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LIGHT-INDUCED VARIATION IN PHENOLIC LEVELS IN FOLIAGE OF RAIN-FOREST PLANTS. I. Chemical Changes

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Abstract—Levels of phenolic secondary metabolites in the leaves of four west African rain-forest plants, *Acacia pennata*, *Cynometra leonensis*, *Diopyros thomasii*, and *Trema guineensis*, were correlated with incident light intensity at both the inter- and intraindividual level. Enhanced phenolic levels under high light intensity appeared to be due to production of both polyphenolics (condensed and hydrolyzable tannins) and simple phenolics. In *Trema guineensis*, where it is possible to separate leaves in terms of both their age and the light incident upon them, condensed tannin production progressed differently during the development of "sun" and "shade" leaves, suggesting continuing production of new oligomers in the former but not in the latter. The results of this study suggest that the production of phenolics in relation to variation in incident light is a finely tuned process, which must be explained in terms of plant physiology and intermediate metabolism rather than in terms of resource allocation or a direct response to herbivory.

Key Words—Phenolics, tannins, light enhancement, overflow metabolism, *Acacia pennata, Cynometra leonensis, Diopyros thomasii, Trema guineensis.*

INTRODUCTION

Considerable emphasis has been placed on the role of phenolic compounds, particularly polyphenolics (e.g., tannins), in the defense of plants against herbivores (Feeny, 1970; 1976; Rhoades and Cates, 1976; Rhoades, 1979; Baldwin and Schultz, 1983; Zucker, 1983; Harborne, 1985). The mode of action

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generally associated with tannins has been that of digestibility reduction, providing plants with a quantitative defense (sensu Feeny, 1976) against herbivory by sequestering the protein in herbivore diets as insoluble tannin–protein complexes or by inhibiting digestive enzymes (Rhoades, 1979; Pierpoint, 1985). While there is considerable evidence for avoidance of high levels of tannins in food selection by many mammalian herbivores (Mole and Waterman, 1986), convincing support for digestibility-reducing effects has not generally been forthcoming from in vivo studies (Bernays, 1978; Berenbaum, 1983; Mole and Waterman, 1986), and evidence at the molecular level now indicates that animal digestive systems have evolved ways to counter the potential antinutritional effects of tannins on dietary proteins (Martin and Martin, 1984; Mehansho et al., 1985; Mole and Waterman, 1985). In short, the notion (Feeny, 1976; Rhoades and Cates, 1976) that tannins provide plants with a costly but relatively impregnable defense against herbivores is compromised by currently available evidence. While the production of tannins by a species may be optimized at a constant constitutive level, there is evidence that the quantities produced by an individual are variable and may be under metabolic control (Schultz, 1983; Baldwin and Schultz, 1983). On the assumption that such variation is of adaptive value in terms of plant fitness, then an understanding of the conditions that influence the production of tannins may help us appreciate their role(s).

If it is accepted that tannins have no metabolic or physiologic roles, it is in terms of constraints upon availability of fixed carbon and energy for their metabolism that explanations for their level of production will have to be sought. The starting point for the present work is the report (Waterman et al., 1984) that individuals of the tree *Barteria fistulosa* (Passifloraceae) growing in rainforest clearings respond to the higher light intensity of that environment by an increase in levels of phenolics in their foliage in comparison to individuals growing under the forest canopy. Specifically, the condensed tannin component of foliar total phenolics was shown to be enhanced by high light intensity. Waterman et al. (1984) suggested possible explanations for this phenomenon in terms of passive or active response of biosynthetic processes directly to variation in the light environment or indirectly to variability of herbivore pressure on *B. fistulosa* situated in different light environments. Given the highly effective defense *B. fistulosa* obtains against insect herbivores through an obligate mutualistic association with pseudomyrmicine ants (Janzen, 1972) a direct relationship between light intensity and condensed tannin production was considered the more likely explanation in this case.

A positive association between increasing light intensity and levels of phenolics has been recognized for some time (Hillis and Swain, 1959; McClure, 1985), but only in the last few years has it received attention in the ecological literature. Woodhead (1981) showed that the total phenolic content of *Sorghum*

bicolor increases with high light intensity. Hanley et al. (1986) found that black-tailed deer prefer sympatric shaded conspecifics over their insolated counterparts and that tissues from shaded foliage had lower phenolic concentrations. Waring et al. (1985) and Larsson et al. (1986) both reported decreases in foliar tannin and phenolic glycosides in shaded willow foliage preferred by chrysomelid beetles. Bryant (personal communication) studied the effect of light on the phenol chemistry of *Salix alaxensis* and found that shading contributes to a lowering of phenolics and an increase in palatability, the eventual result of which is the replacement of the willow by less palatable alder (*Alnus* sp.). One plausible explanation for the apparent association between light intensity and phenol production arises from the "resource allocation hypothesis" (Coley et al., 1986; Bryant, 1986). On this basis it can be argued that a reduction in light intensity will lead to a lower level of photosynthesis and consequent decline in carbohydrate production. As nutrient uptake is not similarly influenced, the result is a net decrease in the C/N balance (Bryant et al., 1983) in the shaded leaf, resulting in lower levels of C-based metabolites, including phenolics.

If a plant's light environment does exert a major influence on the output of phenolic metabolites, then the impact of herbivores in determining the level of investment in plant secondary metabolism may not be as great as is generally thought (Bell, 1978; Fraenkel, 1959; Harborne, 1985; McKey, 1979), at least for phenolic compounds. We have sought to test the importance of light intensity in influencing the relative and absolute quantities of phenolic allelochemicals accumulated by plants. The subjects of our study were individuals of four west African rain-forest species: (1) *Acacia pennata* DC (Mimosaceae), a spiny climber found growing in light gaps in recently regenerated secondary forest; (2) *Cynometra leonensis* Hutch. & Dalz. (Caesalpiniaceae), a large forest tree that produces abundant epicormic shoots and thus provides easily accessible foliage; (3) *Diospyros thomasii* Hutch & Dalz. (Ebenaceae), a small understory tree growing 5–10 m high; and (4) *Trema guineensis* (Schum. & Thonn.) Ficalho (Ulmaceae), a small tree found in open secondary regrowth on disused farmland.

Our analysis extends beyond that of Waterman et al. (1984) in two ways. First, we examined responses to light within, as well as between, individuals of a species. The reason for this approach lies in the many reports indicating the adaptive within-plant distribution of chemical defenses (McKey, 1979) which thus make it important to demonstrate a response to the physical environment on a similar scale. Second, we examined the influence of light over a much narrower variation in intensity than the gross insolated–shaded comparison employed for *Barteria fistulosa*. The species chosen for the study were generally restricted to either closed or open canopy vegetation types. Thus, by the criteria adopted for *Barteria*, all individuals of both *D. thomasii* and *C.*

leonensis would be regarded as shaded and all individuals of *T. guineensis* as insolated. *A. pennata* was intermediate, occurring in the widest range of light intensities.

METHODS AND MATERIALS

The study site was located in Sierra Leone, on Tiwai Island (12,000 hectares, 7°33′N 11°21′W) situated in the Moa River, some 60 km inland from the Atlantic Ocean. The island carries no permanent settlement, and about half its area supports 40- to 60-year-old secondary high forest (Cole, 1968), in which leguminous species predominate (e.g., *Cynometra leonensis*, *Piptadeniastrum africanum*, *Pentaclethra macrophylla*). The remaining vegetation is a mosaic of swamp, riverine, and young secondary forest types, together with some recently farmed land. Mean annual rainfall in the area is 3300 mm. Species identifications were made with reference to previously identified specimens growing on site and to Hutchinson and Dalziel (1958) and Savil and Fox (1966).

Measurements of Light Intensity. Individual plants were selected for their possession of branches in relatively sunny and/or shaded positions that could be reached from a 6-m ladder. Once located, suitable groups of leaves were tagged for reference. Whenever possible, shaded and unshaded leaves were chosen from the same plant, but this was not always feasible and single sets of leaves were occasionally taken from individual plants. Tagged leaves were made the object of light-intensity measurements over a period of five days. On each day, measurements of the light incident upon each set of leaves studied were made by placing a piece of white card over them and recording the f-stop and shutter speed required to expose 50 ASA film in a Minolta X300 camera fitted with a 50-mm lens and aimed at the card. These measurements were timed at each set of leaves, and taken at intervals of about 2 hr throughout the daylight period. They were used for comparison with recordings made at 10-min intervals during the day using an identical camera aimed at an identical piece of card placed in a large open clearing. This second set of readings was defined to be a measure of total light incident upon the vegetation. By converting camera settings to foot-candles, using a Weston light meter dial, these data allowed an estimation of the percentage of available light (LIGHT) that reached the leaves under study. This percentage of available light was obviously not constant throughout the day for a given sample of leaves, as it was dependent upon solar movements in relation to openings in the surrounding canopy. However, by averaging all readings taken during each day, for all five days of sampling, an estimate of the percentage of available light reaching each set of leaves was calculated. This is not intended as an absolute measure, or even one linearly

related to solar irradiance, but one that will allow variation in light intensity to be assessed on a continuous scale.

Collection and Preparation of Foliage. On completion of light intensity measurements, leaves were collected and sun dried, the drying process being initiated within 1 h of collection. For *T. guineensis*, which has simple alternate leaves borne on long straight branches with the youngest leaves at the single apex, leaves were separated into the following age classes: "young," the six leaves over 2 cm long closest to the apex; "mature," the next six older leaves; and "old," the remaining older leaves if present. For the other three species, leaf sets were matched for age only to the extent that selection was restricted to visibly mature leaves. Dried leaves were sealed in air-tight bags and returned to the laboratory for chemical analysis. In the laboratory, samples were ground to give a maximum particle size of 1 mm before chemical analysis.

Analysis for Phenolics, Fiber, and Protein. Samples were assayed for their content (% dry wt) of total phenolics (TP), condensed tannins (CT), and nitrogen (N). Total phenolics were estimated using the Folin-Denis method on extracts prepared in 80% aqueous methanol under reflux and are expressed in terms of tannic acid. Condensed tannins were estimated using a sample of the same extract and the proanthocyanidin method, with results expressed in terms of quebracho tannin. The details of the assay procedures have been described by Gartlan et al. (1980). The nitrogen content of the ground samples was measured by the Kjeldahl method with crude protein (PROT) being estimated as N% $\times$ 6.25. Acid detergent fiber was estimated by the standard technique (Waterman et al., 1984).

An additional estimation of CT was made using the vanillin method following Butler et al. (1982). Extracts were prepared by adding 10–20 ml of the milled dry leaf to a 100-ml conical flask containing 25 ml of 70% aqueous acetone to wet the material. Then 25 ml of diethyl ether was added, and the flask was stoppered and set to shake on an orbital mixer for 24 h. At the end of this time, the resulting solution was filtered and then separated with the lower aqueous layer being returned to the flask and freeze dried. The lyophilized powder was then analyzed for CTs by the proanthocyanidin method used above, and also by the vanillin assay using both methanol (Burns, 1971) and glacial acetic acid (Butler, 1982) as solvent.

Chromatography of Extracts. Approximately 100 mg of the powdered extract was added to 4 ml 2 M hydrochloric acid and the mixture heated to 100°C for 30 min. The resulting hydrolysates were cooled and extracted with amyl alcohol (1 ml). The amyl alcohol extract was then chromatographed on cellulose (paper, Whatman No. 3; or thin layer) and developed with Forestal solvent (glacial acetic acid–water–conc. hydrochloric acid, 30:10:3). Red-colored spots that turned blue in daylight when exposed to ammonia vapor were iden-

tified as cyanidin (R_f 0.45–0.55) or delphinidin (R_f 0.30–0.37). Ellagic acid gave a spot intermediate in R_f between cyanidin and delphinidin, which appeared blue under UV light and yellow under UV after brief exposure to ammonia vapor. Gallic acid was identified by its high R_f value and by turning brown on long exposure (30 min) to ammonia vapor. Where gallic acid was detected, a repeat chromatogram was obtained for an unhydrolyzed sample so as to check for the occurrence of free gallic acid in the extract. All named compounds were compared directly with authentic markers obtained from Koch-Light Ltd., Edinburgh.

Estimate for Hydrolyzable Tannin (HT). This estimate of HT levels is described in detail by Mole (1986). It makes use of a blue-colored complex (max. 660 nm) formed between ferric chloride and HTs (cf. green color produced with CTs and the hydrolysis products of HTs). The reduction in absorbance at 660 nm on hydrolysis of an HT-containing extract thus reflects the amount of HT that was present in the original extract. Measuring the absorbance of hydrolyzed and unhydrolyzed extracts at 660 nm in the presence of a standard ferric chloride solution permits an estimation of HT content to be made. A major drawback in the technique is that the green complex formed by HT breakdown products also absorbs at 660 nm at about 10% of the intensity of the blue complex. To compensate, the result of the assay is considered to give a loss of absorbance about 10% less than in fact occurs, and final results are modified accordingly.

The estimate is performed using the following method. An accurately weighed portion of the powdered extract prepared for CT polymer length analysis (see above) was dissolved in water and divided into two equal portions of about 5 ml. To one sample two drops of 4 M sodium hydroxide was added; this mixture was heated in a boiling water bath for 2 hr, and the extract was then cooled and neutralized by the addition of two drops of 4 M hydrochloric acid. Both hydrolyzed and unhydrolyzed samples were then assayed as follows.

At time zero, 500 μl of the extract was added to 3.5 ml of ferric chloride solution (1.62 g anhydrous ferric chloride dissolved in 1 liter 0.001 M hydrochloric acid) in a cuvette. The contents of the cuvette were mixed rapidly and the absorbance read at 660 nm, 15 sec after addition of the extract. Reagent-only blanks were used to zero the spectrophotometer. Readings were only considered valid in the range of 0.05–1.00 absorbance units; all results were duplicated.

RESULTS

The amount of incident light received by leaves of the four species varied from an average of 7% for *D. thomasii* to 81% for the shade leaves and, by definition, 100% for the sun leaves of T. *guineensis*. The data for the shade leaves of *T. guineensis* (sampled exclusively from the basal foliage of the trees)

is approximate as inclement weather prevented a full five-day sampling program. On the available data, a statistical analysis (Mann-Witney U test) revealed that the median values of available light (LIGHT) were significantly different for every possible interspecies comparison ($P < 0.05$). The following is a description by species of the relationships found between light intensity and the chemical measurements made on the samples of whole leaf material. Basic statistics for levels of TP, CT, ADF, PROT, and incident LIGHT are given in Table 1.

Acacia pennata. Samples were taken from a total of 10 individual plants which provided eight paired samples for within plant comparisons of the effect of light intensity on leaf chemical composition. Scatter graph plots for LIGHT versus TP and CT levels are presented in Figure 1a and b. Positive correlations are clearly evident, indicative of phenolic production increasing as light intensity increases. The correlation coefficients among these three variables are (1) LIGHT vs. TP, $r = +0.73$; (2) LIGHT vs. CT, $r = +0.81$; and (3) TP vs. CT, $r = +0.86$; all these being associated with a high degree of statistical confidence ($P < 0.001$). The above analysis could simply reflect gross interplant differences. However, paired samples taken from the same plant (paired sample t test; Campbell, 1974) show that there are significantly more total phenolics and condensed tannins in the leaves receiving the higher light intensity within any given individual ($P < 0.01$). No correlations were found between LIGHT and either PROT or ADF.

Cynometra leonensis. A total of 11 individual trees, which again provided eight paired samples for within plant comparisons, were studied. Scatter graph plots for the whole data set are given in Figure 1c and d, where positive correlations are again evident between LIGHT, TP, and CT (LIGHT vs. TP, $r = +0.69$, $P < 0.01$; LIGHT vs. TP, $r = +0.72$, $P < 0.01$; TP vs. CT, $r = +0.84$, $P < 0.001$). The analysis of the paired data also showed the same within-plant variations as before ($P < 0.01$). However, for this species, significant negative correlations (in the whole data set) were found between PROT and TP ($r = -0.65$, $P < 0.05$) and between PROT and CT ($r = -0.62$, $P < 0.05$) but not between LIGHT and PROT ($r = -0.30$). There was no correlation between LIGHT and ADF.

Diospyros thomasii. Samples were taken from 25 trees with 12 paired samples. The scatter graphs for the data set are presented in Figure 1e and f. Correlations between TP, CT, and LIGHT were: TP vs. LIGHT, $r = +0.65$, $P < 0.001$; CT vs. LIGHT, $r = +0.79$, $P < 0.001$; TP vs. CT, $r = +0.74$, $P < 0.001$; showing the same pattern as *A. pennata* and *C. leonensis*. The analysis of paired samples also confirmed the relationship noted above ($P < 0.01$). No significant correlation was found between PROT or ADF and any other variable.

Trema guineensis. In the absence of light measurements on a continuous

TABLE 1. MEANS (AND RANGES) FOR LEVELS OF TOTAL PHENOLICS (TP), CONDENSED TANNINS (CT), ACID DETERGENT FIBER (ADF), AND PROTEIN (PROT) IN FOUR STUDY SPECIES (AS % DRY WEIGHT) AND INCIDENT LIGHT MEASURED ON THEIR LEAVES

Measure	*Acacia pennata*	*Cynometra leonensis*	*Diospyros thomasii*	*Trema guineensis*
Number of samples	18	15	31	48
TP	5.16 (2.30–8.23)	6.20 (4.91–7.26)	5.81 (2.63–11.54)	1.92 (0.78–3.62)
CT	13.29 (3.83–31.37)	13.16 (6.27–25.59)	5.57 (2.94–12.73)	8.23 (1.59–17.99)
ADF	56.1 (49.9–60.1)	44.7 (38.7–50.8)	51.5 (30.0–64.4)	45.2 (31.8–55.6)
PROT	18.43 (15.18–29.90)	18.43 (16.07–21.39)	13.07 (10.70–15.77)	15.11 (6.92–25.43)
LIGHT	28.10 (4.65–57.93)	14.41 (2.57–39.78)	6.43 (1.03–27.90)	[a]

[a] See text.

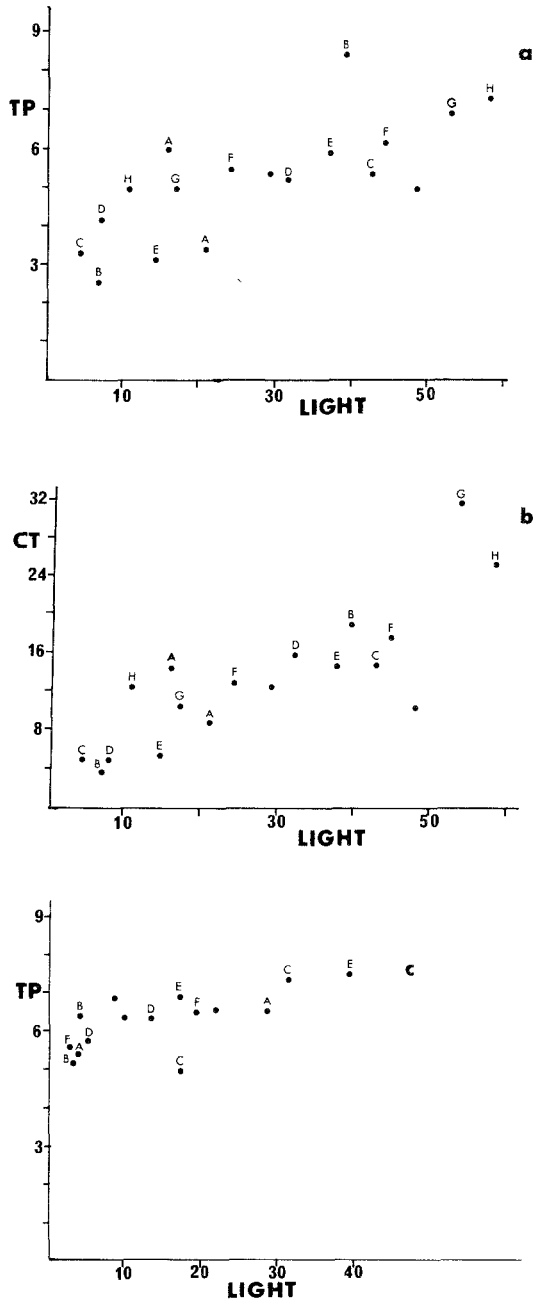

FIG. 1. Scatter graphs of foliar TP and CT (% dry weight) against light intensity (LIGHT) for *Acacia pennata* (a and b), *Cynometra leonensis* (c and d) and *Diospyros thomasii* (e and f). Paired samples, taken from high and low light intensity on an individual tree, are identified by the letter over the point.

