Larvae were collected as second instars from wild parsnip and brought back to the laboratory, where they were placed on artificial diet and allowed to develop until they reached sixth instar; these larvae were not visibly pigmented. A second group of larvae was collected at the same site several weeks later as late fourth and early fifth instars; these were brought back to the laboratory and maintained on umbels collected from the same sites until they reached sixth instar. Both pigmented and unpigmented sixth instars, matched for approximately equal

weight and size, were dissected and fat body removed for extraction. The fat body in pigmented larvae was a distinctive bright yellow color; the fat body in unpigmented larvae was white in color. Although the yellow fat body surrounds the gut in its entirety, the yellow color is visible only on the ventral side of the abdomen; the presence of densely distributed microspinules on the dorsum may reduce the transparency of the cuticle (which is dull gray in color). Fat bodies were pooled, placed in Eppendorf tubes, and frozen until they were extracted.

Solubility of the plant pigment was tested in hexane, methanol, ethanol, distilled water, alkaline solution, and chloroform. Approximately 10 mg of dried, ground plant material was placed in solvent, agitated, and left to stand for 10 min. The resulting color intensity of the solvent was used as an indication of solubility. This preliminary analysis indicated that the yellow pigment was extremely lipophilic and was most efficiently and rapidly extracted in chloroform or hexane.

Plant and insect samples were extracted with chloroform and characterized by thin-layer chromatography (TLC). Extracts of plant and insects were applied to C18-F silica plates (J. T. Baker Chemical, Phillipsburg, New Jersey). Two-dimensional analysis was conducted with hexane–ethyl acetate–acetic acid (72:27:1) as the first solvent system and acetic acid–methanol–water (3:3:4) as the second system (Heimler, 1986). Under these conditions, the yellow color in both flower petal extracts and extracts of fat bodies from caterpillars consuming umbels tended to travel with the solvent front. Individual bands were scraped off TLC plates and eluted in methanol; after filtering through cotton wool, extracts were placed in quartz cuvettes and scanned from 250 to 850 nm (Perkin Elmer UV/Vs spectrophotometer). The presence of distinct peaks in the 400 to 450-nm range suggested that the yellow compounds were carotenoids.

After preliminary analysis confirmed the carotenoid nature of the pigments, plant and insect samples were extracted again for definitive characterization. The basic procedure for pigment extraction was that of Valadon and Mummery (1967, 1973, 1978). Fresh plant material was extracted twice in methanol in a blender and then agitated at 10°C with methanol–ethyl ether until no more color was extracted. Extracts were combined and partitioned with ethyl ether. Water was added until all color had transferred to the ether layer, which was then evaporated in darkness under vacuum. The resulting oil was taken up in methanol and saponified with 60% (w/v) aqueous potassium hydroxide. The alkaline mixture was kept in darkness under helium overnight, after which time it was diluted with three volumes of distilled water and one volume ethyl ether. The mixture was firmly shaken and allowed to stand until two phases appeared. Ether extraction was repeated twice and the resulting extracts combined and washed free of potassium hydroxide by shaking with water. This procedure continued until no alkalinity was detectable in the ether extract. The ether was then evaporated in the dark under vacuum and the resulting oil dissolved in 20

ml hexane. The hexane solution was centrifuged at −20°C to remove precipitated sterols, and carotenoids in the hexane were then partitioned with 90% methanol. The upper phase, containing carotenes and monohydroxy xanthophylls, was washed three times with water to remove the methanol and then was dried with sodium sulfate (0.5 g/10 ml). Sodium sulfate in turn was removed by centrifuging for 10 min in a table-top centrifuge. The hexane extract was concentrated in darkness under vacuum and taken up in acetonitrile–methanol–ethyl acetate (Tee and Lim, 1992).

The lower phase of this separation, containing di- and polyhydroxy xanthophylls, was further processed by the addition of ethyl ether. All color transferred to the epiphasic layer, which was washed three times with water to remove methanol. The washed layer was next reduced in darkness under vacuum, taken up in hexane, and dried with sodium sulfate; sodium sulfate was removed as described. The hexane phase was evaporated under vacuum in darkness and taken up in acetonitrile–methanol–ethyl acetate. Both acetonitrile–methanol–ethyl acetate fractions were stored at −80°C until analyzed.

High-pressure liquid chromatography (Waters model 991 photodiode array system, Milford, Massachusetts) was used to characterize and identify pigments in purified extracts. A 5-μm C18 stainless steel column (Vydac 201TP54 C18 reverse phase; The Separations Group, Hesperia, California), preceded by a precolumn filter, was used to effect separation; the mobile phase was methanol–acetonitrile–water (88:9:3) with 1% 2,2,4-trimethyl pentane added as a solvent modifier. Flow rate was 2 ml/min; peaks were scanned by a photodiode array detector and compounds were identified by comparisons of retention time of standards; UV/Vs spectra were compared with spectra run on authentic standards for identification.

Behavioral Assays. For both pigment identification and behavioral bioassays, we took advantage of the fact that, when parsnip webworms are raised from egg hatch on artificial diets (Nitao and Berenbaum, 1987) lacking host-plant material, they possess no visible trace of yellow pigment. When larvae are raised on plant material for the first four or five stadia and then switched to artificial diet for the penultimate and/or ultimate stadium, they retain the yellow color despite the absence of host-plant pigments in the artificial diet. The advantage of conducting bioassays with pigmented larvae that had been switched to artificial diet is that such an arrangement minimizes the potential confounding effects of phototoxic reactions. Wild parsnip fruits and flowers are rich sources of furanocoumarins (Berenbaum et al., 1986), which are phototoxic to a wide range of insects in the presence of ultraviolet light (Berenbaum, 1995). In these experiments, our objective was to determine responses to ultraviolet light (Beard, 1972), as well as responses to ultraviolet light-induced furanocoumarin pathology.

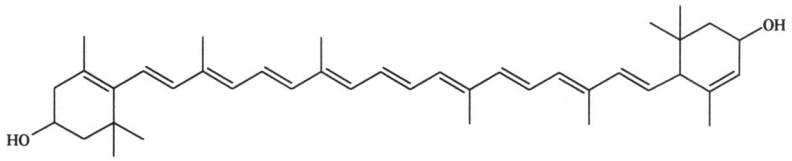
For behavioral bioassays, neonate larvae were transferred onto artificial diet and allowed to feed until second instar, at which point they were transferred to one of three diets: unamended artificial diet, artificial diet supplemented with 5 μg lutein/g diet, or flowers from parsnip plants grown locally. Lutein, as obtained from Sigma (St. Louis, Missouri), was actually a mixture of carotenoids, approximately 70% of which was lutein. Larvae were maintained on these diet treatments through sixth instar. Larvae fed either the lutein-supplemented diet or flowers developed the yellow-striped coloration; by contrast, larvae fed artificial diet lacking a carotenoid supplement did not develop the yellow stripe. Insects were kept at 20°C with a 16L:8D photoperiod.

To test responses to visible light, 12 sixth instars raised from second instar on lutein-supplemented artificial diet, 17 sixth instars raised from second instar on unamended artificial diet, and 14 sixth instars raised from second instar on female flowers were used. Larvae were placed individually in UVA-transparent Pyrex Petri dishes. Half of each Petri dish was covered with black construction paper fitted to shade any underlying larva from direct visible light. The dishes were placed beneath a bank of Phillips Cool-white fluorescent bulbs and covered by a UV-opaque Plexiglas shield to block any incidental UVA light. The position of each larva was recorded every 5 min for 60 min. Larval distributions in the shaded and illuminated halves were compared across treatments by a chi-square test for each observation period.

A similar bioassay design was used to determine whether webworms can sense and avoid ultraviolet (UVA) light. As before, larvae were raised from second instar on three different diets: lutein-supplemented artificial diet (N = 26), unaugmented artificial diet (N = 34), and female parsnip flowers (N = 28). Larvae were placed individually in UVA-transparent Pyrex Petri dishes. A UV-opaque Plexiglas shield was placed over the Petri dishes and positioned so that half of each dish was shielded from UVA light. The dishes were placed under a bank of Phillips 40W BLB bulbs. Light intensity was measured with a UVX radiometer (San Gabriel, California) and averaged approximately 300 mW/cm^2 on the exposed half of the dish for the duration of the experiment. No UVA was detectable under the shielded half of the Petri dishes. The position of each larva was recorded every 5 min for 55 minutes. Larval distributions in UVA-exposed and UVA-protected halves of the dishes were compared across treatments by a chi-square test for each observation period.

RESULTS

Characterization of Pigment. Although no spectra were obtainable from extracts of fat bodies removed from caterpillars raised on unaugmented artificial diet, several compounds were found in abundance in extracts of fat bodies



lutein

FIG. 1. Structure of lutein.

removed from caterpillars raised on parsnip umbels. Xanthophylls predominated in this yellow-colored extract. The principal compound, as confirmed by comparison with authentic material, was lutein (Figure 1) (with peaks at 422, 443, and 472 nm, retention time 4.82); present in smaller amounts were mutatoxanthin (lutein epoxide) (400, 424, and 442 nm, retention time 2.76 min) and two compounds tentatively identified as α-cryptoxanthin (423, 445, and 472 nm, retention time 8.04 min) and β-cryptoxanthin (427, 449, and 475 nm, retention time 9.66 min). Peaks corresponding to those in fat body extracts were

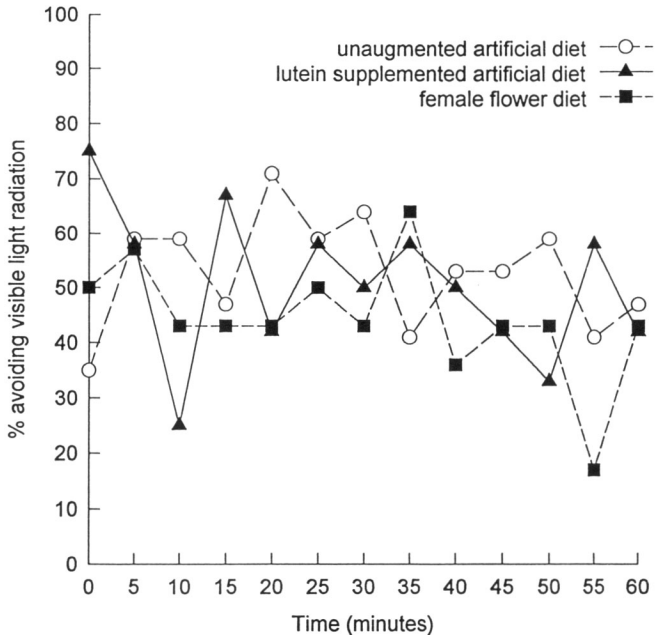


FIG. 2. Avoidance of visible light by sixth-instar parsnip webworms over time.

also found in extracts of flower petals; among the other compounds confirmed in flower petals was β-carotene (428, 452, 480 nm, retention time 18.84 min).

Behavior. Light in the visible range does not stimulate avoidance behavior in caterpillars feeding on either artificial diet, artificial diet supplemented with lutein, or furanocoumarin-rich parsnip flowers (chi-square test, $P > 0.05$ for all 13 time intervals) (Figure 2). In contrast, UVA radiation does elicit avoidance behavior in unpigmented larvae (i.e., those raised on unaugmented artificial diet), but not in pigmented larvae (raised on lutein-supplemented diet or on parsnip flowers) (Figure 3). For 9 of 11 time intervals, there were fewer unpigmented larvae in the side of the dish exposed to UVA than pigmented larvae (chi square test, $P < 0.05$). The dietary source of the pigment did not affect UVA-avoidance behavior; larvae fed lutein-supplemented diets did not differ significantly in their avoidance response from larvae fed flowers (chi square test, $P > 0.05$ for all 11 time intervals). However, unpigmented larvae avoided UVA light more often than either lutein-fed larvae (chi square test, $P < 0.05$ for 8 of 11 time intervals) or flower-fed larvae (chi square test, $P < 0.05$ for 8 of 11 time intervals).

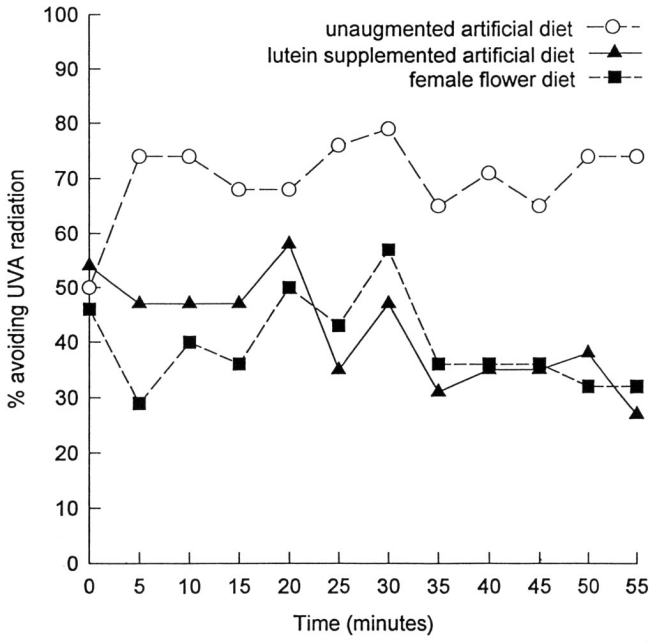


FIG. 3. Avoidance of ultraviolet light by sixth-instar parsnip webworms over time.

DISCUSSION

The parsnip webworm has developed successful defenses against phototoxins in its diet, but it is by no means immune to the effects of these compounds. Some furanocoumarins are metabolized poorly if at all (Berenbaum and Zangerl, 1992), and degradation or inactivation of phototoxin-metabolizing enzymes upon exposure to furanocoumarins (Zangerl and Berenbaum, 1993) compromises the efficacy of metabolic detoxification as a defense mechanism. Moreover, these defenses are activated only after exposure to phototoxins has occurred. In contrast, reliance on visual cues to stimulate negative phototaxis, independent of the consumption of phototoxins, allows insects to avoid UVA-associated damage entirely. Behavioral avoidance of ultraviolet light also may be less prone to circumvention than is metabolic detoxification by the evolution of novel plant secondary metabolites (e.g., Ehrlich and Raven, 1964).

While UV-light avoidance confers apparent benefits to the webworm, this behavior is not without cost; avoiding sunlight results in restriction of foraging behavior and reduction of internal temperature. Webworms are often forced to search for new umbels due to consumption of fruit and flowers at their immediate web site (Berenbaum and Zangerl, 1992). Webworms limited to foraging in UVA-free areas would presumably be at a disadvantage relative to webworms capable of finding food sources irrespective of light exposure. Moreover, UV avoidance also removes insects from direct sunlight. Solar irradiation can be beneficial in that it can act to increase insect metabolic rates (Ali et al., 1990). Larvae of the monarch butterfly (*Danaus plexippus*), for example, increase their internal temperature 3–8°C by basking, significantly reducing development time (Rawlins and Lederhouse, 1981). In general, for caterpillars, rapid growth rates are beneficial in that they reduce the risk of encounters with predators and parasites (Price et al., 1980).

By sequestering carotenoid pigments from their host plants, parsnip webworms may be able to extend the amount of time they are able to remain exposed to ultraviolet light and thus offset, to some degree, the disadvantages of light sensitivity. Previous investigators have documented the presence of sequestered carotenoids in both larval and adult Lepidoptera (Valadon and Mummery, 1967, 1978; Rothschild et al., 1986). These sequestered pigments are thought to be important in protecting insects against photooxidative stress. This protection may be particularly important in species associated with plants containing phototoxic natural products. Fields et al. (1990) suggested that β-carotene present in the fat body of *Chrysolina hyperici* protects this specialist against the phototoxicity of hypericin, the principal anthroquinone pigment in its *Hypericum* host plants. Furthermore, Davidson et al. (1991) reported the presence of the novel carotenoid 3,4-didehydro-*b*,*b*-caroten-2-one in the eggs of the walking stick, *Anisomorpha buprestoides*, which also feeds on *Hypericum* species.

Sequestration of lutein, a nonprovitamin carotenoid, by the parsnip webworm is consistent with such a physiological function. In the parsnip webworm, however, carotenoid sequestration may serve not only to ameliorate the toxicity of furanocoumarins but also to influence the foraging behavior of this insect. By sequestering lutein, a powerful antioxidant (Hirayama et al., 1994), the parsnip webworm is able to withstand exposure to ultraviolet light for a longer period of time even in the absence of phototoxins in the diet.

The ubiquity with which lutein is sequestered by Lepidoptera suggests that the physiological ability to store this and similar compounds and to retain them through metamorphosis may have facilitated the acquisition and led to the proliferation of diurnal habits among the butterflies. Light stress may be widespread and independent of the occurrence of phototoxins in plants. Although arthropods appear to use provitamins, such as β-carotene, as well as some nonprovitamin carotenoids, for the production of visual pigments used in detecting light and responding to photoperiod (Goldsmith, 1964; Goldsmith and Warner, 1964; Rothschild et al., 1975; Veerman, 1980; Dadd, 1985), carotenoids with no provitamin activity may be sequestered for different reasons—namely, to reduce the body burden of toxic oxygen species generated by exposure to ultraviolet light and to prolong the period of time during which exposure to ultraviolet radiation can be withstood.

The behavioral importance of carotenoid sequestration is consistent with the observation that many species sequester greater amounts of carotenoids in the larval stage, during which time considerably more movement in search of food takes place, than in the sedentary pupal stage. Also consistent with this protective function is the observation that lutein and β-carotene are the carotenoids most widely sequestered by Lepidoptera (Feltwell and Valadon, 1972; Feltwell and Rothschild, 1974; Feltwell, 1978; Czeczuga, 1986). The degree of hydroxylation, in general, affects the likelihood of sequestration; in a survey of 102 species, the most frequently sequestered carotenoids, β-cryptoxanthin, lutein epoxide, zeaxanthin, canthaxanthin, and astaxanthin, are all at least monohydroxylated. In that attachment of a hydroxy group there is "a positive effect" on the antioxidant properties of carotenoids in general (Hirayama et al., 1994); the preponderance of hydroxylated carotenoids among the sequestered pigments of insects argues for a protective antioxidant function.

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Neotyphodium coenophialum MYCELIAL PROTEIN AND HERBAGE MASS EFFECTS ON ERGOT ALKALOID CONCENTRATION IN TALL FESCUE

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Abstract—Livestock grazing endophyte-infected tall fescue (*Festuca arundinacea* Schreb.) pastures often suffer from ergot poisoning. The endophyte, *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon, and Hanlin, comb. nov., also provides drought-tolerant, insect-resistant, and disease-resistant qualities to the plant. Therefore, producers are faced with a biological dilemma of risking loss of pasture by using endophyte-free tall fescue pasture or animal losses with endophyte-infected tall fescue pasture. One potential solution is to breed endophyte-infected tall fescue with lower levels of alkaloids. However, breeding could select for plants that are antagonistic to the endophyte, resulting in reduced plant vigor as a consequence of disruption of the mutualistic association between the organisms. The objectives of this study were to determine the relationship between plant mass and endophyte mycelial proteins versus ergot alkaloid concentration. To examine the endophyte effect on mycelial mass, tissue culture regenerants from tall fescue genotype PDN2 were infected with endophyte isolates EDN11, EDN12, and EDN2 to eliminate confounding effects of multiple plant genotypes. Crosses with PDN11 as the maternal parent and plant genotypes DN2, DN12, and DN15 as paternal parents were used to produce a population of different plant genotypes, all containing the same endophyte. Fungal mycelial protein was extracted from lyophilized tall fescue leaf sheath tissue and immunochemically quantified with monoclonal antibodies specific to *N. coenophialum* proteins. Ergot alkaloid concentration was also immunochemically measured. Quantities of mycelial protein and ergot alkaloids were calculated by regressing experimental values against standards of each. There was no correlation between herbage mass and alkaloid concentration or fungal protein quantity and alkaloid concentration when different fungal isolates were inserted into the same plant genotype. Coefficients of determination (r^2) were low (0.31 and 0.17) between leaf sheath and leaf blade alkaloid concentrations and

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endophyte protein when different plant genotypes contained the same endophyte isolate. Likewise, r^2 values were low between leaf sheath and leaf blade alkaloid concentrations and herbage mass. These data suggest that little or no antagonism occurred to the endophyte among plants low in alkaloid concentration.

Key Words—*Neotyphodium*, *Acremonium*, ergot alkaloids, mutualism, antagonism, fescue toxicosis, tall fescue.

INTRODUCTION

The mutualistic association between *Neotyphodium coenophialum* and tall fescue has been studied extensively. Benefits imparted by the fungus to the plant have been well documented and include increased plant vigor (Hill et al., 1991a), resistance to insects (Siegel et al., 1990), nematodes (West et al., 1988), diseases (Siegel and Latch, 1991), and drought (Arachevaleta et al., 1989); and tolerance to grazing (Stuedemann and Hoveland, 1988). Therefore, the presence of *N. coenophialum* aids the persistence of tall fescue in the agricultural ecosystem (Bouton et al., 1993). The encumbrance to this mutualistic association is that the endophyte also produces ergot alkaloids, which are the causal agents of the livestock disorder known as fescue toxicosis (Hill et al., 1994). Tall fescue breeding efforts should, therefore, focus on developing endophyte-infected cultivars that are persistent, but produce little or no ergot alkaloids.

Ergot alkaloid concentration varies depending on the genotype of the tall fescue plant (Agee and Hill, 1994; Roylance et al., 1994a; Adcock et al., 1997). Although the endophyte produces ergot alkaloids, the plant can modify the expression of the endophyte's potential (Hill et al., 1991b), presumably via chemical, nutritional (Roylance et al., 1994a), or genetic interaction. Adcock et al. (1997) demonstrated that recurrent selection for the low alkaloid trait was highly heritable. However, information is needed to define plant–endophyte relationships to determine if selection for decreased ergot alkaloids may be a result of antagonistic effects by the plant to the endophyte or vice versa (Adcock et al., 1997). Endophyte isolates also vary in alkaloid production and can be selected and inserted into existing germplasm for low-toxicity cultivar development (Davies et al., 1993).

Within plants, multigenic alleles control tolerances to parasitic organisms (Day, 1974; Hooker, 1983; Russel, 1978). Similarly, parasites exhibit genetic changes of their own, designed to overcome the genetic defenses of the plant. This gene-for-gene interaction develops a dynamic equilibrium whereby tolerant and intolerant plant forms are exposed to virulent and avirulent parasitic forms. In mutualistic associations, such as with *Neotyphodium* and tell fescue, the plant diminishes the virulence of the inhabitant by genetic accumulation of tolerance

genes and inhabitants supply the host with protection, nutritional supply, and/or vigor (Law, 1985). *Neotyphodium* has lost its capacity to sexually reproduce (Bacon and Siegel, 1988) and can no longer rapidly respond to further accumulation of plant tolerance genes. One concern when breeding *Neotyphodium*-infected tall fescue for reduced ergot alkaloid concentration is that the balance between plant tolerance genes and virulence of the endophyte is disrupted by breeding plants that become antagonistic to the endophyte.

One way to detect virulence of a plant fungal parasite is to measure mycelium or mycelial components. Enzyme-linked immunosorbent assays (ELISA) have been used to detect and quantify fungal mycelia in the genera *Abguillospora* (Bermingham et al., 1995), *Alatospora*, and *Tetracladium* (Bermingham et al., 1996). Monoclonal antibodies have been developed for ELISA quantification of *N. coenophialum* mycelia in tall fescue (Hiatt et al., 1997). Therefore, the relationship between plant and endophyte mass, and ergot alkaloid concentration, can be evaluated to assess whether plant antagonism causes reduced ergot alkaloids in *Neotyphodium*-infected tall fescue.

The specific objective of this experiment was to determine the relationship between plant mass and ergot alkaloid concentration and between *N. coenophialum* mass and ergot alkaloid concentration in tall fescue. Because of the confounding effects of plant and endophyte genotypes on ergot alkaloid production, the role of antagonism by each mutualist was evaluated by examining the relationships between plant mass and ergot alkaloid concentration and *N. coenophialum* mass and ergot alkaloid concentration in: (1) tissue culture regenerants of plant genotype PDN2 containing different endophyte isolates (to remove confounding by plant), and (2) progeny of an open-pollinated tall fescue genotype containing a common endophyte (to remove confounding by endophyte).

METHODS AND MATERIALS

Experiment 1: PDN2 Regenerants Containing Different Endophyte Isolates

The tall fescue plant material used in this experiment was that from the work of Kearney et al. (1991) and Roylance et al. (1994b). Briefly, calli from tall fescue genotype PDN2 were inoculated with one of three endophyte isolates cultured from tall fescue genotypes PDN2, PDN11, and PDN12. Plantlets were removed from the calli and eventually transferred to pots, hardened, and transferred to the greenhouse (Kearney et al., 1991). Regenerants were evaluated for the presence of endophyte (Clark et al., 1983) and somoclonal variation (Roylance et al., 1994b). Regenerants were maintained in the greenhouse prior to use in this experiment.

Two tall fescue genotype PDN2 regenerants each were infected with endophyte isolates EDN12 or EDN11. One PDN2 regenerant was infected with endophyte isolate EDN2 as a control to determine whether plant regeneration affected endophyte growth. The five tall fescue PDN2 regenerants, one endemic PDN2/EDN2 association, and an endophyte-free form were grown in a greenhouse in a randomized complete block design consisting of three replications. Vegetative propagation was performed, and replication obtained, by splitting the plants into three ramets of three tillers each. The ramets were planted into 4-liter containers filled with 1:1:1 Cecil sandy clay loam (Typic Hapludult, clayey, kaolinitic, thermic)–shredded peat moss–and perlite. Plants were watered daily and fertilized weekly with a commercial greenhouse liquid fertilizer. Plants were harvested after 111 days and divided into leaf blade and leaf sheath tissue. Tissue was lyophilized and ground through a 1-mm screen using a Cyclone-type mill.

Fungal Protein Analysis. Fungal proteins were extracted by placing 15 mg of ground, lyophilized leaf sheath tissue into a 1.5-ml microcentrifuge tube and adding 1 ml phosphate buffered saline (PBS; 2.70 g NaH_2PO_4, and 8.77 g NaCl/liter distilled H_2O, pH 7.3). The samples were vortexed and incubated for 1 hr at room temperature. The tubes were centrifuged for 15 min at 12,000*g* to remove cellular debris and the supernatant was removed and placed in another microcentrifuge tube. Polyethylene glycol (PEG) was added to the supernatant until PEG concentration equaled 100 g/liter, and proteins were precipitated by chilling the mixture overnight at 4°C.

The solution was centrifuged for 15 min at 12,000*g* and the supernatant discarded. The protein pellet was resuspended in 250 μl PBS containing 0.1% sodium dodecyl sulfate (SDS) and incubated for 5 min at 60°C. The SDS solution was centrifuged for 15 min at 12,000*g* to remove undissolved material, and the supernatant was diluted 1:4 (v/v) in cold acetone (−20°C). After mixing, the protein was precipitated by chilling the solution overnight at −20°C. The solution was centrifuged for 15 min at 12,000*g*, the supernatant discarded, and the protein pellet washed in 1 ml of cold acetone. Following centrifugation (15 min, 12,000*g*), excess acetone was decanted and the pellet air dried.

ELISA quantification of mycelia was conducted by suspending the pellet in 100 μl borate saline solution (6.19 g H_3BO_3, 9.50 g $Na_2B_4O_7 \cdot 10\ H_2O$, and 4.90 g NaCl/liter distilled H_2O, pH 8.5). The dissolved protein was diluted 1:50 (v/v) in borate saline solution and 50 μl was placed into wells of an Immulon 4 microtiter plate (Dyanatech Co., Chantilly, Virginia). After permitting the protein to anneal overnight at 4°C, the plate was washed three times with ELISA wash (1.21 g Tris, 500 μl Tween 20, and 0.20 g NaN_3/liter distilled H_2O, pH 8.0), blocked with 100 μl of bovine serum albumin blocking solution (10 g bovine serum albumin, 1.17 g Na_2HPO_4, 0.24 g NaH_2PO_4, 8.20 g NaCl/liter distilled H_2O), and the plate incubated for 30 min at 21°C. The plate was

washed three times with ELISA wash. *Neotyphodium coenophialum*-specific monoclonal antibodies from hybridoma cell lines 15D7, 4H2, and 5C7 (Hiatt et al., 1997) were mixed and diluted equally to a final total antibody dilution of 1:20 in ELISA diluent (1 liter blocking solution with 500 μl Tween 20). Fifty microliters was added to each well and the antibodies were allowed to anneal to proteins coated to the microtiter plate wells for 2 hr at 21°C. The plate was 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), diluted 1:500 in ELISA diluent. After incubating at 21°C for 2 hr, the plate was washed three times with ELISA wash. Fifty microliters of substrate solution (1 g *p*-nitrophenyl phosphate, 0.10 g $MgCl_2$, and 96 ml-diethanolamine/liter distilled H_2O, pH 9.8) was added to each well, incubated for 30 min at 21°C and optical density measured spectrally at 405 nm in a Bio-Tek EL311 (Bio-Tek Instruments, Winooski, Vermont) microplate reader.

Fungal proteins were quantitated by regressing ELISA values to those of a standard dilution of purified proteins from each fungal isolate used in this experiment. Proteins from fungal isolates EDN2, EDN11, and EDN12 were diluted to 2.00×10^{-2}, 1.00×10^{-2}, 5.00×10^{-3}, 2.50×10^{-3}, 1.25×10^{-3}, 6.25×10^{-4}, 3.12×10^{-4}, and 1.56×10^{-4} g/liter in borate saline and 50 μl used to coat wells in the microtiter plates.

Ergot Alkaloid Analysis. Plant tissue was immunochemically analyzed for ergot alkaloids with the competitive ELISA assay described by Hill and Agree (1994). Immulon 4 microtiter plate wells were incubated overnight at 4°C coated with 188 pg of human serum albumin-glutaryl-lysergol (HSA-LYS) conjugate diluted in 50 μl borate saline. The plates were washed three times with ELISA wash using a Denley Wellwash 5000 (Denley Instruments Ltd., Billingshurst, England) to remove excess conjugate. The microtiter plate wells were blocked with 100 μl bovine serum albumin blocking solution for 30 min at 21°C and washed three times with ELISA wash.

Ergot alkaloids were extracted from ground, lyophilized leaf sheath and leaf blade tissue by vortexing 0.10 g tissue in 8 ml ELISA phosphate buffer–Tween solution (EPBST) (1.17 g Na_2HPO_4, 0.243 g NaH_2PO_4, 8.175 g NaCl, and 500 μl Tween 20/liter distilled H_2O, pH 7.35–7.45), incubating for 15 min at room temperature, and vortexing a second time prior to sampling the liquid fraction. Both leaf sheath and leaf blade extractions were diluted to a final concentration of 1:320 in EPBST and 50 μl was added to each microtiter plate well. The antibody from hybridoma cell line 15F3.E5 supernatant (Hill and Agee, 1994) was diluted 1:80 in borate saline and 50 μl of the diluted antibody was added to each well containing the alkaloid extracts. The antibody was permitted to competitively bind with extracted ergot alkaloids and the lysergic moiety of HSA-LYS for 2 hr at 21°C. Plates were washed three times with

ELISA wash. Fifty microliters of rabbit anti-mouse antibody alkaline phosphatase conjugate diluted 1:500 in ELISA diluent were added and incubated at 21°C for 2 hr. The plates were washed three times with ELISA wash and 50 μl of chromogen solution added to each well. Plates were incubated at 21°C and color development was stopped after 25 min by adding 50 μl of 3 M NaOH. The optical density of each well was measured at 405 nm in a Bio-Tek Instruments EL311 microplate reader.

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 concentrations in EPBST. Absorbance values of the standards were fit to a logit/log regression equation and used to quantitate ergot alkaloid content in each sample.

Experiment 2: Tall Fescue Progeny Containing a Common Endophyte

The material used in this study was progeny from cross-fertilizations using DN11 as the female parent and DN2, DN12, and DN15 as the male parents (Adcock et al., 1997). Seeds from the crosses were planted in flats containing a 1:1:1:1 Cecil sandy clay loam (Typic Hapludult, clayey, kaolinitic, thermic)–shredded peat moss–perlite–fritted clay mixture. Three weeks after emergence, plants were transferred to 0.5-liter pots containing the same 1:1:1:1 soil mix. The plants were maintained in the greenhouse. Five F_1 plants from each of the three crosses were chosen for the experiment. The experiment therefore consisted of 15 plant genotypes, all containing endophyte isolate EDN11. Experiment 2 replications were established and plant maintenance performed as described in experiment 1. Endophyte protein and ergot alkaloid analyses were conducted comparably to experiment 1, except leaf sheath extractions for ergot alkaloid analysis were diluted to 1:800 in EPBST, and leaf blade extractions were diluted to 1:480 in EPBST.

Statistical Analysis

In experiment 1, data were analyzed to determine the effects of endophyte on leaf blade, leaf sheath, and total herbage mass, endophyte mycelial protein mass, and alkaloid concentration within the leaf sheath and blade. Data were analyzed by ANOVA using a randomized complete block model with replication and endophyte isolate as the treatment variables. In experiment 2, data were analyzed to determine the effects of plant genotype on the same response variables mentioned for experiment 1. Data were analyzed by ANOVA using a randomized complete block model with replication and plant genotype as the treatment variables (PROC ANOVA; SAS Institute, Cary, North Carolina). When ANOVA indicated significant differences among means, means were com-

pared using LSDs. Linear regression analysis was used to determine the relationships between alkaloid concentrations of leaf sheath and blade tissues with mycelial protein of the endophytes and between alkaloid concentration and total herbage mass in both experiments. Only significant regression statistics were reported.

RESULTS

Experiment 1: PDN2 Regenerants Containing Different Endophyte Isolates

Examination of alkaloid–endophyte relationships using endophytes inserted into tissue culture regenerants of a single tall fescue genotype remove confounding effects of the plant genotype on growth and expression of alkaloid concentration by the endophyte (Hill et al., 1996). There were no differences for leaf sheath, blade, herbage, or endophyte protein mass or for leaf sheath or blade alkaloid concentrations among regenerants with the same endophyte ($P = 0.05$). This was interpreted to indicate an absence of somoclonal variation among regenerants containing the same endophyte and that variation among regenerants for plant or endophyte mass and for alkaloid content was due to the endophyte effect in this experiment. Hence, data for regenerants containing the same endophyte were nested and analysis of variance reevaluated to determine endophyte effects.

Leaf sheath, leaf blade, and total aboveground herbage mass were similar for PDN2 regenerants containing endophytes EDN12 and EDN2 (Table 1). Leaf

TABLE 1. DRY MASS, FUNGAL PROTEIN MASS, AND ERGOT ALKALOID CONCENTRATION IN LEAF COMPONENTS OF REGENERANTS FROM TALL FESCUE GENOTYPE PDN2 CONTAINING ENDOPHYTE ISOLATES OF EDN2, EDN11, AND EDN12

	Mass				Alkaloids (μg/g)	
Endophyte	Leaf sheath (g)	Leaf blade (g)	Total herbage (g)	Fungal protein (mg)	Leaf sheath	Leaf blade
EDN12	1.44	1.69	3.13	1.18	4.71	1.13
EDN11	2.65	2.74	5.39	0.61	5.50	3.30
EDN2	1.59	1.45	3.04	0.22	5.96	2.23
LSD (0.05)[a]	0.62	0.65	1.19	0.38	0.92	0.49

[a] Least significant difference between two means at the 0.05 level of probability.

sheath, leaf blade, and total aboveground mass were greater for PDN2 regenerants containing endophyte EDN11. These data were consistent with other experiments using these regenerants (Hill et al., 1996).

Ergot alkaloid concentration was lowest in leaf sheath from PDN2 regenerants containing EDN12 and greatest in sheath tissue containing endophyte EDN2. Sheath alkaloid concentration was not different from regenerants containing EDN11 and those containing EDN2 or EDN12 (Table 1). Ergot alkaloid concentration was lowest in leaf blades of regenerants containing EDN12 and highest among regenerants containing EDN11.

Monoclonal antibodies used to quantify endophytic proteins are specific to proteins located in the cell walls of *Neotyphodium* sp. (Hiatt et al., 1997). Therefore, the mass of fungal protein is stoichiometric with the mycelial mass of *N. coenophialum* and can be used as a quantifier of the mycelia. Mycelial protein was greatest in leaf sheath tissue from PDN2 regenerants containing endophyte EDN12 and least in regenerants containing EDN2 (Table 1). It is significant to note that the regenerants with the lowest leaf sheath and leaf blade alkaloid concentration had the highest mass of mycelial protein.

There was no correlation between alkaloid concentration and endophytic mycelial protein, suggesting that low alkaloid concentration was independent of endophyte mass in this experiment (Figures 1 and 2). As with mycelial protein mass, there was no correlation between herbage mass and either leaf sheath alkaloid or leaf blade alkaloid concentrations among PDN2 regenerants containing different endophyte isolates (Figures 3 and 4).

Experiment 2. Tall Fescue Progeny Containing a Common Endophyte

Inasmuch as the endophyte passes from the maternal parent into the developing seed, all progeny used in experiment 2 contained endophyte EDN11 because the female parent of each was DN11 (Adcock et al., 1997). The maternal parent, DN11, had less leaf sheath, leaf blade, total herbage, and mycelial protein mass than all progeny (Table 2). There was variability among all genotypes for herbage traits even when they had the same paternal parent. Interestingly, genotypes that had DN12 as the paternal parent did not differ for mycelial protein while genotypes that had either DN2 or DN15 as the paternal parents varied in mycelial protein. Progeny 24 had DN2 as the paternal parent and had the greatest ergot alkaloid concentration in its leaf sheath. The greatest leaf blade alkaloid concentration was in progeny 11, which was not different from progenies 14 or 24. Progenies 11, 14, and 24 all had genotype DN2 as their paternal parent.

Progeny 3 had DN15 as its paternal parent and had the lowest leaf sheath mass, as well as the lowest leaf sheath and leaf blade alkaloid concentrations

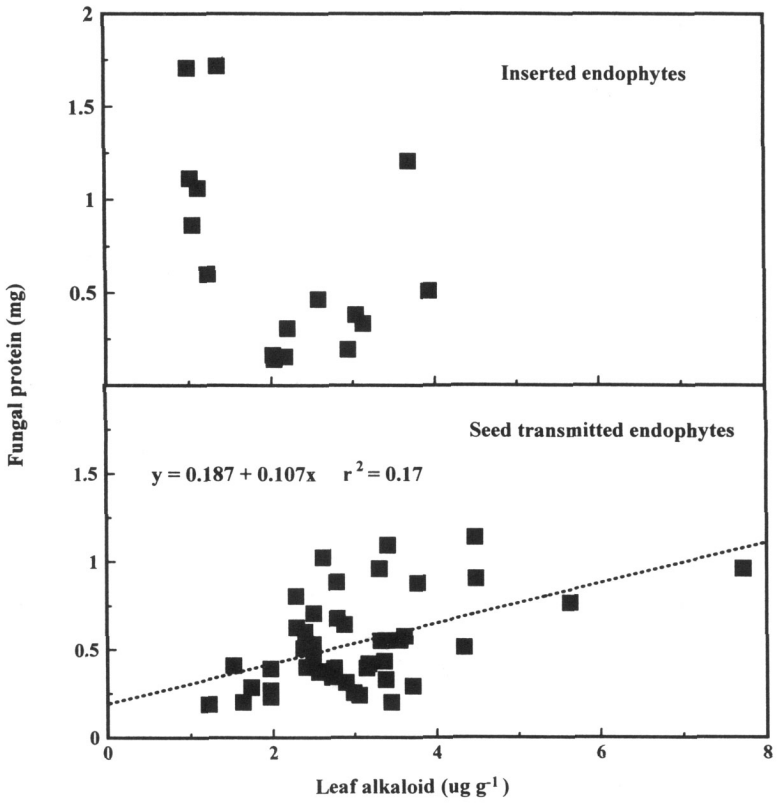


FIG. 1. Relationship between leaf blade alkaloid concentration and fungal protein mass in tall fescue plants containing either inserted endophytes (top) or seed transmitted endophytes (bottom).

(Table 2). Progeny 14a had DN12 as its paternal parent and had the lowest leaf blade mass. Progeny 15 had DN12 as its paternal parent and had the lowest total herbage mass. Progeny 23 had DN12 as its paternal parent and had the lowest endophyte mycelial protein mass.

Leaf sheath and leaf blade alkaloid concentrations were associated with endophyte protein (Figures 1 and 2). Although significant, the r^2 values were low (0.31 and 0.17, respectively), suggesting the association was weak and that other plant variables affected alkaloid concentration in leaf sheath and blade. Leaf sheath and leaf blade alkaloid concentration were associated with herbage mass (Figures 3 and 4) but, likewise, had low r^2 values.

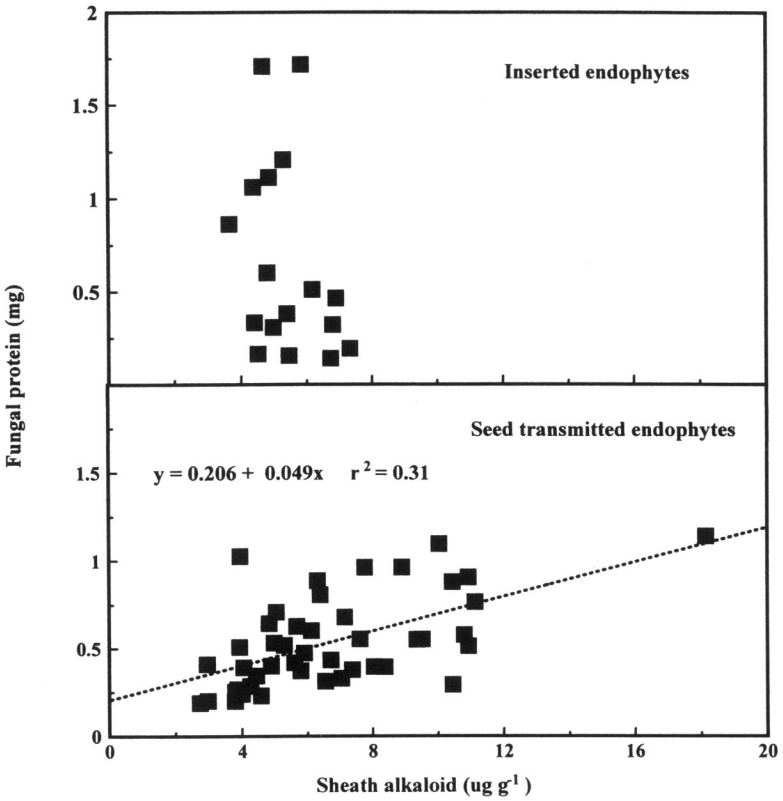


FIG. 2. Relationship between leaf sheath alkaloid concentration and fungal protein mass in tall fescue plants containing either inserted endophytes (top) or seed transmitted endophytes (bottom).

DISCUSSION

Some regenerants from PDN2 had low herbage mass but high endophyte mycelial protein. Low herbage mass could be a result of antagonism of the endophyte toward the plant. In this case, endophyte isolates could be viewed as unique individuals or races of asexual fungi, each having varied pathogenic tendencies toward the plant. Systems such as these are common in other fungi (Kenaga, 1974) and are likely to occur in tall fescue populations as remnants of the host–pathogen interactions from which this mutualistic relationship evolved (Hill, 1995). Some fungi may also be more vigorous, resulting in aggression to the plant. In previous experiments, we found that some endophytes completely engulfed developing embryos of tall fescue during tissue culture regeneration,

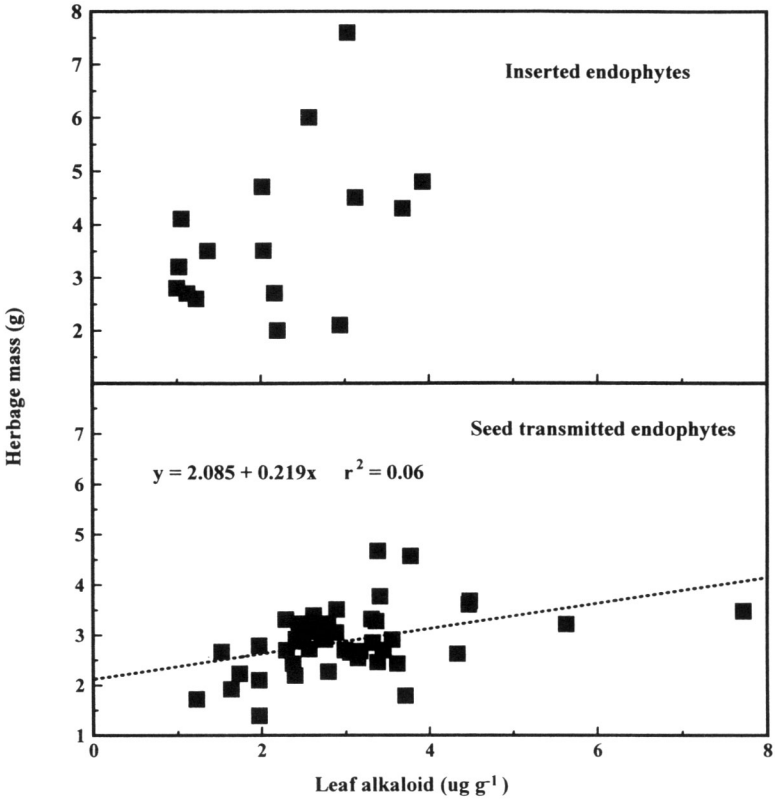


FIG. 3. Relationship between leaf blade alkaloid concentration and total herbage mass of tall fescue plants containing either inserted endophytes (top) or seed transmitted endophytes (bottom).

while other endophytes could not infect the developing embryos (Kearney et al., 1991). Christensen (1995) also found differing capacities for endophytes to infect perennial ryegrass.

We examined the relationship between alkaloid concentrations and fungal mass because (1) alkaloid concentration may be a controlling variable toward livestock toxicity (Adcock et al., 1997), and (2) we interpret the mass of mycelial protein as one measure of the antagonistic, mutualistic, or commensalistic associations between specific plant–endophyte combinations. By testing a common endophyte within various tall fescue genotypes, the confounding effect of endophyte genotype was removed, and the effect of plant genetics on the endophyte was examined. Therefore, when plants contain less mycelial protein than

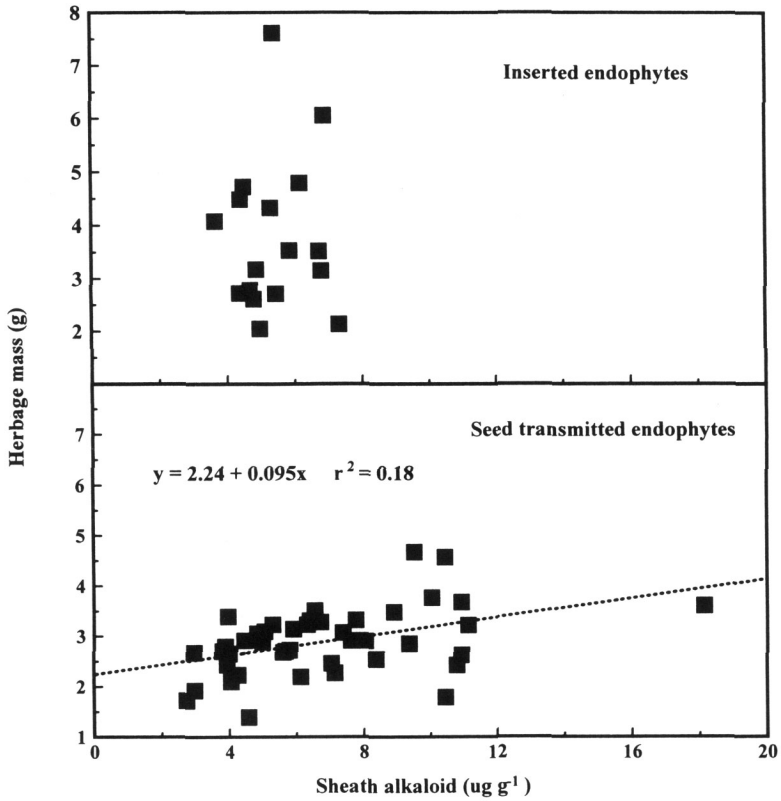


FIG. 4. Relationship between leaf sheath alkaloid concentration and total herbage mass of tall fescue plants containing either inserted endophytes (top) or seed transmitted endophytes (bottom).

others, the phenomenon can be interpreted as antagonism of the plant toward the endophyte.

Plant morphology may also affect the quantity of fungal mycelia within a plant. The endophyte resides within the leaf sheath (Bacon and Siegel, 1988) so plants with more sheath tissue have greater potential for endophyte mycelium than those with less leaf sheath tissue. To test this hypothesis, a plant population derived from one maternal parent and several paternal parents was utilized. Hence, each plant genotype had the same endophyte, but plant genetics altered plant morphology. We regressed the mass of leaf sheath tissue with endophyte mycelium protein and found a low r^2 value between the two ($r^2 = 0.30$) (data not shown). Because the r^2 value for the association between leaf sheath mass

TABLE 2. DRY MASS, FUNGAL PROTEIN MASS, AND ERGOT ALKALOID CONCENTRATION IN LEAF COMPONENTS OF TALL FESCUE GENOTYPES CONTAINING ENDOPHYTE EDN11

	Parent		Mass				Alkaloids (μg/g)	
Progeny number	Male	Female	Leaf sheath (g)	Leaf blade (g)	Total herbage (g)	Fungal protein (mg)	Leaf sheath	Leaf blade
2	DN2	DN11	1.26	1.70	2.96	0.51	5.44	2.85
9	DN2	DN11	1.23	1.71	2.94	0.84	7.07	2.96
11	DN2	DN11	1.81	2.13	3.94	0.98	9.80	4.96
14	DN2	DN11	1.58	1.35	2.92	0.51	8.09	4.13
24	DN2	DN11	1.52	1.79	3.31	0.85	13.33	4.43
7	DN12	DN11	1.70	1.85	3.55	0.40	7.70	3.22
14a	DN12	DN11	1.05	1.09	2.73	0.39	6.35	2.70
15	DN12	DN11	0.89	1.10	1.97	0.38	4.19	2.10
17	DN12	DN11	1.15	1.23	2.38	0.47	9.62	3.62
23	DN12	DN11	1.13	1.59	2.71	0.25	3.89	2.67
3	DN15	DN11	0.87	1.23	2.10	0.27	2.88	1.46
10	DN15	DN11	1.48	1.72	3.19	0.79	5.11	2.46
17a	DN15	DN11	1.11	1.49	2.60	0.52	5.41	2.47
22	DN15	DN11	1.17	1.68	2.85	0.50	4.87	2.23
23a	DN15	DN11	1.49	1.59	3.09	0.40	7.37	2.90
DN11			0.32	0.45	0.77	0.15	6.72	3.29
LSD (0.05)[a]			0.43	0.43	0.77	0.27	2.80	1.27

[a]Least significant difference between two means at the 0.05 level of probability.

and fungal protein mass was similar to that between fungal protein mass and leaf sheath alkaloid concentration ($r^2 = 0.31$) (Figure 1), it can be concluded that plant morphology had a minor effect on the alkaloid producing capabilities of the endophyte residing within.

The data presented here support the hypothesis that antagonism exists between endophyte and plant, and variations in plant morphology are partly responsible for reduced ergot alkaloid concentration. However, weak coefficients of determination (low r^2 values) suggest other sources of variation are responsible for alkaloid phenotype. Other mechanisms are possible to obtain plant–endophyte associations with low-alkaloid phenotypes, besides a gene-for-gene interactive effect for virulence and avirulence. Researchers in New Zealand have identified endophytes that are void of alkaloid production (Fletcher and

Sutherland, 1993). When forage cultivars are infected with endophyte strains that are alkaloid deficient, the resulting populations have nil-alkaloid phenotypes. Therefore, endophyte genotype is a third source of variability for expression of alkaloids in tall fescue–endophyte associations.

A fourth mechanism for low alkaloid phenotypes is the ability of the plant to supply endophytes with the nutrients necessary for alkaloid production. Calcium is a coenzyme for dimethylallyl tryptophan synthase (DMATase), the first pathway-specific enzyme for ergot alkaloid biosynthesis (Lee et al., 1976; Cress et al., 1981). Tall fescue genotypes vary in calcium concentration, and calcium concentration is a highly heritable trait ($h = 0.70$) (Sleper, 1979). We have examined the effect of calcium nutrition on ergot alkaloid concentration and found that calcium fertilization increases alkaloid concentration independent of soil pH (Hill, 1995). L-Tryptophan is also an essential nutrient for the enzyme DMATase (Lee et al., 1976; Cress et al., 1981), but genotypic variability for tryptophan is unknown in tall fescue.

We propose that manipulating a population of tall fescue plants by selecting for the low-alkaloid trait is likely to result in a population deficient in alkaloids because of multiple regulating effects including: (1) antagonism between plant and endophyte, (2) endophyte-specific alkaloid-producing capabilities, (3) deficiencies of specific plant metabolites needed for alkaloid production, and (4) plant morphology. With these alkaloid regulating systems in mind, it would be expected to have low regression coefficients between ergot alkaloid concentration and any one trait. These data suggest that breeding for low ergot alkaloid concentrations may not affect the mutualistic relationship between plant and endophyte other than removal of undesirable effects of the alkaloids on desirable grassland animals.

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WARNING ODOR OF THE NORTH AMERICAN PORCUPINE (*Erethizon dorsatum*)

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Abstract—Volatile compounds in the lipid coating of the lower-back quills of the North American porcupine, *Erethizon dorsatum*, were collected using headspace trapping on Tenax and analyzed by gas chromatography–mass spectrometry (GC-MS), GC with organoleptic detection, and GC with a chiral stationary phase. Over 50 components were isolated, primarily oxygenated aliphatics, lactones, and isoprenoids. The most abundant constituent was tetradecyl acetate, which does not contribute significantly to the odor of the quill lipids. GC with a wide-bore capillary column and a human nose as detector was used to determine the retention time of the peak with the characteristic porcupine odor. Comparison of this organoleptic chromatogram with those obtained with a flame ionization detector and GC-MS showed the compound to be a δ-decalactone. The racemic mixture of this optically active substance does not possess the unique odor. Resolution of the enantiomers of δ-decalactone by GC with a chiral cyclodextrin phase shows that the isomer possessing the characteristic porcupine odor is the (*R*)-δ-decalactone. We suggest the biological function of this compound is to serve as a warning odor, which, in conjunction with an initial quill strike, produces a conditioned aversion in potential predators.

Key Words—*Erethizon dorsatum*, porcupine, quill lipid volatiles, warning odor, enantiomeric separation, δ-decalactone.

INTRODUCTION

Aposematism (warning behavior) is common in well-defended animals that are subject to predation. By limiting mutual injury, the warning behavior benefits

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both the predator and its potential prey. Warnings may be transmitted in any of the sensory modalities; thus examples are known of aposematic coloration (Cott, 1957, McIver and Latkin, 1990) and of acoustic aposematism (Dunning and Roeder, 1965; Mohr, 1965; Masters, 1979). Olfactory aposematism has been cited less often. Rothschild and coworkers (Rothschild et al., 1984; Guilford et al., 1987; Moore et al., 1990) described a Mullerian complex of warning odoration in insects; Dumbacher et al. (1992) noted that chemically defended birds of the genus *Pitohui* emit a strong sour odor.

We report on olfactory aposematism in the North American porcupine, *Erethizon dorsatum*. The warning odor is released in conjunction with quill erection and is part of a trimodal signal. The porcupine sends a visual signal in the form of contrasting black-and-white areas along its lower back and tail. An acoustic signal may be added in the form of tooth-clacking. Finally, the olfactory signal consists of a pungent odor released from the skin of the lower back (Roze, 1989). All three signals are transmitted effectively in the porcupine's natural travel habitat, the nocturnal forest floor.

A histological study of porcupine skin shows well-developed sebaceous glands associated with quills of the rosette (lower back) region (Chapman and Roze, 1997). The rosette quills are also supplied with powerful piloerector muscles, which, upon quill erection, cause a dimpling of the skin and possible sebum expression, with which the warning odor is associated. In addition, rosette quills possess osmetrichial modifications for odor dispersal (Roze and Chapman, unpublished data). Because of these osmetrichial modifications, the quills of the lower back become saturated with the warning odor upon arousal. We used the odor-saturated quills to isolate the odor components and to identify the active principle.

METHODS AND MATERIALS

Quill Samples. The porcupines sampled were resident in the Catskill Mountains, Greene Country, New York. Approximately 50 individuals were caught by hand and becalmed by injection with 100 mg/ml ketamine HCl at a dose of 10 mg/kg (Parke-Davis Co., Morris Plains, New Jersey). Approximately 100 odoriferous quills (approximately 1.5 g) were removed with tweezers from the rosette area, sealed in 40-ml Teflon-capped vials, and immediately stored in Dry Ice in a styrofoam cooler. Later the vials were transferred in the laboratory to a $-20°C$ freezer for storage before analysis. When warmed to room temperature, the quill samples released the intense characteristic odor of the porcupine.

Collection of Volatiles. Volatile components emitted from the quill samples

were collected in a laboratory-built headspace sampler. Defrosted quills (ca. 1 g) were placed in a 150-ml vacuum filtration flask. The flask was placed on a hot plate to warm the sample to approximately 40°C to facilitate release of volatiles from the quill lipids. A 6 × 70-mm charcoal cartridge trap (SKC #226-01, SKC, Eighty Four, Pennsylvania) was placed in the air inlet to remove any impurities in the laboratory air. The purified air was drawn over the quills using an Airchek portable air sampling pump (SKC, model 224-PCXR7) at 1 liter/min, and passed through a Tenax cartridge (SKC #226-35; contains 45 mg Tenax) for 5–10 hr. The Tenax resin was transferred into a thinly drawn disposable glass pipet tip with a glass wool plug in the bottom and eluted with 500 μl of methanol (Baker HPLC grade, Fisher Scientific, Springfield, New Jersey) into a chilled 1-ml Reacti-vial (Supelco, Bellefonte, Pennsylvania). The container was sealed and stored in the −20°C freezer until the contents were ready for analysis.

Transmethylation of Triglycerides and Esters and Methylation of Acids in Volatiles. Free fatty acids in the methanol extract of the Tenax trap were methylated with diazomethane. Diazomethane was generated by mixing 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wisconsin) with cold 1 N NaOH solution in a special diazomethane generator (Aldrich). The gaseous diazomethane was collected in cold ether, and 0.5 ml was mixed with 0.5 ml of the methanol solution to produce the fatty acid methyl esters.

Triglycerides and other esters were transesterified to methyl esters with methanolic (*m*-trifluoromethylphenyl)trimethyl ammonium hydroxide (Meth-Prep II, Alltech Assoc., Deerfield, Illinois). The contents of the 1-ml vial of Meth-Prep II were added to 0.5 ml of the methanol solution and allowed to stand for 30 min before injection into the GC. A blank solution was prepared by addition of the same volume of Meth-Prep II to methanol.

Isolation of Acidic Components from Volatiles. To a chilled Reacti-Vial containing 100 μl of the methanol extract of the Tenax trap was added 100 μl of 1 N NaOH solution. After 24-hr, the nonhydrolyzed basic and neutral compounds were extracted into *n*-hexane (2 × 500 μl) and the hexane solution removed by micropipet. The aqueous layer was cooled and acidified to pH < 2 with 1 N HCl. This solution was extracted with *n*-hexane (2 × 500 μl). Each of the hexane extracts was concentrated in a gentle stream of nitrogen to 50 μl prior to analysis. The odor qualities of each of the solutions were noted.

Solvent Extraction and Silica Gel Fractionation of Quill Lipid. n-Hexane (15 ml) (Baker HPLC grade, Fisher Scientific) was added to a sample vial containing approximately 1.5 g of thawed quills; the vial was shaken vigorously for 2 min and allowed to stand 30 min with occasional shaking. The vial was then centrifuged for 5 min and the upper clear solution removed with a pipet. Another 15 ml *n*-hexane was added to the quills in the vial for reextraction as

above. The hexane extracts were combined and added to a silica gel (Fisher Scientific) (deactivated by addition of 5% water) column about 5 cm long dry-packed into a short buret. The column was eluted sequentially with 10 ml each of hexane, hexane–diethyl ether (50:50, v/v) and methanol. The fractions were concentrated individually to 1 ml under a gentle stream of nitrogen. The odor of each was noted prior to GC-MS analysis.

GC-MS Analysis. Both a Hewlett-Packard (HP) 5988A GC-MS with a HP 1000 data system with RTE-6 software, and a HP 5971A GC-MSD with HP Chemstation software were used for separation and identification. Both 50 m × 0.32-mm-ID with cross-linked Carbowax 20 M (0.25-μm film thickness) (HP, Wilmington, Delaware) and 30-m × 0.25-mm-ID DB-5 bonded methyl–5% phenyl polysiloxane (0.25-μm film thickness) (J&W Scientific, Folsom, California) columns were used. For all these columns a typical temperature program was an initial 2 min hold at 60°C, 10°C/min to 280°C, and a final hold at 280°C until all peaks eluted. Sample injection (2 μl) was done in the splitless mode, with the split valve opened at 0.75 min. The carrier gas was He at 1 ml/min (at 60°C). Tentative peak identification was made by computer searching of the NBS-Wiley mass spectral libraries installed in the software. In the absence of a strong match, mass spectra were interpreted manually. Wherever possible, authentic standards were run; mass spectral identifications were confirmed by coincidence of retention times and mass spectra. In some cases, retention time–carbon number plots were used to substantiate identification of members of homologous series.

GC-Organoleptic Detection. To facilitate identification of the compound(s) responsible for the characteristic odor, a sampling device was constructed to enable the human nose to evaluate the organoleptic qualities of the GC effluent. A schematic diagram is shown in Figure 1. To prevent condensation of eluted components on the wall of the GC column outside the oven, a 1/8-in.-OD Teflon tube was placed in a 5/32-in. hole drilled through the HP 5890A GC oven wall. The external end was attached to a copper tee. The exit end of the 30-m × 0.53-mm-ID Supelco SPB-5 column (0.50-μm film thickness) was threaded through the Teflon tube and penetrated a silicone septum in the right-hand side of the tee, to extend a few millimeters beyond. A Tygon tube was attached to the bottom of the tee and connected to the house vacuum. The vacuum pulled heated air from the oven over the capillary to prevent condensation. Although not indicated in Figure 1, a plastic funnel was placed over the capillary end and attached to the tee to focus the eluting vapors and to prevent damage to the nose detector. A 10-μl extract aliquot was injected, the temperature program started, and the individual whose nose was being used gave a running report to a second person with a clock who recorded the time and odor description. The experiment was done in duplicate for each detector, and all three authors served as detectors.

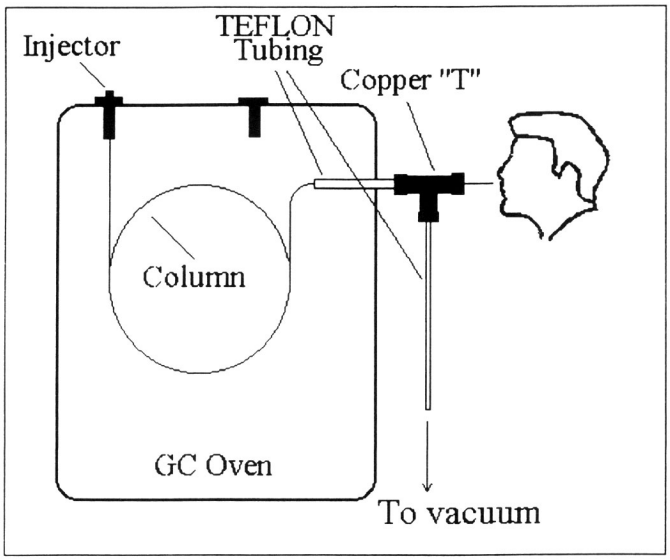


FIG. 1. Schematic diagram of the GC setup for organoleptic testing of the effluent vapors.

Determination of Enantiomeric Composition of Decalactones. GC separation of the enantiomers of γ- and δ-decalactones was studied on α-, β-, and γ-permethylated cyclodextrin (CD) capillary columns. The stationary phases contain 20% (w/w) CD dissolved in SPB-35 (35:65 phenyl-methyl polysiloxane). The columns were supplied by Supelco and are designated α-, β-, and γ-DEX 120 columns. Both the HP 5988A GC-MS and the HP 5890A GC-FID were used. Racemic mixtures (Aldrich) were used to establish the best GC columns and conditions. More careful selection of chiral columns is required for enantiomeric separations than is the case with conventional GC capillary columns. Thus, for the γ-decalactones, the β-DEX column gave the best separation with a temperature program of 70°C to 130°C at 10°C/min with a final 130°C hold until the solutes all eluted. However, the best separation of the δ-decalactones was achieved on the γ-DEX 120 column with a temperature program of 70°C to 115°C at 10°C/min with a final 115°C hold until the solutes eluted. The elution order of the *R* and *S* isomers was established using the only pure enantiomer available to us, the (*S*)-δ-decalactone. For the extracts of the quill samples, after the γ-decalactones eluted, the temperature was raised at 10°C/min to 220°C and held at that temperature until the rest of the organics eluted.

RESULTS

Identification of Volatile Components in Head Space. The odoriferous compound(s) were present in the methanol extract of the Tenax trap. A representative GC-FID chromatogram is shown in Figure 2, and a list of the identified compounds corresponding to the numbered peaks is given in Table 1.

The volatiles are comprised primarily of oxygenated aliphatic compounds, lactones, and isoprenoids. The most abundant compound is tetradecyl acetate; other acetates and lactones are also major peaks; altogether they comprise about 40% (w/w) of the headspace volatiles. Acetates have been found in some insects but are not often observed in mammalian lipids (Albone and Shirley, 1984). One exception is (Z)-7-dodecen-1-yl acetate, used by female Asian elephants as a sex pheromone (Rasmussen et al., 1996). Acetates have weak, fruity, sweet odors and are not major contributors to the porcupine odor.

Many of the compounds found are members of homologous series, which in temperature programmed GC follow a linear net retention time–carbon number relationship. Figure 3 illustrates this relationship for several classes of compounds, which helps to verify compound identification. The origin of these compounds is presumably the result of bacterial action on the quill lipids.

Our initial qualitative impression of the characteristic odor was that of a carboxylic acid (Roze, 1989), so attention was paid first to the acidic components in the volatiles. The acids were methyl esterified and analyzed by GC-MS. A number of acids were identified as shown in Table 2. However, the methanol extract solution after methylation with diazomethane retained the porcupine odor and gained the fruity, sweetish smell of methyl esters. Thus carboxylic acids were eliminated as principal porcupine odor components, although they may contribute to the overall odor characteristics of the quill lipid. This was confirmed by treatment of the quill volatiles extract with 1 N NaOH. Initially, there was no change in the odor, but after 24 hr the odor disappeared. After removal of the neutral and basic compounds and treatment with 1 N HCl, the characteristic odor was restored, suggesting it might be caused by a lactone or other ester.

Identification of Compounds After Silica Gel Fractionation. The hexane fraction had no particular odor, and few peaks were found by GC; there is not much nonpolar material in the quill lipids. The hexane–ether fraction was odoriferous but not porcupine-like. The compounds identified by GC-MS were the same types of compounds (alcohols, aldehydes, ketones, acetates) found in the volatiles. The characteristic porcupine odor was found in the methanol fraction. This was further concentrated under a gentle stream of nitrogen to 100 μl and analyzed by GC-MS. The important part of the chromatogram and peak identifications are given in Figure 4. The peak at 23.6 min was identified by MS as

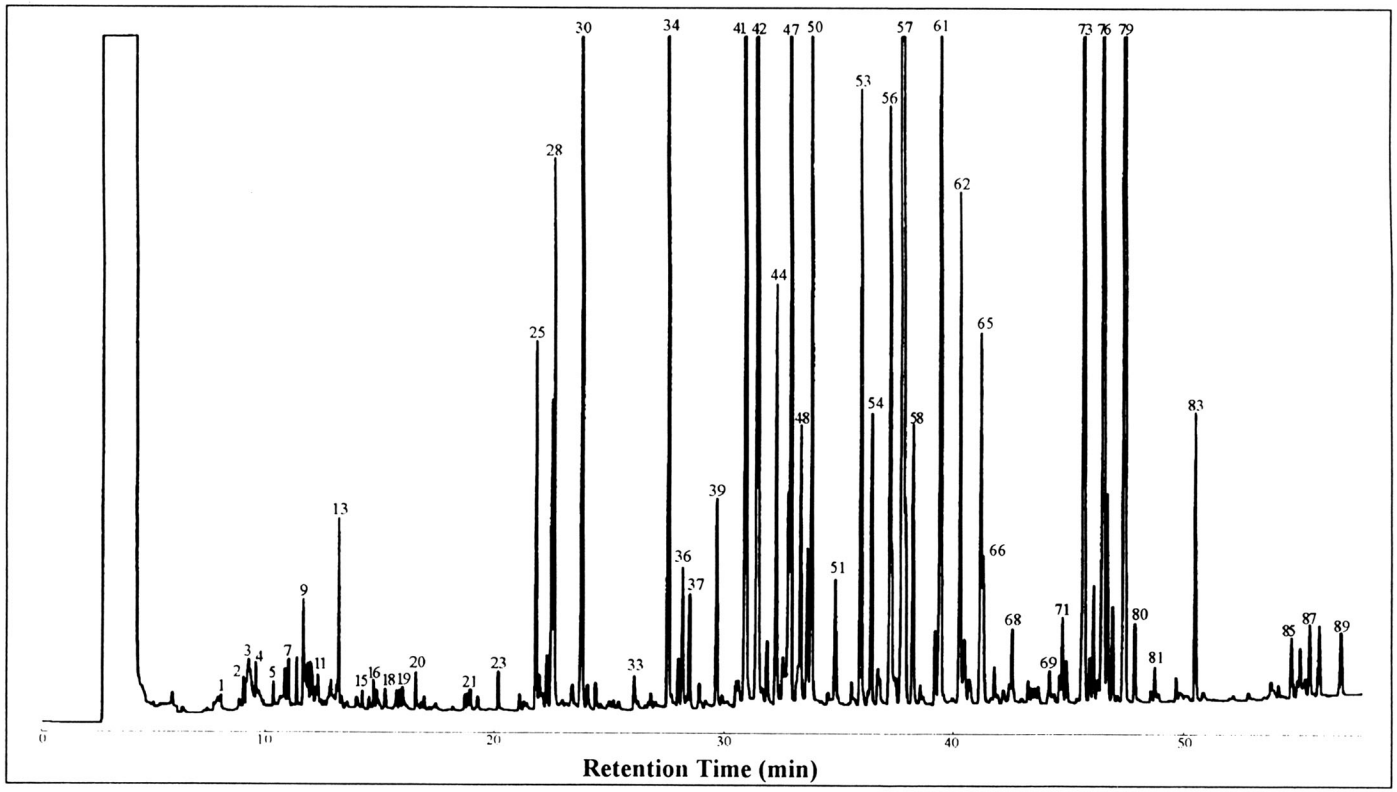


FIG. 2. Chromatogram of volatile components in the headspace of porcupine quills. Sample: methanol desorption solution from head space Tenax trap; column: 60 m × 0.25 mm DB-5; detector: FID; GC temperature program: 65°C initial for 2 min, 10°C/min to 150°C, 2 min hold, 2°C/min to 270°C.

TABLE 1. IDENTIFICATION OF VOLATILE COMPONENTS IN THE HEADSPACE OF PORCUPINE QUILLS

Peak	Peak identification	Area (% of total)
1	Heptanal	<0.1
2	2-Heptenal	<0.1
3	Hexanoic acid[a]	0.1
5	6-Methyl-5-hepten-2-one	<0.1
7	Heptanoic acid[a]	0.1
8	2-Nonenal	0.1
9	Unidentified fatty acid	0.2
13	Octanoic acid[a]	0.4
18	γ-Octalactone	0.1
19	Nonanoic acid[a]	0.1
20	δ-Octalactone	0.2
21	Decanoic acid[a]	0.1
23	Dodecanal	0.1
25	Geranylacetone[a]	1.1
27	1-Dodecanol[a]	0.9
28	γ-Decalactone[a]	1.8
30	δ-Decalactone[a]	2.8
34	Dihydroapofarnesol	5.3
36	1-Dodecanol acetate[a]	0.5
37	Tetradecanal	0.4
39	1-Tetradecene	0.8
41	*cis*-9-Tetradecen-1-ol	3.8
42	1-Tetradecanol[a]	5.3
43	γ-Dodecalactone	0.2
44	Unidentified terpenoid	1.6
47	Unidentified terpenoid	3.3
48	δ-Dodecalactone[a]	1.2
50	Farnesol[a]	3.2
51	Farnesal	0.5
53	Unidentified	2.4
54	Unidentified	1.1
56	1,13-Tetradecadiene	3.0
57	1-Tetradecanol acetate[a]	14.2
58	Hexadecanal	1.2
61	Farnesyl acetate	4.0
62	1-Octadecene	2.1
65	1-Hexadecanol[a]	1.7
73	Alkyl (C16) acetate	6.8
76	Alkyl (C16) acetate	8.9
79	1-Hexadecanol acetate[a]	8.7

[a]Confirmed by mass spectrum and retention time of authentic compounds.