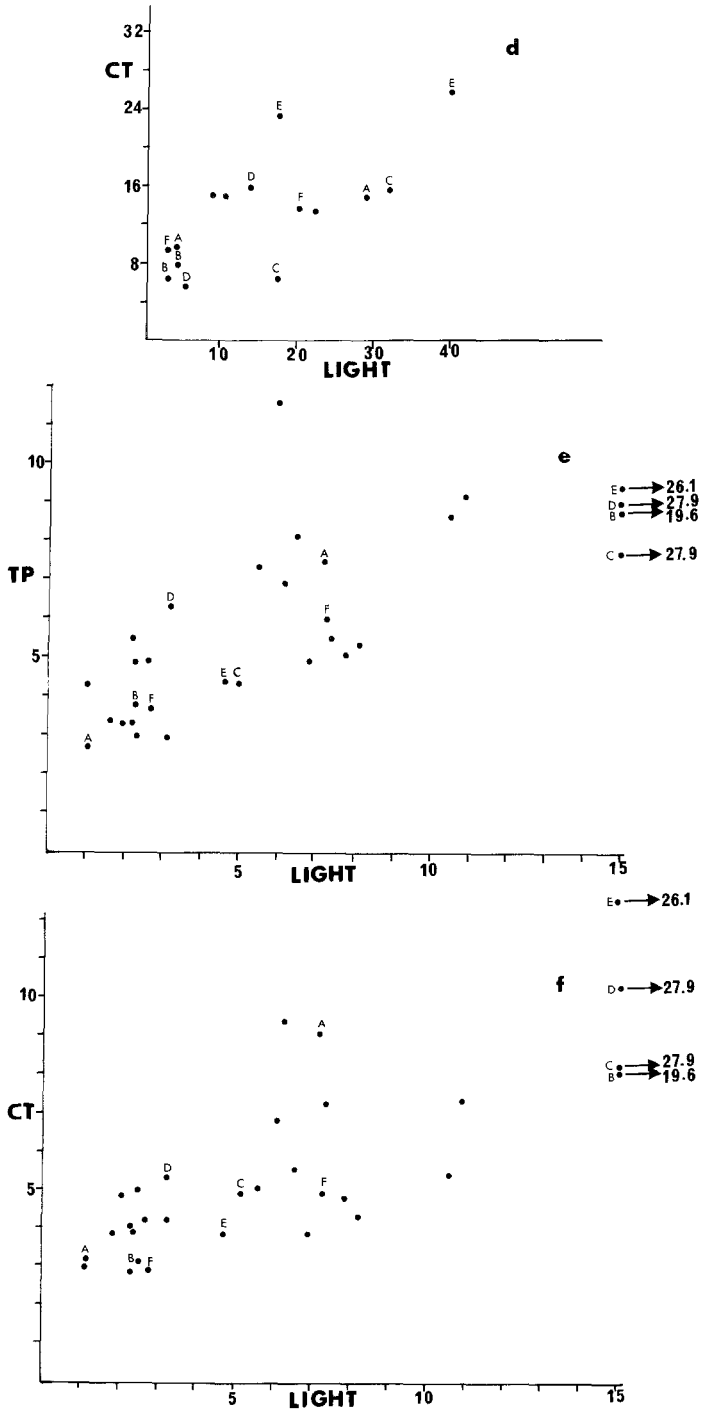

FIG. 1. (*Continued*)

scale, paired samples of leaves were taken from "sun" and "shade" positions on each of eight trees. Table 2 gives summary statistics for leaves in each light environment and for each of the age classes previously defined. Irrespective of leaf age class, "sun" leaves have greater quantities of TP and CT than "shade" leaves. With the single exception of CT in "old" leaves, all these differences are statistically significant ($P < 0.05$). The correlation coefficient between TP and CT was again high ($r = +0.73$, $P < 0.001$) for the whole data set. The correlation coefficients for the age–light classes showed considerable variation (Table 2). ADF levels, as would be anticipated, increase with age.

Identification of Foliar Polyphenolics. The first step in the analysis of the extracts was to examine their acid hydrolysates by chromatography, and a species by species analysis of the results follows.

For *Acacia pennata*, as only 19 extracts were available, each was analyzed. In every case distinctive spots for cyanidin (Pc) and delphinidin (Pd) were evident and typically of an approximately equal color intensity (relative to each other as gauged by eyesight). This means that, for the whole species sample, the CT estimate will be higher than in species where the CT is wholly or predominately Pc, because Pd gives a higher A_1^1 than Pc. Consequently a

TABLE 2. COMPARISON OF LEVELS OF TP, CT, ADF, AND PROT IN "SUN" AND "SHADE" LEAVES OF *Trema guineensis*, BY AGE CLASSES OF LEAVES (EIGHT PAIRED SAMPLES IN EACH AGE/LIGHT CLASS COMPARISON; STANDARD DEVIATIONS IN PARENTHESES)

Measure[a]	Leaf age	"Sun" leaves	"Shade leaves"
TP	young	2.72 (0.39)	1.42 (0.46)
	mature	2.32 (0.22)	1.75 (0.57)
	old	2.03 (0.43)	1.49 (0.20)
CT	young	14.11 (2.73)	5.96 (1.72)
	mature	9.56 (1.30)	5.54 (2.04)
	old	7.99 (2.01)	6.19 (2.05)
r[b]	young	+0.51	+0.72[c]
	medium	−0.25	+0.33
	old	+0.90[d]	+0.35
ADF	young	40.7 (5.66)	41.3 (4.67)
	medium	43.8 (3.61)	46.9 (3.59)
	old	49.2 (3.82)	49.2 (4.17)
PROT	young	18.59 (2.21)	18.03 (1.47)
	medium	14.46 (1.23)	15.30 (2.54)
	old	11.10 (1.53)	12.55 (1.84)

[a] Abbreviations as Table 1.
[b] Pearson's correlation between TP and CT.
[c] $P < 0.05$.
[d] $P < 0.01$.

CT/TP ratio will overestimate the CT contribution. For plant-to-plant comparisons within the species, on the other hand, the CT/TP measure is not greatly biased by the Pd/Pc ratio of the tannins present. However, four extracts yielded an overwhelming predominance of Pd. Two of these were from relatively insolated leaves and two from relatively shaded leaves. Interestingly, these represented collections from four individuals in which the paired collection did not exhibit a corrspondingly high Pd/Pc ratio. Such samples thus constituted a real but unassignable cause of variation. All the extracts also gave a faint spot that was tentatively identified as ellagic acid, suggesting the presence of a trace amount of HT in the extract.

For *Cynometra leonensis*, a random sample of 15 extracts was selected for chromatography, and in each case a distinct spot for Pc was observed together with a very faint spot for Pd. This suggests that Pd does not influence the analysis to any great extent in this species.

For *Diospyros thomasii*, a subset of extracts was analyzed, and all gave the same pattern. A strong spot was observed for Pc, but not Pd could be detected. All samples also gave intense spots for both ellagic acid and gallic acid, indicative of the presence of hydrolyzable tannins. Neither gallic acid nor ellagic acid were observed in chromatograms run prior to hydrolysis.

Trema guineensis gave the same pattern for anthocyanins as *C. leonensis*. The spot indicative of ellagic acid was present in all extracts, both hydrolyzed and unhydrolyzed. *T. guineensis* may therefore contain free ellagic acid rather than hydrolyzable tannin.

Condensed Tannin Polymer Lengths. To complete the analysis of CTs in the extracts, their polymer lengths were estimated by the method of Butler et al. (1982) and by use of the ratio of proanthocyanidin to vanillin (in methanol) assays (Goldstein and Swain, 1963), so as to produce the estimates P1 and P2 (for definition of P1 and P2 see Table 3). Data for mean polymer lengths of the CTs grouped according to species, irrespective of other variables (e.g., light or leaf age) are given in Table 3.

The first point of note is the considerable variability of the data as seen from the standard deviations. While the use of ratios in polymer length estimation will compound errors over individual measurements, their use has been shown to be of practical value, and it is reasonable to assume that the observed variability does reflect the presence of a wide range of polymer lengths within the CTs of each species. As a consequence, interspecific variation is not generally significant, the only exception being for P1 between *A. pennata* and *C. leonensis*. On further inspection of the data, no clear trend in polymer lengths dependent on LIGHT could be found for any species. However, with *T. guineensis*, leaf age was recorded as well as LIGHT and, when the interaction between these two factors was taken into account, a more subtle pattern in polymer length variation was uncovered. Given that the data set was incom-

TABLE 3. ESTIMATES (P1 AND P2) OF CONDENSED TANNIN POLYMER LENGTHS (STANDARD DEVIATIONS IN PARENTHESES[a])

Species	N(P1)	P1	N(P2)	P2
Acacia pennata	18	0.77 (0.09)	18	12.3 (2.99)
Cynometra leonensis	23	14.8 (2.54)	25	16.5 (5.76)
Diospyros thomasii	51	8.8 (1.22)	52	6.9 (9.65)
Trema guineensis	32	8.6 (2.12)	29	29.9 (10.9)

[a] P1 = $CF \times L$, where $CF = A_1^1$ of tannin/A_1^1 of standard CT from *Pinus radiata*, $L = A_1^1$ catechin/A_1^1 of tannin. P2 = A_1^1 of tannin in procyanidin assay/A_1^1 of tannin in vanillin assay.

plete, an ANOVA analysis was not possible, and so differences in polymer length (ΔP1 and ΔP2) were calculated between leaf samples of different age and light classes taken from individual trees. Table 4 shows the mean difference in polymer length between sun and shade leaves with change in leaf age. Also given are the mean differences between sun and shade leaves within the three age classes. From these data, it can be seen that, for both measures, there is a considerable difference in polymer length in relation to leaf age. The other suggestion that emerges from these results is that, in high insolation, polymer length appears to increase consistently with age; in shade, average polymer length, while possibly greater in young leaves, does not increase as much during maturation. This may be interpreted in terms of light-enhanced CT production leading initially to the formation of the greater number of short polymers, which then elongate and surpass the polymer length in shade leaves as higher input of CT occurs in light environments.

Hydrolyzable Tannins. According to the analyses employed, only *D. thomasii* was a significant HT-producer. If HTs were present in the other species, quantities were so small as to be masked by CTs in the assay procedure. As

TABLE 4. CHANGES IN CONDENSED TANNIN POLYMER LENGTHS FOR "SUN"/"SHADE" AND LEAF AGE SUBGROUPS IN *Trema guineensis* (STANDARD DEVIATION IN PARENTHESES)

Leaf type	Comparison	N(P1)	ΔP1	N(P2)	ΔP2
Sun	young to old	15	8.10 (2.63)	13	11.40 (4.98)
Shade	young to old	11	4.55 (2.75)	10	−7.23 (7.99)
Young	sun to shade	7	21.80 (3.45)	5	5.49 (1.59)
Mature	sun to shade	5	1.37 (0.35)	7	3.80 (7.25)
Old	sun to shade	5	−5.04 (1.57)	7	−6.04 (5.69)

[a] ΔP1 and ΔP2 refer to mean change in polymer length value in going from the first to the second variable.

CTs were known to increase as a function of the light intensity incident upon the leaves, and if HTs were to increase in step with this, then the CT/HT ratio should be a constant, independent of LIGHT. The ratio CT/HT was calculated for *D. thomasii* extracts and its dependence on LIGHT investigated. The CT values employed were from measurements made upon the same lyophilized extract as the methanol extract used for the previous CT assays.

The mean CT/HT ratio for extracts from leaves exposed to the lowest light intensities was 4.34 (SD = 1.49, n = 13) and for leaves exposed to the highest light intensity it was 3.20 (SD = 1.58, n = 14). The inference is that under higher levels the quantity of CTs actually decreases in proportion to HTs, suggesting that a greater rise in HT (versus CT) occurs in sun leaves. However, the difference between the means for CT/HT just fails to reach significance at the 5% level, and it can only be concluded that net HT production increases approximately in proportion to CT production as ambient leaf light intensity increases.

DISCUSSION

The results show that for all four species there is, without exception, an increase in levels of TP and CT with increased light intensity. This occurs within and between individual plants. These differences are found in species restricted to near maximal or to relatively low ambient light intensities. Thus, this phenomenon holds within and between individuals that would all be classed as either shaded or insolated by the subjective standards of the *Barteria fistulosa* study (Waterman et al., 1984). As with *B. fistulosa*, there is no comparable relationship apparent between light intensity and fiber levels. Unlike *B. fistulosa*, there is no significant correlation between protein and light intensity. A possible explanation of this is that in these examples differences were examined in terms of light levels in a single environment, while the *B. fistulosa* study used individuals from two distinct environments (light gaps and understory) in which edaphic nutrient levels could be appreciably different.

Waterman et al. (1984) assumed that the response to the light environment by *B. fistulosa* was not due to genetic differences between individual plants. Clearly, for the four species studied here, this assumption is correct because the response has been observed within a single plant. It would thus seem that the induction of phenolics observed in this study is a physiologic response by individual leaves or branches to their position on the plant. Furthermore, the results for *T. guineensis* show that the response occurs for leaves of all ages and that, on a more subtle level, the light environment affects the difference in chemical composition between leaves of different ages.

These findings indicate that light-induced variation in the production of

phenolics is a finely tuned phenomenon. The hypothesis that increased investment in phenolics is a function of resource availability (Coley et al., 1985), while offering a plausible explanation for interplant variation, does not satisfactorily explain the intraplant variability observed here. At this detailed level it seems more appropriate to consider the phenomenon in relation to plant physiology per se, rather than in terms of microenvironment or plant–animal interactions. This is not to discount the resource allocation hypothesis, but rather to view the intraplant variation we observe as taking place within the broader metabolic parameters laid down by that hypothesis.

The significant correlations between LIGHT and CT, and TP and CT are strongly suggestive that the increase in TP is primarily one of increase in CT. However, a first indication that this does not satisfactorily explain all variation is seen in the wide variability in the correlation coefficients between CT and TP for the different light–age classes of *T. guineensis* (Table 2). This suggests changes in other phenolics may well be involved for this species at least. Given that CT is not the only phenolic component of plant material, then if only CT increased with light, the ratio of the two variables (CT/TP) should also increase, as CT becomes a greater proportion of the TP. For the three species where such an analysis is possible, the correlations of LIGHT versus (CT/TP) are as follows: *A. pennata*, $r = +0.77$, $P < 0.001$; *C. leonensis*, $r = +0.66$, $P < 0.01$; *D. thomasii*, $r = +0.12$, NS. In the first two instances, it would indeed seem that the proportion of CT does increase in higher light intensity, but for *D. thomasii* no such correlation occurs. In the latter there is a more general and coordinated increase in the production of phenolics, including HTs. The conlusion that the increase in other phenolics is a coordinated one follows because a disproportionate increase in the other phenolics would have led to a negative value of the correlation coefficient for this species.

Table 5 presents mean values of CT/TP ratios for all four species, and the different age–light classes of *T. guineensis*. By inspection it can be seen that for *T. guineensis* the values for "sun leaves" are not consistently higher than those of "shade leaves." So, applying the arguments used above, other phenolics may be involved in the response to light in this species as well. Of the four species, *T. guineensis* has the greatest proportion of CTs among its phenolics, with the two leguminous species intermediate, and *D. thomasii* with by far the least. On this basis, if other phenolics are involved with *T. guineensis*, then light-dependent changes in other phenolics cannot be ruled out for any of the species.

The hypothesis (Haslam, 1977) that the polymerization of the CTs occurs as an unregulated nonenzymic reaction may be due for review, as Putnam and Butler (personal communication) have found (in vitro) "procyanidin polymerase" activity in *Sorghum* grain. It is thus untimely to attempt a mechanistic interpretation of our results concerning CT polymer lengths. In the present con-

TABLE 5. RATIOS BETWEEN CT AND TP MEASUREMENTS FOR LEAVES FROM EACH SPECIES, WITH DATA FOR *Trema guineensis* FURTHER SUBDIVIDED ACCORDING TO "SUN"/"SHADE" AND LEAF AGE (STANDARD DEVIATIONS IN PARENETHESES)

Species	CT/TP ratio
Acacia pennata	2.44 (0.77)
Cynometra leonensis	2.63 (0.68)
Diospyros thomasii	0.99 (0.26)
Trema guineensis	
All leaves	4.35 (1.46)
Young sun leaves	5.23 (0.99)
Mature sun leaves	4.18 (0.83)
Old sun leaves	3.89 (0.53)
Young shade leaves	4.31 (0.98)
Mature shade leaves	3.42 (1.39)
Old shade leaves	5.06 (1.82)

text, their importance lies in their indication that a plant's light environment affects more than simply the quantity of CT produced. With regard to HTs, this is the first report that light or any other ecological factor affects their production.

At this point it becomes reasonable to consider alternative explanations as to why plants might generally increase their production of phenolics in higher light intensities, particularly as the effect is now established for species in a range of different plant families. The pathways involved in the synthesis of phenolics are related to the three major routes of intermediate metabolism (i.e., the glycolytic and phosphogluconate pathways and the citric acid cycle). The relationships among these processes and the two major types of tannin are slightly different. HTs contain glucose and so may draw directly on the products of primary metabolism (i.e., the Calvin cycle), while their phenolic constituents are derived from an early stage of the shikimate pathway. CTs are produced by the further metabolism of C6–C3 derivatives of the shikimate pathway which consume additional products of intermediate metabolism. About 60% of the carbon in CTs must pass through the reaction catalyzed by the enzyme phenylalanine ammonium lyase (PAL). This reaction is, in metabolic terms, irreversible, and the enzyme is thought to be a point at which metabolic control is exerted (Camm and Towers, 1973).

Hanson and Havir (1981) note that up to 20% of fixed carbon may be subject to metabolism by PAL. Light intensity is included in the factors thought to promote PAL activity either directly or indirectly due to high levels of sugar accumulating in the leaves (McClure, 1985). With respect to the indirect effect,

Margna (1977) provides evidence to suggest that PAL is under substrate level control, which, if it is the case, would explain increased tannin production in high light intensity when there is abundant fixed carbon available. The evidence is therefore favorable to the notion that phenolics, including both types of tannin, could build up in highly insolated plant parts for purely "economic" reasons.

The possible mechanisms involved in the response to light, indicated above, correspond to the hypothesis of Haslam (1985) that secondary metabolites may result from "overflow metabolism" into which excess metabolites may be shunted in times of stress. Haslam (1985) cites evidence that metabolic disorders such as those caused by metal-ion imbalances can result in the accumulation of metabolites, particularly organic acids. An early example was provided by Chesters and Robinson (1951), who regarded the build up of citrate in a zinc-deficient fungal culture as evidence of the organism's inability to completely oxidize carbon to CO_2; this was taken to be a symptom of metabolic malfunction. With regard to tannins, boron deficiency can result in their accumulation (Rajaratnam and Hock, 1975), possibly because this element is involved in the regulation of 6-phosphogluconate dehydrogenase and thus in the regulation of carbon flux into the phsophogluconate, and subsequently into the shikimate pathways (Rajaratnam and Hock, 1975). The similarity of this situation to that reported by Chesters and Robinson (1951) is striking. Further direct evidence that plant nutrient status is important in regulating tannin production is provided by Wilson (1955), Barry and Forss (1983), and Bryant (1987), who have studied tannin production as a function of fertilizer applications. More circumstantially, the evidence that vegetation on poor soils is likely to be rich in phenolics (Janzen, 1974; Waterman, 1983) also supports this case. These effects have been viewed at the level of the whole plant. The intraplant "overflow" observed in this study can be viewed as reflecting a directional stress (rather than a general stress) that leads to the synthesis of compounds that are not readily translocated from the site of formation.