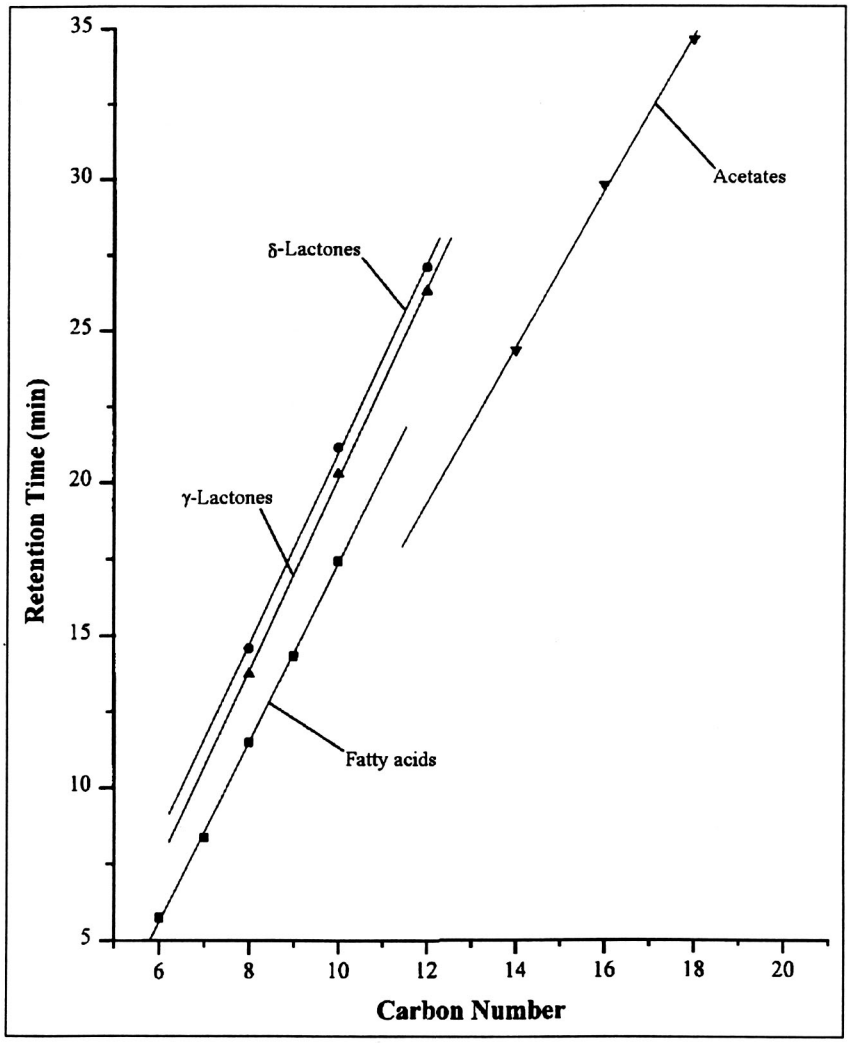
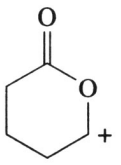


FIG. 3. Retention time *vs.* carbon number for homologous series represented in headspace volatiles. Column: 30 m × 0.25 mm DB-5; detector: MS; GC temperature program: 60°C for 2 min, 10°C/min to 280°C.

TABLE 2. ACIDIC COMPONENTS IN HEADSPACE OF PORCUPINE QUILLS IDENTIFIED AS THEIR METHYL DERIVATIVES

Peak	Retention time (min)	Acids (identified as methyl esters)
1	8.19	Hexanoic
2	9.35	4-Oxopentanoic
3	10.02	Heptanoic
4	11.13	6-Methylheptanoic
5	11.51	Benzoic
6	11.75	Octanoic
7	12.21	Methyl nicotinate
8	12.76	Benzeneacetic
9	13.35	Nonanoic
10	14.85	Decanoic
11	17.10	Unidentified
12	17.58	Dodecanoic
13	19.59	Unidentified
14	20.01	Tetradecanoic
15	22.20	Hexadecanoic
16	24.19	Octadecanoic

δ-decalactone. The experimental and library mass spectra are given in Figure 5A and B, respectively. The base peak at $m/z = 99$ ($C_5H_7O_2$) is characteristic of δ-lactones and results from cleavage of the alkyl side chain from the lactone ring to give a stable six-membered ring,



$[C_5H_7O_2]^+$, $m/z = 99$

The retention time and mass spectrum of a δ-decalactone standard obtained from Aldrich were identical to that in the lipid extract. The odor, however, was a combination of that of the porcupine and of coconut, with coconut predominating.

GC-Organoleptic Detection. To confirm the association of δ-decalactone with the porcupine odor, we set up the apparatus to enable the GC effluent to

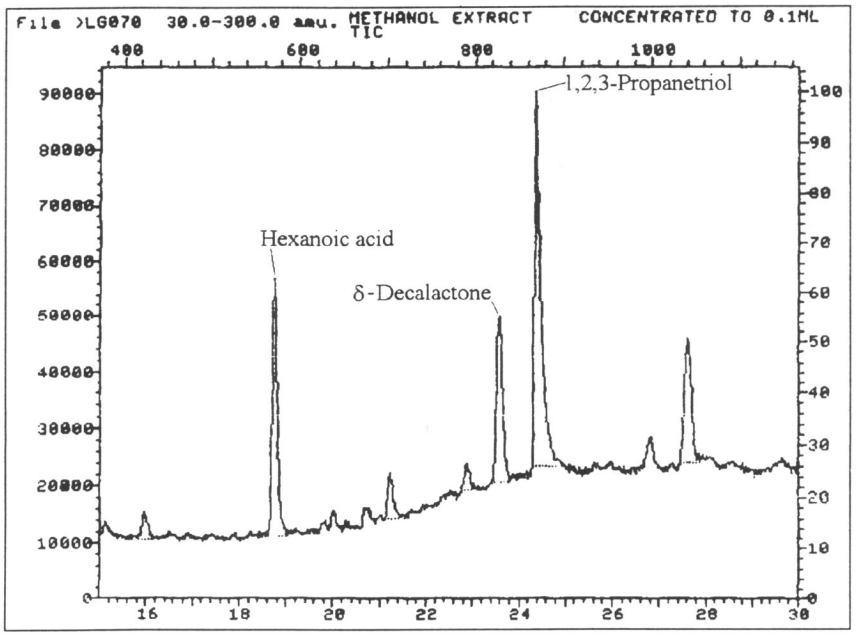


FIG. 4. Total ion chromatogram of methanol fraction from silica gel cleanup.

be monitored by smell. This is a technique used in flavor and fragrance research (Zeng et al., 1991; Mosandl, 1992) and is especially useful for complex samples. It is necessary to use wide-bore capillary columns because of their larger capacity, in order to be able to inject sample sufficient to be detectable. The results are shown in Figure 6. A number of odoriferous peaks were detected. A weak porcupine odor quality was found in the peaks eluting near δ-octalactone, but the strong, characteristic odor was associated with the peak eluting at the retention time of δ-decalactone. The δ-octalactone probably contributes to the overall odor, but is not the principal source.

Enantiomeric Composition of δ-Decalactone in Extract. The combination of odors in the commercial δ-decalactone and the recognition that the compound has a chiral center (Mosandl and Gessner, 1988) led us to determine the enantiomeric composition of the δ-decalactone in the quill extract. In Figure 7 are shown the δ-decalactone peaks in a racemic mixture (the Aldrich sample) and in the extract. Identification of the *R* and *S* isomers was achieved by injection into the γ-DEX 120 chiral GC column of a sample of the pure *S* isomer, kindly donated to us by T. Haffner. The retention time of the *S* isomer was 77.33 min under the conditions used. The sole isomer present in the extract and the com-

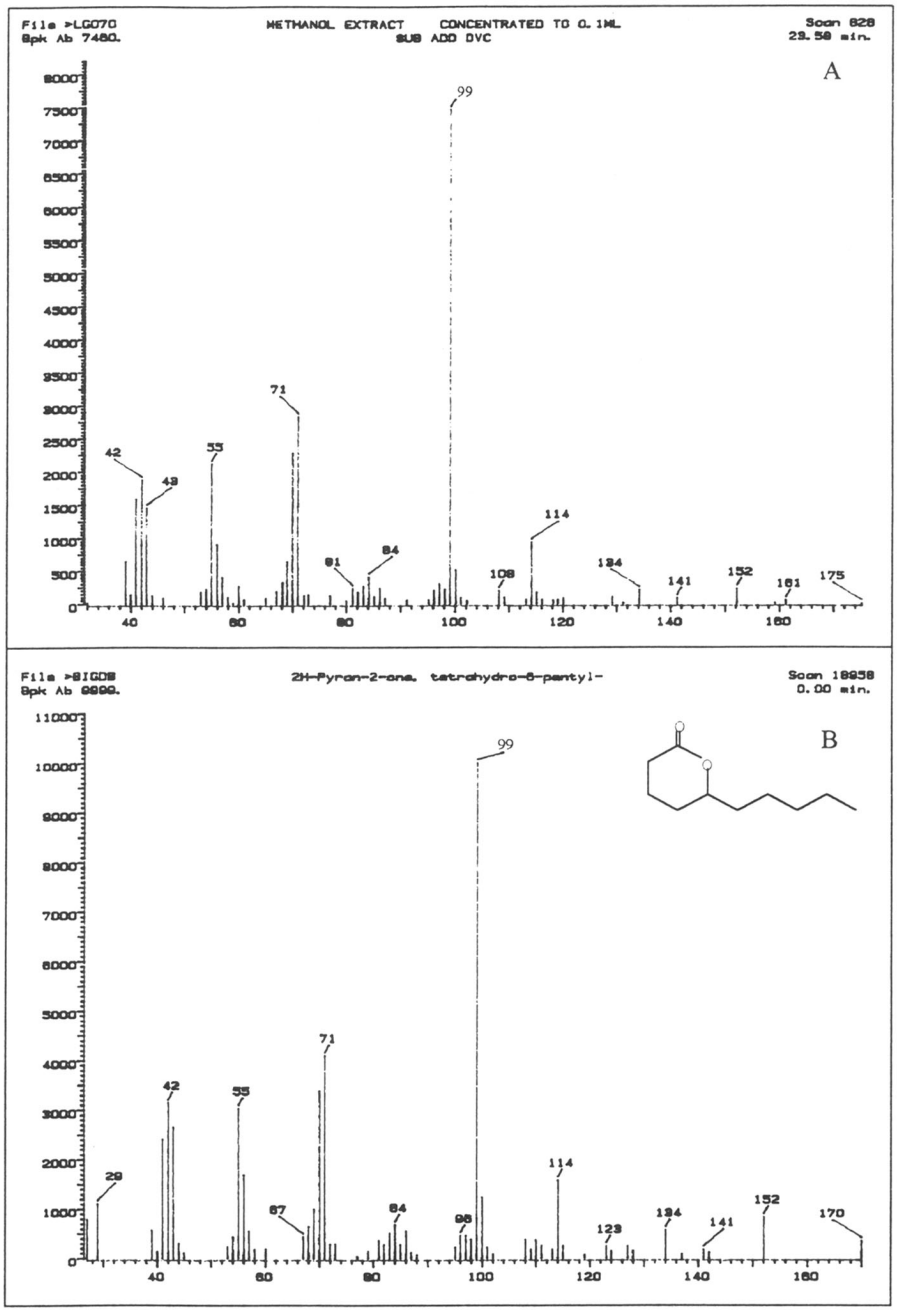


FIG. 5. Mass spectra of δ-decalactone: (A) from quill volatiles chromatogram, (B) reference spectrum from NBS-Wiley mass spectral library.

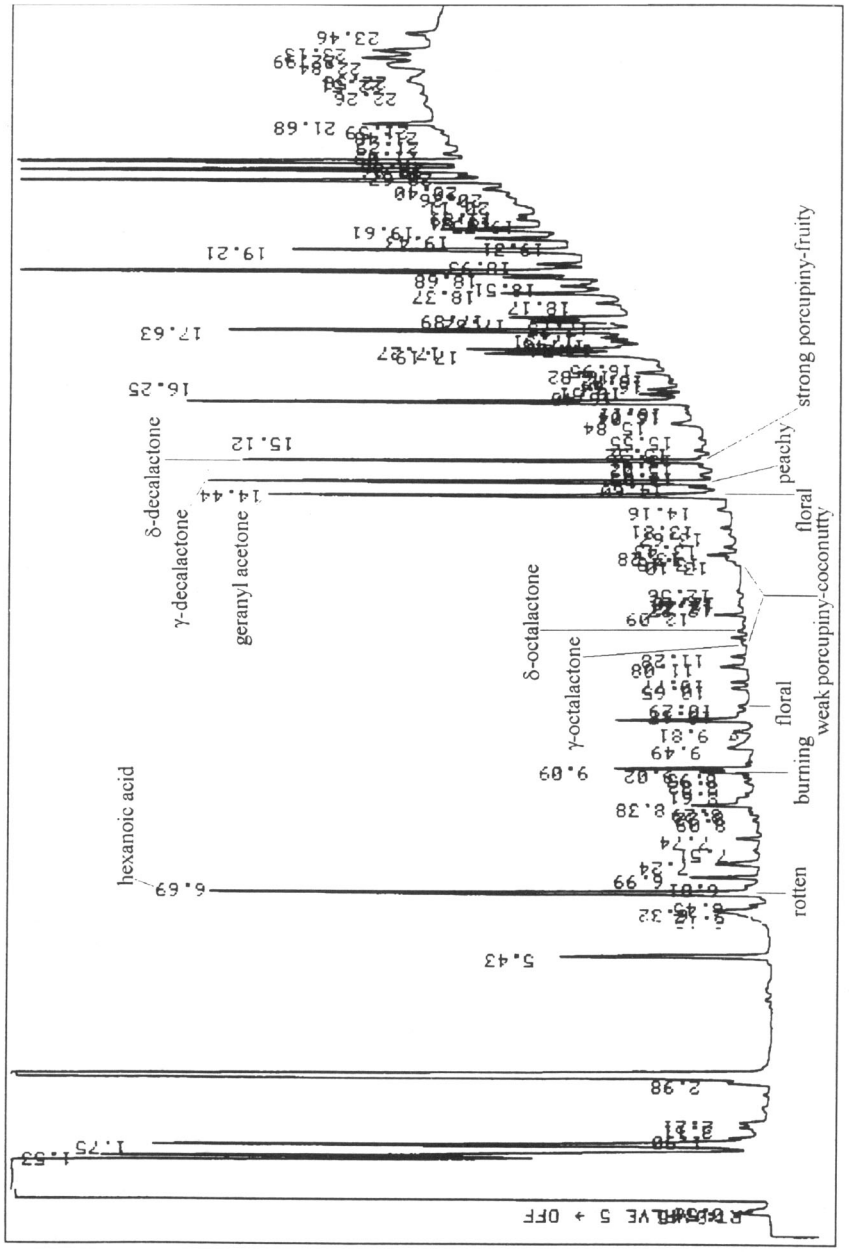


FIG. 6. Chromatogram from GC–organoleptic evaluation of quill headspace volatiles in methanol.

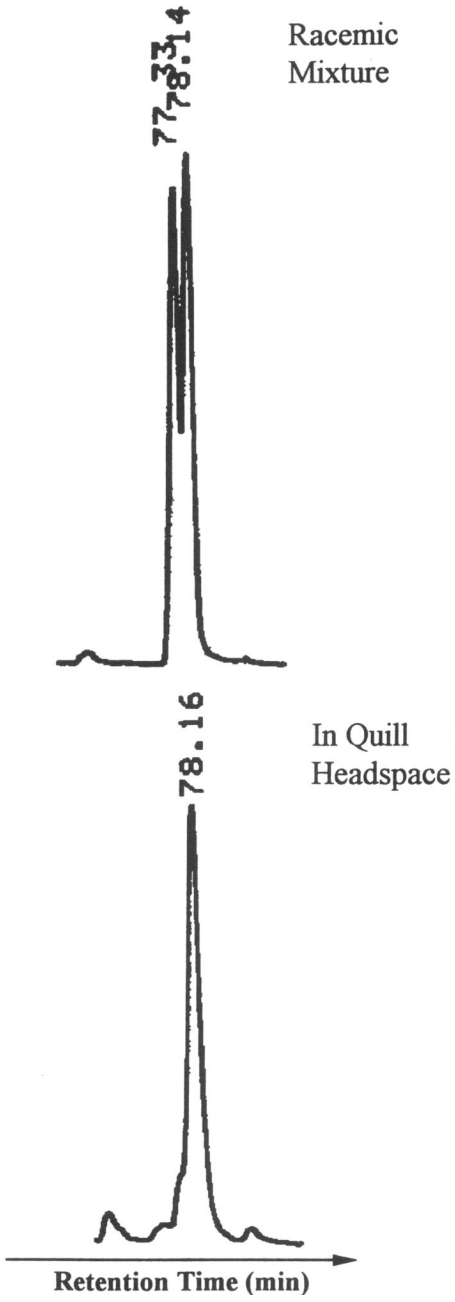


FIG. 7. Chiral separation of δ-decalactone enantiomers. Upper chromatogram: racemic mixture (Aldrich Chemical Co.); lower chromatogram: quill headspace sample; column: Supelco γ-DEX 120 column; GC temperature program: 70°C to 115°C at 10°C/min; detector, FID.

pound to which we attribute the principal odor qualities of the porcupine quill lipid is therefore the (*R*)-δ-decalactone.

DISCUSSION

The porcupine warning odor is released as a consequence of quill erection. Since porcupines are solitary animals that rarely interact with conspecifics (Roze, 1989), it follows that both quill erection and odor release are largely directed to heterospecifics. However, agonistic interactions among porcupines do occur and under such conditions the odor may play an intraspecific role as well.

To the (heterospecific) human olfactory sense, the porcupine odor appears to carry no information about individual or sex differences, nor does it change with the seasons. Whether the same is true for conspecific recipients can not be answered without additional information about variability of odor components between individuals and across seasons.

For optimal efficiency in such a heterospecific signal, it should be source-specific, because other, undefended organisms emitting the same odor would function as Batesian mimics. The greater the proportion of mimics to models, the larger is the time required for predator learning, and the greater the potential cost to the model (Pianka, 1978). While lactones, including δ-decalactone, have been previously reported in biological products, they have been largely confined to fruits, e.g., apricots (Takeoka et al., 1990), coconuts (Lin and Wilkens, 1970), peaches (Do et al., 1969), and raspberries (Casabianca and Graff, 1994). The odor characteristics are described as coconutty, fruity sweet, and peachy (Maga, 1976). This suggests the predominant isomer in these sources is the (*S*)-δ-decalactone. We are not aware of any reports of (*R*)-δ-decalactone in animal secretions. The lactone disseminated by blacktailed deer tarsal glands is a γ-lactone (Brownlee et al., 1969).

One strategy for generating specificity is via enantiomeric excess. It is well established that enantiomers of the same compound can have quite different odor characteristics (Boelens and van Gemert, 1993). Biogeneration can lead to significant enantiomeric excess. For example, the formation of δ-decalactone by microorganisms fed either (*S*)- or (*R*, *S*)-coriolic acid (an unsaturated 13-hydroxy C_{18} carboxylic acid) produces 80% (*S*)-δ-decalactone (Cardillo et al., 1991; Fronza et al., 1993). In the present case, the porcupine δ-decalactone has near-complete *R* specificity.

The sensory quality of the porcupine lactone is not pleasant, and in a closed space can cause wheezing and tearing in a human subject (Roze, 1989). However, it is unlikely that under natural conditions the concentration of the (*R*)-δ-lactone can ever reach levels high enough to act as a primary repellent, as with

the alkyl mercaptans and sulfides of skunk defensive secretions (Andersen et al., 1982). Rather, it is probable that the unique porcupine odor may serve in the formation of a conditioned reflex. A predator receiving the warning odor followed by a painful quill strike should learn to associate the odor with the unpleasant consequences of the latter. Rothschild and coworkers have demonstrated the formation of conditioned aversion to 2-methoxy-3-isobutylpyrazine, an insect warning odor, in naive hatchling chicks (Guilford et al., 1987) and Norway rats (Kaye et al., 1989).

An alternate or supplemental role for the pyrazine odor suggested by Kaye et al. (1989) is that it might potentiate the learning of context-associated averse stimuli. There is, in fact, evidence that predators perceive a complex of warning signals as a gestalt, with signals of different modalities reinforcing each other (Ikin and Turner, 1972). However, in the nocturnal, forest-floor context of most porcupine–predator interactions, an olfactory signal would function more efficiently than associated visual signals. A factor that should promote the formation of a conditioned aversion to the porcupine odor is the fact that the odor is released during the act of quill erection. Thus the potential predator receiving an olfactory warning is already within range of a follow-up quill strike.

Headspace analysis of the porcupine odor revealed no 5-hydroxydecanoic acid, the presumed precursor of the δ-lactone. This suggests the equilibrium towards lactone production lies far to the right under acid to neutral conditions. The hydroxy acid in question is probably a product of bacterial metabolism, as are most of the other free acids listed in Figure 3 (Nicolaides, 1974). Porcupines are atypical mammals in that they have high concentrations of free fatty acids in their skin lipids (Roze et al., 1990). It is notable that baby porcupines a few hours old, with fully developed quills and the ability to erect them, lack the characteristic warning odor (U. Roze, personal observation). Presumably, their sebaceous glands have not yet been colonized by the requisite bacteria.

Acknowledgments—We thank T. Haffner, Technische Universität Berlin for a gift of (*S*)-δ-decalactone; Supelco for donation of the α-, β-, and γ-DEX 120 chiral GC columns; and the Hewlett-Packard Corp. for the donation of the 5880A GC. This research was supported in part by the PSC-CUNY Faculty Research Award Program.

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BIOISOSTERIC APPROACH TO ELUCIDATION OF BINDING OF THE ACETATE GROUP OF A MOTH SEX PHEROMONE COMPONENT TO ITS RECEPTOR

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Abstract—A number of analogs of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, in which the acetate group has been replaced by functional groups that may function as bioisosters, have been synthesized and tested using single-cell electrophysiology. The activities have been interpreted in terms of the molecular electrostatic potentials of the polar functional group as calculated by *ab initio* quantum mechanical calculations. It is concluded that both oxygens of the acetate group in (*Z*)-5-decenyl acetate contribute to the interactions between the pheromone component and its receptor. Furthermore, the results indicate that the crucial interaction between the carbonyl group and the receptor, which is most probably a hydrogen bonding interaction, takes place in a direction pointing away from the hydrocarbon chain of the pheromone component.

Key Words—Lepidoptera, Noctuidae, *Agrotis segetum*, (*Z*)-5-decenyl acetate, pheromone analogs, structure-activity, bioisosteres, single-sensillum recordings, receptor interaction, molecular electrostatic potential, quantum mechanical calculations, *ab initio* calculations.

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INTRODUCTION

A model for the interaction between (Z)-5-decenyl acetate (**1**), a pheromone component of the turnip moth, *Agrotis segetum*, and its receptor has previously been developed, and a number of structure–activity studies based on this model have been reported (Liljefors et al., 1985, 1987; Bengtsson, 1988; Bengtsson et al., 1987, 1990; Jönsson et al., 1991 a,b, 1992, 1993; Gustavsson et al., 1995). The proposed bioactive conformation of **1** is shown in Figure 1. In the development of the model, three pharmacophore elements, i.e., structural elements of decisive importance for the activity of **1**, have been identified: the terminal methyl group, the double bond, and the acetate group. Due to its polarity and hydrogen-bonding capability, the acetate group should be of great importance for the recognition and binding of the pheromone component to its receptor. Thus, it is of interest to investigate the details of the interaction between the acetate group and the receptor. In the present study, a number of analogs of (Z)-5-decenyl acetate have been synthesized and tested by single-cell electrophysiology. In these analogs, the acetate group has been replaced by a functional group that mimics some aspect of the electrostatic properties of the acetate group and that may function as a bioisostere to the acetate group. The purpose of this study was to find out how the acetate group and the receptor interact. In particular, we were interested in studying the directions in which the interactions between the acetate group and the receptor take place. It is reasonable to assume that the acetate group is interacting with the receptor *via* hydrogen bonding involving one or both oxygen atoms. Various possible sites and directions of hydrogen bond interactions are shown in Figure 2.

Previously, modifications of the acetate group of (Z)-7-dodecenyl acetate, another pheromone component of *Agrotis segetum*, were studied by single-cell

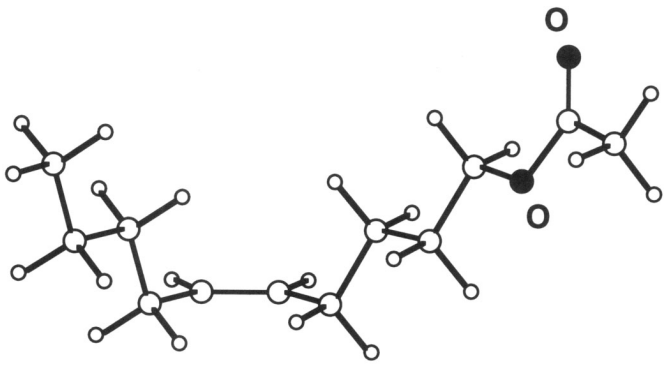


FIG. 1. (Z)-5-Decenyl acetate in its proposed bioactive conformation.

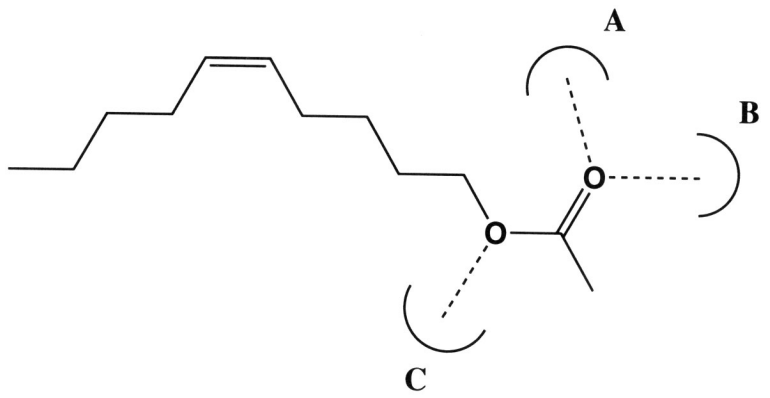


FIG. 2. Possible hydrogen bonding sites and directions for the acetate group of **1**.

electrophysiology (Liljefors et al., 1984). The results from that study imply that, although the presence of the carbonyl group is crucial for the activity, both oxygens in the ester function of (*Z*)-7-dodecenyl acetate are important for full biological activity. In a recent study by Hoskovec et al. (1996), mimics of the acetate function in a pheromone component of the Oriental fruit moth, *Cydia molesta*, (*Z*)-8-dodecenyl acetate, were investigated. The acetate group was replaced with a chloroformate and a lactone function. These authors found that the chloroformate and the alkene-4-olide functional group successfully mimicked the acetate function of the natural pheromone component.

Priesner et al. (1975) and Tamaki et al. (1984) also studied acetate group analogs of pheromone components of various moth species. However, the biological assays used in those studies, EAG and field trapping, preclude drawing conclusions about interactions with a specific receptor.

The analogs studied in the present work are shown in Figure 3. These analogs were selected on the basis of electrostatic potential maps calculated by quantum mechanical *ab initio* calculations. Warthen et al. (1995) recently employed calculated electrostatic potentials in a structure–activity study on (*Z*)-11-tetradecen-1-ol acetate and a series of fluoro analogs.

Bioisosteric replacement of an ester by a heterocyclic analog as in compounds **5** and **6** has to our knowledge not previously been attempted for pheromone components. However, such replacements have been successful in other areas. For instance, Orlek et al. (1991) studied the replacement of an ester group by heterocyclic analogs in compounds interacting with the muscarinic acetylcholine receptor and found that an oxadiazole could successfully replace an ester function *in vitro*. Furthermore, Brown et al. (1996) in a study of analogs of the antibacterial agent pseudomonic acid, replaced an ester function by an oxazole

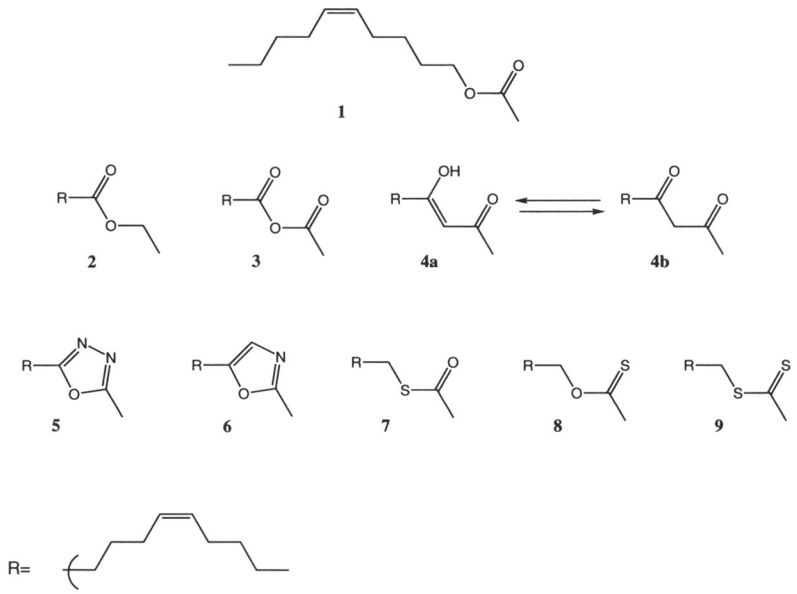


FIG. 3. Structure of compounds synthesized and studied. Names of compounds are in text.

in order to avoid ester hydrolyses *in vivo*. For a recent review on bioisosterism see Patani and LaVoie (1996).

METHODS AND MATERIALS

Synthesis. All reactions of air- and water-sensitive materials were performed under a nitrogen or argon atmosphere with oven-dried glassware. Anhydrous diethyl ether (ether) and tetrahydrofuran (THF) were distilled prior to use from dark blue solutions of the sodium benzophenone radical anion. *N*,*N*′-Dimethyl-*N*,*N*′-propylene urea (DMPU), purchased from Fluka Chemie AG, and toluene was distilled under vacuum from calcium hydride. The dimethyl sulfide complex of cuprous bromide was prepared according to House et al. (1975).

Thin-layer chromatography (TLC), on silica gel 60 F254, was used for the monitoring of the reactions. An anisaldehyde mixture (550 ml ethanol, 20.7 ml sulfuric acid, 6.3 ml acetic acid, and 15.3 ml 4-methoxy-benzaldehyde) or UV-light was used for detection. Flash chromatography was performed on TLC-grade silica gel 60H with eluting solvents indicated in the text.

Analytical GLC was performed with a Varian 3400 capillary GLC fitted

with a DB-Wax 30-m column. Preparative GLC was performed on a 6-m × 4-mm OV-351 column. The purity of the isolated final products was ≥99% except for compound **4**, for which the purity was only 88% (see Discussion). No sample contained any detectable amount of the natural pheromone component **1**. ^{1}H and ^{13}C NMR spectra were determined on a Varian XL-300 or 200 or on a Bruker DRX 400 spectrometer. High-resolution mass spectra were recorded on a Jeol JMS-SX 102 spectrometer. Infrared spectra were recorded on a Perkin Elmer 298. The synthetic schemes used for the preparation of **2–6** are shown in Figure 4. The synthesis for compounds **1** and **7–9** have previously been reported (Olsson et al., 1983; Liljefors et al., 1984; Hansson et al., 1996).

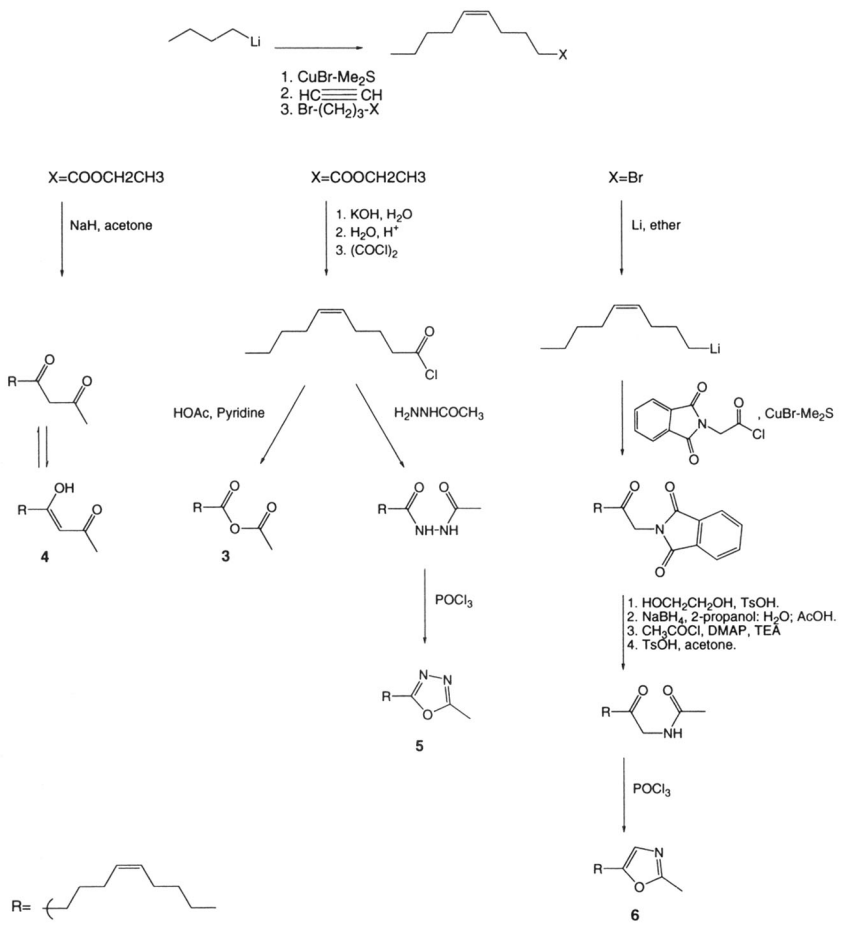


FIG. 4. Scheme for the preparation of analogs **2–6**.

Preparation of Lithium Bis[(Z)-hex-1-enyl] Cuprate. This compound was prepared by carbocupration of acetylene according to Gardette et al. (1985). To a well-stirred suspension of CuBr-Me_2S complex (8.2 g, 40 mmol) in ether (200 ml) at −50°C, 40.0 ml of 2.1 M butyl lithium was slowly added. The solution was allowed to reach −30°C and stirred for 30 min at this temperature. The almost clear solution was cooled to −50°C, and acetylene (2 liter, 80 mmol) was gently bubbled into the reaction mixture. The resulting solution was allowed to reach −25°C and then stirred for 30 min.

(Z)-5-Ethyl-decenoate (2) (X = $COOCH_2CH_3$ in Figure 4). To a stirred solution of lithium bis[(Z)-hex-1-enyl] cuprate (40 mmol) at −30°C, DMPU (14 ml) in 100 ml THF and ethyl-4-bromobutanoate (5.8 ml, 40 mmol) in 16 ml THF were successively added. The reaction mixture was slowly allowed to reach room temperature and stirred over night. After cooling to −20°C, the reaction was quenched with 5 M HCl (100 ml). After stirring for 15 min with air bubbling into the mixture, 100 ml pentane was added and the precipitate was filtered off with celite. The aqueous layer was extracted with ether, and the combined organic extracts were washed once with 17% NH_3, once with saturated NH_4Cl solution, and then dried over $MgSO_4$. The solvents were removed, and the crude product was purified by flash chromatography (pentane–ethyl acetate, 40:1). Compound **2** was afforded in two fractions: 3.5 g, 82% pure by GLC and 0.73 g, 70% pure by GLC. Total yield was 42%. The fraction to be analyzed and biologically tested was further purified by argentation chromatography (Houx et al., 1974), δ_H (300 MHz) 0.88 (t, 3H, $\mathbf{CH_3}$), 1.25(t, 3H, $\mathbf{CH_3}(CH_2O)$, 1.27–1.33 (m, 4H, $\mathbf{CH_2CH_2}$), 1.62–1.72 (m, 2H, $\mathbf{CH_2}$), 1.96–2.10 (m, 4H, $\mathbf{CH_2}CH{=}CH\mathbf{CH_2}$), 2.28 (t, 2H, $\mathbf{CH_2}CO$), 4.11 (q, 2H, $CH_3\mathbf{CH_2}O$), 5.26–5.44 (m, 2H, J = 10.9 Hz, $\mathbf{CH}{=}\mathbf{CH}$). δ_C (75.4 MHz) 14.0, 14.2, 22.3, 24.9, 26.5, 26.9, 31.9, 33.7, 60.2, 128.4, 131.0, 173.7. MS (high resolution) for $C_{12}H_{22}O_2$ calculated 198.1620, found 196.1618.

(Z)-5-Decenoic Acid. KOH (37 g) was dissolved in water (37 ml) and (Z)-5-ethyl-decenoate (**2**) (0.50 g, 2.6 mmol) was added. The reaction mixture was allowed to reflux over night. After cooling in an ice bath, the mixture was acidified with 5 M HCl, extracted with ether, and dried ($MgSO_4$). The solvent was removed, and the acid (0.43 g, 2.5 mmol) was isolated in 97% yield. δ_H (300 MHz) 0.88 (t, 3H, $\mathbf{CH_3}$), 1.24–1.35 (m, 4H, $\mathbf{CH_2CH_2}$), 1.62–1.74 (m, 2H, $\mathbf{CH_2}$), 1.97–2.12 (m, 4H, $\mathbf{CH_2}CH{=}CH\mathbf{CH_2}$), 2.36 (t, 2H, $\mathbf{CH_2}COOH$), 5.26–5.46 (m, 2H, $\mathbf{CH}{=}\mathbf{CH}$). IR: 3400–2500 (−OH), 1725–1690 (C=O).

(Z)-5-Decenoyl Chloride. (Z)-5-Decenoic acid (1 g, 5.9 mmol) was dissolved in dry ether and cooled to 0°C. Oxalyl chloride (3.0 ml, 16.2 mmol) and dimethyl formamide (60 μl, 0.9 mmol) were successively added. The reaction mixture was stirred for 30 min at 0°C and 3 hr at room temperature. The mixture was evaporated and CCl_4 was added, followed by further evaporation.

This procedure was repeated three times. The acid chloride was isolated in quantitative yield. δ_H (300 MHz) 0.88 (t, 3H, CH_3), 1.22–1.36 (m, 4H, CH_2CH_2), 1.71–1.80 (m, 2H, CH_2), 1.96–2.14 (m, 4H, $CH_2CH{=}CHCH_2$), 2.88 (t, 2H, CH_2COCl), 5.22–5.50 (m, 2H, CH=CH. IR: 1770–1830 (C=O).

1-Oxa-(Z)-5-Decenyl Acetate (3). Compound **3** was prepared according to Penn et al. (1993). (*Z*)-5-Decenoyl chloride (0.66 g, 3.5 mmol) in dry toluene (10 ml) was added to a solution of pyridine (0.29 ml, 3.5 mmol) and toluene. Stirring was continued until a white precipitate, the acylhalide–pyridinium complex, was formed (about 30 min). The reaction mixture was cooled to −10°C, and freshly distilled acetic acid (0.20 ml, 3.5 mmol) was added with stirring for 4 hr. The mixture was filtered through celite, rinsed with toluene, and the solvent was then removed. The residue (0.64 g) contained a mixture of the desired unsymmetric anhydride and the (*Z*)-5-decenoic acid anhydride. This mixture was distilled with a Kugel-Rohr apparatus, at approximately 3 mm Hg and 125°C. Great losses occurred and an 88% (NMR) pure product **3** was obtained. δ_H (300 MHz) 0.88 (t, 3H, CH_3), 1.22–1.35 (m, 4H, CH_2CH_2), 1.66–1.76 (m, 2H, CH_2), 1.97–2.14 (m, 4H, $CH_2CH{=}CHCH_2$), 2.20 (s, 3H, CH_3CO), 2.44 (t, 2H, CH_2CO, in unsym. anhydride) 2.43 (t, 4H, CH_2CO, in sym. anhydride); 88% versus 12%, 5.25–5.47 (m, 2H, J = 10.9 Hz, CH = CH). δ_C (75.4 MHz) 13.9, 22.2, 22.3, 24.1, 26.2, 26.9, 31.8, 34.5, 127.9, 131.6, 166.6, 169.3. IR: 1740–1760, 1800–1830 (C=O). MS (high resolution) for $C_{12}H_{20}O_3$ calculated 212.1412, found 212.1412.

*Preparation of (Z)-8-Tridecene,2,4-dione, 4-Hydroxy-(Z)-3,8-tridecadien-2-one (**4a, 4b**).* To a mixture of NaH (80% in mineral oil, 0.81 g, 27 mmol) and (*Z*)-5-ethyl decenoate, **2** (2.69 g, 13.5 mmol), freshly distilled acetone (2.0 ml, 27 mmol) was slowly added. When 30% of the acetone had been added, three drops of ethanol were added to start the reaction, followed by addition of the remaining acetone. The mixture was occasionally cooled on an ice bath to keep the temperature between 30 and 40°C. Dry ether (20 ml) was added, and the reaction mixture was stirred over night at room temperature. The unreacted NaH was destroyed by adding ethanol (1.5 ml), and, after stirring for 15 min, ice cooled acetic acid (1.7 ml, 30 mmol) and water (15 ml) were slowly added. The ether phase was separated, and the aqueous phase was extracted with ether. The combined organic extracts were washed three times with $NaHCO_3$ solution, once with water, and then dried ($CaSO_4$). The solvent was removed, and the residue was purified by flash chromatography (petroleum ether–ethyl acetate, 10:1) affording compound **4** (0.53 g, 2.5 mmol) in 19% yield. The amount to be biologically tested was further purified by flash chromatography (petroleum ether–ethyl acetate, 30:1), followed by preparative GLC. **4a**: δ_H (400 MHz) 0.91 (t, 3H, CH_3), 1.29–1.36 (m, 4H, $CHCH_2$), 1.64–1.72 (m, 2H, CH_2), 2.00–2.12 (m, 4H, $CH_2CH{=}CHCH_2$), 2.07 (s, 3H, CH_3CO), 2.29 [t, 3H,

$CH_2C(OH)=$], 5.30–5.46 (m, 2H, J = 10.8 Hz, CH=CH), 5.51 (s, 1H, [C(OH)=CHCO]. δ_C (100 MHz) 14.4, 22.8, 25.4, 26.1, 27.0, 27.4, 32.3, 38.1, 100.2, 128.9, 131.5, 191.9, 194.5. **4b**: δ_H (400 MHz) 2.25 (s, 3H, CH_3), 2.52 (t, 2H, CH_2CO), 3.58 (s, 2H, $COCH_2CO$). δ_C (100 MHz) 23.7, 26.7, 27.0, 31.3, 43.6, 58.4, 128.8, 131.7. Enol form to keto form ratio was 85:15 ($CDCl_3$). MS (high resolution) for $C_{13}H_{22}O_2$ calculated 210.1620, found 210.1621.

Acethydrazide. This compound was synthesized according to Henecka and Kurtz (1952). Hydrazine hydrate (15 ml, 0.3 mol) was heated to approximately 100°C, and ethyl acetate (22 ml, 0.22 mol) was added dropwise. The mixture was refluxed for two days and then evaporated. The residue was cooled, the crystals formed were treated with ether and chloroform (1:1), and the solvents were then removed. The crystalline acethydrazide (11.5 g, 0.15 mol, 68%) was allowed to dry, melting point 54–59°C (lit. 67°C). δ_H (300 MHz) 1.95 (s, 3H, CH_3CO), 3.85 (broad s, 2H, $NHNH_2$), 6.90 (broad s, 1H, $NHNH_2$).

N'-(Z)-5-Decenoic-N"-Acethydrazide. Acethydrazide (0.54 g, 7.3 mmol) was diluted in acetic acid (4 ml) and cooled on an ice bath. (Z)-5-Decenoyl chloride (100 g, 5.3 mmol) was slowly added (Brown et al., 1961). The mixture was stirred for 1 hr at room temperature, poured into water, and neutralized with $NaHCO_3$ solution. The aqueous solution was extracted with ether (three times), and the combined organic extracts were washed twice with K_2CO_3 solution, once with water, and then dried (Na_2CO_3). The solvent was removed, and the resultant crystals (0.70 g, 3.1 mmol, 58%) had a melting point of 75–77°C after recrystallization in petroleum ether (60/70). δ_H (300 MHz) 0.86 (t, 3H, CH_3), 1.21–1.34 (m, 4H, CH_2CH_2), 1.62–1.73 (m, 2H, CH_2), 1.94–2.08 (m, 4H, $CH_2CH=CHCH_2$), 2.03 (m, 3H, CH_3CONH), 2.26 (t, 2H, CH_2CONH), 5.23–5.42 (m, 2H, CH=CH), 9.45 (broad s, 1H, NH), 9.77 (broad s, 1H, NH).

2-Methyl-5-[(Z)-4-Nonenyl]-oxadiazole (5). The hydrazide (100 mg, 0.44 mmol) was treated with $POCl_3$ (0.5 ml) and heated to reflux. After 3 hr the mixture was quenched with water, made basic with K_2CO_3 solution, and then extracted with ether. The combined ether extracts were dried ($Na_2CO_3/MgSO_4$), and the solvent was removed. The residue was purified by flash chromatography (ethyl acetate), and compound **5** was obtained (60 mg, 0.29 mmol) in 64% yield. The compound was further purified by preparative GLC. δ_H (400 MHz) 0.90 (t, 3H, CH_3), 1.30–1.36 (m, 4H, CH_2CH_2), 1.81–1.89 (m, 2H, CH_2), 2.00–2.05 (m, 2H, $=CHCH_2$), 2.16 (m, 2H, $=CHCH_2$), 2.51 [s, 3H, $CH_3C(O)=N$], 2.82 [t, 2H, $CH_2C(O)=N$], 5.31–5.48 (m, 2H, J = 10.8 Hz, CH=CH). δ_C (100 MHz) 11.4, 14.4, 22.8, 25.2, 26.84, 26.85, 27.4, 32.3, 128.2, 132.0, 164.0, 167.5. High-resolution CI mass spectrum for $C_{12}H_{21}N_2O$ ($M+H^+$), calculated 209.1654, found 209.1654.

1-Bromo-(Z)-4-nonene. This compound (X = Br in Figure 4) was synthesized in the same manner as compound **2** from lithium bis[(*Z*)-hex-1-enyl] cuprate (0.08 mol) and 1,3-dibromopropane (20 ml, 0.20 mol) (Gardette et al., 1983). The crude product was distilled and the halide (9.0 g, 0.044 mol) was obtained at 90–100°C/12 mm Hg and in 55% yield. δ_H (300 MHz) 0.90 (t, 3H, CH_3), 1.25–1.38 (m, 4H, CH_2CH_2), 1.86–2.24 (m, 6H, $CH_2CH{=}CHCH_2CH_2$), 3.42 (t, 2H, CH_2Br), 5.26–5.49 (m, 2H, J = 10.8 Hz, $CH{=}CH$).

1-Lithium-(Z)-4-nonene. Lithium (30% in mineral oil, 0.81 g, 34.7 mmol) and ether (4 ml) were cooled to −10°C, and 1-bromo-(*Z*)-4-nonene (3.00 g, 14.6 mmol) in ether (14 ml) was slowly added. After the addition was complete, the reaction mixture was stirred for 1 hr at 0°C. The 1-lithium-(*Z*)-4-nonene was titrated according to Watson and Eastham (1967) and found to be 0.5 M, 71% yield.

2-(2-Ethanoic acid)-isoindole-1,3-dione. This compound was prepared according to Billman and Harting (1948). Phthalic anhydride (20.0 g, 0.135 mol) and glycine (10.0 g, 0.133 mol) were placed in a large sublimation apparatus and heated to 180°C. The phthalic anhydride that sublimed on the cold finger was pushed down into the reaction mixture three times during 15 min. Thereafter, the excess of phthalic anhydride was allowed to sublime on the cold finger, and the residue was recrystallized from ethanol and water (9:1). The phthalimide-protected acid (25.3 g, 0.123 mol) was isolated in 92% yield. δ_H (200 MHz) 4.50 (s, 2H, CH_2), 7.70–7.78 (m, 2H, Ar**H**), 7.85–7.92 (m, 2H, Ar**H**).

2-(2-Ethanoyl chloride)-isoindole-1,3-dione. The acid halide was prepared as described above for (*Z*)-5-decenoyl chloride from phthalimide ethanoic acid (0.53 g, 2.6 mmol) in ether (40 ml) and by the addition of oxalyl chloride (1.3 ml, 7.0 mmol) and dimethyl formamide (26 ml). The product was isolated in quantitative yield. δ_H (200 MHz) 4.82 (s, 2H, CH_2), 7.75–7.82 (m, 2H, Ar**H**), 7.88–7.95 (m, 2H, Ar**H**).

2-[2-Oxa-(Z)-6-undecenyl]-isoindole-1,3-dione. This compound was synthesized according to Posner (1975). 1-Lithium-(*Z*)-4-nonene (10.0 mmol) was slowly added to a cooled (−40°C) suspension of CuBr–Me_2S (1.00 g, 4.8 mmol) and ether (20 ml). The mixture was allowed to reach −20°C and stirred for 0.5 hr. After cooling to −65°C, phthalimide ethanoyl chloride (2.40 g, 10.7 mmol) in ether (100 ml) was added. The reaction mixture was stirred for 1 hr, then quenched with water, and allowed to reach room temperature. The organic phase was separated, and the aqueous phase was extracted with ether. The combined organic extracts were washed with saturated NH_4Cl and dried ($MgSO_4$). After evaporation, the crude product was purified by flash chromatography (heptane–ether; 4:1). The product (0.59 g, 1.9 mmol) was obtained in 40% yield. δ_H (300 MHz) 0.88 (t, 3H, CH_3), 1.24–1.36 (m, 4H, CH_2CH_2),

1.66–1.75 (m, 2H, CH_2), 1.96–2.12 (m, 4H, $CH_2CH{=}CHCH_2$), 2.52 (t, 2H, CH_2CO), 5.25–5.46 (m, 2H, J = 10.8 Hz, CH=CH), 7.70–7.78 (m, 2H, ArH), 7.82–7.90 (m, 2H, ArH).

2-{2-[(Z)-4-Nonenyl]-[1,3]-dioxolan-2-yl methyl}-isoindole-1,3-dione. The β-keto function was protected as an acetal by refluxing the compound (540 mg, 1.72 mmol) with ethylene glycol (1.2 ml, 2.1 mmol) and *p*-toluene sulfonic acid monohydrate (20 mg, 0.10 mmol) in benzene (100 ml) with a Dean-Stark apparatus over night. After cooling to room temperature, ether was added, and the organic phase was washed with $NaHCO_3$ solution and brine and dried ($NaSO_4$). The solvents were removed, and the product (550 mg, 1.54 mmol) was isolated in 90% yield. δ_H (300 MHz) 0.88 (t, 3H, CH_3), 1.22–1.37 (m, 4H, CH_2CH_2), 1.42–1.55 (m, 2H, CH_2), 1.60–1.68 (m, 2H, CH_2), 1.95–2.10 (m, 4H, $CH_2CH{=}CHCH_2$), 3.80 (s, 2H, CH_2N), 3.90–4.15 (m, 4H, OCH_2CH_2O), 5.28–5.40 (m, 2H, CH=CH), 7.68–7.76 (m, 2H, ArH), 7.82–7.90 (m, 2H, ArH).

{2-[(Z)-4-Nonenyl]-[1,3]dioxolan-2-yl methyl}-amine. The phthalimide protecting group was removed by a method developed by Osby et al. (1984). The compound (500 mg, 1.40 mmol) was dissolved in 2-propanol (14 ml) and water (3.2 ml). $NaBH_4$ (0.55 g, 14.5 mmol) was added in small portions, and the reaction was allowed to stir over night. Then acetic acid (5 ml) was carefully added. When foaming had subsided, the mixture was heated to 80°C for 2 hr. After cooling to room temperature, it was made basic with Na_2CO_3 and then extracted with ether. The organic phase was dried (Na_2SO_4), and the solvents were removed. The crude amine was purified by flash chromatography (ethyl acetate–triethyl amine, 4:1), and the pure product (210 mg, 0.92 mmol) was isolated in 66% yield. δ_H (300 MHz) 0.88 (t, 3H, CH_3), 1.22–1.68 (m, 8H, CH_2), 1.96–2.10 (m, 4H, $CH_2CH{=}CHCH_2$), 2.72 (s, 2H, CH_2N), 3.96 (s, 4H, OCH_2CH_2O), 5.28–5.42 (m, 2H, CH=CH).

N-{2-[(Z)-4-Nonenyl]-[1,3]-dioxolan-2-yl methyl}-acetamide. The amine (200 mg, 0.88 mmol), 4-dimethylaminopyridine (10 mg, 0.08 mmol), and triethyl amine (0.5 ml) in dichloromethane (10 ml) were cooled on an ice bath, and acetic anhydride (0.15 ml, 1.60 mmol) was added. The reaction was allowed to reach room temperature and stirred for 6 hr. It was then poured onto ice water and extracted with ether. The organic phase was washed with brine, dried ($NaSO_4$), and concentrated. The crude amide was purified by flash chromatography (ethyl acetate), and the product (210 mg, 0.78 mmol) was obtained in 89% yield. δ_H (300 MHz) 0.88 (t, 3H, CH_3), 1.22–1.48 (m, 6H, CH_2), 1.57–1.66 (m, 2H, CH_2), 1.94–2.08 (m, 4H, $CH_2{=}CHCH_2$), 2.00 (s, 3H, CH_3CO), 3.39 (d, 2H, CH_2NH), 3.95 (s, 4H, OCH_2CH_2O), 5.25–5.40 (m, 2H, CH=CH), 5.60 (broad s, 1H, NH).

N-[2-Oxo-(Z)-6-undecenyl]-acetamide. In order to remove the acetal, the amide (200 mg, 0.74 mmol) was refluxed in acetone (50 ml) with *p*-toluene

sulfonic acid monohydrate (20 mg, 0.10 mmol) over night. After cooling, ether was added, and the organic phase was washed with saturated $NaHCO_3$ solution and brine, dried ($NaSO_4$), and concentrated. The product (135 mg, 0.60 mmol) was obtained in 81% yield. δ_H (300 MHz) 0.89 (t, 3H, CH_3), 1.24–1.38 (m, 4H, CH_2CH_2), 1.62–1.74 (m, 2H, CH_2), 1.94–2.10 (m, 4H, $CH_2CH{=}CHCH_2$), 2.02 (s, 3H, CH_3CO), 2.45 (t, 2H, CH_2CO), 4.13 (d, 2H, CH_2NH), 5.23–5.46 (m, 2H, J = 10.8 Hz, $CH{=}CH$), 6.24 (broad s, 1H, NH).

2-Methyl-5-[(Z)-4-nonenyl]-oxazole (6). Compound **6** was synthesized by treating the β-keto amide (120 mg, 0.53 mmol) with $POCl_3$ in the same manner as in the synthesis of compound **5.** The crude product was purified by flash chromatography (ethyl acetate), and the oxazole (60 mg, 0.29 mmol) was isolated in 53% yield. The oxazole was further purified by preparative GLC. δ_H (400 MHz), 0.90 (t, 3H, CH_3), 1.30–1.36 (m, 4H, CH_2CH_2), 1.65–1.73 (m, 2H, CH_2), 1.99–2.14 (m, 4H, $CH_2CH{=}CHCH_2$), 2.41 [s, 3H, $CH_3C(O){=}N$], 2.61 [t, 2H, $CH_2(CO){=}N$], 5.32–5.46 (m, 2H, J = 10.8 Hz, $CH{=}CH$), 6.59 [s, 1H, $CH(N){=}C$]. δ_C (100 MHz) 14.37, 14.41, 22.8, 25.4, 26.9, 27.4, 28.0, 32.3, 122.4, 128.9, 131.4, 152.8, 160.6. High-resolution CI mass spectrum for $C_{13}H_{22}ON$ (MH^+), calculated 208.1701, found 208.1702.

Electrophysiology. The activities of compounds **1–9** were determined by single-cell electrophysiology (Kaissling, 1974). The olfactory receptor-cell specifically tuned to (*Z*)-5-decenyl acetate, present in the antennal sensilla type SW1 of *Agrotis segetum*, was used (Hallberg, 1981; Löfstedt et al., 1982; van der Pers and Löfstedt, 1983). The method was modified according to van der Pers and den Otter (1978) and has been previously described (Liljefors et al., 1987; Bengtsson et al., 1990).

The stimulus amounts used for pheromone component **1** were 10^{-4}–10^{-1} μg and for analogs **2–6**, 10^{-1}–10^{2} μg in decadic steps. For each loading, 10 replicates were recorded and the mean value of the action potentials generated during 1 sec from the onset of stimulation was used in the construction of the dose–response curves. The errors were expressed as standard errors of the mean (SEM). The electrophysiological activity of each compound in relation to **1** is expressed as the reciprocal of the relative quantities required to elicit the same response from the receptor as the natural pheromone component **1**.

Differences in volatilities for compounds **2**, **3**, and **4** were taken into account by correcting the activities by using relative vapor pressures as previously described (Liljefors et al., 1985; Bengtsson et al., 1990; Gustavsson et al., 1995). The correction added to the logarithm of the relative activity for compound **2** is based on vapor pressure data (Dykyj and Répaš, 1979) for propyl acetate, ethyl propanoate, butyl acetate, and ethyl butanoate and was −0.06. The correction for compound **3** is based on vapor pressures for acetic anhydride and ethyl acetate and was 1.26. The correction added for compound **4** was 1.01,

based on data for acetyl acetone and ethyl acetate. The correction used for **6** was based on vapor pressure data for oxazole (DIPPR Database, American Institute of Chemical Engineers) and for methyl formate (Dykyj and Répaš, 1979) and was 0.64. As no vapor pressure data could be found for oxadiazole, this correction was also employed for compound **5**. For compounds **7–9** the uncorrected activities, taken from Hansson et al. (1996), were used.

Quantum Mechanical Calculations. Electrostatic potentials for the polar functional groups in compounds **1–9** (R = CH_3 in Figure 3) were calculated using quantum mechanical *ab initio* calculations. The electrostatic potential V at a point r is defined by equation 1 (Politzer and Murray, 1991):

$$V(r) = \sum_{\alpha=1}^{N} \frac{Z_\alpha}{|R_\alpha - r|} - \int \frac{\rho(r')}{|r' - r|} dr' \quad (1)$$

where N is the number of nuclei, Z_α is the charge of the αth nucleus located at R_α, and $\rho(r)$ is the electron density function of the molecule. Integration is carried out over all space. The electrostatic potential at point r is calculated by letting a unit positive charge at that point interact with the nuclei and electrons of the molecule. Thus, an electrophile will be attracted to negative regions of $V(r)$, most strongly to the point where $V(r)$ has its most negative value.

For the calculations of the electrostatic potentials, the HF/6-31G* basis set was employed with geometries calculated by using the HF/3-21G(*) basis set. For compounds **3**, **4**, and **7**, additional conformational analysis were performed using the 6-31G* basis set. The calculations were performed on a Silicon Graphic Indy Workstation using the Spartan computer program, (Wavefunction Inc.).

RESULTS

Chemicals. In order to obtain the *Z* double bond, a selective carbocupration of acetylene, a method developed by Gardette et al. (1985), was used. This method has previously been employed for the syntheses of a number of (*Z*)-5-decenyl acetate analogs (Jönsson et al., 1991a,b, 1992, 1993; Gustavsson et al., 1995). The anhydride, compound **3**, was synthesized from the acylpyridinium complex and acetic acid according to Penn et al. (1993). However, the anhydride could not be satisfactorily purified by distillation with Kugel-Rohr apparatus (due to the small amounts available, an ordinary distillation could not be undertaken). The final product obtained was a mixture, which, according to NMR spectroscopy, contains 88% of the desired unsymmetric anhydride **3** and 12% of the symmetric (*Z*)-5-decenoic acid anhydride. As the vapor pressure of this compound should be much lower than that of **3**, this symmetric anhydride should not significantly influence the result of the electrophysiological testing. Compound **4** was synthesized *via* a Claisen acylation according to Swamer and

Hauser (1950). To obtain the heterocyclic compounds **5** and **6**, cyclization of the hydrazide compound and the β-keto amide, respectively, was performed with the dehydrating reagent phosphorus oxychloride according to Hayes et al. (1955).

Molecular Electrostatic Potentials. The electrostatic potentials for the functional groups of compounds **1–9** (Figure 3, R = CH_3) were calculated using conformations in which all heavy atoms are located in the same plane, corresponding to the conformation of the acetate group in the proposed bioactive conformation of **1** (Figure 1). For all analogs, except compounds **3**, **4b**, and **7**, the planar conformation is the lowest energy conformation found by the *ab initio* calculations. The conformation of acetic anhydride corresponding to the polar functional group in compound **3** is, according to gas-phase electron-diffraction experiments, a twisted structure with a dihedral angle between the two carbonyl groups of 79° (Vledder et al., 1971). Energy minimizations, using the 6-31G* basis set, gave two geometries of low conformational energies, one with a dihedral angle between the two carbonyl functions of 51° and one with 90°, which is 0.4 kcal/mol less stable. The experimental value is a mean value of the interchange between the two low-energy conformations. The planar *cis* conformation used in the calculations of electrostatic potentials is computed to be 1.1 kcal/mol higher in energy than the most stable conformation.

Compound **4** may exist in two tautomeric forms, **4a** and **4b** (Figure 3). Experimental data (Iijima et al., 1987) show that the enol form **4a** predominates in the gas phase (ca. 98% enol form). However, the keto–enol equilibrium is sensitive to the polarity of the environment. Thus, in water, the keto form is the dominating tautomer (84%) (Emsley, 1984). In our calculations (6-31G* basis set), the most stable keto form is found to be twisted with an angle of 96° between the carbonyl groups. This is in agreement with experimental results. Infrared and Raman spectroscopy give an angle of 90° (Ernstbrunner, 1970; Buemi and Gandolfo, 1989), while electron diffraction gives a corresponding angle of 49° (Lowery et al., 1971). The planar *cis* conformation was found to be as much as 6.6 kcal/mol less stable than the twisted conformation.

For compound **7**, the calculated lowest energy conformation, using the 6-31G* basis set, was found to be a structure with a C-S-C-C dihedral angle of 80°. However, the planar conformation used in the calculations of electrostatic potentials is only 0.3 kcal/mol higher in energy.

The calculated electrostatic potentials are shown in Figure 5. The potentials are displayed as contours in the plane of the heavy atoms of the various functional groups. The local minima, i.e., the points of maximum negative electrostatic potentials, are indicated by arrows, and the interaction energies (in kilocalories per mole) with a unit positive charge in these points are shown.

In the case of the acetate group (**1**), there are three regions of strong negative electrostatic potentials, two in the vicinity of the carbonyl oxygen in

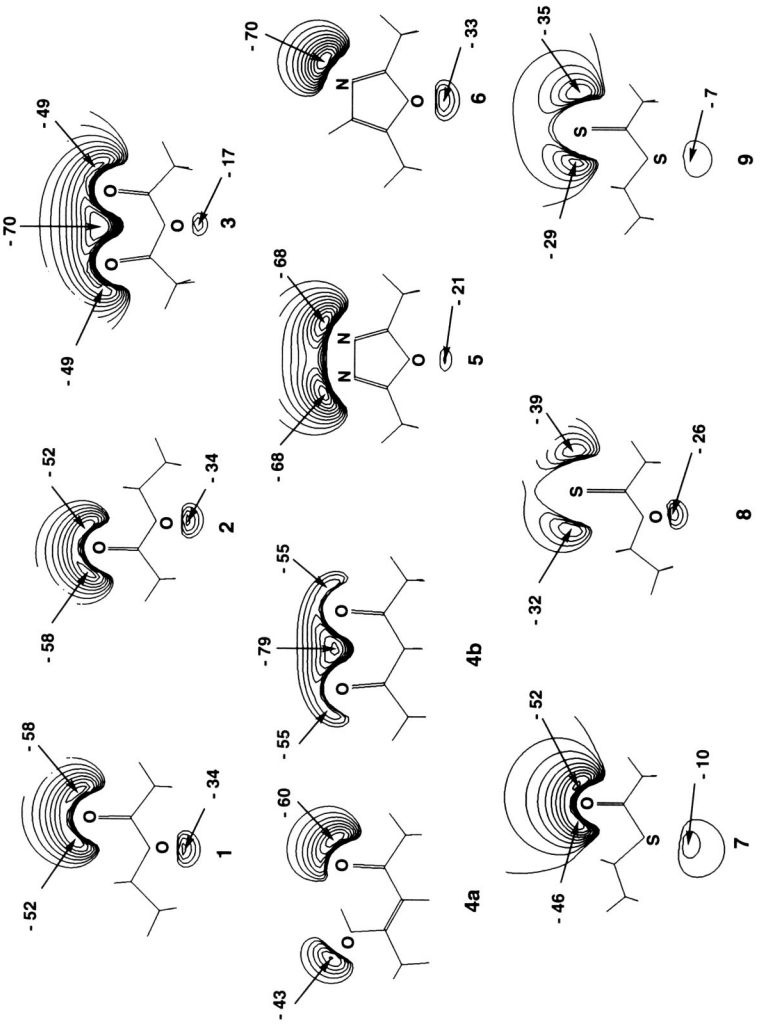


FIG. 5. Molecular electrostatic potentials in the plane of the polar functional groups of compounds **1–9** calculated by *ab initio* HF/6-31G*//HF/3-21G(*) calculations. Interaction energies are in kilocalories per mole.

the directions of the oxygen lone pairs and one located around the ether oxygen. These regions correspond to regions A, B, and C in Figure 2.

The sulfur analogs **7–9** display qualitatively the same regions of negative electrostatic potentials as for **1**, but the exchange of oxygen by sulfur significantly decreases the sizes of the interaction energies. As shown by compounds **7** and **8**, the electrostatic potentials, compared to those of **1**, are not only affected in the areas in the vicinity of the exchanged atom but also in other areas. For compound **7**, an exchange of the oxygen in the ether position by a sulfur decreases not only the size of the maximum interaction energy in area C, but also lowers the energies of attraction in areas A and B. For compound **8**, the exchange of a carbonyl group for a thiocarbonyl group affects not only areas A and B but also C.

The "reversed" ester group **2** has areas A and C in common with the acetate group (**1**) but introduces a new area of strong negative electrostatic potentials, D, which does not have a correspondence in **1**. The anhydride **3**, the enol and keto forms of **4** (**4a** and **4b**), and the oxadiazole **5** also display strong negative electrostatic potentials in this area (D). It should be noted that the enol form **4a** only has area B in common with the acetate (**1**). The oxazole **6** has only two regions of strongly negative electrostatic potentials corresponding to areas B and C in **1**.

In summary, the electrostatic potentials of the sulfur analogs **7–9** mimic the electrostatic potentials in areas A, B, and C of **1**. The reversed ester **2** mimics **1** with respect to areas A and C and introduces area D. The anhydride **3** and the oxadiazole **5** display similarities with **1** in areas A, B, and C, and **4b** mimics **1** in areas A and B. The enol form of **4** (**4a**) has only a strong negative electrostatic potential in area B in common with **1**. Furthermore, **3–5** have negative electrostatic potentials in an area D which is not present in **1**. Finally, the oxazole **6** mimics **1** with respect to the electrostatic potentials in areas B and C.

Receptor Cell Responses. Relative single-cell activities for compounds **1–9** are shown in Figure 6. The activities for compounds **1–6** are corrected for differences in volatility (see Methods and Materials). The data for **7–9** are taken from Hansson et al. (1996). Due to lack of vapor pressure data, the activities for these analogs are uncorrected. Since these compounds should be less volatile than **1**, the activities for **7–9**, shown in Figure 6, may be somewhat underestimated.

The most active analog is the thiocarbonyl analog **8**, which is found to be 90 times less active than **1**. The thioether analog **7** also displays significant activity, while the dithio analog **9** is much less active. Interestingly, the oxazole analog **6** displays moderate activity, whereas the structurally similar oxadiazole analog **5** is essentially inactive. Compound **4** (enol form is assumed, see Dis-

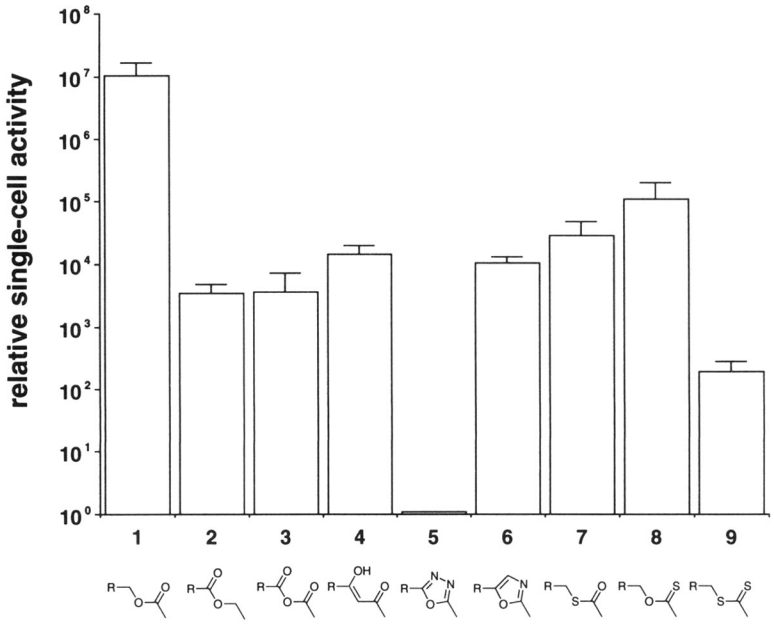


FIG. 6. Relative electrophysiological single-cell activities (+SEM) for compounds **1–9**. The activities for compounds **1–6** are corrected for differences in volatility. The data for **7–9** are taken from Hansson et al. (1996).

cussion) shows a somewhat higher activity than the equally active analogs **2** and **3**.

DISCUSSION

It is reasonable to assume that the acetate group of the natural pheromone component **1** is interacting with the receptor *via* hydrogen bonding involving one or both oxygen atoms. This type of interaction has previously been proposed for the binding of pheromone components to pheromone binding proteins (Prestwich et al., 1995; Prestwich, 1996). In a study of the effects on the activity of modifications of the acetate group of (Z)-7-dodecenyl acetate (Liljefors et al., 1984), it was shown that the removal of the carbonyl group results in a virtually inactive compound. The substitution of the ether oxygen by a CH_2 group decreases the activity by a factor of 100.

Various combinations of hydrogen bond interactions and hydrogen bond directions are possible for the acetate group in **1**. These possibilities correspond

to maximum negative electrostatic potentials in the plane of the acetate group. The electrostatic potential has previously been successfully used to predict the sites and directionality of hydrogen bonds in a variety of systems. In addition, good correlation has been found between calculated hydrogen bond energies and the value of the electrostatic potential in appropriate areas (Politzer and Murray, 1991).

The calculated electrostatic potentials indicate three areas (A, B, and C) of possible hydrogen bond interactions between **1** and hydrogen-bond-donating amino acid residues in the receptor. The calculated electrostatic potentials of compounds **1–9** may, in conjunction with their single-cell activities, be employed to draw conclusions about the most probable sites and directions of the assumed hydrogen bond interactions.

Sulfur is a weaker hydrogen bond acceptor than oxygen (Chang et al., 1991; Hansson et al., 1996), which is reflected in the smaller size of the maximum negative electrostatic potentials in the vicinity of the sulfur atom. Thus, the reduced activities of the sulfur analogs **7–9** may be understood in terms of weaker hydrogen bond interactions with the receptor. Compounds **1** and **7** have similar electrostatic potentials in the vicinity of the carbonyl group. However, in contrast to the relatively strong potential caused by the ether oxygen in **1**, the corresponding thioether in **7** only gives rise to a weak electrostatic potential. This difference rationalizes the lower activity of **7**, which, furthermore, implies both ester oxygens in **1** are contributing to the interaction between the acetate group and the receptor. The same conclusion may be drawn by comparing the activities and the calculated electrostatic potentials of compounds **8** and **9**. These compounds have the same magnitude of the electrostatic potential in the thiocarbonyl areas A and B, but have significantly different values in area C where **9** displays a much less negative potential than **8**. This difference shows up in a lower activity of **9** by a factor of about 600. These results are in line with previously reported results for the interaction between (*Z*)-7-dodecenyl acetate and its receptor (Liljefors et al., 1984).

Analogs **7** and **9** have similar electrostatic potentials in areas C, but **7** has a much stronger ability to interact *via* a hydrogen bond to the carbonyl group (areas A and B). This is reflected in the significantly higher activity of **7** compared to that of **9**. Compound **9** has weak electrostatic potentials in each of the three areas A, B, and C, and has consequently the lowest activity of the sulfur analogs.

Compound **2** is 2800 times less active than **1**. The most notable feature of the electrostatic potential of **2** is that there is no local potential minimum in **2** corresponding to area B in compound **1**, but instead there is a local minimum D that does not have a correspondence to **1**. The strongly reduced activity of **2** is most probably not due to the presence of area D for the following reasons. Compound **4**, which is somewhat more active than **2** also has a strongly negative

electrostatic potential in area D in the enol (**4a**) as well as in the keto form (**4b**). However, the decreased activity of **4** compared to that of **1** is clearly due to the lack of a negative electrostatic potential corresponding to that in the vicinity of the ether oxygen in **1** (area C). For this reason, an activity of **4** somewhat lower than that of **7**, which has a weak electrostatic potential in area C, is expected. This is also observed. Thus, the low activity of **2** can probably be attributed to the lack of a negative electrostatic potential in area B.

The lowest energy conformation on the diketo group in **4b** is highly twisted. Such a comformation is not compatible with a bioactive conformation corresponding to that of **1** in Figure 1. A more or less planar conformation is required for **4b** to fit the model. However, this type of conformation has a very high conformational energy. The planar conformation is calculated to be 6.6 kcal/mol higher than the lowest energy one (see above). This high energy makes it less probable that **4** interacts with the receptor in its keto form **4b**. The more probable enol form (**4a**) only displays two areas of large negative electrostatic potentials corresponding to areas B in Figure 2 and D as defined above. According to the discussion above, the activity decrease of **4b** compared to **1** is completely rationalized by the lack of a negative electrostatic potential in area C. Thus, as **4a** lacks an area of negative electrostatic potential corresponding to area A of **1**, it may be concluded that area A is of less importance for the interaction of **1** with its receptor.

The anhydride analog **3** displays a weak electrostatic potential in area C but a potential of the same magnitude as that of **7** in area B and a very strong one in area A. In addition, **3** also has a strong negative potential in area D. The decrease in activity of **3** by a factor of 2800 compared to that of **1** indicates that the presence of a very strong negative electrostatic potential in area A may negatively influence the activity. However, it should be noted that the anhydride **3** is easily hydrolyzed, which may affect the amount of compound that is actually reaching the receptor resulting in a lower than expected activity.

According to the discussion above, the oxazole gorup of compound **6** should be a good bioisostere to the acetate group of **1**. The activity of this compound supports the conclusion that a negative electrostatic potential in area B is crucial for the activity. A lack of a strong negative electrostatic potential in area A is of minor importance for the activity, and the decreased activity of **6** compared to **1** is most probably due to repulsive steric interactions between the hydrogen of the oxazole ring and the receptor. Such a steric repulsion may also be the reason why **8** displays a somewhat higher activity than **6**, despite the fact that the electrostatic potentials in areas B and C are more negative in **6** than in **8**.

In view of the moderate activity of **6**, the essential inactivity of compound **5** is unexpected and difficult to rationalize. However, the strongly negative electrostatic potential in the area between areas A and D in Figure 5 is unique

among the present analogs with respect to its position as well as its directional properties and may be detrimental to the activity of this compound.

CONCLUSIONS

None of the modifications of **1** studied above result in a fully active bioisosteric replacement of the acetate group in **1**. However, by analyzing the electrophysiological single-cell activities in terms of the molecular electrostatic potentials of the polar functional groups, as calculated by *ab initio* quantum mechanical calculations, it may be concluded that both oxygens of the acetate group in **1** contribute to the interaction between the pheromone component and its receptor. Furthermore, the results strongly indicate that the crucial interaction between the carbonyl group and the receptor, which is most probably a hydrogen bonding interaction, takes place in a direction pointing away from the hydrocarbon chain of the pheromone component, i.e., in the direction of the maximum negative electrostatic potential in area B in Figure 2.

The calculated weaker electrostatic potentials in the vicinity of a sulfur atom compared to those at the corresponding positions about an oxygen atom rationalize the lower activity of the sulfur analogs. The replacement of the acetate group in **1** by an oxazole ring system gives a pheromone component analog with a moderate electrophysiological activity. However, the corresponding replacement by an oxadiazole ring leads to a virtually inactive compound. Thus, the pattern of the electrostatic potential about the oxazole ring is compatible with a productive interaction with the receptor, whereas that about the oxadiazole ring is not.

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STRUCTURAL REQUIREMENTS OF JASMONATES AND MIMICS FOR NICOTINE INDUCTION IN *Nicotiana sylvestris*

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Abstract—Jasmonic acid (JA) is strongly implicated in the long-distance signal transduction cascade increasing nicotine synthesis in the roots of plants after leaf wounding. In order to explore the structural requirements of the inducing signal, we examined jasmonates, mimics, and a biosynthetic precursor for nicotine-inducing activity (NIA). We examine the importance of the keto group on the five-membered ring and the double bond in the *n*-pentenyl chain by comparing the NIA of methyl jasmonate (MJ) with that of cucurbic acid, 1,3-dithiolane-MJ, 1,3-dioxolane-MJ, methyl dihydrojasmonate (DHMJ), 1,3-dioxolane-DHMJ, 1-oxo-indan-4-carboxylic acid ILE-methyl ester, and 1-hydroxyl-indan-4-carboxylic acid ILE-methyl ester. We found that: 1,3-dioxolane MJ, cucurbic acid, and 1,3-dioxolane DHMJ were less active than MJ and that the isoleucine (ILE) conjugates of 1-oxo- and 1-hydroxyindanon-4-carboxylic acid had the same NIA as MJ. The activities of these indanon amino acid conjugates may be due to the structural similarity of their keto or hydroxyl groups on the five-membered ring to MJ or to the keto-enolized MJ. These results support the hypothesis that the enolization of the keto group during or prior to its interaction with the putative JA receptor is required for activity. We explore the importance of the esterification of the carboxyl functional group by comparing the NIAs of cucurbic acid and cucurbic acid methyl ester, 1-oxo-indan-4-carboxylic acid, 1-oxo-indan-4-carboxylic acid methyl ester, and 1-oxo-indan-4-carboxylic acid ILE-methyl ester. In all cases, the esters were more active than the free acids. We compared the NIA of MJ of different epimeric composition (8% and 20% 3*R*,7*S*-MJ); 12-oxophytodienoic acid (12-oxo-PDA) methyl ester, an important precursor

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of JA; and coronatine (a well-known phytotoxin and putative structural mimic of 12-oxo-PDA). We found that: (1) the epimeric composition of MJ did not affect its NIA; (2) 12-oxo-PDA methyl ester had lower NIA than MJ; and (3) coronatine significantly inhibited plant growth but did not increase nicotine biosynthesis. In summary, JA, rather than its biosynthetic precursor, 12-oxo-PDA, is likely the endogenous signal in *Nicotiana sylvestris*, and the keto functional group on the five-membered ring and the double bond in the *n*-pentenyl side chain are crucial components of JA for NIA.

Key Words—*Nicotiana sylvestris*, nicotine, nicotine-inducing activity, jasmonates.

INTRODUCTION

Products of the octadecanoid pathway, which include jasmonic acid (JA), its methyl ester (MJ), and 12-oxo-phytodienoic acid (12-oxo-PDA), have been implicated in the signal-transduction cascade that mediates wound- and elicitor-induced responses (reviewed in Hamberg and Gardner, 1992; Sembdner and Parthier, 1993; Farmer, 1994; Gross and Parthier, 1994). Evidence for JA's involvement comes from (1) the elicitation of induced responses by the exogenous addition to octadecanoid-derived metabolites; (2) correlated changes in endogenous metabolite pools and the induced responses; and (3) inhibition of the pathway producing the metabolites with the commensurate inhibition of the response. For example, the exogenous additions of JA analogs mimic the following wound- and elicitor-induced responses (Table 1): increases in volatile emission in the lima bean (Krumm et al., 1995; Boland et al., 1995), in benzophenanthridine alkaloid concentrations in cell cultures of *Eschscholtzia californica* and *Agrostis tenuis*, in indole alkaloid concentrations in cell cultures of *Rauvolfia serpentina*, in isoflavone concentrations in cell cultures of *Glycine max* (Weiler et al., 1994); tendril curvature in *Bryonia dioica*; induction of tubers in cultures of potato stems (Koda et al., 1995, 1996); senescence of oat leaves; and growth of soybean callus (Koda et al., 1995).

The exogenous feeding of metabolites to elicit a response is a valuable technique for determining structure–activity relationships, and important advances have been made for some responses. For example, Blechert et al. (1995) examined the benzophenanthridine alkaloid-inducing activity of JA-related molecules with the keto group on the five-membered ring and side chains modified and determined that the keto group was required for activity; however, the length of the alkyl side chain was not important. Krumm et al. (1995) proposed that the enolization of the keto group, which results in two sp^2 centers and planarizes the "upper half" of the molecule, is required for the elicitation of volatiles. Here we examine these conclusions regarding jasmonate activity with induced nicotine biosynthesis in *Nicotiana sylvestris*.

TABLE 1. CONCENTRATIONS OF JASMONATES MIMICS REQUIRED FOR ACTIVITY IN SIX DIFFERENT INDUCED RESPONSE BIOASSAY SYSTEMS

	Bioassay Systems					
Compounds	Induction of nicotine	Induction of volatiles (Krumm et al., 1995)	Induction of Benzophenanthridine (Blechert et al., 1995)	Induction of Benzophenanthridine (Weiler et al., 1994)	Induction of Tendril coiling (Weiler et al., 1994)	Induction of 3-indolymethyl glucosinolate (Bodnarky and Yoshiharra, 1995)
MJ (**6**) (μM)	0.5	100	2	100	50	500
Cucurbic methyl ester (**3** and **5**) (μM)	1 71% activity of MJ	NT[a]	inactive	NT	NT	NT
DHMJ (**9**) (μM)	1 70% activity of MJ	NT[a]	10	NT	NT	NT
12-oxo-PDA (**15**) (μM)	1 80% activity of MJ	NT[a]	1	NT	5	NT
Coronatine (**1**) (μM)	inactive	1	NT	100	5	500
1-Oxo-indan-4-carboxylic ILE-methyl ester (**11**) (μM)	1	20	NT	NT	NT	NT
1-Hydroxyl-indan-4-carboxylic ILE-methyl ester (**12**) (μM)	1	20	NT	NT	NT	NT

[a] NT = not tested.

The results of exogenous feeding experiments are frequently difficult to interpret for the following reasons. First, for many of the bioassay systems, tissues are wounded prior to the addition of chemicals (in excised leaves and tissue culture); hence it is difficult to disentangle the interactive effects of prior damage and the added chemicals. Second, because many of the bioassays involve excised tissues, they are necessarily short-term. With such short-term bioassays, it is difficult to determine whether a compound is toxic or inhibits growth in addition to activating a response. Since much of secondary metabolism is dramatically influenced by plant growth rate (Baldwin, 1994; Waterman and Mole, 1989), it is essential to distinguish between induction and growth-inhibition effects of putative signals. Third, for most bioassay systems the concentrations of exogenously added metabolites required to elicit responses are substantially higher than the endogenous wound-induced changes in jasmonates that are thought to mediate the responses in intact plants (Table 1). This may be due to poor uptake of the chemicals, but this explanation has not been verified in most systems.

The induced increase in nicotine synthesis in *N. sylvestris* is one of the few induced defenses that is well understood on a whole-plant (WP) basis. It is known that: (1) additions of MJ to roots increase de novo nicotine synthesis just as leaf damage does (Baldwin et al., 1994); (2) leaf damage rapidly (<0.5 hr) increases shoot JA pools, which reach peak induction 90 min after wounding, and more slowly (<2 hr) increases root JA pools, which reach peak induction at 180 min (Baldwin et al., 1997); (3) there are strong, positive relationships between the level of leaf wounding, the induced-endogenous JA response, and WP nicotine response (Baldwin et al., 1997; Ohnmeiss et al., 1997); (4) exogenous ^{14}C-labeled JA, when applied in a lanolin paste to a leaf, is transported to roots and increases root JA pools at the same time and in the same amounts as does wounding (Zhang and Baldwin, 1997). Together, these results strongly support the model that JA is a systemic signal involved in the wound-induced nicotine response. Moreover, the nicotine induction activity (NIA) in *N. sylvestris* may be an ideal system for structure–activity relationshp research, because (1) it requires no prewounding; (2) the nicotine response requires five days for its full expression, during which time plants grow vigorously (hence any effects on growth rate are easy to discern); (3) it is more sensitive than other bioassay systems (Table 1) and exogenous additions of JA quantitatively mimic wound-induced endogenous JA flux in plants (Baldwin et al., 1994, 1997; Zhang and Baldwin, 1997).

Because in our previous structure–activity studies we found that linolenic acid or its methyl ester and abscisic acid did not have NIA, we established that the nicotine system differs in response characteristics from the well-studied proteinase-inhibitor system of tomato (Baldwin et al., 1996). In order to test the

keto-enolization hypothesis, we compared the NIA of several jasmonates with modification in the keto functional group [1,3-dithiolane MJ (**7**), 1,3-dioxolane MJ (**8**), 1,3-dioxolane DHMJ (**10**), α-cucurbic acid (**4**), β-cucurbic acid (**2**), α-cucurbic acid methyl ester (**5**), β-cucurbic acid methyl ester (**3**)], with structural mimics of jasmonates [1-oxo-indan-4-carboxylic acid (**13**), 1-oxo-indan-4-carboxylic acid methyl ester (**14**), 1-oxo-indan-4-carboxylic acid ILE-methyl ester (**11**), 1-hydroxyl-indan-4-carboxylic acid ILE-methyl ester (**12**)]. In addition, we examined the NIA of 12-oxophytodienoic acid methyl ester (**15**), an important precursor for JA biosynthesis, and coronatine (**1**), a phytotoxin that is thought to be a structural mimic of 12-oxo-PDA (Weiler et al., 1994).

METHODS AND MATERIALS

Materials. Mass spectra were taken with HP GC-MS (5890 Series II GC and HP 5971). TLC analyses were performed on Merck 60F 254 silica gel plates. Silica gel 60 (240–400 mesh) was used for column chromatography, 3*R*,7*S*-MJ (8%) and DHMJ were obtained from Bedoukian Research (Danbury, Connecticut). 3*R*,7*S*-MJ (20%), α-cucurbic acid, and β-cucurbic acid were obtained as gifts from Cayman Chemical (Ann Arbor, Michigan). Reagents and solvents were dried and purified before use, and reactions were carried out under dry, oxygen-free argon. The isomeric compositions of 8% 3*R*,7-*S*-MJ and 20% 3*R*,7*S*-MJ were determined by GC-MS before use.

Synthesis of 1-Oxo-indan-4-carboxylic Acid and Its Methyl Ester. 1-Oxo-indan-4-carboxylic acid was synthesized according to the procedure of Aono et al. (1978). Pure compound (95% of GC-MS) was obtained by crystallization from acetone at an overall yield of 85%. The acid was methylated with CH_2N_2 to give its methyl ester.

Synthesis of 1,3-Dioxolane MJ. MJ (1.05 g, 4.8 mmol) and chlorotrimethylsilane (1.4 ml, 11.0 mmol) were added to a mixture of dry ethylene glycol (3 ml, 54 mmol) and dry methanol (20 ml) under a nitrogen atmosphere. The mixture was stirred for 16 hr at room temperature. The pH was adjusted to 6.0 by adding a 5% solution of sodium methoxide in methanol, and the solvent was removed under reduced pressure. The residue was dissolved in ether (20 ml) and filtered through silica gel (5 g). Subsequently, the silica gel was washed with ether (2 × 10 ml) and the ether was removed under reduced pressure. The crude product was purified by flash chromatography (silica gel, elution solution: ethyl acetate–hexane, 1 : 10 v/v) to give the pure product as a colorless oil; yield: 0.52 g (53%). Purity >97% by GC-MS. MS: *m/z* 268 (M), 237, 195, 153, 99, 86.

Synthesis of 1,3-Dioxolane DHMJ. The synthesis procedure is the same as that of 1,3-dioxolane MJ. However, DHMJ was used as the starting material.

Overall yield was 58% with a purity of >95% by GC-MS. MS: *m/z* 270 (M), 239, 197, 155, 99, 86, 55.

Synthesis of 1,3-Dithiolane MJ. MJ (1.02 g, 4.8 mmol) was dissolved in 15 ml methylene chloride to which 0.6 g (6.0 mmol) of 1,2-ethane-dithiol was added, followed by 0.5 ml of boron trifluoride etherate. The solution was stirred at room temperature overnight and 5 ml of 5% sodium hydroxide was added. The organic layer was separated, washed with H_2O, dried ($MgSO_4$), and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silca gel, eluent: ethyl acetate–hexane, 1:10, v/v) to give a colorless oil. Overall yield was 0.68 g (63%) at a purity >95% by GC-MS. MS: *m/z* 300 (M), 272, 239, 207, 165, 131.

Plant Culture. Plants were grown in hydroponic solution as described in Baldwin and Schmelz (1994). Seeds that were the progeny of one greenhouse-grown plant were sown in Cornell mix A (Boodley and Sheldrake, 1977) and allowed to germinate and grow for 16 days. Following this, the seedlings were transferred to 40-liter communal hydroponic boxes filled with a complete nutrient solution. After four days, plants were placed in individual 1-liter cups filled with the nitrogen-free hydroponic solution and 2 ml of a 1 M solution of KNO_3. Plants were grown in these 1-liter cups in a glass house with supplemental lighting from sodium vapor lamps for 16 hr/day. An additional 1 ml of a 1 M solution of KNO_3 was added to each plant's hydroponic container eight days later when the experiment started. Over the course of the experiment, the liquid level in the 1-liter cups was maintained at 1 liter by the addition of the nitrogen-free hydroponic solution.

Determination of NIA. We compared with NIA of jasmonates, structural mimics of jasmonates, 12-oxo-PDA methyl ester (a biosynthetic precursor of JA), and standardized leaf damage. Each treatment had five replicate plants. To control for variation in plant size, plants were sorted by mass at the start of the experiment (Baldwin and Schmelz, 1994). Reproducible amounts of leaf damage were produced by rolling a fabric pattern tracing wheel across three leaves at phylotatically adjacent positions 3, 4, and 5 (with the apical leaf designated as position 1). Each leaf received eight rows of evenly distributed pattern-wheel damage. Chemicals (in aqueous solutions) were added to the hydroponic growth media. The chemicals used are depicted in Figure 1. Most chemicals were tested at two concentrations. The high concentration (1 μM) was chosen as the upper limit because previous studies suggested a saturation of nicotine accumulation at this concentration of MJ (Baldwin et al., 1994). Plants were treated or damaged on day 0; on day 5, they were harvested for nicotine analysis. Plants were separated into roots and shoots, frozen in liquid nitrogen, lyophilized, weighed (to 0.1 mg), and ground to a fine powder (850-μm mesh) in a Wiley mill (Thomas Scientific, Swedesboro, New Jersey). Nicotine analysis was modified

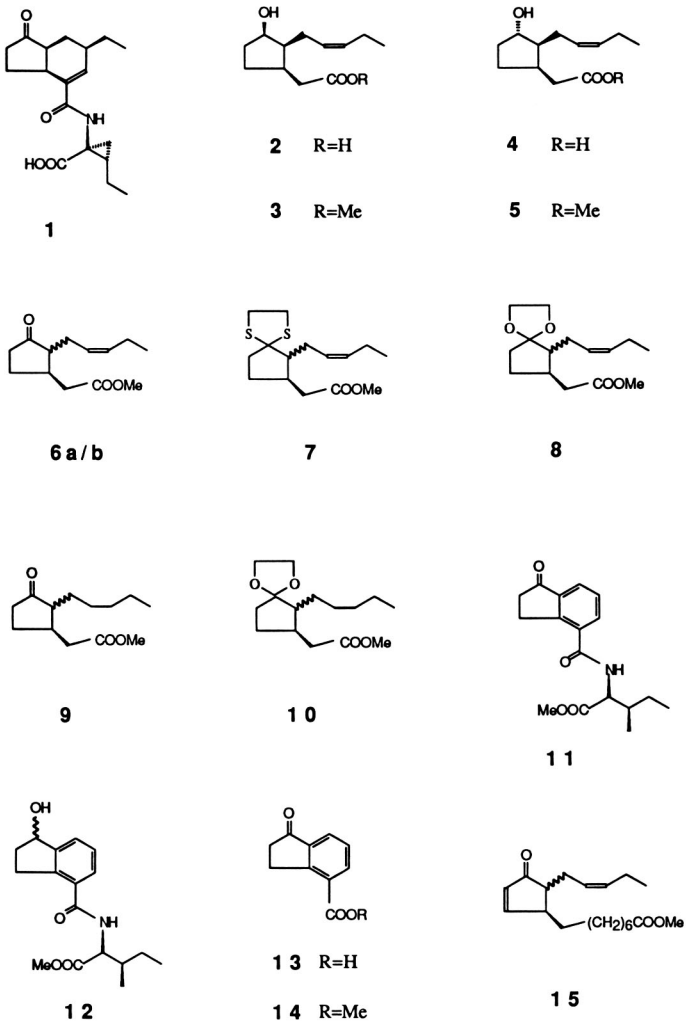


FIG. 1. Structures of jasmonates and mimics tested for nicotine-inducing activity (NIA) in *Nicotiana sylvestris*, **1,** coronatine; **2,** β-cucurbic acid; **3,** β-cucurbic acid methyl ester; **4,** α-cucurbic acid; **5,** α-cucurbic acid methyl ester; **6a,** MJ with 8% 3*R*,7*S*-MJ epimer (epi-MJ); **6b,** MJ with 20% 3*R*,7*S*-MJ epimer (epi-MJ); **7,** 1,3-dithiolane methyl jasmonate; **8,** 1,3-dioxolane methyl jasmonate; **9,** DHMJ; **10,** 1,3-dioxolane methyl dihydrojasmonate; **11,** 1-oxo-indan-4-carboxylic acid ILE-methyl ester; **12,** 1-hydroxyl-indan-4-carboxylic acid ILE-methyl ester; **13,** 1-oxo-indan-4-carboxylic acid; **14,** 1-oxo-indan-4-carboxylic acid methyl ester; and **15,** 12-oxo-PDA methyl ester with 33% 9-*trans* isomer.

from Baldwin (1988) to obtain WP nicotine measures. A weighed portion (approximately 50 mg) of lyophilized and homogenized tissue was extracted for three days in 10.0 ml of aqueous alkaloid extraction solution (40% methanol, 0.1% HCl). Nicotine content of these extracts was determined by HPLC. WP nicotine concentrations were calculated from biomass values and WP nicotine pools.

RESULTS AND DISCUSSION

Figure 2 depicts WP nicotine concentrations of untreated plants (control), wounded plants (damage), and plants treated with three different concentration ($\sim$0.05, 0.5, and 1 μM) of the chemicals depicted in Figure 1. Figure 3 depicts WP nicotine pools of the same plants. The pattern of NIA as seen in the WP nicotine concentration data mirrored that found in WP nicotine pool for all chemicals assayed except coronatine (**1**). These results demonstrate that none of the chemicals, except coronatine, inhibited plant growth at the concentrations used in our bioassay. Recently several research groups have reported that coronatine has similar or higher biological activity than JA or MJ (Boland et al., 1995; Koda et al., 1996; Weiler et al., 1994) or 12-oxo-PDA and dihydro-12-oxo-PDA in a number of plant tissue culture bioassay systems (Weiler et al., 1994). Our results demonstrate that coronatine significantly inhibits the growth of tobacco plants but does not significantly affect nicotine biosynthesis. The apparent increase in WP nicotine concentration is due to the fact that smaller plants have higher nicotine concentrations (Baldwin and Schmelz, 1996). We conclude that coronatine is more likely acting as a phytotoxin that inhibits plant growth than as a mimic of a signal in the nicotine defense response in tobacco.

Krumm et al. (1995) proposed that the activity of MJ requires the enolization of the keto group on the five-membered ring during or prior to its interaction with the putative JA receptor. This model was derived from the activity of indanon isoleucine conjugates, structures which have two sp^2 centers without the reduction of the keto group. In order to test this proposal in our system, we compared the NIA of the molecules in which the keto group had been protected by 1,3-dioxolane, 1,3-dithiolane, or reduced to a hydroxyl group. We found β-cucurbic acid (**2**) and α-cucurbic acid (**4**) to be inactive (Figure 2). 1,3-Dithiolane MJ (**7**) and 1,3-dioxolane MJ (**8**) with protected keto functional groups that cannot be enolized have 60% of NIA of MJ. There is no significant difference between dioxolane MJ and dithiolane MJ. Usually, the 1,3-dioxolanes are of limited stability. However, in our experimental conditions these compounds clearly retained their activity. DHMJ (**9**), which lacks a double bond in the *n*-pentenyl side chain, had only 70% of NIA of MJ (**6a**). Furthermore, when we protected the keto group of DHMJ, the activity of 1,3-dioxolane DHMJ (**10**)

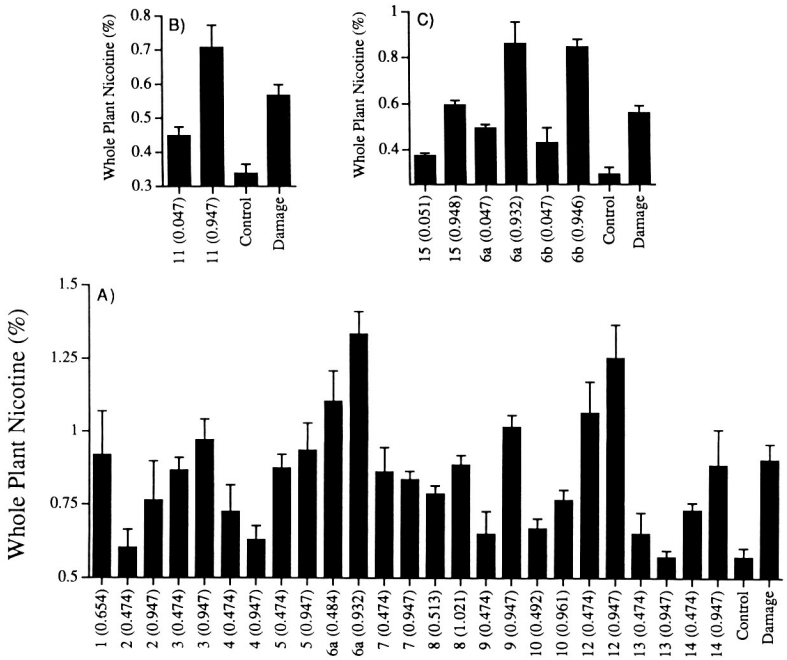


FIG. 2. (A) Mean (±SEM) whole-plant (WP) nicotine concentrations of undamaged plants (control), plants with leaves damaged by a pattern wheel (damage), and undamaged plants treated with test compounds **1–14** at indicated dosages (μmole): coronatine (**1**), β-cucurbic acid (**2**), β-cucurbic acid methyl ester (**3**), α-cucurbic acid (**4**), α-cucurbic acid methyl ester (**5**), MJ with 8% 3*R*,7*S*-MJ epimer (epi-MJ) (**6a**), 1,3-dithiolane methyl jasmonate (**7**), 1,3-dioxolane methyl jasmonate (**8**), DHMJ (**9**), 1,3-dioxolane methyl dihydrojasmonate (**10**), 1-hydroxyl-indan-4-carboxylic acid ILE-methyl ester (**12**), 1-oxo-indan-4-carboxylic acid (**13**), and 1-oxo-indan-4-carboxylic acid methyl ester (**14**) added to the hydroponic solution of each plant. (B) Mean (±SEM) WP nicotine concentrations of undamaged plants (control), plants with leaves damaged by a pattern wheel (damage), and undamaged plants that had 0.047–0.947 μmol of 1-oxo-indan-4-carboxylic acid ILE-methyl ester (**11**) added to the hydroponic solution of each plant. (C) Mean (±SEM) WP nicotine concentrations of undamaged plants (control), plants with leaves damaged by a pattern wheel (damage), and undamaged plants that had 0.047–0.948 μmol of MJ with 8% 3*R*,7*S*-MJ epimer (epi-MJ) (**6a**), MJ with 20% 3*R*,7*S*-MJ epimer (epi-MJ) (**6b**), and 12-oxo-PDA methyl ester with 33% 9-*trans* isomer (**15**) added to the hydroponic solution of each plant. Each treatment group contained five plants that were harvested for alkaloid analysis five days after treatment applications.

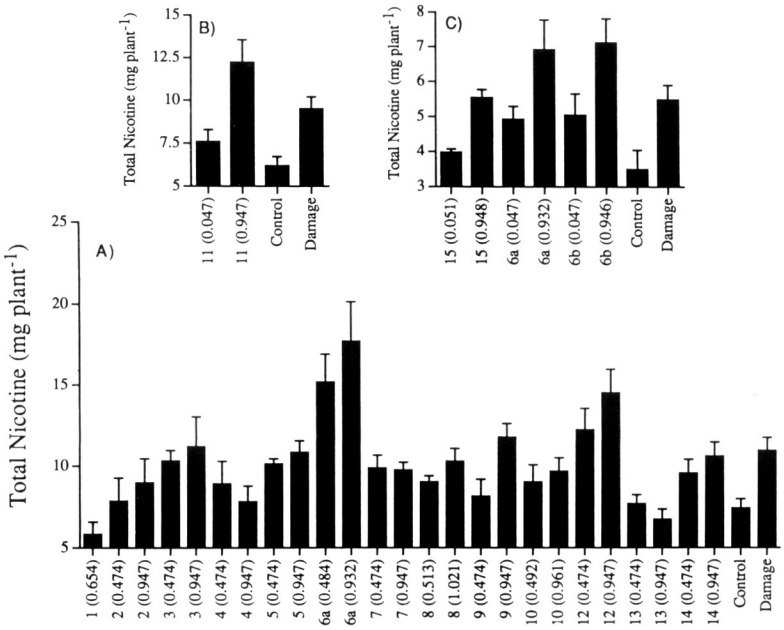


FIG. 3. Mean (±SEM) whole-plant (WP) nicotine (milligrams per plant) pools of all the treatments depicted in Figure 2A–C. WP nicotine pools reflect WP nicotine concentration in all cases except when plants were treated with 0.654 μmol coronatine (**1**), which dramatically inhibited plant growth over the five-day test period and thereby reduced WP nicotine pools.

was reduced significantly to 50% NIA of MJ. Moreover, both the 1-oxo-indan-4-carboxylic acid ILE-methyl ester (**11**) and the 1-hydroxyl-indan-4-carboxylic acid ILE-methyl ester (**12**) had the same level of NIA ad MJ (Figure 2A and 2B). These results confirm that the biological activity of indanon amino acid conjugates may be attributable to the upper part of their structures, which are similar to the enolized MJ. We concluded that the keto group and the double bond in the *n*-pentenyl chain are the crucial components of JA's NIA.

We explored the importance of esterification and conjugation of the carboxyl functional group by comparing the NIA of cucurbic acid and cucurbic acid methyl ester and of indanon acid, indanon acid methyl ester, and indanon acid isoleucine methyl ester conjugate. β-Cucurbic acid (**2**) and α-cucurbic acid (**4**) were inactive, but when esterified, the NIA of each was comparable to that of wounding. Similar results were found with indanon acid. When the indanon's carboxyl functional group was conjugated with isoleucine methyl ester, its NIA

was comparable to that of MJ. Esterification and amino acid conjugation may play a role in the transport of chemicals across cell membranes.

Weiler et al. (1994) and Blechert et al. (1995) showed that 12-oxo-PDA had higher biological activity than MJ (Table 1). However, in the nicotine system, 12-oxo-PDA methyl ester was less active than MJ at all concentrations tested (Figure 2C: at 0.05 μmol/plant 12-oxo-PDA methyl ester was 79% as active as MJ; at 0.94 μmol/plant, 80% as active). Since the MJ and 12-oxo-PDA methyl ester used in the experiment differed in their isomeric ratios (3*R*,7*R*-MJ and 3*R*,7*S*-MJ in a ratio of 92:8 and 80:20; 9-*trans*-12-oxo-PDA methyl ester and 9-*cis*-12-oxo-PDA methyl ester in a ratio of 33:67), the possibility exists that the difference in activities is due to the differences in epimeric ratio. We find this possibility unlikely because mixtures of MJ differing in their epimeric compositions did not differ significantly in their NIA (Figure 2C, contrast $F_{1,56} \leq 3.911$, $P \geq 0.053$). It is not clear whether the epimeric composition of a solution of either MJ or 12-oxo-PDA methyl ester would be preserved when taken up by the roots because JA is known to be epimerized in the presence of globular proteins in plant tissues (Mueller and Brodschelm, 1994). We conclude that JA, rather than 12-oxo-PDA, is an important intermediate in this whole-plant signal cascade due to JA's higher molar activity in inducing nicotine synthesis (Figure 2C).

In summary, JA, rather than its biosynthetic precursor, 12-oxo-PDA, is likely a systemic signal in *Nicotiana sylvestris*. Moreover, the keto functional group in the five-membered ring and the double bond in the *n*-pentenyl side chain are crucial components of JA for NIA. However, all studies that measure plant responses after exogenous addition without determining the plant's endogenous JA responses must be interpreted with caution. The JA biosynthetic pathway is very sensitive to external stimuli, and some responses may be the result of the stimulation of the endogenous JA cascade by the exogenous application.

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IDENTIFICATION OF SEX PHEROMONE OF ADZUKI BEAN BORER, *Ostrinia scapulalis*[1]

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Abstract—By means of gas chromatography with electroantennographic detection (GC-EAD), gas chromatography–mass spectrometry (GC-MS), and a series of bioassays, (*Z*)-11-tetradecenyl acetate (*Z*11-14:OAc) and (*E*)-11-tetradecenyl acetate (*E*11-14:OAc) at a ratio of 100:3 were identified as the female sex pheromone of the adzuki bean borer, *Ostrinia scapulalis*. The average amounts of *Z*11-14:OAc and *E*11-14:OAc in a single sex pheromone gland were 6.6 ± 2.4 ng and 0.2 ± 0.1 ng, respectively. In a wind-tunnel bioassay, the binary blend of *Z*11- and *E*11-14:OAc elicited almost the same male behavioral responses as did virgin females and sex pheromone gland extract. In field trapping experiments, rubber septa impregnated with the binary blend (50 μg/septum) attracted more males than virgin females. The sex pheromone of *O. scapulalis* thus turned out to be similar to that of the Z-type European corn borer, *O. nubilalis*, in both components and their ratio.

Key Words—*Ostrinia scapulalis*, sex pheromone, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, GC-EAD, field trapping.

INTRODUCTION

Eight *Ostrinia* species (Lepidoptera: Pyralidae) have been recorded in Japan (Mutuura and Munroe, 1970). One species, *O. sanguinealis*, has not been

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[1]Studies on sex pheromones of *Ostrinia* spp. in Japan[1].

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observed in Japan for at least 30 years, and thus seven *Ostrinia* species inhabit Japan at the present time. The seven species are *O. furnacalis*, *O. orientalis*, *O. palustralis*, *O. latipennis*, *O. zealis*, *O. zaguliaevi*, and the species of current interest, the adzuki bean borer, *O. scapulalis*.

Ostrinia scapulalis is a serious pest of the adzuki bean plant, *Vigna angularis*, cowpea, *V. sinensis*, and hops, *Humulus lupulus*, in Japan. This species is very polyphagous, and many noncrop host plants from various plant families are known, e.g., docks, *Rumex* spp., a knotweed, *Polygonum sachalinense*, a butterbur, *Petacites japonicus*, and a cocklebur, *Xanthium canadense* (Hattori and Mutuura, 1987). Despite its importance as a pest, research on *O. scapulalis* has been hindered by the presence of the morphologically difficult to distinguish species, *O. zealis* and *O. zaguliaevi*. Overlap among the host plants of these three species also complicates the situation. To counter these difficulties, we have recently started research on the biology of *Ostrinia* species in Japan employing multiple methodologies that include morphology, molecular biology, and chemical ecology.

In the genus *Ostrinia* of the world, sex pheromones of three species, *O. nubilalis*, *O. obumbratalis*, and *O. furnacalis*, have been reported. The European corn borer (ECB), *O. nubilalis*, uses (*E*)- and (*Z*)-11-tetradecenyl acetates as sex pheromone components and is known to be polymorphic with respect to female sex pheromone composition: E-type ECB females produce an *E*:*Z* blend of 97:3, whereas Z-type females produce a 3:97 blend (Klun et al., 1975). The sex pheromone components of the smartweed borer, *O. obumbratalis*, were found to be the same as those of *O. nubilalis*, but the ratio of the two components is 1:1 (Klun and Robinson, 1972). In contrast to these two species, the Asian corn borer, *O. furnacalis*, employs the positional isomers (*E*)- and (*Z*)-12-tetradecenyl acetates as its sex pheromone components, at a ratio of 1:1 (Cheng et al., 1981; Kou et al., 1992) to 2:3 (Ando et al., 1980), depending upon the geographical population. The objective of the present study was to identify the sex pheromones of *O. scapulalis* for comparison with other *Ostrinia* species.

METHODS AND MATERIALS

Insects. Female moths of *Ostrinia* spp. were captured by insect net in a field in Matsudo, Chiba Prefecture, Japan, in 1995 and 1996. Eggs produced by each female were reared, with the mother's identity maintained. Identification of offspring to species was done based on the morphology of the male moths, which are easier to determine compared to the females (Mutuura and Munroe, 1970). Larvae of *O. scapulalis* were mass-reared on a general diet for insects (Insecta LF, Nihon Nosan Co. Yokohama, Japan) in an environment cabinet maintained at 25°C, 70% relative humidity, and a 15L:9D photoperiod. Male

and female pupae were segregated based on the morphology of the 8th–10th abdominal segments, and maintained under the same conditions as mass rearing. For observation of female calling behavior, females were put individually in clear plastic cups (120 ml) placed upside down on a damp towel. Since preliminary observations indicated that no moths called during the photophase, we observed behaviors of 20 virgin females (2 to 4 days old) during the scotophase at 30-min intervals. In order to observe copulation, 10 pairs each of the same age were put into two transparent containers (25 × 25 × 25 cm), and copulating pairs were counted at 30-min intervals.

Sex Pheromone Extraction. During the calling period in the late scotophase, the abdominal tips, which include the sex pheromone glands, were excised from 2- to 4-day-old virgin female moths and immersed in redistilled hexane (8 μl/tip) for 30 min. Then, the tips were removed and the extract was kept at 0°C until analysis.

Chemicals. Tridecyl acetate (13:OAc, purity 99.7%) used as an internal standard was prepared conventionally by acetylating tridecanol and purified by silica gel chromatography. Tridecanol (97%), tetradecanol (14:OH, 97%), tetradecyl acetate (14:OAc, 97%), and hexadecyl acetate (16:OAc, 96%) were purchased from Wako Pure Chemical Industries (Kyoto, Japan). (*Z*)-9-Tetradecenyl acetate (*Z*9–14:OAc), (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc), (*E*)-11-tetradecenyl acetate (*E*11–14:OAc), and (*Z*)-11-hexadecenyl acetate (*Z*11–16:OAc) were purchased from Sigma Chemical Co. (St. Louis, Missouri). (*E*)-12-Tetradecenyl acetate (*E*12–14:OAc), and (*Z*)-12-tetradecenyl acetate (*Z*12–14:OAc) were purchased from the Research Institute for Plant Protection (Wageningen, The Netherlands). The purity of these compounds was confirmed by gas chromatography to be more than 99.5% with respect to isomers.

Chemical Analysis. Gas chromatography of the extracts was performed on a Shimadzu 17-A GC (Kyoto, Japan) fitted with FID, a splitless injector, and a fused silica capillary column (DB-Wax or DB-1, 60 × 0.25 mm ID, J&W Scientific, Folsom, California). The oven temperature was maintained at 100°C for 2 min, programmed at 5°C/min to 200°C, maintained for 23 min, and again increased at 5°C/min to 240°C to purge any low-volatility compounds remaining on the column. The same temperature program was used for GC-FID-EAD and GC-MS. The carrier gas was nitrogen. GC-FID-EAD was performed on an HP-5890II plus Gas chromatograph equipped with a splitless injector and a 30-m × 0.25-mm capillary column (HP-Innowax, Hewlett-Packard, Wilmington, Delaware). The effluent from the column was split to FID and EAD at a ratio of 1:1. An antenna was excised from a male moth at the base, and a few distal segments were cut off. The antenna was arranged for EAD under a binocular so as to make the sensilla face the airflow from GC effluents. GC-MS analysis was conducted with an HP-5972 mass spectrometer (Hewlett-Packard) in the electron impact (EI) mode. Spectra were recorded at 70 eV with the ion source

at 250°C. The column was the same as that used in GC-FID-EAD. Helium was used as the carrier gas. The chemicals in the extract were identified by analysis of the mass spectra and by comparison of the GC retention times of the components on polar (DB-Wax) and apolar (DB-1) columns with those of authentic standards.

Pheromone Titer Analysis. For pheromone titer determination, the sex pheromone gland of a 3-day-old female moth was cut during the calling period in the late scotophase and immersed for 30 min in 8 μl hexane containing 13:OAc as an internal standard (1 ng/μl). The extract was concentrated under nitrogen, and injected into the gas chromatograph. The quantity of each component was calculated by comparing the peak area with that of 13:OAc.

Electroantennogram (EAG) Analysis. The EAG equipment used in this experiment was based on Ishikawa (1988) with a slight change in stimulator. An order delivery system with an electric solenoid valve was used in place of the hand-controlled 5-ml injector. Hexane solution of a test chemical (10 ng/μl) was applied directly to the inner surface of a disposable glass pipet. Air was puffed (0.5 sec) through the pipet into an airstream that passes continuously over the antenna. Before the bioassay, each antenna was pretested with 10 ng of *E*11–14:OAc to check its responsiveness. Components identified from female sex pheromone glands and several other structurally similar chemicals were tested at a dose of 10 ng to examine the sensitivity spectrum of male antenna.

Wind-Tunnel Bioassay. An indoor wind tunnel (30 cm diam. × 2 m) (Sugie et al., 1991) was used to test the biological activity of the identified chemicals. The wind-tunnel test was performed under the conditions of 25 ± 1°C, 40–50% relative humidity, 0.6 lux (red light), and wind velocity of 0.3 m/sec. For odor delivery, a triangle-shaped piece of filter paper (3 mm base × 8 mm height) was used as the pheromone release substrate. Males (2 to 3 days old) were individually released from a cylindrical screen cage placed 1.5 m downwind from the pheromone source. Four criteria were used to judge the behavioral response: starting flight within 1 min (SF), orientation (OR), reaching the source (RS), and landing on the filter paper (LA). A male moth that came within 20 cm of the source was scored as showing RS behavior. LA behavior was recorded when the male's antennae touched the source or the male landed on the filter paper.

Field Trapping. Field-trapping experiments were done in the Experimental Farm of Tokyo University (Tanashi, Tokyo). Test chemicals in hexane were loaded on rubber septa (Aldrich Chemical Company, Milwaukee, Wisconsin). Sticky traps (Nitto Denko K.K., Osaka, Japan) were hung 50 cm above the ground at 10-m intervals, and insects trapped were checked every three days. Three 2-day-old virgin females were put in a small screen cage with a supply of sucrose solution and employed as bait for comparison. The females were

changed every three days. For each formulation, three replicates were made of the field test. The experiments were designed to examine attractancy of different chemical compositions and the optimum dosage of the binary blend of *E*11- and Z11-14:OAc.

RESULTS

Diel Rhythms of Female Calling and Copulation. Diel rhythms of female calling and copulation are shown in Figure 1. Virgin females started calling at the fourth hour of the scotophase (D4), and the incidence of calling increased towards the end of the scotophase (Figure 1A). All of the 3- and 4-day-old virgin females showed calling behavior at hours D8 and D9. A similar rhythm of calling was observed with 2-day-old females, although their incidence of calling was always lower compared to the 3- and 4-day-old females. This suggests that one to two days may be necessary for the sexual maturation of females. The incidence of copulation basically corresponded with that of the calling (Figure 1B). Copulation was observed from hour D6 to the end of the scotophase.

Sex Pheromone Chemistry. Among the compounds found in the female sex pheromone gland extract, only three compounds (A, B, and C) elicited EAD responses from male antenna (Figure 2). The largest EAD response was elicited by compound C, the major component in the female sex pheromone glands. The amount of compound A was larger than that of compound B, but the EAD response to this compound was marginal. In contrast, the EAD response to compound B was large, although the amount of B was very small. Thereafter, only the EAD-active compounds, A, B, and C, were subjected to analysis as sex pheromone candidates.

By using the same column, the crude extracts were analyzed by GC-MS. EI-MS at 70 eV [*m/z* (%)]: compound A—55 (100), 56 (61), 57 (69), 61 (85), 69 (86), 70 (63), 82 (44), 83 (90), 84 (38), 97 (71), 168 (11), 196 (7). Compound B—54 (31), 55 (82), 61 (18), 67 (67), 68 (82), 69 (50), 81 (53), 82 (100), 95 (43), 96 (55), 110 (19), 166 (3), 194 (14). Compound C—54 (31), 55 (89), 61 (18), 67 (78), 68 (96), 69 (51), 81 (64), 82 (100), 83 (25), 95 (47), 96 (59), 109 (20), 166 (3), 194 (16). All three compounds produced an ion fragment of *m/z* 61 ($CH_3COOH_2^+$), a characteristic of straight-chain acetates (Brown et al., 1988). Compound A had an ion fragment of *m/z* 196 (M^+-60), whereas both B and C had an ion fragment of *m/z* 194 (M^+-60). This indicated that A was a saturated 14-carbon acetate (14:OAc), and B and C were monounsaturated 14-carbon acetates. By the relative intensities of five diagnostic pairs of the predominant ions—[$(m_1/z)/(m_2/z)$] 54/55, 67/68, 81/82, 81/95, and 95/

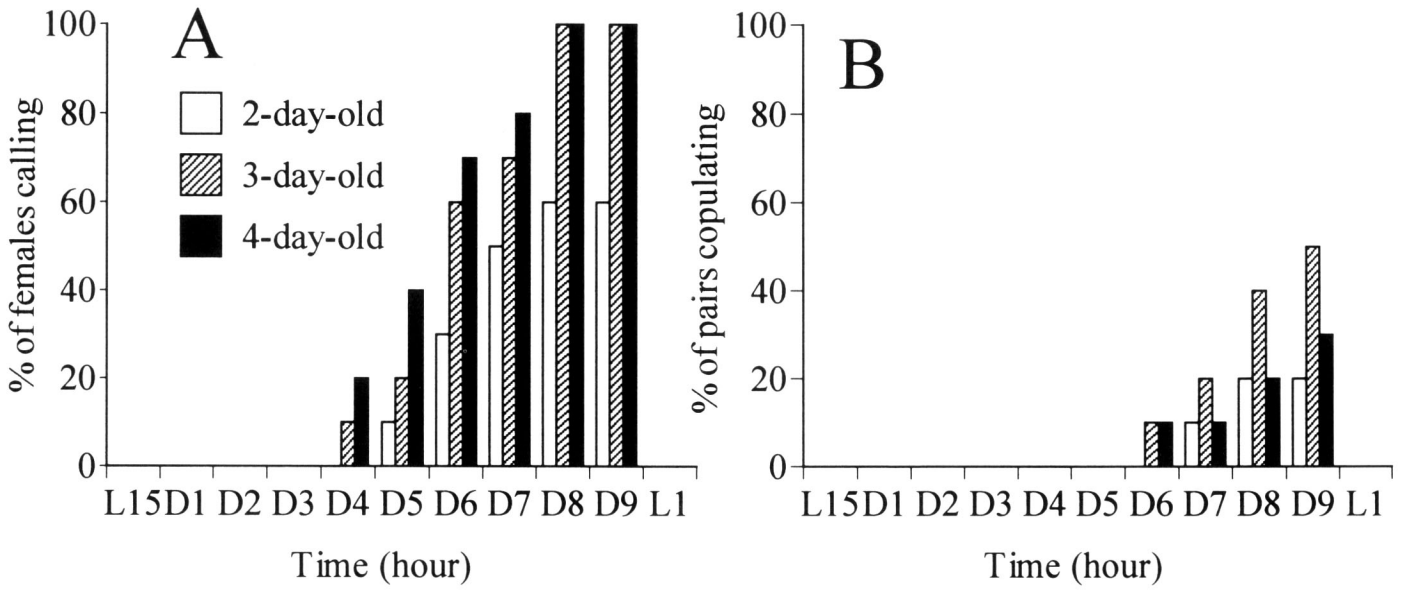


FIG. 1. The calling (A) and copulation (B) rhythms of *Ostrinia scapulalis*. Numbers with L and D represent hours in the light and dark period, respectively.

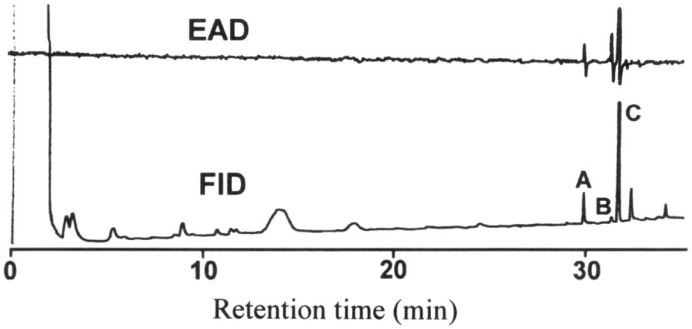


FIG. 2. GC-FID-EAD analysis of crude extract from female sex pheromone glands of *Ostrinia scapulalis*. The three compounds A, B, and C elicited EAD responses.

96—the similarity indices between compounds B and C and each of the double-bond positional isomers of tetradecenyl acetates were calculated according to Horiike et al. (1991). The comparison of these indices indicated that both B and C were delta 11–14:OAc. Finally, the comparison of the GC retention times (on DB-Wax and DB-1, min) of A (30.08, 29.39), B (31.77, 29.10), and C (32.29, 29.24) with those of authentic 14:OAc (30.08, 29.39), *E*11–14:OAc (31.76, 29.09), and *Z*11–14:OAc (32.30, 29.24) enabled us to conclude that A, B, and C were 14:OAc, *E*11–14:OAc, and *Z*11–14:OAc, respectively.

Titers of Sex Pheromone Components. The analysis of sex pheromone gland extracts from ten 3-day-old virgin females showed that the titers (ratio) of 14:OAc, *E*11–14:OAc, and *Z*11–14:OAc were 0.9 ± 0.6 ng (14), 0.2 ± 0.1 ng (3), and 6.6 ± 2.4 ng (100), respectively.

EAG Responses. The EAG responses of male antennae to compounds identified from female sex pheromone glands and their analogs differing in carbon chain length, double-bond position, and functional group are summarized in Figure 3. Male antennae produced strongest responses to *Z*11–14:OAc (2.5 mV) and *E*11–14:OAc (2.2 mV), the major and minor components of the sex pheromone gland extract, respectively. The response to 14:OAc, another component found in the extract, was moderate (ca. 1.1 mV). It should be noted that *Z*9–14:OAc also exhibited very strong EAG responses comparable to *Z*11–14:OAc. The male antennae also responded moderately to *E*12- and *Z*12–14:OAc, the sex pheromone components of the Asian corn borer, *O. furnacalis*. Acetates with 16 carbons (16:OAc, *Z*9–16:OAc, *Z*11–16:OAc) and an alcohol (14:OH) did not elicit significant responses.

Wind-Tunnel Bioassay. The indoor wind tunnel was employed to study the behavioral responses of *O. scapulalis* males to virgin females and three sex

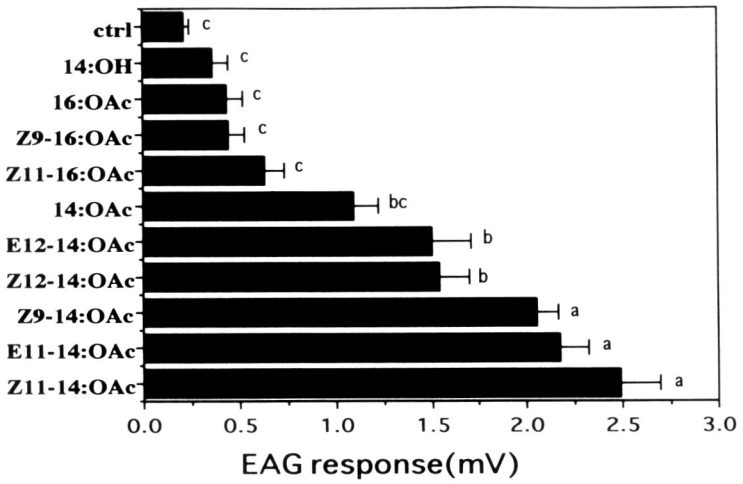


FIG. 3. EAG responses of male *Ostrinia scapulalis* to compounds identified from the sex pheromone gland and their analogs differing in carbon chain length, double-bond position, and functional group at a dose of 10 ng. Bars with the same letters are not significantly different from each other according to Tukey-Kramer's multiple range test at the level of 5%; ctrl = control.

pheromone candidates in different compositions (Table 1). No males showed oriented flight when 14:OAc or *E*11-14:OAc or a mixture of these two compounds was used as the lure. Behaviors up to landing were elicited when *Z*11-14:OAc was included as a component in the lure. Seventy percent of the males tested showed landing behavior toward the binary blend of *Z*11- and *E*11-14:OAc, and the addition of 14:OAc to this binary blend did not enhance the behavioral responses. No significant differences were observed among male responses to the binary blend, to virgin females, and to the sex pheromone gland extract (Table 1). It was demonstrated that the binary blend was sufficient to elicit all male behavioral responses in the wind tunnel.

Field-Trapping Experiments. Among the three components, 14:OAc and *E*11-14:OAc showed no attractancy to males in the field (Table 2). *Z*11-14:OAc alone or with 14:OAc attracted a few males. When *E*11-14:OAc and *Z*11-14:OAc were mixed based on the ratio in the sex pheromone gland, a large number of males were captured. The addition of 14:OAc to this blend decreased the number of males caught (Table 2).

As to the dose of sex pheromone (binary blend), the number of males captured was maximum at a dose of 50 μg/lure (Figure 4). At this dose, significantly more males were captured by synthetic lure than by virgin females. A further increment of the dose sharply decreased the number of males captured.

TABLE 1. BEHAVIORAL RESPONSES OF MALE *Ostrinia scapulalis* TO SYNTHETIC CHEMICALS, CRUDE EXTRACTS, AND VIRGIN FEMALES IN WIND-TUNNEL EXPERIMENTS (N = 20)

Composition of lures (ng)[a]			Behavioral response (%)[b]			
14:OAc	*E*11-14:OAc	Z11-14:OAc	ST	OR	RS	LA
50	0	0	100 a	0 d	0 c	0 c
0	50	0	100 a	0 d	0 c	0 c
0	0	50	100 a	45 c	25 bc	25 bc
41	9	0	100 a	0 d	0 c	0 c
6	0	44	100 a	50 bc	30 bc	30 bc
0	1.5	48.5	100 a	90 a	90 a	70 ab
6	1.3	42.7	100 a	85 ab	85 a	65 ab
Crude extracts (4 FE)			100 a	95 a	80 a	75 a
3 Virgin females			100 a	60 bc	60 ab	60 ab

[a]The amount of synthetic chemicals in each lure was 50 ng in total. The ratios of chemicals in multicomponent lures were based on the ratios found in the female sex pheromone gland.
[b]Numbers in the same column with the same letters are not significantly different at the 5% level by Ryan's multiple comparison test on proportions (Ryan, 1960).

TABLE 2. FIELD CATCHES OF *Ostrinia scapulalis* MALES WITH SYNTHETIC LURES OF DIFFERENT COMPOSITIONS AND VIRGIN FEMALES[a]

Composition of lures (μg)				
14:OAc	*E*11-14:OAc	Z11-14:OAc	Total numbers caught[b]	Males/trap
Control			0 b	0
50	0	0	0 b	0
0	50	0	0 b	0
0	0	50	6 b	2
41	9	0	0 b	0
6	0	44	5 b	1.7
0	1.5	48.5	40 a	13.3
6	1.3	42.7	29 a	9.7
3 Virgin females			6 b	2

[a]The experiments were conducted in Tanashi, Tokyo, August 26–September 7, 1996.
[b]Numbers with the same letters are not significantly different according to Tukey-Kramer's multiple range test at the level of 5%.

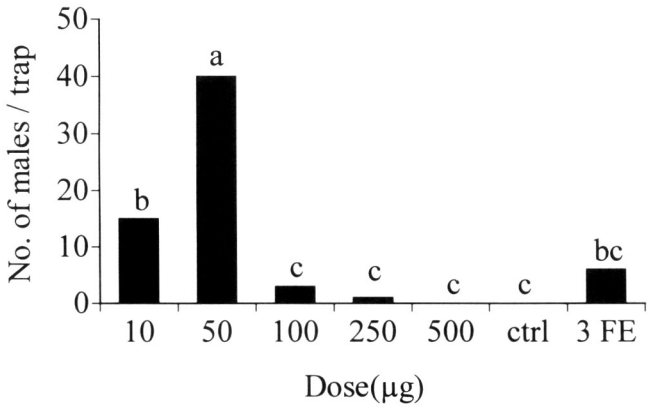


FIG. 4. Field catches of males of *Ostrinia scapulalis* with the binary blends of *Z*11-14:OAc and *E*11-14:OAc (100:3) at different doses. Experiments were conducted in Tanashi, Tokyo, August 26–September 7, 1996. Bars with the same letters are not significantly different according to Tukey-Kramer's multiple range test at the level of 5%.

At a dose of 500 μg/lure, attractancy was reduced to a level not significantly different from the control (Figure 4).

DISCUSSION

In the field trapping tests, results comparable with those of the indoor wind-tunnel tests were obtained except that traps baited with three virgin females captured considerably fewer males than those baited with 50-μg binary lures. All of the females were confirmed to be alive at the end of the experiments. The reason for this result may be that the calling of females was limited to a specified period, whereas the lures released sex pheromone continuously and at a higher rate. No males were captured when 14:OAc was used alone, moreover, it did not enhance the attraction when mixed with the other two components. No specific response to 14:OAc was found in the wind-tunnel tests. At present, the role of 14:OAc in the pheromone communication system of *O. scapulalis* is not clear.

Based on the laboratory analysis and field trapping experiments, the sex pheromone of *O. scapulalis* was identified as a combination of *Z*11-14:OAc and *E*11-14:OAc at a ratio of 100:3. This composition is similar to the sex pheromone of the Z-type European corn borer, *O. nubilalis*, in both components

and ratio. It is an interesting phenomenon that *O. scapulalis*, distributed in Asia, and *O. nubilalis*, distributed mainly in Europe and in United States, utilize almost the same signal system. Studies on the independent evolution of the two species in separated geographical areas might reveal useful information, which may be extended to other species.

According to the classification of the genus *Ostrinia* by Mutuura and Munroe (1970), both *O. scapulalis* and *O. nubilalis* were classified into the same trilobed uncus species group. Within the species group, *O. nubilalis* belongs to the "small tibia" subgroup together with *O. furnacalis*, whereas *O. scapulalis* belongs to the "large tibia" subgroupd together with *O. zealis* and *O. zaguliaevi*. The small tibia subgroup was considered to be more primitive compared with the large tibia subgroup (Mutuura and Munroe, 1970). The isolated geographical condition of Japan offers good opportunities to study the evolution of the seven *Ostrinia* species. In particular, it is expected that analyses of their communication systems will contribute information to test and extend the taxonomic interpretation.

From the electrophysiological data obtained in the present study, some implications were obtained regarding the phylogeny of the *Ostrinia* species. Antennae of male *O. scapulalis* produced very strong responses to *Z*9–14:OAc, although this compound was not detected by GC-FID-EAD analysis. Antennae of male *O. nubilalis* have also been shown to respond to *Z*9–14:OAc (Hansson et al., 1987), and this compound is known as a very strong inhibitor of the pheromone in this species (Glover et al., 1989). Why is the physiological response of the males not strictly tuned only to the female sex pheromone components? In the parallel study of *O. zaguliaevi* and *O. zealis* in our laboratory, *Z*9–14:OAc was identified as a sex pheromone component in addition to *Z*11- and *E*11–14:OAc (Huang, unpublished data). This finding indicates that the sex pheromone of the genus *Ostrinia* evolved from a two-component to a three-component system, and the third component (*Z*9–14:OAc) is working as a congeneric inhibitor to the species with a two-component system.

It is well known that *O. nubilalis* has polymorphism in the female sex pheromone (Cardé et al., 1978; Kochansky et al., 1975). In the present study, the sex pheromone of *O. scapulalis* from a geographic population (Matsudo) was found to be similar to that of the Z-type European corn borer. However, we have found that sex pheromone polymorphism similar to *O. nubilalis* exists in *O. scapulalis*. The details on this phenomenon and *E/Z* ratio of the pheromone components will be reported elsewhere.

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PREMATING ISOLATION IS DETERMINED BY LARVAL REARING SUBSTRATES IN CACTOPHILIC *Drosophila mojavensis*. III. EPICUTICULAR HYDROCARBON VARIATION IS DETERMINED BY USE OF DIFFERENT HOST PLANTS IN *Drosophila mojavensis* AND *Drosophila arizonae*

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Abstract—Adult epicuticular hydrocarbon profiles of male and female *Drosophila mojavensis* have been implicated as determinants of mate choice leading to premating isolation between geographically isolated populations. Hydrocarbon profiles of a Baja California and a mainland Sonora population of *Drosophila mojavensis*, a *yellow* body mutant strain of *D. mojavensis*, and a population of *D. arizonae* were compared among flies that had been reared on two cactus substrates and a synthetic laboratory growth medium in order to assess the degree to which natural rearing substrates influence adult hydrocarbon composition. Twenty epicuticular hydrocarbon components, ranging from C_{29} to C_{41}, were recovered by gas chromatography that represented major classes of alkanes, alkenes, and alkadienes. We found differences in relative amounts of epicuticular hydrocarbons among Baja and mainland *D. mojavensis*, and the *yellow* body mutants. There were few differences between *D. mojavensis* and *D. arizonae*. The effects of rearing substrates were remarkable: most of the differences were due to the effects of lab food vs. cactus, but there were significant rearing substrate effects due to differences in the two cacti used. Eleven hydrocarbon components differed in abundance between males and females or showed significant sex × rearing substrate interactions from ANOVA. The effects of rearing substrates on epicuticular hydrocarbon composition in *D. mojavensis* are concordant with changes in the intensity of premating isolation between populations, implicating host ecology as a major determinant in patterns of mate choice in this species.

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Key Words—Incipient speciation, sexual selection, mating behavior, cuticular hydrocarbons, cactus, Sonoran Desert, *Drosophila*.

INTRODUCTION

The origins of reproductive isolation in organisms that share a common fertilization system remain an unresolved issue in the study of speciation. In outcrossing species, variation in mating systems has been hypothesized to be a potent factor in the reduction of gene flow among demes, particularly when success in mating depends upon the interaction of multiple cues exchanged between the sexes. These may include chemical, behavioral, tactile, and acoustic signals exchanged prior to successful fertilization. Paterson (1978) argued that the evolution of a specific mate recognition system, composed of multiple "coadapted stages," is essential to intersexual signaling and should be maintained by strong stabilizing selection. This, however, was described as a species-specific recognition system (Carson, 1995) and describes Paterson's definition of a species (Paterson, 1985).

Variation in mate recognition systems among isolated demes within species may be promoted by local ecological conditions, leading to the evolution of new equilibrium signaling systems (Butlin, 1995). The effects of local conditions leading to divergence in mating systems may lead to premating isolation should such demes ever come into secondary contact. These causes for divergence are essential to the understanding of species formation.

Carson (1987, 1995) has pointed out that the evolution of common mate recognition systems via coadaptation of male–female signaling systems and sexual selection may be a major cause of speciation in animals. These interactions between potential mates within demes must be the driving force of sexual selection with only secondary consequences for reproductive isolation, consistent with Paterson's recognition model (Paterson, 1985). However, most analyses of premating isolation among presumed species "in statu nascendi" (Dobzhansky and Spassky, 1959; Dobzhansky and Pavlovsky, 1967) require assessment of the kinds of changes in mate choice between groups (Stalker, 1942; Malagolowkin-Cohen et al., 1965; Wasserman and Koepfer, 1977; Zouros and D'Entremont, 1980; Ehrman and Wasserman, 1987; Wu et al., 1995). There is clearly a lack of data addressing the connections between intrademic and interdemic facets of sexual selection (but see Jamart et al., 1993) and patterns of mate choice, i.e., are the mechanisms driving mating success within demes, sensu Carson, related to patterns of premating isolation between demes?

Before we can approach the relationships between intra- and interdemic mating behavior, cases of consistent premating isolation between populations

judged to be incipient species must be identified. Within-deme mating success can then be directly compared to mating success between individuals of different demes. As a first step towards this goal, premating isolation in *Drosophila mojavensis* has been intensively analyzed (Zouros and D'Entremont, 1974, 1980; Wasserman and Koepfer, 1977; Markow, 1981, 1991; Markow et al., 1983; Koepfer, 1987a,b; Etges, 1992; Brazner and Etges, 1993). The present study concerns variation in premating isolation and the epicuticular hydrocarbon profiles as presumed contact pheromones of adult flies (Markow and Toolson, 1990; Toolson et al., 1990).

Allopatric populations of *D. mojavensis* have been described as incipient species because of low, but significant levels of premating isolation between Baja California and mainland Sonora, Mexico populations. Behavioral isolation has been characterized as "one-way" premating isolation because mainland females tend to discriminate against Baja males in multiple choice tests, but Baja California females tend not to discriminate between Baja and mainland males. Courtship attempts are somewhat reduced in Baja males, but the major decrease in copulation frequency is due to a lack of mainland female receptivity once courtship has been initiated (Krebs and Markow, 1989). So far, little is known about the intensity of sexual selection within demes of *D. mojavensis*.

Intersexual signaling includes tactile stimulation by males and exchange of chemical cues through sensory reaction to the epicuticular hydrocarbons (Markow and Toolson, 1990; Toolson et al., 1990). Cuticular hydrocarbon variation has been implicated as a critical factor influencing mate choice in *D. melanogaster* group species (Antony and Jallon, 1982; Scott and Richmond, 1988; Ferveur, 1991; Coyne et al., 1994), *D. pseudoobscura* and *D. persimilis* (Noor and Coyne, 1996), *D. virilis* (Bartelt et al., 1986; Oguma et al., 1992), *D. pallidosa* (Nemoto et al., 1994), several Hawaiian *Drosophila* (Thompkins et al., 1993), house flies, *Musca domestica* (Reed et al., 1996), and tetranychid mites, *Tetranychus urticae* and *Oligonychus pratensis* (Margolies and Collins, 1994).

Because premating isolation in *D. mojavensis* is influenced by larval rearing substrates (Etges, 1992; Brazner and Etges, 1993) variation in epicuticular hydrocarbon profiles of flies reared on two natural hosts was compared with hydrocarbons from flies reared on laboratory medium. In this study, we tested the hypothesis that larval rearing substrates determine the quantity and variety of cuticular hydrocarbons in a mainland and Baja population of *D. mojavensis*, *yellow* body *D. mojavensis* (Etges, 1993) which are characterized by reduced male mating success with nonmutant females (Etges, 1997), and *D. arizonae*, a sibling species that sometimes shares host plants with *D. mojavensis* in nature (Ruiz and Heed, 1988).

METHODS AND MATERIALS

The two wild populations of *D. mojavensis* used in this study were collected in 1994. Flies from Mission San Borja, Baja California Norte, were descended from 43 wild-caught adults, and mainland flies originated from 44 wild-caught adults and 181 flies that emerged from an organ pipe cactus, *Stenocereus thurberi*, rot from Cerro Colorado, Sonora. The *D. mojavensis yellow* body (*y*) strain was derived from an outbred population from Punta Onah, Sonora, in which the X-linked mutation arose spontaneously in the lab (Etges, 1993). A stock of the sibling species, *D. arizonae*, was derived from 534 adults that emerged from a *S. standleyi* cactus rot collected in Tomatlan, Jalisco in 1982 by W. B. Heed.

All flies were reared on banana–malt–yeast–agar food (Brazner and Etges, 1993) in 8-dr shell vials at room temperature (ca. 22°C) until the controlled growth experiment began. Each stock was then reared on lab food and fermenting pitaya agria, *S. gummosus*, and organ pipe cactus tissues. The four stocks of flies were cultured in an incubator programmed for a 14L:10D photoperiod and a temperature cycle of 27°C during the day and 17°C at night. Laboratory-food-reared adults were produced by allowing several hundred adults to oviposit directly onto lab food for several days in ½-pint bottles. Cactus-reared flies were cultured using standard methods (Etges, 1989); eggs were collected from several hundred adults and washed in deionized water, 70% ethanol, and again in sterile deionized water. Eggs were counted out in groups of 200, transferred to a 1-cm^2 piece of filter paper, and placed on fermenting cactus. Cactus cultures were established in plugged ½-pint bottles with 75 g of aquarium gravel at the bottom covered with a 5.5-cm-diameter piece of filter paper. Bottles were then autoclaved, and after 60 g of either agria or organ pipe tissue were in place, autoclaved again for 10 min. After cooling to room temperature, each culture was inoculated with 0.1 cc of a pectolytic bacterium, *Erwinia cacticida* (Alcorn et al., 1991), and 0 .1 cc of a mixture of the following seven species of yeasts common in natural agria and organ pipe rots (Starmer, 1982; Fogleman and Starmer, 1985); *Pichia cactophila, P. mexicana, P. amethionina* var. *amethionina*, *Cryptococcus cereanus*, *Candida valida*, *C. ingens*, and *C. sonorensis*. Eight replicate cultures of each food type were started for each of the four stocks of flies.

All flies were collected every day from each culture bottle, separated by sex, and aged in the incubator described above on lab food in vials for at least 12 days because cuticular hydrocarbon profiles change until adults are 8–10 days old (Toolson et al., 1990). Epicuticular hydrocarbons were extracted from counted groups of aged females and males (usually 20–30) in Biosil mini-columns. Each column consisted of a Pasteur pipet that contained packed glass wool and Biosil (silica gel, Sigma S-4133) washed several times with HPLC-

grade hexane. Flies were then added, washed in 8 ml of hexane, and the hydrocarbons were collected in hexane-rinsed vials. After the hexane was evaporated with nitrogen, each sample was sealed and stored at room temperature. Each hydrocarbon sample was redissolved in hexane (2.5 μl/fly) containing 382 ng of docosane (C_{22}) per microliter as an internal standard. One microliter of each sample was analyzed by capillary gas–liquid chromatography using a Shimadzu G14 fitted with a 30-m DB-1 fused-silica column. Injector and detector temperatures were set at 345°C with the injector port in split mode. Running temperatures started at 200°C and increased to 345° at 10°/min with a hold at 345°C for 7 min.

Peak identity was confirmed and equivalent chain lengths (ECLs) were calculated for each hydrocarbon component by coinjection of sample hydrocarbons with paraffin wax consisting of C_{19} to C_{38} straight-chain alkanes and docosane (C_{22}), hexatriacontane (C_{36}), and tetracontane (C_{40}) standards. Retention times of alkanes used to calculate ECLs were linearly related to their chainlengths (slope = 0.676, $r = 0.997$, 14 df, $P < 0.001$). Equivalent chain lengths for all hydrocarbon components were calculated by linear interpolation between alkane peaks of known chainlengths, i.e.,

$$ECL_{\text{sample}} = HC_n + (RT_{\text{sample}} - RT_n)/(RT_{n+1} - RT_n)$$

where HC_n is an alkane of known hydrocarbon length n and RT is the retention time of each component. Alkane, alkene, and alkadiene peaks were identified by sequential elution of a sample using silver nitrate-impregnated Biosil in a minicolumn with successive washes of hexane, 2% diethyl ether in hexane, and 25% ether in hexane (Jackson et al., 1974; Toolson et al., 1990). At least two to four replicates of each group of flies grown on each rearing substrate were analyzed.

Hydrocarbon amounts were estimated by analysis of peak integrations using EZCHROM software (ver. 2.1) provided by Shimadzu. All data were expressed as nanograms per fly of cuticular hydrocarbons and were analyzed with ANOVA using PROC GLM in SAS (SAS Institute, 1985) with population, rearing substrate, and sex as main effects and all interactions between main effects. All significant main effects were further assessed by Duncan's multiple-range tests.