Considering the effects of both light and mineral nutrition on phenol production, a scenario can be envisaged where a plant subject to an external stress that results in imbalances in carbohydrate metabolism might then accumulate an overflowing metabolite (sensu Haslam, 1985). The accumulation of tannin would fit the need for an oxidized product, and, unlike most simple organic acids, tannins may have beneficial effects as allelochemicals. In engineering terminology, tannins are thus viewed as the strain evident as a result of some external stress. Such a view of plant responses to the environment has been proposed by Ayres (1984), and it is one that seems to provide an attractive framework for considering tannins.

Some caution has to be exercised in adopting this position, as metabolic overflow does not necessarily lead to the accumulation of metabolites. For in-

stance, while Haslam (1985) and Hoffman (1985) agree that stress can lead to the citric acid cycle being overloaded by both glycolytic and pentosephosphate pathway metabolites, Hoffman indicates that, due to cyanide-resistant respiration of these metabolites, the accumulation of fixed carbon as phenolics could be prevented. Indeed, not all stresses lead to the accumulation of phenolics or other carbon-rich products, as might be expected from the work of Coley et al. (1985) or Bryant (1987). The accumulation of proline, betaine, or putrescine by water-stressed plants provides a case in point (Treichel et al., 1984).

One final question that should be addressed is why there is no comparable increase in fiber levels related to insolation, particularly in view of the fact that cellulose and lignin formation stems from the carbon-based sector of the nutrient cycle, in the case of lignin via PAL mediation. For the formation of cellulose and related polysaccharides, there appears to be a complex and specific series of control mechanisms (Northcote, 1985), and so there may well be a level of conservatism in these processes that is not seen in simpler PAL-mediated reactions. Lignin production is linked to the process of secondary thickening during cell wall formation, and its rate of synthesis would, therefore, appear to be governed primarily by the formation of the cell wall polysaccharide. In short, cell wall production is a primary process and as such cannot be allowed to exhibit the flexibility afforded to stress metabolic processes.

The arguments presented here are not intended to suggest that all secondary products are overflow metabolites produced in time of stress or that, once produced, phenolics represent a waste of fixed-carbon resources to the plant. Both the possible allelochemical function and possible reentry into metabolism, via turnover, make such assumptions about secondary products unwarranted. Furthermore the production of metabolites as allelochemicals in circumstances of low metabolic stress is not inconsistent with their accumulation as overflow metabolites during stress. The work reported here shows that both HT and CT may be produced for reasons independent of any allelochemical function. The following paper will consider whether these light induced accumulations of polyphenolics are likely to be adaptive at the plant herbivore level.

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LIGHT-INDUCED VARIATION IN PHENOLIC LEVELS IN FOLIAGE OF RAIN-FOREST PLANTS. II. Potential Significance to Herbivores

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Abstract—The allelochemic activity of the polyphenolics isolated from the leaves of four west African rain-forest plants, *Acacia pennata*, *Cynometra leonensis*, *Diospyros thomasii*, and *Trema guineensis*, was examined by means of protein precipitation experiments to estimate their specific activity as precipitants. Results indicated that light-induced phenol synthesis in the more heavily insolated foliage of a species led to greater protein-precipitating capacity in these leaves. It is considered doubtful that this produced a quantitative difference in protein availability to an herbivore as, on average, even in the most shaded leaves there was sufficient tannin present to precipitate all the foliar protein. However, taking into account the considerable variability inherent in the results obtained and the adaptations herbivores possess to circumvent the antinutritional properties of tannins, it was concluded that shaded foliage was generally likely to present a nutritionally more acceptable food package on a statistical basis but that acceptable leaves could be found from throughout the light continuum encountered in any of the species studied.

Key Words—Foliar phenolics, condensed tannins, light enhancement, protein precipitation, herbivory, variable plant chemistry.

INTRODUCTION

In the preceding paper (Mole et al., 1988) evidence was presented for a positive correlation between the production of phenolic secondary metabolites and incident light intensity in the leaves of four west African plants, *Acacia pennata*, *Cynometra leonensis* (both Leguminosae), *Diospyros thomasii* (Ebenaceae), and

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Trema guineensis (Ulmaceae). In all four species enhanced phenolic levels in insolated leaves seemed to carry a significant contribution from increased condensed tannin (CT) synthesis; in the case of *D. thomasii* it also included increased production of hydrolyzable tannins (HT).

The ability of tannins to act as antifeedants has been widely considered to occur through their interaction with proteins (Zucker, 1983). The antifeedant activity could be due to astringency of tannins in the mouth (Bate-Smith, 1972; Lea and Arnold, 1978), but most authors assume that tannins exert their activity through inhibiting the action of proteases and other digestive enzymes in the gut and by generally insolubilizing proteins as precipitates (Rhoades, 1979; Mitaru et al., 1984; Sekiya et al., 1984; Beart et al., 1985). Thus it has been widely assumed that animals avoid feeding on tannin-containing plants to avoid inhibiting the processes of digestion.

Martin and Martin (1984) and Mole and Waterman (1985) have shown how tannin-protein precipitates may be solubilized by surfactants naturally present in the gut. While indicating potential adaptations for feeding on tannin-containing plants, this evidence does not show that tannins do not combine with proteins in vivo. Indeed, there is clear evidence that tannins do bind to proteins to form soluble complexes (Van Buren and Robinson, 1969), and so the process of digestion may still be subject to inhibition under conditions where precipitates do not form. Furthermore, evidence indicating that tannins and other phenolics can promote digestion in soluble systems (Neucere et al., 1978; Mole and Waterman, 1985; Oh and Hoff, 1986a) may now need to be reassesssed in light of evidence that tannins have the potential to disrupt sequential proteolytic systems involving inactive zymogen precursors (Oh and Hoff, 1986b).

Thus, none of the available evidence contradicts the notion that tannins will bind to proteins in vivo as they do in vitro, and there is evidence supporting complex formation in vivo (Mitaru et al., 1984). Analysis of feces shows a particular tendency for tannins to prevent digestion of proline-rich proteins ingested in the diet (Eggum and Christensen, 1975; Mole et al., unpublished results). It has recently been proposed (Butler et al., 1986) that mammals might counteract the effects of tannins by means of proline-rich proteins secreted in saliva, binding preferentially with them and so preventing interaction with other proteins.

Recent reviews (Bernays, 1981; Mole, 1986; Mole and Waterman, 1986) suggest that typical insect or mammalian herbivores tend to avoid consuming levels of tannin above an acceptable level for that species. This holds whether the herbivore has a high average intake (tannin specialist) or a low to zero average intake (nonspecialist). There are exceptions, but this generalization holds in the majority of cases.

Thus, despite evidence for counteradaptations (Martin and Martin, 1984; Mole and Waterman, 1985; Butler et al., 1986), the quantitative argument concerning the effects of tannins in vivo still holds true. An excessive intake of

tannin (for a given species) is still liable to overwhelm any endogenous process capable of either nullifying their antinutritional activity or turning tannin–protein binding to the benefit of the herbivore. Given that this is the case, assessment of the concentration of tannins in a food item and its protein-binding capacity remain valuable indicators of their potential as antifeedants.

In this paper we attempt to assess the effects of light-induced variation in tannin levels in the leaves of our four study plants in terms of the potential antifeedant effects of the tannins and the overall value of those leaves as food for a herbivore. To do this we employ in vitro chemical and biochemical assays to illustrate some major variables that, according to present thinking, seem likely to influence selection of food items. The herbivore we consider here could be either vertebrate or invertebrate; the only requirement is that it is deterred from feeding by tannins that are present in food items to a higher extent than they are in the normal diet of that herbivore.

METHODS AND MATERIALS

Experiments were performed on freeze-dried leaf material. The collection and preparation of this material and the assay procedures used for chemical analyses were reported in the previous paper (Mole et al., 1988).

Protein-Precipitating Ability of Extracts. Measurements of the protein-precipitating activity of the extracts followed Hagerman and Butler (1980). Bovine serum albumin (BSA, Sigma fraction V) was prepared as a 5 mg/ml stock solution in a buffer of pH 4.9, which is the protein's isoelectric point. The buffer (170 mM NaCl and 200 mM acetic acid brought to pH 4.9 with NaOH) contained more than sufficient salt to ensure that ionic strength did not limit precipitation.

Measurements of protein-precipitating ability of individual extracts were carried out as follows: A sample of each tannin extract was made up as an aqueous solution in 5 ml of water (concentrations in milligrams per milliliter were uncontrolled and results are expressed in terms of concentration of total phenolics), and 0.5-ml aliquots of this solution were added to each of two centrifuge tubes, one containing 2 ml of "blank" pH 4.9 buffer and the other containing 2 ml of the BSA solution (pH 4.9). The tubes containing the BSA were then centrifuged and the results of total phenolic assays of the supernatant solutions were compared with total phenolic assays (Hagerman and Butler, 1980) of the "blank" buffer so as to calculate the proportion of phenolics present that were not precipitated by BSA. Assays for the proportion of BSA precipitated (%PPT) were also made by the Moore and Stein method (1954, 1968).

After being used for this assay and the HT assay reported in the preceding paper, the aqueous solutions of the extracts for each species that remained unused were bulked and freeze dried again for use in a standard specific activity

assay, where the need to vary the phenolic concentration at will necessitated the use of a powdered extract as starting material.

For each specific activity assay, two sets of 11 test tubes containing 0, 0.5, 1.0, 1.5, ... , 5.0 ml of BSA stock solution were prepared, each tube being made up of 0.5 ml by the addition of "blank" buffer solution. The tannin extract to be assayed was then dissolved in more buffer solution and any insoluble material was removed by decanting or centrifugation. A 0.5-ml aliquot of the clear tannin solution was then added to each of the 22 tubes containing BSA–buffer solution. Fifteen minutes was then allowed for precipitate formation after which the tubes were centrifuged for 5 min at the top speed of a bench centrifuge to pellet the precipitates. The supernatants were assayed for phenolics using the Hagerman and Butler (1980) method and were then discarded. The tube with zero addition of BSA gave no precipitation, and the result obtained from this tube was taken to indicate the total phenolic substances (TPS) present in the assay. The pelleted material was redissolved in 4 ml of 4 M NaOH and heated for 4 hr in a boiling water bath. At the end of this period the hydrolysate produced was neutralized with 4 M HCl and assayed for amino nitrogen by the method of Moore and Stein (1954, 1968). None of the tannin extracts (hydrolyzed or unhydrolyzed) gave detectable reactions for amino nitrogen when tested at the concentrations employed in the experiments.

Experiments were repeated with tannin solutions of an appropriately altered concentration where previously either no precipitation had occurred or where no leveling off in the amount of protein precipitated was seen as the higher BSA concentrations were reached. The 0 to 5 mg/ml concentration range for BSA employed in the experiments was thus used as a "window" through which maximal levels of precipitation of protein by the tannin were found by adjusting the concentration of tannin added to the assays. This resulted in a considerable amount of trial-and-error experimentation.

The specific activity ratio was calculated from the above data following Hagerman and Butler (1980): SA = maximum percentage of BSA precipitated/total phenolics added.

RESULTS AND DISCUSSION

Figure 1 plots the protein-precipitating ability of extracts from the foliage of each of 68 individual collections of *D. thomasii* leaves. This species was chosen for illustration because the extracts showed a considerable range in concentration of phenolics and gave protein precipitation from near zero to a maximum level at which all available protein was precipitated. Total precipitation appeared to be attained with phenolic levels equivalent to about one absorbance unit but variation was considerable. With the assay procedure used, one absorbance unit was equivalent to 0.91% tannic acid, 2.22% *Pinus radiata* CT,

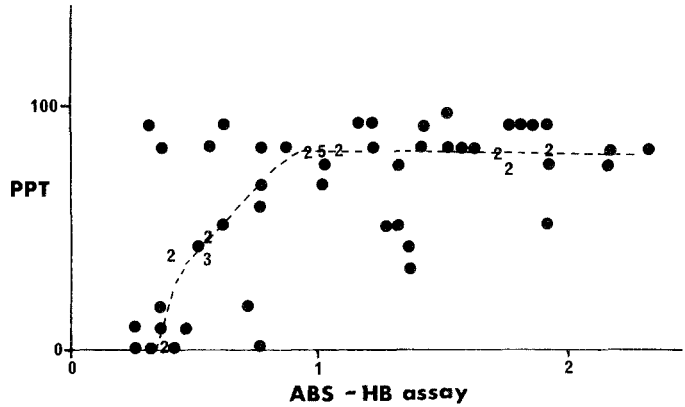

FIG. 1. Precipitation of bovine serum albumin by extracts of *Diospyros thomasii*. The percentage of the maximum recorded amount of protein precipitated (%PPT) is plotted against the phenolic content of the extract as estimated from the Hagerman and Butler (1980) assay for total phenolics (values as absorbance units). Dots represent single results, numbers indicate more than one coincident points.

or 3.3% quebracho CT. Variability in astringency as a function of a single chemical measure is presumably due to intraspecific variation in the qualitative composition of the plant phenolics, which obscures any consistent relationship between the two variables. Within this data set a comparison of high and low light-intensity collections from within an individual tree failed to show any consistent differences other than those anticipated from the relative amounts of phenolics they contained.

Among the other three species, analyses showed a similar pattern in *T. guineensis* but a much narrower range of phenolic levels in the other two species. *A. pennata* samples gave consistently high absorbance values (>0.75) and precipitation values approaching 100%, whereas *C. leonensis* gave low values (<0.75) and generally low but variable levels of precipitation. Table 1 gives average precipitation levels and percentages of phenolics left in solution after precipitation for each species after they had been separated into two groups according to whether the absorbance value in the phenolics assay was greater than 0.75 (sufficient to give near maximum precipitation of protein) or less than 0.75.

Table 2 gives the figures for the specific activity (SA) of the phenolics for each species together with values for the overall level of phenolics present (T/PS) and the percentage of those phenolics that enter into the precipitate (%T). It must be remembered that these results are obtained for bulked extracts for each species and thus represent very crude means. Evidence from the preceding experiments suggest that, at least for *D. thomasii* and *T. guineensis*, the standard deviation in SA values measured for each collection would be very large.

TABLE 1. MEANS AND 95% CONFIDENCE LIMITS (IN PARENTHESES) FOR PHENOLIC CONTENT OF EXTRACT SOLUTIONS; QUANTITIES OF BSA THEY PRECIPITATE (PPT); AND PROPORTION OF NONPRECIPITATING PHENOLICS IN EXTRACTS (NPP)

Species	Phenolics[a]	PPT (%)	NPP (%)
Samples with high levels of phenolics			
Acacia pennata	1.00 (0.32)	71 (26)	41 (8)
Diospyros thomasii	1.00 (0.10)	87 (5)	19 (12)
Trema guineensis	0.87 (0.10)	66 (23)	40 (16)
Samples with low levels of phenolics			
Cynometra leonensis	0.36 (0.09)	11 (18)	57 (53)
Diospyros thomasii	0.34 (0.05)	30 (34)	78 (24)
Trema guineensis	0.38 (0.08)	19 (14)	79 (18)

[a] Phenolics given as terms of absorbance values using the ferric chloride assay of Hagerman and Butler (1980).

However, evidence from experiments on protein precipitation (Figure 1, Table 1) and from *T/P* values (see below) does not indicate that this variability is a function of the light–shade continuum in which these leaves grow.

At an approximate level, the results for SA can be used to estimate the amount of tannin required to precipitate the entire complement of foliar protein (*T/P**). The calculation of *T/P** for a given species assumes that foliar protein is equivalent to the BSA used in the SA assay and that the tannins of the species have the same A_1^1 as quebracho tannin. On this basis *T/P** can be computed from:

$$T/P^* = \left(SA \times \left[\frac{A_1^1}{10}\right]\right)^{-1}$$

TABLE 2. VALUES FOR OVERALL LEVEL OF PHENOLICS PRESENT (TPS), PERCENTAGE OF PHENOLICS ENTERING INTO PRECIPITATION REACTIONS WITH BSA (%T), AND SPECIFIC ACTIVITY (SA) FOR BULKED EXTRACTS FROM EACH SPECIES

Species	TPS[a]	%T	SA
Acacia pennata	0.32	55	8.50
Cynometra leonensis	0.45	13	4.79
Diospyros thomasii	0.36	74	14.42
Trema guineensis	0.24	32	10.67

[a] This value is based on the concentration of the bulked phenolic extract used in the specific activity experiment and does not reflect the actual phenolic content of the bulked leaf samples (Table 1). Values are in absorbance units for the Hagerman and Butler (1980) assay.

TABLE 3. CALCULATED MINIMUM TANNIN–PROTEIN RATIO (T/P*) FOR COMPLETE PROTEIN PRECIPITATION, CALCULATED T/P VALUES (MEAN AND RANGES) FOR INDIVIDUAL LEAF COLLECTIONS FROM EACH SPECIES WITH *Trema Guineensis* SAMPLES SUBDIVIDED ACCORDING TO AGE AND INSOLATION

Species	T/P*	T/P	
		Mean	Range
Acacia pennata	0.026	0.70	0.16–1.57
Cynometra leonensis	0.046	0.74	0.29–1.47
Diospyros thomasii	0.016	0.44	0.20–1.25
Trema guineensis	0.028		
Young sun leaves		0.76	0.36–1.06
Mature sun leaves		0.67	0.30–0.82
Old sun leaves		0.71	0.34–1.31
Young shade leaves		0.33	0.15–0.50
Mature shade leaves		0.36	0.09–0.62
Old shade leaves		0.49	0.19–0.70

where T/P^* = mg tannin/mg BSA required for complete precipitation of the BSA; SA = specific activity of the tannin; A_1^1 for quebracho tannin; and the division by 10 takes account of the different units of SA and A_1^1.

The values for T/P^* based on SA are given in Table 3 and suggest that the phenolics of *D. thomasii* are most effective at protein precipitation and those of *C. leonensis* least effective.

Individual *T/P* values for each extract of *A. pennata*, *C. leonensis* and *D. thomasii*, calculated from the ratio of CT to protein levels are plotted in Figure 2. The tannin element of the *T/P* calculation employed in these figures relates to the procyanidin assay. Similar analyses using the results of Folin-Denis and ferric chloride assay procedures produced comparable plots. Average values and ranges for these *T/P* figures are given in Table 3. Once again there is a large scatter in the results, and in each case these show a significant relationship to the incident light so that as light levels decrease so does the *T/P* value. *T/P* values for sun and shade leaves of *T. guineensis* subdivided into their different age classes are also presented in Table 3. Once again there is a clear distinction between *T/P* levels for sun and shade leaves, this being particularly obvious in the young and mature leaf categories but less so in old leaves. No distinction on the basis of *T/P* can be made for different age classes, except perhaps for the old leaves, in which *T/P* generally rises, presumably due to removal of protein.