RESULTS

The 20 most abundant epicuticular hydrocarbons from *D. mojavensis* and *D. arizonae* ranged from C_{29} to C_{39}, but hydrocarbons with chain lengths greater than C_{37} were usually variable in abundance and rare, including C_{41} chain lengths (Table 1, Figure 1). The most abundant hydrocarbons consisted of molecules with chain lengths of C_{29}, C_{31}, C_{33}, C_{35}, and C_{37}, with the C_{35} group accounting

TABLE 1. EPICUTICULAR HYDROCARBONS OF *D. mojavensis* AND *D. arizonae* IN THIS STUDY[a]

Number of carbons	Type	Equivalent chain length
29	Branched alkane	28.65
31	Branched alkane	30.65
	n-alkene	30.78
33	Branched alkane	32.47
	Alkadienes	32.63–32.70
	n-alkene	32.40–32.79
34	Alkadienes	33.50
	n-alkene	33.60
35	Alkadienes	34.59–34.66
	Branched alkene	34.45
	n-alkene	34.73
36	Branched alkene	35.60
37	Alkadiene	36.50
	Branched alkene	36.40
	n-alkene	36.60
38	Alkenes	37.40
39	Alkenes	38.50

[a]The types of alkanes, alkenes, and alkadienes are described in Toolson et al. (1990).

for 30–50% of total epicuticular hydrocarbons. Identification of alkanes, alkenes, and alkadienes and calculated equivalent chain lengths confirmed the molecular compositions and relative retention times of these hydrocarbons given in Toolson et al. (1990) with few exceptions. Because of the strong influence of rearing substrates on variation in epicuticular hydrocarbons, abundance of C_{33}, C_{35}, and C_{37} branched alkenes could not always be reliably scored, so we have not included them in this analysis. They account for a small proportion ($< 10\%$) of total adult cuticular hydrocarbons in lab food reared flies (Toolson et al., 1990), and were occasionally absent in cactus reared flies.

The predominant classes of hydrocarbons were C_{35} positional isomers of *n*-pentatriacontadiene (Toolson et al., 1990). We recovered two different peaks of these C_{35} alkadienes with equivalent chainlengths of $C_{34.59}$ and $C_{34.66}$, depending on the group of *D. mojavensis* being analyzed. There was a striking correlation between the presence of $C_{34.59}$ and one of the two C_{33} alkadiene peaks: if $C_{34.59}$ alkadiene was abundant, so was the $C_{32.63}$ alkadiene peak (Figures 2 and 3). Such correlations in the abundance of hydrocarbon components within different groups of *D. mojavensis* strongly suggest a simple chain-length-

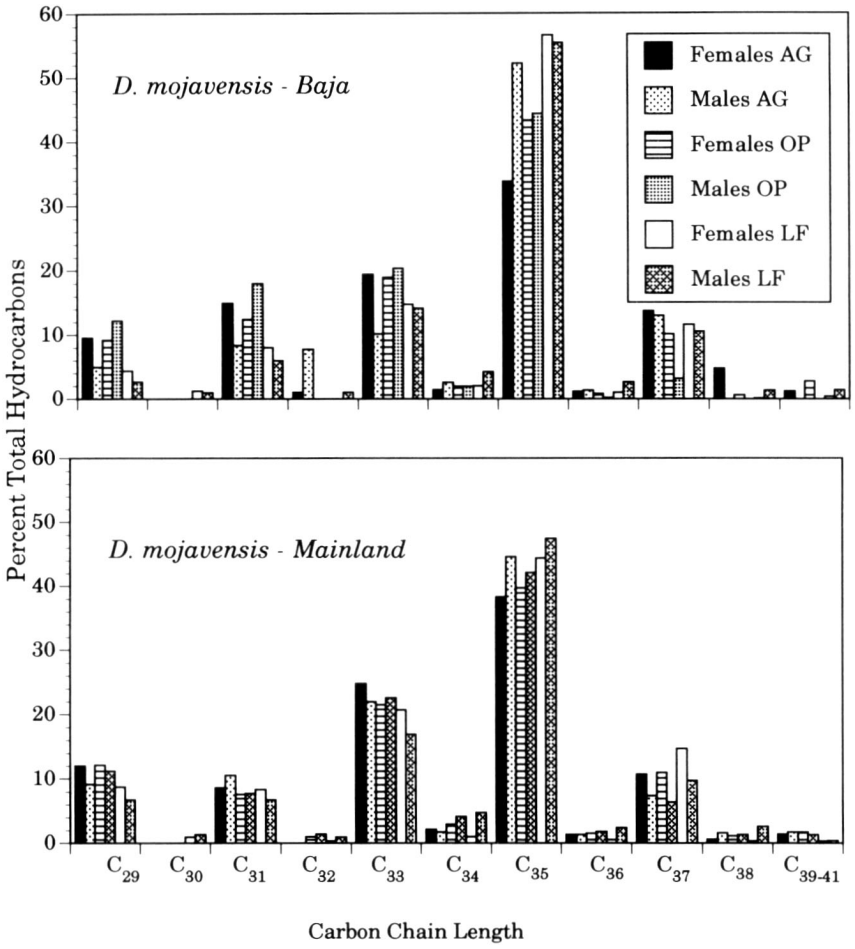


FIG. 1. The percent of total hydrocarbons (per fly) of each of the epicuticular hydrocarbon components observed in a population of *D. mojavensis* from Baja California, mainland Sonora, the *yellow* body (*y*) mutant strain, and a population of *D. arizonae*. The bars represent the average percent of each chain length for two to four replicates separated by sex and rearing substrate. The three rearing substrates are agria cactus (AG), organ pipe cactus (OP), and laboratory food (LF).

ening biosynthetic pathway for production of the variety of epicuticular hydrocarbons in this species (Toolson et al., 1990).

Variation Due to Rearing Substrates. Rearing *D. mojavensis* and *D. arizonae* on fermenting agria or organ pipe cactus tissues and laboratory food

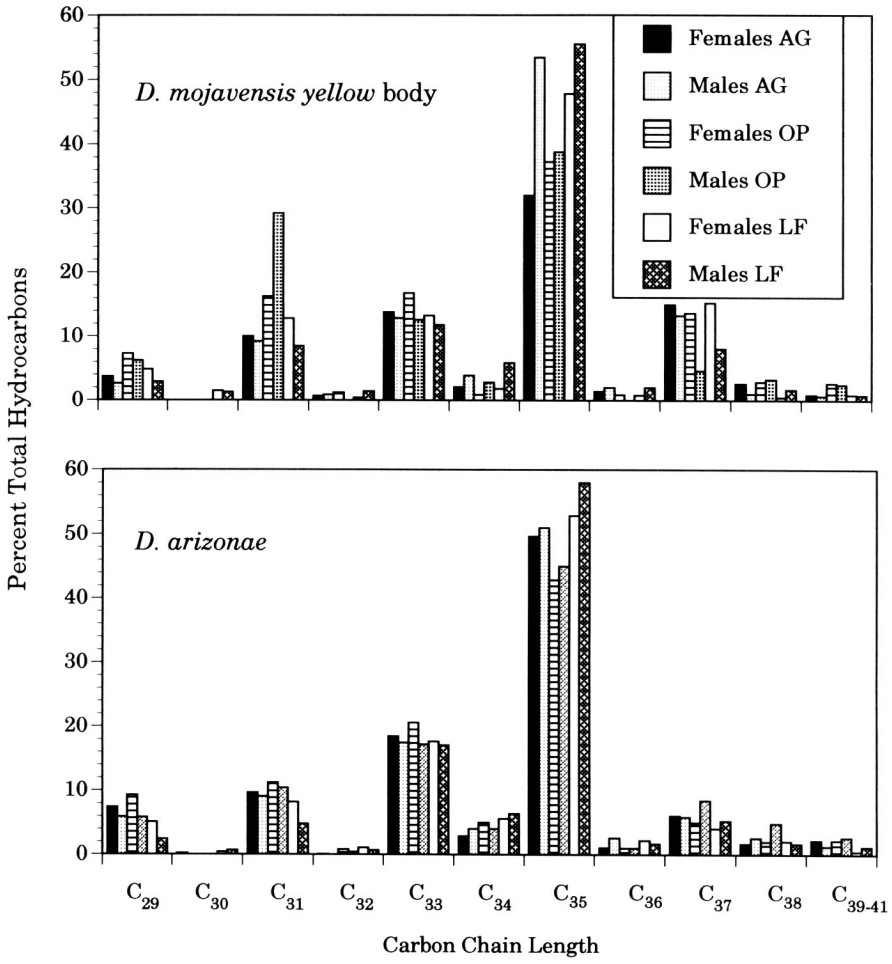


FIG. 1. Continued.

caused both qualitative and quantitative differences in the amounts of epicuticular hydrocarbons (Figures 1 and 2). These differences were apparent even though the flies had been fed lab food as young adults. Amounts of C_{29} branched alkanes (C_{29br}, ECL = 28.65) were significantly greater in flies reared on organ pipe than agria or lab food (Figure 4; $F = 15.64$, $P < 0.0001$), and there was a significant food × sex interaction ($F = 6.19$, $P = 0.006$) due in part to the large increase in C_{29br} in females reared on organ pipe (Figure 5). The small amounts of C_{30} *n*-alkane were only observed in flies reared on lab food (Figure

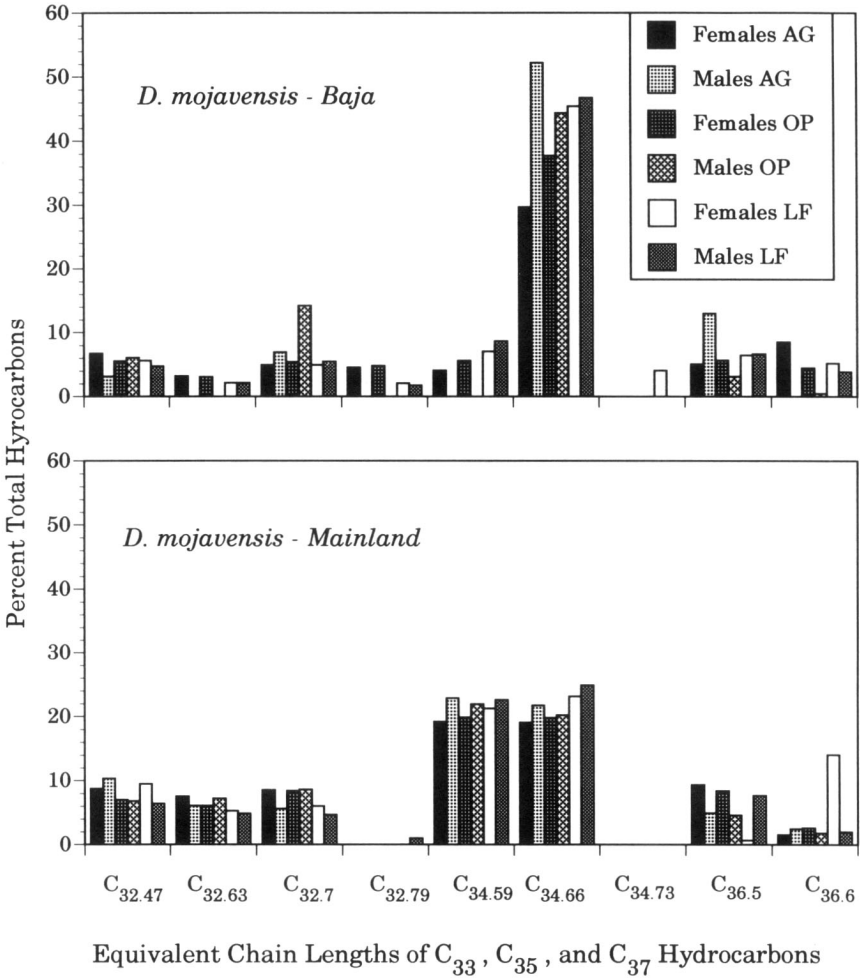


FIG. 2. The percent of total hydrocarbons (per fly) of each of the C_{33}, C_{35}, and C_{37} epicuticular hydrocarbon components observed in the four groups of *D. mojavensis* and *D. arizonae* in this study designated by equivalent chain lengths. The labels are the same as in Figure 1.

1), thus some hydrocarbons in *D. mojavensis* and *D. arizonae* are manufactured only under laboratory conditions. The most abundant alkane, C_{31br} (ECL = 30.65), was generally more abundant in flies reared on organ pipe tissues than either agria or lab food (Figure 4; $F = 8.25$, $P < 0.002$). There were no differences in amounts of any of the C_{33} components due to rearing substrates.

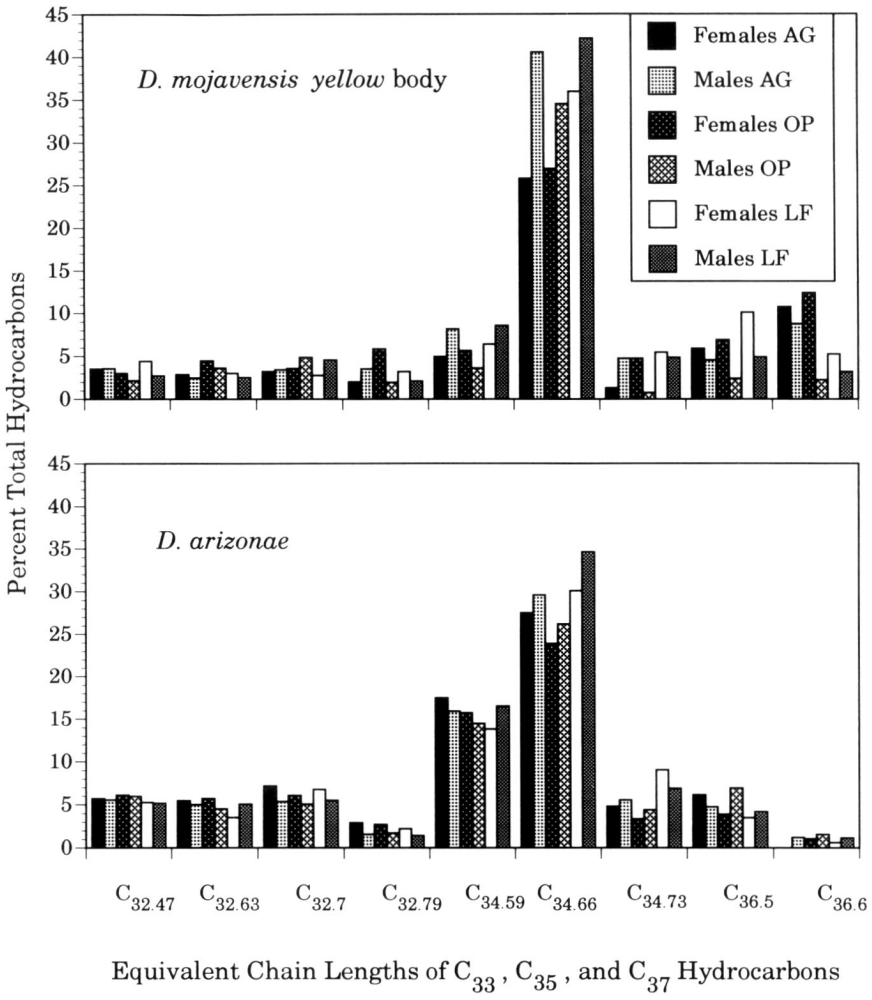


FIG. 2. Continued.

Both C_{34} alkenes and alkadienes were present in significantly greater amounts in lab food-reared flies than those reared on either cactus substrate ($F = 4.43$, $P = 0.022$ and $F = 10.55$, $P < 0.001$, respectively, Figure 5). All three major C_{35} components, both $C_{34.59}$ and $C_{34.66}$ dienes and $C_{34.73}$ *n*-alkene, were also significantly more abundant in lab food-reared flies (Figures 2, and 5; $F = 10.21$, $P = 0.0005$; $F = 5.64$, $P < 0.01$; and $F = 16.73$, $P < 0.0001$,

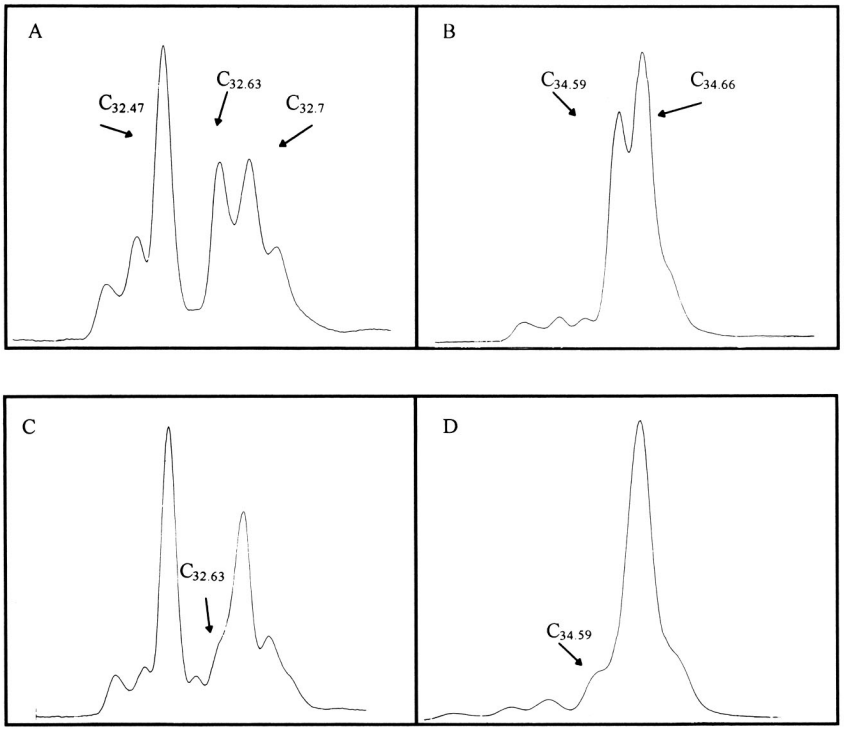


FIG. 3. Gas chromatograms of the C_{33} and C_{35} groups of epicuticular hydrocarbons showing the difference between mainland (A) and Baja (C) C_{33} profiles, and the difference between mainland (B) and Baja (D) C_{35} profiles. The equivalent chainlengths of each component designated in Table 1 are shown.

respectively). We routinely recovered a single C_{36} alkene peak, which Toolson et al. (1990) identified as a straight-chain *n*-alkene, $C_{36:1}$. This hydrocarbon was also in greater abundance in flies reared on lab food ($F = 10.42$, $P = 0.0004$). Amounts of the $C_{36.5}$ alkadiene component did not differ between food types but amounts of $C_{36.6}$ *n*-alkene were present in higher quantities in lab food-reared flies ($F = 16.82$, $P < 0.0001$) than agria- or organ pipe-reared flies. There were no significant food effects on any of the hydrocarbons longer than C_{37}. Total epicuticular hydrocarbon amounts were greater in lab food-reared flies, 1443.6 ng/fly, than either organ pipe- or agria-reared flies, 1061.9 and 1013.1 ng/fly, respectively ($F = 6.13$, $P = 0.0064$).

In order to assess the degree to which variation in host use in nature may cause differences in epicuticular hydrocarbon profiles, these data were reana-

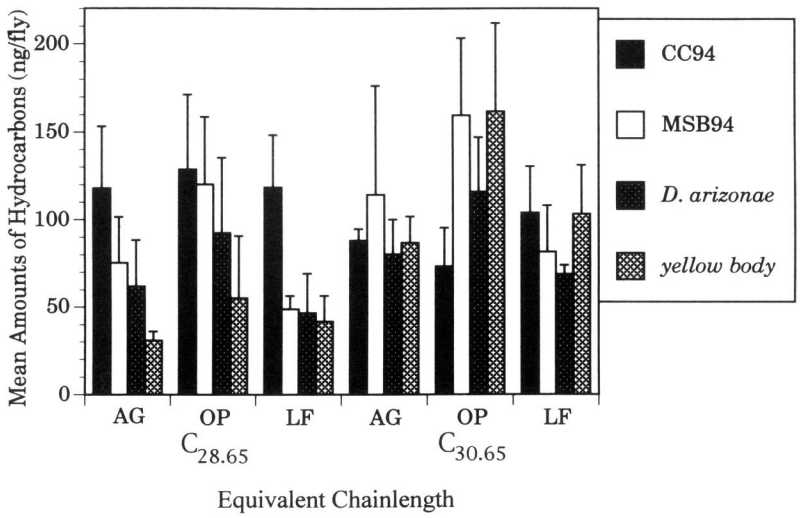


FIG. 4. Mean (+ 1 SD) amounts of the two major alkanes of the four groups of *D. mojavensis* and *D. arizonae* in this study showing the effects of rearing substrates on their relative abundances. Labels for the three rearing substrates are explained in Figure 1.

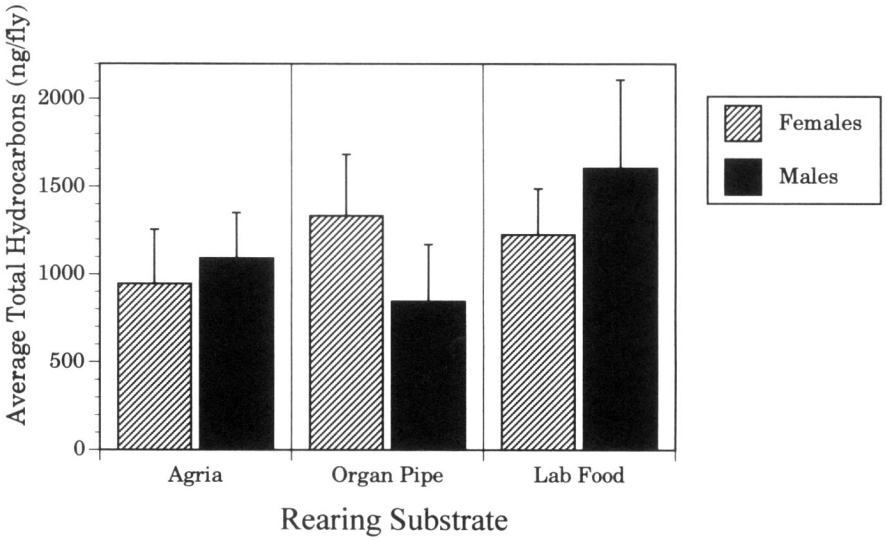


FIG. 5. The influence of rearing substrates (both cacti vs. lab food) on differences between males and females in average total amounts (+ 1 SD) of epicuticular hydrocarbons in this study.

lyzed after eliminating the lab food-reared flies. This way, differences between the effects of agria and organ pipe substrates on adult hydrocarbons were directly assessed. Amounts of C_{29} branched alkanes, C_{31br} alkanes, and C_{34} alkenes were greater in organ pipe-reared flies than those reared on agria ($F = 15.68$, $P = 0.001$; $F = 7.83$, $P = 0.012$; and $F = 5.85$, $P = 0.027$, respectively). Thus, changes in the composition of epicuticular hydrocarbons in adult *D. mojavensis* due to different rearing substrates can be caused by their host cacti.

Variation Among Populations and Species. Although most of the hydrocarbon components differed in abundance among Baja and mainland *D. mojavensis*, *y* body *D. mojavensis*, and *D. arizonae* (Figures 1 and 2), the differences between mainland and Baja populations are of most interest due to the presumed role of epicuticular hydrocarbons as contact pheromones (Markow and Toolson, 1990). Levels of C_{29} and C_{33} branched alkanes and $C_{32.63}$ and $C_{34.59}$ dienes (Figure 3) were greater in mainland adults than Baja adults ($F = 11.48$, $P < 0.005$; $F = 6.79$, $P = 0.021$; $F = 55.0$, $P < 0.0001$; and $F = 91.26$, $P < 0.0001$, respectively), and C_{31br} alkanes, C_{33} *n*-alkenes, C_{34} and $C_{34.66}$ dienes, and $C_{34.73}$ *n*-alkenes were greater in Baja adults than mainland adults ($F = 5.60$, $P < 0.05$; $F = 20.12$, $P = 0.0005$; $F = 22.34$, $P = 0.0003$; $F = 26.45$, $P < 0.0001$; and $F = 18.68$, $P < 0.001$, respectively). Total hydrocarbon amounts were not different between these two populations.

Since the *y* body mutation affects the formation of black melanin and its placement in the cuticle in *D. melanogaster* (Walter et al., 1991), we hypothesized that there would be little difference in amounts of epicuticular hydrocarbons between *y D. mojavensis* and normally pigmented flies from the mainland. The *y* body adults produced the smaller quantities of C_{29} branched alkanes than any of the other groups (Figure 1): 121.5 ng/fly, *D. mojavensis* mainland; >86.8 ng/fly, *D. mojavensis* Baja; >66.8 ng/fly, *D. arizonae*; >42.4 ng/fly *y D. mojavensis* (Duncan's multiple-range test, $P < 0.05$). Amounts of C_{31br} alkanes were intermediate when compared to the other groups, but there was a significant population × food interaction ($F = 2.81$, $P < 0.03$; Figure 4). Thus, amounts of C_{31br} alkanes differed among groups, but these differences were not predictable across rearing substrates. Of the other most abundant hydrocarbon components, *y* adults had comparatively less C_{33} branched alkanes, $C_{32.63}$ and $C_{32.7}$ dienes, and C_{34} alkenes, but larger amounts of $C_{32.79}$ *n*-alkenes than the other populations (Duncan's multiple-range tests, all $P < 0.05$). Average total hydrocarbon amounts of *y* adults were not significantly lower than those of the other groups (941 ng/fly vs an average of 1243 ng/fly of the other three groups).

Overall, differences in hydrocarbon components between *D. mojavensis* and *D. arizonae* were unremarkable (Figures 1 and 2). In most cases, when there was significant variation among groups, *D. arizonae* were intermediate in amounts of cuticular hydrocarbons, particularly for the most abundant classes.

D. arizonae had the highest amounts of C_{34} and $C_{34.73}$ *n*-alkenes, and the lowest amounts of $C_{36.6}$ *n*-alkenes than the other groups (Duncan's multiple range tests, all $P < 0.05$). For hydrocarbons greater in size than C_{37}, *D. arizonae* possessed greater amounts of C_{38} and C_{40} chain lengths than the other groups.

Hydrocarbon Variation Among Males and Females. The potential utility of any one or a suite of epicuticular hydrocarbons to serve as contact pheromones requires that these compounds alter intersexual recognition. If epicuticular hydrocarbon variation caused by rearing substrates is involved with decreased premating isolation between Baja and mainland adults (Etges, 1992; Brazner and Etges, 1993), then the cactus-reared flies should show alterations in hydrocarbon composition that break down behavioral isolation relative to lab food-reared flies. Thus, any sex × food interactions in the ANOVAs are of considerable interest. Eleven of the 20 major hydrocarbon components differed between sexes and/or showed sexual dimorphism that changed with rearing substrate (Table 2). Based only upon body size, total epicuticular hydrocarbons should be greater in females than males because female *D. mojavensis* are larger (Etges, 1990) and thus have a greater surface area. However, total hydrocarbons amounts did not differ among males and females (Table 2), so differences in amount of component hydrocarbons were likely due to sex and not body size. Adult males

TABLE 2. DIFFERENCES BETWEEN MALES AND FEMALES FOR AMOUNTS OF EPICUTICULAR HYDROCARBONS IN THIS STUDY[a]

	Amount (ng/fly)		F^b	
Hydrocarbon	Male (mean ± SD)	Female (mean ± SD)	Sex	Sex × rearing
C_{29Br} alkane	63.48 ± 31.56	93.81 ± 48.87	28.66***	6.19**
C_{C31Br} alkane	96.90 ± 36.95	113.56 ± 47.00	4.77*	1.20 ns
$C_{32.63}$ diene	36.88 ± 33.57	53.55 ± 28.86	5.47*	8.15**
$C_{32.79}$ *n*-alkene	15.90 ± 16.67	28.93 ± 29.73	7.79**	7.51**
C_{34} diene	14.20 ± 17.28	6.78 ± 8.28	11.99**	6.17**
C_{34} *n*-alkene	34.47 ± 32.70	23.16 ± 26.64	3.09 ns	4.17*
$C_{34.59}$ diene	129.95 ± 120.34	149.63 ± 110.56	0.02 ns	6.60**
$C_{34.66}$ diene	435.62 ± 242.75	347.77 ± 161.06	3.04 ns	3.54*
C_{36Br} alkene	19.07 ± 15.55	12.82 ± 8.25	8.30**	13.69***
$C_{36.5}$ diene	65.76 ± 35.51	66.50 ± 54.03	0.06 ns	8.16**
$C_{36.6}$ *n*-alkene	66.85 ± 62.30	27.97 ± 30.36	58.64***	6.75**
Total hydrocarbons	1157.20 ± 488.49	1172.79 ± 427.06	0.00 ns	5.56**

[a] All means are based on 26 observations.
[b] *F* statistics for the main effect of sex and significant sex × rearing substrate interaction terms from ANOVAs are given. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: not significant.

that were reared on agria tended to have more total epicuticular hydrocarbons then females, but this was reversed when the flies were reared on organ pipe (Figure 6). Total hydrocarbons were greater in lab food-reared flies (see above) with the smaller males having greater amounts of total hydrocarbons than females.

Females had significantly greater amounts of both of the major alkanes, C_{29Br} and C_{31Br}, than males and the pattern of expression was similar across rearing substrates (Figure 5). Females expressed greater amounts of $C_{32.63}$ dienes and $C_{32.79}$ *n*-alkenes than males consistent across cactus hosts, but reversed on lab food, leading to a significant sex × rearing substrate interaction (Table 2). A similar pattern was apparent for both C_{34} components and $C_{34.59}$ and $C_{36.5}$ dienes, but not for $C_{34.66}$ dienes. Although overall amounts of $C_{34.66}$ were not different in males and females, males possessed more of this component than females when reared on agria and lab food, but not on organ pipe (Figure 5). Relative amounts of $C_{36.6}$ *n*-alkene were also dependent on substrate type. Thus, for many of the epicuticular hydrocarbons that differed in amounts between adult male and female *D. mojavensis*, lab food caused a reversal in the relative amounts expressed by males and females compared to cactus-reared flies.

DISCUSSION

Epicuticular hydrocarbon profiles of adult *D. mojavensis* and *D. arizonae* are dependent on larval rearing substrates. Thus, the presumed chemical cues involved with mate recognition in these species are sensitive to the type of larval substrates used in nature that could cause local shifts in mate signaling (Butlin, 1995). The relevance of these results to patterns of reproductive isolation between *D. mojavensis* and *D. arizonae*, while not the focus of this study, are tied to the hypothesized cause for behavioral premating isolation between geographically isolated populations of *D. mojavensis*. The presence of *D. arizonae* on the mainland is thought to be responsible for causing reproductive character displacement in mainland *D. mojavensis* populations (Zouros and D'Entremont, 1974; Wasserman and Koepfer, 1977). Although the extent to which *D. arizonae* is fully sympatric with *D. mojavensis* is unclear (Brazner and Etges, 1993), these species have been documented to occasionally share both agria in coastal Sonora and cina, *S. alamosensis*, in southern Sonora (Markow et al., 1983; Ruiz and Heed, 1988; Etges and Heed, unpublished data).

In general, past studies have shown that low but significant premating isolation exists between mainland Sonora populations and Baja California populations of *D. mojavensis* (Zouros and D'Entremont, 1980; Markow, 1981; Koepfer, 1987a,b; Krebs and Markow, 1989). Unfortunately, most past studies have been conducted with lab food-reared flies or flies reared on lab food sup-

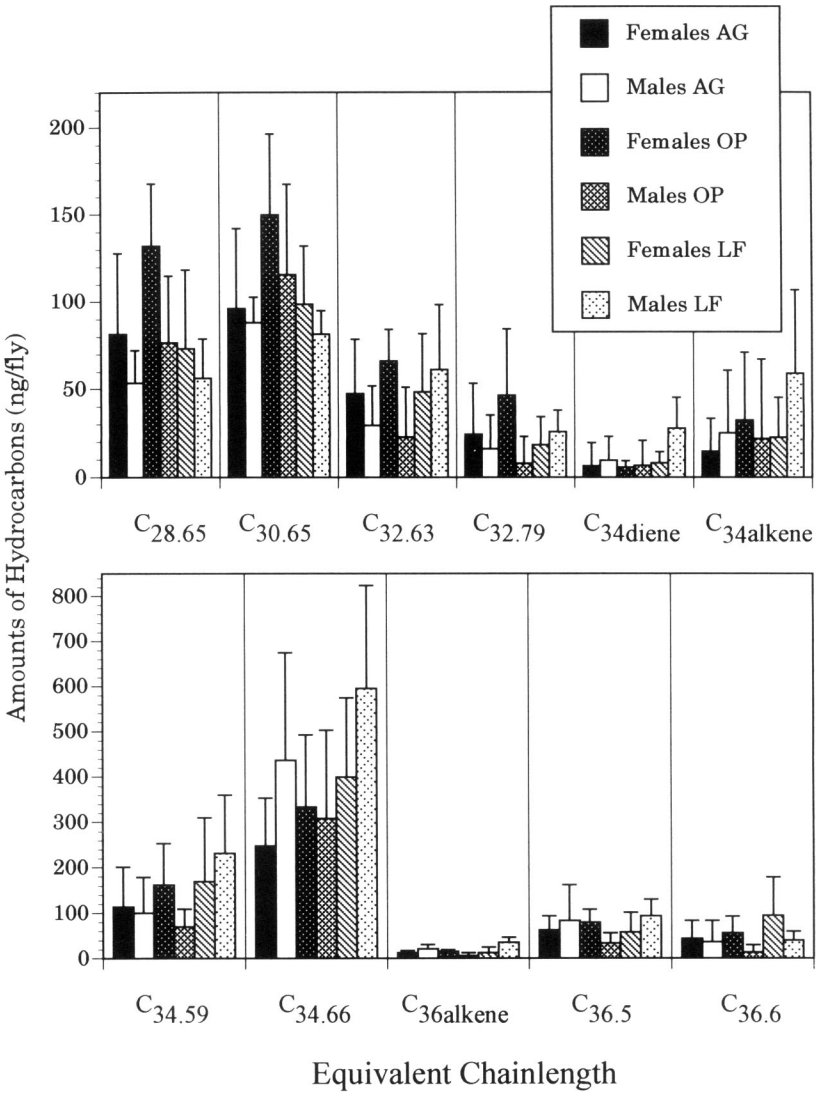


FIG. 6. The influence of rearing substrates on those hydrocarbon components that differed between males and females or showed sex × rearing substrate interactions in this study. Substrate labels are explained in Figure 1.

plemented with cactus (Markow et al., 1983), and these rearing conditions artificially induce significant premating isolation, increase mainland female-based assortative mating, and increase levels of mainland male mating propensity (Brazner, 1983; Brazner and Etges, 1993). Toolson et al. (1990, p. 1174) reported that flies reared on agria tissues were "only about half as large" and yielded only 33% as much total hydrocarbons as compared to flies reared on lab food. Unfortunately, they provided no information on culture conditions or any further information about these experiments, but pointed out that "compounds in *Stenocereus* tissue can directly affect synthesis and deposition of epicuticular hydrocarbons." We agree. Larval growth on artificial medium, "lab food," causes multiple significant differences in *D. mojavensis* and *D. arizonae* epicuticular hydrocarbons and premating isolation between peninsular and mainland populations of *D. mojavensis* as compared to flies reared on cactus substrates.

Environmentally induced shifts in insect cuticular hydrocarbons have also been associated with temperature and humidity experienced by larvae or adults (Toolson and Kuper-Simbron, 1989; Markow and Toolson, 1990; Reidy et al., 1991). A shift towards increased proportions of longer-chain hydrocarbon components at higher temperatures has been shown to result in decreased cuticle permeability, a response to thermal stress (Toolson and Hadley, 1979; Toolson, 1982). Thus, epicuticular hydrocarbon profiles in wild populations of *D. arizonae* and *D. mojavensis* are likely to be influenced by both rearing substrates and ambient temperatures.

Markow and Toolson (1990) selected total $C_{35:2}$ and $C_{37:2}$ alkadienes as the most likely components to serve as contact pheromones because of their abundance and degree of sexual dimorphism. They found that the ratio of $C_{35:2}$ to $C_{37:2}$ alkadienes, "*R* values," increased in adults held at 17°C for eight days after eclosion and that males with higher *R* values experienced greater mating success than males with lower *R* values caused by holding them at 34°C for eight days (Table 3). Overall amounts of total $C_{35:2}$ and $C_{37:2}$ alkadienes did not differ among males and females in the present study, but because there were sex × rearing substrate interactions, $C_{35:2}$ and $C_{37:2}$ alkadiene differences between males and females were not predictable across substrates. Analyses of flies reared on agria and organ pipe substrates with the lab food data deleted revealed that amounts of $C_{34.59}$ alkadiene were 67% greater in females than males ($F = 9.91$, $P < 0.01$), but this was dependent on cactus type. Analysis of *R* values by ANOVA with just the mainland and Baja populations revealed several interesting interaction terms involving rearing substrates. *R* values ranged from 4 to 9, with two prominent outliers caused by very small amounts of $C_{37:2}$ alkadienes (Figure 7). This pattern of *R* values is inconsistent with the observations made by Markow and Toolson (1990), who suggested that lab food-

TABLE 3. ANALYSIS OF VARIANCE RESULTS FOR RATIOS OF $C_{35:2}$ TO $C_{37:2}$ ALKADIENES, *R* VALUES (TOOLSON ET AL., 1990), FOR MAINLAND AND BAJA CALIFORNIA POPULATIONS OF *D. mojavensis* REARED ON LAB FOOD AND BOTH AGRIA AND ORGAN PIPE CACTUS TISSUES IN THIS STUDY

Source	*df*	Type IV SS	*F*	*P*
Model	11	621.12	3.29	0.026
Population	1	3.37	0.20	0.666
Food	2	96.71	2.82	0.099
Sex	1	0.23	0.01	0.910
Population × food	2	137.97	4.02	0.046
Population × sex	1	33.59	1.96	0.187
Food × sex	2	224.78	6.55	0.012
Population × food × sex	2	121.78	3.55	0.062
Error	12	206.06		

reared males with higher R values (up to 8) were more readily accepted by females during courtship. Cactus-reared mainland males generally had larger *R* values than females, similar to observations reported by Toolson et al. (1990) for wild caught adults from San Carlos, Sonora. *R* values for Baja adults reared on agria were not consistent with the greater mating activity of agria-reared Baja

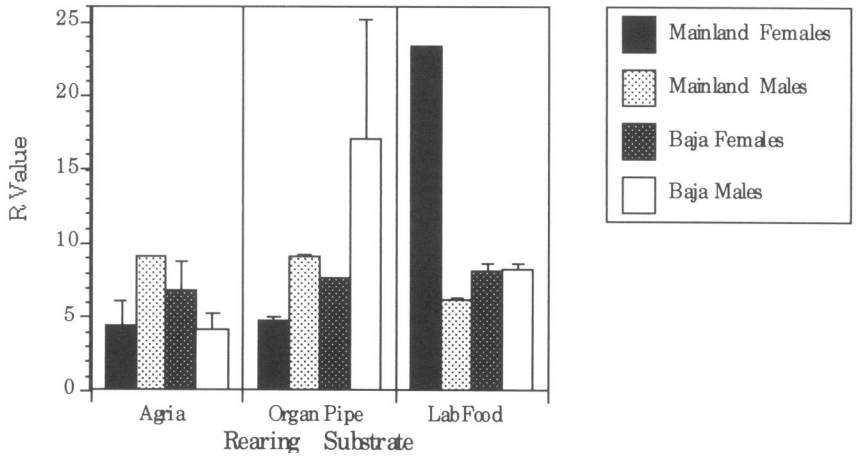


FIG. 7. Plot of $C_{35:2}/C_{37:2}$ alkadiene ratios, *R* values (+ 1 SD), of males and females reared on cactus and lab food in this study.

males and decreases in premating isolation with mainland adults (Brazner and Etges, 1993). More data for a larger number of natural populations are certainly required, but it is clear that if both $C_{34.59}$ and $C_{36.5}$ alkadienes are involved in mate recognition, their utility as cues will differ depending on the cactus used for larval growth and development.

The number of epicuticular hydrocarbon components that differed among sexes in a host-specific fashion suggests that the chemical basis of mate recognition in *D. mojavensis* involves a suite of contact pheromones as opposed to the relatively simple sexual differences in *D. melanogaster* (Jallon, 1984; Scott, 1994). Any interpretation of the contributions of particular hydrocarbons to mate discrimination, including female and male choice, must include the variation due to cactus rearing substrates in this species if we are to understand the role of epicuticular hydrocarbons in premating isolation and sexual selection outside of the laboratory. Further analysis of the role of particular hydrocarbon profiles will be required before we can conclude which components are responsible for the shifts in premating isolation. Also necessary will be analyses of cuticular hydrocarbon composition among wild-caught flies and the effects of cina cactus on epicuticular hydrocarbons of both *D. mojavensis* and *D. arizonae*. Since use of particular hosts varies across the range of *D. mojavensis* (Heed and Mangan, 1986; Etges et al., 1997) chemical cues important in mate discrimination will vary among populations that use different hosts.

Obviously, the results of our studies of premating isolation and the role of cuticular hydrocarbon variation in mating systems could not have been conducted in *D. mojavensis* without knowledge of their ecology. If these results are in any way general to other species, one conclusion from the present study concerning the role of cuticular hydrocarbons in premating studies carried out with flies reared on artificial substrates is: Proceed with caution.

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EFFECTS OF ENANTIOMERIC BLEND OF VERBENONE ON RESPONSE OF *Ips paraconfusus* TO NATURALLY PRODUCED AGGREGATION PHEROMONE IN THE LABORATORY

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Abstract—The aggregation pheromone produced by male *Ips paraconfusus* (Coleoptera: Scolytidae) tunneling in Monterey pine, *Pinus radiata*, logs was trapped on Porapak Q. A concentration of an extract of trapped volatiles that attracted beetles was determined in the laboratory through a concentration–response analysis of walking behavior of males and females. The interruptant effects of four concentrations of verbenone on response to a constant concentration of this naturally produced aggregation pheromone were tested with males and females. Independent of its enantiomeric composition [99.5% (*S*)-(−), 93.1% (*S*)-(−), and 98.3% (*R*)-(+)], verbenone significantly reduced the percentage of females (but not males) reaching the attractant source. However, when the time required for beetles of both sexes taken together to reach the attractant source was considered, verbenone of higher enantiomeric purity had a greater effect on beetle behavior. Solutions of 99.5% (*S*)-(−)- and 98.3% (*R*)-(+)-verbenone increased the time required for beetles to reach the attractive source when compared to 93.1% (*S*)-(−)-verbenone. When pooled across enantiomeric blends, increasing concentrations of verbenone resulted in slower responses in beetles that reached the attractant source within 2 min. Males and females did not respond to verbenone alone.

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INTRODUCTION

Verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) has been implicated in the interruption of the response of *Ips paraconfusus* Lanier (Coleoptera: Scolytidae) to its aggregation pheromone, [(4*S*)-(−)-ipsenol, (4*S*)-(+)-ipsdienol, and (1*S*,2*S*)-(+)-*cis*-verbenol) (Silverstein et al., 1966; Byers and Wood, 1980, 1981; Light, 1983). While verbenone has not been isolated from *I. paraconfusus*, it has been found in several species of *Dendroctonus* (Renwick, 1967; Rudinsky et al., 1974; Hughes et al., 1976; Libbey et al., 1985), *Pityophthorus pityographus* Ratzeburg (Francke et al., 1987), and in other species of *Ips* (Francke et al., 1980; Lanier et al., 1980; Birgersson et al., 1984) including *I. confusus* (LeConte) (Young et al., 1973), a close relative of *I. paraconfusus* (Lanier, 1970). Thus, verbenone acting as an allomone may regulate heterospecific cocolonization or niche separation in suitable hosts (Byers and Wood, 1980, 1981; Borden et al., 1992; Miller et al., 1994, 1995; Borden, 1997).

Conspecifically produced verbenone may also regulate scolytid host colonization by interrupting response to aggregation pheromones in many *Dendroctonus* spp. (Renwick and Vité, 1969; Rudinsky, 1973; Payne et al., 1978; Bedard et al., 1980; Byers and Wood, 1980, 1981; Livingston et al., 1983; Ryker and Yandell, 1983; Tilden and Bedard, 1988; Lindgren et al., 1989; Schmitz and McGregor, 1990; Paine and Hanlon, 1991; Shore et al., 1992; Bertram and Paine, 1994; Hayes and Strom, 1994; Miller et al., 1995) and *I. typographus* (L.) (Bakke, 1981; Schlyter et al., 1989). The ultimate natural source of verbenone perceived by scolytids is unknown. It could be one or more combinations of a *de novo* or oxidative product of scolytid tissues, of fungal associates (Brand et al., 1976; Leufvén et al., 1984; Hunt and Borden, 1990), or an autoxidative product of α-pinene (Hunt et al., 1989; Grosman, 1995).

In all previous field and laboratory studies in which verbenone was used to interrupt the response to an attractant, the source of attractant was either synthetic, or natural but variable, since pheromone production from logs or trees colonized with conspecific scolytids varies over time (Wood and Vité, 1961; Byers et al., 1984; Bedard et al., 1985). Furthermore, verbenone occurs as two enantiomers, and bark beetles generally show enantio-specific responses to attractant semiochemicals. For example, the response of *I. pini* (Say) to (*R*)-(−)-ipsdienol was interrupted by a small amount of (*S*)-(+)-ipsdienol (Birch et al., 1980; Seybold, 1992). Sensitivity to the enantiomeric composition of ver-

benone has been found in some cases, e.g., *D. ponderosae* Hopkins (Ryker and Yandell, 1983), *D. brevicomis* LeConte (Shea, 1990; Paine and Hanlon, 1991), and *I. paraconfusus* (Light, 1983), but not in others, such as *I. typographus* (Bakke, 1981). Tests utilizing verbenone of high enantiomeric and chemical purity have not been reported.

In the laboratory experiments described here, we determined an effective concentration of naturally derived aggregation pheromone of *I. paraconfusus* and tested the effect of verbenone of high enantiomeric and chemical purity on the percentage of *I. paraconfusus* walking upwind in response to this constant source of naturally derived aggregation pheromone and the time taken for responding beetles to reach the source of naturally derived aggregation pheromone.

METHODS AND MATERIALS

Insects. The first population of *I. paraconfusus* used to produce the attractant for laboratory assays was collected in *Pinus radiata* D. Don on March 25, 1988, near the intersection of Highways 1 and 17, Santa Cruz County, California. A second population of *I. paraconfusus* used in the laboratory assays to develop a full concentration–response profile for the attractant was collected in *P. radiata* on August 22, 1988, in Tilden Regional Park, Contra Costa County, California. A third population of *I. paraconfusus* used in all experiments with verbenone was collected from *P. radiata* on June 9, 1989, on the University of California, Berkeley Campus, Alameda County, California. The insects were reared from the host material (Browne, 1972) and stored on moist filter paper at 4°C until used for volatile collection or laboratory assays.

Production of Attractant. Volatiles associated with tunneling adult male *I. paraconfusus* were collected in the laboratory on May 2–9, 1988, by colonizing *P. radiata* logs (cut in Orinda, Contra Costa County, California, in April 1988) with *I. paraconfusus* males and trapping the volatiles from a 4-liter/min airflow on 15 g Porapak Q 50/80 mesh size (glass Porapak column: 20 cm × 2.5 cm ID) (Supelco, Inc., Bellefonte, Pennsylvania) (Byrne et al., 1975; Seybold, 1992). The Porapak was extracted with 350 ml pentane, which was subsequently concentrated to 10 ml. This extract was serially diluted in pentane for the assays. The same source of attractant was used for all experiments reported in this paper. During the 168-h collection period, 86 males released on average 29 ng ipsenol per male per minute. Concentration was expressed as nanograms of ipsenol per microliter of extract. Concentrations tested ranged from 0.04 to 400 ng/μl.

Verbenone. The (*R*)-(+) and (*S*)-(−) enantiomers of verbenone were synthesized from the respective optical isomers of α-pinene (chemical purity 99+%,

optical purity 98+%; Aldrich Chemical Co., Milwaukee, Wisconsin) (Seybold, 1992). The enantiomeric composition of verbenone was determined by injection on capillary GC with a chiral stationary phase (column: 50 m × 0.22 mm ID fused silica Cydex-B, SGE, Inc., Austin, Texas; carrier gas: helium, flow rate = 21.6 cm/sec; temperature: 110°C isothermal) (Seybold, 1992). The enantiomeric compositions of the (*R*)-(+)- and (*S*)-(−)-verbenone were 98.3% and 99.5%, respectively. The (*R*)-(+)- and (*S*)-(−)-verbenone samples contained chemical impurities of 3.7% and 5.4%, respectively. Commercially available "(*S*)-(−)-verbenone" (Bedoukian Research Inc., Danbury, Connecticut; product number P802) was determined to be 80% (*S*)-(−)-verbenone. After conducting the laboratory assays, it was discovered that a solution that was considered to be racemic was actually 93.1% (*S*)-(−)-verbenone with 99+% chemical purity.

Behavioral Assays. Attractants were tested in an open arena olfactometer (Wood and Bushing, 1963; Seybold, 1992), which consisted of a 75 × 50-cm board with five air ports along one side and an exhaust system at the opposite side to facilitate air movement and to eliminate volatile odors from the room. A plastic tube carrying air opened into a metal port with a screen facing toward the center of the board (Wood and Bushing, 1963). Solutions of attractants at various concentrations were allowed to evaporate from a filled 5-μl capillary tube mounted on clay and placed in the central port. A single beetle was placed 28 cm from the source of volatiles and timing of the experiment was initiated after the beetle began to walk. Each beetle was used only once, and 30 females and 30 males were exposed to each concentration. A clean 5-μl capillary tube was filled with attractant solution and placed in the port for each beetle assayed. A positive response was scored if the beetle arrived within 0.5 cm of the central port within a 2-min period. After 2 min the assay was stopped and the empty capillary tube was removed. The results were expressed in terms of the percentage of positive responses. The time for beetles that responded to the attractant to reach the source was also recorded.

The concentration–response relationships for female and male *I. paraconfusus* were determined over five orders of magnitude of the natural attractant beginning at 0.04 ng/μl ipsenol equivalent. This lowest concentration tested is equivalent to 1.4×10^{-3} beetle-minutes/μl (Browne et al., 1979). Thus, the amount of ipsenol loaded in each capillary tube at the beginning of each assay ranged from 0.2 ng to 2 μg. This experiment also contained a negative control of pentane solvent alone. The responses to one concentration (10^2 ng/μl) of three enantiomeric blends of verbenone [99.5% (*S*)-(−), 93.1% (*S*)-(−), and 98.5% (*R*)-(+)] alone were also tested in the laboratory assay. These responses were also compared to a negative pentane control.

Behavioral assays of the attractant together with verbenone employed concentrations of verbenone of each enantiomeric blend over four orders of magnitude increasing from 10^2 ng/μl. An attractant concentration of 40 ng/μl (ipsenol

equivalent) was chosen because, in the concentration–response profile, it was the minimal concentration that elicited a near-maximal, repeatable response from both sexes (Table 1). Assays were performed as they were for the attractant, but included the additional release of 5 μl of verbenone solution in pentane. The capillary tube with verbenone was placed in the clay holder adjacent to the tube containing the attractant. Concentration–response profiles for attractant together with each of the three enantiomeric blends of verbenone were developed for both females and males. Assays for the attractant concentration–response profile were conducted between October 18 and November 7, 1988, while the experiments with verbenone were conducted between July 3 and August 25, 1989.

Data Analysis. Hierarchical log-linear analyses were used to examine relationships between the factors in each experiment (Zar, 1984). In the experiment with varying concentrations of attractant (verbenone absent), the factors were concentration, sex, and response. In the experiment testing the attractiveness of the three enantiomeric blends of verbenone alone, the factors were enantiomeric blend, sex, and response. When verbenone was released in the presence of a constant concentration of attractant (40 ng/μl), the factors were concentration of verbenone, enantiomeric blend, sex, and response.

In the analysis of varying concentrations of attractant, Spearman's rank correlations were carried out between the ranks of concentrations and the ranks of responses for males and females (Siegel, 1956). If these correlations were not significant, the effects of the attractant were investigated by making a priori comparisons between high and low concentrations for each sex using G tests (Sokal and Rohlf, 1981). Similarly, the effects of verbenone on the response to the attractant were investigated by making a priori comparisons. If, in either

TABLE 1. PERCENT RESPONSE OF MALE AND FEMALE *Ips paraconfusus* TO CONCENTRATIONS OF A PENTANE EXTRACT OF PORAPAK-TRAPPED VOLATILES FROM MALE-INFESTED *Pinus radiata* LOGS[a]

	Concentration (ng/μl ipsenol)					
Sex	0.00	0.04	0.40	4.00	40.0	400.00
Male	6.7	6.7	3.3	30.0	26.7	23.3
Female	3.3	26.7	30.0	50.0	56.6	73.3

[a]Concentrations of the extract are expressed as the concentration of ipsenol (ng/μl) in the extract. Concentration of 0.00 is a negative control of pentane alone. Response is dependent on sex and concentration (hierarchical log-linear analysis, $P = 0.018$). Contrasts between responses to various concentrations are reported in the text. In all cases $N = 30$.

case, these comparisons did not maintain orthogonality, Bonferroni's correction was used (Winer et al., 1991).

Analysis of variance (Sokal and Rohlf, 1981) was used to analyze the data on the time required for beetles to arrive at the source. Concentration of verbenone, enantiomeric blend of verbenone, and sex were treated as qualitative variables in the analysis. Significant effects were partitioned using user-defined a priori contrasts to compare high and low concentrations of verbenone and high and low enantiomeric purities of verbenone.

RESULTS

The response to increasing concentrations of attractant was dependent on the sex of the responding beetles and on the concentration of the attractant (response × concentration × sex, $P = 0.018$) (Table 1). For females, the rank of the percent positive response showed a positive correlation with the rank of the concentration of attractant ($R_S = 1.00$, $N = 6$, $P < 0.01$). For males, this correlation was not significant. Response of males to the control (zero concentration) was not significantly different from the response to all the other concentrations combined, but the combined response to the three highest concentrations was significantly higher than the combined response to the two lowest (i.e., 0.04 and 0.40 ng/μl) concentrations (26.7% vs. 5.0%, $G = 12.95$, 1 *df*, $P < 0.01$). Comparisons within the three highest concentrations and within the two lowest concentrations were not significant. Thus, there was a trend for increased attraction to the source of volatiles with increasing concentration of attractant for females. Furthermore, higher concentrations of attractant were more attractive to males than lower concentrations (Table 1).

The responses to the three enantiomeric blends of verbenone and to pentane alone were not dependent on sex or the enantiomeric blend tested, indicating no attraction to the three enantiomeric blends of verbenone (Table 2).

TABLE 2. PERCENT RESPONSE OF MALE AND FEMALE *Ips paraconfusus* TO PENTANE (CONTROL) AND THREE ENANTIOMERIC BLENDS OF VERBENONE IN PENTANE[a]

		Enantiomeric blend of verbenone		
Sex	Pentane	98.3% (*R*)-(+)	99.5% (*S*)-(−)	93.1% (*S*)-(−)
Male	6.7	0.0	0.0	6.7
Female	3.3	3.3	6.7	6.7

[a]Response is not dependent on sex or enantiomeric blend of verbenone (hierarchical log-linear analysis, $P > 0.05$). In all cases $N = 30$.

The response to increasing concentrations of three enantiomeric blends of verbenone in the presence of 40 ng/μl attractant was dependent on the sex of the responding beetle and on the concentration of verbenone, but was independent of the enantiomeric blend of verbenone (response × concentration of verbenone × sex, $P = 0.018$) (Table 3). Since the enantiomeric blend of verbenone

TABLE 3. PERCENT RESPONSE OF MALE AND FEMALE *Ips paraconfusus* TO INCREASING CONCENTRATIONS OF VERBENONE OF THREE ENANTIOMERIC BLENDS IN PRESENCE OF ATTRACTIVE CONCENTRATION (40 ng/μl) OF PENTANE EXTRACT OF PORAPAK-TRAPPED VOLATILES FROM FEEDING MALES[a]

Verbenone concentration (ng/μl)	Sex	Enantiomeric blend of verbenone	Percent responding ($N = 30$)	Response time (sec, mean ± SE)
0	Male	None	33.3	57.1 ± 7.63
	Female	None	63.3	58.8 ± 6.03
10^2	Male	98.3% (*R*)-(+)	43.3	63.7 ± 7.50
		99.5% (*S*)-(−)	30.0	64.2 ± 7.45
		93.1% (*S*)-(−)	33.3	78.1 ± 8.09
	Female	98.3% (*R*)-(+)	56.7	68.2 ± 6.16
		99.5% (*S*)-(−)	56.7	69.1 ± 6.35
		93.1% (*S*)-(−)	76.7	58.2 ± 5.36
10^3	Male	98.3% (*R*)-(+)	23.3	78.3 ± 12.16
		99.5% (*S*)-(−)	20.0	87.0 ± 10.21
		93.1% (*S*)-(−)	16.7	70.0 ± 12.72
	Female	98.3% (*R*)-(+)	63.3	60.1 ± 4.42
		99.5% (*S*)-(−)	73.3	72.6 ± 5.74
		93.1% (*S*)-(−)	66.7	66.5 ± 4.27
10^4	Male	98.3% (*R*)-(+)	43.3	87.0 ± 5.12
		99.5% (*S*)-(−)	26.7	82.5 ± 6.18
		93.1% (*S*)-(−)	10.0	65.7 ± 22.15
	Female	98.3% (*R*)-(+)	63.3	78.9 ± 4.49
		99.5% (*S*)-(−)	56.7	75.3 ± 4.91
		93.1% (*S*)-(−)	43.3	74.7 ± 6.26
10^5	Male	98.3% (*R*)-(+)	20.0	82.0 ± 7.83
		99.5% (*S*)-(−)	6.7	96.5 ± 31.00
		93.1% (*S*)-(−)	23.3	58.0 ± 10.32
	Female	98.3% (*R*)-(+)	36.7	85.8 ± 6.67
		99.5% (*S*)-(−)	26.7	87.9 ± 8.47
		93.1% (*S*)-(−)	16.7	70.2 ± 16.31

[a] Mean time for responding beetles to reach the source of the combined semiochemicals is also given. Response is dependent on sex and concentration of verbenone (hierarchical log-linear analysis, $P = 0.0181$). Time to respond is dependent on concentration of verbenone ($P = 0.012$: ANOVA) and on enantiomeric blend of verbenone ($P = 0.016$: ANOVA).

was not a significant factor in the model, data for the three enantiomeric blends of verbenone were pooled (Figure 1). For females, the combined response to the two highest concentrations of verbenone and the natural pheromone blend was significantly lower when compared to the combined response to the two lowest concentrations of verbenone and the attractant (40.5% vs. 65.5%, G = 22.74, 1 *df*, $P < 0.01$) (Figure 1). The higher of the two highest concentrations (10^5 ng/μl) significantly reduced attraction to the natural pheromone blend when compared with the lower concentration (10^4 ng/μl) (26.6% vs. 54.4%, G = 14.51, 1 *df*, $P < 0.01$). There was no significant difference between the responses to the two lowest concentrations (10^2 and 10^3 ng/μl). A similar set of contrasts was carried out using the data for male beetles, but none of the comparisons was significant. Male response was lower than female response in all cases (Table 3, Figure 1), although a priori statistical comparisons between male and female responses at each concentration were not made.

An ANOVA comparing the response to the control (attractant alone) and the combined response to all treatments with verbenone showed that the time taken (in seconds) for beetles to reach the control (attractant alone) was significantly shorter [58.2 $\pm$ 4.51 sec, (SE)] than the time to reach the attractant when verbenone was present (72.1 $\pm$ 1.37; F = 9.55, 1; 307 *df*, P = 0.002). This result was independent of the sex of the beetles.

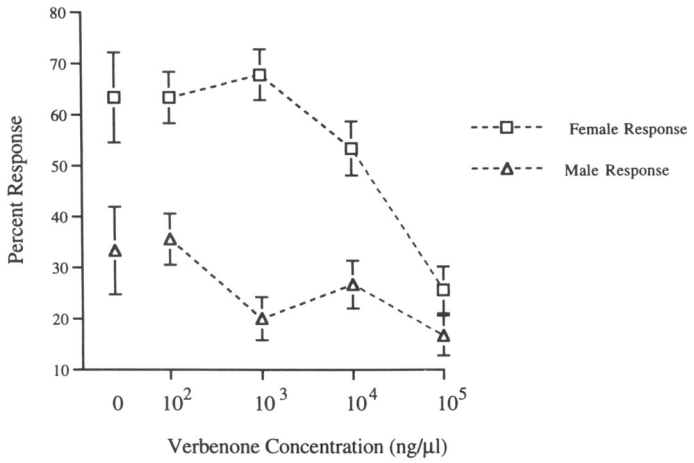


FIG. 1. Percent response of male and female *Ips paraconfusus* to increasing concentrations of verbenone in the presence of an attractive concentration (40 ng/μl) of a pentane extract of Porapak-trapped volatiles from feeding males. Response data from three enantiomeric blends of verbenone (Table 3) were pooled for this figure. Bars indicate standard errors. See text for contrasts.

The time required for beetles to arrive at the source in treatments where verbenone was released was compared across treatments (concentration of verbenone, enantiomeric blend, sex; Table 3). Both concentration of verbenone ($P = 0.012$) and enantiomeric blend ($P = 0.016$) were significant effects in the ANOVA model. The effect of sex, and the interaction terms in the analysis were not significant. Since sex was not a significant factor in the model, data for both sexes were pooled (Figures 2 and 3). When response data to the three enantiomeric blends of verbenone were combined, time to respond increased with increasing concentration of verbenone (Figure 2). Times for the combined response to the two higher concentrations of verbenone were significantly longer than times for the combined response to the two lower concentrations (78.8 vs. 67.5 sec; $F = 15.1$, 1, 279 *df*, $P < 0.001$). Contrasts within the two highest concentrations and within the two lowest concentrations were not significant. Hence, beetles took longer to respond to higher concentrations of verbenone.

When response data from both sexes and all concentrations of verbenone were combined, time to respond when the two more enantiomerically pure blends of verbenone were present [99.5% (*S*)-(−) and 98.5% (*R*)-(+)] was signifi-

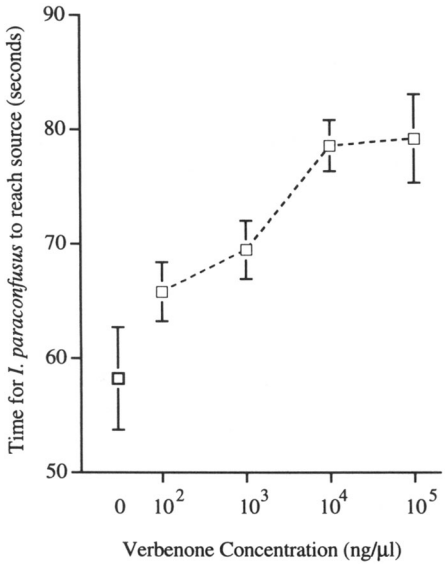


FIG. 2. Time for *Ips paraconfusus* to reach source with increasing concentrations of verbenone in the presence of an attractive concentration (40 ng/μl) of a pentane extract of Porapak-trapped volatiles from feeding males. Response data from both sexes to three enantiomeric blends of verbenone (Table 3) were pooled for this figure. Effect of concentration is significant ($P = 0.012$). Bars indicate standard errors. See text for contrasts.

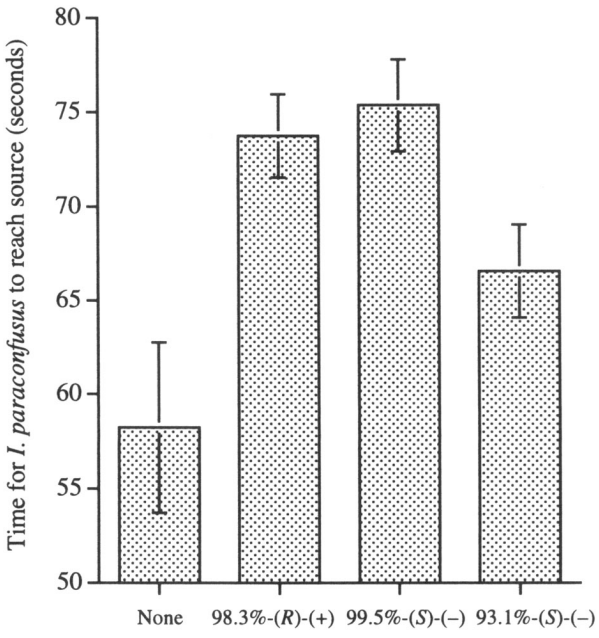


FIG. 3. Time for *Ips paraconfusus* to reach source with one of three enantiomeric blends of verbenone in the presence of an attractive concentration (40 ng/μl) of a pentane extract of Porapak-trapped volatiles from feeding males. Response data from both sexes to all concentrations of verbenone (Table 3) were pooled for this figure. Effect of enantiomeric blend of verbenone is significant ($P = 0.016$). Bars indicate standard errors. See text for contrasts.

cantly longer than the time to respond to the less enantiomerically pure [93.1% (*S*)-(−)] blend (74.5 vs. 66.6 sec; $F = 5.5$; 1, 279 *df*, $P = 0.019$) (Figure 3). The contrast between the two more enantiomerically pure blends of verbenone was not significant. Hence, responding beetles took longer to reach the source of attractant in the presence of higher concentrations of verbenone and longer when the enantiomeric purity of verbenone was higher.

DISCUSSION

This study demonstrates that verbenone interrupted the attraction of *I. paraconfusus* to an extract containing its naturally produced pheromone components in a laboratory assay. Increasing the concentration of verbenone

decreased the percentage of female beetles arriving at the attractant source. Independent of the sex of the beetle assayed, increasing the concentration of verbenone also increased the time required to reach the source.

In an earlier laboratory study, Byers and Wood (1981) found that a verbenone concentration of 10^3 ng/μl was necessary to interrupt the walking response of *I. paraconfusus* to 1 ng/μl of each synthetic component of its pheromone (1000:1 ratio). In our study, females required a similarly high concentration of verbenone (between 10^4 and 10^5 ng/μl = 250–2500:1 ratio to the principal attractant compound ipsenol) to elicit interruption. These high concentrations of verbenone required for activity in the laboratory may not be ecologically relevant in view of the relative quantities of verbenone and principal pheromone components produced by bark beetles, e.g., 10 ng verbenone/beetle compared to 500 ng methylbutenol/beetle for *I. typographus* (Birgersson et al., 1984) and 30 ng verbenone/beetle compared to 241 ng *trans*-verbenol/beetle for *D. ponderosae* (Hunt and Borden, 1989).

The different enantiomeric blends of verbenone in our laboratory assay were not a significant factor in the percentage of beetles reaching the source of attractants. However, the time required for both sexes of *I. paraconfusus* pooled to arrive at the source was significantly longer for more enantiomerically pure verbenone [98.3% (*R*)-(+)- and 99.5% (*S*)-(−)] than for a less pure enantiomeric blend [93.1% (*S*)-(−)]. Hence, more enantiomerically pure formulations of verbenone [i.e., >99.5% (*S*)-(−) or >98.3% (*R*)-(+)] may be more effective at slowing the response to the attractant.

Light (1983) found that female antennae of *I. paraconfusus* responded more to (*S*)-(−)-verbenone than to (*R*)-(+)-verbenone in a study of electroantennograms (EAGs); however, male antennae responded equally to both enantiomeric blends at >90% enantiomeric purity. In EAG studies with *I. avulsus*, *I. calligraphus*, and *I. grandicollis*, Smith et al. (1988) suggested that verbenone and several other behavioral chemicals act in short-range beetle behavior, such as orientation and/or arrestment. Our study demonstrates that 98.3% (*R*)-(+) and 99.5% (*S*)-(−) verbenone increased the time required to reach the attractant source in an assay of walking behavior, which we suggest supports a close-range function for verbenone. In studies using synthetic pheromone components, Akers and Wood (1989) showed the walking rate and deviation from the upwind direction decreased and the turning rate increased as female *I. paraconfusus* approached the pheromone source. Our data indicate that verbenone acts to further decrease the walking rate in the presence of aggregation pheromone. Thus, after landing, a female beetle may be deterred from finding a male gallery if sufficient amounts of verbenone are present.

Verbenone has also been shown to interrupt response in field studies that assayed flight behavior of *I. paraconfusus*. Byers and Wood (1980) and Light (1983) demonstrated interruption of response to male-infested logs with verbe-

none of low enantiomeric purity [approx. 68% (*R*)-(+), (Byers and Wood, 1980); >90% (*R*)-(+) and >90% (*S*)-(−), (Light, 1983)]. Paine and Hanlon (1991) found that 84% (*S*)-(−)-verbenone interrupted the field response of *I. paraconfusus* to traps containing synthetic aggregation pheromone components. Thus, verbenone can lower the percentage of *I. paraconfusus* reaching a source of naturally produced or synthetic pheromone in the laboratory and can lower the number responding to naturally produced (but variably released) or synthetic pheromone components in the field. Our laboratory study, which utilized verbenone of high enantiomeric purity, showed no enantio-specific effect on the percentage of beetles responding. However, field studies with pure enantiomers of verbenone are still required to determine whether or not enantiomeric ratios are important in reducing flight response. Enantiomeric purity can be very important in the effectiveness of aggregation pheromones. For example, Seybold (1992) showed in field studies in California that as little as 1% of the (*S*)-(+) isomer will decrease the response of *I. pini* to the attractive pheromone component, (*R*)-(−)-ipsdienol.

Verbenone has been shown to decrease response to pheromone sources for many bark beetle species. For this reason verbenone is being actively pursued as a potential candidate for the protection of individual trees and forest stands (Shea, 1990; Vité and Baader, 1990; Amman, 1994; Borden, 1994, 1997). Studies aimed at optimizing the concentration and enantiomeric composition of verbenone have significant potential benefits for developing treatments utilizing this broadly active compound.

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PHOTOOXIDATION OF NIMBIN AND SALANNIN, TETRANORTRITERPENOIDS FROM THE NEEM TREE (*Azadirachta indica*)

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Abstract—Nimbin and salannin, major triterpenoids accompanying azadirachtin in extracts of neem (*Azadirachta indica*) seeds, were photooxidized by UV light in the presence of oxygen to more polar, unstable intermediates that rearranged on silica gel to two final products in which the furan ring had been oxidized to isomeric hydroxybutenolides. The isomeric hydroxybutenolides were also readily formed when a crude extract of triterpenoids from neem seeds was irradiated, and both isomers of salannin have been isolated from seeds. Photooxidation of nimbin and salannin proceeded much faster than that of azadirachtin. All photoproducts showed some biological activity against *Spodoptera littoralis*, *Locusta migratoria*, and *Schistocerca gregaria*. Isonimbinolide was as potent as azadirachtin at inhibiting feeding in all three species, and it also inhibited the growth of *S. littoralis*. Isosalanninolide showed potent antifeedant and growth inhibitory activity against *S. littoralis*.

Key Words—Azadirachtin, nimbin, salannin, antifeedant, growth inhibition, *Azadirachta indica*, *Spodoptera littoralis*, *Locusta migratoria*, *Schistocerca gregaria*, photooxidation, photoisomerization.

INTRODUCTION

Neem (*Azadirachta indica* A. Juss, Meliaceae) seeds contain the complex triterpenoid azadirachtin (**1**) (Figure 1), a powerful insect antifeedant (Butterworth

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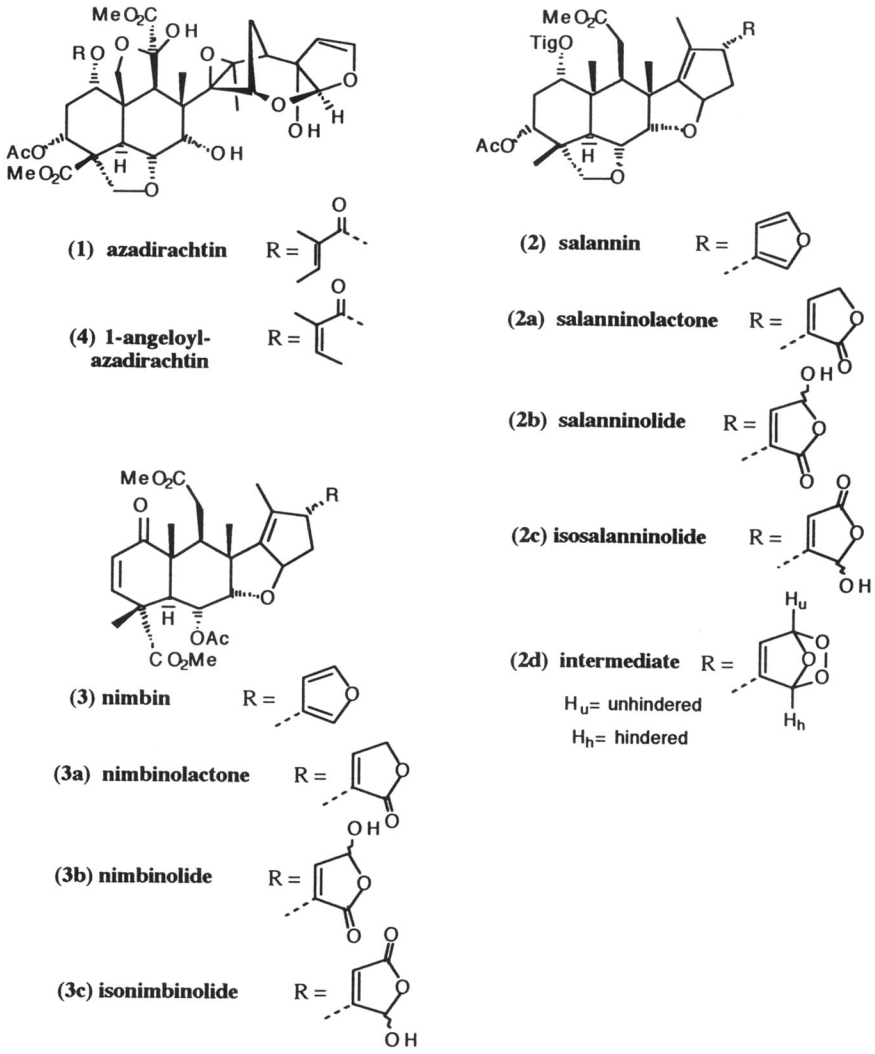


FIG. 1. Chemical structures of compounds isolated and identified.

and Morgan, 1968) and growth-regulating substance (Ruscoe, 1972). There are many other triterpenoids in the seeds (Jones et al., 1989), some of which, for example, salannin (**2**) and nimbin (**3**), also have antifeedant properties (Blaney et al., 1990; Kraus, 1995).