Finally, mention should be made of two other measures made by Mole et al. (1987) which are of significance to an assessment of the palatability and food value of a leaf: protein and acid detergent fiber. Neither showed any sig-

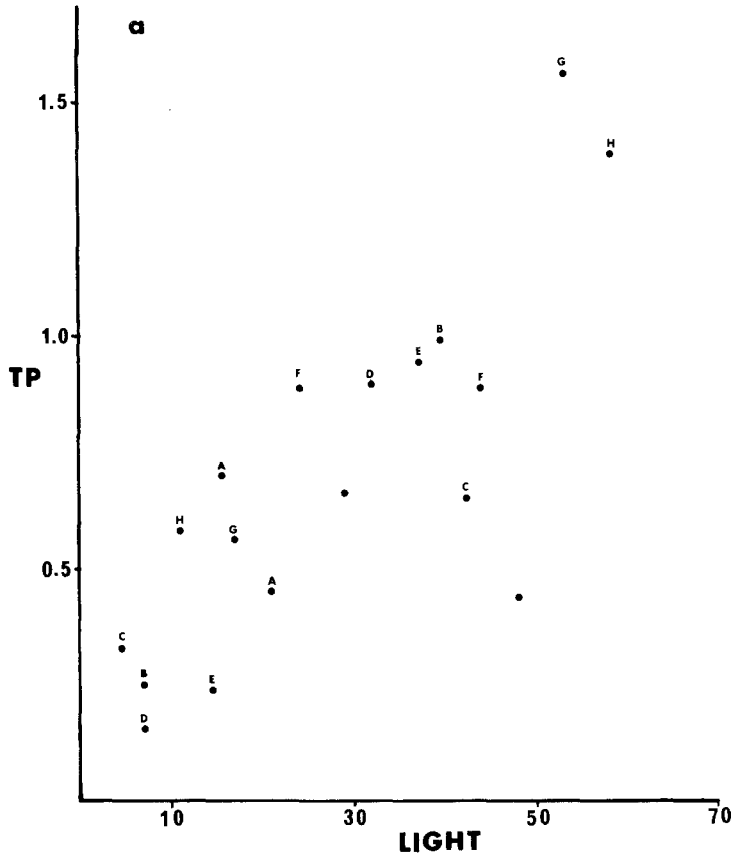

FIG. 2. Plots of tannin–protein ratios (T/P) for individual leaf collections against incident light, estimated as a percentage of the total insolation, on those leaves. Tannin estimates are based on the proanthocyanidin assay for condensed tannins. Plots are for: (a) *Acacia pennata*, (b) *Cynometra leonensis*, and (c) *Diospyros thomasii*. Paired samples taken from high- and low-light environments on a tree are indicated by the same letter.

nificant correlation with light intensity, although in most cases marginally higher protein levels did tend to occur in the more shaded leaves. The coefficients of variation calculated for these measures in relation to all samples from *A. pennata*, *C. leonensis*, *D. thomasii*, and the three age classes of *T. guineensis* were generally low (9–24% for protein and 8–26% for fiber; cf. 13–40% for total phenolics and 36–54% for CTs). By contrast, in *T. guineensis* both measures showed a relationship to leaf age, fiber increasing with age and protein decreasing. Only in *C. leonensis* did there appear to be a significant negative correlation between protein and fiber ($r = -0.66$, $N = 15$, $P < 0.05$).

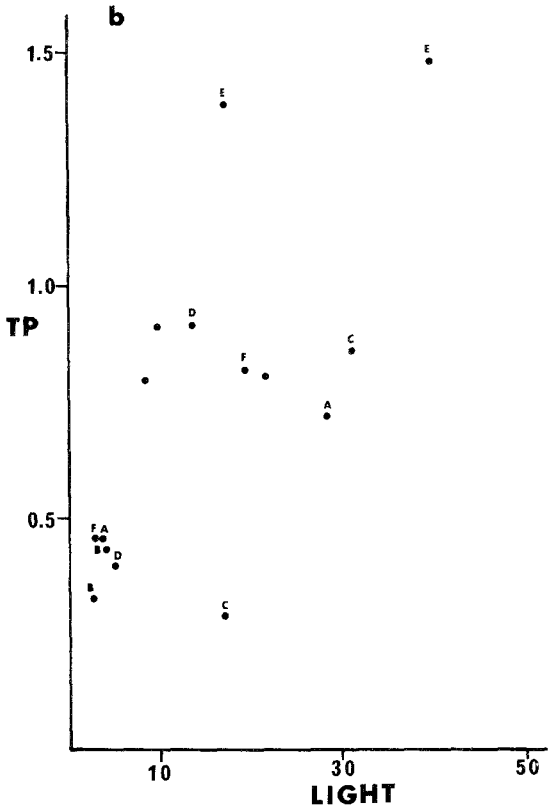

FIG. 2. Continued.

CONCLUSIONS

Because of the approximations necessary in obtaining some of the data reported here, conclusions can only be tentative. What is clear is that in each of the four species *T/P* approaches *T/P** most closely in leaves that come from the more shaded environments and that in an intraspecific comparison these leaves represent a better "nutrient package" than corresponding more insolated leaves. However, taking the computed values for *T/P** and *T/P* for individuals at face value, it would appear that in all situations in each species there is more than sufficient CT present to precipitate all foliar protein. Indeed, in all but a very few cases there is at least an order of magnitude difference between *T/P** and the lowest *T/P* values. One striking feature is that the pattern observed within all four species seems to be very similar, despite their apparent preference for different niches within the light–shade continuum. One possible exception to this argument is *D. thomasii*, which appears comparable to the others

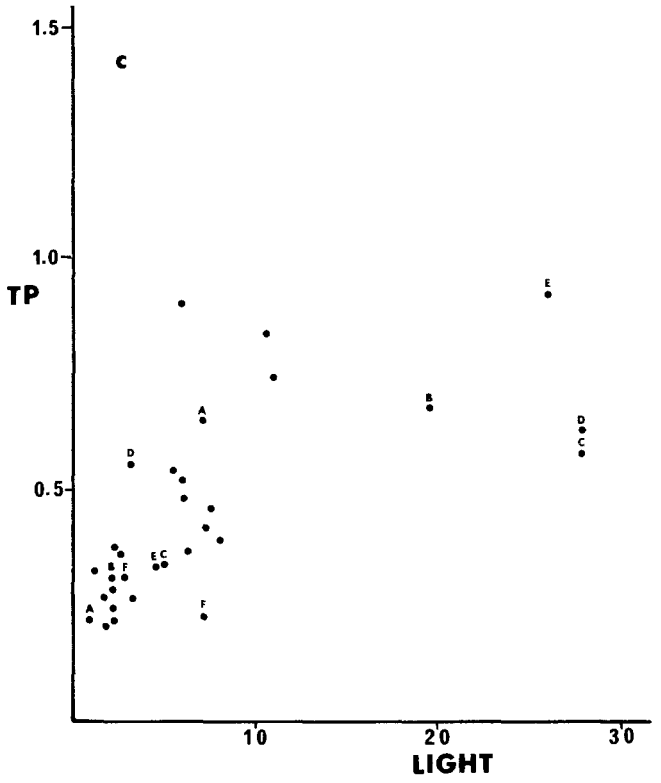

FIG. 2. Continued.

without taking into account the contribution to its phenolics made by HT, which will increase its *T/P* values (Figure 2c) significantly.

Arguments based on the assumption that protein precipitation in vitro reflects on the in vivo process of digestibility reduction are, as we indicated in the Introduction, probably naive. However, choosing as an in vitro assay a procedure optimized to detect protein precipitation does provide a direct measure of tannin–protein binding. Following from this, quantifying the amount of tannin in a leaf by reference to its capacity to bind to the protein complement of that leaf provides a useful framework within which to make predictions concerning allelochemic effects. As *T/P* becomes relatively greater than *T/P**, then if those tannins do bind to the leaf proteins to form soluble or insoluble complexes, there remains an increasing excess of tannin available to bind with other proteins.

What is apparent from this survey is that within each species and within each individual there is appreciable variation in quantitative phenol chemistry that appears to be related to the degree of shading. There is substantial further

variation superimposed on this due to the nonuniformity of qualitative chemical structure and protein-binding activity of the polyphenolic component. On the assumption that the tannin-protein binding mediates the allelochemic activity of tannins, then the biochemical changes that relate to light intensity suggest that more shaded leaves are likely to be preferable to a herbivore as a food source. These data thus lend support to a number of recent reports (Woodhead, 1981; Waring et al., 1985; Hanley et al., 1986; Larsson et al., 1986; Bryant, personal communication) suggesting that shading leads to greater levels of herbivory. On the other hand, the variability in leaf chemistry does indicate that a selective herbivore could obtain an acceptable diet from sun leaves. This, plus the range of herbivore digestive adaptations relating to polyphenolics, allows for the presence of many alternatives for folivores to exploit the variable chemistry of partially insolated/shaded plants.

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NOVEL 2-ETHYL-5-ALKYLPYRROLIDINES IN THE VENOM OF AN AUSTRALIAN ANT OF THE GENUS *Monomorium*

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Abstract—Novel 2-ethyl-5-alkylpyrrolidines and their corresponding 1-pyrrolines have been identified as poison gland products from an unidentified Australian species of *Monomorium*. The major alkaloids present in the venom of this ant are *trans*-2-ethyl-5-undecylpyrrolidine and *trans*-2-ethyl-5-(12-tridecen-1-yl)pyrrolidine. The position of the double bond in the latter was established from its dimethyl-disulfide adduct after the amine function had been protected, and the stereochemistry of the alkyl groups was determined by direct comparison with synthetic compounds. The corresponding 1-pyrrolines were also detected in varying amounts in this venom. The pyrrolidines and 1-pyrrolines possess considerable insecticidal activity when evaluated against termite workers. The alkaloidal venoms of *Monomorium* appear to be an important factor contributing to the success of these small ants both as competitors and as predators.

Key Words—Ants, *Monomorium*, Hymenoptera, Formicidae, venom, 2,5-dialkylpyrrolidines, 2,5-dialkyl-1-pyrrolines, dimethyldisulfide adducts, insecticidal activity, chemical ecology.

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INTRODUCTION

A variety of 2,5-dialkylpyrrolidines have been identified in the venoms of ants from the two related myrmicine genera, *Solenopsis* and *Monomorium*. While these compounds have been found in some *Solenopsis* (*Diplorhoptrum*) species (*Solenopsis molesta*, *S. fugax*, and *S. punctaticeps*), they are particularly characteristic of *Monomorium* and have been found in every species of this genus in which alkaloids have been detected (Jones et al., 1982a, and references therein).

This paper reports two novel 2-ethyl-5-alkylpyrrolidines and their pyrrolines identified from an undescribed Australian species of *Monomorium*. This species, henceforth referred to as *Monomorium* sp. #1066, belongs to a major radiation of Australian *Monomorium* that consists of numerous small (1.5–2 mm), dark-colored species, almost all of which are undescribed. They are diurnal, predominantly summer-foraging unspecialized predators and scavengers, most common in semiarid and seasonally arid regions, where they are often among the most abundant ants (e.g., *Monomorium* spp. 1 and 3; Andersen, 1983). Although most *Monomorium* species are quite diminutive ($\sim$2 mm) they are generally effective competitors against any species that are considerably larger (Baroni Urbani and Kannowski, 1974) primarily because of the toxic and repellent activities of their venoms. It thus appears that the great success of *Monomorium* species is in no small way correlated with the evolution of alkaloidal venoms that are effective toxins and deterrents to the wide variety of insect species that constitute both food and competitors for these ants. In order to gain some insights into the chemical ecology of this ant genus, the toxicities of these alkaloids to termite workers, which are common prey of *Monomorium* species, have also been determined. *Monomorium* sp. #1066 were collected near Melton, Victoria, and voucher specimens are deposited under this number in the British Museum (Natural History), London.

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic analyses were performed on a Gow-Mac model 750P, using a 2-m $\times$ 2-mm-ID glass column packed with 5% SP-1000 on 100–120 mesh Supelcoport. This instrument was programed from 40°C to 200°C at 10°C/min as soon as the solvent had eluted. Retention temperatures were found to be reproducible within one degree on a given day. Preparative gas chromatography was performed on a Varian model 920 using a 2-m $\times$ 5-mm-ID aluminum column packed with either 10% SP-2100 or 10% SP-1000 on 100–120 mesh Supelcoport. Infrared spectra were obtained from neat liquid films with a Perkin-Elmer 1320 grating infrared spectrophotometer.

[^{1}H]NMR spectra were obtained using a Varian FT-80 spectrometer. Mass spectra were obtained using a LKB-9000 GC-MS at an ionizing voltage of 70 eV and fitted with a 2-m × 2-mm-ID glass column packed with 1% SP-1000 on Supelcoport. High-resolution mass spectra were obtained using a VG 7070F in the EI mode at an ionizing voltage of 70 eV. Melting points are uncorrected.

3,6-Heptadecanedione (1). Condensation of 2.9 g of dodecanal (18.4 mmol) and 1.55 g of ethylvinyl ketone in the presence of triethyl amine and 0.5 g of 5-(2′-hydroxyethyl)-4-methyl-3-benzylthiazolium chloride in the usual manner (Jones et al., 1979) gave 4.0 g of the diketone as a waxy solid (75% yield), mp (GC prep) 65–67°C; MS *m/z* (rel. intensity) 268(3, M^+), 239(7), 211(9), 183(14), 141(10), 129(10), 128(100), 113(38), 85(32), 71(25), 57(20), and 43(6). Calculated for $C_{17}H_{32}O_2$ 268.2404 (M^+), observed 268.2411.

cis and trans-2-Ethyl-5-undecylpyrrolidine (2). Reductive amination of 2.0 g (7.2 mmol) of the diketone 1 in the usual manner with sodium cyanoborohydride and ammonium acetate (Jones et al. 1980) gave 1.6 g of a 1 : 1 mixture of *cis* and *trans* isomers that was 90% pure by GLC analysis. The retention times of the isomers on a 5% SP-1000 column were 4.8 min and 5.8 min, respectively, at 190°C. Both isomers had the following mass spectrum: MS *m/z* (rel. intensity) 253(1, M^+), 252(2), 225(9), 224(49), 114(2), 111(4), 99(7), 98(100), 82(2), 74(1), 57(2), and 43(1). Calculated for $C_{17}H_{34}N$ 252.4694 (M^+-1), observed 252.4714; Calculated for $C_{15}H_{30}N$ 224.2378 (M-C_2H_5), observed 224.2380.

2-Ethyl-5-undecyl-1-pyrrolines (5 and 6). A solution containing 0.4 g (1.6 mmol) of 2, and 5 ml of 5% NaOCl solution in 15 ml of methanol was stirred for 1 hr. After the addition of 1.5 g of NaOH, the mixture was heated to reflux for 3 hr. After the solvent was removed, 20 ml of water was added, and the mixture was extracted with ether (3 × 25 ml). The combined ether washings were dried over anhydrous K_2CO_3, and the solvent was removed in vacuo to provide 0.3 g of a mixture of the two 1-pyrrolines that was ca. 90% pure by GLC analysis; IR 1640 cm^{-1}. The two 1-pyrroline isomers could be separated (SP-1000) and had the following mass spectra: First eluting—(5) MS *m/z* 251 (2, M^+), 250(2), 222(6), 208(2), 194(2), 180(2), 166(3), 138(5), 124(35), 111(100), 82(40), 71(5), 70(6), and 57(6); second eluting—(6) MS *m/z* (rel. intensity) 251(22, M^+), 236(7), 222(8), 208(9), 194(9), 180(6), 166(15), 152(30), 138(20), 124(30), 111(90), 110(60), 97(100), 96(75), 82(18), 71(10), and 57(12).

18-Nonadecen-3,6-dione (3). A well-stirred solution containing the Grignard reagent formed from 7.0 g of 4-tetrahydropyranyloxybutyl bromide (Ferdinandi and Just, 1971) and 0.8 g of magnesium in 70 ml of tetrahydrofuran, cooled to 0°C under a nitrogen atmosphere, was treated with 2.0 ml of a freshly prepared 0.2 M solution of Li_2CuCl_4 in tetrahydrofuran (Fouquet and Schlosser,

1974). Subsequently, a solution containing 5.0 g (21 mmol) of 9-decen-1-yl mesylate (from the commercially available alcohol and methanesulfonyl chloride in the usual manner) in 10 ml of tetrahydrofuran was added dropwise to the Grignard solution. The resulting mixture was allowed to warm to 25°C overnight, carefully acidified with 10% HCl and extracted with ether (4 × 50 ml). The combined ether extracts were dried over anhydrous $MgSO_4$, then filtered and distilled to give 3.4 g (75% yield) of colorless 13-tetradecen-1-ol, bp 135–145°C (0.4 mm Hg), having infrared and NMR spectra matching those previously reported (Yamanaka and Imai, 1981). This alcohol (3.0 g) was immediately oxidized with pyridinium chlorochromate (Corey and Suggs, 1975), and after the usual work-up, distillation gave 1.9 g of 13-tetradecenal (63% yield), bp 95–100°C (0.1 mm Hg): IR 3080, 2960, 2720, 1715, 1640, 990, and 910 cm^{-1}; NMR δ 9.76 (1H, t, J = 1.9 Hz), 5.8 (1H, d of d of t, J = 18, 10, and 6 Hz), 5.0 (1H, br d, J = 18 Hz), 4.9 (1H, br d, J = 10 Hz), 2.4 (2H, br t, J = 7 Hz), 2.0 (2H, m), 1.3 (18H, br s); MS *m/z* (rel. intensity) 210(2, M^+), 192(4), 181(2), 167(3), 166(8), 135(10), 121(14), 112(12), 111(18), 110(14), 109(13), 98(30), 97(26), 96(26), 95(30), 84(20), 83(38), 82(40), 81(40), 70(24), 69(48), 68(30), 67(30), 67(40), 57(38), 55(100), 43(34), and 41(66). This aldehyde (0.8 g/3.8 mmol) was condensed with ethyl vinyl ketone as described above to give 1.0 g of diketone 3 (83% yield), mp 55–56°C; NMR δ 5.8 (1H, d of d of t, J = 18, 10 and 6Hz, 5.0 (1H, br d, J = 18 Hz), 4.9 (1H, br d, J = 10 Hz), 2.7 (4H, s), 2.44 (4H, m), 2.0 (2H, m), 1.26 (18H, br s), 1.1 (3H, t, J = 7Hz); MS *m/z* (rel. intensity) 294(2, M^+), 265(6), 237(5), 209(5), 141(12), 129(10), 128(100), 113(48), 95(14), 85(40), 71(30), 69(20), 67(10), 57(37), 55(33), 43(17), and 41(25). Calculated for $C_{19}H_{34}O_2$ 294.2559 M^+), observed 294.2551.

cis and trans-2-Ethyl-5-(12-tridecen-1-yl)pyrrolidine(4). Reductive amination of diketone 3 (0.735g/2.5 mmol) in the manner described above gave 0.6 g of a 1:1 mixture of *cis* and *trans* isomers that was nearly 80% pure by GLC analysis. Their retention times were 14 min and 17.6 min, respectively, on a 5% SP-1000 column at 190°C. Pure samples of the isomeric mixture were obtained by preparative gas chromatography: NMR δ 5.8 (1H, d of d of t, J = 18, 10, and 6 Hz), 5.0 (1H, br d, J = 18 Hz), 4.9 (1H, br d, J = 10 Hz), 2.9 (2H, br m), 2.0 (2H, m), 1.7–1.1 (26H, m), and 0.85 (3H, t, J = 7Hz); MS *m/z* (rel. intensity) 279(2, M^+), 278(2), 277(2), 251(7), 250(37), 152(2), 138(1), 124(5), 111(14), 99(7), 98(100), 97(6), 96(6), 82(10), 69(5), 68(4), 67(3), 55(12), and 41(11). Calculated for $C_{19}H_{27}N$ 279.2928 (M^+), observed 279.2915; calculated for $C_{19}H_{36}N$ 278.2849 (M-1), observed 278.2841; calculated for $C_{17}H_{32}N$ 250.2535 (M-C_2H_5), observed 250.2533.