Neem seed extracts containing these complex triterpenoids meet many of

the criteria necessary for a natural insecticide (van Beek and de Groot, 1986) and its development into a commercially successful product, although field trials have shown that activity can last for as little as four to eight days (Schmutterer, 1988). This loss of biological activity has been attributed to the destruction of azadirachtin (**1**) in sunlight (Stokes and Redfern, 1982), and UV light (Barnby et al., 1989; Sundaram and Curry, 1996). The triterpenoids usually present in the greatest amount in seeds are salannin (**2**) and nimbin (**3**). Both absorb UV light, so their environmental fate may be important in determining the stability of insecticidal preparations of neem.

We have already shown that azadirachtin is slowly photooxidized to a mixture of unknown polar products and is readily photoisomerized in UV light to 1-angeloylazadirachtin (**4**) (Johnson et al., 1994). Here we describe the results of our experiments on the UV irradiation of salannin (**2**) and nimbin (**3**) and discuss the biological activity of the compounds formed, including that of 1-angeloylazadirachtin (**4**).

Salannin (**2**) has two UV-absorbing chromophores, an α,β-unsaturated ester and β-substituted furan ring, both of which could undergo photochemical reactions. Nimbin (**3**) also has two UV-absorbing chromophores, an α,β-unsaturated ketone and a β-substituted furan ring. Connolly et al. (1968) isolated autooxidized products of salannin and nimbin, where the furan ring had been converted to an α,β-unsaturated lactone (**2a** and **3a**, respectively). A more highly oxidized product containing a 23-hydroxybut-20(22)-ene-21,23-γ-lactone (δ-hydroxybutenolide) was formed by reacting salannin with UV light and oxygen (Purushothaman et al., 1984). This compound (**2b**), which has also been found in the seeds of *Melia dubia* (Purushothaman et al., 1984) and named compositolide, was isolated from *Azadirachta indica* by Garg and Bhakuni (1984), who referred to it as salannolide, and by Johnson (1995), who attempted to introduce some system to the names of these derivatives by calling it salanninolide. An analogous nimbin photoproduct, nimbinolide (**3b**), was isolated from seeds and called isophotonimbin by Thiele (1991). An isomer, a 21-hydroxybut-20(22)-ene-21,23-γ-lactone product (**3c**) has been found in the stem bark of *Azadirachta indica* (Ara et al., 1988).

METHODS AND MATERIALS

Solvents. Benzene was spectroscopic grade (Fisons Scientific Equipment, UK), and methanol was redistilled GPR grade. Light petroleum was the fraction with bp 60–80°C. All other solvents were GPR grade and used as received.

Isolation of Salannin and Nimbin. Salannin (**2**) and nimbin (**3**) were isolated by the procedure described by Johnson et al. (1994).

Chromatographic Analysis. An LDC supercritical fluid chromatograph,

consisting of a cooler (Haake C), liquid CO_2 pump (CP 3000), modifier pump (ConstaMetric 3000), gradient programmer (GM 4000), high-pressure mixer and pulse dampener (Negretti, Southampton, UK), variable wavelength UV detector (SpectroMonitor 3100) set at 217 nm, an oven containing a model 7125 loop injector fitted with a 20-μl loop (Rheodyne), backpressure regulator (Tescom), and the analytical column, was used to monitor the irradiation of salannin and nimbin. Analysis was carried out with a Spherisorb cyanopropyl silica column (150 mm × 4.6 mm ID) of 5-μm particle size (HPLC Technology, Macclesfield, UK). Separations were performed under isocratic conditions at a flow rate of 2 ml/min, with a mobile phase of CO_2–methanol (94 : 6 v/v) for salannin analysis, CO_2–methanol (95 : 5 v/v) for nimbin analysis, and CO_2–methanol (96 : 4 v/v) for neem seed extracts. A pressure of approximately 3000 psi and an oven temperature of 55°C was used.

Photooxidation of Nimbin. A solution of nimbin (270 mg) in benzene (45 cm^3) was irradiated with UV light (Hanovia, 90W) while oxygen was bubbled through the reaction mixture. An aliquot of the reaction mixture was taken at 15-min intervals and analyzed by supercritical fluid chromatography (SFC). After 30 min, when all the nimbin had reacted, the reaction was stopped. The solvent was removed by rotary evaporation, the residue dissolved in dichloromethane (~ 1 cm^3), and it was chromatographed on a Chromatotron (Harrison Research) plate (2 mm thick, 65 g silica gel, E. Merck). The products were eluted with chloroform (100%), followed by chloroform–methanol (95 : 5), and methanol (100%).

Isolation of Nimbinolide and Isonimbinolide. The chloroform–methanol fraction was rechromatographed on a fresh plate (1 mm thickness, 50 g of silica), and eluted with light petroleum–ethyl acetate (3 : 2) to give the two photoproducts. The faster running of the two compounds was purified further by recrystallizing from methanol, to give nimbinolide (**3b**) as colorless crystals (32 mg from MeOH), mp 193–196°C, CI-MS $M^{+\cdot}$ at *m/z* 572; IR (KBr disc) 3418, 2950, 1764, 1722, 1686, 1246, 1080 cm^{-1}. The slower running compound, identified as isonimbinolide (**3c**), was obtained as a white powder (8 mg), mp 141–145°C, CI-MS $M^{+\cdot}$ at *m/z* 572; IR (KBr disc) 3450, 2960, 1730, 1688, 1638, 1242, 1026 cm^{-1}. For ^1H NMR spectral data see Table 1.

Photooxidation of Salannin and Isolation of Salanninolide. A solution of salannin (300 mg) in benzene (45 cm^3) was irradiated as described for nimbin. When all the salannin had reacted (30 min), the solution was evaporated to dryness, the residue redissolved in dichloromethane (~ 1 cm^3), and chromatographed on a Chromatotron plate as above. The plate was eluted with chloroform (100%) followed by methanol (100%). The methanol fraction was evaporated to dryness and the residue crystallized from methanol to give salanninolide (**2b**) as a white amorphous powder (80 mg from MeOH), mp 282–286°C, λ_{max}

(MeOH) 205 nm, ϵ 37,300; CI-MS $M^{+\cdot}$ at *m/z* 628; IR (KBr disc) 3322, 2925, 1758, 1730, 1704, 1437, 1374, 1270 and 1162 cm^{-1}. For ^1H NMR spectral data, see Table 1.

Isolation of Photooxidation Products of Salannin from Seeds. Neem seeds (2 kg) of Indian origin were treated initially as described by Johnson et al. (1994), i.e., they were deoiled with light petroleum, extracted with methanol, and the triterpenoids extracted into dichloromethane from aqueous methanol. The dichloromethane solution was concentrated under vacuum and redissolved in the same solvent (1 cm^3/g) and chromatographed in 5-cm^3 portions on a Biotage 40M flash column (90 gm silica, Biotage). Elution of the column with a mixture of light petroleum–ethyl acetate (3:7) gave a mixture of compounds from which salanninolide (**2b**) was isolated by crystallization from light petroleum–ethyl acetate (1:1) as colorless crystals (125 mg), mp 278–279°C. The compound was shown by its IR and NMR spectra and its chromatographic properties to be identical with the product from photooxidation of salannin.

Ethyl acetate eluted a single compound from the column. This was purified by recrystallization from ethyl acetate, to give isosalanninolide (**2c**) as colorless crystals (50 mg), mp 104–106°C; UV λ_{max} 207 nm, ϵ 27,000; IR (KBr disc) 3450, 2923, 1733, 1690, 1264, 1051 cm^{-1}, accurate mass analysis gave *m/z* 629.2962 ($M+H^+$) corresponding to $C_{34}H_{44}O_{11}$.

Isolation of Isosalanninolide. The irradiation of salannin (100 mg) in benzene (45 cm^3) was repeated with the addition of triethylamine (225 ml). The reaction was followed by SFC, and when all the salannin had reacted (75 min), the reaction was stopped. Benzene was removed and replaced by dichloromethane ($\sim$ 1 cm^3). The solution was chromatographed on a Chromatotron plate as before and eluted with chloroform and then methanol. Two compounds were present in the methanol fraction. These were separated by rechromatography on a Chromatotron plate (1 mm thick, 50 g silica gel), which was eluted with light petroleum–ethyl acetate (3:2). The first eluted compound was identified as salanninolide (**2b**) (16 mg) and the second was identified as isosalanninolide (**2c**) (22 mg). The IR and NMR spectra and chromatographic properties were identical with the substances isolated from seeds (above).

UV Irradiation of Neem Seed Extract. Neem seeds (15 g) were extracted in a domestic food liquidizer (Moulinex, France) with methanol (6 $\times$ 100 cm^3). The solvent was removed by rotary evaporation, and the residue redissolved in methanol–water (2:1, 15 cm^3). The aqueous methanol was partitioned against dichloromethane (3 $\times$ 30 cm^3). The dichloromethane fraction was evaporated to dryness and the residue redissolved in benzene (45 cm^3). This benzene solution was irradiated with UV light while oxygen was bubbled through the solution. An aliquot of the reaction mixture was taken at 10-min intervals and analyzed by SFC (see below). After 30 min the reaction was stopped, the solvent

TABLE 1. ^{1}H NMR (AT 270 OR 500 MHz) SPECTRAL DATA OF SALANNIN (2), NIMBIN (3), AND THEIR PHOTOOXIDATION PRODUCTS[a]

Signal	Salannin (2)	Salanninolide (2b)	Isosalanninolide (2c)	Nimbin (3)	Nimbinolide (3b)[b]	Isonimbinolide (3c)[b]
1-H	4.80 (t)	4.90 (t)*	4.68 (s)			
2-H				5.88 (d)	5.88 (d)	5.89 (d)
2-Ha,b	2.05–2.35 (m)	2.05–2.45 (m)	Δ			
3-H	4.97 (t)	4.99 (t)*	4.95 (br s)	6.34 (d)	6.34 (d)	6.37 (d)
5-H	2.83 (d)	2.73 (d)	2.75 (d)	3.70 (d)	3.59 (d)	3.62 (d)
6-H	4.00 (dd)	3.97 (dd)	3.98 (dd)	5.22 (dd)	5.19 (dd)	5.20 (dd)
7-H	4.18 (d)	4.26 (d)	4.25 (d)	4.05 (d)	4.11 (d)	4.11 (d)
9-H	2.76 (dd)	2.53 (dd)	2.72 (ddd)	2.87 (m)	2.63 (t)	2.69 (m)
11-Ha			2.16 (s)	2.25 (m)	2.23 (dd)	2.15 (m)
11-Hb			2.32 (m)	2.94 (m)	2.91 (dd)	2.85 (dd)
11-Ha,b	2.05–2.35 (m)	2.05–2.45 (m)				
15-H	5.44 (m)	5.43 (m)	5.59 (t)	5.57 (m)	5.52 (m)	5.40(br s)**
16-Ha			2.22 (m)	2.02 (m)	†	2.05 (m)**
16-Hb			2.16 (m)	2.19 (m)	2.35 (dd)	2.38 (br s)
16-Ha,b	2.05–2.35 (m)	2.05–2.45 (m)				

17-H	3.66 (d)	3.65 (d)**	3.84 (d)	3.61 (m)	3.52 (br d)	††
18-H	1.67 (d)	1.83 (d)	1.71 (d)	1.67 (d)	1.87 (d)	1.93 (br s)
19-H	0.99 (s)	0.94 (s)	1.04 (s)	1.28 (s)	1.27 (s)	1.30 (s)
21-H	7.33 (m)		5.88 (br s)	7.32 (m)		5.77 (br s)**
22-H	6.30 (m)	6.77 (s)	5.96 (s)	6.33 (m)	6.98 (s)	5.94 (s)
23-H	7.26 (m)	5.97 (d)		7.23 (m)	6.08 (d)	
28-Ha	3.72 (m)	3.49 (d)**	3.70 (d)			
28-Hb	3.58 (m)	3.73 (d)**	3.59 (d)			
29-H	1.22 (s)	1.21 (s)	1.23 (s)	1.36 (s)	1.34 (s)	1.34 (s)
30-H	1.30 (s)	1.30 (s)	1.27 (s)	1.34 (s)	1.36 (s)	1.37 (s)
OH		5.37 (d)	5.41 (br s)		5.12 (d)	5.65 (br s)**
OAc	1.94 (s)	2.01 (s)	1.57 (s)	2.04 (s)	2.04 (s)	2.05 (s)
12-CO_2CH_3	3.24 (s)	3.45 (s)	3.56 (s)	3.65 (s)	3.67 (s)	3.65 (s)
28-CO_2CH_3				3.73 (s)	3.75 (s)	3.80 (br s)
3′-H	6.92 (m)	6.96 (m)	6.93 (m)			
4′-H	1.78 (dd)	1.85 (dd)	1.84 (ddd)			
5′-H	1.86 (s)	1.91 (s)	1.92 (s)			

[a]Recorded in $CDCl_3$ with TMS at δ 0.00.
[b]Recorded on a Varian unity 500 MHz instrument.
[c]*These assignments may be reversed. **Identifications uncertain. †Masked by signal at δ 2.04. ††Masked by signal at δ 3.80. ^Signals hidden by H-16a and H-16b signals.

removed, and the residue applied to a Chromatotron plate (2 mm thickness). The plate was eluted with chloroform (100%) followed by chloroform–methanol (19:1) and finally with methanol (100%). SFC analysis of the chloroform–methanol fraction showed it to contain a large number of UV-absorbing substances, which were not investigated further.

Biological Testing. A choice bioassay was used to assess the antifeedant activity of the compounds (Blaney et al., 1990), which were applied to glassfiber discs (Whatman GF/A, 2.1 cm diam.) made palatable by the addition of 100 μl of sucrose solution (50 mM). Control discs carried just sucrose, whereas the treatment discs carried sucrose and 100 μl of one of the test compounds at 1×10^{-10} M to 1×10^{-4} M. The discs were left to dry and then weighed. For the bioassay, final instars of *Spodoptera littoralis* were placed individually in Petri dishes (8.5 cm diam.) or individual fifth instars of either *Locusta migratoria* or *Schistocerca gregaria* were placed in clear plastic containers (17 × 12 × 6 cm). Each insect had a control and treatment disc. Every concentration of a compound was tested against 5–20 insects. The bioassay terminated after 50% of either disc was eaten or after 18 hr if the insects had not eaten 50% of either disc. The insects were removed and the discs dried and reweighed. The antifeedancy index $\{AI = [(C - T)/(C + T)] * 100\}$ was calculated where C and T represent the mass eaten of control and treatment discs, respectively. Regression analysis was used to establish the concentration required to obtain an antifeedant index of 50% (AI_{50}).

The responses of gustatory neurons in the styloconic sensilla of *S. littoralis* to the compounds were recorded (Simmonds et al., 1995). The medial sensilla of final instars were stimulated with each compound at one concentration (1×10^{-6} M). Each sensillum was stimulated with the electrolyte (KCl, 50 mM) for 1 sec and after 1 min the sensillum was restimulated with one of the compounds. The sensillum was then left for 10 min and restimulated with the electrolyte for 1 sec. After a 1-min break, it was stimulated with another compound. Each sensillum was stimulated with up to five of the compounds. After 2 hr, or when the sensillum failed to respond to the electrolyte, the experiment was terminated. Each compound was tested against four to six larvae and the total neural response in the first second of stimulation was used in the analysis.

The growth-regulating effects on third instar *S. littoralis* or fourth instar locust nymphs were determined by three bioassays (Simmonds et al., 1990). The compounds were administered either by topical application (1 μl per *S. littoralis* larva and 2.5 μl per locust nymph), by oral cannulation into the midgut (1 μl per *S. littoralis* larva and 3 μl per locust nymph), or by injection into the hemolymph (2 μl per *S. littoralis* larva and 3 μl per locust nymph). Each compound was tested at four or more concentrations in the range 1 μl/g to 100 μl/g with 10–50 replications per concentration. After treatment, insects were

reared individually on their culturing diet and mortality recorded after 72 hr. This was the time required for the untreated control insects to molt.

RESULTS AND DISCUSSION

Nimbin. The progress of the irradiation of nimbin (**3**) under oxygen was followed by periodic analysis of an aliquot of the reaction mixture by supercritical fluid chromatography (SFC), which has been shown to be a simple and fast technique for analyzing extracts from neem seeds (Huang and Morgan, 1991; Johnson et al., 1994). The SFC chromatogram of the reaction mixture before irradiation is shown (Figure 2a), with nimbin eluting at ~3 min. Figure 2b (shown at a higher integrator setting than 2a) indicates that after irradiation for 15 min, there was an increase in polar compounds, although most of the nimbin remained unreacted. After 30 min (Figure 2c, same integrator setting as 2b), essentially all the nimbin had reacted, leaving a number of more polar reaction products. The initial products were unstable on silica gel chromatography. When separation was attempted on the Chromatotron, two final products were formed in approximately a 7:3 ratio (Figure 2d), both of which were isolated and their structures determined.

The compound eluting after 11 min (Figure 2d) gave a CI mass spectrum with an ion at *m/z* 590 ($M+NH_4^+$) corresponding to a molecular ion of *m/z* 572, i.e., 32 mass units more than nimbin. The ^1H NMR spectrum of the purified compound (Table 1) was essentially unchanged from that of nimbin (Bokel et al., 1990) except for the absence of the furan ring protons and the presence of three new signals at δ 5.12 (d), δ 6.08 (d), and δ 6.98 ppm (s). On D_2O exchange, the doublet at δ 5.12 disappeared and the doublet at δ 6.08 collapsed to a singlet. An IR spectrum showed the presence of an OH absorption (3418 cm^{-1}) and several additional absorptions in the carbonyl region (~1764 cm^{-1}) compared with nimbin. These data all fit the conversion of the furan ring of nimbin to a 21-hydroxy-20(22)-ene-21,23-δ-lactone. This compound is named nimbinolide (**3b**).

The second photoproduct from nimbin, eluting after ~16 min (Figure 2d), had a ^1H NMR spectrum that showed many of the resonances of nimbin, but again the furan ring signals were absent and the signals of the C-15, C-16, and C-17 protons were broadened. There were new signals at δ 5.95 (s) and two very broadened signals at δ 5.65 and δ 5.77. The IR spectrum showed a broad OH absorption (3450 cm^{-1}) and there was an additional absorption at 1638 cm^{-1}. The CI mass spectrum gave an ion at *m/z* 590 ($M+NH_4^+$) corresponding to a molecular mass of 572. From this evidence, the compound was identified as isonimbinolide (**3c**).

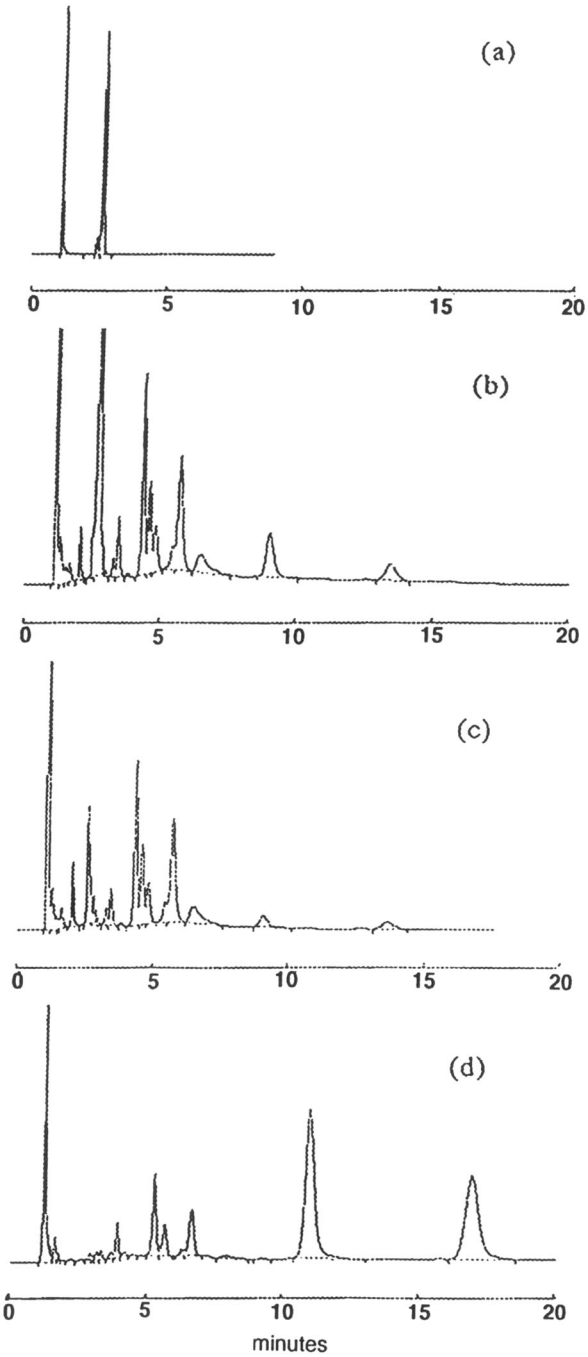


FIG. 2. SFC chromatograms from the UV irradiation reaction of nimbin in an oxygen atmosphere: (a) before reaction; (b) after 10 min; (c) after 20 min; and (d) after passing the products through silica gel of a Chromatotron plate.

Salannin. The progress of the reaction was followed in the same way as for nimbin, monitoring the reaction using SFC. Salannin eluted at ~3.5 min, and after irradiation for 15 min there was an increase in more polar compounds eluting between 5 and 11 min, although most of the salannin remained unreacted. After 30 min essentially all of the salannin had reacted, leaving three major reaction products (t_R ~ 5.0, 5.9, and 6.6 min).

Chromatotron separation of the reaction products showed them to be unstable and degraded on silica gel. A compound that formed during chromatography was isolated and its CI mass spectrum gave an ion at *m/z* 646, corresponding to a molecular mass of 628, i.e., 32 mass units more than salannin. The ^{1}H NMR spectrum of the purified compound (Table 1) was essentially unchanged from that of salannin (Kraus et al., 1987), except that there was an absence of the furan ring protons and the presence of three new signals at δ 5.36 (d), δ 5.97 (d), and δ 6.77 ppm (s). On D_2O exchange, the doublet at δ 5.36 disappeared and the doublet at δ 5.97 ppm collapsed to a singlet. An IR spectrum showed the presence of an OH absorption (3322 cm^{-1}) and an additional carbonyl group (1768 cm^{-1}) not present in salannin. These results all fit with the previously reported compound **2b** except the position of the OH absorption in the ^{1}H NMR spectrum was not quoted by either Purushothaman et al. (1984) or Garg and Bhakuni (1984). The OH signal is shifted further downfield than seen in similar γ-hydroxybutenolide ring structures (Siddiqui et al., 1986a,b).

The mechanism for the conversion of the furan ring to a γ-hydroxybutenolide is thought to occur via an intermediate ozonide-like peroxide, formed by a [4p + 2p] cycloaddition reaction between the furan ring and singlet oxygen (Foote et al., 1967). To show that singlet oxygen was involved in the reaction, the photooxidation of salannin was repeated in the presence of ~10% (w/w of salannin) of 1,4-diazabicyclo[2.2.2]octane (DABCO), a singlet oxygen quencher (Kearns, 1971). The reaction was not stopped completely, but the reaction was slowed, so that 60 min was required for all the salannin to react (Figure 3). A small amount (~10 mg) of a second compound was isolated from the reaction mixture and tentatively identified as isosalanninolide (**2c**). It was proposed that DABCO was acting as a hindered base and removing the less hindered proton from the intermediate (**2d**). To determine whether a bulky base would promote the formation of more isosalanninolide, the irradiation of salannin was repeated with various amines, of which triethylamine gave the best results. With triethylamine, isosalanninolide (22 mg) was formed preferentially over salanninolide (16 mg). The ^{1}H NMR spectrum of isosalanninolide (Table 1) gave three new signals at δ 5.41 (s), δ 5.88 (d), and a broad singlet at δ 5.96. The IR spectrum showed OH absorption (3450 cm^{-1}) and carbonyls at 1761 and 1741 cm^{-1}.

Neem Extract. Since salannin and nimbin are both readily photooxidized in a pure state, their photooxidation in a crude extract of triterpenoids from neem seeds was investigated. The SFC chromatogram of the extract before irradiation

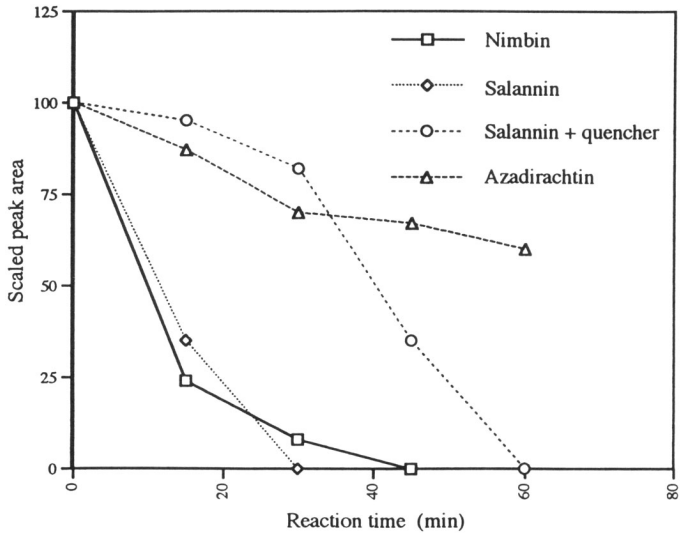


FIG. 3. The rate of photooxidation for azadirachtin (**1**), salannin (**2**), salannin + DABCO quencher, and nimbin (**3**) in an oxygen atmosphere.

showed nimbin eluting at 2.6 min, salannin at 3.6 min, and azadirachtin at 13.6 min. After a 10-min irradiation with oxygen, all the nimbin and salannin had disappeared, leaving an increased number of new compounds eluting before azadirachtin, which itself was essentially unchanged. The chromatogram at 10, 20, and 30 min of irradiation was about the same, although many of the peaks had decreased in height. The crude mixture from irradiation was passed across a Chromatotron plate, and the SFC chromatogram showed two new compounds eluting at 9.3 and 12.4 min. These were identified by cochromatography as nimbinolide and salanninolide. There was evidence of the presence of isonimbinolide, eluting as a shoulder on the peak of azadirachtin. The proportion of isosalanninolide expected would have been too small to observe. It is noteworthy that there was no evidence of 1-angeloylazadirachtin (**4**) (Johnson et al., 1994) being formed on this time scale.

Biological Activity. The photooxidation products of nimbin and salannin and the photoisomerized product of azadirachtin differed in their effects on insects. Overall, isonimbinolide and isosalanninolide were the most active of the photooxidation products tested (Tables 2 and 3; Figures 4 and 5). Isonimbinolide and isosalanninolide were potent antifeedants against *S. littoralis* and, like azadirachtin, they retained their antifeedant activity over the range of concentrations tested (Figure 4). Overall there was a positive relationship between the neural responses from the medial styloconic sensilla and the behavioral responses to the eight compounds tested ($R^2 = 0.532$, $P < 0.01$; Figure 5).

TABLE 2. EFFECT OF COMPOUNDS ON FEEDING BEHAVIOR OF *Spodoptera littoralis*, *Schistocerca gregaria*, and *Locusta migratoria*[a]

Compound	Concentration (M) required to give AI_{50}		
	S. littoralis	*S. gregaria*	*L. migratoria*
Azadirachtin	4.4×10^{-9}	4.1×10^{-7}	4.1×10^{-6}
Angeloylazadirachtin	1.1×10^{-6}	3.4×10^{-4}	2.0×10^{-2}
Salannin	2.6×10^{-5}	2.0×10^{-3}	$>1 \times 10^{-2}$
Salanninolide	$>1 \times 10^{-2}$	2.1×10^{-3}	$>1 \times 10^{-2}$
Isosalanninolide	2.9×10^{-9}	nt	nt
Nimbin	1.4×10^{-2}	nt	nt
Nimbinolide	9.1×10^{-5}	nt	nt
Isonimbinolide	1.7×10^{-8}	2.0×10^{-5}	1.3×10^{-5}

[a] AI_{50} represents the concentration of compounds required to elicit an antifeedant index of 50% in a choice test (estimated from regression analysis). Compounds tested from 10^{-4} to 10^{-9} M, N = 5–20, nt = not tested.

Thus the medial sensillum contains a neuron responsive to azadirachtin (Simmonds et al., 1995), and it was also responsive to isonimbinolide and isosalanninolide. Both these iso compounds had similar arrangements of the functional groups in the butenolide rings, and both showed very potent antifeedant activity (Table 2, Figures 4 and 5). The behavioral and neural data (Figure 5) suggest that the arrangements of the lactone and hydroxyl groups in the butenolide ring are critical for the biological activity of the photooxidation products of nimbin and salannin (Figure 1). Why the iso compounds should give the greater activity is unknown, but the same effect has been observed with similar compounds (Siddiqui et al., 1986a).

The antifeedant activity of azadirachtin recorded in this experiment against *S. littoralis* was lower than expected (Simmonds et al., 1995). Whether this was due to variations among generations of insects or some other cause is not known. In order to investigate whether the activity of isonimbinolide would vary among generations, five generations of *S. littoralis* larvae were tested with isonimbinolide. The same batch of compound was used throughout, but the solutions were freshly prepared for each generation. The results show that there was significant variation in activity among generations, but the compound was always very active (Table 4). Isonimbinolide also showed potent antifeedant activity against both species of locust (Table 2). However, we did not have enough isosalanninolide to compare its activity with isonimbinolide against the locusts.

Azadirachtin caused mortality by growth inhibitory effects in all three bioas-

TABLE 3. EFFECTS OF COMPOUNDS ON GROWTH OF *Spodoptera littoralis*, *Schistocerca gregaria*, and *Locusta migratoria*[a]

Compound	*Spodoptera littoralis*			*Schistocerca gregaria*			*Locusta migratoria*		
	Topical	Cannulation	Injection	Topical	Cannulation	Injection	Topical	Cannulation	Injection
Azadirachtin	15	10	12	45	40	35	35	25	20
Angeloylazadirachtin	>250	>250	>250	134	100	149	>250	>250	>250
Salannin	>250	>250	>250	>250	>250	>250	>250	>250	>250
Salanninolide	>250	>250	>250	>250	>250	>250	>200	>250	>250
Isosalanninolide	99	nt	nt	>250	nt	nt	nt	nt	nt
Nimbin	>250	>250	>250	nt	nt	nt	nt	nt	nt
Nimbinolide	150	100	105	nt	nt	nt	nt	nt	nt
Isonimbinolide	30	12	10	nt	nt	nt	68	nt	>250

[a]Values represent the LD_{50} dose in $\mu g/g$ required to kill 50% of the insects (estimated from Probit analysis). Compounds were tested from 1 $\mu g/g$ to 100 $\mu g/g$, $N = 10–50$. nt = not tested.

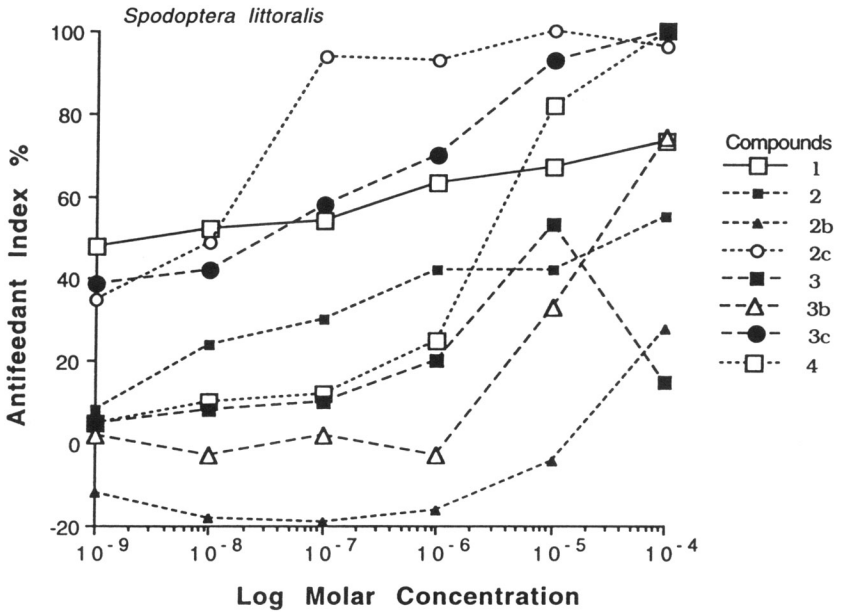


FIG. 4. Effects of different concentrations of the compounds on the feeding behavior of *Spodoptera littoralis* larvae. Responses reflect the mean antifeedant index calculated from data obtained in the no-choice test. For clarity the SEM associated with each mean has not been plotted, they vary from 0 to 38%. Antifeedant index means between +20 and −20 often had high SEM values, reflecting the behavioral variability among larvae (N = 5–20 per concentration). Compound numbers correspond to those in Figure 1.

says against the three insect species. *S. littoralis* was more responsive to azadirachtin than *L. migratoria* or *S. gregaria* (Table 3). 1-Angeloylazadirachtin, the photo-isomerized product of azadirachtin was only active against *S. gregaria* and showed greatest activity after cannulation into the gut (Table 3). Salannin and salanninolide did not show any growth inhibitory effect in the experiments, although isosalanninolide caused mortality to *S. littoralis* larvae after topical application. Nimbin was only tested against *S. littoralis*, and it caused no mortality, whereas both its photooxidation products increased larval mortality, and isonimbinolide was as active as azadirachtin in all three bioassays. Isonimbinolide caused mortality to *Locusta* nymphs when applied topically but not when injected. The differences in activity of these compounds against *S. littoralis* suggest that further experiments with isonimbinolide and isosalanninolide should be undertaken to establish their range of activity.

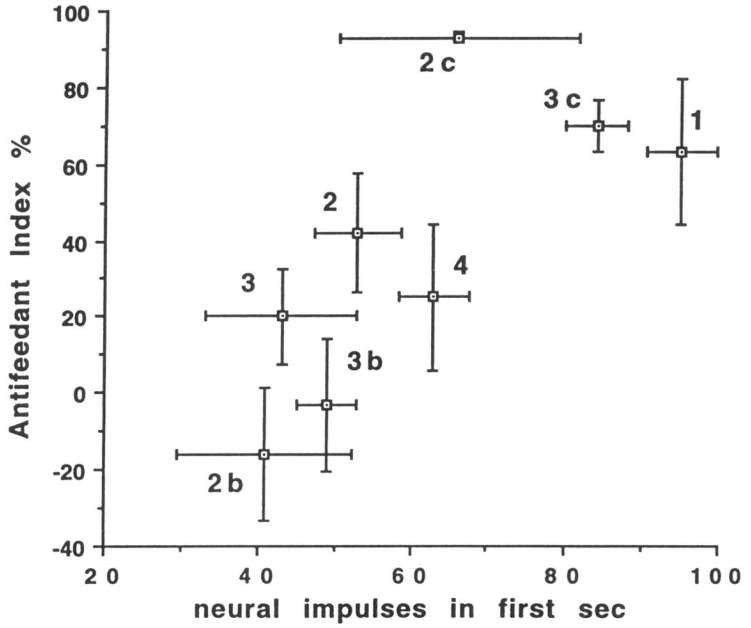


FIG. 5. Relationship between the neural and behavioral responses of *Spodoptera littoralis* larvae to the compounds. Responses (impulses in the first second; mean ± SEM; N = 4–6) from the medial styloconic sensilla to stimulation with 1×10^{-6} M of each compound are compared with the behavioral response to the same concentration (antifeedant index; mean ± SEM; N = 10). Compound numbers correspond to those in Figure 1.

TABLE 4. VARIABILITY IN BEHAVIORAL RESPONSES OF DIFFERENT GENERATIONS OF *Spodoptera littoralis* TO ISONIMBINOLIDE

Generation	AI_{50}[a]	Antifeedant index (mean ± SEM)[b]
1	1.7×10^{-8} a	70 ± 6.2 a
2	1.3×10^{-10} b	100 ± 0.0 b
3	3.6×10^{-11} b	69 ± 3.7 a
4	3.1×10^{-10} b	95 ± 0.2 b
5	8.4×10^{-10} b	100 ± 0.0 b

[a] AI_{50} represents the concentration (molar) of compounds required to elicit an antifeedant index of 50% in a choice test (estimated from regression analysis). Compounds tested from 10^{-6} to 10^{-10} M except for generation 1, which was tested with 10^{-4} to 10^{-9} M, N = 5–20 per concentration.
[b] Antifeedant index (mean ± SEM; N = 10) of larvae to glass-fiber discs treated with 10^{-6} M solutions are also presented. Among generations, differences in the AI_{50} and AI to 10^{-6} M were compared (ANOVA, Minitab); different letters within a column indicate the values differ significantly ($P < 0.01$).

TABLE 5. DEGRADATION OF SALANNIN (2) AND AZADIRACHTIN (1) BY UV LIGHT OR SUNLIGHT UNDER VARIOUS CONDITIONS

Compound	Light source	Light intensity (w)	Dist. from light (m)	Time (hr)	% Degraded	References
Salannin[a]	UV	[c]	0.1	0.17	20	Ermel et al. (1987)
Salannin[a]	UV	[c]	0.1	14	95	Ermel et al. (1987)
Salannin[a]	UV	90	0.0[d]	0.17	100	Johnson (1995)
Salannin[b]	UV	450	[c]	3	100	Purushothaman et al. (1984)
Salannin[b]	UV	90	0.0[d]	0.5	100	Johnson (1995)
Azadirachtin[a]	UV	[c]	0.1	14	65	Ermel et al. (1987)
Azadirachtin[a]	UV	90	0.0[d]	0.5	0	Johnson (1995)
Azadirachtin[b]	UV	90	0.0[d]	24	78	Johnson (1995)
Azadirachtin[b]	UV	30	1.8	200	95	Barnby et al. (1989)
Azadirachtin[b]	sun	[c]		4	20	Ermel et al. (1987)
Azadirachtin[b]	sun			168	50	Stokes and Redfern (1982)
Azadirachtin[b]	sun	0.0142[e]		192	75	Sundaram and Curry (1996)

[a] In crude extract.
[b] Pure compound.
[c] Not quoted in paper.
[d] Light source placed directly into the reaction mixture.
[e] W/cm^2 at the irradiated surface.

CONCLUSIONS

Both nimbin and salannin are photooxidized to a mixture of two isomeric hydroxybutenolides. Both are more rapidly oxidized under these conditions than azadirachtin and more rapidly when in a crude mixture than in the pure state. The latter is probably a result of a higher concentration of singlet oxygen stimulated by other components in the mixture. The peroxide intermediate (**2d**), formed during the photooxidation reaction can rearrange to form mono- and bisepoxides that, under acidic conditions of silica gel chromatography, can be converted to hydroxybutenolides (Takeshita et al., 1973). These epoxides may have been formed during the reaction, and observed by SFC, but subsequently rearranged on attempted isolation. Therefore the mono- and bisepoxides may be regarded as the true photooxidation products of nimbin and salannin, with the hydroxybutenolides as mainly formed during chromatography.

It is evident that any insecticidal preparation made from neem triterpenoids would require the addition of UV blockers in the formulation. Sundaram and

Curry (1996) have recently demonstrated the remarkable effectiveness of two UV absorbers on a neem insecticide preparation. However, we believe the UV spectra of their compounds and the conclusions drawn from them may be in error. We suggest that the compound detected, but not isolated, by Barnby et al. (1989) from irradiating azadirachtin may be 1-angeloylazadirachtin.

Rates of Degradation. Previous studies concerning the photolysis of salannin and azadirachtin, (to our knowledge no studies have been carried out on nimbin) have shown the compounds to be unstable in both sunlight (Stokes and Redfern, 1982; Ermel et al., 1987) and UV light (Ermel et al., 1987; Purushothaman et al., 1985; Barnby et al., 1989; Sundaram and Curry, 1996). The results obtained by different experimenters, in general, cannot be directly compared due to different experimental conditions, such as sample concentration, UV light intensity, distance of light source from the reaction mixture, and different reaction times. However, previous studies have repeatedly shown that salannin, whether pure or within a crude extract, is more susceptible to photodegradation than azadirachtin.

In this study, except for possible minor variations in light intensity, all the reactions were carried out under identical conditions and, therefore, the rates of degradation can be compared. To present this graphically, the peak area corresponding to the amount of each compound present at time zero was normalized to 1 and the amounts of each compound present during the reaction scaled accordingly. With pure compounds in an oxygen atmosphere, salannin reacts completely and nimbin almost completely in just 30 min, showing that both these compounds are very readily degraded in UV light.

The results obtained for the salannin reaction in the presence of the singlet oxygen quencher DABCO clearly show a large decrease in degradation rate of the salannin, with 84% of the salannin still present in the reaction mixture after 30 min. Azadirachtin degrades at a much slower rate compared to nimbin and salannin and only degrades by 27% in 30 min and by 78% after 24 hr.

All the triterpenoids in whole seed kernels were stable over the 72-hr irradiation time. This is probably due to the lack of penetration by the UV light and oxygen through the seed, although it may partly be due to an antioxidant action of the neem oil (Stokes and Redfern, 1982). In contrast, exposing a crude methanol extract of the seeds to UV light and oxygen caused complete degradation of the nimbin and salannin in under 10 min, compared to 30 min in the case of the pure compounds. The azadirachtin was degraded to a lesser extent in the crude extract than when pure, suggesting that the nimbin and salannin may protect the azadirachtin from photodegradation.

Few compounds from neem or related plants have shown insecticidal activity comparable with azadirachtin. Nimbin and salannin are generally considered to be less active feeding (Blaney et al., 1990) and growth inhibitors (Simmonds et al., 1990) than azadirachtin. Yet they are the major tetranortriterpenoids in

the neem seeds from many areas (unpublished data). The ability to transform these compounds into highly active products is an interesting discovery. The structure–activity relationships among nimbin, salannin, and their photooxidation products warrants further investigation.

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SYSTEMIC INDUCTION OF TERPENOID ALDEHYDES IN COTTON PIGMENT GLANDS BY FEEDING OF LARVAL *Spodoptera exigua*

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Abstract—Pigment glands in cotton contain terpenoid aldehydes that are toxic and deterrent to feeding of several generalist lepidopteran insects. We hypothesized that previously observed systemically induced feeding deterrence may be associated with pigment glands. We conducted experiments to determine the dynamics and chemical nature of inducible feeding deterrents in leaves of cotton, *Gossypium hirsutum* L, to larvae of the beet armyworm, *Spodoptera exigua*. Production and/or filling of pigment glands was influenced by physiological age of Deltapine 90 cotton plants. In undamaged plants, successively formed leaves contained more pigment glands, up to the seventh or eighth true-leaf developmental stage. Feeding choice tests conducted one or seven days after initial feeding damage revealed that third instars of *S. exigua* consumed more of the two youngest leaves from control cotton plants than from plants whose two oldest leaves had been fed on previously for 24 hr by *S. exigua*. The preference for leaves from control plants was significant one day after initial damage and highly significant seven days after damage. Consumption of mature foliage (leaf immediately above initially damaged leaves) from control plants and damaged plants did not differ. More pigment glands were counted on the youngest leaf of damaged plants than on the youngest leaf of control plants one day after initial damage. HPLC analysis revealed greater amounts of hemigossypolone, heliocides 1 and 2 (H_1 and H_2), and total terpenoid aldehydes per gland in young foliage of damaged plants than control plants one day after initial injury. By seven days after initial injury, greater quantities of hemigossypolone and all heliocides except

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H_4 were detected in young foliage from damaged plants compared to control plants. Concentrations of H_1 per gland in young leaves from damaged plants increased the most of all terpenoid aldehydes measured (3.4× the amount found in leaves from control plants). Mature leaves from damaged plants did not contain more terpenoid aldehydes than mature leaves from control plants. We suggest that systemically induced feeding deterrence to *S. exigua* in young leaves of glanded cotton was due to increased amounts of terpenoid aldehydes in pigment glands.

Key Words—*Gossypium hirsutum*, cotton, *Spodoptera exigua*, beet armyworm, gossypol, heliocides, terpenoid aldehydes, pigment glands, systemic induction, plant defense, plant-insect interactions, plant resistance.

INTRODUCTION

Plants have evolved several means of chemical defense against herbivory. Insects attacking plants may face preformed (i.e., constitutive) allelochemicals with antibiotic effects, such as toxins or digestibility reducers, or others that alter host utilization behaviors to the benefit of the plant. In addition to preformed allelochemicals, herbivores may encounter allelochemicals whose production and/or release has been induced by their actions (e.g., feeding) or those of a previous generation on the plant. Induced allelochemicals may be expressed at the site of damage or systemically.

Cultivated upland cotton, *Gossypium hirsutum* L., possesses preformed allelochemicals with antibiotic effects, such as terpenoid aldehydes (Hedin et al., 1992), and expresses localized (Croxford et al., 1989) and systemic induced responses to herbivory (Karban and Carey, 1984; Alborn et al., 1996). Feeding by larvae of *Spodoptera exigua* Hübner and *Spodoptera littoralis* Boisduval on cotton plants resulted in systemic induction of feeding deterrents in undamaged portions of those plants (Alborn et al., 1996). Feeding deterrence was observed in choice tests where larvae fed preferentially on foliage from undamaged plants. In no-choice tests, young larvae starved to death rather than feed on foliage from previously damaged plants. Older larvae fed foliage from previously damaged plants grew more slowly than larvae fed foliage from control plants (Alborn et al., 1996).

The basis for the feeding deterrence observed in our previous study (Alborn et al., 1996) was unknown. However, observations made during the course of the experiments suggested the involvement of the foliar pigment glands. *Gossypium hirsutum* and other members of the malvaceous tribe Gossypieae have characteristic pigment glands in vegetative and reproductive tissues (Lukefahr and Fryxell, 1967). Terpenoid aldehydes, such as hemigossypolone, gossypol, and the sesterterpenoid heliocides, have been identified in glands from several *Gossypium* species, exhibiting both qualitative and quantitative differences within

the tribe (Altman et al., 1990). Young larvae of generalist-feeding insects such as tobacco budworm, *Heliothis virescens* (F.), and cotton bollworm, *Helicoverpa zea* Boddie, avoid eating pigment glands, thus avoiding ingestion of terpenoid aldehydes (Parrott et al., 1983; Hedin et al., 1991). When ingestion of terpenoid aldehydes is unavoidable through their incorporation into artificial diet, larvae exhibit antibiotic symptoms such as increased developmental time, increased mortality, and reduced larval and pupal weight (Bottger and Patana, 1966; Shaver and Lukefahr, 1969; Lukefahr et al., 1977; Montandon et al., 1986). It has been suggested that increasing constitutive levels via breeding or genetic engineering is possible and will prove useful in host-plant resistance (Williams et al., 1987). However, it has not been suggested previously that production of these allelochemicals may be systemically induced by insect feeding injury.

Knowing the importance of terpenoid aldehydes in pigment glands as toxins and feeding deterrents to generalist insects, we investigated the possibility that induction of feeding deterrence in cotton foliage (Alborn et al., 1996) was due to changes in quality or quantity of terpenoid aldehydes. We hypothesized that feeding damage may induce production and/or filling of pigment glands in developing young foliage. Alternatively, or perhaps in addition, qualitative or quantitative changes in terpenoid aldehyde content of glands may occur after feeding damage. The specific objectives of this study were: (1) to determine the density of pigment glands within leaves of cotton plants at various ages, and (2) to correlate feeding deterrence, as measured in feeding preference bioassays, with changes in gland density and gland terpenoid aldehyde content at two intervals after initial feeding damage.

METHODS AND MATERIALS

Plants. Deltapine 90 cotton plants were grown in a greenhouse in 4-liter pots filled with a commercial soil mix (MetroMix 220, Grace Sierra, Milpitas, California). Natural light was not supplemented and the light cycle ranged from 14L:10D to 12L:12D (80 $\pm$ 10% relative humidity, and 30 $\pm$ 10°C). Plants were watered as necessary and fertilized once at the first-true-leaf stage with 5 ml of slow-release-formulation fertilizer (Osmocote 14-14-14, Scotts-Sierra, Marysville, Ohio).

Insects. Beet armyworm larvae, *S. exigua*, were reared on an artificial diet, based on pinto beans, according to established methods (King and Leppla, 1984). Third instars were used in all experiments and were starved for 8 hr prior to experiments to encourage immediate feeding.

Foliar Pigment Gland Density Within Plants at Selected Developmental Stages. Twenty insect-free, undamaged cotton plants were harvested at each of

five developmental stages (2-, 3-, 4-, 5-, or 8-leaf). Lateral shoots were beginning to form in the leaf axils of 8-leaf-stage plants. The area of each true leaf was measured with a leaf area meter (model 3000A, Li-Cor, Lincoln, Nebraska). Two impressions were made with a 5.5-mm-diameter cork borer (area = 23.76 mm^2) on opposite halves of the abaxial surface. Pigment glands were counted within these circles using 16× magnification.

Infliction of Initial Feeding Damage on Cotton Plants. Plants used in experiments had six to eight true leaves and were 1 month old. Two thirds instars of *S. exigua* were placed on each of the two oldest leaves of 60 plants. Leaves with larvae were enclosed in perforated plastic bags (Ziploc vegetable bags, DowBrands, Indianapolis, Indiana) sealed with a pipe cleaner twisted gently around the leaf petiole. The two oldest leaves of 60 control plants received plastic bags and a pipe cleaner but no larvae. Larvae and bags were removed 24 hr later. Half the plants were used for feeding bioassays, gland counts, and terpenoid aldehyde analysis using high-pressure liquid chromatography (HPLC) 24 hr after larvae were removed (one-day bioassay). The remaining plants were used for feeding bioassays, gland counts, and HPLC analysis one week after larvae were removed (seven-day bioassay).

Feeding Preference Bioassays and Changes in Gland Densities. Plants used in feeding bioassays were matched for number and size of leaves. Leaves were cut and their petioles placed immediately in water-filled plastic 1.5-ml Eppendorf vials sealed with parafilm. Leaves were photocopied and arranged symmetrically in clear plastic rectangular boxes (24 cm long × 32 cm wide × 11 cm high) with lids. Three third instars were placed in the center of the leaf array. After 24 hr, larvae were removed and leaves were again photocopied. Leaf photocopies were scanned and imported into an imaging program (ImagePC alpha version 7, Scion Corporation, Frederick, Maryland) where area eaten and leaf area remaining were quantified.

Two feeding bioassays were set up at each time period (day 1 and day 7). In one, larvae were given a choice between two mature leaves: the leaf immediately above the two larvae-damaged leaves and the corresponding leaf from the control plant. In the other, larvae were given a choice among four young leaves: the two youngest leaves (Y1, youngest and Y2, next youngest) on the control and damaged plants. Both bioassays were replicated 15 times at each time period.

The remaining 30 plants were used for quantification of pigment glands and HPLC analyses. All undamaged leaves were removed from plants and measured with a leaf area meter. Glands were counted in two 23.76-mm^2 circles on the abaxial leaf surface. During gland counts, leaves were stored at 4°C. After all leaves had been processed, young (Y1 and Y2) and mature leaves (leaf immediately above initially damaged older leaves) were immediately frozen at

−70°C until preparation for HPLC analysis. The remaining leaves were discarded.

HPLC Analysis of Terpenoid Aldehydes. Young and mature leaves from control and damaged plants were prepared separately for HPLC analysis. Lyophilized frozen foliage was ground to a fine powder in a blender. For each sample, 0.5 g of dried foliage was extracted with 6 ml hexane–ethyl acetate (3:1) and 0.4 ml 10% HCl. Samples were extracted a second time with 4 ml hexane–ethyl acetate. The combined organic fraction was concentrated to dryness with N_2 and 4 ml of HPLC mobile phase was added [20.0% ethanol, 5.5% methanol, 14.5% isopropyl alcohol, 24.2% acetonitrile, 25.0% water, 4.6% ethyl acetate, 6.1% dimethylformamide, and 0.1% phosphoric acid; modified from Stipanovic et al. (1988)]. After 10 min in an ultrasonic bath, the solid residue was removed by filtering through a 0.45-μm nylon filter. Samples were analyzed on a Shimadzu LC-600 liquid chromatograph equipped with a UV–visible spectrophotometric detector set at 272 nm, and a 25-cm × 4-mm-ID C18 column (ODS2 8/5 packing, SGE, Austin, Texas). Mobile phase flow rate was 1.25 ml/min. Chemicals were quantified in terms of gossypol equivalents using commercially available gossypol (Sigma Chemical Company, St. Louis, Missouri). Five replicate extractions were analyzed for each sample by injecting 20 μl onto the column.

Gossypol was identified via coelution with commercially available gossypol. Hemigossypolone and the heliocides were tentatively identified based on comparison with published chromatograms (Stipanovic et al., 1988; Altman et al., 1990). To further support the assignments, HPLC peaks presumed to be hemigossypolone, H_1, and H_2 were collected and analyzed using HPLC-MS. Negative chemical ionization mass spectrometric analyses were performed using a Hewlett-Packard 5989A mass spectrometer. The mass spectrometer interfaced with a Hewlett Packard model 1050 HPLC pump via a Hewlett Packard 59980B particle beam LC-MS interface. The system was operated in the flow injection mode using a 20-μl injection loop, and the mobile phase was methanol with a flow rate of 0.4 ml/min. Particle-beam introduction was chosen because the thermal instability of these compounds precluded the use of direct-probe or GC-MS introduction. Negative chemical ionization was chosen because of its greater sensitivity for these compounds, which were virtually undetectable using conventional electron impact or positive chemical ionization.

Statistical Analyses. Gland density and leaf area were used to estimate total glands per leaf. Leaf area eaten in feeding preference bioassays was expressed directly as area eaten in square centimeters and as the proportion of each leaf eaten. Residual plots indicated that the variance increased with the mean, so area eaten was transformed via $\sqrt{(x + 0.01)}$ and proportion eaten was transformed via arcsine $\sqrt{x}$. Analysis of variance was performed with the MIXED

procedure of SAS (SAS Institute, 1995) using leaf area as a covariate. Quantities of cotton allelochemicals were compared using *t* tests. Data presented in tables and figures are nontransformed means with their standard errors.

RESULTS

Foliar Gland Density Within Plants at Selected Developmental Stages. Gland density (number per 23.76 mm^2) was greatest on the youngest leaf of each plant, regardless of the developmental stage of the plant (Figure 1A). This finding was expected, as the youngest leaves had small areas (Figure 1B) and were still expanding. The estimated number of glands per leaf, obtained by multiplying gland density and leaf area, indicated that individual leaves were formed with their final number of glands, and there was no trend for increase or decrease in number of glands on that leaf as plants grew (Figure 1C). Leaves 1–5 each contained statistically similar numbers of glands at each plant developmental stage (*F* tests; $P > 0.05$). However, within each developmental stage, successively younger leaves, up to the seventh leaf, had significantly more glands than the older leaves on the same plant (Figure 1C).

Feeding Preference Bioassays and Changes in Gland Densities. The area of young leaves consumed during the bioassay conducted one day after initial feeding damage did not differ significantly by treatment ($F = 3.65$; $df = 1,41$; $P = 0.0632$) or by leaf age ($F = 1.47$; $df = 1, 42$; $P = 0.2317$) (Table 1). However, the proportion of each young leaf eaten was influenced significantly by treatment ($F = 6.34$; $df = 1,42$; $P = 0.0157$) and by leaf age ($F = 16.27$; $df = 1,42$; $P < 0.001$) (Table 1). A significantly greater proportion of the oldest control leaf (Y2) was eaten compared to the other three leaf types (Table 1). During bioassays conducted seven days after initial feeding damage, treatment × leaf age interactions were significant for both the area of young leaves eaten ($F = 24.10$; $df = 1,41$; $P < 0.001$) and the proportion of each leaf eaten ($F = 20.88$; $df = 1,42$; $P < 0.001$) (Table 1). The interactions can be explained by our observation that larvae ate more of the Y2 leaf than of the Y1 on control plants, while Y1 and Y2 from the damaged plant were equally nonpreferred (Table 1). Larvae did not show a statistically significant preference between mature leaves from control or damaged plants either one or seven days after feeding injury (Table 1).

Pigment glands on the youngest leaves of control and damaged plants differed in appearance. Many glands on the youngest leaves of damaged plants were black, large in diameter, and easily punctured, yielding a viscous material. While some glands on control leaves were similar, most were pale to dark brown and appeared smaller in diameter and less full of material. The greater ease of seeing pigment glands on leaves from damaged plants compared to control plants probably accounted for the greater gland density recorded on leaves from dam-

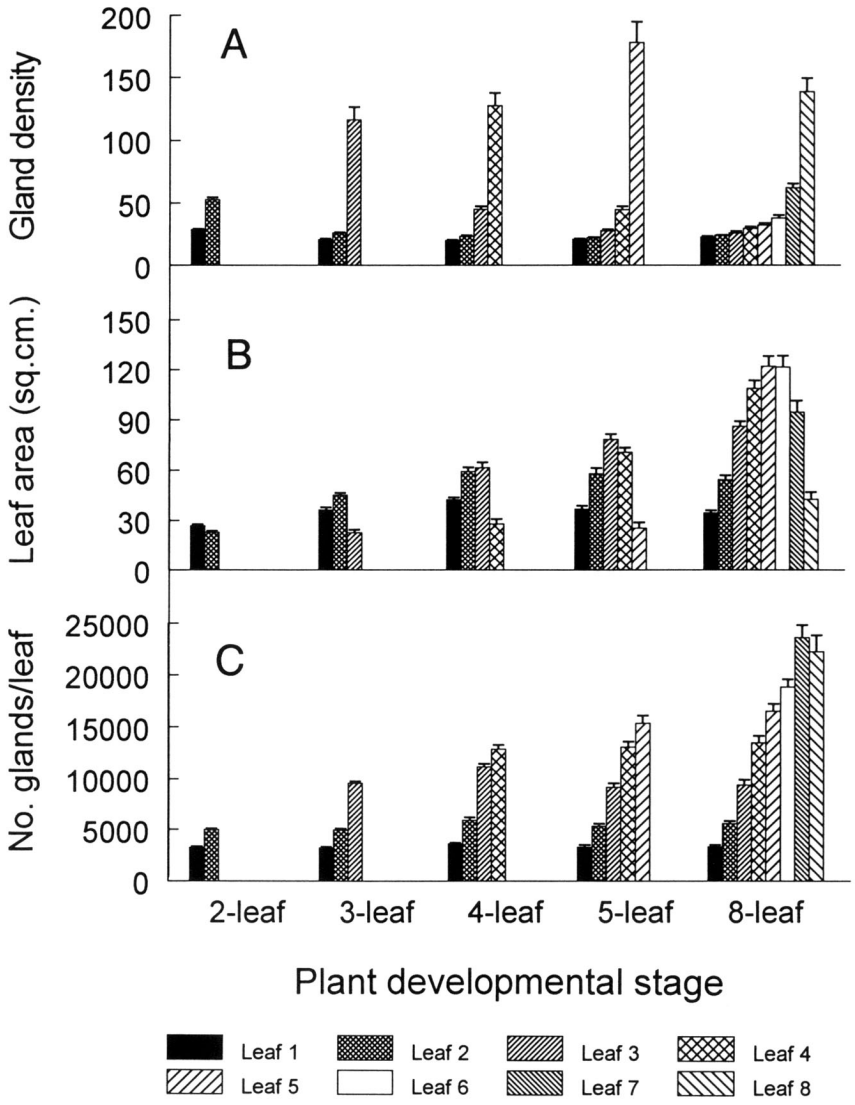


FIG. 1. (A) Average gland density (glands per 23.76 mm^2), (B) leaf area, and (C) estimated total glands per leaf on leaves of undamaged cotton plants at selected developmental stages. Leaf 1 is the oldest leaf on a plant. Histogram bars represent means of 20 plants and error bars indicate one standard error of the mean.

Table 1. 24-Hour Feeding Preferences of Third Instars of *S. exigua* Presented Choices Among Undamaged Leaves from Control and Previously Damaged Cotton Plants 1 or 7 Days After Damage

Day	Treatment	Leaf age[a]	Mean ± SEM	
			Leaf area consumed (cm^2)	Proportion of leaf eaten
1	Control	Y1	0.90 ± 1.83	0.014 ± 0.011
	Control	Y2	6.31 ± 1.80	0.073 ± 0.011
	Damaged	Y1	0.46 ± 1.82	0.001 ± 0.011
	Damaged	Y2	2.65 ± 1.85	0.030 ± 0.011
1	Control	Mature	17.69 ± 2.56	0.150 ± 0.020
	Damaged	Mature	14.91 ± 2.56	0.110 ± 0.020
7	Control	Y1	0.42 ± 0.59	0.007 ± 0.003
	Control	Y2	3.66 ± 0.52	0.039 ± 0.003
	Damaged	Y1	0.19 ± 0.62	0.001 ± 0.003
	Damaged	Y2	0.20 ± 0.70	0.003 ± 0.003
7	Control	Mature	8.81 ± 1.24	0.064 ± 0.009
	Damaged	Mature	6.46 ± 1.24	0.049 ± 0.009

[a]Youngest leaf, Y1, or second youngest leaf, Y2, on each plant. Mature leaf was immediately above the two larvae-damaged leaves on damaged plants and was the corresponding leaf on control plants.

aged plants. The eighth leaf (i.e., the youngest leaf) of plants that had been injured by larval feeding one day previously had significantly greater gland density than did the eighth leaf of control plants ($F = 14.01$; $df = 1,5$; $P = 0.0134$) (Figure 2A). Similarly, on a second set of plants whose pigment glands were counted seven days after feeding injury, damaged plants had significantly higher gland densities on the eighth leaf than did control plants ($F = 14.24$; $df = 1,10$; $P = 0.0036$) (Figure 2B). The eighth leaf of damaged plants also averaged more glands per leaf than the eighth leaf of control plants seven days after feeding injury ($F = 39.22$; $df = 1,10$; $P < 0.001$) (Figure 3B) but not one day after injury (Figure 3A). Leaf 5 on the control plants had significantly greater gland density ($F = 6.87$; $df = 1,13$; $P = 0.0211$) (Figure 2A) and more glands per leaf ($F = 5.78$; $df = 1,13$; $P = 0.0320$) (Figure 3A) than leaf 5 of damaged plants one day after feeding injury. This was not seen on plants examined seven days after injury.

HPLC Analysis of Terpenoid Aldehydes. Mass spectral fragmentation patterns of collected HPLC peaks supported their assignments as hemigossypolone, heliocide H_1, and heliocide H_2. The characteristic fragmentation patterns were

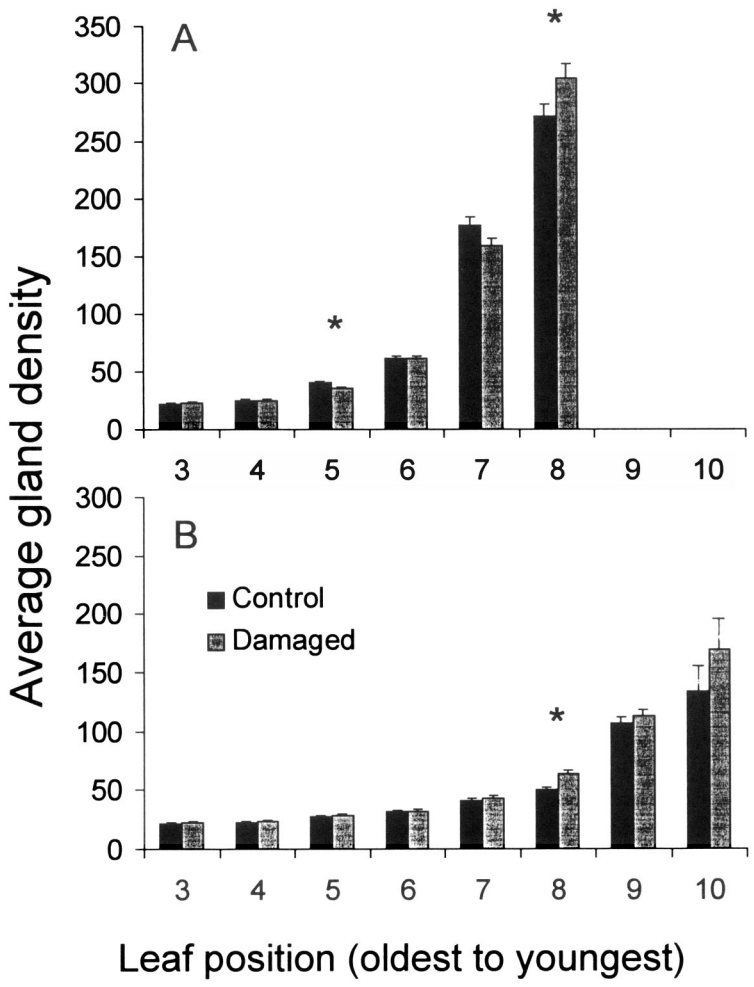


FIG. 2. Average gland density (glands per 23.76 mm^2) at various leaf positions within control cotton plants and plants that had been damaged by larval feeding either (A) one day or (B) seven days previously. Glands were not counted on leaves 1 and 2, which were subjected to the initial feeding damage. Plants examined at day 7 were not the same as those examined on day 1. Means were adjusted for leaf area as a covariate and bars indicate one standard error of the mean. Asterisks indicate those leaf positions where control and damaged plants differed significantly ($P < 0.05$).

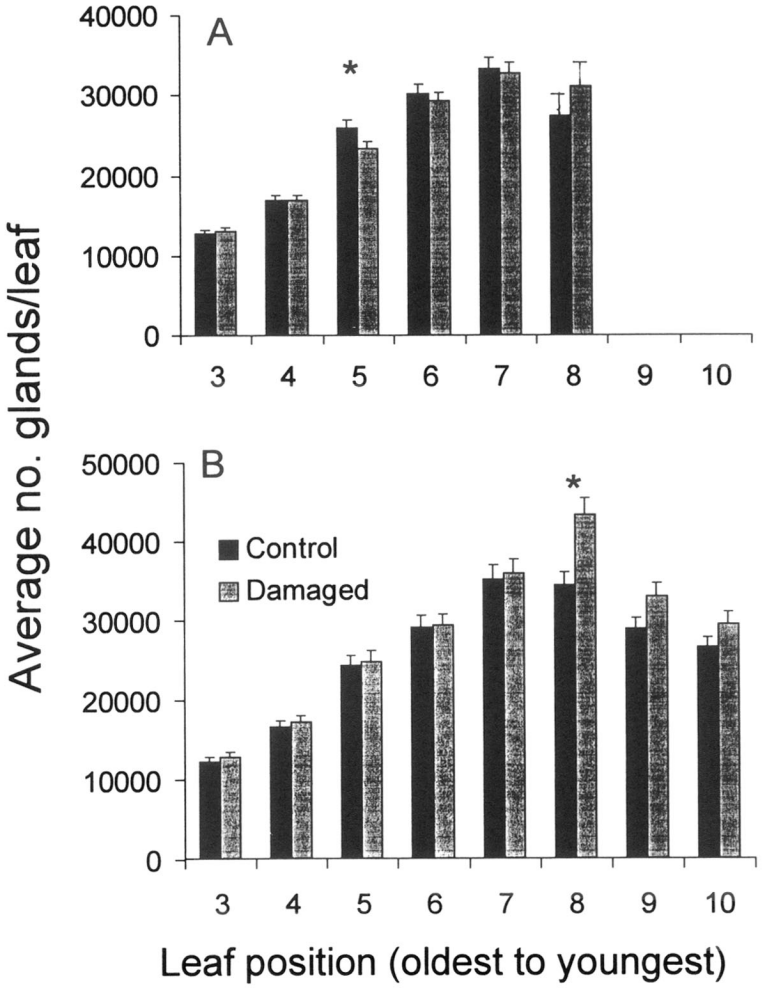


FIG. 3. Estimated total number of glands at various leaf positions within control cotton plants and plants that had been damaged by larval feeding either (A) one day or (B) seven days previously. Glands were not counted on leaves 1 and 2 that were subjected to the initial feeding damage. Plants examined at day 7 were not the same as those examined on day 1. Means were adjusted for leaf area as a covariate and bars indicate one standard error of the mean. Asterisks indicate those leaf positions where control and damaged plants differed significantly ($P < 0.05$).

as follows: hemigossypolone *m/z* (% relative abundance)—150 (15), 178 (32), 194 (100), 258 (12), 274 (12, M^-), 288 (5); H_1—150 (15), 178 (25), 194 (100), 410 (12, M^-); H_2—150 (16), 178 (6), 194 (100), 292 (1), 410 (17, M^-). The HPLC peak in extracts of young leaves from control plants that cochromatographed with gossypol was broader in extracts from damaged plants. The mass spectrum of the broad peak from damaged plants did not match that of gossypol, but suggested instead a terpenoid aldehyde of molecular weight 426. This could mean that the electronegativity of the unknown terpenoid so exceeds that of gossypol as to suppress detection of gossypol by electron depletion; it could also mean that the coelution of this peak with gossypol is coincidental and that the latter is not present. For this reason, pending further investigation, the peak that cochromatographs with gossypol is hereafter referred to simply as "gossypol coelutant."

Terpenoid aldehyde content of mature leaves from control and damaged plants did not differ significantly either one or seven days after feeding injury (Tables 2 and 3). Significant differences were apparent in young leaves, however. One day after feeding injury, hemigossypolone, H_1, H_2, H_3, and total terpenoid aldehydes were found in significantly greater quantities in young leaves of damaged plants than in control plants (Table 2). It was difficult to quantify H_4 because this heliocide was present in concentrations near the detection limit. Increases in chemical quantities in leaves from damaged plants ranged from 126%, for the amount of gossypol coelutant per square centimeter leaf area, to 19%, for the amount of H_2 per gland. Ratios among terpenoid aldehydes had also changed significantly within one day of feeding injury (Table 2). The ratio of hemigossypolone to all heliocides was significantly greater in damaged plants than in control plants. Likewise, the ratio of $H_1 + H_4$ to $H_2 + H_3$ was higher in damaged plants than in control plants.

Significantly greater quantities of all terpenoid aldehydes, except H_4, were detected in the young leaves that developed during the week following feeding injury than in young leaves from control plants (Table 3). Increases over chemical amounts present in control plants ranged from 338%, for the amount of H_1 per square centimeter leaf area, to 13%, for the amount of hemigossypolone per gland. Damaged plants had a significantly greater ratio of $H_1 + H_4$ to $H_2 + H_3$ than did control plants (Table 3), as was the case one day after injury (Table 2). However, seven days after injury, damaged plants had a lower ratio of hemigossypolone to heliocides than did control plants (Table 3), the opposite of what we observed one day after damage (Table 2).