Derivatization of Pyrrolidine 4. A solution containing 10 mg of pure *cis/trans 4*, 10 mg of 4-dimethylaminopyridine, and 200 mg of trifluoroacetic anhydride in 1 ml of pyridine was stirred under a nitrogen atmosphere for 4 hr at

25°C. The mixture was diluted with pentane and acidified with 10% HCl. The organic phase was separated, dried (anhydrous $MgSO_4$), and concentrated in vacuo to give a solution with two major isomeric components having retention times of 11.2 min and 12 min, respectively, at 200°C on 5% SP-1000 column. MS *m/z* (rel. intensity) 375(4, M^+), 348(6), 347(5), 346(22), 306(2), 262(1), 207(1), 195(11), 194(100), 166(6), 140(6), 95(5), 83(4), 81(14), 69(8), 67(6), 55(5), 44(3), 43(3), and 41(10). This solution was treated with 0.1 ml of dimethyldisulfide and 0.01 ml of an ethereal iodine solution (50 mg in 1 ml of ether) and allowed to stand at 25°C for 48 hr. In a separate experiment, a trace of BF_3 etherate, used as a catalyst instead of iodine, was more efficient. After washing with a 5% $Na_2S_2O_3$ solution, the organic phase was concentrated and gas chromatographic analysis revealed the presence of two major high-molecular-weight isomers (retention times = 32.4 min and 33.6 min, respectively, at 250°C on a 5% SP-2100 column). MS *m/z* (rel. intensity) 469(8, M^+), 422(5), 410(5), 409(10), 408(100), 396(2), 374(5), 373(3), 360(5), 346(2), 295(2), 195(5), 194(38), 95(10), 81(12), 69(8), 67(8), 61(20), 55(20), and 41(10).

Analysis of Monomorium, sp. #1066. Gas chromatographic examination of the methylene chloride extracts of six collections of 50–100 workers of these ants revealed the presence of two components in an 8 : 1 ratio. The mass spectra of these compounds had important ions at *m/z* 253(M^+), 224, and 98(100) and at *m/z* 279(M^+), 250, and 98(100), respectively, and were otherwise identical to those of authentic samples of pyrrolidines 2 and 4. In addition, direct comparison (5% SP-1000 and 5% SP-2100) showed that they had retention times identical to the second eluting isomers of 2 and 4.

GC-MS analysis of the extracts of all of the collections of ants showed two components eluting before 2 whose mass spectra and gas chromatographic retention times were identical to those of an authentic sample of pyrrolines 5 and 6. In addition, there were two analogous compounds eluting before pyrrolidine 4: MS *m/z* (rel. intensity) 277(3, M^+), 276(5), 248(10), 166(10), 152(3), 138(5), 124(30), 112(10), 111(100), 82(35), 71(10), 70(8), and 57(15); and MS *m/z* (rel. intensity) 277(25, M^+), 276(5), 252(10), 268(9), 236(15), 194(15), 180(5), 166(10), 152(20), 148(15), 124(8), 111(70), 110(45), 97(100), 96(50), 82(18), 71(15), and 57(25). In one collection, the total quantity of each isomeric pyrroline mixture was nearly equivalent to the amount of corresponding pyrrolidine present. In the other five collections, all of the pyrrolines combined were not more than 15% of the total alkaloid mixture. As with other collections of ants (Jones et al., 1982b), it was possible to obtain an estimate that each ant contained between 0.5 μg and 2.0 μg of the major pyrrolidine 2 by comparison with standardized solutions of synthetic 2. There were correspondingly less of the other alkaloids present.

Treatment of a sample of one of the extracts first with a drop of trifluora-

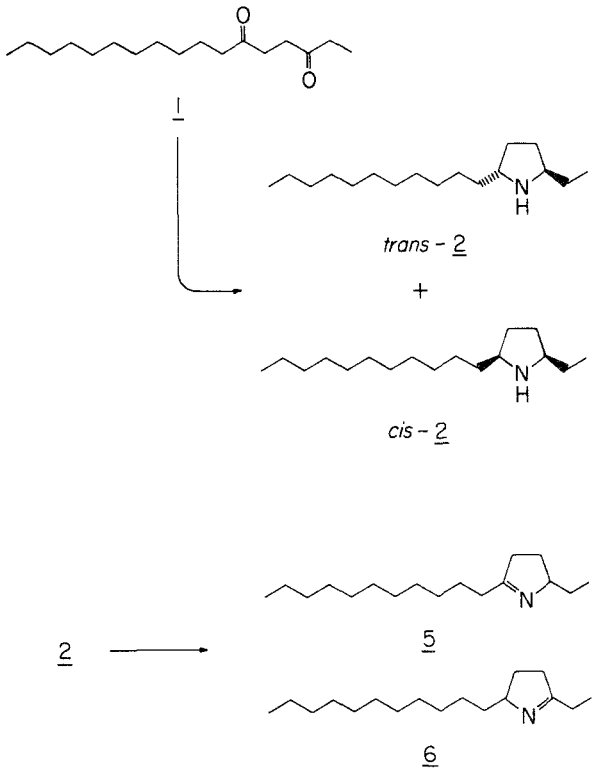
cetic anhydride and three to four drops of pyridine and then, after careful work-up with pentane and 10% HCl, with dimethyldisulfide and iodine gave a complex mixture. GC-MS analysis showed one major high-molecular-weight component (retention time 40 min on a 1% SP-1000 column at 200°C), whose mass spectrum had important ions at m/z = 469(M^+), 408(100), and 194 and was identical to that of the di(thiomethyl)-*N*-trifluoracetyl derivative of 4.

Toxicity Determinations. The toxicities of the alkaloids were determined utilizing workers of an undescribed species of *Reticulitermes* collected from Athens, Georgia. Individual termites (instars 6–8), placed in groups of 10 in 5-cm glass Petri dishes, were each treated with 1 μl of an acetone solution of the test compound. Three replicates per dosage were evaluated, and termite workers treated with acetone served as controls. The treated termites were provided with a moist filter paper and toxicity determinations were made 20 hr later. The resultant sigmoid dosage–mortality curve was linearized by a statistical treatment (Pogits) and the data treated by a computer program in order to obtain the LD_{50} for each compound expressed as micrograms per milligram termite. Values for the insecticides nicotine and methomyl were determined for comparison.

RESULTS

Gas chromatographic analysis of the methylene chloride extracts from all the collections of *Monomorium* sp. #1066 revealed the presence of one major component, whose mass spectrum (m/z = 253 M^+, 224, and 98) indicated that it was 2-ethyl-5-undecylpyrrolidine (2). Comparison with an authentic sample obtained by the reductive amination of the corresponding diketone 1 (Scheme 1) (Jones et al., 1980) confirmed this structure. This synthetic methodology produces the *cis* and *trans* 2,5-disubstituted pyrrolidines in a 1:1 ratio, and these isomers can be separated on a 5% SP-1000 column. It has been shown (Pedder et al., 1976; Jones et al., 1979) that the first eluting isomer has the *cis* configuration. In the case of 2, the natural material had an identical retention time with the second-eluting (*trans*) synthetic isomer.

A second, minor component (present in 10–15% of the amount of *trans*-2) was also found in each of the four samples. This alkaloid had a mass spectrum (m/z = 279 M^+, 250, and 98) indicating a 2-ethyl-5-tridecenylpyrrolidine. The position of the double bond was determined by means of the dimethyl disulfide adduct (Buser et al., 1983). A sample of the extract was first treated with trifluoroacetic anhydride in the presence of pyridine, and then the resulting *N*-trifluoroacetamides were treated with dimethyl disulfide in the presence of iodine. The mass spectrum of the dithiomethyl-*N*-trifluoroacetamide derivative of the minor pyrrolidine (M^+ = 469) had M-61 ($-CH_2SCH_3$) as the base peak

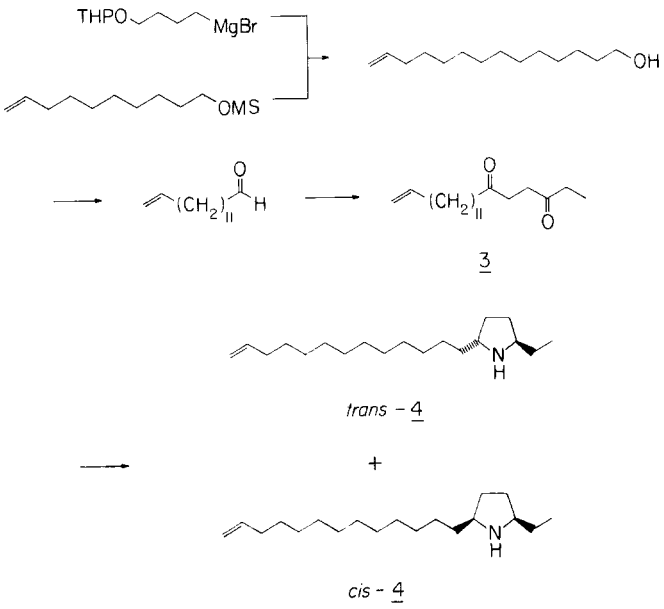


SCHEME 1

(Scheme 2), indicating the presence of a terminal double bond, so that this alkaloid was 2-ethyl-5-(12-tridecen-1-yl)pyrrolidine (4).

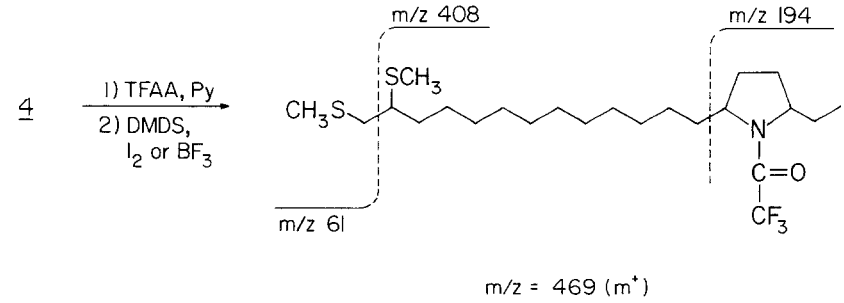
In order to confirm this assignment and to determine its stereochemistry, pyrrolidine 4 was synthesized as shown in Scheme 3. Synthetic 4 is produced as a 1 : 1 mixture of *cis* and *trans* 2,5-disubstituted pyrrolidine isomers by this method, and as before, the second eluting (*trans*) isomer had a retention time and mass spectrum identical to that of the natural alkaloid. A small sample of the purified isomeric mixture was first converted to its *N*-trifluoroacetamide, and then treated with dimethyl disulfide in the presence of iodine to give the dithiomethyl adduct which had a mass spectrum identical to that obtained by derivatizing the ant alkaloid (Scheme 2).

The methylene chloride extracts of the ants contained four additional alkaloidal components. The first two of these components, which eluted before 2, had mass spectra and retention times identical to those of synthetic 5 and 6,



SCHEME 2

prepared from 2 by *N*-chlorination, and subsequent dehydrochlorination (Scheme 1) (Jones et al., 1979). Two similar alkaloids were also present, eluting before 4, and having mass spectra [m/z = 277(M^+), 111(100), and 82; m/z = 277(M^+), 262, 97(100), and 82] indicating the 1-pyrroline homologs of 4. The mass spectra of 2-ethyl-5-alkyl-1-pyrrolines are characterized by important ions at m/z = 96 and m/z = 97, whereas the mass spectra of 2-ethyl-5-alkyl-5-pyrrolines have important fragments at m/z = 82 and m/z = 111 (Pedder et al., 1976).



SCHEME 3

The LD_{50} of 2-ethyl-5-undecylpyrrolidine (2) was 0.17 ± 0.02 μg/mg termite and that of 2-ethyl-5-(12-tridecen-1-yl)pyrrolidine (4) was 0.23 ± 0.07. A mixture of the Δ^1 and Δ^5 pyrrolines of 2-ethyl-5-undecylpyrroline was about half as toxic (0.46 ± 0.03 μg/mg termite) as the corresponding pyrrolidine (2). For comparison, LD_{50s} of the insecticides nicotine and methomyl were 0.5 ± 0.02 and 0.25 ± 0.02, respectively.

DISCUSSION

Although 2-ethyl-5-alkylpyrrolidines have been found in a *Solenopsis* species (Pedder et al., 1976), this is the first report of their occurrence as major venom components in a species of *Monomorium*. The overall carbon–nitrogen skeleta of the 2-ethylpyrrolidines 2 and 4 could be assigned from their mass spectra, and were confirmed by unambiguous syntheses (Schemes 1 and 3). This synthetic methodology also permits the assignment of stereochemistry of the alkyl groups about the pyrrolidine ring, since both *cis* and *trans* isomers are formed (Jones et al., 1980) having identical mass spectra but different gas chromatographic retention times. As has been found to be the case in other ants producing 2,5-dialkypyrrolidines, only the *trans* isomers of the 2-ethyl-5-alkylpyrrolidines 2 and 4 are found in *Monomorium* sp. #1066.

Although terminally unsaturated side chains have also been previously reported in *Monomorium* (Jones et al., 1982a, b), this is the first report of their assignment in an alkaloid by the formation of a dimethyldisulfide adduct. It was found that the trifluoroacetamide derivitive had to be formed before the dithiomethylation reaction (Buser et al., 1983) could be successfully carried out. Although it happens to be irrelevant in this case, an important advantage of forming the dimethyl disulfide adduct for the determination of double-bond location over the previously utilized methoxymercuration–demercuration methodology (Jones et al., 1982b) is the formation of a single derivative for any given vicinally disubstituted double bond.

In the case of pyrrolidine 4 the mass spectrum of the dithiomethyl-*N*-trifluoroamide derivitive shows a parent ion at m/z = 469(M^+), a base peak at m/z = 408(M-61) for the loss of CH_3SCH_2— fragment, and a peak at m/z = 61, which result from C–C bond cleavage between two vicinal thiomethyl groups, and indicate the position of the double bond. Other important ions appear at m/z = 422 (M-CH_3S), m/z = 374 [M-(CH_3S + CH_3SH)], m/z = 360 [M-(CH_3SCH_2— + CH_3SH)], and m/z = 194 [M-$(CH_3S)_2C_{13}H_{25}$] (Scheme 2). Using synthetic 4, it was found the formation of this derivative could be catalyzed by iodine or boron trifluoride etherate (Caserio et al., 1985).

In one of the collections of *Monomorium* sp. #1066, quantities equivalent to those of the pyrrolidines present of the four possible 1-pyrrolines correspond-

ing to 2 and 4 were found, easily identifiable by their mass spectra, in which the important fragmentation and rearrangement ions were the same as those reported for similar ethyl-1-pyrrolines (Pedder et al., 1976). These compounds were also present in much smaller amounts in the other three collections of ants. Although it has been shown that 1-pyrrolines can be formed as artifacts in GC-MS systems (Fales et al., 1980), the fact that there was so great an observable difference in the amounts of these compounds from collections of ants from different nests indicates otherwise. The 1-pyrrolines could be either decomposition products of 2 and 4, or they could have been present in all the extracts initially and were much more subject to decomposition themselves than the concomitant pyrrolidines. The latter seems more likely, since the protonated forms of 1-pyrrolines would be strong electrophiles, subject to attack by a variety of biological and nonbiological nucleophiles.

The 2,5-dialkylpyrrolidines and 2,5-dialkyl-1-pyrrolines present in the venom of *Monomorium* sp. #1066 provide this ant with an insecticidal venom that possesses considerable toxicity when applied topically. Significantly, the tip of the string of *Monomorium* workers is spatulate and adapted for the topical application of venom products (Clement, 1981), rather than being the pointed hypodermic structure characteristic of related ants. Since European species of *Monomorium* commonly utilize termites (*Reticulitermes* spp.) as prey (Clement, 1981), and since we have observed the same thing with *M. minimum* and *Reticulitermes* spp. in the southeastern United States, termites must be considered major food items of these ants. Furthermore, the availability of an alkaloid-rich venom with great topical toxicity appears to be the major evolutionary development that has enabled these diminutive ants to facilely exploit much larger insects such as termites as prey.

In addition to being highly toxic to termites, the dialkylpyrrolidines act very rapidly after topical administration, paralysis of treated termites occurring almost instantaneously. Thus, these alkaloids can enable *Monomorium* workers to speedily immobilize insects with small dosages of topically applied venom. Since dialkylpyrrolidines have been previously demonstrated to be highly effective insect repellents (Blum et al., 1980), these compounds emerge as allomones par excellence.

Monomorium species #1066 belongs to a group of small species, no member of which we have previously examined. Species in this group, which contain a palpal formula of PF 1,2 (Bolton, personal communication) are not uncommon in Australia, although the groupings of Australian *Monomorium* are much more complex than palpal formulae. We are attempting to obtain other members of this group for comparative analyses. The venoms of *Monomorium* species in the PF 3,3 group (*M. latinode*) and species in the PF 2,2 group (*M. minimum*) are qualitatively rich in terms of dialkylpyrrolidines and pyrrolines (Jones et al., 1982a, b) compared to *Monomorium* sp. #1066. Analyses of ad-

ditional species in the PF 1,2 group should demonstrate whether the venom of species #1066 is typical of those of other members of this heretofore unanalyzed group of myrmicine ants.

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SPECIFICITY OF MALE RESPONSE TO MULTICOMPONENT PHEROMONES IN NOCTUID MOTHS *Trichoplusia ni* AND *Pseudoplusia includens*

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Abstract—The response of male cabbage looper (CL) and soybean looper (SBL) moths was observed in the flight tunnel and measured in field tests to the six-component CL pheromone, the five-component SBL pheromone, and to Z7-12:OAc, the major component common to each pheromone. In both the flight tunnel and the field, male CL exhibited significantly greater levels of response to their six-component blend than to Z7-12:OAc alone. A low level of cross-attraction of male CL to the SBL pheromone was observed in both the flight tunnel and the field, but it was quantitatively and qualitatively similar to their response to Z7-12:OAc alone. Thus the minor components of the SBL blend did not appear to disrupt the flight behavior of male CL. With respect to SBL, in the flight tunnel males also exhibited a greater level of response to the five-component blend compared to Z7-12:OAc, but in the field their response was not significantly different to either treatment. There was also a low level of cross-attraction of male SBL to the CL blend, but this appeared to involve a significant arrestment effect on the upwind flight of males, as well as a difference in male sensitivity to the blend of components compared with Z7-12:OAc alone. The observed arrestment effect may have been due to male perception of one or more minor components of the CL pheromone. The results show that the multicomponent pheromones of these species function effectively as specific mating signals and that discrimination of odor quality by male moths can occur as the result of minor components affecting male sensitivity or their upwind flight response to the pheromone.

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Key Words—Sex pheromone, Lepidoptera, Noctuidae, *Pseudoplusia includens*, *Trichoplusia ni*, multicomponent pheromone blends, sustained-flight tunnel, behavioral thresholds.

INTRODUCTION

Successful mate location is dependent on a communication channel that is highly efficient and specific. In part, the communication channel is represented by a set of ecological factors, including temporal periodicity of activity (both seasonal and diel), unique microhabitat requirements, energy requirements, and predation (Cardé and Baker, 1984). In many moth species, the communication channel also involves the use of a sex pheromone. Studies have shown that species within a taxonomic group (family or subfamily) often utilize the same chemicals, as a result of common biosynthetic pathways (Roelofs and Brown, 1982). In sympatric species with similar activity patterns, this could lead to significant levels of cross-attraction, decreasing the efficiency of the communication channel. In these cases specificity is presumably effected by the use of unique minor components or similar compounds in different ratios, resulting in different chemical signals for each species.