DISCUSSION

We observed that foliar pigment gland production and/or filling changed over the course of development of Deltapine 90 cotton seedlings. Bell (1984)

TABLE 2. TERPENOID ALDEHYDE CONTENT OF UNDAMAGED MATURE AND YOUNG LEAVES FROM CONTROL COTTON PLANTS AND PLANTS DAMAGED 1 DAY PREVIOUSLY BY *S. exigua*

Terpenoid aldehyde[a]	Mature leaves			Young leaves		
	Control	Damaged	P^b	Control	Damaged	P^b
	Gossypol equivalents (ng/gland, mean ± SE)					
HGQ	13.5 ± 1.8	14.0 ± 0.9	ns	13.4 ± 0.53	22.0 ± 0.67	***
G coelutant	0.37 ± 0.06	0.40 ± 0.05	ns	0.19 ± 0.05	0.41 ± 0.06	*
H_4	0.19 ± 0.10	0.12 ± 0.06	ns	0.08 ± 0.04	0.15 ± 0.06	ns
H_1	0.93 ± 0.06	0.93 ± 0.12	ns	0.37 ± 0.02	0.63 ± 0.07	*
H_3	0.95 ± 0.07	1.21 ± 0.23	ns	0.32 ± 0.02	0.40 ± 0.03	ns
H_2	2.26 ± 0.21	2.21 ± 0.12	ns	0.77 ± 0.03	0.92 ± 0.04	*
TTA	18.2 ± 1.7	18.8 ± 1.0	ns	15.2 ± 0.55	24.5 ± 0.67	***
	Gossypol equivalents (ng/cm^2 leaf area, mean ± SEM)					
HGQ	1247 ± 164	1335 ± 90	ns	5121 ± 203	8702 ± 266	***
G coelutant	34.2 ± 6.0	37.8 ± 5.0	ns	71.4 ± 20.5	161.6 ± 22.4	*
H_4	18.0 ± 8.9	11.1 ± 6.2	ns	32.7 ± 13.5	59.8 ± 22.7	ns
H_1	86.2 ± 6.1	89.0 ± 11.3	ns	140.4 ± 7.7	248.4 ± 26.4	*
H_3	88.2 ± 6.8	115.9 ± 21.7	ns	123.9 ± 7.6	159.4 ± 11.7	*
H_2	209.2 ± 19.2	211.1 ± 11.0	ns	294.1 ± 10.2	363.1 ± 17.8	**
TTA	1683 ± 159	1800 ± 99.8	ns	5783 ± 211	9694 ± 265	***
	Ratios among terpenoid aldehydes (mean ± SEM)					
HGQ:H_4 + H_1 + H_2 + H_3	3.16 ± 0.50	3.15 ± 0.21	ns	8.68 ± 0.30	10.60 ± 0.62	*
H_1 + H_4:H_2 + H_3	0.36 ± 0.04	0.31 ± 0.04	ns	0.42 ± 0.03	0.59 ± 0.04	**

[a] Terpenoid aldehyde abbreviations: HGQ, hemigossypolone; G coelutant, gossypol coelutant; H_1 to H_4, heliocides 1–4; TTA, total terpenoid aldehydes (= sum of HGQ, G coelutant, and heliocides).

[b] Student's *t* tests were performed after ensuring that variances were equal (*F* test, $P > 0.05$). Means for control and damaged leaves were compared within an age group. Abbreviations for significance levels are as follows: ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

TABLE 3. TERPENOID ALDEHYDE CONTENT OF UNDAMAGED MATURE AND YOUNG LEAVES FROM CONTROL COTTON PLANTS AND PLANTS DAMAGED 7 DAYS PREVIOUSLY BY *S. exigua*

Terpenoid aldehyde[a]	Mature leaves			Young leaves		
	Control	Damaged	P^b	Control	Damaged	P^b
	Gossypol equivalents (ng/gland, mean ± SEM)					
HGQ	17.3 ± 1.5	18.8 ± 1.4	ns	41.2 ± 1.8	46.5 ± 1.2	*
G coelutant	0.58 ± 0.12	0.69 ± 0.18	ns	2.09 ± 0.26	3.89 ± 0.29	***
H_4	0.04 ± 0.04	0.00 ± 0.00	ns	0.36 ± 0.03	0.78 ± 0.22	ns
H_1	1.65 ± 0.11	1.87 ± 0.08	ns	6.09 ± 0.40	20.6 ± 1.0	***
H_3	2.08 ± 0.18	2.22 ± 0.12	ns	2.84 ± 0.21	5.14 ± 0.34	***
H_2	4.98 ± 0.41	5.45 ± 0.37	ns	6.77 ± 0.48	9.31 ± 0.59	**
TTA	26.6 ± 2.1	29.0 ± 2.2	ns	59.4 ± 3.2	86.2 ± 2.7	***
	Gossypol equivalents (ng/cm^2 leaf area, mean ± SEM)					
HGQ	1515 ± 136	1734 ± 134	ns	11590 ± 510	16998 ± 450	***
G coelutant	51.2 ± 10.2	63.6 ± 17.1	ns	589 ± 72	1422 ± 108	***
H_4	4.0 ± 4.0	0.0 ± 0.0	ns	102 ± 8.8	285 ± 79	ns
H_1	145 ± 10.0	172 ± 7.4	ns	1713 ± 113	7524 ± 382	***
H_3	182 ± 16	204 ± 11	ns	799 ± 59	1879 ± 123	***
H_2	437 ± 36	503 ± 34	ns	1903 ± 134	3402 ± 215	***
TTA	2335 ± 183	2677 ± 200	ns	16697 ± 891	31509 ± 996	***
	Ratios among terpenoid aldehydes (mean ± SEM)					
HGQ:$H_4 + H_1 + H_2 + H_3$	1.99 ± 0.17	1.97 ± 0.06	ns	2.58 ± 0.07	1.31 ± 0.05	***
$H_1 + H_4$:$H_2 + H_3$	0.24 ± 0.01	0.24 ± 0.01	ns	0.67 ± 0.01	1.48 ± 0.04	***

[a] Terpenoid aldehyde abbreviations: HGQ, hemigossypolone; G coelutant, gossypol co-elutant; H_1 to H_4, heliocides 1–4; TTA, total terpenoid aldehydes (= sum of HGQ, G coelutant, and heliocides).

[b] Student's *t* tests were performed after ensuring that variances were equal (*F* test, $P > 0.05$). Means for control and damaged leaves were compared within an age group. Abbreviations for significance levels are as follows: ns, not significant $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

indicated that constitutive defenses are often least expressed in the first true leaves and then increase in intensity with each leaf formed until maximum expression occurs between leaves 8 and 12. In our experiments, successively formed leaves on undamaged plants contained more pigment glands, up to the seven-true-leaf stage (Figure 1C). These data indicate the importance of choosing leaves matched for developmental age and size (due to greater gland density in unexpanded leaves, Figure 1A) for use in feeding bioassays if we suspect that glands may influence feeding behavior. Pigment glands, once formed, did not disappear nor were their contents visibly reduced as leaves aged. However, as revealed with HPLC analysis, the composition of terpenoid aldehydes present in glands of young and mature leaves differed (Tables 2 and 3).

Feeding for 24 hr by four *S. exigua* larvae on the two oldest true leaves of cotton plants caused no significant changes in mature leaves for any parameter measured (i.e., gland density, total glands, or quality and quantity of terpenoid aldehydes). Third instars of *S. exigua* did not prefer mature leaves from control plants to mature leaves from damaged plants in feeding bioassays (Table 1). These data are in contrast to those of Alborn et al. (1996), who found that feeding for 16 hr by two third instars of *S. littoralis* on the oldest true leaf of a cotton plant caused third instars to almost completely reject mature leaves from the previously damaged plant when they were presented to them five days later in a choice feeding bioassay. No obvious explanation accounts for this difference, except that different species of *Spodoptera* were used in these two experiments.

In contrast to mature leaves, previous feeding injury on the two oldest leaves of cotton seedlings caused profound differences in the young leaves developing on these plants compared to young leaves on control undamaged plants. Gland density was significantly greater on the youngest leaf of plants that had been fed on one day previously than on control plants (leaf 8, Figure 2A). One week after initial feeding damage, the eighth leaf on damaged plants still had significantly greater gland density (Figure 2B) and total glands per leaf (Figure 3B) than the eighth leaf on control plants. Leaf 7 had the maximum number of glands per leaf on the control plants whereas the maximum gland number had been shifted to leaf 8 on the damaged plants (Figure 3B). Total glands per leaf declined on leaves 9 and 10 and did not differ significantly between damaged and control plants (Figure 3B).

Feeding deterrence was observed in young leaves one day after 24 hr of continuous feeding damage had ceased (one-day bioassay, Table 1). This is similar to the deterrence observed in feeding bioassays induced by either 6 or 30 hr of continuous feeding by *S. exigua* (Alborn et al., 1996). Deterrence was even greater in young leaves that developed during a one-week period after the cessation of feeding (seven-day bioassay, Table 1). In general, for both control and damaged plants, Y1 was less preferred than Y2, probably due to the greater

gland density. In addition, Y2 from damaged plants was less consumed than Y2 from control plants, again due possibly to greater gland density and/or to greater allelochemical content. Larvae probably could not avoid biting into these glands when they were so densely arranged. Many studies have indicated that early instars of *H. virescens* avoid feeding on pigment glands (Parrott et al., 1983, 1989; Hedin et al., 1991), as does the cotton leafworm, *Alabama argillacea* (Hübner) (Johnson, 1984). There are no published reports indicating that *S. exigua* larvae avoid feeding on pigment glands, but larvae do preferentially feed on glandless varieties of cotton (Bottger et al., 1964; McAuslane and Alborn, unpublished data).

Feeding deterrence observed in Y2 from damaged plants could perhaps be explained by gland density but was more likely due to significantly greater amounts of terpenoid aldehydes in these glands than in glands on control plants (Tables 2 and 3). Within one day of cessation of feeding injury, some unknown signal had elicited quantifiable changes in allelochemicals more than four leaves distant from the site of initial feeding injury. Hemigossypolone and all of the heliocides, except H_4, were more abundant in young leaves from damaged plants on both per gland and per square cm leaf area bases (Table 2). Young leaves formed during the seven-day period after cessation of feeding damage also contained significantly greater quantities of terpenoid aldehydes than the young leaves on control plants (Table 3).

Many studies have indicated antibiotic effects of gossypol on *H. virescens*, *Pectinophora gossypiella* (Saunders) (Shaver and Lukefahr, 1969; Shaver and Parrott, 1970), and *S. exigua*, *H. zea*, and *Trichoplusia ni* (Hübner) (Bottger and Patana, 1966). These effects included increased developmental time, reduced larval and pupal weight, and reduced survival to adulthood. Originally it was thought that gossypol was the only terpenoid aldehyde responsible for these effects. However, when hemigossypolone and the sesterterpenoid heliocides were identified from pigment glands, feeding assays revealed the following reduction in growth of larval *H. zea*, from most to least growth reducing: gossypol > H_1 > hemigossypolone > H_2 (Lukefahr et al., 1977; Stipanovic et al., 1977a). The feeding deterrence of individual terpenoid aldehydes is not known. However, if deterrence is related to toxicity, it is significant that H_1 was more induced in foliage from damaged cotton plants than were H_2 and the biosynthetically related H_3. One day after feeding injury, the ratio of $H_1 + H_4$ to $H_2 + H_3$ was 0.59 in damaged plants and 0.42 in control plants (Table 2). Seven days after injury, the ratio was 1.48 in damaged plants and 0.67 in control plants (Table 3).

It is not possible to compare statistically allelochemical concentrations on day 1 and day 7; however, comparisons between control and damaged plants at each time period can give us an idea of the time course of induction of the terpenoid aldehydes. In cotton plants, hemigossypol (not quantified in these

experiments) is either dimerized via a peroxidase to gossypol (Veech et al., 1976) or oxidized by an unknown enzyme to hemigossypolone (Bell and Stipanovic, 1976). In tissue exposed to light, such as leaves, conversion to hemigossypolone is more prevalent than conversion to gossypol (Bell and Stipanovic, 1976). Hemigossypolone then reacts via Diels-Alder recombinations with either *trans*-β-ocimene to produce H_1 and H_4 (Stipanovic et al., 1978a) or with myrcene to produce H_2 and H_3 (Stipanovic et al., 1977b, 1978b). In young leaves, hemigossypolone is more abundant than the heliocides (Bell and Stipanovic, 1976). However, as individual leaves age, hemigossypolone reacts with available diene monoterpenes to produce a greater abundance of heliocides (Bell and Stipanovic, 1976). One day after cotton plants were injured in our experiments, young leaves had a significantly higher ratio of hemigossypolone to heliocides than did leaves from control plants (Table 2). This may indicate an increase in synthesis of hemigossypolone in damaged plants. However, seven days after damage, young leaves from injured plants had a lower ratio of hemigossypolone to heliocides than did leaves from control plants (Table 3). This decline in hemigossypolone relative to heliocides likely reflects the reaction of hemigossypolone with diene monoterpenes that were increased in availability after feeding damage.

It is known that only certain monoterpenes in cotton are emitted in increased amounts after damage by insect herbivores. Increased emission of β-ocimene, but not myrcene, was induced within three days of feeding by *S. exigua* on Deltapine 90 (Loughrin et al., 1994). The induced production of β-ocimene was systemic in nature because the monoterpene was released from undamaged portions of damaged plants (Röse et al., 1996). In addition, increased emission was due to de novo synthesis of β-ocimene rather than synthesis from preformed building blocks (Paré and Tumlinson, 1997). This increased pool of β-ocimene likely led to the increased production of H_1 in damaged cotton seedlings in our experiments. Since myrcene emission is not known to be increased after larval feeding injury, H_1 was produced in greater amounts in damaged plants than were H_2 and H_3 (Tables 2 and 3).

It has not been demonstrated previously that insects can systemically induce production of terpenoid aldehydes in cotton. However, localized induction of terpenoid aldehydes has been shown in cotton attacked by root knot nematodes (Khoshkhoo et al., 1994) or treated with certain plant growth regulators (Hedin and McCarty, 1991; Khoshkhoo et al., 1993). Similarly, cotton infected with pathogens produces increased amounts of biosynthetically related compounds, such as desoxyhemigossypol (Stipanovic et al., 1991) in fungal-infected tissue and 2,7-dihydroxycadalene and related sesquiterpenes (Essenberg et al., 1982) in bacterial-infected tissue. It is not known how these various localized induction phenomena are related to each other or to the systemic induction of terpenoid

aldehydes and cotton volatiles. There are many questions to pursue in the basic study of cotton plant responses to biotic stressors.

In addition to these interesting basic questions that must be addressed, we can think about the application of induced feeding deterrence to host-plant resistance. Bell et al. (1987) suggested that genetically increasing the ratio of β-ocimene to myrcene in cotton cultivars would increase plant resistance to herbivores due to increased levels of the more toxic H_1. Our data indicate that feeding damage to Deltapine 90 leads preferentially to induction of increased H_1 levels. One could screen germplasm and select for those genotypes that are most sensitive to feeding injury or that respond to feeding injury with a large production of terpenoid aldehydes. This could lead to cultivars that are capable of reacting strongly to herbivore attack when necessary but that do not bear the cost of producing these allelochemicals constitutively when they may never be needed.

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VARIATION OF FLORAL SCENT EMISSION AND POSTPOLLINATION CHANGES IN INDIVIDUAL FLOWERS OF *Ophrys sphegodes* subsp. *sphegodes*

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Abstract—We investigated the scent composition of individual flowers of *Ophrys sphegodes*, its alteration following pollination, and of picked flowers by day and at night. Odor samples were collected by headspace sorption and analyzed by gas chromatography and mass spectrometry. To evaluate the function of postpollination odor changes, we carried out behavioral tests on the pollinator *Andrena nigroaenea* with pollinated and unpollinated flowers. We identified 27 volatiles in the flower scents. Aldehydes and alkanes were most frequently found. Aldehydes were the most abundant class of compounds (40–50%). When flowers were picked, they emitted significantly lower total amounts of volatiles than unpicked flowers, and their odor bouquets were significantly different. Comparison of scents released by day and at night showed no decrease in scent emission during nighttime, but the odor bouquets were significantly different. Pollinated flowers produced significantly different odor bouquets, and the total amount of scent emitted two to four days after pollination was significantly lower compared with unpollinated flowers. In addition, behavioral tests with *A. nigroaenea* males showed that flowers were significantly less attractive three days after pollination. This reduced attractiveness is hypothesized to guide pollinators to the unpollinated flowers within an inflorescence, and thus increase the reproductive success of the plant.

Key Words—*Ophrys sphegodes* subsp. *sphegodes*, *Andrena nigroaenea*, floral scent, floral semiochemicals, headspace sorption, postpollination changes, nocturnal scent emission.

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INTRODUCTION

The Mediterranean orchid genus *Ophrys* is pollinated by means of sexual deception, mostly by male bees and wasps. Lured by the odor and insectlike shape of the flower, the pollinator alights on the labellum and attempts to copulate with it (Kullenberg, 1961). During these so called "pseudocopulations," the male contacts the pollinia, which become attached to its head or abdomen. Numerous behavioral tests have shown that the *Ophrys*–pollinator relationship is highly specific: each *Ophrys* species is pollinated by males of usually only one or a few pollinator species (Kullenberg, 1961, 1973; Paulus and Gack, 1986, 1994). These studies also showed that the flower's odor is the most important cue for eliciting male mating behavior and for ensuring the pollinator specificity in the male attracting communication signal.

In behavioral experiments it was shown that males of the sweat bee *Lasioglossum malachurum* (Kirby) use odor signals to learn the identity of female bees with which they have attempted to copulate, thereby avoiding these females in further encounters (Smith and Ayasse, 1987). In *Ophrys* flowers, pollinating male bees also learn the odor bouquet of a visited flower and subsequently modify their response to it. *Andrena nigroaenea* (Kirby) males learn the odors of individual *O. sphegodes* Miller flowers and avoid copulatory attempts in further visits (Ayasse et al., 1996). Although the flower odors of numerous *Ophrys* species have been chemically analyzed (Borg-Karlson, 1987, 1990; Borg-Karlson et al., 1985, 1987), none of the studies have addressed the variation of odor bouquets emitted by single flowers. Our experiments (Ayasse et al., 1996) suggest that there is some inter- and intraindividual variation within floral odor bouquets of *O. sphegodes* that may influence the behavior of the male pollinator.

In bees, postmating changes of the females' mate-attracting odor signals have been shown, which direct males to virgin females (Ayasse et al., 1993; Engels et al., 1993). Similarly, in *Ophrys* orchids, a decrease and alteration of floral odor bouquets after pollination might lead to a lower attractiveness of these flowers. Besides the advantage of reduced costs, this may be of selective value if one plant bears more than one flower, since the plants' overall reproductive success can be increased by directing pollinators to the unpollinated flowers of an inflorescence (Tollsten, 1993). Although some studies have documented postpollination changes in scent emission of orchids (e.g., Tollsten and Bergström, 1989; Tollsten, 1993), none dealt with *Ophrys*.

Few studies proved an alteration in floral volatile production of orchids after injury. In orchids of the genus *Cycnoches*, excised labella emit considerably altered fragrance profiles more than 8 hr after excision (Gregg, 1983). In *Ophrys* orchids, alteration of odor emission of picked flowers has not yet been investigated. However, behavioral tests showed that picked plants are generally still

attractive for pollinators over several days (Paulus and Gack, 1990). This first study dealing with changes of scent emission in picked *Ophrys* plants is a base for further scent investigations and behavioral experiments.

In orchids pollinated by nocturnal animals, an increased scent production during the night was reported (e.g., Nilsson, 1978; Kaiser, 1993). In contrast, apple flowers, which are primarily bee pollinated during the daytime, produce lower amounts of scent during the night (Loughrin et al., 1990). Since orchids of the genus *Ophrys* are also pollinated by day-active insects, we expect flowers to emit lower amounts of volatiles during the night versus the day. In the absence of pollinators, the production of biologically active fragrances can be regarded as redundant. This study is a first approach to understand changes in odor profiles emitted by *Ophrys* orchids during the night.

In order to obtain information about intraspecific variation of *Ophrys* flower odor emission under various conditions, the following aspects are addressed: (1) quality of odors—none of the recent studies dealing with odor emission of *Ophrys* flowers includes *O. sphegodes* subsp. *sphegodes*; (2) postpollination changes in flowers with respect to their odor and color and in terms of their attractiveness for pollinators; (3) effect of picking on odor emission; and (4) comparison of odor emission during day and night.

METHODS AND MATERIALS

Plant Material. Ophrys sphegodes subsp. *sphegodes* grows in the Mediterranean region and Central Europe. It is pollinated by *Andrena nigroaenea* males (Godfery, 1925; Kullenberg and Bergström, 1973; Paulus and Gack, 1986, 1990), a solitary bee species that is common in the area of our study. We investigated individual plants of *O. sphegodes* growing in the "Bisamberg" area in Lower Austria. Plants were dug up, numbered, and transferred to pots in the laboratory. The scent of 36 single flowers was collected over two weeks. In addition, 25 plants were picked and kept in glass tubes filled with water. These freshly picked plants were investigated over three subsequent days. The scent of 17 unpicked flowers that were hand pollinated immediately after anthesis was collected during the following four days. Furthermore, 17 picked flowers were left unpollinated and their scent was collected during the night (21:00–05:00 hr) in a completely dark room.

Flower Odor Headspace Sampling. For the collection of volatiles, single flowers were placed in glass tubes (approx. 5 ml) open at both ends, which were plugged with cotton wool. Air was pulled over the flowers (30 ml/min for 6–8 hr) by a battery-operated membrane pump. Air coming into the flower chamber was cleaned by an adsorbent tube filled with 120 mg activated charcoal (SKC). The air exited through a cartridge that contained 1.5 mg activated charcoal

(CLSA Filter) and onto which the volatiles were trapped. Contamination from the environment was checked by collecting volatiles from the laboratory air (control sample). The fragrance was eluted with 20 μl carbon disulfide (Merck Uvasol) according to the technique described in Habich and Grob (1984).

Chemical Analysis. Analyses of volatiles were carried out by combined capillary gas chromatography–mass spectrometry. A mass spectrometer VG 70-250 SE (Vacuum Generators) was linked to a gas chromatograph HP 5970 (Hewlett-Packard) equipped with a DB5 capillary column (30 m × 0.32 mm) operating splitless at 50°C for 30 sec, followed by opening the split and programming to 220°C at a rate of 4°C/min. Helium served as the carrier gas. A combination of a quadrupole mass spectrometer (Fisons MD 800) and a gas chromatograph (Fisons 8060) was also used. Compound identifications are based on comparison of mass spectra and gas chromatographic retention times of natural products with authentic reference samples. To determine the enantiomeric composition of 2-nonanol and *endo*-brevicomin, a capillary column (25 m × 0.25 mm) coated with a 1:1 mixture of octakis (6-*O*-methyl-2.3-di-*O*-pentyl)-γ-cyclodextrin and OV-1701 was used while hydrogen served as carrier gas. For determination of absolute amounts collected, *n*-octadecane was added as an internal standard to all samples. GC analyses were performed with a Hewlett-Packard 5890 Series II gas chromatograph under the conditions described above.

Behavioral Tests. In seven *O. sphegodes* plants from Bisamberg, one flower of an inflorescence was hand pollinated in the field, and plants were picked after three days. In *Ophrys* orchids, it can be easily determined if a flower is pollinated by checking the stigma for deposited pollen massulae. Behavioral experiments were then carried out in Oberweiden/Lower Austria, where *A. nigroaenea* males regularly patrol. In a dual-choice experiment, one pollinated and one unpollinated flower were offered together on the same stem for 3 min to *A. nigroaenea* males in a patrolling area. On three plants the pollinated flower was older than the unpollinated one, and on four plants it was reverse. Copulation attempts with the flower were recorded.

Postpollination Flower Color Change. Twenty flowers were pollinated in the field and their color was compared with 20 unpollinated flowers on the same plants. The color change of 10 flowers was observed after three days and that of another 10 flowers after seven and 10 days. Flowers with dark-brown color were regarded as unchanged, and flowers with a yellow-whitish appearance were regarded as wilted.

Statistics. Relative and total amounts of single compounds and of chemical classes of compounds were tested for significant differences between unpollinated and pollinated flowers, picked and unpicked flowers, and day and night scent emission of picked flowers by means of Mann-Whitney *U* test (nonnormal distributed data), *t* tests (normal distributed data), and stepwise discriminant

function analysis (Norusis, 1993). To approach normality and reduce heterogeneity of variances, all data for discriminant analysis were transformed by $\ln(1 + x)$. The standardized discriminant function coefficient was used to assess the importance of variables within the discriminant functions.

RESULTS

Odor of Unpicked Flowers. We identified 27 compounds in the headspace samples of *O. sphegodes* (Table 1, Figure 1). The volatiles consisted of a blend of nine straight-chain aldehydes, nine straight-chain alkanes, six terpenoids, and three nonane derivatives [nonanone, nonanol, and (−)-*endo*-brevicomin]. Aldehydes were the most abundant class of compounds, having a relative proportion of 40–50% in all samples. Among the aldehydes, nonanal and decanal were most prominent. 2-Nonanol was detected as a mixture of enantiomers in a ratio of $R:S = 85:15$. *endo*-Brevicomin was found to be represented by the almost pure (−) enantiomer. GC analysis used in this study was not able to separate the following substances: heptanal + nonane, octanal + decane, or 2-nonanol + undecane.

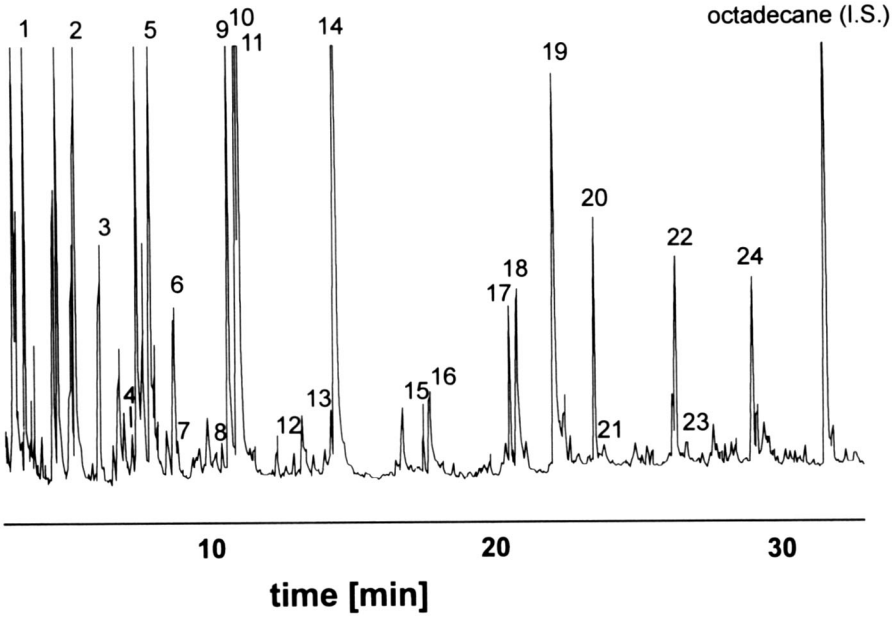


FIG. 1. Gas chromatogram of the headspace sample of an unpollinated, unpicked *Ophrys sphegodes* flower. For names of compounds, see Table 1.

TABLE 1. RELATIVE AMOUNTS (% ± SE) OF EACH VOLATILE COMPOUND IDENTIFIED IN FLORAL SCENT OF SINGLE *O. sphegodes* FLOWERS[a]

Compound[b]	Intact flowers[c]		Unpollinated flowers[c]	
	Unpollinated (N = 36)	2–4 days after pollination (N = 17)	Picked plants (N = 25)	Picked plants during the night (N = 17)
1 Hexanal	2.02 ± 0.37	2.30 ± 0.21*	1.95 ± 0.23	4.91 ± 0.38**
2 Heptanal + nonane	7.20 ± 0.66	8.27 ± 0.57	9.42 ± 1.01	11.30 ± 0.60*
3 α-Pinene[d]	3.50 ± 0.56	5.55 ± 1.26*	4.03 ± 0.82	9.81 ± 1.10**
4 6-Methyl-5-hepten-2-one	5.62 ± 0.51	8.01 ± 0.45*	7.93 ± 0.47*	14.68 ± 0.53**
5 Octanal + decan	2.54 ± 0.43	0.62 ± 0.34*	1.27 ± 0.37*	0.18 ± 0.18*
6 Limonene[d]	6.98 ± 1.14	4.75 ± 0.54	3.44 ± 0.27	7.62 ± 0.42**
7 1,8-Cineole[d]	0.90 ± 0.13	0.98 ± 0.13	1.03 ± 0.08	1.64 ± 0.06**
8 (−)-*endo*-Brevicomin	0.12 ± 0.10	0.04 ± 0.00*	0.03 ± 0.00*	0.06 ± 0.00**
9 2-Nonanone	8.00 ± 1.17	5.72 ± 1.25	3.92 ± 0.97*	2.89 ± 0.37
10 2-Nonanol[e] + undecane	8.02 ± 1.03	6.29 ± 0.91	5.50 ± 1.07	6.83 ± 0.62*
11 Nonanal	16.67 ± 2.09	18.55 ± 1.32*	20.48 ± 0.81**	12.41 ± 0.90**
12 Camphor[d]	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00**
13 Dodecane	0.60 ± 0.08	0.80 ± 0.06	0.76 ± 0.06	1.25 ± 0.04**
14 Decanal	16.92 ± 1.84	26.29 ± 2.37*	29.16 ± 2.11**	10.46 ± 1.64**
15 Tridecane	0.63 ± 0.09	0.60 ± 0.09	0.50 ± 0.06	1.20 ± 0.05**
16 Undecanal	0.39 ± 0.09	0.74 ± 0.17*	0.62 ± 0.15	0.35 ± 0.07
17 Tetradecane	2.20 ± 0.77	1.33 ± 0.11	0.99 ± 0.07	2.63 ± 0.09**
18 Dodecanal	0.96 ± 0.15	1.05 ± 0.23	1.06 ± 0.26	1.26 ± 0.23
19 Geranylacetone	1.75 ± 0.44	1.52 ± 0.43	1.21 ± 0.32	0.67 ± 0.34
20 Pentadecane	1.79 ± 0.22	1.59 ± 0.11	1.49 ± 0.09	2.64 ± 0.10**
21 Tridecanal	0.58 ± 0.14	0.80 ± 0.48	0.25 ± 0.07	0.66 ± 0.19*
22 Hexadecane	8.96 ± 3.15	1.91 ± 0.11*	1.87 ± 0.28*	3.26 ± 0.38**
23 Tetradecanal	1.87 ± 0.90	0.24 ± 0.09*	1.19 ± 0.43	0.74 ± 0.14
24 Heptadecane	1.90 ± 0.29	2.11 ± 0.10	1.93 ± 0.11	2.60 ± 0.17*

[a] Floral emission of pollinated flowers, cut flowers and emission during the night was compared with those of unpollinated flowers.
[b] Compounds listed in the order of retention times.
[c] Mann Whitney U test; *$P < 0.05$; **$P < 0.001$.
[d] Unknown enantiomeric composition.
[e] Enantiomers $R:S$ = 85:15.

Postpollination Changes in Floral Scents. Three groups of flowers were compared: unpollinated flowers, flowers one day after pollination, and flowers two to four days after pollination. Two to four days after pollination, the relative amounts of some hydrocarbons and 6-methyl-5-hepten-2-one decreased significantly, while those of most aldehydes and α-pinene increased (Mann-Whitney U test, $P < 0.05$, Table 1). A discriminant analysis revealed that there was a significant difference between the odor bouquets of unpollinated and pollinated flowers (discriminant function 1: $\chi^2 = 46.6$; $df = 8$; $P < 0.001$; discriminant function 2: $\chi^2 = 6.2$; $df = 3$; $P > 0.05$, Figure 2). According to the standardized discriminant function coefficients, dodecane, tridecane, undecanal, and 6-methyl-5-hepten-2-one contributed most to the discriminant function (i.e., they showed the largest values for the standardized canonical discriminant function coefficient).

The mean total amount of scent released by single flowers of unpicked, unpollinated plants was 66 $\pm$ 7 ng/hr/flower (Figure 3). Flowers 24 hr after pollination showed a slight decrease in the total amount of scent and flowers two to four days after pollination emitted significantly lower total amounts of volatiles compared to unpollinated flowers (t test, $t = 2.04$, $P < 0.05$, Figure 3). Almost all compounds were affected by this decrease, but the reductions

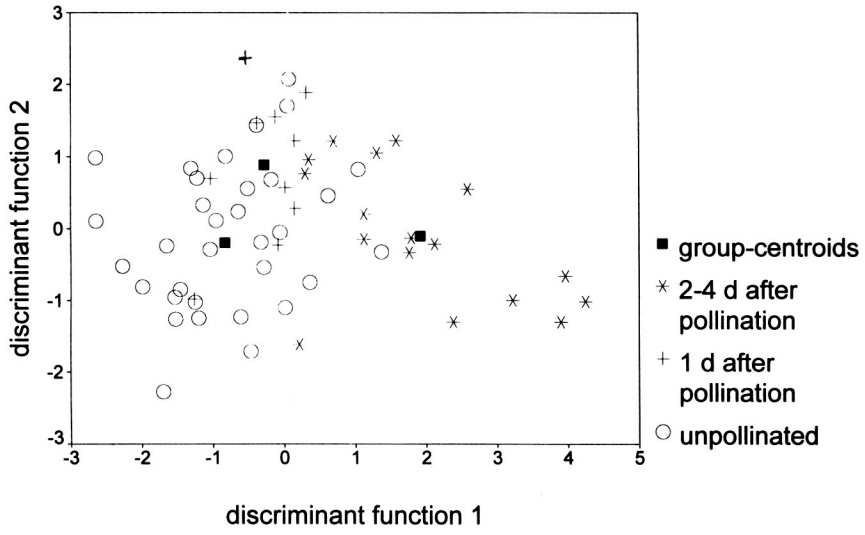


FIG. 2. Comparison of odor bouquets of unpollinated and pollinated *O. sphegodes* flowers by means of a discriminant analysis. Odor bouquets of unpollinated flowers were significantly different from those of pollinated flowers as discussed in the text.

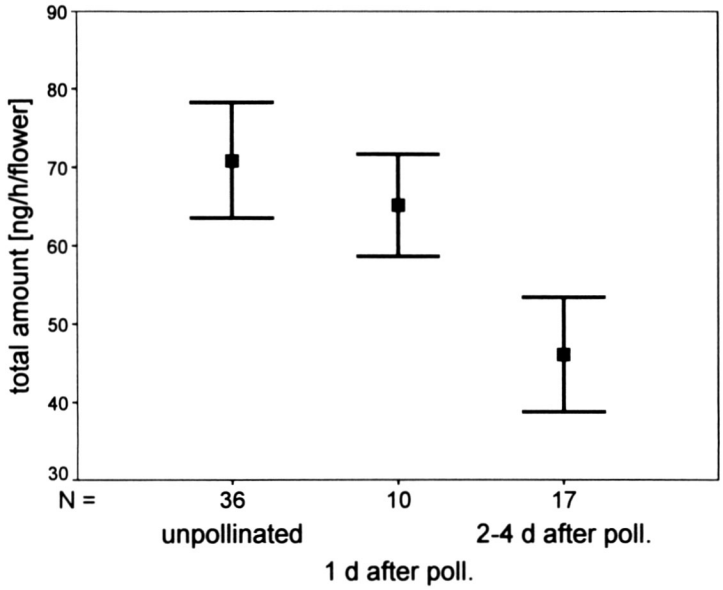


FIG. 3. Mean (±SE) of the total amounts of volatiles emitted by unpollinated and pollinated *O. sphegodes* flowers. Flowers two to four days after pollination produced significantly lower amounts of volatiles compared with unpollinated flowers (*t* test, $t = 2.04$, $P < 0.05$).

were significant only for 6-methyl-5-hepten-2-one and 2-nonanol+undecane (Mann-Whitney *U* test, $P < 0.05$).

Odors of Picked Versus Unpicked Flowers. Picked flowers released smaller relative amounts of some hydrocarbons and 2-nonanone and higher relative amounts of some aldehydes (Mann-Whitney *U* test, $P < 0.05$, Table 1). Multivariate analysis showed significant differences between the odor bouquets of picked and unpicked flowers ($\chi^2 = 45.5$; $df = 7$; $P < 0.001$). Dodecane, tridecane, heptanal, and 2-nonanone contributed most to the differences. Furthermore, the total amount of scent released by picked flowers was significantly lower compared with unpicked flowers (Mann-Whitney *U* test, $P < 0.05$, Figure 4). All components were affected by this decrease in volatile emission.

Odor Emitted During Night Versus Day. Floral odor collected during the night contained significantly lower relative amounts of most aldehydes and 6-methyl-5-hepten-2-one and significantly higher relative amounts of most hydrocarbons, α-pinene, limonene, 1,8-cineole, and 2-nonanol than samples collected during the day (Mann-Whitney *U* test, $P < 0.05$, Table 1). A dis-

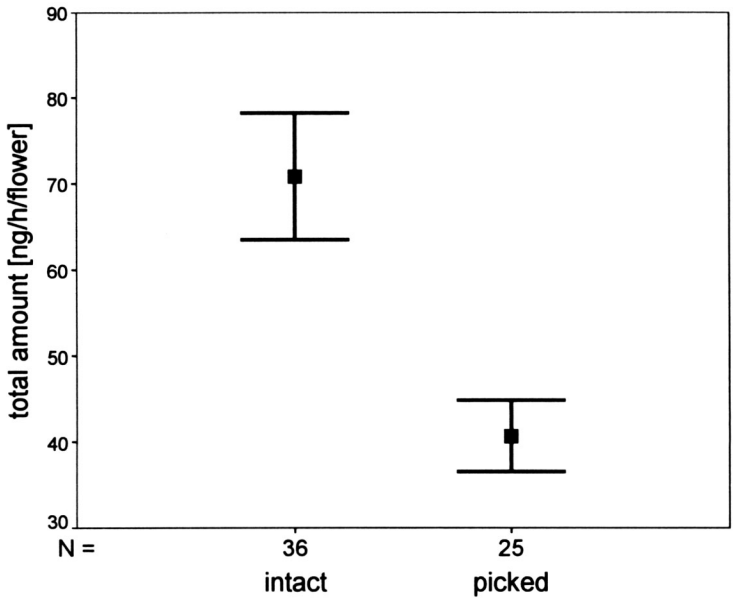


FIG. 4. Mean (±SE) of the total quantity of volatiles emitted by flowers of unpicked and picked *O. sphegodes* flowers. Flowers of picked plants released significantly less volatiles (Mann-Whitney U test, $P < 0.05$).

criminant analysis showed a significant difference between the composition of odor bouquets emitted during night versus day ($\chi^2 = 91.1$; $df = 5$; $P < 0.001$). Tetradecane, heptadecane, hexanal, and 1,8-cineole contributed most to the discriminant function. Furthermore, the total amount of scent emitted increased during the night (Mann-Whitney U test, $P > 0.05$).

Bee Behavioral Tests. In the behavioral tests, males of *A. nigroaenea* made significantly fewer copulation attempts on *O. sphegodes* flowers three days after pollination than on unpollinated flowers (Mann-Whitney U test, $P < 0.001$, Figure 5).

Postpollination Change in Flower Color. In terms of color change, 80% of flowers had wilted three days after pollination compared to only 50% of unpollinated flowers (Mann-Whitney U test, $P > 0.05$). Within the other sample, flowers were checked seven and 10 days after pollination. After seven days, 100% of the pollinated flowers had wilted compared to only 20% of the unpollinated controls (Mann-Whitney U test, $P < 0.05$). After 10 days, 60% of unpollinated flowers had wilted.

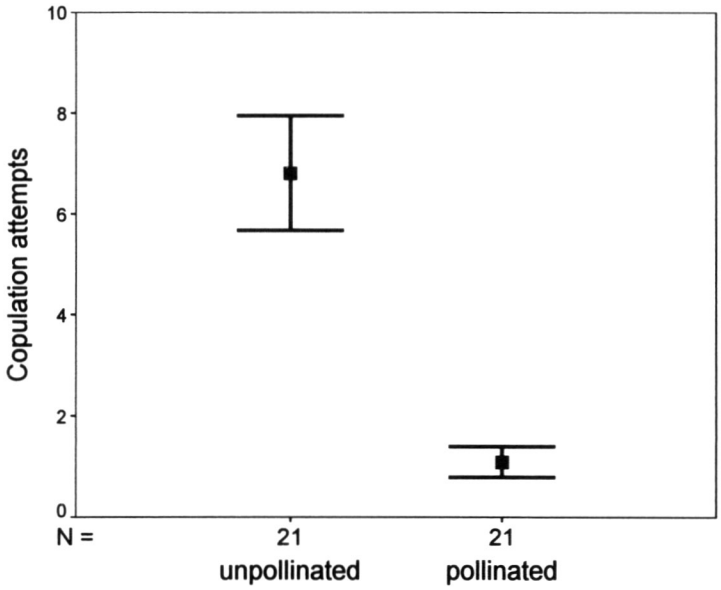


FIG. 5. Mean (±SE) number of copulation attempts by *Andrena nigroaenea* males on unpollinated and pollinated *O. sphegodes* flowers. Pollinated flowers were significantly less attractive than unpollinated ones (Mann-Whitney U test, $P < 0.001$).

DISCUSSION

Among *Ophrys* orchids, which are usually interfertile, floral odor is the most important factor for ensuring the reproductive success and isolation of species in sympatry (Kullenberg, 1961, 1973; Paulus and Gack, 1986, 1994). The pollinator male insect apparently perceives the floral odor as the sex pheromone of the female insect since both elicit the same behavioral response. The odor triggers searching behavior in the male and releases male copulatory movements on the flower labellum, which result in the attachment of the pollinia to the bee (Kullenberg, 1961; Kullenberg and Bergström, 1975, 1976a; Bergström, 1978). *Ophrys* flowers usually produce complex bouquets of volatiles (Borg-Karlson, 1990), although presumably not all components are important for the attraction of male pollinators. Behavioral experiments have shown that only certain compounds are active in stimulating mating behavior (Kullenberg and Bergström, 1976b). Besides the odor, the size and pilosity of the labellum also contribute to the species specificity of pollinator attraction (Kullenberg, 1961).

Depending on the conditions, the quantitative compositions of the odor patterns of the flowers show characteristic alterations (Table 1). Changes can

not be generally attributed to any specific class of compounds, neither with respect to carbon skeletons (acetogenins vs. mevalogenins) or to functional groups (hydrocarbons vs. alcohols vs. carbonyl compounds). The concentration of α-pinene is lowest in intact flowers, of which the concentration of the non-terpenoids 6-methyl-5-hepten-2-one and geranylacetone are highest. Changes among the acetogenins seem to be slightly more obvious.

2-Nonanol is widespread in the plant and animal kingdoms, and a defined mixture of enantiomers serves as part of the queen pheromone in the stingless bee, *Scaptotrigona postica* Latr. (Engels et al., 1990, 1993). Both the *exo-* and *endo-* isomers of brevicomin are well known pheromones of bark beetles of the genus *Dendroctonus* (Byers, 1989). However, *endo*-brevicomin was also found in the odor of *Ophrys speculum* Link (Francke et al., 1995), and it may play a role in the pollination of *O. sphegodes* by *A. nigroaenea*.

Scent production and maintenance by flowers is expensive in terms of energy resources (Vogel, 1962; Arditti, 1979). Therefore, many orchid species have evolved various mechanisms for energy conservation, such as cessation of scent production and fast wilting immediately after pollination (Arditti, 1979). Few investigations have examined the changes of floral odor following pollination. In *Platanthera bifolia* (L.) Rich., Tollsten and Bergström (1989) reported a decrease in scent production 24 hr after pollination, and Tollsten (1993) also found changes in the floral odor bouquets of flowers two and five days after pollination. These changes were interpreted as causing a reduced attractiveness in pollinated flowers. Neiland and Wilcock (1995) studied *Ophrys lutea* Cav. flowers and found them to be visibly unchanged for at least three days after pollination. They assumed that the flowers remained attractive for several days after pollination and thus promoted additional insect visitation; however, they did not investigate the changes in floral odors.

In contrast to these results, we found the color of pollinated *O. sphegodes* flowers to fade three days after pollination, whereas many of the unpollinated flowers remained unchanged. The changes in scent involved a decrease of total amount of scent produced as well as an alteration of the odor bouquet. Besides reduced costs for scent production in pollinated flowers, a second advantage of altered odor bouquets is the directing of pollinators to unpollinated flowers of the plant. Behavioral tests with male *A. nigroaenea* confirmed that pollinated flowers have a reduced attractiveness compared to unpollinated ones. The advantage of reducing floral attractiveness after pollination (Faegri and van der Pijl, 1979) may be enhanced by the generally low visitation rates by pollinators that characterize *Ophrys* orchids (Paulus, personal communication). Moreover, in orchids with pollinia, such as *Ophrys*, most or all of the pollen of one flower can be removed by a single pollinator. A pollinated flower, therefore, no longer functions as a pollen donor and benefits only little by a visit of a second pollinator (Casper and La Pine, 1984). Therefore, if a plant bears more than one

flower, as is usual in *O. sphegodes* (Hutchings, 1987), its reproductive success can be enhanced by guiding the few pollinators to the unpollinated flowers of an inflorescence. This can be achieved by reducing the attractiveness of pollinated flowers. Our findings very clearly confirm these theoretical assumptions for *O. sphegodes*. Pollinated flowers change their odor bouquets and also their color, and are thus less attractive to potential pollinators.

Little attention has been paid to floral scent emission of orchids after picking. Gregg (1983) found some differences in odor profiles of intact and excised labella of *Cycnoches* spp. investigated after more than 8 hr. However, as long as they were analyzed within 8 hr after excision, no great changes were found. Picked *Ophrys* flowers often have been used for both floral odor and pollinator behavioral studies, and our findings here show that odor can be significantly altered following picking. Picked *O. sphegodes* flowers showed a great decrease in total amount of volatiles and odor bouquets differed considerably from intact flowers. Nevertheless, these picked flowers are still attractive to pollinators over several days (Ayasse, unpublished data), which suggests that behaviorally important volatiles are not immediately affected by picking.

Nocturnal scent emission has been investigated mostly in orchids pollinated by nocturnally active animals. Nilsson (1978) found great differences in scent production during day and night of *Platanthera chlorantha* (Cluster) Rchb. flowers, which are pollinated predominantly by night-active moths. *Malus* × *domestica* Borkh. and *Nicotiana otophora* Gresebach flowers are pollinated primarily by day-active insects and emit greater amounts of volatiles during the day (Loughrin et al., 1990). Pollinators of *O. sphegodes* are also active only during the day and one could expect the scent production to decrease or even to cease during the night. In our samples, scent bouquets collected during day and night were significantly different. However, we found no decrease in nocturnal scent production. Methodological reasons might in part account for these findings. Under natural conditions, lower temperatures during the night may cause a reduced fragrance emission. We collected our samples under laboratory conditions, where no or a smaller temperature decrease occurred. Nevertheless our findings prove that scent emission does not completely cease during the night due to diurnal rhythms. Since odor bouquets are significantly different during day and night, a reduced production of biologically active compounds during the night may be possible. In future studies on nocturnal scent emission of *Ophrys* flowers, samples should be collected under constant light and temperature conditions or under natural conditions in the field.

This first study dealing with odor variability of individual flowers in *Ophrys* confirms that odor variability is an important mechanism for influencing the behavior of the pollinators. Its alteration after pollination reduces the attractiveness of these flowers, and pollinators are guided to unpollinated flowers. This mechanism of maximization of the reproductive success of a plant appears to

be especially important in the genus *Ophrys*, which is characterized by a low visitation rate. Our present investigations focus on the identification of the male-attracting compounds within the odor bouquets by conducting electrophysiological recordings and behavioral tests with synthetic compounds. Knowledge about these biologically active compounds would allow much more complete interpretation of changes in scent emission and its functional significance.

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CHEMICALS ATTRACTIVE TO MEXICAN FRUIT FLY FROM *Klebsiella pneumoniae* AND *Citrobacter freundii* CULTURES SAMPLED BY SOLID-PHASE MICROEXTRACTION

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Abstract—Headspace above tryptic soy broth culture filtrates of *Klebsiella pneumoniae* contained greater amounts of ammonia, methylamine, 3-methylbutanamine, 1-pyrroline, 2,3,4,5-tetrahydropyridine, and two pyrazines than were found above tryptic soy broth. It also contained chemicals not found above tryptic soy broth, including dimethyldisulfide and several alcohols and ketones. Headspace above tryptic soy broth culture filtrates of *Citrobacter freundii* contained greater amounts of ammonia, 1-pyrroline, and several pyrazines than were found above tryptic soy broth. It also contained chemicals not found above tryptic soy broth including dimethyldisulfide, some of the same alcohols as above *K. pneumoniae* filtrates, a different ketone, and phenol. Additional chemicals were detected above filtrates that were saturated with sodium chloride or had their pH adjusted up or down. In laboratory bioassays with protein-starved, sugar-fed Mexican fruit flies, chemicals that did not contain protonizable nitrogen were not attractive. All chemicals containing protonizable nitrogen, except 2-methylpyrazine, were attractive. Synthetic mixtures of ammonia, trimethylamine, 1-pyrroline, 3-methylbutanamine, pyrazine, 2,3,4,5-tetrahydropyridine, 2,5-dimethylpyrazine, and trimethylpyrazine in concentrations similar to those in filtrates of the two bacteria were 73–87% as attractive as bacterial filtrates.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, bacteria, amines, solid-phase microextraction.

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INTRODUCTION

Reports that bacteria are attractive to fruit flies have increased in recent years (Gow, 1954; Bateman and Morton, 1981; Drew and Fay, 1988; Jang and Nishijima, 1990; Robacker et al., 1991; MacCollom et al., 1992, 1994; Martinez et al., 1994), but little progress has been made in identifying the chemicals responsible for the attractiveness. Several papers have demonstrated the attractiveness to fruit fly species of ammonia emanating from bacterial cultures (Gow, 1954; Drew and Fay, 1988; Robacker and Flath, 1995). Drew (1987) showed that 2-butanone and 1-butanol produced by bacteria were attractive to *Bactrocera tryoni*, and Robacker and Flath (1995) demonstrated attractiveness to *Anastrepha ludens* of six chemicals identified from headspace above bacterial filtrates. In addition, Lee et al. (1995) and DeMilo et al. (1996) identified over 20 chemicals in headspace above cultures of two bacteria species, but attractiveness of the chemicals was not reported.

Most attempts to identify attractants from bacteria employed procedures that have been used successfully to identify pheromones and host-plant kairomones in thousands of published reports. Typically this involves extraction with an organic solvent or volatile collection on a carbon-based adsorbent, solvent reduction relying on lower boiling points of solvents compared with principals, and GC-MS using GC columns optimized for nonpolar compounds with boiling points above the solvent. Attractiveness bioassays sometimes have been performed without understanding why fruit flies are attracted to bacteria and how this relationship may impact bioassay results.

Robacker and Flath (1995) developed a new approach to identification of volatiles from bacteria attractive to *A. ludens*. The first step was to understand two critical links between the hunger of the flies and their attraction to the bacteria. First, all bacteria tested to date are most attractive to flies hungry for protein (Robacker and Garcia, 1993; Martinez et al., 1994; Robacker and Moreno, 1995). Second, attraction of flies to bacterial odors decreases sharply as sugar starvation increases (Robacker and Garcia, 1993). We do not know how generally these rules apply to all bacteria–fruit fly relationships. However, there is evidence that hunger for protein may be important in other fruit flies (Drew et al., 1983; Drew and Lloyd, 1989).

Also important was knowledge of chemical functional groups involved in the attraction response. Robacker et al. (1993) characterized the attractive principals in headspace above filtrates of *Staphylococcus aureus* cultures as low molecular weight, water-soluble chemicals, most of which contained protonizable nitrogen. Because the attractive chemicals could not be extracted from water, which is a poor solvent to inject on GC columns, it was necessary to remove the water without losing the active chemicals. An additional complication was that the GC method had to be capable of resolving ammonia and

other low-molecular-weight chemicals from each other and from the solvent front. Robacker and Flath (1995) used solid-phase microextraction to sample attractive chemicals from headspace above *S. aureus* filtrates and to load them without solvent onto a thick-film GC column that provided ample resolution and low adsorption of most of the low-molecular-weight compounds.

The work reported herein was conducted to identify volatiles in headspace above filtrates of cultures of two additional bacteria species by the same methods that were used for *S. aureus*, to construct synthetic mixtures of the chemicals that matched the headspace profile of the bacterial filtrates with regard to chemicals that are individually attractive to flies, and to evaluate the synthetic mixtures relative to the bacterial filtrates for attractiveness to Mexican fruit flies. The bacteria species selected for the work were *Klebsiella pneumoniae* and *Citrobacter freundii*. These species were chosen because they were highly attractive to laboratory cultured and wild Mexican fruit flies in laboratory and field experiments, respectively (Martinez et al., 1994).

METHODS AND MATERIALS

Insects and Test Conditions. Flies used to test attractiveness of bacterial preparations and chemicals identified from the preparations were from a culture that originated from yellow chapote fruit, a native host of the fly, collected in Nuevo Leon, Mexico, in 1987. Fly handling and laboratory maintenance were as described in Robacker and Flath (1995). For bioassays, flies were sugar-fed and protein-starved (since eclosion) because previous work indicated this physiological state maximizes attraction to bacteria volatiles (Robacker and Garcia, 1993).

Bacterial Preparations. The two bacterial cultures were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland). *Klebsiella pneumoniae* (ATCC strain 13883) and *Citrobacter freundii* (ATCC strain 8090) were grown in tryptic soy broth (Difco Laboratories, Detroit, Michigan) in a shaker for 144 hr at 30°C. Bacterial cultures were centrifuged, and the resulting supernatants were filtered to remove bacterial cells as described previously (Robacker and Flath, 1995). Martinez et al. (1994) demonstrated that filtered and unfiltered cultures of these two bacteria species were equally attractive, indicating that the attractants were dissolved in the filtrate.

Solvent–Solvent Extraction. Bacterial filtrates were extracted with dichloromethane (Baxter Healthcare Corporation, Muskegon, Michigan) to determine if the attractive chemicals would partition into an organic solvent. For each bacteria species, 2 ml of filtrate was extracted consecutively with two 6-ml volumes of dichloromethane. The first 6-ml dichloromethane extract and the extracted aqueous filtrate were saved for bioassay. The extraction procedure was performed on two fermentations of each bacteria species.

Two experiments were conducted to evaluate attractiveness of the dichloromethane extracts using cage-top bioassays described below. In the first experiment, bioassays were conducted by placing four filter paper triangles (3 cm/side), two containing 30 μl of dichloromethane extract and two containing 30 μl of dichloromethane, near the corners on the top of an insect cage (30 cm/side, aluminum-screened). The two papers containing extract were positioned diagonally from each other on two corners of the cage top, and the two papers containing solvent were positioned diagonally from each other on the other two corners. The filter papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction, not contact chemoreception, was solely responsible for the response of the flies. Each bioassay cage contained 180–200 flies. The number of flies beneath each filter paper was counted once each minute for 10 min following application of the extracts to the papers. Ten bioassays were conducted for each bacteria species.

The second experiment was done to evaluate the effect of recombining the extract with the extracted filtrate. Bioassays were modified from those described above. The four filter paper triangles were loaded with the following treatments: (1) 30 μl of dichloromethane extract plus 10 μl of water, (2) 10 μl of extracted filtrate plus 30 μl of dichloromethane, (3) 30 μl of dichloromethane extract plus 10 μl of extracted filtrate, and (4) 10 μl of unextracted filtrate plus 30 μl of dichloromethane. Samples and solvents were loaded as separate spots on the papers. Sixteen bioassays were conducted for *K. pneumoniae* extracts and 32 for *C. freundii* extracts.

Effects of pH on Attractiveness of Bacterial Filtrates. Filtrates of both bacteria species had pH near 8.8–9.0. The filtrates of the two species were adjusted to pH 9 and 11 with saturated sodium hydroxide (Fisher Scientific, Fair Lawn, New Jersey) and to pH 2, 4, 5, 6, 7, and 8 with 85% phosphoric acid (Fisher). Each pH treatment was tested for attractiveness using cage-top bioassays like those described for testing dichloromethane extracts, except that test quantities were 10 μl of pH treatment and 10 μl of water. Bioassays for the two bacteria species were conducted in two randomized complete block experiments (replication of pH treatments blocked by time). Ten replications of each experiment were conducted.

Volatile Collections. Chemicals were sampled in the headspace above filtrates of bacteria and uninoculated tryptic soy broth for quantitative analysis by GC-MS. Sodium chloride (Fisher), sodium hydroxide (Fisher), or hydrochloric acid (Mallinckrodt, Paris, Kentucky) were added to aliquots of the filtrates to increase headspace concentrations of volatiles and thus improve spectrum quality. Sodium chloride increases headspace concentrations of many organic chemicals by salting out (Page and Lacroix, 1993), while sodium hydroxide and hydrochloric acid increase headspace concentrations of bases and acids, respectively, by converting them into nonionized and more volatile forms. Volatiles

were collected by solid-phase microextraction (SPME) with a 100-μm polydimethylsiloxane coated fiber (Supelco, Inc., Bellefonte, Pennsylvania). The fiber was inserted through a septum into the headspace above 1 ml of bacterial preparation in a 4-ml vial for 30 min at 25–27°C before analysis.

Chemical Identifications. The primary method of chemical identification was GC-MS. GC-MS data were acquired using a Hewlett Packard 5890 GC with a HP 5970 mass selective detector (electron energy = 70 eV). Mass spectral data were acquired over a mass range of 20–300 amu, at 2 scans/sec. GC-MS identifications were based on computer matching of unknown spectra with those in the Wiley 138K Mass Spectral Database (John Wiley & Sons, New York) and with spectra of authentic samples in many cases. All samples were also examined by GC-FID.

Injection of volatiles for GC-MS analyses was by thermal desorption from the SPME fiber at 200°C in a split/splitless injector containing a glass injector liner (0.75 mm ID) designed for use with SPME (Supelco). The injector was operated in the splitless mode and the purge valve was opened after 1 min. Injection for GC-FID analyses was by thermal desorption at 200°C in an on-column injection port. Because the inner diameter of the analytical column was smaller than the diameter of the SPME fiber, on-column injection was accomplished by inserting the fiber into a 10-cm length of deactivated fused silica (0.53 mm) (Supelco) in the injection port connected to the analytical column by a GlasSeal connector (Supelco). The analytical column in all analyses was a DB-1 (J&W Scientific, Folsom, California) fused-silica capillary column (30 m $\times$ 0.32 mm ID, 5-μm film). Linear velocity of helium carrier gas was 60 cm/sec. Column oven temperature was 35°C for 1 min, then programmed at 10°C/min to 200°C.

Bioassay of Identified Chemicals. Selected chemicals identified from headspace above unaltered filtrates of the two bacteria species were obtained and dissolved in water or methanol for attractiveness testing. Chemicals that had been tested in previous work were not retested. All previously untested chemicals containing protonizable nitrogen were selected for bioassay because chemical characterization tests indicated that the attractive components generally fit this profile. No other chemicals were selected for bioassays from *K. pneumoniae* filtrates. With the exception of 2-methylbutanol, all previously untested chemicals from unaltered *C. freundii* filtrates that had concentrations in headspace of 0.01 ng/ml or greater were tested. 2-Methylbutanol was not selected because it is similar in structure to 3-methylbutanol, a chemical that had a higher concentration in the headspace and that was selected for bioassay. 3-Methylbutanoic acid was bioassayed because it was a major component of the filtrates of both bacteria species, although it was not present in headspace of unaltered filtrates. Piperidine was also tested because it was present as a contaminant (5%) in 2,3,4,5-tetrahydropyridine.

Chemicals dissolved in water were 2-butanone, pyrazine, methylpyrazine, trimethylpyrazine, 2,3,4,5-tetrahydropyridine, phenol, and 3-methylbutanoic acid. Chemicals dissolved in methanol were 3-methylbutanal, 3-methylbutanol, 2-ethylhexanol, 2-phenylethanol, and benzaldehyde. Sources and purities of the chemicals are shown in Table 1. Four concentrations of each chemical were prepared and subsequently four quantities (10 ng, 100 ng, 1 μg, 10 μg) of each chemical were tested, each in 10 μl of solvent. The control for each chemical was 10 μl of water or methanol for chemicals dissolved in water or methanol, respectively. Cage-top bioassays were the same as those used to test dichloro-

TABLE 1. CHEMICAL NAMES, SOURCES, AND PURITIES OF CHEMICALS USED AS GC STANDARDS AND/OR TESTED IN CAGE-TOP BIOASSAYS

Chemical	Source[a]	Purity (%)
Ammonium carbonate	Aldrich	unknown[b]
Methylamine HCl	Sigma	99
Trimethylamine HCl	Sigma	99
2-Methylpropanamine	Aldrich	99
3-Methylbutanamine	Aldrich	99
2-Methylbutanamine	Aldrich	>97
1-Pyrroline	synthesized	solution[c]
Pyrazine	Aldrich	99
2,3,4,5-Tetrahydropyridine	synthesized	solution[d]
Piperidine	M, C & B	98
Methylpyrazine	Aldrich	99
2,5-Dimethylpyrazine	Aldrich	99
Trimethylpyrazine	Aldrich	99
3-Methylbutanol	Aldrich	99
2-Ethylhexanol	Alltech	99
2-Phenylethanol	Aldrich	99
Phenol	Aldrich	99
2-Butanone	Aldrich	99
3-Methylbutanal	Aldrich	97
Benzaldehyde	Sigma	99
3-Methylbutanoic acid	Aldrich	99

[a] Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; Sigma Chemical Company, St. Louis, Missouri; Matheson, Coleman & Bell (M, C & B), Norwood, Ohio; Alltech Associates, Inc., Deerfield, Illinois.

[b] Mixture with ammonium bicarbonate and ammonium carbamate.

[c] 1.4% by GC analysis in basic, aqueous solution freshly prepared; ethanol (1.9%), a reaction byproduct, was the principal volatile contaminant identified by GC-MS.

[d] 5% by GC analysis at maximum yield 2 days after preparation; piperidine (0.25%) was the principal contaminant by GC-FID.

methane extracts against dichloromethane controls except that test quantities were 10 μl instead of 30 μl. Ten replications were conducted for each of the four concentrations of all of the chemicals.

FTD Analysis of Headspace Volatiles. Relative amounts of nitrogen-containing chemicals in the headspace of bacterial cultures (at unaltered pH 8.8–9.0), tryptic soy broth (pH raised to 8.9 with NaOH), and mixtures of synthetic chemicals were determined. Analyses were conducted using a Shimadzu GC-17A (Shimadzu Scientific Instruments, Inc., Columbia, Maryland) with a flame thermionic detector (model FTD-17). The FTD was used to establish the presence of C–N bonds and thus confirm identification of many of the bacterial volatiles as amines and to provide highly sensitive detection of trace quantities of several of the compounds. Volatiles were thermally desorbed in an on-column injector into a 10-cm length of deactivated fused silica connected to the analytical column as described above. The analytical column was a DB-1 (60 m × 0.32 mm ID, 5-μm film). Analyses were at 100°C. Carrier gas was helium at a linear velocity of 30 cm/sec. GC peak heights were measured using Millennium 2010 Chromatography Manager software (Waters Corporation, Milford, Massachusetts). Three headspace analyses were conducted for each of five fermentations of the two bacteria species and the tryptic soy broth that was used to culture the bacteria. One headspace analysis per fermentation was also done using flame ionization detection to quantify the amount of phenol in the *C. freundii* headspace.

Quantification of Chemicals in Bacterial Filtrates. Major components identified from headspace above bacterial filtrates that were attractive to flies in cage-top bioassays were mixed together in aqueous solutions for the purpose of producing synthetic solutions that would match the headspace profiles of the bacterial filtrates. This was done by comparing SPME samplings in headspace above known concentrations of mixtures of standards with SPME samplings in headspace above the filtrates. Standard solutions were adjusted to pH 8.8 with aqueous NaOH to equal the pH of unaltered bacterial filtrates. A trial-and-error approach was used in which chemicals were mixed together in a vial in various concentrations until FTD peak heights on the DB-1 column matched those obtained from a vial containing bacterial filtrates.

Concentrations in the final synthetic mixture that matched a composite filtrate containing equal portions of the five fermentations of *K. pneumoniae* that were used to quantify attractive chemicals were: ammonium carbonate, 1.6 mg/ml; trimethylamine HCl, 66 ng/ml; 1-pyrroline, 220 ng/ml; 3-methylbutanamine, 500 ng/ml; pyrazine, 400 ng/ml; 2,3,4,5-tetrahydropyridine, 80 ng/ml; 2,5-dimethylpyrazine, 2.8 μg/ml; and trimethylpyrazine, 200 ng/ml. Concentrations in the final synthetic mixture that matched a composite filtrate containing equal portions of the five fermentations of *C. freundii* were: ammonium carbonate, 1.6 mg/ml; trimethylamine HCl, 40 ng/ml; 1-pyrroline, 160 ng/ml;

3-methylbutanamine, 75 ng/ml; pyrazine, 350 ng/ml; 2,5-dimethylpyrazine, 4.8 μg/ml; and trimethylpyrazine, 350 ng/ml. A second synthetic mixture of *C. freundii* volatiles was prepared containing the above chemicals at the same concentrations and phenol at 51 μg/ml. This concentration of phenol produced a peak by FID that matched the phenol peak in the *C. freundii* composite filtrate headspace. Phenol was tested as a possible synergist of *C. freundii* volatiles because it was present in headspace above *C. freundii* but not *K. pneumoniae* and because methylene chloride extraction of *C. freundii* filtrate, but not *K. pneumoniae* filtrate, apparently removed a synergistic component.

Bioassay of Synthetic Mixtures. The synthetic mixtures for each bacteria species were tested in cage-top bioassays with water as the control. Separate bioassays of filtrates with water as the control were conducted concurrently with flies from the same cohort. Furthermore, synthetic mixtures were tested in cage-top bioassays against their respective composite filtrates as the controls. Test quantities used on filter papers were 10 μl. Fifteen replications were conducted for the *K. pneumoniae* synthetic mixture against water, its filtrate against water, and its synthetic mixture against its composite filtrate. Twelve replications were conducted for each of the two *C. freundii* synthetic mixtures against water, each filtrate against water, and each synthetic mixture against its composite filtrate.

Chemicals. Sources of chemicals used in this work are shown in Table 1. 1-Pyrroline was synthesized by acid hydrolysis of 4-aminobutyraldehyde diethyl acetal (Aldrich, 90%) according to methods of Schopf and Oechler (1936). The concentration of 1-pyrroline in this product was determined from an FID calibration curve for pyrrolidine (Sigma). 2,3,4,5-Tetrahydropyridine was synthesized by reaction of *N*-chlorosuccinimide (Aldrich, 98%) with piperidine (Matheson, Coleman & Bell, Norwood, Ohio, 98%) to form *N*-chloropiperidine, followed by elimination of HCl from *N*-chloropiperidine with KOH (Fisher) (Quick and Oterson, 1976). The concentration of 2,3,4,5-tetrahydropyridine in the product was determined with an FID calibration curve for 1,2,3,6-tetrahydropyridine (Aldrich).

Statistical Analyses. Two-way analysis of variance (ANOVA) was conducted for the dichloromethane extract recombination experiment to separate effects of replication (each bioassay) and treatments applied to filter papers using SuperANOVA (Abacus Concepts, 1989). Data used in the analyses were total counts at each treatment paper. Responses of flies to the bacterial filtrates at the different pHs were compared with each other in a randomized complete block experiment. Data used in the analyses were differences between total counts at papers containing pH treatments and papers containing water within each bioassay. Two-way ANOVA was conducted to separate replication effects and pH effects. Paired *t* tests were used to analyze results of experiments evaluating dichloromethane extracts or individual chemicals against solvent blanks, or syn-

thetic mixtures of chemicals against either water or bacterial filtrates within bioassays. For paired t tests, counts of flies at filter papers containing test samples were compared to counts at solvent-blank or control papers. Also, t tests were used to compare counts at filter papers containing synthetic mixtures with counts, obtained in separate bioassays, at papers containing composite filtrates. Analysis of FTD peak heights of chemicals in the bacterial filtrates and TSB filtrate was conducted by two-way ANOVA to separate replication effects and filtrate-type effects. Means separations were done by Fisher's protected least significant difference (LSD) method in all ANOVAs.

RESULTS AND DISCUSSION

Solvent–Solvent Extractions. Methylene chloride extracts of filtrates of the two bacteria species were not significantly more attractive than solvent controls. For *K. pneumoniae*, mean counts ($\pm$ SE) at papers with extracts were 17.6 $\pm$ 1.4 and mean counts at papers with solvent were 17.6 $\pm$ 3.1. For *C. freundii*, mean counts ($\pm$ SE) at papers with extracts were 23.8 $\pm$ 2.4 and mean counts at papers with solvent were 19.5 $\pm$ 1.1. These results indicate that chemicals attractive to protein-starved *A. ludens* cannot be readily extracted into methylene chloride from the aqueous filtrates.

Results of bioassays of recombinations of methylene chloride extracts with the extracted bacterial filtrates are shown in Table 2. Extracted filtrates of *K. pneumoniae* were equal in attractiveness both to combinations of extracts with extracted filtrates and to unaltered filtrates. This indicates that methylene chloride did not remove any chemicals from the filtrate that affected attractiveness of other chemicals in the filtrate.

For *C. freundii*, extracted filtrates were significantly less attractive than both unaltered filtrates and recombinations of methylene chloride extracts with extracted filtrates ($F = 73.0$; $df = 3,124$; $P < 0.0001$) (Table 2). These results indicate that methylene chloride removed one or more chemicals that enhanced attractiveness of other chemicals in the filtrate.

Effects of pH on Attractiveness of Bacterial Filtrates. Attractiveness of filtrates of both bacterial species was greatly affected by filtrate pH (Figure 1). Filtrates were most attractive above pH 6, indicating that most of the attractive chemicals, or the most attractive ones, are ionized and relatively nonvolatile at pH < 6. These results suggest that the attractive principals mostly are amines or other compounds containing protonizable nitrogen. However, filtrates at pH 2–5 also were more attractive ($P < 0.05$) than water according to paired t tests, except for the *K. pneumoniae* pH 4 treatment ($t = 2.1$, $df = 9$, $P = 0.07$). Robacker et al. (1993) obtained similar pH effects upon attractiveness with *Staphylococcus aureus* filtrates.

TABLE 2. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING METHYLENE CHLORIDE EXTRACTS OF BACTERIAL FILTRATES OF TWO BACTERIA SPECIES, FILTRATES AFTER EXTRACTION, RECOMBINATIONS OF EXTRACTS WITH EXTRACTED FILTRATES, OR UNALTERED BACTERIAL FILTRATES, IN CAGE-TOP BIOASSAYS[a]

	N	Extract	Extracted filtrate	Extract + extracted filtrate	Filtrate (unaltered)
Klebsiella pneumoniae	16	12.6a	56.4b	55.7b	54.5b
Citrobacter freundii	32	9.6a	43.2b	50.5c	52.5c

[a]For each bacteria species, mean counts followed by the same letter were not significantly different from each other by LSD ($P < 0.05$).

Chemical Identifications by GC-MS. Chemicals identified by a combination of GC-MS and GC retention time in headspace above tryptic soy broth filtrates and filtrates of the two bacteria species are listed in Table 3. Fifteen chemicals with concentrations of at least 0.01 ng/ml of headspace were identified by

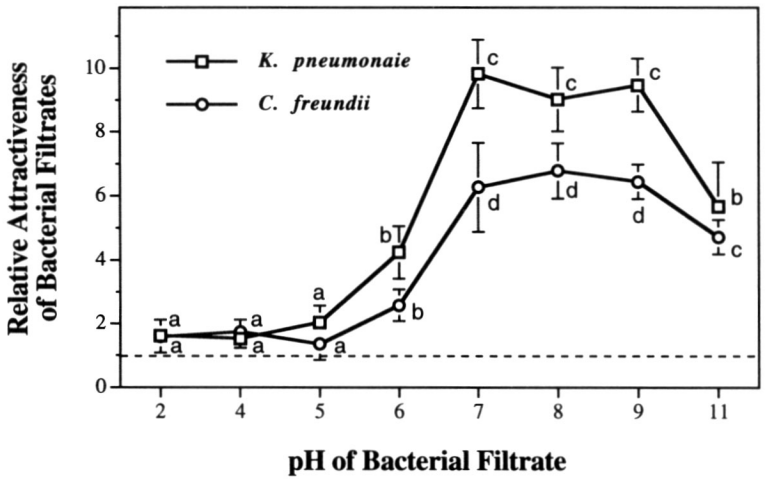


FIG. 1. Mean counts (± SE) of *A. ludens* at filter papers containing filtrate of tryptic soy broth cultures of two bacterial species at various pH levels, divided by counts at papers containing water, in cage-top bioassays. The dotted line indicates the no-response level. Means on the same line followed by the same letter are not significantly different from each other at the 5% level by Fisher's protected LSD.