Within the subfamily Plusiinae of the noctuid moths, there are a number of closely related species that utilize the compound (Z)-7-dodecenyl acetate (Z7–12 : OAc) as the major pheromone component (Tamaki, 1985). Two such species are the cabbage looper (CL), *Trichoplusia ni* (Hubner), (Berger, 1966), and the soybean looper (SBL), *Pseudoplusia includens* (Walker) (Tumlinson et al., 1972). Both the CL and SBL have overlapping seasonal and geographic distributions, similar host plants, and similar circadian rhythms of activity (Mitchell, 1973; Leppla et al., 1979; Leppla, 1983). In field-trapping studies, Mitchell (1972) observed in two of three tests that, whereas females of both species attracted predominantly conspecific males, CL females reduced the capture of SBL males by SBL females or synthetic Z7–12 : OAc. Similarly, Leppla (1983) reported that in release–recapture studies male CL and SBL responded selectively to conspecific females, although there was some cross-attraction. His studies suggested that the SBL and CL are reproductively isolated, in part, by a number of factors related to their chemical communication systems, including long-range attraction, close-range courtship behavior, and male released pheromones.

Recently we identified several new components of the sex pheromone of the cabbage looper (Bjostad et al., 1984) and the soybean looper (Linn et al., 1987). In addition to Z7–12 : OAc as their major, or most abundant, pheromone component, both species produce two common minor components: dodecyl acetate (12 : OAc) and 11-dodecenyl acetate (11–12 : OAc). In addition to these, the sex pheromone of CL includes (Z)-5-dodecenyl acetate (Z5–

12:OAc), (Z)-7-tetradecenyl acetate (Z7–14:OAc), and (Z)-9-tetradecenyl acetate (Z9–14:OAc). Two additional components for the SBL are (Z)-7-dodecenyl propionate (Z7–12:Prop) and (Z)-7-dodecenyl butanoate (Z7–12:But).

With the identification of more complete pheromone blends for these two noctuid species, we initiated behavioral tests in the flight tunnel and in a field situation to compare the response of males to conspecific blends, to Z7–12:OAc, and to heterospecific blends. These tests were designed to show, in two species that share a common major component, how the different complements of minor components affect male sensitivity and specificity of response to the chemical signal.

METHODS AND MATERIALS

Insects. Both species were reared on a semisynthetic medium (Shorey and Hale, 1965) at 25–27°C, 16:8 light–dark photoperiod. For CL the relative humidity was 30–50%; for the SBL 70–80% relative humidity was required for optimal mating, egg laying, and male response (Leppla et al., 1979; see Results). Males were separated from females as pupae and kept under conditions similar to those during rearing. Adults were provided with 8% sucrose solution.

Chemicals. The synthetic chemicals used in this study were the same as those used by Linn et al. (1984, 1987). For CL the proportions of each compound in the six-component blend were: 12:OAc (6.8%), Z5–12:OAc (7.6%), Z7–12:OAc (100%), 11–12:OAc (2.3%), Z7–14:OAc (0.9%), and Z9–14:OAc (0.6%). The proportions of compounds for the five-component SBL blend were: 12:OAc (2.3%), Z7–12:OAc (100%), 11–12:OAc (2.2%), Z7–12:Prop (1.3%), and Z7–12:But (5.0%). These mixtures of compounds, and the relative ratios indicated, represent blends produced by female moths as determined from capillary GLC analysis of female glands. Solutions of these blends were prepared in Skelly B (predominantly *n*-hexanes) and checked on capillary GLC (45-m Carbowax 20 M column) to ensure purity (>99%), with a detection limit of 0.1%. A dilution series was then prepared for each treatment to be applied to polyethylene cap sources. The same solutions were used for both flight tunnel and field tests. Caps (OS-6 closures, American Scientific Products, Rochester, New York) were prepared by adding solutions (10 or 100 $\mu g/\mu l$) to the inside surface of the cap. After 1 hr the caps were closed, and those for the flight tunnel were allowed to sit in a laboratory hood for 36 hr, then placed in glass vials, and held at −10°C when not in use. For the field tests, caps were used within one week of preparation.

Males of both species were tested in the flight tunnel and in the field to 1- and 3-mg dosages of Z7–12:OAc alone and to the same dosages of Z7–12:OAc plus the additional components of the two pheromone blends. These dosages were selected on the basis of previous tests with CL (Linn et al., 1984) and

SBL (Linn et al., 1987) in which dosage–response curves were established for each species to Z7–12:OAc and the female-produced blend.

Flight Tunnel Test Procedures. Individual 3- to 4-day-old males of both species were tested in the sustained-flight tunnel described by Miller and Roelofs (1978), during the fifth and sixth hours of the 8-hr scotophase period (Linn et al., 1984; Leppla, 1983). Males were placed in the room housing the tunnel at the beginning of scotophase to acclimate to scotophase light and temperature conditions: 0.3 lux, 23–25°C. Other flight tunnel conditions were 75–80% relative humidity, and 50–55 cm/sec air velocity. The high relative humidity in the present study was necessary for optimal flight behavior by male SBL. Preliminary tests utilizing flight tunnel conditions that would normally elicit high levels of response from male CL (50% relative humidity or less) failed to elicit significant levels of flight activity in SBL. Subsequent tests revealed that 70% relative humidity or greater was necessary for optimal flight behavior.

Males were handled during testing as previously described (Linn et al., 1984). Males were allowed 1 min to respond and were scored for the following behaviors: taking flight, stationary oriented flight in the odor plume space near the point of release, initiation of upwind flight, source contact, and attempted copulations. Males were used once and then discarded.

Flight tunnel tests were conducted during one 2-hr period each day. During each period five to 10 males of each species were tested to the three treatments at one of the two dosages. For each experiment 100 males were tested to each treatment. Analysis was based on the total response to each treatment according to the method of adjusted significant levels for proportions (Ryan, 1960).

Field Test Procedures. SBL and CL sex pheromones, along with Z7–12:OAc, were field tested during the growing seasons of 1983, 1984, and 1985, at the Louisiana State Penitentiary farm, Angola, Louisiana. Pherocon-IC (Zoecon Corp., Palo Alto, California) traps were baited with the desired treatments and placed 30 m apart on aluminum poles ca. 1.5 m high. Trap height was no greater than 20 cm above the crop canopy. Tests were conducted in a variety of crops, including soybean, cabbage, and broccoli. Each treatment was replicated from three to five times, and treatments were randomized each collection day. Captured specimens were returned to the laboratory and identified to species, using genitalia characteristics described by Eichlin (1975). Data were analyzed by ANOVA, and treatment means were separated using Duncan's multiple-range test (Steel and Torrie, 1960).

RESULTS

Response to Z7–12:OAc and Blends: Flight Tunnel. In the flight tunnel males of both species responded at each dosage in greater percentages to con-

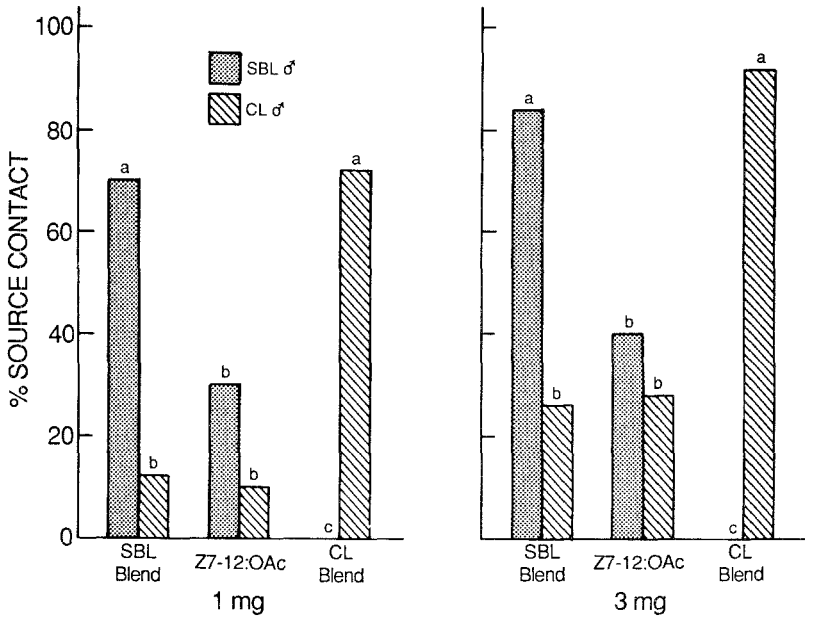

FIG. 1. Response (as percentage of source contacts) of male SBL and CL in the sustained-flight tunnel to the five-component SBL blend, Z7-12:OAc, and the six-component CL blend, at 1- and 3-mg dosages. $N = 100$ for each treatment and dosage. Analysis was performed separately on response values for each species to the three treatments, and the values with different letters are significantly different ($P < 0.05$), according to the method of adjusted significance levels for proportions (Ryan, 1960).

specific blends than to Z7-12:OAc alone (Figure 1). The lower levels of response to Z7-12:OAc were as expected based on earlier dosage–response tests. Male response to heterospecific blends was also much lower than to conspecific blends. Male CL completed flights to the SBL blend in percentages approximating those to Z7-12:OAc. In contrast, none of the SBL males completed flights to the CL blend.

Details of the flight responses of males to the 3-mg treatment in Figure 1 are presented in Figure 2. The lower level of source contacts by male CL to Z7-12:OAc or the SBL blend compared to the six-component blend was due to lowered levels of flight activation, orientation, and initiation of upwind flight. With respect to the SBL, low levels of cross-attraction were also the result of lowered response levels in the initial behaviors of the sequence, but in addition there was significant arrestment of the upwind flight of those males that did initiate this behavior.

Significant differences also were observed in the temporal aspects of the

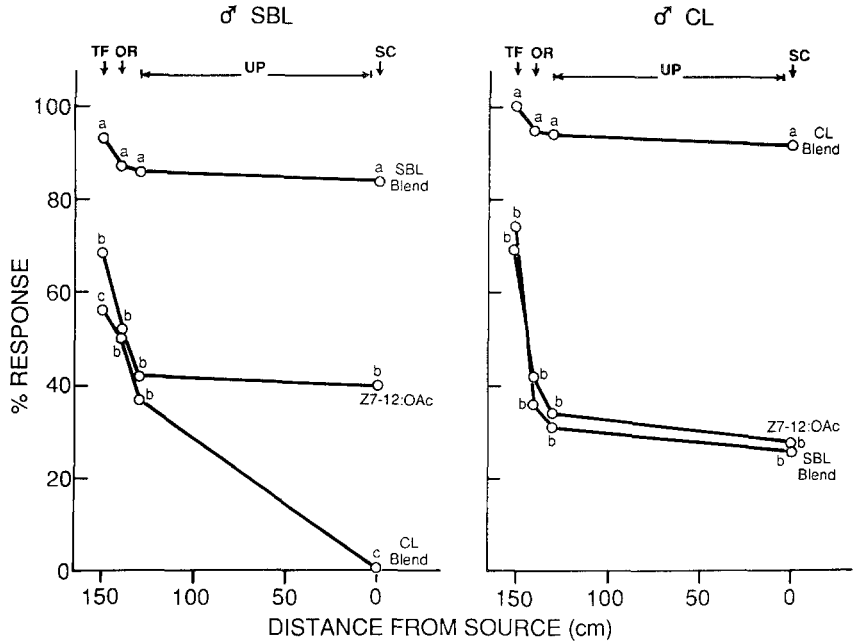

FIG. 2. Details of male SBL and CL response to the 3-mg dosage of the treatments shown in Figure 1. Behaviors are: taking flight (TF), plume orientation (OR), upwind flight (UP), and source contacts (SC). Values with different letters within each behavior are significantly different ($P < 0.05$) according to the method of adjusted significance levels for proportions (Ryan, 1960).

flight responses. The flight of males to Z7-12:OAc was significantly slower than that to their conspecific blends (5 ± 2 SD sec for SBL over the 1.5 m distance to the blend compared to 22 ± 9 SD sec to Z7-12:OAc; 6 ± 2 SD sec, and 28 ± 8 SD sec, respectively, for CL males). Furthermore, the flight of male CL to the SDL blend was not significantly different from that to Z7-12:OAc alone (25 ± 9 SD vs. 28 ± 8 SD sec).

Response of Males to Z7-12:OAc and Blends: Field: In field tests there was no significant difference in the number of SBL males trapped with Z7-12:OAc compared to the five-component blend (Figure 3). Trap capture of SBL males with the CL blend, however, was significantly reduced over that observed to the SBL blend or Z7-12:OAc.

In contrast, male CL were attracted in significantly greater numbers with the six-component blend compared to Z7-12:OAc or the SBL blend. In addition, there was no significant difference in the response of male CL to the SBL blend or to Z7-12:OAc.

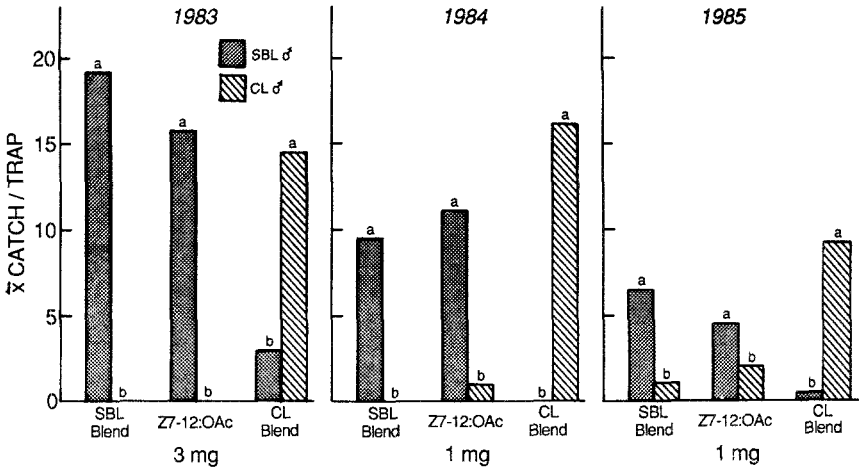

FIG. 3. Trap capture of male SBL and CL to three treatments in Figure 1. Replicates were three in 1983, four in 1984, and five in 1985. Responses for each species within each year were analyzed by one-way ANOVA, and values with different letters are significantly different ($P < 0.05$) according to Duncan's multiple-range test.

DISCUSSION

One of the objectives of the present study was to test the hypothesis that, in species possessing a common major component that is essential for chemically mediated behavior to occur, unique sets of minor components provide the basis for discrimination by males between conspecific and heterospecific females. The results reported here support this hypothesis in that low levels of cross-attraction occurred between male CL and SBL, compared with the response to conspecific blends. The results also show, however, that discrimination of odor quality was the result of two different effects of the minor components on male perception. These involve (1) males possessing a significantly lower threshold for response to the complete blend compared to the major component alone, and (2) male detection of a chemical that is not in the species pheromone blend, but is found in that of the related species, and results in significant arrestment of upwind flight.

In the case of the CL, the data show that a lower proportion of males initiated a response to the SBL blend, compared with the conspecific pheromone. A careful analysis of the flight tunnel results further shows that the response to the SBL blend was both quantitatively and qualitatively like that to Z7–12 : OAc. From this we conclude that (1) the minor components of the SBL

blend did not adversely affect the upwind flight of male CL, and (2) the low level of cross-attraction by male CL to the SBL blend is the result of the majority of the male CL population possessing a higher sensitivity to the six-component blend than to Z7–12:OAc alone.

In the case of the SBL, males took flight, oriented, and initiated upwind flight in the flight tunnel to the CL blend in approximately the same proportions as they did to Z7–12:OAc alone (Figure 2), a result similar to that obtained with CL. However, the level of male SBL response to Z7–12:OAc in the flight tunnel was slightly higher (30% with 1 mg, 40% with 3 mg) than that of CL males (10% with 1 mg, 24% with 3 mg), and in the field it was much higher.

In further contrast to the response of male CL, we observed in flight tunnel tests a significant degree of arrested upwind flight of male SBL responding to the CL blend, suggesting that the upwind flight response of males that initiated a response to Z7–12:OAc was adversely affected by one or more of the CL minor components. Recently, Grant et al. (1987) reported the existence of single olfactory receptor neurons on the antenna of male SBL specifically sensitive to low dosages of Z5–12:OAc, a minor component of the CL blend and one not found in the SBL. They suggested that Z5–12:OAc may be an important element in male SBL discrimination between female SBL and the CL blend.

The types of discrimination exhibited by CL and SBL males are not unique to these species. In a previous study we showed that the sensitivity of male CL, Oriental fruit moths, *Grapholita molesta* Busck, and the red-banded leafroller *Argyrotaenia velutinana* Walker, to the female-produced blend of components was significantly greater than to the major component(s) alone (Linn et al., 1986). Similarly, a number of other species have been reported to be able to detect compounds that are not part of the conspecific pheromone blend but that result in significant reductions in trap catch when presented with the pheromone (Priesner 1980, 1985). This is, in fact, the case with the CL, in which the compound Z7–12:OH causes significant levels of arrested upwind flight when present in the CL blend in amounts as low as 0.5% of the major component (Linn et al., 1984). Interestingly, Z7–12:OH has been proposed as a pheromone component in the related noctuid, *Autographa californica* (Steck et al., 1979).

Our results and conclusions are also in basic agreement with other published studies. Mitchell (1972) found in two of three field tests that CL females caught significantly more CL males than SBL males, and that while SBL females caught approximately equal numbers of male CL and SBL, the number of CL caught was significantly lower than to CL females and was approximately equal to the number caught by Z7–12:OAc alone. This supports our conclusion that minor components of the CL blend reduce the response of SBL males to Z7–12:OAc and that the Cl males responding to the SBL pheromone represent

a small proportion of the population that possess a lower degree of sensitivity to the six-component blend compared to Z7–12 : OAc. In another study, Leppla (1983) also showed, in the laboratory and the greenhouse, that female CL attracted many more male CL than SBL, but also that female SBL caught more SBL than CL. He showed further that when females were placed together male capture was approximately equal. These results suggest that males are able to disciminate between respective blends, a conclusion similar to our flight tunnel results but in disagreement with the study by Mitchell (1972).

Although the selection pressures that have led to the evolution of multicomponent pheromones are not well understood, our results support the idea that both interspecific and intraspecific interactions are important. Male CL, and to a lesser extent male SBL, are clearly more sensitive to the female-produced blend than to Z7–12 : OAc alone. This suggests that there is, or has been, selection for increased male discrimination of pheromone quality, possibly as a result of intraspecific competition between males for unmated females. The increased selectivity of male CL in turn provides a mechanism for discrimination of heterospecific signals, because the majority of the male population requires the complement of minor components for a response to occur. However, a more obvious interspecific effect relates to the perception by males of compounds that are not components of the conspecific female pheromone, but rather are part of the chemical signal of a closely related species. Male SBL exhibited significant levels of arrested upwind flight to the CL blend and, based on the studies of Grant et al. (1987), we would concur with their proposal that Z5–12 : OAc provides the basis for discrimination by male SBL between female SBL and CL. This phenomenon appears to be widespread in the moths and is possibly associated with selection pressures between closely related sympatric species to prevent cross-attraction.

In conclusion, our study demonstrates that the multicomponent pheromones of the CL and SBL serve as effective signals for mate location as well as for discriminating between heterospecific blends. It is also clear that male discrimination can result from at least two processes affecting male perception, one (an intraspecific effect) associated with a heightened sensitivity and specificity for the female-produced blend, and the other (an interspecific effect) involving perception of a component that is not a part of the conspecific pheromone signal but which is a component in a related species blend. The results also indicate that the communication channel is noisy, as in both species a certain proportion of the population did not discriminate betwen the common major component of the two systems and the heterospecific signals. In this regard, Leppla (1983) suggested that close-range courtship behaviors could provide an additional source of information by which discrimination between species can occur. Future studies should elucidate the details of pheromone

composition in other related noctuid moths, as well as the response of males to those pheromones and the effects of related species signals on their behavior.

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TOXICITY OF TERPENOID DETERRENTS TO THE LEAFCUTTING ANT *Atta cephalotes* AND ITS MUTUALISTIC FUNGUS

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Abstract—Four natural products, of varying activity as deterrents of leafcutter attack, were tested for their effects on ant survival and on the growth of the mutualistic attine fungus. The substances were incorporated into an artificial liquid diet for bioassays on the ants or included in an agar culture medium for fungus growth-inhibition studies. Three of the four compounds exhibited deleterious effects on either adult leafcutting ants or their mutualistic fungus, and there appeared to be some correlation between deterrency and activity in these toxicity assays. The implications of these findings for leafcutting ant foraging patterns are discussed.

Key Words—*Atta cephalotes*, Hymenoptera, Formicidae, Attini, leafcutter ants, mutualism.

INTRODUCTION

Leafcutting ants of the genus *Atta* (Formicidae: Attini) are common herbivores in successional forests throughout the Neotropics. A single colony of these ants may harvest many kilograms of leaf material in the course of a year, while attacking 50 or more plant species in the vicinity of the colony (Cherrett, 1968; Lugo et al., 1973; Rockwood, 1976; Haines, 1978). Adult ants are known to imbibe liquid from cut and crushed leaf tissues to satisfy some of their metabolic

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requirements (Littledyke and Cherrett, 1976; Quinlan and Cherrett, 1979). The ants use solid leaf material to culture a mutualistic fungus, which is believed to be the sole food of the ant larvae (Quinlan and Cherrett, 1979) and the source of proteolytic enzymes which are redistributed in the fungal gardens via the ant's fecal fluid (Boyd and Martin, 1975; Febvay and Kermarrec, 1981). This fungus metabolizes large amounts of cellulose (Martin and Weber, 1969) and may also detoxify some inhibitory or potentially deleterious plant substances (Littledyke and Cherrett, 1976). These characteristics contribute significantly to the abundance of leafcutter ants in neotropical habitats, and to the enormous size of mature ant colonies (in excess of one million workers; Weber, 1972; Schade, 1973).

Although leafcutting ants attack many different plants, at any given time most of the harvested material comes from only a few species (Cherrett, 1968; Rockwood, 1976). Recent laboratory and field studies of the Central American leafcutter *Atta cephalotes* have shown that diet selection is closely related to plant secondary chemistry (Hubbell et al., 1984; Howard, 1987). Numerous terpenoid compounds, isolated from plants unpalatable to *A. cephalotes*, are known to repel these ants or to deter the harvest of otherwise acceptable substrates (Wiemer, 1985). Furthermore, the crude lipid extracts of many other avoided or unpalatable plants are significantly deterrent (Hubbell et al., 1984; Howard, 1987), although specific active compounds have not yet been identified.

Why should terpenoids and other lipids deter or repel leafcutting ants? One possible explanation is that these substances are harmful to the ants, to their mutualistic fungus, or to both; but little evidence currently exists in support of this hypothesis. While lipid extracts of plant tissues have been shown to deter or repel leafcutting ants (Littledyke and Cherrett, 1978; Mudd et al., 1978), the physiological impact of these deterrents on the ants and their fungus has not been investigated. To evaluate the hypothesis that deterrent lipids are generally deleterious to leafcutter ants and/or to their mutualistic fungus, we undertook a preliminary investigation of the effects of several terpenoid deterrents on the survival of the ants and the growth of their fungus.

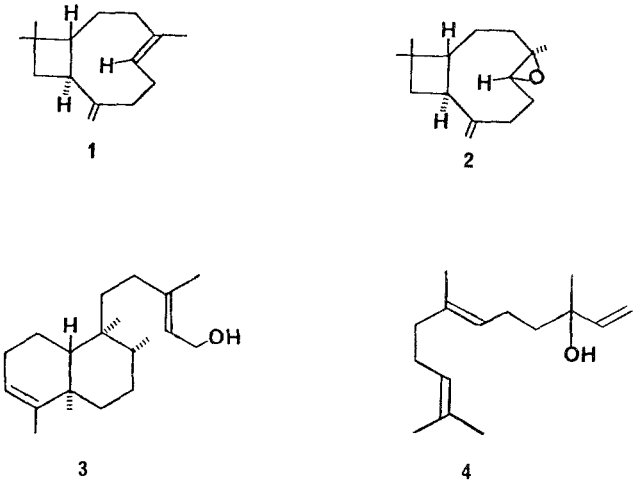
METHODS AND MATERIALS

The leafcutting ants and the fungus used in the study were obtained from a captive colony at the University of Iowa. The colony was originally collected in Santa Rosa National Park, Costa Rica, in August 1982, and was approximately 1 year old at the time of collection. The colony has been maintained in 15 interconnected Plexiglas boxes (12 × 12 × 24 cm), in a temperature- and humidity-controlled room in the Department of Biology, University of Iowa.

Ambient conditions were maintained at 23°C and 40–60% relative humidity. The colony has been fed lilac leaves daily (freshly picked in season, and frozen for use during the winter months), and maintained on a 12 hr: 12 hr light–dark cycle. Under these conditions the colony grew from a Petri dish-sized ball of fungus at the time of collection to completely fill five of the Plexiglas boxes with fungus.

Deterrent Chemicals Tested. Four deterrent terpenoids were tested for potential toxic effects on *A. cephalotes* and its mutualistic fungus: caryophyllene (1), caryophyllene epoxide (2), kolavenol (3), and nerolidol (4) (Scheme 1). All four compounds have been isolated from plants native to the range of *A. cephalotes*, including *Hymenaea courbaril* (1 and 2; Hubbell et al., 1983), *Melampodium divaricatum* (2 and 3; Hubert and Wiemer, 1985), and *Vismea baccifera* (2, 3, and 4; Cancilla, 1985). They were chosen for this study either because they are commercially available or because a large quantity was available from a plant extract. In most cases, natural concentrations of these compounds in plants have not been established; but caryophyllene and caryophyllene epoxide are estimated to occur in leaves of *Hymenaea courbaril* in concentrations of 6.7 mg/g and 8.5 mg/g dry weight, respectively (Hubbell et al., 1983). These concentrations represent approximately 3.0 mg/g and 3.8 mg/g in fresh leaf material, given an average moisture content of 55% for leaves of this species (Howard, 1987).

A laboratory bioassay, which has been described in detail elsewhere (Hubbell and Wiemer, 1983), was used to measure the deterrent activity of these compounds. Bioassays were conducted by placing 60 rye flakes soaked in a



SCHEME 1.

solution of the test compounds on a computer-generated random grid; 60 rye flakes soaked in solvent alone served as controls. Ants were allowed to forage on the grid until half the control flakes were harvested, and the number of test flakes harvested at this time was counted. The significance of the difference between the number of control and test flakes harvested (C − T) was determined using a modified binomial test (Hubbell et al., 1984).

Ant Survivorship Bioassays. Three concentrations of each terpenoid deterrent were tested for their effect on the survivorship of adult ants: 1, 0.1, and 0.01 mM. Each compound was incorporated into an artificial liquid diet consisting of 5% glucose, 1% bacteriological peptone (Nutritional Biochemicals Corp., Cleveland, Ohio), and 0.1% Vanderzant vitamin mixture (Nutritional Biochemicals) (Boyd and Martin, 1975). The diet was autoclaved and then, after it had cooled for 20 min, the test compound was added as an ether solution (1 ml). The ether was removed by heating the diets in a hot water bath (80°C), and the resulting solution was stored in a refrigerator until needed. A control diet was prepared by adding ether (1 ml) to the glucose–peptone–vitamin mixture and then removing the ether in the same manner.

Each concentration of the four deterrents was presented to 60 worker ants in a no-choice bioassay. Worker ants ($\bar{X}$ = 2.2 mm ± 0.14 mm head width) were collected from the colony and assigned to treatment groups using a random number table (Rohlf and Sokal, 1969). Each treatment group of 60 ants was distributed among 10 disposable 100 × 15-mm Petri dishes, and each dish of ants was given the liquid diet in small dishes packed with cotton. Dishes were checked daily and the number of surviving ants noted. Petri dishes and the diet were regularly changed to prevent mold growth on the diet. All dishes were kept in a temperature-controlled incubator, evenly distributed among the shelves.

In addition to tests involving deterrent compounds and the controls, survival under conditions of starvation was measured using 60 ants divided equally among 10 Petri dishes. Ants were collected and assigned to dishes at random, but only a small amount of water was offered in place of food. These dishes also were distributed evenly among the shelves of the incubator.

The effect of each deterrent compound on ant survival was tested using a one-way analysis of variance (ANOVA). The average number of days ants survived in each Petri dish was calculated, and this mean value was used as a single observation in the ANOVA. Each Petri dish was treated as a single observation for the purposes of statistical analysis, yielding 10 observations per treatment.

Fungal Growth Bioassays. Fungus growth bioassays were conducted by comparing the relative inhibition of fungus growth on an agar medium containing deterrent compounds with the amount of growth on control media. A pure culture strain of the mutualistic ant fungus, originally taken from a laboratory colony, was the source of inoculum for these bioassays. The fungus was maintained in culture on potato dextrose agar (PDA) at 25°C. Confirmation that this

fungus isolate was the mutualistic ant fungus was achieved by offering pure cultures to the ants and observing that they readily incorporated the artificial cultures into existing fungus gardens in the colony.

Each of the deterrent compounds was dissolved in diethyl ether at a concentration 10-fold higher than that required for the assay. A 1.0-ml volume of each test solution was added to 10 ml sterile melted PDA contained in a 20 × 150-ml plastic cap-stoppered test tube. Each deterrent compound was tested at final concentrations of approximately 0.1 mg/ml and 0.01 mg/ml medium. The medium was immediately vortexed to distribute the compound evenly throughout, and the tubes were kept in a 50°C water bath for 2 hr to allow evaporation of the diethyl ether. The tubes of media were allowed to cool and solidify to provide agar slants, which were stored for two days at room temperature to allow the last traces of solvent to evaporate. One set of control media was prepared by adding 1 ml ether to each tube of agar and going through the same evaporation procedures as described for the test media. A second set of control media contained only PDA and went through the same evaporation procedures as described for the other media.

Both final concentrations of each of the four test compounds and the control media were inoculated in triplicate with a small center-point inoculum (ca. 1 mm diam.) of the mutualistic ant fungus. The cultures were incubated at room temperature (24°C), and growth readings were made on days 3, 5, 7, 10, 12, 14, 21, and 28. Growth in the two test media and in the solvent control was compared with the amount of growth seen in the PDA controls. The relative growth inhibition by the deterrents was scored as follows: 4+ = no growth; 3+ = growth 25% that of controls; 2+ = growth 50% that of controls; 1+ = growth 75% that of controls; 0 = growth equal to that of controls.

RESULTS

The four compounds tested differed both in their deterrent activity and in their effect on ant survival. Nerolidol and caryophyllene epoxide are highly deterrent to leafcutting ants, even at very low concentrations, while kolavenol is slightly less deterrent, and caryophyllene is deterrent only at relatively high concentrations. Neither caryophyllene nor kolavenol had any consistent effects on the survival of adult ants at the concentrations tested, and ants given a 0.01 mM solution of caryophyllene survived longer than did control ants (Figure 1). In contrast, a 1.0 mM solution of nerolidol significantly decreased the survival of adult ants, and intermediate concentrations of 0.1 and 0.01 mM resulted in lower survival rates, although these were not statistically significant. All concentrations of caryophyllene epoxide tested resulted in significant decreases in survival compared to controls.

In contrast to our observations with caryophyllene epoxide, kolavenol, and

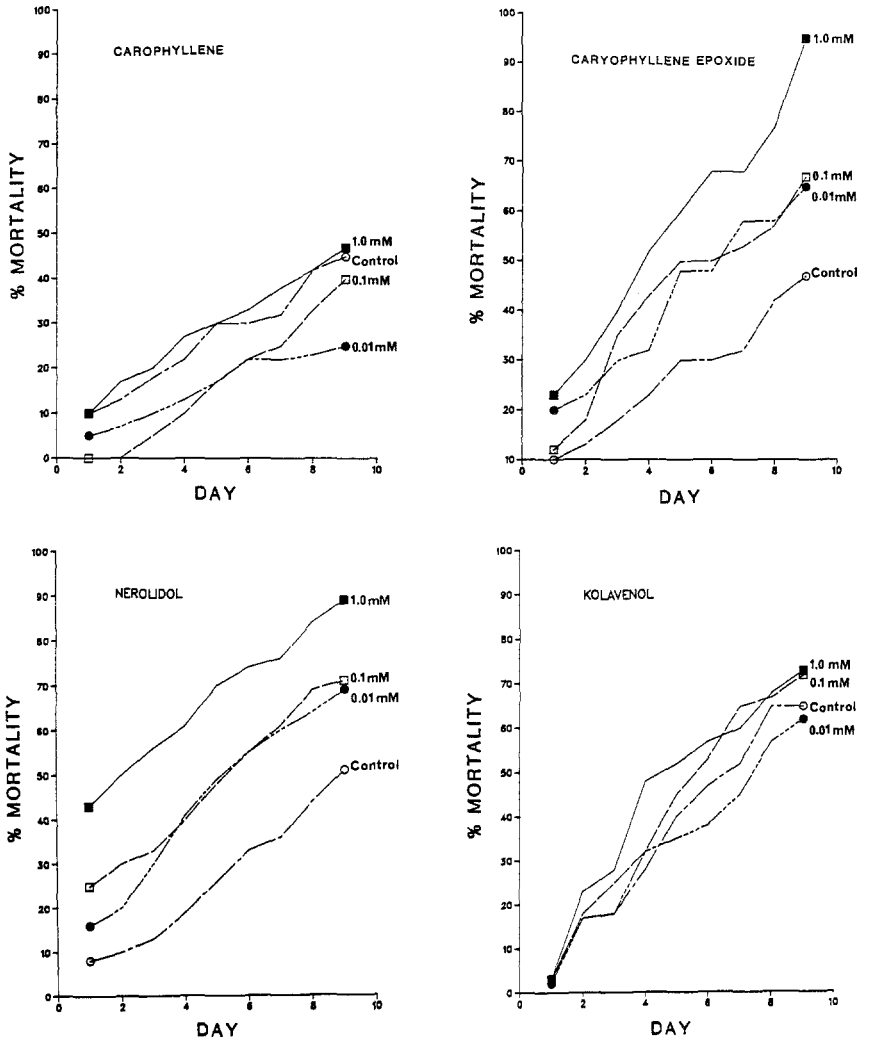


FIG 1. Ant Mortality on artificial diets containing terpenoid deterrents.

nerolidol, the high concentrations of caryophyllene are not completely soluble in the normal assay medium. Therefore a second experiment was conducted in which 2% ethanol was added to both caryophyllene test and control plates. Although caryophyllene is much more soluble in this modified medium, there was still no significant difference in toxicity between the caryophyllene and control experiments.

The decreases in ant survival produced by caryophyllene epoxide and nerolidol were not caused by simple feeding inhibition induced by these com-

TABLE 1. ANT DETERRENT ACTIVITY, ANT TOXICITY, AND FUNGISTATIC ACTIVITY OF FOUR TERPENOID NATURAL PRODUCTS ISOLATED FROM TROPICAL WOODY PLANTS

	Ant deterrency			Ant toxicity		Fungus inhibition	
Compound	mg/ml	C/T[a]	*P*	mM	Mean survival (days)[b]	mg/ml	7-day[c]
Caryophyllene	52.5	35/16	<0.01	1.0	7.5[a]	0.14	2+
				0.1	10.0[ab]	0.014	1+
				0.01	12.1[b]		
				0	9.9[ab]		
Caryophyllene epoxide	3.0	31/6	<0.001	1.0	3.9[a]	0.10	4+
				0.1	5.9[b]	0.01	1+
				0.01	6.9[bc]		
				0	9.9[c]		
Kolavenol	5.5	28/10	<0.005	1.0	5.6[a]	0.11	0
				0.1	6.9[a]	0.011	0
				0.001	7.8[a]		
				0	6.7[a]		
Nerolidol	2.4	29/1	<0.001	1.0	3.5[a]	0.14	4+
				0.1	6.1[ab]	0.014	2+
				0.001	6.6[ab]		
				0	8.7[b]		

[a] C/T is the ratio of control flakes to test flakes taken in a deterrency bioassay; at lower concentration caryophyllene is not significantly deterrent.

[b] Mean survival time in days is given for the 60 ants in each ant toxicity assay. Column entries followed by the same superscript letter are not significantly different (Tukey's HSD test, $P < 0.05$).

[c] Relative growth inhibition in the fungal bioassays was scored as follows: 4+ = no growth; 3+ = growth 25% that of controls; 2+ = growth 50% that of controls; 1+ = growth 75% that of controls; 0 = growth equal to that of controls).

pounds. Even with water freely available to prevent desiccation, all ants in a starvation treatment died within two days of the start of the bioassay. Ants given 1.0 mM concentrations of nerolidol and caryophyllene epoxide survived nearly twice as long. These results suggest that ants did ingest some amount of the liquid diet offered, even when this contained deterrent compounds. The effects of caryophyllene epoxide and nerolidol are thus due, at least in part, to direct intoxication of ants rather than simple starvation.

Similar patterns of variation were found in the effects of these compounds on fungal growth. Kolavenol had virtually no effect on fungal growth, but each of the other three compounds produced detectable inhibition of the fungus (Table 1). At the lower concentration tested, caryophyllene and caryophyllene

epoxide reduced fungal growth by about 25% and nerolidol reduced growth by about 50%. At the higher of the two concentrations tested, caryophyllene decreased growth of the ant fungus by as much as 50% compared to controls, and both nerolidol and caryophyllene epoxide completely inhibited ant-fungus growth.

DISCUSSION

The bioassays reported here represent relatively crude methods for determining the impact of plant natural products on leafcutter ants and their mutualistic fungus, measuring only dramatic effects (e.g., ant death or macroscopic evidence of fungal growth inhibition) produced by relatively short-term exposure. In addition, for bioassays on the ants, we used concentrations approximately an order of magnitude lower than those known for caryophyllene and caryophyllene epoxide from fresh leaves of *Hymenaea courbaril*. Nonetheless, three of the four deterrent compounds tested exhibited some degree of deleterious activity against either the leafcutting ants or their fungus. Caryophyllene had no detectable impact on the survival of adult ants but did decrease fungal growth. Both nerolidol and caryophyllene epoxide showed strong deleterious effects on both ant survival and fungal growth.

Among the three compounds that were active against ants and the fungus, there is a rough correlation between deterrency and the magnitude of toxic or inhibitory activity. Nerolidol is the most deterrent of these compounds, and caryophyllene epoxide is almost as deterrent, while caryophyllene is much less active (Table 1). Caryophyllene epoxide showed the strongest activity against adult ants, and nerolidol was also demonstrably active, but no significant effects were produced by caryophyllene on the ants. Nerolidol and caryophyllene epoxide had strong and approximately equal effects on fungal growth, while caryophyllene decreased fungus growth to a lesser extent. Therefore, the two most deterrent compounds had strong effects on ants and fungus, while the less-deterrent compound exhibited only a somewhat weak inhibitory effect on the fungus.

The results also show that all deterrent compounds do not have parallel effects on ants and fungus. Caryophyllene epoxide decreased survival of adult ants at all concentrations tested; it also strongly decreased fungal growth. In contrast, low levels of nerolidol did not significantly reduce survival of adult ants, but it did inhibit fungus growth more than did low levels of caryophyllene epoxide (50% reduction in growth vs. 25%, respectively). Caryophyllene decreased fungus growth but did not significantly affect ant survival.