TABLE 3. KOVATS INDICES AND CONCENTRATIONS OF CHEMICALS IDENTIFIED BY GC-MS IN HEADSPACE ABOVE UNALTERED BACTERIA AND TRYPTIC SOY BROTH FILTRATES AND MAXIMUM CONCENTRATIONS DETECTED IN FILTRATES WITH ADDED NaCl, NaOH, OR HCl (ng/ml HEADSPACE)[a]

	Kovats index	Kp	Cf	TSB	Maximum detected, filtrate/additive
Trimethylamine	419	nd	nd	nd	1.8, TSB/NaOH
Ethanol	436	nd	nd	4.0	16.0, TSB/NaCl
2-Butanone	575	nd	0.53	nd	1.6, Cf/NaCl
2-Methylpropanamine	591	nd	nd	nd	0.23, TSB/NaOH
2-Methylpropanol	611	nd	nd	nd	0.81, Cf//NaCl
3-Methylbutanal	635	nd	0.96	0.77	5.6, TSB/NaCl
1-Pyrroline	656	nd	nd	nd	0.23, Kp/NaOH
Propanoic acid	659	nd	nd	nd	0.68, Kp/HCl
2-Pentanone	666	0.11	nd	nd	0.65, Kp/NaCl
3-Methylbutanamine	696	0.05	nd	nd	2.7, Kp/NaOH
2-Methylbutanamine	701	nd	nd	nd	0.21, Kp/NaOH
Pyrazine	711	0.06	0.06	0.04	0.17, Cf/NaOH
3-Methylbutanol	717	0.04	2.0	nd	16.0, Cf/NaCl
2-Methylbutanol	722	0.19	1.0	nd	8.2, Cf/NaCl
2-Methylpropanoic acid	723	nd	nd	nd	0.71, Kp/HCl
Dimethyldisulfide	736	2.3	6.2	nd	34.0, Kp/NaOH
Butanoic acid	746	nd	nd	nd	0.19, Cf/HCl
2-Hexanone	770	0.04	nd	nd	0.21, Kp/NaCl
2,3,4,5-Tetrahydropyridine	796	0.03	nd	nd	0.29, Kp/NaOH
Methylpyrazine	803	0.15	0.11	0.04	0.33, Kp/NaCl
3-Methylbutanoic acid	804	nd	nd	nd	8.3, Kp/HCl
2-Methylbutanoic acid	813	nd	nd	nd	1.6, Kp/HCl
2-Heptanone	872	nd	nd	nd	0.07, Cf/NaOH
2,5-Dimethylpyrazine	895	0.58	0.66	0.24	2.2, Cf/NaCl
Benzaldehyde	950	0.14	0.32	0.09	0.4, Cf/NaCl
Phenol	951	nd	2.4	nd	12.0 Cf/NaCl
Trimethylpyrazine	988	0.04	0.05	0.01	0.35, Cf/NaCl
2-Ethylhexanol	1015	0.01	0.01	nd	0.15, Cf/NaCl
2-Methyl-5-isopropylpyrazine	1044	nd	nd	nd	0.02, Cf/NaCl
1-Phenylethanone	1057	nd	nd	nd	0.01, Kp/Cf/NaCl
2-Phenylethanol	1105	0.02	0.04	nd	0.31, Cf/NaCl
1-Phenyl-2-propanone	1116	0.04	nd	nd	0.18, Kp/NaCl

[a]Kp = *Klebsiella pneumoniae*, Cf = *Citrobacter freundii*, TSB = tryptic soy broth, nd = not detected or <0.01 ng/ml.

GC-MS above unaltered filtrates of *K. pneumoniae*. Thirteen chemicals with concentrations of at least 0.01 ng/ml were identified in headspace of unaltered *C. freundii* filtrates. Concentrations were determined using a method developed by Bartelt (1997).

The chemicals in Table 3 accounted for approximately 94% and 98% of the volatiles by FID area counts for *K. pneumoniae* and *C. freundii*, respectively. Unidentified substituted pyrazines accounted for approximately 4% and 2% of the FID area counts for the two bacteria. Generally, alcohols were most prevalent in headspace above filtrates saturated with NaCl, amines were most prevalent in headspace above filtrates at pH > 12, and carboxylic acids were most prevalent in headspace above filtrates at pH < 2. Headspace above tryptic soy broth contained mostly pyrazines but also contained small amounts of 1-pyrroline and the same amines found in headspace of the two bacteria. Alcohols (except ethanol), ketones, and carboxylic acids were nearly absent from headspace above the tryptic soy broth.

FTD Analysis of Headspace Volatiles. Analyses by FTD of 12 nitrogen-containing chemicals in headspace above unaltered filtrates of the two bacterial cultures and tryptic soy broth (pH 8.9) are shown in Table 4. These 12 chemicals included six that were not detected by GC-MS in unaltered filtrates. One of

TABLE 4. PEAK HEIGHTS (mV) BY FLAME THERMIONIC DETECTION OF CHEMICALS IN HEADSPACE ABOVE FILTRATE OF UNINOCULATED TRYPTIC SOY BROTH AT pH 8.9 AND UNALTERED FILTRATES OF TWO BACTERIA SPECIES GROWN ON TRYPTIC SOY BROTH (pH = 8.8–9.0)[a]

	Tryptic soy broth	*K. pneumoniae*	*C. freundii*
Ammonia[b]	−0.32 ± 0.04 a	−0.86 ± 0.07 b	−0.85 ± 0.05 b
Methylamine	0.18 ± 0.02 a	0.52 ± 0.04 b	0.31 ± 0.08 a
Trimethylamine	7.1 ± 0.6 b	7.8 ± 0.6 b	5.6 ± 0.4 a
2-Methylpropanamine	0.67 ± 0.10 a	0.78 ± 0.03 a	0.75 ± 0.10 a
1-Pyrroline	4.0 ± 0.3 a	26.1 ± 3.8 c	13.2 ± 0.6 b
3-Methylbutanamine	1.4 ± 0.1 a	9.2 ± 0.4 b	1.5 ± 0.1 a
2-Methylbutanamine	0.39 ± 0.03 a	0.45 ± 0.04 a	0.43 ± 0.04 a
Pyrazine	9.5 ± 1.1 a	11.7 ± 1.2 b	11.1 ± 1.3 b
2,3,4,5-Tetrahydropyridine	1.1 ± 0.1 a	9.9 ± 0.8 b	1.7 ± 0.1 a
Methylpyrazine	12.2 ± 2.1 a	15.5 ± 2.1 b	15.4 ± 2.3 b
2,5-Dimethylpyrazine	146 ± 18 a	134 ± 19 a	254 ± 16 b
Trimethylpyrazine	7.5 ± 0.9 a	7.5 ± 1.0 a	13.4 ± 1.0 b

[a] For a given chemical, mean peak heights followed by the same letter were not significantly different from each other by LSD ($P < 0.05$, $N = 5$ fermentations).
[b] Ammonia produced negative peaks by FTD. Larger absolute values indicate greater amounts.

these six chemicals, ammonia, had not been monitored by GC-MS due to its low molecular weight, although it had been observed by FID and identified by its retention time. Identifications of all 12 chemicals were confirmed by comparisons of retention times and FTD/FID ratios with authentic samples.

FTD peak heights indicated that many of these nitrogen-containing chemicals were present in greater amounts in headspace of the bacterial filtrates than in that of the tryptic soy broth. Table 4 shows that ammonia and 1-pyrroline were found in headspace in much higher concentrations in both bacterial species than in the tryptic soy broth. Pyrazine and methylpyrazine also were found in greater concentrations in the two bacterial species. Methylamine, 3-methylbutanamine, and 2,3,4,5-tetrahydropyridine were found in greater concentrations in headspace of *K. pneumoniae* than in tryptic soy broth, and 2,5-dimethylpyrazine and trimethylpyrazine were found in greater concentrations in headspace of *C. freundii* than in tryptic soy broth. No differences were found in headspace for 2-methylpropanamine and 2-methylbutanamine, while trimethylamine was actually found in lower concentration in headspace of *C. freundii* than in tryptic soy broth.

Quantification of Chemicals in Bacterial Filtrates. Trial-and-error FTD and FID analyses of solutions containing various amounts of standards showed that composite *K. pneumoniae* filtrate contained the following concentrations of chemicals: ammonia, 600 μg/ml; trimethylamine, 41 ng/ml; 1-pyrroline, 220 ng/ml; 3-methylbutanamine, 500 ng/ml; pyrazine, 400 ng/ml; 2,3,4,5-tetrahydropyridine, 80 ng/ml; 2,5-dimethylpyrazine, 2.8 μg/ml; and trimethylpyrazine, 200 ng/ml. Concentrations of chemicals in composite *C. freundii* filtrate were: ammonia, 600 μg/ml; trimethylamine, 25 ng/ml; 1-pyrroline, 160 ng/ml; 3-methylbutanamine, 75 ng/ml; pyrazine, 350 ng/ml; 2,5-dimethylpyrazine, 4.8 μg/ml; trimethylpyrazine, 350 ng/ml; and phenol, 51 μg/ml. Synthetic solutions for bioassay (Methods and Materials) were prepared with this information.

Attractiveness of Individual Chemicals. Cage-top bioassays showed that only chemicals containing protonizable nitrogen were significantly more attractive to flies than solvent controls (Table 5). Of the five chemicals in Table 5 that contain protonizable nitrogen, pyrazine, trimethylpyrazine, 2,3,4,5-tetrahydropyridine, and piperidine were significantly attractive. Piperidine, a contaminant of 2,3,4,5-tetrahydropyridine that was not present in bacterial headspace, was not attractive at test quantities of 10 and 100 ng. This finding suggests that the attractiveness of 2,3,4,5-tetrahydropyridine was not due to piperidine contamination, at least at the 100-ng and 1-μg test quantities that contained about 5 and 50 ng of piperidine, respectively.

Other nitrogen-containing chemicals identified in headspace above the two bacterial filtrates had proven attractive in earlier work. These include ammonia and methylamine (Robacker and Warfield, 1993); trimethylamine, 2-methylpro-

TABLE 5. MEAN COUNTS OF *A. ludens* AT SOLVENT BLANKS OR TEST CHEMICALS IDENTIFIED FROM HEADSPACE ABOVE FILTRATES OF *Klebsiella pneumoniae* AND *Citrobacter freundii*, IN CAGE-TOP BIOASSAYS

	10 ng		100 ng		1 μg		10 μg	
	Test	Solvent	Test	Solvent	Test	Solvent	Test	Solvent
Pyrazine	33.1	29.6	38.3[a]	26.6	29.1	28.0	30.9	29.5
2-Methylpyrazine	19.0	19.6	14.3	18.4	16.2	19.1	15.1	16.1
Trimethylpyrazine	15.7	14.9	18.6	17.7	21.9[a]	17.1	19.2	20.5
2,3,4,5-Tetrahydropyridine	20.6	19.2	26.3[a]	18.9	27.9[a]	19.1	26.4[a]	18.4
Piperidine[b]	16.1	21.6	18.3	20.4	38.8[a]	21.4	65.0[a]	20.8
3-Methylbutanol	14.5	15.6	17.1	16.9	17.2	17.3	14.6	14.9
2-Ethylhexanol	15.8	15.4	15.8	15.1	16.1	18.4	15.8	14.9
2-Phenylethanol	16.9	20.6	18.2	20.1	20.6	20.8	20.3	18.7
Phenol	31.7	28.3	30.0	30.8	26.0	25.3	25.3	28.4
2-Butanone	16.5	18.0	14.9	16.4	17.7	18.0	13.0	16.6
3-Methylbutanal	13.4	14.8	13.9	15.4	13.0	14.8	11.7[a]	16.6
Benzaldehyde	27.6	22.8	25.1	27.1	23.1	25.5	21.8[a]	28.8
3-Methylbutanoic acid	19.5	19.9	16.8	19.8	18.8	16.8	21.5	20.3

[a]The mean response to a chemical was significantly different from the response to solvent by paired *t* tests ($P < 0.05$).
[b]Not found in headspace of any filtrates but was a contaminant of the 2,3,4,5-Tetrahydropyridine reaction product.

panamine, 3-methylbutanamine, 2-methylbutanamine, and 2,5-dimethylpyrazine (Robacker and Flath, 1995); and 1-pyrroline (Robacker et al., 1997).

Attractiveness of Synthetic Mixtures. Synthetic mixtures of the two bacterial filtrates were about four to five times more attractive than water controls in cage-top bioassays (Table 6). Synthetic mixtures generally were significantly less attractive than their filtrates. Synthetic *K. pneumoniae* mixture was 81% as attractive as its filtrate when comparing counts at treatment filter papers obtained in separate bioassays, and 73% as attractive when comparing counts obtained within the same bioassays. For the *C. freundii* synthetic mixture without phenol, analogous comparisons were 81% and 84%, respectively. For the *C. freundii* synthetic mixture containing phenol, analogous comparisons were 87% and 78%,

TABLE 6. COUNTS (± SE) OF *A. ludens* AT FILTER PAPERS CONTAINING SYNTHETIC MIXTURE OF CHEMICALS IDENTIFIED FROM HEADSPACE ABOVE TWO BACTERIAL SPECIES COMPARED WITH COUNTS AT PAPERS CONTAINING WATER OR FILTRATES OF TWO BACTERIA, USING CAGE-TOP BIOASSAYS

Test	Test preparation	Control	Synthetic/filtrate
K. pneumoniae synthetic vs. water control	108.1 ± 3.2[a,b]	22.3 ± 2.2	0.81
K. pneumoniae filtrate vs. water control	133.6 ± 4.8[a]	21.5 ± 1.5	
K. pneumoniae synthetic vs. filtrate control	79.8 ± 2.8[c]	109.5 ± 4.6	0.73
C. freundii synthetic vs. water control	99.8 ± 5.1[a,b]	17.8 ± 1.7	0.81
C. freundii filtrate vs. water control	123.9 ± 7.4[a]	18.7 ± 1.7	
C. freundii synthetic vs. filtrate control	80.5 ± 3.8[c]	95.9 ± 4.7	0.84
C. freundii synthetic + phenol vs. water control	98.8 ± 5.4[a]	28.5 ± 2.7	0.87
C. freundii filtrate vs. water control	113.4 ± 4.6[a]	27.3 ± 2.5	
C. freundii synthetic + phenol vs. filtrate control	78.8 ± 6.5[c]	101.5 ± 5.9	0.78

[a] Mean response to synthetic mixture or bacterial filtrate was significantly greater than response to water by paired t tests ($P < 0.01$).

[b] Mean response to synthetic mixture was significantly lower than response to bacterial filtrate in independent bioassays by t test ($P < 0.05$).

[c] Mean response to synthetic mixture was significantly lower than response to bacterial filtrate within the same bioassay by paired t test ($P < 0.01$).

respectively. The *C. freundii* synthetic mixture containing phenol was nearly identical in attractiveness to the synthetic mixture without phenol, indicating that addition of phenol had no effect.

Possible reasons for the lower responses to the synthetic mixtures than to the filtrates are: (1) chemicals that do not contain protonizable nitrogen may play some undetermined roles; (2) several amines found in trace quantities were not included in synthetic mixtures; (3) methyl pyrazine, a major component of the volatiles, was not included because it was not attractive in cage-top bioassays; and (4) several pyrazines were not completely identified and were not included in synthetic mixtures.

Comparison with S. aureus. Attractive chemicals identified in headspace of *S. aureus* were ammonia, trimethylamine, 3-methylbutanamine, 2-methylbutamine, 2,5-dimethylpyrazine, and acetic acid (Robacker and Flath, 1995). Because fermentations were conducted in tryptic soy broth (now known to contain 1-pyrroline), 1-pyrroline was probably present but was not identified. This profile of chemicals is similar to that of *K. pneumoniae* and *C. freundii*.

Comparison with Standard Methods of Semiochemical Analyses. Isolates of *K. pneumoniae* and *C. freundii* used in this work were the same as those used by Lee et al. (1995) and DeMilo et al. (1996) to identify headspace volatiles. The original isolates were obtained from ATCC by the Weslaco laboratory and shared with A. J. Martinez for attractiveness bioassays (Martinez et al., 1994) and with Lee and DeMilo for their work. Furthermore, bacteria were cultured on tryptic soy broth in all of the work. Thus, the current work provides an opportunity to compare results obtained with methods that differed in three principal ways. We used solid-phase microextraction to sample headspace volatiles and load them onto a GC column. Lee et al. (1995) and DeMilo et al. (1996) collected volatiles on activated charcoal, a standard method in this type of work. Moreover, we used on-column injection onto a thick-film GC column for our analyses compared with split injection (Grob-type injector) onto thin-film GC columns used in the other two studies. The studies also differed in that Lee et al. (1995) and DeMilo et al. (1996) used autoclaved supernatants of bacterial cultures whereas we used filtered supernatants.

Results differed in several ways. First, Lee et al. (1995) and DeMilo et al. (1996) reported that 3-methylbutanol was the principal component, making up >90% of the volatiles (by FID area counts), in headspace of both bacteria species. We found 3-methylbutanol in much lower amounts (Tables 3 and 4). Furthermore, many chemicals identified by their methods were not found by our methods or were found in amounts below 500 area counts, the lower limit for positive identification that we imposed in our analyses. The only major component in this category was 3-(methylthio)propanol found in *K. pneumoniae* (Lee et al., 1995). Minor component differences included several alcohols,

ketones, and esters that we did not observe and several pyrazines that we observed but for which we did not authenticate isomeric identities.

The major difference in results between the two methods was that neither Lee et al. (1995) nor DeMilo et al. (1996) identified ammonia, 1-pyrroline, 2,3,4,5-tetrahydropyridine, or any amines in the bacterial volatiles. Although these chemicals are relatively minor components, this is a critical difference because these chemicals were the most attractive. It is not surprising that Lee et al. (1995) and DeMilo et al. (1996) did not observe ammonia, methylamine, and trimethylamine because these chemicals would elute at or near the solvent front by their methods. Reasons that 2-methylpropanamine, 3-methylbutanamine, 2-methylbutanamine, 1-pyrroline, and 2,3,4,5-tetrahydropyridine were not seen are less evident. Possibly, they were not trapped by activated charcoal or were adsorbed on active sites in the injector ports or the thin-film columns used in their studies. These types of problems with low-molecular-weight amines were discussed in the Introduction and in previous work (Robacker and Flath, 1995). It is noteworthy that SPME with polydimethylsiloxane fibers is especially sensitive to nitrogen-containing compounds (Bartelt, 1997).

Lee et al. (1995) and DeMilo et al. (1996) autoclaved the bacterial cultures before collecting and identifying chemicals in the headspace. To test the effects of autoclaving, we conducted GC-MS analyses of headspace above autoclaved filtrates of each bacteria species using the same SPME headspace sampling procedures that we had used for nonautoclaved filtrates. We observed little difference between the autoclaved and nonautoclaved samples, except there were reduced amounts of ammonia, 3-methylbutanol, and 2-methylbutanol in the autoclaved filtrates. We concluded that differences in results between our work and studies by Lee et al. (1995) and DeMilo et al. (1996) were not caused by autoclaving bacterial cultures.

Significance of the Method. This is the second investigation to identify volatile chemicals produced by bacterial cultures attractive to an insect species, to test attractiveness of the chemicals to the insect, to construct synthetic mixtures of the attractive chemicals at the same concentrations as found in the bacterial preparations, and to test the attractiveness of the mixtures relative to the attractiveness of the bacterial preparations. As in the first such investigation with *S. aureus* cultures (Robacker and Flath, 1995), the synthetic mixtures were highly attractive but less so than the bacterial filtrates. We believe that our method represents the best current protocol for conducting these types of studies. Nevertheless, we acknowledge that improvements in the methods are needed.

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A 3D-QSAR STUDY OF ANALOGS OF (Z)-5-DECENYL ACETATE, A PHEROMONE COMPONENT OF THE TURNIP MOTH, *Agrotis segetum*

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Abstract—The methodology of three-dimensional quantitative structure–activity relationships (3D-QSAR) has been employed to develop a quantitative model for the relationship between single-cell activities for a large set of analogs of (Z)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, and the structural characteristics of the analogs. The method of partial least squares (PLS) projections to latent structures has been used to delineate the relationships between measured activities and chemical structures. The training set includes 42 compounds, and a test set of seven molecules has been employed to assess the predictivity of the model. The model derived is able to calculate the activities of the seven test compounds with an average unsigned error of 0.3, close to the experimental error. Contour maps of regression-like coefficients from transformed PLS components display electronic and nonbonded features of high importance for the observed variation in the activities of compounds in the training set. The contours for the nonbonded (van der Waals) field support previous conclusions that the interaction between the pheromone component and its receptor is characterized by a high steric complementarity.

Key Words—Pheromone analogs, 3D-QSAR, partial least squares, *Agrotis segetum*, Noctuidae, (Z)-5-decenyl acetate analogs, structure–activity relationship.

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INTRODUCTION

A large number of analogs of (Z)-5-decenyl acetate (**1**), a pheromone component of the turnip moth, *Agrotis segetum*, have in previous studies been synthesized and tested by single-cell electrophysiology, and structure–activity relationships have been investigated (Liljefors et al., 1985, 1987; Bengtsson et al., 1987, 1990; Jönsson et al., 1991a,b, 1992, 1993; Gustavsson et al., 1995). In these studies, three pharmacophore elements in **1**, i.e., structural features of high importance for the activity, have been identified: the terminal methyl group, the (Z)-double bond, and the acetate group. Furthermore, on the basis of extensive structure–activity studies, a bioactive conformation of the pheromone component **1** has been proposed (Liljefors et al., 1987, Bengtsson, 1988; Jönsson, 1991a,b). This conformation is shown in Figure 1. In the previously reported structure–activity studies, the analogs are proposed to adopt this type of bioactive conformation, i.e., a conformation in which structural elements corresponding to the pharmacophore elements in **1** are located in the relative positions in space defined by the proposed bioactive conformation of the natural component **1**.

A number of structure–activity relationships have been established for particular series of analogs. For instance, in some series of analogs, a quantitative relationship between the conformational energy required to adopt the bioactive conformation and the electrophysiological single-cell activity has been established (Liljefors et al., 1985, 1987; Bengtsson et al., 1987; Gustavsson et al., 1995). In other series, steric repulsive interactions between the analog and the receptor that decrease the activity have been identified (Jönsson et al., 1991a, 1992, 1993, 1996). However, in these cases, a quantification of the steric repul-

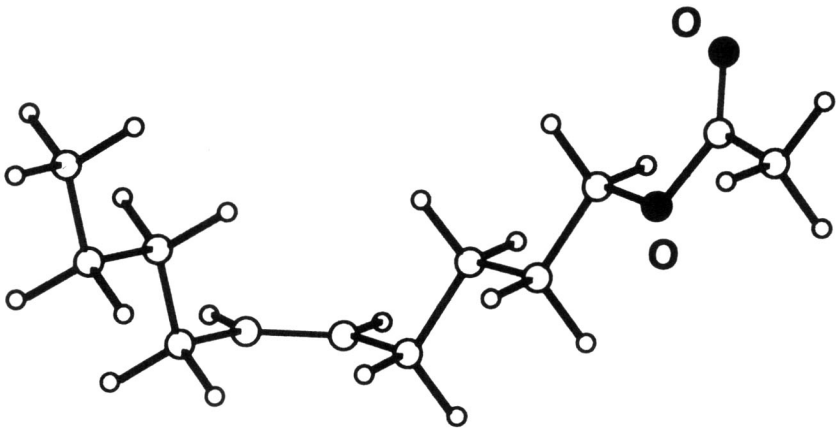


FIG. 1. (Z)-5-Decenyl acetate (**1**) in its proposed bioactive conformation.

sive interactions has not been possible. Thus, previous structure–activity studies have established local relationships of a quantitative, mixed quantitative/qualitative, or pure qualitative nature. Considering the large amount of data available for analogs of **1** obtained by the same methodology (single-sensillum recordings), it is of interest to attempt to establish a quantitative relationship between the single-cell activities of a large and structurally varied set of analogs and the structural characteristics of the analogs. Furthermore, it is of interest to investigate if such a relationship is capable of predicting activities of compounds not used in the development of the model. Such an attempt, based on the methodology of three-dimensional quantitative structure–activity relationships (3D-QSAR) (Norinder, 1993 and references therein; Kubinyi, 1993) is presented in this work. To our knowledge, this methodology has not previously been used in structure–activity studies of pheromone component analogs.

Models aimed at delineating the relationships between the structure of a compound and its biological activity or property are referred to as QSARs (quantitative structure–activity relationships) (Jonsson et al., 1991). The principles behind QSARs are that the properties of a structure are translated into a row vector using a pertinent set of numerical descriptors. A number of parameterized structures together form a matrix X (Figure 2). The structural information in this matrix is subsequently correlated to quantitative indices of the corresponding biological activities, by means of multivariate methods such as partial least

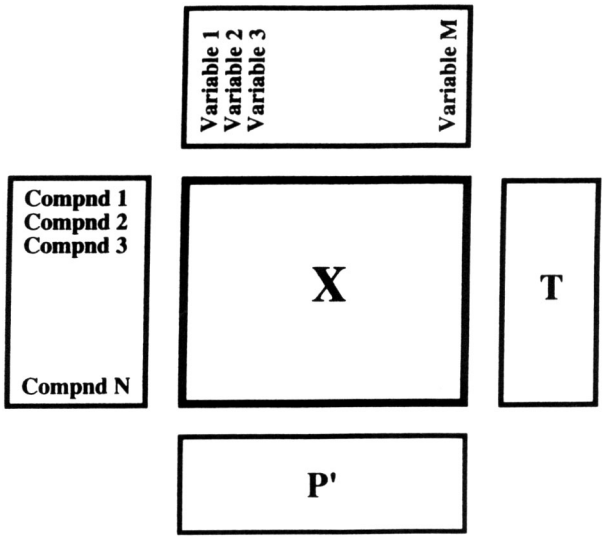


FIG. 2. Overview of the PLS method whereby the matrix X is partitioned into a column score matrix T and a row loading matrix P'.

squares (PLS) (Wold et al., 1993), which extracts and condenses the systematic information in the multiproperty matrix X into a few orthogonal components T (Jackson, 1991; Jolliffee, 1986; Martens and Naes, 1991; Malinowski, 1991) (for further details, see Methods and Materials). In summary, PLS reexpresses a matrix X (e.g., a set of data collected for a number of compounds) as a mean vector plus the product of a few column score matrix T times a few row matrix P' (Figure 2). The scores t contained in T provide the best linear summary of X and may be seen as the underlying factors of the studied system. These variables, often referred to as principal properties, are well suited as descriptors since they are few and orthogonal (independent) and are possible to interpret in terms of the particular aspects of the structures they reflect. The established model may thereafter be used to predict the biological activities for additional structures belonging to the class. Another objective may be to interpret the model in terms of which features of a particular class of compounds are most influential on biological activity. The models may subsequently be used to generate new synthetic structures that are predicted to have improved biological properties/affinities.

The analogs selected for this study are shown in Figure 3.

METHODS AND MATERIALS

Single-Cell Activities. All activity data used in the present study were obtained by single-sensillum recordings. The data for the analogs were taken from previous studies: compounds **2–7** from Liljefors et al. (1987), **8–12** from Wenqui et al. (1993), **13** and **14** from Jönsson et al. (1996), **15–18** from Gustavsson et al. (1995), **19–29** from Jönsson et al. (1991a, 1992, 1993), **30–34** from Bengtsson et al. (1987), **35** and **36** from unpublished results (B. S. Hansson, Lund University), **37–39** from Gustavsson et al. (1997), **40–43** from Jönsson et al. (1991a, 1992), **44–46** from Jönsson et al. (1991b), and **47–49** from Bengtsson et al. (1990). The experimental data used are given in Table 1. All experimental data are corrected for differences in volatility by using relative vapor pressures as previously described (Liljefors et al., 1985; Bengtsson et al., 1990).

Molecular Mechanics Calculations. As conformational energies and energy optimized geometries in previous studies were calculated by using different versions of the molecular mechanics programs MM2 and MM3 (Burkert and Allinger, 1982; Allinger et al., 1989), the energies and geometries of compounds **1–49** have been recalculated using the MM3(92) program as implemented in the molecular modeling package MacMimic/MM3(92) (Instar Software AB, Ideon Research Park, S-223 70 Lund, Sweden). In particular, the conformational energy

difference (ΔE) between the bioactive conformation and the lowest energy conformation has been calculated. The bioactive conformation for each analog was obtained, as previously described in detail (Liljefors et al., 1987), by using the proposed bioactive conformation of **1** (Figure 1) as a template. For each of the compounds **1–43**, except **35** and **37**, the terminal carbon atom, the carbonyl carbon, the carbonyl oxygen, and the carbon atom bonded to the carbonyl carbon were fixed to the same positions as in **1**. As compound **35** does not have a carbon atom bonded to the carbonyl carbon, only the first three atoms were used. The carbonyl group of compound **37** is not in the same position as that of **1**, and in this case only the terminal methyl carbon and the two carbons of the ethyl group bonded to the ester oxygen were used. In compounds **44–49**, the last carbon in the nonpolar chain was fixed to the same position as the corresponding carbon in **1**. In all analogs, the C−C=C−C fragment was fixed to the plane of the corresponding fragment in **1**. The geometries of all analogs were energy optimized with the constraints described above giving the final alignment of the compounds. The calculated conformational energies (ΔE) of the bioactive conformations are shown in Table 1.

Descriptor Generation. A 3D grid was spanned around the aligned structures (2873 points with an internal separation of 1.5 Å), and two values related to nonbonded and electrostatic (charge–charge) interactions were computed for each grid point. A methyl probe was used for the nonbonded calculations using the equation of Del Re et al. (1977). A positive point charge probe (charge = +1) was used for the electrostatic computations using the AM1 atomic charge distributions of each structure calculated by using the semiempirical quantum chemical AM1 method as implemented in the MOPAC package (MOPAC Version 6.0, QCPE program No. 455, University of Indiana, Bloomington, Indiana 47405). An upper cutoff value of 50 kcal/mol was used for the nonbonded interactions. A "missing value" was assigned to a grid point in the charge–charge calculations if the distance between the closest atom and the methyl probe was less than the sum of the van der Waals radii. The two sets (also called fields in 3D-QSAR terminology) of interactions (nonbonded and charge–charge) were mean centered for each column and transformed to a common scale using block scaling whereby each field (block) was divided by the square root of the total sums of squares of all variations (columns) belonging to the respective field prior to any statistical operation.

Three additional parameters, the partition coefficient (logP, Table 1) calculated using the Ghose-Crippen method (Ghose et al., 1988) as implemented in the SPARTAN program (Wavefunction Inc., Irvine, California 92715), the corresponding quadratic term, and the conformational energy of the bioactive conformation (ΔE), described above, were added as descriptors for each compound.

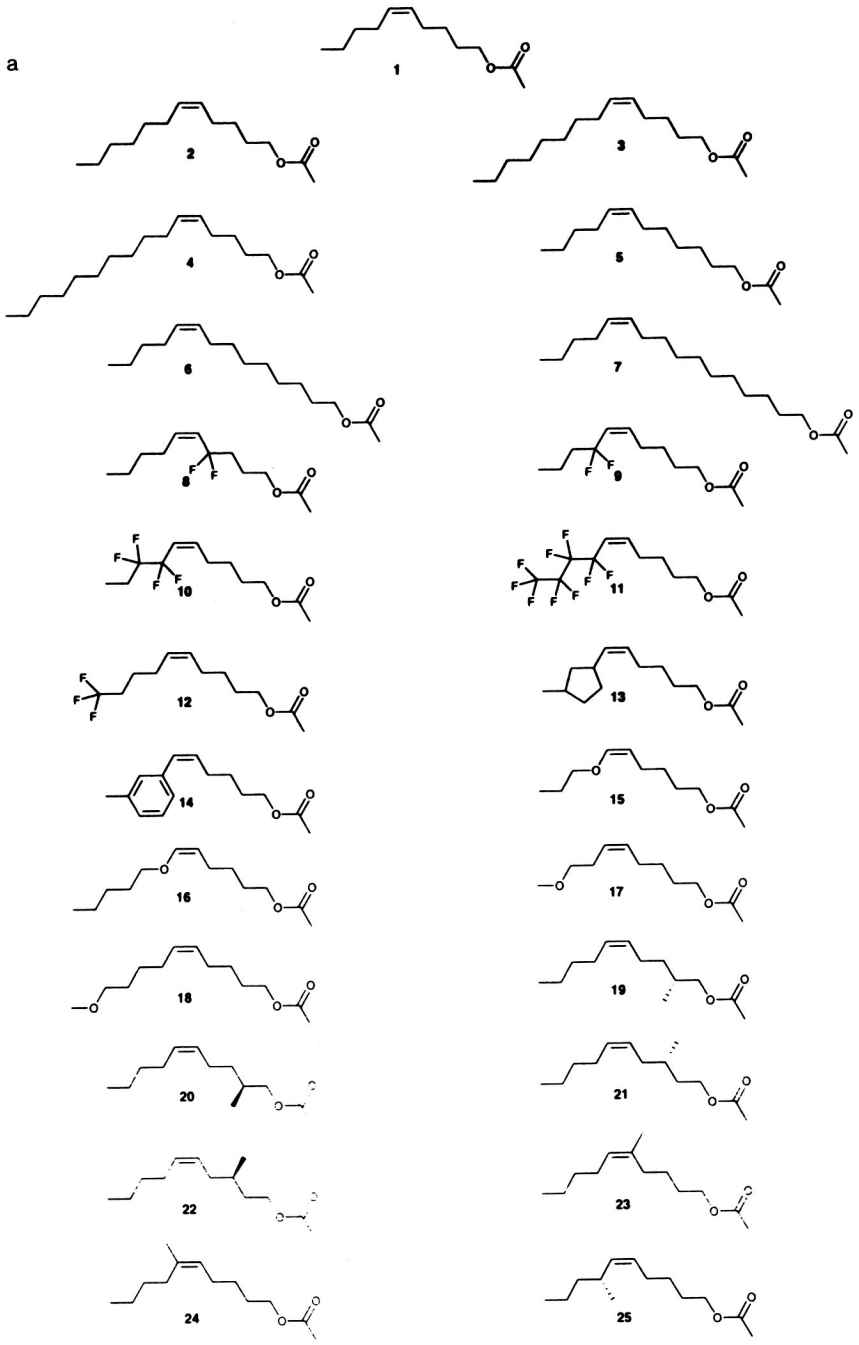


FIG. 3. Compounds included in the present study.

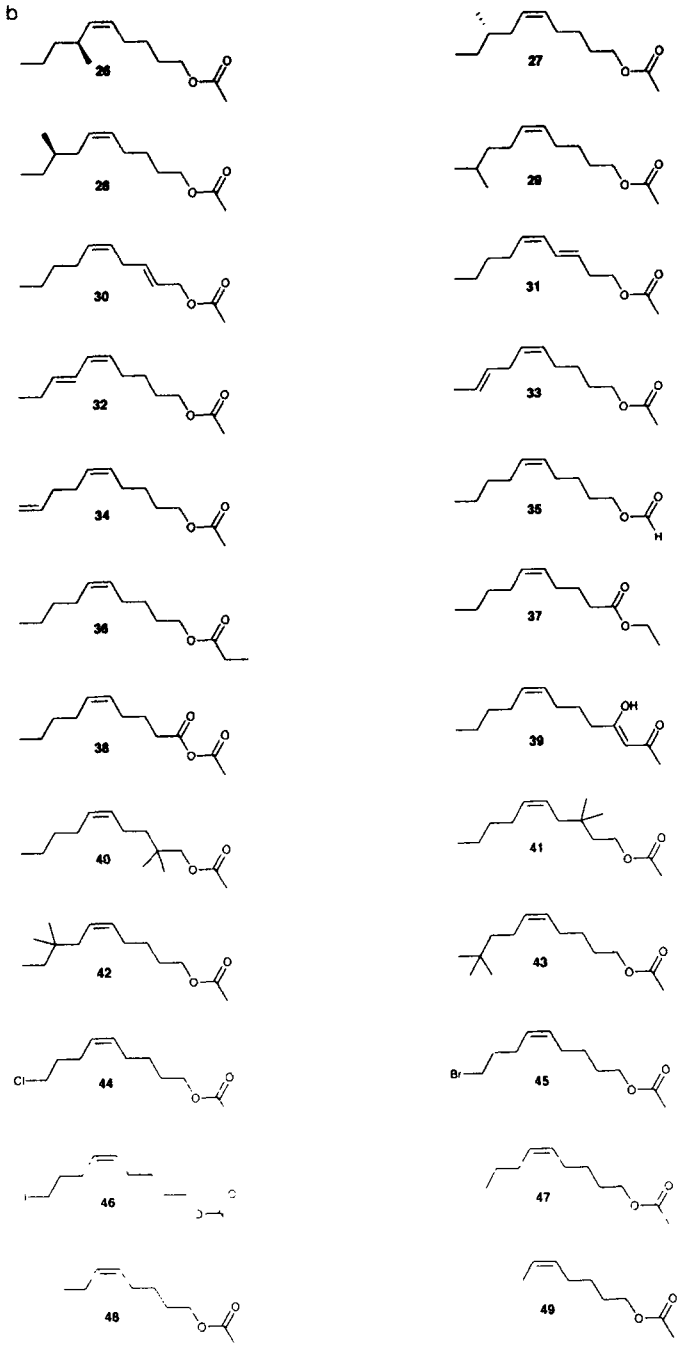


FIG. 3. Continued.

TABLE 1. CALCULATED CONFORMATIONAL ENERGIES (ΔE) FOR BIOACTIVE CONFORMATIONS, PARTITION COEFFICIENTS (log$P$), AND EXPERIMENTAL AND PREDICTED BIOLOGICAL ACTIVITIES FOR COMPOUNDS **1–49**[a]

Compound number	Conformational energy (ΔE)	log$P$	log(exp. activity)	log(calc. activity)
Training set				
1	0.6	3.38	7.0	5.7
2	6.8	4.22	4.2	3.9
3	11.7	5.05	2.5	2.5
4	9.3	5.89	4.8	5.0
5	6.9	4.22	4.5	4.6
6	10.4	5.05	2.0	2.0
7	10.7	5.89	4.0	3.7
8	0.7	3.26	4.9	4.8
9	0.5	3.26	5.1	4.4
10	0.6	3.44	5.1	5.0
11	0.6	4.37	2.0	2.6
12	1.2	3.68	4.9	4.6
13	1.8	3.54	4.9	5.1
14	1.5	3.81	4.0	3.8
15	2.2	2.15	4.8	4.6
16	2.8	2.98	5.0	4.7
17	0.5	1.71	6.2	5.9
18	4.0	2.54	4.3	4.7
19	1.7	3.78	4.8	5.0
20	1.6	3.78	4.6	4.7
22	2.5	3.71	5.4	5.2
23	0.5	3.56	4.1	4.8
24	0.4	3.56	6.0	6.1
26	3.3	3.71	4.1	4.7
31	3.1	3.06	5.8	5.6
32	4.4	3.06	4.1	4.5
33	1.4	3.06	6.2	6.0
35	0.4	3.12	4.6	5.0
36	0.8	4.04	5.6	5.8
37	1.5	3.55	3.5	3.0
38	1.7	2.92	4.7	4.8
39	4.2	3.23	5.4	5.7
40	0.7	4.32	4.2	4.0
41	3.0	4.18	3.8	3.8
42	2.2	4.18	5.0	5.1
43	0.8	4.18	5.1	5.3
44	1.2	2.99	5.3	5.5
45	1.2	3.11	5.6	5.4
46	1.2	3.50	5.1	5.2
47	[b]	2.97	5.5	5.5
48	[b]	2.55	4.8	4.7
49	[b]	2.13	3.0	3.2

TABLE 1. Continued

Compound number	Conformational energy (ΔE)	log$P$	log(exp. activity)	log(calc. activity)
Test set				
21	1.7	3.71	4.6	4.5
25	1.1	3.71	5.3	5.0
27	0.6	3.71	5.1	5.6
28	2.1	3.71	5.1	5.2
29	0.7	3.71	6.0	5.9
30	1.5	3.20	5.0	5.6
34	2.0	3.11	6.0	5.5

[a]The experimental activities are relative to the natural pheromone component **1** for which the log(activity) is set to 7. Conformational energies are in kcal/mol.

[b]These chain-shortened analogs do not fit our conformational energy model as their terminal methyl group can not be superimposed with the terminal methyl group of **1**. A conformational energy value for these analogs is therefore not meaningful.

Training Set Selection. In order to select a training set of compounds that well covered the available chemical property space of the 49 compounds studied, we used a distance-based D-optimal design protocol (Marengo and Todeschini, 1992). The method works, in contrast to other D-optimal procedures using matrix computations, through an exchange algorithm where, in each cycle, a substitution is selected to provide the maximum increase of the minimum distance between the currently selected compounds. The procedure provides a final uniform distribution of the selected compounds from all available structures. The number of training set compounds was set to 42, and 10 random starting points were used. The solution of 42 selected compounds with the largest minimum distance between two compounds was used as a training set. The remaining seven compounds (compounds **21**, **25**, **27–30**, and **34**) were used as an external test set to assess the predictivity of the derived model and the randomly generated models (see section on partial least squares analysis for further details).

Partial Least Squares Analysis. The method of partial least squares (PLS) projections to latent structures (Wold et al., 1993) was used to delineate the relationship between measured biological activities and chemical structures. The PLS method used calculates one component at a time and stops when the added information becomes insignificant as determined by the cross-validation procedure (see below for details). In this way, PLS summarizes the original variables stored in the X matrix as a few orthogonal new variables called scores (t), which are collected in the (few) column score matrix T (Figure 2). The scores, which are linear combinations of the original variables, provide the best linear summary of X and can be seen as the underlying factors of the studied system. The PLS

method solves the problems of forming the model $Y = f(T)$ and finding the ts, i.e., the coefficients (loadings) of the original variables that form each t, at the same time. The loadings are collected in the row matrix P'. The PLS model, as expressed through the score matrix T, can subsequently be transformed into "regression" coefficients of the original variables for comparison purposes so that the influence of each variable can be analyzed in a straightforward manner as is the case for ordinary multiple regression methods. The number of significant components was determined using a leave-one-out cross-validation procedure (LOO-cv) (Wold, 1979). In such a procedure, each compound is removed from the data set once, and the remaining compounds are used to develop the respective model. The left-out compound is then predicted from the developed model. The sum of the squared differences between predicted activity and experimental activity for the left-out compounds (predictive residual sum of squares, PRESS) is computed. If the PRESS value is smaller for the latest calculated PLS component compared to the previous component, then the former component is judged to be significant and kept in the model. Thus, the model with the smallest computed PRESS value is used. In order to avoid overfitting of the data, a maximum of five PLS components was used. To further substantiate the statistical quality of the derived model, 15 randomized models with scrambled biological activities were generated. The PLS components of the resulting model were transformed into regression-like coefficients for contour generation.

Weight Factor Estimation for logP and ΔE Descriptors. Since the variables logP, $\log P^2$, and ΔE are single descriptors among 5746 variables of nonbonded as well as charge–charge interaction type, these three variables have to be given additional impact in order to potentially be able to influence the statistical outcome. In order to assess the magnitude of the weights of logP, $\log P^2$, and ΔE, we performed a study involving eight statistical experiments based on a fractional factorial design scheme (Box et al., 1978) (Table 2) where PRESS was used as a measure of predictivity.

Contour Maps. 3D contour maps were obtained by using the Insight II program (Molecular Simulations Inc., Cambridge, UK). The coefficients were scaled so that the largest absolute value was set to +100 or −100 depending upon the sign; the rest of the values were scaled accordingly.

RESULTS AND DISCUSSION

The evaluation of the experimental design scheme resulted in the following equation:

$$\text{PRESS} = 0.03144 * \text{const}(\Delta E) - 0.02033 * \text{const}(\log P) + 0.00009 * \text{const}(\log P^2) + 36.37860 \quad (1)$$

TABLE 2. FRACTIONAL FACTORIAL DESIGN SCHEME FOR DETERMINING MULTIPLICATION FACTORS FOR ΔE, log P AND log P^2

Experiment	ΔE^a	log P	log P^2	PRESS[b]
1	+ +	+ +	+ +	37.16
2	− +	+ −	− −	35.63
3	+ −	+ −	− +	36.70
4	− −	+ +	+ −	35.25
5	+ +	− −	+ −	38.76
6	− +	− +	− +	36.57
7	+ −	− +	− −	38.05
8	− −	− −	+ +	36.28

[a]Factorial design levels for the factors: + + = 75, + − = 50, − + = 25, − − = 0.
[b]PRESS value using a leave-one-out cross-validation procedure.

TABLE 3. PLS STATISTICS OF DERIVED 3D-QSAR MODEL

Cross-validated q^2	0.177
Ordinary R^2	0.881
Number of PLS components	4
Number of compounds	42
Standard deviation	0.379
F value[a]	68.25

[a]Quota between explained and unexplained variance of the dependent variable.

Equation 1 reveals that the ΔE (conformational energy) parameter and the quadratic logP term should not be used at all, while logP should be multiplied by a factor of 75 (the largest value investigated by the experimental design scheme).

The final PLS model contains four significant components. A summary of the statistical results is given in Table 3. The cross-validated squared correlation coefficient (q^2) defined as

$$q^2 = 1 - (\text{PRESS/SS2}) \quad (2)$$

where SS2 is the sum of squares of the mean centered experimental biological activities and is 0.177 for the derived model, while none of the corresponding values for the 15 randomized models reaches a positive value (Table 4). On the other hand, with respect to the ordinary squared correlation coefficient (R^2) based on fitting the model to the dependent variable, i.e., the biological activity,

several randomized models obtained positive values of considerable magnitude compared to the corresponding value for the "true" model (Table 4).

The q^2 value of 0.177 for the derived PLS 3D-QSAR model may, at first, appear to be rather low compared to the values (0.3–0.8) obtained in other 3D-QSAR investigations (Kubinyi, 1993). However, both Wakeling and Morris (1993) and Clark and Cramer (1993) have shown using randomized training sets that q^2 values, based on a LOO-cv procedure, as low as 0.10 for a training set of 42 compounds with some 5700 variables and four significant components are statistically significant at the 95% level, i.e., there is only a 5% chance that the derived model could be the result of a chance correlation. Furthermore, the somewhat low q^2 value in this investigation is markedly influenced by the nature of the investigated compounds where many compounds have substituents, e.g., methyl groups, not found in other structures, or where only a small number of compounds contain the corresponding substituents or substructures. This situation naturally influences the outcome of the LOO-cv procedure in an unfavorable manner when a compound with an uncommon/unique substituent, e.g., the methyl group in position 3 in compound **22**, is removed from the set of compounds that are used to derive the model and the activity of **22** then is predicted

TABLE 4. PLS STATISTICS OF DERIVED 3D-QSAR MODEL AND 15 RANDOM GENERATED TRAINING SET MODELS

Model name	q^2	R^2 fitted training set/random sets
PLS Model	0.177	0.8852
RAN1	−0.985	0.6609
RAN2	−0.260	0.7823
RAN3	−0.965	0.7335
RAN4	−0.936	0.6977
RAN5	−0.533	0.6887
RAN6	−0.527	0.6391
RAN7	−0.419	0.7607
RAN8	−0.552	0.7612
RAN9	−0.365	0.7153
RAN10	−0.899	0.6672
RAN11	−0.779	0.7331
RAN12	−0.084	0.7203
RAN13	−0.419	0.7607
RAN14	−0.150	0.7688
RAN15	−0.840	0.6504

by that same model. The lack of a more uniform distribution of substructural features among the investigated compounds is also the reason why we have used a large training set (42 compounds) as compared to the test set (seven compounds). The training set must cover the available structural property space in a good manner in order to serve as a well-balanced set for which a model with reasonable predictivity may eventually be derived (Box et al., 1978).

The importance of a proper validation of models is clearly shown in this investigation in which random models may obtain appreciable R^2 values (Table 4) from simply fitting the randomized dependent variable (biological activity) to the independent variables, but where the cross-validation procedure unequivocally detects the lack of predictivity of these random models by their negative q^2 values (Table 4).

The calculated activities for the training set and the test set are given in Table 1. Figure 4 displays the relationship between the experimental single-cell

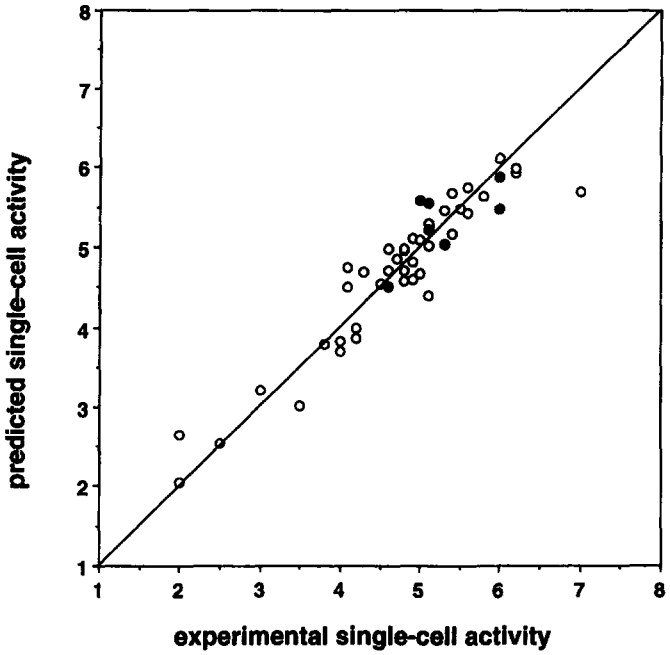


FIG. 4. Experimental vs. predicted single-cell activities for compounds **1–49**. The 42 compounds in the training set are shown by open circles; the seven compounds in the test set are denoted by filled circles. The line in the plot has a slope equal to 1 and indicates a perfect agreement between experimental and calculated activities.

activities and the calculated ones for the two sets of compounds: training set and test set. The straight line in Figure 4 has a slope equal to 1 and denotes a perfect agreement between calculations and experiments. The activities of the training set (unfilled circles in Figure 4) are well modeled by the PLS 3D-QSAR model. Furthermore, the calculated activities of the external test set used to assess the predictivity of the derived model (filled circles) are in good agreement with the experimental data. The average unsigned error of the predicted activities of the seven test compounds is 0.3, which is close to the experimental error in the activities.

A notable feature of the derived PLS model is that the calculated activity of the natural pheromone component **1** deviates significantly more from the experimental value than the calculated activity for any of the other compounds (Table 1 and Figure 4). An inspection of the molecular structures in Figure 3 reveals that the great majority of structural variations of **1** are confined to the nonpolar chain. This may indicate that the information obtained by the structural variations in the polar chain is insufficient to provide a PLS model that is able to accurately calculate the activity of compound **1.** To investigate this, a new PLS model was derived from those 31 compounds in Figure 3 that have polar chains identical to compound **1** and in which the structural variations are confined to the nonpolar chain. The q^2 value for this test model is calculated to be 0.352 and the squared correlation coefficient (R^2) 0.91. However, also in this model, the activity of the natural pheromone component was underpredicted to approximately the same extent as in the original model. Thus, the reason for the underestimation of the activity of compound **1** is most probably not due to lack of structural variation in the training set.

Recent studies on pheromone binding proteins (PBPs) (Prestwich et al., 1995; Prestwich, 1996) may offer an alternative explanation to the underestimation of the activity of **1** in the 3D-QSAR model. The PBP recognizes the pheromone molecule and transports it to its receptor. It has recently been shown that a natural pheromone component binds to the PBP with a higher affinity than is the case for analogs (Prestwich et al., 1995). Thus, even if the pheromone component and an analog have identical concentrations in the air, the pheromone component may have a significantly higher concentration at the receptor. As the experimental activities are expressed in terms of relative quantities required to elicit the same receptor response, and as these relative quantities refer to the relative amounts in the air, the experimental receptor activities may be somewhat overestimated for high affinity ligands to the PBP. This problem may be solved when more affinity data for ligand binding to the PBP become available.

Figure 5 displays a contour map for the electrostatic field at the levels of +25 and −25 and also the aligned compounds in the training set. At these levels, the contours are, as may be expected, concentrated to areas in the vicinity of the polar functional group in **1**. Some smaller contours are found in other



FIG. 5. Contour map (stereo view) of the electrostatic field at values of +25 and −25 surrounding the aligned analog molecules in their deduced bioactive conformations. The small areas not denoted by a value all have a value of −25. The positive contours surround regions where a molecular fragment with a more negative charge would decrease the activity, while the negative contours show regions where a more negative charge would increase the activity.

areas (all these smaller contours are at the −25 level). Negative contours show regions where a molecular fragment with a more negative charge would increase the activity. Positive contours surround regions where a molecular fragment with a more negative charge would decrease the activity. The major negative contour surrounds the carbonyl group in **1**, which emphasizes the importance of an electronegative atom at the position of the carbonyl oxygen in the acetate group. Interestingly, this contour is not symmetric about the carbonyl group, which indicates that the major interaction between the receptor and the carbonyl group, presumably a hydrogen bond interaction, has a well-defined direction *trans* to the C(carbonyl)–O(ether) bond of the ester group. Note that there are no negative contours at the −25 level in the vicinity of the ether oxygen in the acetate group, which also is a potential hydrogen bonding site. As such negative contours appear at negative levels closer to zero, this indicates that such a hydrogen bonding interaction may exist, but that it is of less importance than the interactions with the carbonyl group. The significant positive contour (+25) in Figure 5 indicates that it is unfavorable for an analog to have an electronegative atom in this area as, for instance, in compound **38**. However, as there are only a few molecules in the training set with structural variations in this part of **1** (compounds **37–39**), this conclusion should be viewed with caution, and more compounds with structural variations in this area are required to be able to draw a firm conclusion.

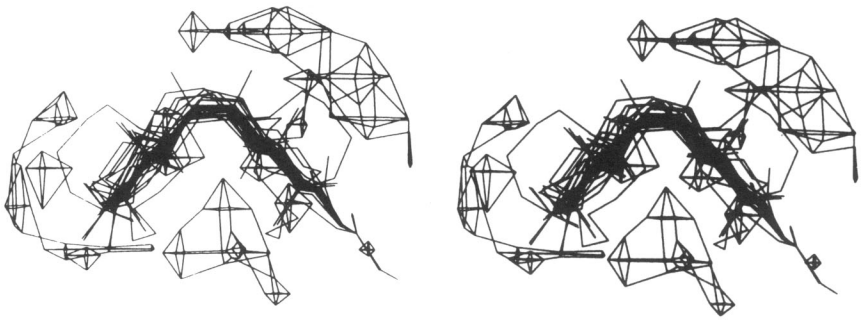


FIG. 6. Contour map (stereo view) of the van der Waals field at a value of −20 surrounding the aligned analogs. The contours indicate areas where a steric interaction would be strongly repulsive and decrease the activity.

Figure 6 shows the contours for the nonbonded (van der Waals) field at a level of −20 surrounding the aligned training set compounds. The corresponding contours at positive levels are essentially featureless and are not shown. The contours in Figure 6 surround areas where a molecular fragment would cause steric repulsions and give an activity decrease. These contours surround most of the aligned molecules, which is in agreement with our conclusions in previous studies on methyl substituted analogs of **1** that there is a very high steric complementarity between the pheromone component molecule and its receptor (Jönsson et al., 1991a, 1992, 1993, 1996). The lack of contours in the area surrounding the acetate methyl group in **1** is a consequence of too little information about this area. Only two molecules, **35** and **37**, with structural variations of the acetate methyl group in **1**, are included in the training set.

CONCLUSIONS

The PLS 3D-QSAR model developed in this study is able to successfully model the relationships between single-cell activities and molecular fields for 42 analogs of (*Z*)-5-decenyl acetate. Furthermore, the model is able to calculate the activities of seven test compounds with an average unsigned error of 0.3, close to the experimental error. Contour maps of regression-like coefficients from transformed PLS components display electronic and nonbonded features of great importance for the observed variation in the activities of compounds in the training set. The contours for the nonbonded (van der Waals) field support previous conclusions concerning the presence of a high steric complementarity between the pheromone component and its receptor.

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COMPARATIVE ANALYSIS OF HEADSPACE VOLATILES FROM DIFFERENT CATERPILLAR-INFESTED OR UNINFESTED FOOD PLANTS OF *Pieris* SPECIES

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Abstract—Plants that are infested by herbivores emit volatile cues that can be used by the natural enemies of the herbivores in their search for hosts. Based on results from behavioral studies, we investigated to what extent intact and herbivore-infested plant species and varieties from the food plant range of *Pieris* herbivore species differ in the composition of the volatile blends. Parasitoids of *Pieris* species, *Cotesia glomerata* and *C. rubecula*, show differential responses towards various herbivore-infested food plants, whereas differences in responses to plants infested by other herbivore species were less clear. Chemical analysis of the headspace samples of red cabbage, white cabbage, and nasturtium plants that were infested by *P. brassicae* or *P. rapae* larvae, or that were intact, yielded 88 compounds including alcohols, ketones, aldehydes, esters, nitriles, terpenoids, sulfides, (iso)thiocyanates, carboxylic acids, and others. The analysis revealed that herbivore-infested plants emit the largest number of compounds in the highest amounts. The plant species affected the volatile blend more than did the herbivore species, and differences between plant varieties were less pronounced than differences between plant species. Differences in headspace composition between plants infested by *P. brassicae* or *P. rapae* were mainly of a quantitative nature. Herbivore-infested nasturtium differed considerably from the cabbage varieties in a qualitative way. Headspace compositions of red and white cabbage varieties were comparable to that of the food plant Brussels sprouts (*Brassica oleracea gemmifera* cv. Titurel) as determined in earlier studies in our laboratory. With respect to plant response to herbivory, nasturtium differed considerably from the cabbage varieties analyzed so far and shows resemblance with Lima bean,

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cucumber, and corn. These plant species produce a greater quantity and variety of volatiles under herbivore attack than intact plants. The results of this study are discussed in relation to behavioral observations on *C. glomerata* and *C. rubecula*.

Key Words—*Pieris*, cabbage, crucifers, *Brassica*, *Tropaeolum*, herbivore-induced synomones, volatile infochemicals, green leaf volatiles, headspace analysis, tritrophic interactions.

INTRODUCTION

Herbivory leads to an increased emission of volatiles by damaged plants. These chemicals can be general plant compounds, toxins, repellents against herbivores, or antibiotics against pathogens, or they can serve in the plant's indirect defense when they are used by natural enemies during host-finding (Price, 1981; Vinson, 1981; Dicke and Sabelis, 1988; Dicke et al., 1990a; Dicke, 1994; Turlings et al., 1990, 1995; Takabayashi and Dicke, 1996).

The headspace volatiles from herbivore-infested plants can vary substantially [see Takabayashi and Dicke (1996) for review]. Relevant factors are plant species, plant cultivar, plant parts under attack, leaf age, growing conditions of plant, time of the day, herbivore species, and herbivore instar.

Volatiles from herbivore-damaged plants are an important source of information for the herbivore's natural enemies during their search for hosts (Vet and Dicke, 1992). Variation in headspace composition that is affected by herbivore species or herbivore instar may be relevant to the natural enemy, since it provides information on the herbivore identity and thus its suitability to serve as a prey or host (Vet and Dicke, 1992). This may also be true for variation due to plant species or cultivar because the plant may affect the herbivore's suitability for the natural enemy (Moraes and McMurtry, 1987). However, the large degree of variation in information from herbivore-infested plants can easily devaluate the role of these infochemicals as the information becomes less reliable. Natural enemies are expected to have developed strategies to utilize those stimuli that most likely lead to host encounter. Apart from innate responses to infochemicals, learning plays an important role. During learning the natural enemy can associate plant volatiles with the presence of hosts (Vet and Dicke, 1992; Turlings et al., 1993; Vet et al., 1995). As a result, associative learning can lead to a temporary specialization, and variation in infochemicals is essential for this. Furthermore, learning to respond to cues from one plant–host complex can facilitate host-finding when other host-infested plants emit similar compounds or volatile blends. By generalization of learned information animals can effectively expand their foraging arena (Smith, 1993).

To assess the type and degree of variation that natural enemies face, it is important to study the variation in headspace composition among plant species

and plant cultivars infested by the same herbivore species, as well as among plants of one cultivar that are infested by different herbivore species. Studies by Dicke et al. (1990b), Turlings et al. (1993), Takabayashi et al. (1991, 1994), and Takabayashi and Dicke (1996) show that variation in headspace composition is largest between different plant species infested by the same herbivore and smallest between plants of one cultivar that are being infested by different herbivore species. In other words: the plant is most important in determining the headspace composition. We have extensively studied the host-finding behavior of two larval parasitoids of the pierids *Pieris brassicae* L. and *P. rapae* L. (Lepidoptera: Pieridae). The food plants used in these studies are the cabbage varieties Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Titurel), white cabbage (*Brassica oleracea capitata* L. var. *alba* cv. Langedijker de Waar), red cabbage [*Brassica oleracea capitata* L. var. *rubra* (DC.) cv. Langedijker vroege rode], and the noncruciferous nasturtium (*Tropaeolum majus*) (Tropaeolaceae). The parasitoids *Cotesia glomerata* (L.) and *C. rubecula* Marshall (Hymenoptera: Braconidae) clearly discriminated between different plant species or cultivars infested by *Pieris* caterpillars (Geervliet et al., 1996). However, discrimination between plants from one cultivar that were infested by caterpillars of either of the two *Pieris* species was not recorded for *C. rubecula* and only occurred in *C. glomerata* after learning (Geervliet et al., 1996, 1997a). In addition to these laboratory studies, field studies also revealed that parasitisation of *Pieris* hosts varied with plant species. Caterpillars on cabbage varieties suffered from high levels of parasitization, whereas caterpillars on nasturtium plants escaped parasitism by both parasitoid species (Geervliet et al., 1997c).

To understand the mechanism underlying these results, we compared the headspace profiles of white cabbage, red cabbage, and nasturtium, either when undamaged or infested by first instars of *P. brassicae* or *P. rapae*. Blaakmeer et al. (1994) and Mattiacci et al. (1994) have already performed comparable headspace analyses of undamaged, artificially damaged, caterpillar regurgitant-treated, and *P. brassicae*- or *P. rapae*-infested Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Titurel), the food plant on which pierid hosts are reared in our laboratory. Their studies revealed that the plants respond to damage with an increased release of "green-leaf volatiles" and that upon caterpillar damage or application of regurgitant, the emission of "green-leaf volatiles" is enhanced (Mattiacci et al., 1994). No significant differences were found between headspace compositions of Brussels sprouts plants infested by either *P. brassicae* or *P. rapae* (Blaakmeer et al., 1994). Our results are compared with these findings.

METHODS AND MATERIALS

Plants. White cabbage (*Brassica oleracea capitata* L. var. *alba* cv. Langedijker de Waar), red cabbage [*Brassica oleracea capitata* L. var. *rubra* (DC.)

cv. Langedijker vroege rode], and nasturtium (*Tropaeolum majus* cv. Mahogeny) (Tropaeolaceae) were reared in a greenhouse compartment at 20–25°C, 50–70% relative humidity, and a 16L:8D photoperiod. When necessary, plants were treated against aphids with DDVP (2,2-dichlorovinyl dimethyl phosphate), but not within a period of four days before using them for experiments.

Insects. Stock colonies of *Pieris brassicae* and *P. rapae* were maintained on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Titurel) in a climate room at 20–22°C, 50–70% relative humidity, and a 16L:8D photoperiod.

Plant Treatments. With each type of food plant, three treatments were used for volatile collection: plants were undamaged (UND), or infested by first instars of either *P. brassicae* or *P. rapae*. To obtain herbivore-infested plants, female butterflies of either host species were allowed to oviposit for 4–6 hr on plants of the desired type. Plants with eggs were kept in the culturing room until hatching of the eggs. Early first instar were gently transferred from the plant to a 4- to 6-week-old plant of the same type one day before headspace sampling. This was done to standardize feeding damage and to use fresh, small food plants that fitted in the headspace collection flask (see below). On each plant 100 *P. brassicae* or 40 *P. rapae* were used, so that the amount of leaf damage inflicted was approximately the same for either plant–host complex.

Collection of Headspace Volatiles. Headspace samples were collected in the period May–November 1995. Odor sources were contained in a 5-liter glass flask with a 10-cm-ID opening, that was closed by a glass lid with an air inlet and an air outlet (Mattiacci et al., 1994). Plants were carefully removed from the pot soil, avoiding damage to the roots and the leaves. The root system was gently rinsed with tap water. The plant was carefully transferred to an Erlenmeyer flask filled with tap water that was placed inside the 5-liter collection flask.

Pressurized air was filtered over silica gel (with anhydrous potassium hydroxide), molecular sieves 4A and 13X (Linde) and activated charcoal before entering the flask (Dicke et al., 1990a; Takabayashi et al., 1991; Mattiacci et al., 1994). The air inlet, air outlet, filters, and sampling jar were connected with 0.8-cm-diam. Teflon tubing. The system was purged for 3 hr at an airflow rate of 500 ml/min to remove volatile contaminants, after which the flow was stopped for 1 hr to allow accumulation of volatiles in the flask. Subsequently, volatiles were collected on Tenax-TA for 1 hr at a flow rate of 500 ml/min (see Mattiacci et al., 1994, for more details of collection procedure).

Analysis of Collected Headspace Volatiles. The collected volatiles were released from the Tenax by heating the trap in a Thermodesorption Cold Trap Unit (Chrompack, Middelburg, The Netherlands) at 250°C for 10 min and flushing with a helium flow of 10 ml/min. The released compounds were cryofocused in a cold trap (0.52 mm ID deactivated fused silica) at an approximate temperature of −85°C (Dicke et al., 1990a). By ballistic heating of the cold

trap to 220°C, the volatiles were transferred to the analytical column (Supelcowax 10, 60 m × 0.25 mm ID, 0.25-μm film thickness), which was connected to a Finnigan MAT 95 mass spectrometer. The column was programmed from 40°C (3 min hold) to 270°C (4 min hold) at 4°C/min and the initial helium velocity was 30 cm/sec. The mass spectrometer was operated in the 70 eV EI ionization mode and scanning was done from mass 24 to 400 at 0.7 sec/dec. Compounds were identified by comparison of the mass spectra with those in the Wiley library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index. The contribution of each compound in a sample to the headspace was calculated as a percentage by dividing the peak area of that compound by the total peak area in that sample. For each compound the percentages determined in the replicates of a treatment were averaged.

RESULTS

Only compounds that were detected in more than one sample per plant species were included in the analysis (Table 1). Among treatments (23 samples), 88 compounds were detected and identified as alcohols, ketones, aldehydes, esters, nitriles, terpenoids, sulfides, (iso)thiocyanates, carboxylic acids, and others.

Herbivore-Infested Plants Versus Intact Plants. Herbivore-infested plants produced the largest number of compounds, although qualitative differences between infested and intact white or red cabbage plants were less pronounced than between infested and intact nasturtium plants (Table 1). Furthermore, the amounts of volatiles released from herbivore-infested plants were larger than from uninfested plants. In the cabbage varieties, the average total peak area was about twice as high in infested plants compared to intact plants, while in nasturtium average total peak areas of infested plants were 10–17 times larger than those of intact plants (Table 1, before last line).

Cabbage Varieties Versus Nasturtium. Qualitative comparison of the headspace profiles of white cabbage and red cabbage shows that they resemble each other more than either resembles nasturtium. Several striking differences in the composition of the volatile blends were found between the two cabbage varieties and nasturtium (compound numbers refer to Table 1 and Figures 1 and 2): (1) In five of five herbivore-infested nasturtium samples two esters (methyl 2-methylpropanoate (34) and methyl 2-methylbutanoate (35) were detected, which were absent in intact nasturtium and in both cabbage varieties (intact and herbivore-infested). (2) A large number of terpenoids (α-cubenene, 59; β-cubenene, 60; β-elemene, 63; β-caryophyllene, 64; α-humulene, 65; γ-muurolene, 66; germacrene-D, 67; β-selinene, 68; α-muurolene, 69; α-selinene, 70;

TABLE 1. PERCENTAGES[a] (±SE) OF TOTAL AREA OF GC PEAKS FOR COMPOUNDS DETECTED IN HEADSPACE OF RED CABBAGE, WHITE CABBAGE, AND NASTURTIUM LEAVES INFESTED BY *P. brassicae* LARVAE, *P. rapae* LARVAE, OR UNINFESTED LEAVES

Compounds[b]	Red cabbage		
	P. rapae (N = 3)	*P. brassicae* (N = 2)	Undamaged (N = 2)
Alcohols			
1 3-Methyl-2-butanol	0.04 ± 0.04^{+}	0.9 ± 0.9^{+}	
2 3-Pentanol$^{\#\triangle}$	0.09 ± 0.09^{+}	0.2 ± 0.2^{+}	0.3 ± 0.3^{+}
3 1-Butanol	0.03 ± 0.03^{+}	0.07 ± 0.07^{+}	2.7 ± 2.7^{+}
4 1-Penten-3-ol$^{\circ\#\triangle}$	0.9 ± 0.4^{+++}	0.6 ± 0.6^{+}	
5 3-Methyl-2-pentanol$^{\triangle}$	0.2 ± 0.2^{+}		–$^{+}$
6 1-Pentanol$^{\#\triangle}$	0.2 ± 0.2^{++}		0.2 ± 0.2^{+}
7 2-Penten-1-ol$^{\triangle}$	0.4 ± 0.4^{+}	0.1 ± 0.1^{+}	
8 1-Hexanol$^{\#\triangle}$	0.8 ± 0.3^{+++}	0.1 ± 0.1^{+}	
9 (*Z*)-3-Hexen-1-ol$^{\circ\#\triangle}$	32.7 ± 14.8^{+++}	10.4 ± 9.5^{++}	0.1 ± 0.1^{+}
10 1-Heptanol$^{\triangle}$	0.2 ± 0.2^{+}		0.5 ± 0.5^{+}
11 1-Octanol$^{\triangle}$	0.6 ± 0.6^{++}	0.2 ± 0.2^{+}	0.7 ± 0.7^{+}
Ketones			
12 2-Pentanone$^{\triangle}$	0.1 ± 0.1^{+}	0.3 ± 0.3^{+}	
13 3-Pentanone$^{\circ\#}$	1.8 ± 0.7^{+++}	2.1 ± 0.06^{++}	2.3 ± 0.2^{++}
14 3-Methyl-2-pentanone$^{\#\triangle}$	0.6 ± 0.4^{++}	0.9 ± 0.9^{+}	0.5 ± 0.5^{+}
15 1-Penten-3-one$^{\#\triangle}$	0.6 ± 0.4^{+++}	0.9 ± 0.9^{+}	
16 2-Heptanone$^{\triangle}$			
17 3-Octanone			
18 1-Octen-3-one			0.1 ± 0.1^{+}
19 6-Methyl-5-hepten-2-one$^{\triangle}$	0.09 ± 0.09^{+}		1.4 ± 0.3^{++}
20 2-Methyl-2-cyclopenten-1-one		0.08 ± 0.08^{+}	
21 2-Nonanone		0.03 ± 0.03^{+}	
22 3,5,5-Trimethyl-3-cyclohexen-1-one	0.07 ± 0.07^{+}		
23 6,10-Dimethyl-5,9-undecadiene-2-one			
Aldehydes			
24 Hexanal$^{\circ\#}$	1.0 ± 0.6^{+++}	0.6 ± 0.6^{+}	0.5 ± 0.5^{+}
25 2-Pentenal$^{\#}$	0.02 ± 0.02^{+}	0.2 ± 0.2^{+}	
26 Heptanal$^{\triangle}$	0.4 ± 0.4^{++}		0.2 ± 0.2^{+}
27 (*E*)-2-Hexenal$^{\#\triangle}$	2.0 ± 1.9^{++}	0.2 ± 0.2^{+}	
28 Octanal$^{\circ\#\triangle}$	1.4 ± 0.9^{+++}	0.4 ± 0.4^{+}	2.7 ± 1.1^{++}
29 Nonanal$^{\#}$	4.5 ± 3.0^{+++}		6.3 ± 6.3^{+}
30 Decanal$^{\#}$	2.6 ± 3.7^{+}	1.6 ± 1.6^{+}	16.3 ± 10.4^{++}
31 Undecanal	0.3 ± 0.4^{+}	0.3 ± 0.3^{+}	1.4 ± 1.4^{+}
32 Phenylacetealdehyde$^{\triangle}$	0.2 ± 0.2^{+}		
33 Dodecanal	0.3 ± 0.3^{+}		1.8 ± 1.8^{+}
Esters			
34 Methyl 2-methylpropanoate			
35 Methyl 2-methylbutanoate			
36 3-Methyl-3-buten-1-yl acetate$^{\#}$	0.8 ± 0.7^{++}	0.5 ± 0.5^{+}	

Treatments[c]					
White cabbage			Nasturtium		
P. rapae ($N = 3$)	*P. brassicae* ($N = 3$)	Undamaged ($N = 3$)	*P. rapae* ($N = 3$)	*P. brassicae* ($N = 2$)	Undamaged ($N = 2$)
$3.3 \pm 3.3^{+}$	$0.06 \pm 0.06^{+}$	$0.6 \pm 0.5^{++}$	$0.1 \pm 0.1^{+}$	$0.7 \pm 0.4^{++}$	
$0.03 \pm 0.03^{+}$	$0.07 \pm 0.07^{+}$		$0.1 \pm 0.1^{+}$	$0.3 \pm 0.3^{+}$	
$0.4 \pm 0.4^{+}$					
$0.1 \pm 0.1^{+}$			$0.2 \pm 0.2^{+}$		
	$0.03 \pm 0.03^{+}$				
$0.08 \pm 0.08^{+}$	$0.08 \pm 0.08^{+}$			$1.0 \pm 0.1^{+}$	
$4.4 \pm 1.8^{+++}$	$8.7 \pm 0.7^{++}$	$0.5 \pm 0.5^{+}$	$1.8 \pm 1.8^{+}$	$1.0 \pm 1.0^{+}$	
	$0.3 \pm 0.3^{+}$	$0.3 \pm 0.3^{+}$	$0.2 \pm 0.2^{+}$	$0.1 \pm 0.1^{+}$	$2.6 \pm 2.6^{+}$
$0.3 \pm 0.3^{+}$	$0.3 \pm 0.2^{++}$	$0.2 \pm 0.2^{+}$	$0.3 \pm 0.2^{++}$	$0.3 \pm 0.3^{+}$	
$0.7 \pm 0.7^{+}$	$-^{+++}$	$-^{+}$			
	$-^{+++}$	$0.3 \pm 0.3^{+}$	$0.2 \pm 0.2^{+}$		
$4.1 \pm 3.6^{++}$	$2.3 \pm 0.9^{+++}$	$1.8 \pm 1.2^{+++}$			
$1.2 \pm 1.6^{++}$	$1.0 \pm 0.1^{+++}$		$0.7 \pm 0.5^{++}$	$0.5 \pm 0.5^{+}$	
	$0.2 \pm 0.2^{++}$	$0.1 \pm 0.1^{+}$		$-^{+}$	
$0.2 \pm 0.2^{+}$	$0.1 \pm 0.1^{+}$				
$0.06 \pm 0.06^{+}$					
$1.3 \pm 0.9^{++}$	$0.7 \pm 0.7^{+}$	$0.2 \pm 0.2^{+}$	$0.2 \pm 0.2^{+}$	$0.4 \pm 0.4^{+}$	
	$0.2 \pm 0.1^{++}$				
	$0.05 \pm 0.05^{+}$				
$0.1 \pm 0.1^{+}$	$0.3 \pm 0.2^{++}$				
			$0.4 \pm 0.4^{+}$	$0.5 \pm 0.5^{+}$	
$1.2 \pm 1.1^{++}$	$0.3 \pm 0.1^{+++}$	$0.1 \pm 0.1^{+}$		$0.3 \pm 0.2^{++}$	
	$0.06 \pm 0.06^{+}$				
	$0.2 \pm 0.2^{++}$			$-^{+}$	
				$0.3 \pm 0.3^{+}$	
$3.3 \pm 3.1^{++}$	$0.6 \pm 0.5^{++}$	$1.0 \pm 1.0^{++}$	$0.8 \pm 0.2^{+++}$	$0.7 \pm 0.4^{++}$	$3.7 \pm 3.7^{+}$
$8.0 \pm 6.1^{++}$	$3.6 \pm 4.3^{++}$	$3.9 \pm 4.3^{++}$	$3.8 \pm 1.5^{+++}$	$-^{++}$	
$12.1 \pm 9.6^{++}$	$4.0 \pm 2.4^{+++}$	$0.9 \pm 0.9^{++}$	$5.4 \pm 2.4^{+++}$	$4.6 \pm 4.2^{++}$	$23.3 \pm 16.6^{++}$
$0.6 \pm 0.6^{+}$	$0.2 \pm 0.2^{+}$		$0.2 \pm 0.2^{+}$	$0.4 \pm 0.4^{+}$	
			$0.2 \pm 0.2^{+}$		$1.3 \pm 1.3^{+}$
$0.7 \pm 0.7^{+}$	$0.5 \pm 0.5^{+}$				
			$10.1 \pm 2.2^{+++}$	$9.9 \pm 3.0^{++}$	
			$20.1 \pm 1.8^{+++}$	$8.5 \pm 1.2^{++}$	
$0.2 \pm 0.2^{+}$	$0.5 \pm 0.3^{+++}$				

TABLE 1. Continued

Compounds[b]	Red cabbage		
	P. rapae (N = 3)	*P. brassicae* (N = 2)	Undamaged (N = 2)
37 Hexyl acetate°#	0.7 ± 0.8^{++}	0.2 ± 0.2^{+}	0.1 ± 0.1^{+}
38 (Z)-3-Hexen-1-yl acetate°#	25.9 ± 9.6^{+++}	24.0 ± 24.0^{+}	0.7 ± 0.2^{++}
39 Methyl benzoate			0.3 ± 0.3^{+}
40 Methyl salicylate	0.4 ± 0.5^{+}		
Nitriles			
41 3-Methylbutanenitrile	0.04 ± 0.05^{+++}	0.06 ± 0.06^{+}	1.7 ± 1.7^{+}
42 2-Methylbutanenitrile			
43 Benzonitrile	1.1 ± 0.8^{+++}	1.7 ± 1.0^{++}	8.9 ± 7.8^{++}
44 Benzyl cyanide	2.6 ± 2.0^{++}	0.5 ± 0.5^{+}	
Terpenoids			
45 α-Pinene°	0.2 ± 0.2^{++}	2.8 ± 2.7^{++}	0.8 ± 0.3^{++}
46 α-Thujene°#	0.7 ± 0.3^{+++}	7.3 ± 6.3^{++}	4.8 ± 1.9^{++}
47 β-Pinene°#	0.1 ± 0.1^{++}	1.6 ± 1.4^{++}	0.7 ± 0.2^{++}
48 *Sabinene*°#	3.0 ± 0.8^{+++}	9.4 ± 6.3^{++}	14.5 ± 6.3^{++}
49 Myrcene°#	1.7 ± 0.8^{+++}	3.7 ± 2.7^{++}	4.8 ± 2.2^{++}
50 α-Terpinene		2.8 ± 2.8^{+}	
51 Limonene°#	4.8 ± 3.1^{+++}	8.2 ± 5.5^{++}	8.9 ± 3.2^{++}
52 β-Phellandrene°	0.05 ± 0.04^{++}	1.1 ± 1.1^{+}	−$^{+}$
53 1,8-Cineole°#	2.4 ± 1.3^{+++}	6.6 ± 3.4^{++}	7.1 + 0.8^{++}
54 γ-Terpinene		7.9 ± 7.9^{+}	0.3 ± 0.3^{+}
55 p-Cymene		1.3 ± 1.3^{+}	
56 (3E)-4,8-Dimethyl-1,3,7-nonatriene	0.06 ± 0.06^{+}		0.2 ± 0.01^{++}
57 α-Copaene			
58 trans-Sabinene hydrate°	0.3 ± 0.2^{++}		0.9 ± 0.1^{++}
59 α-Cubenene			
60 β-Cubenene			
61 Linalool°△			
62 Longifolene			0.5 ± 0.5^{+}
63 β-Elemene#			
64 β-Caryophyllene			
65 α-Humulene			
66 γ-Muurolene			
67 Germacrene-D			
68 β-Selinene			
69 α-Muurolene			
70 α-Selinene			
71 γ-Cadinene			
72 δ-Cadinene			
73 (3*E*,7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene			
74 Calamenene			
75 α-Calacorene			
76 β-Calacorene			

Treatments[c]					
White cabbage			Nasturtium		
P. rapae (*N* = 3)	*P. brassicae* (*N* = 3)	Undamaged (*N* = 3)	*P. rapae* (*N* = 3)	*P. brassicae* (*N* = 2)	Undamaged (*N* = 2)
0.2 ± 0.2^{+}	0.3 ± 0.2^{++}				
7.4 ± 7.4^{+}	30.7 ± 5.1^{+++}	0.5 ± 0.5^{+}			
		0.1 ± 0.1^{+}	0.7 ± 0.5^{+++}	0.9 ± 0.5^{++}	
0.4 ± 0.4^{+}	0.2 ± 0.2^{++}	4.4 ± 0.2^{++}		0.4 ± 0.4^{+}	
0.1 ± 0.1^{+}	0.3 ± 0.3^{+}				
0.4 ± 0.4^{+}	0.05 ± 0.05^{+}				
6.2 ± 5.6^{+++}	1.3 ± 0.8^{+++}	3.6 ± 3.3^{+++}	2.1 ± 0.9^{+++}	3.0 ± 0.6^{++}	53.9 ± 33.7^{++}
	0.1 ± 0.1^{+}		1.3 ± 0.3^{+++}	15.8 ± 10.8^{++}	
1.7 ± 2.4^{++}	1.0 ± 1.0^{+++}	2.1 ± 1.3^{+++}	0.3 ± 0.3^{++}	0.2 ± 0.2^{+}	
3.7 ± 4.3^{++}	5.0 ± 3.3^{+++}	6.7 ± 1.6^{+++}			
0.6 ± 0.7^{++}	0.6 ± 0.3^{+++}	1.0 ± 0.4^{+++}	0.6 ± 0.6^{+++}	0.3 ± 0.3^{++}	
12.5 ± 13.6^{+++}	11.6 ± 5.8^{+++}	22.5 ± 11.1^{+++}	0.5 ± 0.5^{+++}	0.2 ± 0.2^{+}	
3.0 ± 2.5^{++}	4.7 ± 1.8^{+++}	7.5 ± 3.5^{+++}	0.4 ± 0.4^{++}	0.1 ± 0.1^{+}	
		0.05 ± 0.05^{+}			
5.8 ± 4.4^{++}	9.3 ± 1.9^{+++}	16.0 ± 3.8^{+++}	1.8 ± 1.6^{+++}	4.1 ± 3.9^{++}	0.3 ± 0.3^{+}
0.02 ± 0.02^{+}	0.2 ± 0.1^{+++}	0.2 ± 0.2^{++}	0.5 ± 0.7^{++}	0.1 ± 0.1^{+}	
5.1 ± 5.3^{++}	5.5 ± 1.7^{+++}	10.9 ± 1.7^{+++}			
	0.03 ± 0.03^{+}	0.1 ± 0.1^{+}			
	0.6 ± 0.3^{+++}		0.2 ± 0.2^{+}		
4.5 ± 5.9^{++}	2.2 ± 1.4^{+++}	0.6 ± 0.5^{++}	0.2 ± 0.2^{++}		
			3.2 ± 1.0^{+++}	0.7 ± 0.07^{++}	
0.3 ± 0.3^{+}	0.7 ± 0.3^{+++}	1.3 ± 0.7^{+++}			
			0.7 ± 0.6^{++}		
			0.7 ± 0.2^{+++}	0.2 ± 0.2^{+}	
	0.3 ± 0.2^{++}				
		0.2 ± 0.2^{+}			
			9.3 ± 1.8^{+++}	4.4 ± 3.4^{++}	
			7.1 ± 1.2^{+++}	2.0 ± 1.3^{+}	
			1.1 ± 0.2^{+++}	0.3 ± 0.3^{+}	
			0.7 ± 0.6^{++}	0.1 ± 0.1^{+}	
			3.3 ± 2.5^{++}	2.8 ± 2.8^{+}	
			2.4 ± 1.4^{+++}	0.5 ± 0.5^{+}	
			0.8 ± 0.2^{+++}	0.2 ± 0.1^{++}	
			2.5 ± 2.1^{+++}	0.6 ± 0.6^{+}	
			0.9 ± 0.3^{+++}	0.1 ± 0.1^{+}	
			3.3 ± 1.7^{+++}	1.4 ± 0.4^{++}	
	0.04 ± 0.04^{+}	0.4 ± 0.4^{+}			
			0.6 ± 0.4^{++}		
			2.6 ± 1.3^{+++}	2.0 ± 0.2^{+}	
			1.9 ± 0.2^{+++}	−$^{+}$	

TABLE 1. Continued

Compounds[b]	Red cabbage		
	P. rapae (N = 3)	*P. brassicae* (N = 2)	Undamaged (N = 2)
Sulfides			
77 Dimethyl disulfide[°#△]	0.9 ± 0.9[+++]	0.3 ± 0.02[++]	0.5 ± 0.2[++]
78 Dimethyl trisulfide[°#△]		0.02 ± 0.02[+]	
(Iso)thiocyanates			
79 Methyl(iso)thiocyanate[#△]	0.4 ± 0.3[+++]	−[+]	
80 Benzylisothiocyanate			
Carboxylic acids C-Z			
81 Propanoic acid			0.7 ± 0.7[+]
82 Butanoic acid			0.5 ± 0.5[+]
83 3-Methylbutanoic acid			0.5 ± 0.5[+]
84 Pentanoic acid			0.6 ± 0.6[+]
85 Hexanoic acid		0.9 ± 0.9[+]	4.1 ± 4.1[+]
Others			
86 2-Pentylfuran		0.07 ± 0.07[+]	
87 Furfural			0.2 ± 0.2[+]
88 Indole	2.0 ± 2.0[++]		
Number of compounds	47	42	41
Average total peak area	5,783	4,707	2,995
(SE)	(2,534)	(3,099)	(568)

[a] Average peak areas are calculated by dividing the peak area of a compound in every sample by the total peak area of that sample. Percentages are averages of the samples in a treatment.

[b] °Compound detected by Blaakmeer et al. (1994); #compound detected by Mattiacci et al. (1994); △compounds detected by Agelopoulos et al. (1995).

[c] Each + indicates the presence of a compound in one sample; − indicates no peak areas determined.

γ-cadinene, 71; δ-cadinene, 72; calamenene, 74; α-calacorene, 75; and β-calacorene, 76) were detected consistently in headspace samples of *P. brassicae* and *P. rapae*-infested nasturtium plants only (see Figure 1A). (3) On the other hand, the terpenoids 1,8-cineole (53) and *trans*-sabinene hydrate (58) were present in almost all samples of red and white cabbage, in infested and intact plants, but not at all in nasturtium samples. (4) Nasturtium plants infested by *P. brassicae* or *P. rapae* released benzyl isothiocyanate (80) that was absent in the two cabbage varieties and in uninfested nasturtium. (5) In general, fewer ketones were detected in nasturtium plants, both infested and intact, than in infested and intact red and white cabbage plants. The contribution of terpenoids to the total headspace composition of *P. rapae*- and *P. brassicae*-infested nasturtium plants

Treatments[c]					
White cabbage			Nasturtium		
P. rapae (*N* = 3)	*P. brassicae* (*N* = 3)	Undamaged (*N* = 3)	*P. rapae* (*N* = 3)	*P. brassicae* (*N* = 2)	Undamaged (*N* = 2)
0.9 ± 0.9^{++}	0.5 ± 0.3^{+++}	3.3 ± 2.2^{+++}	0.3 ± 0.2^{++}	–$^{++}$	
0.05 ± 0.05^{+}	0.2 ± 0.2^{++}	0.5 ± 0.3^{+++}	0.06 ± 0.06^{+}	0.06 ± 0.06^{+}	
	0.4 ± 0.3^{++}		0.2 ± 0.2^{+}	0.2 ± 0.2^{+}	
			3.3 ± 3.2^{++}	26.6 ± 26.6^{+}	
		0.3 ± 0.3^{+}		0.2 ± 0.2^{+}	
0.2 ± 0.2^{+}		0.3 ± 0.3^{+}			
			0.3 ± 0.3^{+}		
		1.1 ± 1.1^{+}	0.3 ± 0.3^{+}	0.6 ± 0.6^{+}	
4.8 ± 5.3^{++}		6.9 ± 6.9^{+}	0.9 ± 1.0^{++}	2.9 ± 2.9^{+}	7.0 ± 7.0^{+}
				0.1 ± 0.1^{+}	
					7.9 ± 2.3^{++}
	0.03 ± 0.03^{+}				
41	51	37	50	49	8
3,090	4,323	2,530	2,526	4,037	235
(2,633)	(1,085)	(740)	(102)	(1,420)	(151)

(40% of the total number of compounds) was larger than that of herbivore-infested red cabbage (20%) and herbivore-infested white cabbage (25%). Furthermore, (*Z*)-3-hexenol (9) was detected in a few (two of seven) samples of nasturtium and (*Z*)-3-hexenyl acetate (38) was detected in red and white cabbage only and in none of the nasturtium samples. It should be noted that the low number (eight) of compounds detected in intact nasturtium plants accounts for the high percentages for each compound in this treatment.

White Cabbage Versus Red Cabbage. Qualitative differences in composition of volatile blends between red and white cabbage, either herbivore-infested or intact, were restricted to a few alcohols present in samples of red cabbage only (compounds 1 and 2), while (3*E*)-4,8-dimethyl-1,3,7-nonatriene (56) was

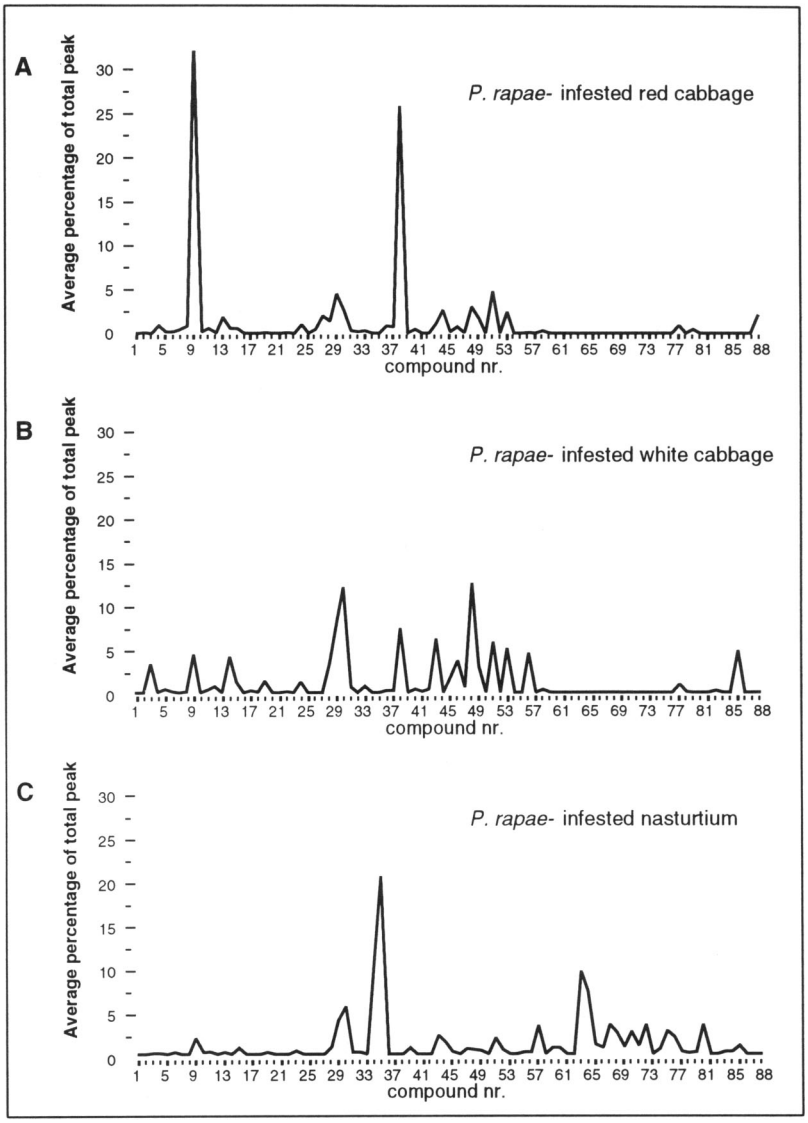


FIG. 1. Average percentages of total area of GC peaks for compounds detected in headspace samples of (A) red cabbage, (B) white cabbage, and (C) nasturtium plants infested by 40 first instars of *P. rapae*. Compound numbers refer to the numbers as presented in Table 1.

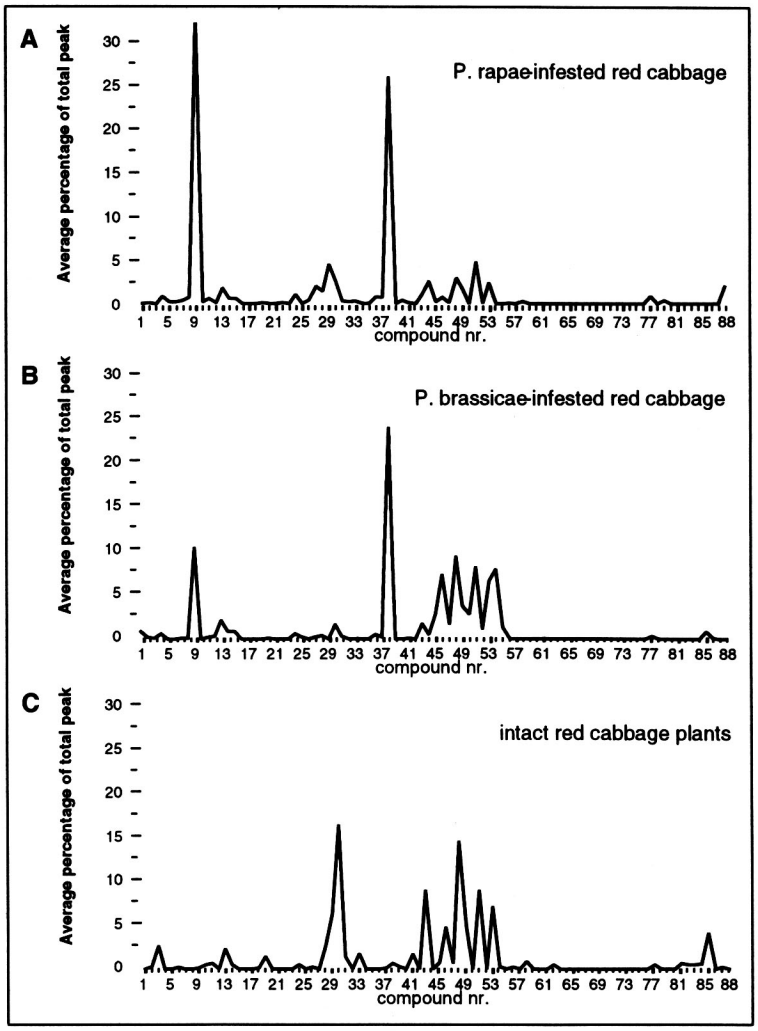


FIG. 2. Average percentages of total area of GC peaks for compounds detected in headspace samples of red cabbage plants infested (A) by 40 first instars of *P. rapae*, (B) 100 first instars of *P. brassicae*, and (C) intact red cabbage plants. Compound numbers refer to the numbers as presented in Table 1.

more pronounced in white cabbage samples (Table 1). Differences were mainly quantitative in nature (Table 1, Figure 1). In *P. rapae*-infested red cabbage plants (Z)-3-hexen-1-ol(9) and (Z)-3-hexenyl acetate (38) were detected in larger proportions than in *P. rapae*-infested white cabbage plants (Figure 1). In con-

trast, decanal (30), benzonitrile (43), sabinene (48), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (56) and hexanoic acid (85) were detected in larger proportions from *P. rapae*-infested white cabbage plants compared to red cabbage samples (Figure 1).

Pieris brassicae *Infested Plants Versus* P. rapae *Infested Plants.* Differences in composition of volatile blends between headspace samples from plants infested by either of the two herbivore species *P. brassicae* and *P. rapae* were minimal (Table 1, Figure 2). Headspace of red cabbage plants infested by *P. rapae* larvae contained larger proportions of (*Z*)-3-hexen-1-ol (9) than *P. brassicae*-infested plants, whereas *P. brassicae*-infested red cabbage plants produced relatively larger proportions of the terpenoids α-pinene (45), α-thujene (46), sabinene (48), limonene (51), and 1,8-cineole (53) (Figure 1B). Except for α-pinene (45), these terpenoid compounds were also detected in relatively large proportions in samples from intact red cabbage plants (Figure 2).

DISCUSSION

Herbivore-Infested Plants Versus Intact Plants. Plant herbivore complexes are known to elicit strong behavioral responses in natural enemies of the herbivores (Dicke et al., 1990a,b; Turlings et al., 1990; Steinberg et al., 1993; Geervliet et al., 1994). Comparisons of chemical blends emitted by herbivore-infested plants and intact plants demonstrated qualitative and/or quantitative changes in the emission of infochemicals by the plant–herbivore complexes (Dicke et al., 1990a,b; Dicke, 1994; Turlings et al., 1990, 1993; Blaakmeer et al., 1994; Mattiacci et al., 1994; Takabayashi et al., 1994, 1995; Takabayashi and Dicke, 1996). Our data also show that herbivore-infested plants produced the highest number of compounds, although qualitative differences between infested and intact cabbage plants are less pronounced compared to the differences between infested and intact nasturtium plants. Differences between intact and infested cabbage plants are mainly of quantitative nature, since infested plants produce similar compounds but in larger amounts than intact plants. In herbivore–plant interactions, two defense options can be recognized (Price et al., 1980; Dicke and Sabelis, 1988; Dicke, 1994, 1998). Plants can have traits by which they directly prevent herbivores from attacking or developing successfully, or they may have indirect defensive traits by which they promote the effectiveness of natural enemies of the herbivores. Cabbage varieties belong to the group with a more direct defense strategy in that they produce glucosinolates that deter many herbivorous species (Chew, 1988). Considering the differences in volatiles between intact and herbivore-infested nasturtium plants, nasturtium seems to employ the extrinsic defense strategy to a larger extent, as do plant species such as Lima bean, cucumber, and corn that produce large amounts of compounds upon herbivory that are not demonstrated in volatile profiles from

intact plants (Dicke et al., 1990b; Turlings et al., 1990, 1993; Dicke, 1994, 1998).

Role of Plant and Herbivore in Determining Headspace Composition. Laboratory and field studies on the parasitoid species *Cotesia glomerata* and *C. rubecula* demonstrated that females of both species differentially respond to food plants infested by pierid hosts. Differences in responsiveness were most clear between different plant species or cultivars infested by the same host species; discrimination between different herbivore species feeding on the same plant cultivar was less clear (Geervliet et al., 1996) and had to be learned (Geervliet et al., 1997a). Herbivore-infested nasturtium elicited low responses in naive *Cotesia* parasitoids that search for hosts compared to the various herbivore-infested cabbage varieties (Geervliet et al., 1996).

Tollsten and Bergström (1988) studied headspace volatiles from intact and mechanically damaged crucifer species and found qualitative and quantitative differences between the various *Brassica* species. The comparative study on Brussels sprouts plants infested by different *Pieris* species by Blaakmeer et al. (1994) revealed minimal differences in volatile profiles that were mainly quantitative in nature. Takabayashi et al. (1991) and Turlings et al. (1995) stated that the plant is more important in affecting the composition of the volatile blend than the herbivore. Our comparative study on the volatile profiles from the cabbage varieties red and white cabbage and the noncruciferous food plant nasturtium supports this view. Samples of plants (cabbage varieties and nasturtium) infested by different herbivore species (*P. brassicae* or *P. rapae*) showed less variability in composition of volatile blends, and differences were mainly quantitative. On the other hand, herbivore-specific responses have been reported for the corn–*Pseudaletia separata* system (Takabayashi et al., 1995), where large differences in volatile compositions were found between plants infested by different instars of the same herbivore species. This difference in herbivore-specific responses between plant species may indicate differences among plant species in the defensive strategies against herbivory (Dicke, 1994, 1998).

Role of Plant Species Versus Plant Cultivar in Determining Headspace Composition. Our data support the suggestion by Takabayashi et al. (1991) that differences between the plant species are more pronounced than those between plant varieties. Composition of volatile blends from herbivore-infested plants or intact plants clearly differed between the two cabbage varieties and nasturtium, both qualitatively and quantitatively, but not so much between the two cabbage varieties, either infested or intact. Samples of plant varieties infested by different herbivore species showed less variability in composition of volatile blends, and variation was mainly quantitative. Furthermore, comparison of the volatile profiles from Brussels sprout plants (Blaakmeer et al., 1994; Mattiacci et al., 1994) with those from red and white cabbage reveals that (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate were also found in high proportions in headspace samples of

P. brassicae-infested Brussels sprouts leaves (Mattiacci et al., 1994). (*Z*)-3-Hexen-1-ol comprised 31% of the total in the headspace of *P. brassicae*-infested Brussels sprouts plants, and only 4–9% in that of undamaged, artificially damaged, or caterpillar-regurgitate-treated leaves (Mattiacci et al., 1994). In our analysis, herbivore-infested red and white cabbage released considerably larger percentages of this compound than intact plants. In our study, green leaf volatiles, such as (*Z*)-3-hexenyl isovalerate, (*Z*)-3-hexenyl butyrate, and (*Z*)-3-hexenyl isobutyrate that were detected in herbivore-infested Brussels sprouts (Blaakmeer et al., 1994; Mattiacci et al., 1994) and that are reported to be involved in the attraction of natural enemies, were not detected. This may account for the reported preference for herbivore-infested Brussels sprouts over infested red cabbage in naive *C. glomerata* and *C. rubecula* (Geervliet et al., 1996).

Nasturtium Versus Cabbage Varieties. In nasturtium samples (*Z*)-3-hexen-1-ol was detected in considerably lower proportions than in red and white cabbage and (*Z*)-3-hexenyl acetate was absent. The latter green-leaf volatile may be important for host-searching by natural enemies (Whitman and Eller, 1990). It was found in high proportions in *P. brassicae*-infested Brussels sprouts plants (Mattiacci et al., 1994) that are highly attractive to *C. glomerata* females. Terpenoids are a major class among herbivore-induced synomones that play a role in host-searching behavior by natural enemies (Dicke et al., 1990a,b; Turlings et al., 1993; Takabayashi et al., 1994). In the emission of this class of compounds, nasturtium showed distinct responses, such as the lack of 1,8-cineole and *trans*-sabinene hydrate, two compounds found consistently in samples of herbivore-infested and uninfested leaves of Brussels sprouts (Blaakmeer et al., 1994; Mattiacci et al., 1994) and red cabbage and white cabbage (this study). On the other hand, in samples of herbivore-infested nasturtium, a high number of terpenoids was detected that were not found in infested red cabbage or white cabbage (this study), Brussels sprouts infested by *P. brassicae* or *P. rapae* (Blaakmeer et al., 1994; Mattiacci et al., 1994), or in cabbage (*Brassica oleracea capitata* cv. Green Coronet) infested by *P. rapae* or *Plutella xylostella* (Agelopoulos and Keller, 1994). The two esters methyl 2-methylpropanoate and methyl 2-methylbutanoate detected in nasturtium were not detected in red or white cabbage, and neither were they reported from cabbage in other studies (Blaakmeer et al., 1994; Mattiacci et al., 1994; Agelopoulos and Keller, 1994), or from other plant–herbivore complexes.

Pieris species feed on plants that contain glucosinolates, which are found in cruciferous plants and in nasturtium (Tropaeolaceae). These secondary plant compounds are the precursors of the volatile isothiocyanates. Methyl (iso)thiocyanate and benzyl isothiocyanate were detected in samples of herbivore-infested nasturtium leaves, while they were absent in the samples of the

cabbage varieties, accounting for another qualitative difference between nasturtium and cabbage.

The qualitatively and quantitatively distinct volatile profiles released by nasturtium in comparison to those known from the cabbage varieties studied so far are likely to account for the different behavior of the parasitoids towards this plant species. The lack or the presence of compounds can make the plant unrecognizable for naive parasitoids, and learning is needed to get increased responses and motivated search by the female parasitoids. In field situations, parasitization of host larvae feeding on nasturtium plants placed among herbivore-infested cabbage varieties rarely occurred, whereas parasitization levels on Brussels sprouts and red cabbage were high (Geervliet et al., 1997c). On the other hand, learning can change the presence of females for certain plant–host complexes, the effect being stronger with initially less attractive plants such as nasturtium. In parasitoids that had their first experience with infested nasturtium, learned responses to nasturtium are not easily overruled by experience with cabbage (Geervliet et al., 1997b). It remains to be tested whether field release of nasturtium-experienced parasitoids would lead to parasitization of *Pieris* hosts on nasturtium plants.

CONCLUSIONS

Our chemical data support the findings of behavioral studies in the system cabbage–*Pieris*–Cotesia. Parasitoids that search for hosts have to deal with variability in the availability of chemical cues emitted by the food plants of their hosts. It was demonstrated that volatile profiles of plants infested by different (related) herbivore species show few, mainly quantitative, differences. It is not known whether the sensory sensitivity of parasitoids is increased with experience, i.e., whether naive parasitoids are less able to detect these differences compared to experienced females. On the other hand, parasitoids may actively choose not to discriminate between such subtle differences until they become more alert to these differences through experience and discriminate (Vet et al., 1997). Based on results found for *Leptopilina heterotoma*, a parasitoid of *Drosophila* flies, a recent hypothesis (Vet et al., 1997) states that qualitative differences in (chemical) information between different substrates are more easily being learned by parasitoids than quantitative differences. Wasps may perceive qualitative differences as relevant information, and use this information to discriminate between different substrates, whereas quantitative differences may be regarded as irrelevant noise. Parasitoids may generalize similarities between herbivore-infested plant varieties to effectively expand their host-foraging arena. Discrimination between substrates with quantitative differences may occur when these differences inform parasitoids about, e.g., high or low host densities

(Geervliet et al., 1997b), or host presence or absence (Papaj et al., 1994). In our system, naive *Cotesia* females were able to discriminate between infested Brussels sprouts and infested red cabbage or infested nasturtium (Geervliet et al., 1996) that showed qualitatively different volatile profiles. *Cotesia glomerata* females did not discriminate between infested Brussels sprouts and infested white cabbage (Vet and van Aaken, unpublished results), although qualitative differences between volatile profiles of Brussels sprouts and white cabbage are found in this study. Despite differences with respect to volatile profiles, Brussels sprouts and white cabbage are very similar with respect to plant color and shape, which may cause female parasitoids to actively decide not to discriminate between these plants during their search for hosts. It is known that parasitoids use visual cues such as color and shape while searching for hosts (Wäckers and Lewis, 1993), and *C. glomerata* females were able to learn to discriminate between herbivore-infested Brussels sprouts and herbivore-infested red cabbage that differ in color (Geervliet et al., 1997a). Additional behavioral experiments with plant–herbivore complexes that only differ quantitatively and/or qualitatively in emitting information are needed to test the hypothesis of Vet et al. (1997) that parasitoids more easily learn to pay attention to qualitative differences than to quantitative differences than to quantitative differences between odor bouquets and that parasitoids initially decide to neglect quantitative differences between substrates until the parasitoid learns through experience that such differences are reliably associated with essential variation in host presence.

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FATE OF HOST-PLANT IRIDOID GLYCOSIDES IN LEPIDOPTERAN LARVAE OF NYMPHALIDAE AND ARCTIIDAE

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Abstract—We compared the ability of larvae of six lepidopteran species to sequester iridoid glycosides. All larvae were fed on a common host plant, *Plantago lanceolata*, which contains two iridoid glycosides, aucubin and catalpol. Four species of arctiids were examined: *Pyrrharctia isabella*, *Spilosoma congrua*, *Spilosoma latipennis*, and *Spilosoma virginica*. For comparison, we also examined two nymphalid species, one of which, *Junonia coenia* (a specialist on plants containing iridoid glycosides), was known to sequester iridoid glycosides, and the other, *Vanessa cardui*, was a generalist that also feeds on *P. lanceolata*. We found that, as expected, *J. coenia* larvae did contain iridoid glycosides, whereas *V. cardui* did not. To our surprise, *Spilosoma congrua* contained substantial amounts of iridoid glycosides (mean = 7.14% dry weight), whereas none of the other arctiids (*S. latipennis*, *S. virginica*, or *P. isabella*) contained detectable levels of iridoid glycosides. We found small amounts of iridoid glycosides in the frass of *S. virginica* but none in the frass of *S. congrua* or *P. isabella*.

Key Words—Arctiidae, Nymphalidae, iridoid glycosides, sequestration.

INTRODUCTION

Sequestration of plant secondary compounds for use as a defense against potential predators is found in many groups of herbivorous insects, most notably the Lepidoptera, Coleoptera, tenthredinid Hymenoptera, and Hemiptera (Duffey,

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1980; Blum, 1981; Brower, 1984; Rothschild, 1985; Bowers, 1992). Most such insects are specialists, feeding on one or a few species of plants from which they acquire their defensive compounds (Bowers, 1988). In contrast, one group of moths, the Arctiidae, are often quite general in their feeding habits (Rothschild, 1985), and yet some species are able to sequester host-plant secondary compounds (Rothschild et al., 1979).

The iridoid glycosides are one group of plant secondary compounds that are sequestered by several insect species, most of which are specialists (Bowers, 1991; Rimpler, 1991). In some of these species, such as members of the genus *Euphydryas* (Bowers, 1991; Rimpler, 1991; Stermitz et al., 1994; Bowers and Williams, 1995), the iridoids are retained through to the adult stage, but in other species, such as the buckeye (*Junonia coenia*, Nymphalidae), compounds are sequestered and retained only in the larval stage (Bowers and Collinge, 1992). Only one generalist species, the gypsy moth *Lymantria dispar* (Lymantriidae), has been examined for iridoid glycoside sequestration, and it does not sequester iridoid glycosides, but eliminates them in the frass (Bowers and Puttick, 1986). Because arctiid caterpillars may feed on plants that contain iridoid glycosides (Tietz, 1975; Dethier, 1988, 1989; Covell, 1984), and some species can sequester secondary compounds from their larval host plants (Teas et al., 1966; Teas, 1967; Rothschild et al., 1979; Ehmke et al., 1990; Hesbacher et al., 1995), we tested several species of arctiids for their ability (or lack thereof) to sequester iridoid glycosides.

We used four different arctiid species, all of which are generalist feeders that include plant taxa containing iridoid glycosides in their diet (Tietz, 1972; Covell, 1984): *Pyrrharctia isabella* (J. Smith), *Spilosoma virginica* (Fab.), *Spilosoma latipennis* Stretch, and *Spilosoma congrua* (Wlk.). For comparison with another generalist, we used larvae of the painted lady, *Vanessa cardui* (L.) (Nymphalidae). For comparison with a specialist, we used *Junonia coenia* (Hubner), also a nymphalid, which sequesters iridoid glycosides in the larval instars (Bowers and Collinge, 1992). Larvae were fed on a host plant that all of these lepidopteran species had in common, narrow-leafed plantain, *Plantago lanceolata* (L.) (Plantaginaceae). This plant contains two iridoid glycosides, aucubin and catalpol (Duff et al., 1965; Bowers and Stamp, 1992; Fajer et al., 1992).

METHODS AND MATERIALS

Study Organisms. The buckeye (*J. coenia*) feeds on plants in four families, Verbenaceae, Scrophulariaceae, Acanthaceae, and Plantaginaceae, all of which contain iridoid glycosides (Bowers, 1984). Two iridoid glycosides, aucubin and catalpol, serve as feeding and oviposition stimulants for buckeyes (Bowers,

1984; Pereyra and Bowers, 1988). Buckeye larvae sequester aucubin and catalpol (Bowers and Collinge, 1992). The amount of iridoid glycosides that a caterpillar contains depends primarily on the iridoid glycoside content of the host plant (Camara, 1996). Larvae used in these experiments were from a laboratory colony at the University of Colorado that was maintained on leaves of *P. lanceolata* (Plantaginaceae).

The painted lady (*V. cardui*) is a cosmopolitan butterfly. Painted ladies are generalist feeders that use plants in over 20 families, and *P. lanceolata* is a common host plant (Scott, 1986; Garrigan, 1994). The caterpillars used in this experiment originated from a colony maintained at Carolina Biological Supply (Burlington, North Carolina).

Pyrrharctia isabella is the common woolly bear, found throughout North America. This species is quite polyphagous in its feeding habits: Shapiro (1968) tested *P. isabella* on 95 plant species in 57 different plant families and found that only one of these species did not elicit a feeding response. *Plantago lanceolata* is a commonly used host plant (Shapiro, 1968). *Pyrrharctia isabella* larvae were reared from eggs deposited from female *P. isabella* collected at Makonikey, Massachusetts.

The three species of *Spilosoma* are all polyphagous feeders. The feeding behavior of *S. virginica* (= *Diacrisia virginica*) has been studied in some detail (Dethier, 1988), but that of the other two species (*S. congrua* and *S. latipennis*) has not. All three of these species are considered to be quite polyphagous (Tietz, 1972; Covell, 1984). *Spilosoma virginica* were obtained from a single female collected in Storrs, Connecticut; *S. latipennis* larvae were reared from eggs deposited by females collected in Storrs, Connecticut; *S. congrua* were reared from eggs obtained from a females collected on Martha's Vineyard, Massachusetts. All of these species will feed on *P. lanceolata* (Tietz, 1972; Covell, 1984; Bowers, personal observation).

The iridoid glycoside content of *P. lanceolata* varies between individual plants and between leaves on an individual plant (Bowers and Stamp, 1992, 1993). The content varies from undetectable in the oldest leaves to as much as 12% in the youngest leaves (Bowers and Stamp, 1992; Bowers et al. 1992; Klockars, et al., 1993). We did not quantify the concentration of iridoid glycosides in the plant material that we fed to the caterpillars in this study.

Feeding Experiments. Because the rearing conditions differed somewhat for each species, depending on when the eggs were obtained, the specifics are described for each species. All larvae were reared in the laboratory in Boulder, Colorado, on *Plantago lanceolata* obtained from plants growing in Boulder; except for *P. isabella* larvae, which were reared in Binghamton, New York, on *P. lanceolata* collected there.

Junonia coenia were reared in the laboratory at 25°C day : 20°C night, with

a photoperiod of 14 hr light. Larvae were reared in plastic Petri dishes (2.5 × 14.5 cm) from hatching until they had just molted to the fifth instar, but had not fed. Larvae were frozen and freeze-dried for chemical analysis.

Vanessa cardui larvae were reared on leaves of *P. lanceolata* from the second instar until they molted to the fifth instar. For the first instar, these larvae were reared on artificial diet obtained from Carolina Biological Supply. This diet contains ground leaf material of another host plant, mallow (*Malva* sp., Malvaceae). Although *V. cardui* larvae will feed and oviposit on *P. lanceolata* in nature (Scott, 1986; Garrigan, 1994), mortality of first instars was very high when larvae were reared on *P. lanceolata*. Frass was collected from each larva during the fourth instar, frozen, and freeze-dried.

Spilosma congrua and *S. virginica* larvae were reared in monospecific groups on leaves of *P. lanceolata* until the third instar, when 15 third instars were placed individually into large Petri dishes (2.5 × 14.5 cm). Larvae were allowed to feed until they had just molted to the fifth instar; at which time each larva was frozen and freeze-dried. Frass was collected from each *S. virginica* larva during the fourth instar, frozen, and freeze-dried. Frass from *S. congrua* was collected from a separate set of larvae during the fifth instar. This allowed us to examine both larval tissue and frass for the presence or absence of iridoid glycosides.

Spilosoma latipennis larvae were reared individually in Petri dishes under the same conditions as the other larvae. As larvae got ready to molt to the fifth instar, they were isolated without food, and after the molt they were frozen and freeze-dried.

We used two different sets of *P. isabella* larvae. The first set of larvae was reared under the conditions described above for the *Spilosoma* species. These larvae were isolated from food as they were about to molt to the fifth instar and were frozen after they had molted. A second set of larvae was collected from a laboratory experiment in which actively feeding fifth instars were killed, their gut dissected out, and the remainder freeze-dried for chemical analysis.

Chemical Analyses. Larvae and frass samples were analyzed for aucubin and catalpol content by gas chromatography (Gardner and Stermitz, 1988; Bowers and Collinge, 1992). Briefly, freeze-dried samples were ground to a fine powder, the powder weighed, extracted overnight in 5 ml methanol, filtered, and the filtrate evaporated to dryness. The residue was taken up in water and partitioned with ether to remove lipophilic substances. The water fraction, containing sugars and iridoid glycosides, was evaporated, the residue taken up in 1 ml methanol, an aliquot of 0.10 ml transfered to a small tube, and the methanol evaporated. Tri-sil Z (Pierce Chemical Company, Rockford, Illinois) was used to derivatize the iridoid glycosides prior to injection on the gas chromatograph.

RESULTS

Newly molted, fifth instar *J. coenia* contained relatively large amounts of iridoid glycosides, over 20% dry weight (Table 1). The ratio of aucubin to catalpol was approximately 4:1. *Vanessa cardui*, a generalist nymphalid, did not contain detectable amounts of iridoid glycosides, nor did the frass (Table 1); suggesting that the ingested iridoid glycosides were metabolized by the larvae. To our surprise, we found that *Spilosoma congrua* larvae sequestered iridoid glycosides, although in amounts less than half those of *J. coenia* (Table 1). The ratio of aucubin to catalpol (1:2) was quite different in *S. congrua* compared to *J. coenia*. We did not detect any iridoid glycosides in the frass from the *S. congrua* larvae. Neither *S. latipennis*, *S. virginica*, nor *P. isabella* contained detectable amounts of iridoid glycosides. Whether *P. isabella* were feeding or not, no iridoid glycoides were detected. We did find low levels of iridoid glycosides in the frass from *S. virginica*, but were unable to sample the frass from *S. latipennis* or *P. isabella*.

DISCUSSION

In contrast to the other four generalist species tested, *S. congrua* was able to sequester substantial amounts of iridoid glycosides from *P. lanceolata*. Indeed, the two congeners of *Spilosoma congrua*, *S. virginica* and *S. latipennis*, did not contain any detectable iridoid glycosides. These data suggest that, although these arctiids are all relatively polyphagous (Tietz, 1972; Covell, 1984), there are some biochemical features of the gut of *S. congrua* that are different from these other taxa. In addition, although *S. congrua* contained a mean of 8% dry weight iridoid glycosides, this was less than half the amount contained in the specialist, *Junonia coenia*. This indicates that *J. coenia* is more efficient than *S. congrua* at sequestering iridoid glycosides from its host plant.

The ratio of aucubin to catalpol was quite different in *J. coenia* (4:1) than in *S. congrua* (1:2). Although we did not measure the aucubin and catalpol content of the plant material we fed to the larvae, in all studies in which we have measured aucubin and catalpol content of *P. lanceolata*, there is typically two to five times more aucubin than catalpol in the plants (Fajer et al., 1992; Bowers and Stamp, 1993; Adler et al., 1995; Stamp and Bowers, 1996). If that were the case with the leaves fed to *S. congrua*, then those larvae are able to selectively sequester catalpol over aucubin. Thus, there may be variation among different sequestering species in the total as well as relative amounts of iridoid glycosides that they are able to store [as has been shown for the monarch, *Danaus plexippus*, and the queen, *Danaus gillippus*, and sequestration of cardenolides (Cohen, 1985)].

TABLE 1. BIOMASS (DRY WEIGHT) AND IRIDOID GLYCOSIDE (AUCUBIN AND CATALPOL) CONCENTRATION FOR FIFTH INSTARS AND FRASS OF SIX LEPIDOPTERAN SPECIES REARED ON *Plantago lanceolata*

Species and stage	*N*	Dry weight (mg) of frass or larvae (mean ± SE)	Percent (mean ± SE)		
			Aucubin	Catalpol	Total iridoids
Junonia coenia					
Fifth instars (newly molted)	10	16.81 ± 1.36	17.61 ± 3.65	4.27 ± 1.18	21.88 ± 4.81
Vanessa cardui					
Fifth instars (newly molted)	11	12.22 ± 1.23	0	0	0
Frass from fifth instars	5	11.42 ± 1.81	0	0	0
Spilosoma congrua					
Fifth instars (newly molted)	15	38.83 ± 1.99	2.80 ± 0.32	4.35 ± 0.33	7.14 ± 0.47
Frass from fifth instars	40	99.60 ± 3.67	0	0	0
Spilosoma latipennis					
Fifth instars (newly molted)	15	34.0 ± 0.87	0	0	0
Spilosoma virginica					
Fifth instars (newly molted)	15	44.53 ± 3.28	0	0	0
Frass from fourth instars	15	47.65 ± 2.08	0.19 ± 0.08	0.20 ± 0.08	0.39 ± 0.16
Pyrrharctia isabella					
Fifth instars (newly molted)	22	84.68 ± 3.96	0	0	0
Fifth instars (actively feeding, guts dissected out)	25	37.51 ± 4.41	0	0	0

Other arctiids have been recorded as sequestering chemical compounds from the plants on which the larvae feed. For example, larvae of *Seirarctia echo*, a specialist on cycads, sequester the azoxyglycoside, cycasin (Teas, 1967; Teas et al., 1966); larvae of the milkweed specialist, *Cycnia tenera*, sequester cardenolides from their host plants in the Asclepiadaceae (Cohen and Brower, 1983), and larvae of a third specialist, *Tyria jacobaeae*, sequester alkaloids from *Senecio* (Asteraceae) (Aplin et al., 1968; Nickisch-Rosenegk and Wink, 1993).

Several other, more polyphagous European species of arctiids reportedly sequester host-plant alkaloids, including *Arctia caja* and, interestingly, *Spilosoma lubricipeda* and *S. lutea* (European species of *Spilosoma*), *Phragmatobia fuliginosa*, *Diacrisia sannio*, and *Callimorpha dominula* (Rothschild et al., 1979; Nickisch-Rosenegk and Wink, 1993). In *Spilosoma lubricipeda*, alkaloids were found in pupae reared on *Senecio vulgaris* at levels of about 120 μg per pupa, and in *Spilosoma lutea*, pupae contained 170–340 μg alkaloid, depending on the host-plant species (*Senecio vulgaris* or *S. jacobaea*) (Rothschild et al., 1979). Smaller amounts were found in the adult moths, 17–27 μg per individual. For these European *Spilosoma* species, however, alkaloids were determined from bulk samples of many individuals, rather than by analyzing each individual separately, thus there is no information available about variation in individual levels of alkaloids (Rothschild et al., 1979). In a more recent and more detailed study, last instar *Spilosoma lubricipeda* were fed a 2-mg dose of the pyrrolizidine alkaloid heliotrine, offered on leaf discs of several different host-plant species (Nickisch-Rosenegk and Wink, 1993). These larvae sequestered 8.2–20.9% of the alkaloid, eliminated 12.0–27.5%, and the remainder (64.3–74.3%) was apparently degraded. Some species of the genus *Spilosoma* may be especially good at sequestering particular groups of plant allelochemicals, as our data with *S. congrua* also suggest.

Another recent paper reported that the adults of 11 species of lichen-feeding arctiids in five genera (out of a total of 20 species in eight genera that were tested) sequestered lichen allelochemicals (Hesbacher et al., 1995). Interestingly, the data indicated that not all individuals of a particular species contained the compounds in amounts that were detectable by HPLC. In addition, in those arctiid taxa that did contain lichen-derived compounds, the amounts were quite low, on the order of micrograms.

In another study (Strohmeyer et al., 1997), buckeyes, painted ladies, and a third species, the tobacco hornworm, *Manduca sexta* (Sphingidae), were fed artificial diets containing dried, powdered leaves of *P. lanceolata*. The iridoid glycoside concentration of these diets ranged from 5.63 to 9.21% dry weight (Strohmeyer et al., 1997). In this study, hemolymph was collected from 6–12 individual larvae and pooled for analysis. When analyzed for iridoid glycosides, the pooled hemolymph of painted ladies and tobacco hornworms contained very

small amounts of iridoid glycosides: painted ladies: 0.43–0.97 μg/μl hemolymph; tomato hornworms: 1.07–1.44 μg/μl (Strohmeyer et al., 1997). In contrast, buckeye larvae contained 63.60–69.96 μg/μl hemolymph. These data, combined with our data from the present experiment, suggest that although very small amounts of iridoid glycosides may pass from the gut to the hemolymph in nonsequestering caterpillar species, only certain species, such as *J. coenia* and *S. congrua*, are able to accumulate large amounts of these compounds.

In the four studies described above (Rothschild et al., 1979; Nickisch-Rosenegk and Wink, 1993; Hesbacher et al., 1995; Strohmeyer et al., 1997), the taxa other than *J. coenia* that contained detectable amounts of host-plant allelochemicals had relatively very low amounts of those compounds (see also Krasnoff et al., 1987). There may have been such low levels of iridoid glycosides in some of the species that we studied, but they may not have been detectable under the conditions that we were using for chromatographic analysis. In such cases, although very small amounts of host-plant compounds may pass through the gut wall, and thus considered to be sequestered (Duffey 1980), it is unlikely such low levels of these compounds will have strong antipredator effects.

The differences in the amount of iridoid glycosides sequestered by larvae of *J. coenia* and *S. congrua* could affect their interaction with potential predators. Although the concentrations of iridoid glycosides in these larvae may be enough to deter certain predators, such as ants (Dyer and Bowers, 1996), other predators, such as wasps and stinkbugs may not be deterred initially (Stamp, 1992). Furthermore, the effect of iridoid glycosides may depend on the amount that an individual caterpillar contains (Dyer and Bowers, 1996), and we did detect variation among individual caterpillars within a species (Table 1). In addition, the amount of chemical defense may be only one part of what determines whether a prey item is discovered and eaten. For example, in a field experiment, *J. coenia* were four times more likely to be killed by predatory wasps than *S. congrua*, apparently because the *J. coenia* were much more apparent on their host plants (Stamp, 1992). However, over time, the wasps learned to avoid *J. coenia*, thus predation rates declined on these caterpillars; while predation rates on *S. congrua* were low but did not decline over time (Stamp, 1992). *Spilosoma congrua* larvae were much more cryptic in their behavior, and this behavior, combined with some degree of chemical defense, afforded them as much protection as the more apparent, yet better chemically defended *J. coenia* larvae. Thus, it is the entire defensive repertoire of a caterpillar and its effectiveness in protecting that individual (including behavioral as well as chemical defenses) that determines the ultimate fate of a potential caterpillar prey.

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PHEROMONAL DISCRIMINATION OF SEX BY MALE AND FEMALE LEOPARD GECKOS (*Eublepharis macularius*)

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Abstract—The ability of male and female *Eublepharis macularius* to discriminate among pheromones of males and females and a blank control was investigated. Stimuli were presented on ceramic tiles in the animal's home cages. Males tongue-flicked at significantly lower rates in response to male stimuli than to female and control stimuli. Males also performed aggressive behaviors toward male, but not female or control, stimuli, and tail vibrations toward female, but not male or control, stimuli. Mean tongue-flick rates by females did not differ significantly among conditions. Discrimination of male pheromones by females was demonstrated by greater performance of labial-licking and chin-rubbing in response to male stimuli than to either female or control stimuli.

Key Words—Pheromone, tongue-flicking, sex recognition, Squamata, Eublepharidae, *Eublepharis macularius*

INTRODUCTION

Pheromonally based sex discrimination has been demonstrated in several species of squamate reptiles (Duvall, 1979; Cooper and Vitt, 1984a; Mason, 1992; Cooper and Trauth, 1992; Cooper et al., 1994). Male *Eumeces laticeps* exhibit differential tongue-flicking rates to male and female conspecific pheromones (Cooper and Vitt, 1984a) and are able to track females by following pheromone trails (Cooper and Vitt, 1986a). Male *Gerrhosaurus nigrolineatus* also tongue-flick at a higher rate to female pheromones than to either male pheromones or distilled water controls (Cooper and Trauth, 1992), as do male *Blanus cinerus*

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(Cooper et al., 1994). Fewer studies address the ability of female lizards to discriminate sex from pheromonal cues. Female *Eumeces laticeps* show no discrimination of sex based on tongue-flick rate (Cooper and Vitt, 1984b), although they do detect conspecific pheromones of both sexes. Female *Cordylus cordylus* tongue-flick at higher rates in response to female, but not male, pheromones than control stimuli (Cooper et al., 1996).

Evidence from several behavioral studies suggests that pheromonal cues are important in sex recognition in *Eublepharis macularius* (Greenberg, 1943; Brillet, 1990, 1993; Mason and Gutzke, 1990). Following surgical exchange of the tails of male and female *Coleonyx variegatus* (close relatives of *Eublepharis*), experimental males courted males with female tails and avoided the male tails of experimental females (Greenberg, 1943), leading to the conclusion that some cue present on the skin relayed information about the sex of the individual. This was an important finding, but possible tactile sex differences between tails were not ruled out (Cooper and Trauth, 1992).

Male *E. macularius* react aggressively to other males at any time and court females except when they are preparing to shed their skins. While females are shedding, however, males react agonistically to them (Mason and Gutzke, 1990). This suggests that the separated skin prior to shedding acts as a barrier, making female sex pheromones unavailable for detection (Mason and Gutzke, 1990). Examination of chemical constituents of the dorsal epidermis of male and female leopard geckos by gas chromatography revealed distinct sex differences in lipid fractions of high molecular weight (Mason and Gutzke, 1990). Some of the lipids that differ between male and female profiles have been shown to serve as sex pheromones in *Thamnophis sirtalis parietalis* (Mason et al., 1989).

Male *E. macularius* perform tail-vibrating behaviors when courting females (Brillet, 1991), but not in other contexts. In empty cages that had previously housed conspecific females, males vibrate their tails, indicating recognition of female pheromones (Brillet, 1990). Experimental males reacted agonistically toward anesthetized males, but courted anesthetized females (Brillet, 1993). Males often attempted to copulate with females, implying that these geckos are not only able to determine the sex of conspecifics in the absence of any behavioral cues, but behave as though they were encountering unanesthetized lizards. Chemical cues may have contributed to sex recognition in Brillet's (1991) study, but were confounded with other cues provided by the lizards.

Brillet (1990) examined lingual responses and other behavioral responses by males and females to both male and female pheromones. He reported that the number of tongue-flicks by males in clean cages did not differ significantly from those in cages previously occupied by males or females. Numbers of tongue-flicks by females did not differ in clean cages and cages previously occupied by females, but Brillet reported that females tongue-flicked at a significantly higher rate in cages that had been occupied by males than in clean

cages. However, Brillet (1990) accepted $P = 0.06$ as significant without making any downward adjustment of α to compensate for the number of significance tests conducted. In addition, the sample sizes were so small ($N = 6$ in all cases) that statistical power was too low to assure detection of real differences.

Here we report further studies of the ability of male and female leopard geckos to detect conspecific chemical stimuli derived from both sexes and to respond differentially to male and female stimuli based solely on chemical cues deposited on a substrate. These abilities were studied experimentally by recording lingual and other behavioral responses to chemical stimuli presented on ceramic tiles.

METHODS AND MATERIALS

Subjects and Maintenance. Adult male ($N = 15$) and female ($N = 28$) leopard geckos were obtained from captive-bred research colonies and kept in an AALAC-accredited animal care facility. They were housed in translucent plastic containers (45 × 25 × 30 cm), each having a plastic shelter site and water bowl. Water was available ad libitum, and animals were fed crickets two times weekly. Any crickets not immediately eaten were left in the cage. Room temperature was maintained at 27°C, with a 12L:12D photoperiod. All experiments were performed between 19:00 hr and 24:00 hr under red light to minimize potential disturbance to these nocturnal lizards due to the investigator's presence and to observe responses during the normal feeding period.

Experimental Design and Analysis. Experiments were conducted to determine whether or not male and female *E. macularius* detect conspecific pheromones and discriminate between pheromones of males and females presented on tiles. Pheromonal stimuli were obtained by placing an unglazed ceramic tile (15 × 15 cm) at the center of one end of each animal's cage for a minimum of two weeks to allow thorough marking. Just before the beginning of a trial, the tile, shelter, and water bowl were removed from the cage of the animal to be tested and placed on the cage lid. After being lightly brushed to remove excessive soiling, an experimental tile was placed in the test animal's home cage in the same position as the one removed. In the control condition for responses to tiles, the tile was a clean, unmarked tile. For the trials requiring marked tiles, the tile was taken from the cage of one of the other individuals in the laboratory population.

In two similar experiments testing tongue-flicking and other behavioral responses of adult males and females, male pheromones or female pheromones on ceramic tiles and clean ceramic tiles were presented to lizards sequentially in randomized blocks designs in which each individual was tested in all three conditions. Order of stimulus presentation was completely counterbalanced for

the 15 males to prevent sequential bias. In the experiment on female responses, trial sequences were completely counterbalanced for 24 females. For the remaining four females, the sequences were randomized with the constraint that all three stimuli occurred at least once in the first, second, and third trials.

After placing a tile in the animal's cage, the investigator moved to a seat 1 m away. Tongue-flicks were recorded for 5 min, beginning with the first tongue-flick directed to the tile. The occurrence of other behaviors was recorded beginning as soon as the tile was placed in the cage until the end of the trial. If the animal failed to tongue-flick the tile in 10 min, the tile was removed and the animal was tested at a later date. Animals were tested only once per day.

At the conclusion of a trial, the experimental animal's tile, shelter, and water bowl were replaced in its cage. If the animal's tile was used as the stimulus in a trial with another gecko, it was replaced by a clean, blank tile that was left to be marked for two weeks for use in future trials. This method allowed tiles to be continuously present in the cages, except during brief intervals before the animals were tested. Each tile was used once, then washed by hand with Alconox and dried.

In the experiment on male response to pheromones, several behaviors were recorded: (1) tongue-flicking; (2) cloacal rubbing—a substrate-marking behavior in which the animal presses the cloacal region to the substrate while moving (Brillet, 1990; Mason and Gutzke, 1990); (3) stilting—behavior whereby the animal lifts its entire body off the substrate by extending all four legs, arches its back, and often waves and coils its tail (Mason and Gutzke, 1990); (4) tail vibration—rapid vibration of the distal third to half of the tail performed only in response to female stimuli while the body and head are held rigidly still (Brillet, 1990); and (5) biting—biting or attempting to bite the experimenter immediately after the trial while the experimental tile was being retrieved and objects were being returned to the cage.

Behaviors recorded in the experiment on female responses to pheromones were tongue-flicks, labial-licks, and chin-rubs. Labial-licks are lingual protrusions in which the tongue wipes the labial scales about the mouth rather than being directed to the air or substrate. Although labial-licks were included in the total tongue-flick count, their occurrence was recorded because their function may differ from tongue-flicks directed to substrates (DePerno and Cooper, 1996). Chin-rubbing is pronounced rubbing of the chin and throat area along the substrate, in this case the cage floor or tile. Only its presence or absence was recorded.

Nonparametric ANOVAs were conducted due to significant heterogeneity of variance (Hartley's F_{max} tests) (Winer, 1962). Friedman two-way analysis of variance was used to determine whether differences between the three conditions were significant. When main effects were significant, multiple comparison procedures (Zar, 1984) were used to determine whether tongue-flick rates differed

between pairs of conditions. Differences in frequency of the other behaviors were analyzed using sign tests and binomial tests (Siegel, 1956). All statistics were assessed at $\alpha = 0.05$, and probability values are two-tailed unless otherwise stated.

RESULTS

Male Responses. Mean tongue-flick rates of males were highest toward control stimuli and lowest toward male stimuli (Table 1). Variances of numbers of tongue-flicks differed significantly among conditions ($F_{max} = 12.34$; $df = 3, 14$; $P < 0.01$). Mean tongue-flick rates by male *E. macularius* differed significantly among conditions ($\chi_r^2 = 12.4$; $df = 2$; $P < 0.006$, Table 1). Tongue-flick rates were significantly lower to male stimuli than to both female stimuli ($P < 0.005$) and blank control stimuli ($P < 0.025$). Tongue-flick rates elicited by female and control stimuli did not differ significantly ($P > 0.05$).

Only a few males stilted, and only during trials with male stimuli (Table 1). In addition, a few males bit the investigator when the tile was removed at the end of the trial involving male stimuli, but none bit after trials in the other conditions (Table 1). Differences between male and other stimulus conditions were not significant for either of these behaviors alone ($P = 0.06$, stilt; and $P = 0.125$, bite; one-tailed sign tests). However, aggressive behavior, either stilting, biting, or both, was performed by five males, all in trials involving male-labeled tiles, but by no male in other trials. Thus, males were significantly more likely to perform aggressive behaviors in response to male stimuli than to either female or control stimuli ($P = 0.03$; one-tailed sign test). This difference is at best only marginally significant. However, assuming that aggression was equally

TABLE 1. RESPONSES BY 15 MALE *Eublepharis macularius* TO CONSPECIFIC CHEMICALS AND CONTROL STIMULI

	Male	Female	Control
Tongue-flicks/5 min			
Mean	29.6	57.0	66.8
SE	4.6	3.7	12.9
Range	5–64	38–78	18–194
Stilting (*N*)	4	0	0
Biting (*N*)	3	0	0
Tail-vibrating (*N*)	0	12	0
Cloacal-rubbing (*N*)	14	10	14

likely in each of the three conditions, the binomial probability that all five instances of aggression occurred in male trials rather than in the combined other trials is <0.004.

Eighty percent of males performed tail vibrations in response to female stimuli, whereas none vibrated their tails in response to male or control stimuli. Thus, significantly more males performed tail vibrations in response to female stimuli than to either of the other two stimuli ($P < 0.0003$ each, one-tailed sign tests).

Males rubbed their cloacal regions over the tile and cage bottom in nearly all trials. There were only seven trials in the entire experiment in which this rubbing behavior did not occur. Five males did not rub during trials with female stimuli, and one each did not rub in the male stimulus and control conditions. No individual failed to perform cloacal rubbing in more than one trial. Differences between pairs of conditions were not significant ($P > 0.10$, sign tests), but the data hint that males may be less likely to perform cloacal rubbing in response to female chemical stimuli than to male or control stimuli.

Female Responses. Females tongue-flicked at substantial rates in all conditions, with slightly higher rates in response to female stimuli than to the other two stimuli (Table 2). Variances of female tongue-flick rates differed significantly among conditions ($F_{max} = 3.98$; $df = 3, 27$; $P < 0.01$). Mean tongue-flick rates by female *E. macularius* did not differ significantly among conditions ($\chi_r^2 = 5.24$; $df = 2$; $0.05 < P < 0.10$, one-tailed).

Twenty-four of 28 females performed labial-licking (Table 2), but only two individuals did so in all three conditions. Thirteen females labial-licked only in response to male stimuli, three only to female stimuli, and none only to control stimuli. Numbers of females labial-licking varied significantly among conditions: more females labial-licked in response to male stimuli than to either female stimuli (sign test, $P < 0.001$) or controls (sign test, $P << 0.001$). There was

TABLE 2. RESPONSES BY 28 FEMALE *Eublepharis macularius* TO CONSPECIFIC CHEMICALS AND CONTROL STIMULI

	Male	Female	Control
Tongue-flicks/5 min			
Mean	46.4	53.2	39.1
SE	3.7	7.4	4.4
Range	23–106	15–211	1–109
Labial-licking (*N*)	21	7	7
Chin-rubbing (*N*)	10	1	0

no significant difference in numbers of females labial-licking between the female and control conditions ($P > 0.10$).

Slightly over one third of females rubbed their chins and throats along the tiles in response to male-labeled tiles. One female chin-rubbed on both male-labeled and female-labeled tiles, but none chin-rubbed a control tile (Table 2). Thus, significantly more females chin rubbed in response to male stimuli in each case (female: $P = 0.002$; control: $P < 0.001$; sign tests).

DISCUSSION

Male Responses. Male *Eublepharis macularius* clearly detected and differentiated between male and female stimuli in tests using marked tiles. Detection is implied by the difference in tongue-flick rates between the male stimulus and control conditions. Discrimination of male from female chemical stimuli is indicated by the significantly lower tongue-flick rate in response to male-labeled than female-labeled tiles. Detection and identification of male chemical stimuli by male *E. macularius* is confirmed by the aggressive responses—stilting and biting—limited to the male stimulus condition. Elicitation of male aggressive behaviors by conspecific male pheromones has also been documented in *Sceloporus occidentalis* (Duvall, 1979) and *Blanus cinerus* (Cooper et al., 1994).

The lower tongue-flick rate to male stimuli than to female and control stimuli confirms a report that male *E. macularius* tongue-flick at a lower rate in cages previously occupied by males than in clean cages (Brillet, 1990). Because tongue-flick rates are reduced rather than increased in male *E. macularius* by contact with conspecific male pheromones, identification must occur within the first few tongue-flicks, with further tongue-flicks serving perhaps for confirmation and reinforcement of the original assessment.

In other lizard species, tongue-flick rates are elevated in response to male pheromones (Cooper and Vitt, 1986b; Cooper and Trauth, 1992; Cooper et al., 1994; Dussault and Krekorian, 1991; Duvall, 1979). Reasons for the difference in effect on tongue-flick rate by male pheromones from other species are obscure. Although little is known about the ecology of *E. macularius*, males of the related *Coleonyx variegatus* may occupy the same sites for some time (Cooper et al., 1985a,b) and are aggressive to conspecific males (Greenberg, 1943). Triggering by pheromones of species-typical aggressive displays and readiness for aggressive biting in the nocturnal leopard gecko might be an evolutionary response to the difficulty of detecting intruding males visually. Readiness for aggression, possibly with improved detection of nonresident males, while in the elevated stilting posture, interferes with further tongue-flicking of the substrate. Such stilting (and presumably vigilance) often persisted for much of the trial in the male stimulus condition. Biting in response to male stimuli also suggests that

males are prepared to respond aggressively to a male whose pheromones have been detected. Diurnal species, readily able to detect other males visually, are free to continue lingual investigation of pheromones.

The frequent marking in all conditions may indicate that leopard geckos mark novel substrates in their home ranges. Although differences among conditions were not significant, the fact that five of the seven instances in which a male did not perform marking behaviors occurred in response to female chemical stimuli hints that there may be a somewhat stronger tendency to overmark pheromonal deposits of other males and to mark unmarked surfaces than to mark female pheromone deposits. By marking the area, a male may signal his occupancy status to intruders of both sexes, allowing nonresident males to avoid interaction, and nonresident females to detect a potential mate.

Males discriminated female chemical stimuli from male chemical stimuli and control stimuli, but this was demonstrated by species-typical sexual behavior rather than by lingual responses. Mean tongue-flicking rates did not demonstrate even detection of female pheromones. Identification of female pheromones by males is confirmed by the significantly greater number of males that vibrated their tails to female stimuli than to other stimuli. These results agree with Brillet's (1990) finding that males performed tail vibrations almost exclusively in cages previously occupied by females, but did not tongue-flick differentially in clean cages and those previously occupied by females.

Performance of social behaviors including stilting, severe biting, and tail vibrations appropriate only for stimuli derived from only one sex (Mason and Gutzke, 1990; Brillet, 1991, 1993) shows that males detect and respond differentially to male and female chemical stimuli even when tongue-flick rates do not differ. Preliminary investigation of conspecifics before performance of these behaviors is brief, often limited to single tongue-flicks, which supports the hypothesis based on the current data that chemosensory identification by males occurs rapidly. Following identification, performance of social behaviors may suppress or reduce further tongue-flicking.

Female Responses. Tongue-flick rates yielded no evidence that females detect conspecific pheromones. Brillet (1990) found no difference in tongue-flick rates in response to female stimuli and clean cages, but he reported that females tongue-flicked significantly more in cages previously occupied by males than in clean cages. However, he did not test for differences among all conditions, accepted $P = 0.06$ as significant, and conducted many individual comparisons without adjusting the level of significance. Appropriate procedures would reveal no significant difference.

The significantly greater numbers of individuals that labial-licked and chin-rubbed when responding to male stimuli than to female or control stimuli shows that females detected male pheromones. There was no indication that females detected female chemical stimuli. These findings differ from those for *Eumeces*

laticeps, in which females clearly detected female chemical stimuli but did not respond differentially to male and female chemical stimuli (Cooper and Vitt, 1984b).

Differential labial-licking indicates that females detected male pheromones and suggests that vomerolfaction or gustation may be involved. Labial-licking might allow an individual to glean more information from a sampled stimulus through redistribution on the tongue, resulting in contact with taste buds or enhancing the transfer to the vomeronasal organs (DePerno and Cooper, 1996). In addition, any molecules that adhere to the anterior labial scales during tongue-flicking might be sampled by labial-licking. *Eublepharis macularius* also performs labial-licks in response to prey chemicals (DePerno and Cooper, 1996), suggesting that labial-licking may function generally in the sampling of chemicals that carry information useful to the animal.

The function of chin-rubbing is uncertain, but two possibilities seem worthy of investigation. First, females may deposit pheromones indicating their presence in response to male pheromones. Chin-rubbing behavior has been found in *Sauromalus o. obesus* (Berry, 1974) and *Sceloporus jarrovi* (Simon et al., 1981). Both sexes of *S. o. obesus* chin-rub the substrate, and chin-rubbed sites elicit species-typical behavioral displays when encountered by conspecifics. Second, because physical manipulation of animal chemical deposits releases more substance (Alberts, 1992), chin-rubbing may release more pheromones for examination. There is no evidence of increased tongue-flicking after chin-rubbing, but chin-rubbing might place chemicals on the labials where they may be transferred to the vomeronasal organs (Graves and Halpern, 1989).

Although there is no strong evidence that females detect female pheromones, neither is there any strong evidence that they do not. Females are not overtly aggressive toward each other and coexist well in the laboratory when housed together (personal observation). A similar lack of overt responsiveness to female pheromones has been reported for female *Eumeces laticeps* (Cooper, 1996), a species also characterized by relatively low levels of interfemale aggression. In contrast, female *Cordylus cordylus*, which are known to be territorial and aggressive toward other females, discriminate females pheromonally as indicated by differential tongue-flicking rates, but do not differentiate male pheromones from controls (Cooper et al., 1996). The low aggression among female *E. macularius* might explain the lack of evidence for detection of female pheromones by tongue-flicking or social behavior. Because little is known of the social structure of *Eublepharis macularius* outside captivity, potential benefits of discriminating female pheromones are unclear.

Geckos have highly developed olfaction as well as vomerolfaction (Gabe and Saint Girons, 1976; Schwenk, 1993). Because tongue-flicking serves to sample chemicals for vomerolfaction (e.g., Halpern and Kubie, 1980; Graves and Halpern, 1989), chemical discriminations based on olfaction might not be

accompanied by changes in tongue-flicking rates. In the related *C. variegatus*, recognition of snake predators may be based on olfaction rather than vomerolfaction (Dial et al., 1989). The olfactory response to pheromones might also account for the low tongue-flick rates of males, especially when responding to other males.

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