Why should kolavenol be deterrent to leafcutting ants but show no activity against either the ants or their fungus? It may be that kolavenol is structurally

similar to a substance which does have such effects and evokes similar sensory responses by the ants. Alternatively, or in addition, it is possible that kolavenol does have some deleterious effect on either ants or fungus, either at higher concentrations or on some aspect of ant and/or fungus survival not readily measured by these bioassays. For example, it might be that kolavenol reduces the fecundity of ant queens, interferes with larval development of ants, or inhibits enzyme systems necessary for fungal growth on leaf material. Furthermore, the natural concentration of kolavenol in its source plants is not yet known, and the concentrations used in these experiments may be lower than those typically encountered by leafcutting ants and their fungus.

Overall, these results support the idea that deterrent terpenoids are deleterious to leafcutting ants and their mutualistic fungus. Furthermore, the degree of deterrency bears a rough relationship to the strength of toxic or inhibitory activity. However, deterrent compounds are not necessarily equally harmful to both ants and fungus. It has often been suggested that leafcutting ants prefer leaf material that provides the best substrate for fungal growth and that they avoid leaves containing compounds which inhibit the fungus. Leafcutting ants do discriminate against caryophyllene, which inhibits fungus growth, but both caryophyllene epoxide and nerolidol are harmful to ants as well. Although none of the compounds tested were dramatically more toxic to the ants than to their fungus, there appears to be no reason why such compounds should not exist.

The deleterious effects documented in this study provide significant insights into the consistent finding that plant lipids deter leafcutting ant attack (Littledyke and Cherrett, 1978; Mudd et al., 1978; Hubbell et al., 1984; Howard, 1987; Wiemer, 1985). The harvest of plant material containing these compounds, either for use as fungus substrate or as a source of ant nutrition, may have serious effects on the viability of both ants and fungus. In contrast, other classes of secondary compounds have little systematic effect on diet selection of leafcutting ants (Howard, 1987), and toxic or inhibitory effects of other plant secondary chemicals have yet to be demonstrated. Studies focusing on tannins, alkaloids, and plant aqueous extracts have established no clear relationship between fungal growth rates and ant preferences for plants or the deterrency of specific chemicals (Quinlan and Cherrett, 1978; Mudd and Bateman, 1978). Although tannic acid inhibits the growth of the fungus of the minor attine *Myrmecocrypta buenzlii* (Seaman, 1984), members of this genus are not generally considered to be "leafcutting ants" as they do not consistently use plant material for fungus garden substrate (Weber, 1946). We suggest that further studies of terpenoid and other lipid ant-deterrent compounds, and elucidation of the mechanisms by which they exert toxic effects, may illuminate many details of the ant–fungus symbiosis and may lead to new and valuable insights into natural mechanisms of plant resistance against attack by insects and fungi.

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EFFECTS OF ALLELOPATHIC COMPOUNDS OF CORN POLLEN ON RESPIRATION AND CELL DIVISION OF WATERMELON[1]

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Abstract—The effects of the allelopathic compounds of an "ethanolic extract" of corn pollen on growth, respiration, and cellular division of watermelon (*Citrullus lanatus*, var. peacock improved) were studied. Bioassays with the ethanolic extract showed an inhibition of radicle and hypocotyl growth. The effects on respiration were observed in isolated watermelon hypocotyl mitochondria. The ethanolic extract acts as an inhibitor of the electron pathway, decreasing oxygen consumption in state 3, with malate and succinate substrates. The specific inhibition site probably is located before the cytochrome *c*. Ascorbate-TMPD as substrate reversed the inhibitory effect of the ethanolic extract. An evaluation of the mitotic index was made in slide preparations of previously treated meristematic cells. A decrease in mitotic activity of more than 50% was found, as well as irregular and pycnotic nuclei. The data obtained from this study reflect an important mechanism of action of the allelopathic compounds of corn pollen.

Key Words—Allelopathy, corn pollen, respiration, cell division, watermelon.

INTRODUCTION

The allelopathic compounds released into the environment by a "producer plant" sometimes cause deleterious effects on other plants. These effects are revealed in growth, development and vigor, or a delay or absence of flowering

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and fruiting. All of these manifestations are a result of alterations in metabolic processes in the affected plant (Horsley, 1977).

Knowledge of the mode of action of allelopathic compounds is rudimentary. However, studies on this subject (Muller, 1965; Muller et al., 1968; Stenlid, 1970; Demos et al., 1975; McCahon, et al., 1973) have shown that allelochemicals can act on several sites within a plant cell, altering processes such as rate of ion uptake, synthesis of proteins, activation or breakdown of hormones, photosynthesis, energy metabolism, and cell division.

Allelopathic compounds can alter the energy metabolism in plant cells, by inhibiting or stimulating respiration. Both actions may be harmful to the energy-producing respiratory process (oxidative phosphorylation) and consequently to other metabolic processes (Horsley, 1977; Putnam, 1983; Rice, 1984).

In a study of the allelopathic potential of corn pollen, Jiménez et al. (1983) confirmed that corn pollen has allelopathic compounds that inhibit the growth of other plants. Peasants from Tabasco, Mexico, assert that fruiting of watermelon is reduced when corn pollen falls over it. This work was undertaken to evaluate the effects of corn pollen on growth, respiration, and cell division of watermelon (*Citrullus lanatus*).

METHODS AND MATERIALS

Bioassays with Corn Pollen and "Ethanolic Extract" of Pollen. Corn pollen was collected from mature male flowers of *Zea mays* L. (Chalquiñocónico, Hernández), in plots located at Mixquic, Mexico City. In an initial bioassay several amounts of pollen (50, 100, and 150 mg) were sprinkled on watermelon seeds (*Citrullus lanatus* (Thunb) Matsum and Nakai, var. peacock improved) in Petri dishes with filter paper as the substrate, and 5 ml of distilled water were added to each dish. The control contained only distilled water. Each treatment was replicated six times. Seeds were germinated at 27°C in the dark. After 168 hr, the radicle length was measured, and data were statistically analyzed as a randomized complete block design.

A second bioassay was carried out with an "ethanolic extract" obtained from corn pollen sonicated in ethanol for 10 min. This extract was filtered and evaporated to dryness in a Buchler flask evaporator under reduced pressure.

The study of Jiménez et al. (1983) shows the preliminary steps to isolate and identify the allelopathic compounds of corn pollen. The authors obtained hexane, ethyl acetate, methanol, and methylene chloride extracts, and also a white and a yellow product from the CH_2Cl_2–MeOH fraction. However, the organic solvents extracted only a small part of the active compounds, and the inhibitory effects of aqueous extract or of remaining pollen were greater than

those of the organic extracts or their products. Considering this result and in order to simplify the process, a new extract was obtained by sonicating pollen with ethanol for 10 min. The ethanolic extract showed a greater inhibitory effect on seeds than that of the methanolic and methylene chloride extracts or of remaining pollen. For this reason, in the present work the ethanolic extract was used for the bioassays; this extract gave a strong positive reaction to preliminary test with phenols.

Five amounts of the ethanolic extract (25, 50, 75, 100, and 125 mg) in a homogeneous suspension with distilled water were tested on the radicle and stem growth of watermelon seedlings. Hereafter, we will call this resuspended ethanolic extract, simply "ethanolic extract" when we refer to these bioassays. Bioassays were conducted in Petri dishes with a filter paper substrate and 11 seeds. The control contained only distilled water. The seeds were germinated under the same conditions as in the first bioassay, with five replicates per treatment. After 168 hr, the radicle and stem length of the seedlings were measured. The data obtained were analyzed using the analysis of variance in a randomized complete block design; $P < 0.01$ and $P < 0.05$ levels of probability were accepted as significant.

Preparation of Mitochondria. The effects of the treatments on cellular respiration were observed on mitochondria isolated from the hypocotyls of 10-day-old watermelon plants grown in the dark in vermiculite at 27°C and a relative humidity of 50%.

The hypocotyls were cut into 1- or 2-cm pieces and placed in a medium containing 0.25 M sucrose–1 mM ethylenediamine tetraacetic acid (EDTA) adjusted to pH 7.3 with Tris base. The tissue was disrupted for 10 sec with a Polytron blender, passed through gauze, and the filtrate was adjusted to pH 7.3 with Tris base. All procedures were conducted at 4°C. The filtrate and supernatant were centrifuged for 10 min at 3000 and 10,000 rpm, respectively. The pellet was resuspended in the same medium with 0.1% (w/v) bovine serum albumin (BSA) and combined to yield a final volume of 50 ml. Two 25-ml aliquots were centrifuged at 10,000 rpm for 10 min, and the pellet was resuspended in 0.5 ml of the medium using a Teflon-tipped tissue grinder. Mitochondrial protein content (mg/ml) was estimated by the method of Lowry et al. (1951) with BSA as the standard.

Oxygen consumption was measured polarographically, with a Clark-type electrode at 25°C. The data obtained were reported as nanoatoms of oxygen consumed per minute per milligram of mitochondrial protein, or as a percent of the control. The reaction medium contained 100 mM KCl, 10 mM phosphate-Tris buffer, pH 7.4, 0.1% w/v BSA, and either 10 mM malate, 10 mM glutamate, or 10 mM succinate. These substrates were adjusted to pH 7.4 with Tris base. At zero time, we added 2 mg of mitochondrial protein. The final volume

was 3 ml. The ethanolic extract used in these experiments with mitochondria was dissolved in ethanol (10 mg/ml); from this stock solution we took the volume necessary for the concentrations tested.

Preparation of Submitochondrial Particles. The mitochondria suspended in 0.25 M sucrose–1 mM EDTA, were adjusted to pH 8.6 with Tris base and subjected to a 60-sec sonication. The small number of unbroken mitochondria were removed by centrifugation at 10,000 rpm for 10 min. The supernatant containing the submitochondrial particles was then centrifuged at 40,000 rpm in an air-driven ultracentrifuge (Beckman, Fullerton, California, Airfuge). The pellet was resuspended in 1 ml of 0.25 M sucrose–1 mM EDTA, pH 7.4. The mitochondrial protein content was estimated using the technique of Lowry et al. (1951), with bovine serum albumin as the standard. We measured the rate of NADH oxidation with the Clark electrode.

Effects on Cell Division. The effects of corn pollen and of the ethanolic extract were observed on meristematic cells from the radicle of dark-grown watermelon seedlings. When the radicle length was 1.5 cm, we randomly chose two seedlings from each Petri dish and from each treatment. The seeds were germinated in the dark, in order to avoid effects of the circadian rhythm.

Root tips 2 mm long were hydrolyzed in 5 N HCl for 15 min, stained with acetorceine, and squashed in a drop of 45% (v/v) acetic acid. Permanent slides were made following the Conger and Fairchild technique (1953).

An evaluation of the mitotic index was carried out on 1000 randomly chosen cells per treatment. The mitotic index (MI) was calculated as the quotient of the total number of dividing cells (DC) with the total number of analyzed cells (AC), ($MI = DC/AC$). The data obtained were statistically analyzed through a test of differences of proportions, in which a Z value is calculated for each treatment vs. control and compared to the Z value of a normal distribution with $P < 0.01$.

RESULTS

Corn pollen had a significant inhibitory effect on the growth of watermelon radicles. Inhibition increased with the amount of pollen sprinkled over the seeds (Figures 1 and 2). Mean contrast test showed no significant difference between 100 and 150 mg of pollen; there was a significant difference between 50 and 150 mg. Fifty milligrams of pollen was enough to reduce radicle growth more than 50%. We also observed swelling and a grayish coloration at the neck base of the root.

The effects of the ethanolic extract of corn pollen on radicle and stem growth of watermelon seedlings are shown in Figures 3 and 4. Significant inhibition was observed with the ethanolic extract. Inhibition increased with in-

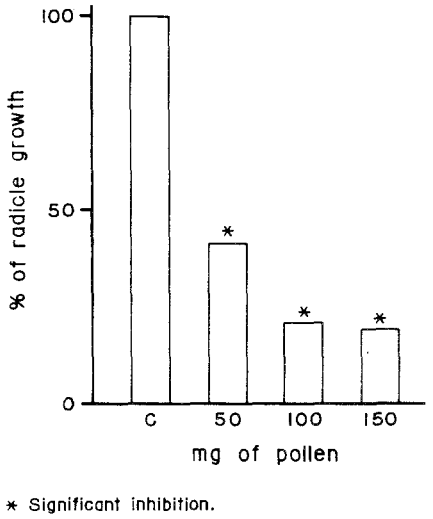

FIG. 1. Radicle growth of watermelon after treatment of seeds with corn pollen ($P < 0.001$).

creasing extract amount. Figure 3 shows that the inhibitory effects on stem growth are greater than those on the radicle. Seventy-five milligrams of extract caused more than 80% inhibition of stem growth.

Effects on Cellular Respiration. Figure 5 shows the traces of oxygen consumption by watermelon mitochondria. Figure 5A shows that ADP stimulated

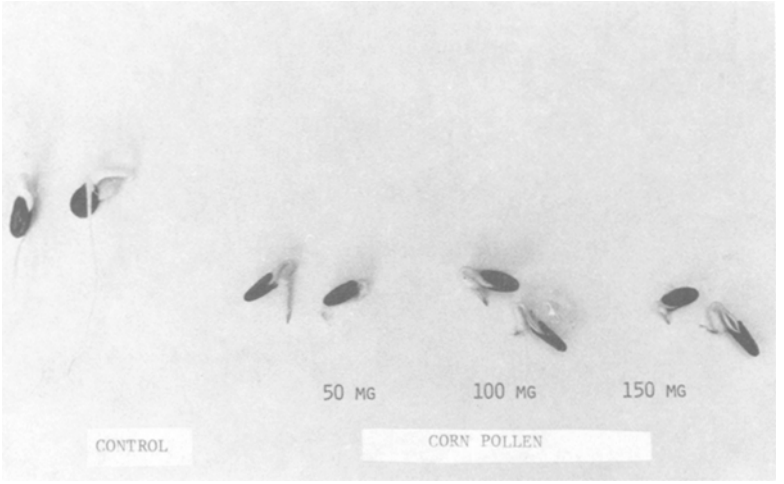

FIG. 2. Radicle growth of watermelon after treatment of seeds with corn pollen.

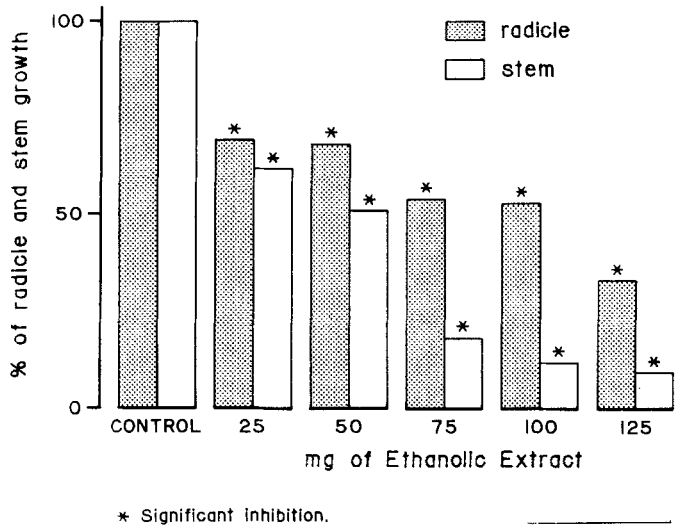

FIG. 3. Radicle and stem growth of watermelon seedlings treated with ethanolic extract (resuspended in water) of corn pollen ($P < 0.001$).

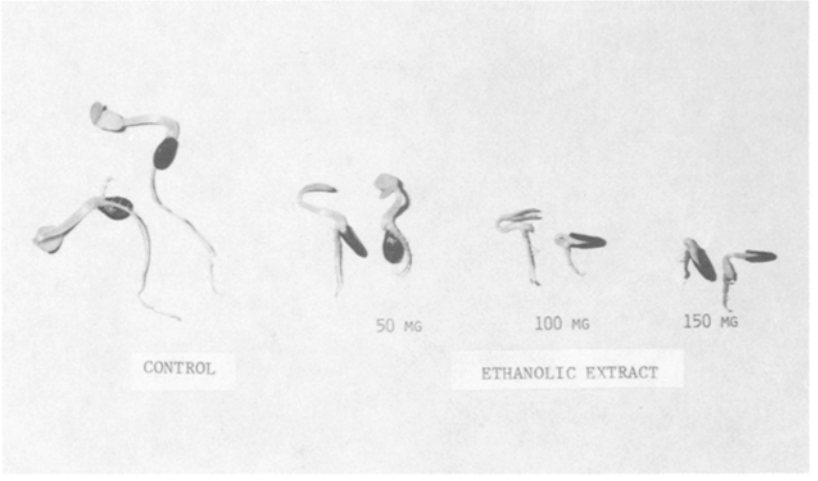

FIG. 4. Radicle and stem growth of watermelon seedlings treated with ethanolic extract (resuspended in water) of corn pollen. The 150-mg concentration is not discussed in this paper.

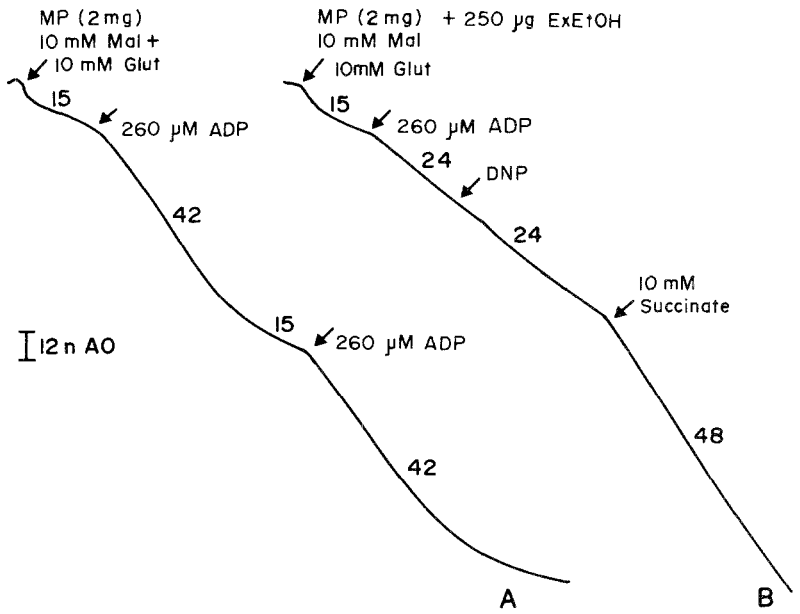

FIG. 5. Oxygen electrode traces: (A) Respiratory control (RC = 2.8) of malate–glutamate oxidation by watermelon mitochondria. (B) Effect of 250 μg of ethanolic extract of pollen added initially on state III of malate–glutamate oxidation. Numbers represent final concentrations in the reaction medium (final volume 3 ml). Numbers on traces refer to nanoatoms of oxygen consumed per minute per milligram of mitochondrial protein.

respiration (state III) with a respiratory control of 2.8 when malate–glutamate was the substrate. Figure 5B demonstrates that the initial addition of 250 μg of the ethanolic extract to mitochondria respiring with malate–glutamate results in a decrease of the state III respiratory rate which was not relieved by DNP. The ethanolic extract acted like an electron transport inhibitor, preventing stimulation of oxygen consumption by ADP and phosphorylation of ADP to ATP. The rate of respiration was the same before and after the addition of DNP. Also, when 10 mM of succinate was added, oxygen consumption was stimulated. This substrate is in the electron pathway at the level of the coenzyme Q (ubiquinone). Ethanol did not produce any effect on the respiratory rate of the mitochondria (data not shown).

Figure 6A and B illustrates the effects of the ethanolic extract on the respiratory rate of watermelon mitochondria with malate and glutamate as substrates. When the extract was added, during state III, the respiratory rate decreased.

Six hundred micrograms of the ethanolic extract added during state III of glutamate–malate oxidation also caused inhibition (Figure 7A). The rate of ox-

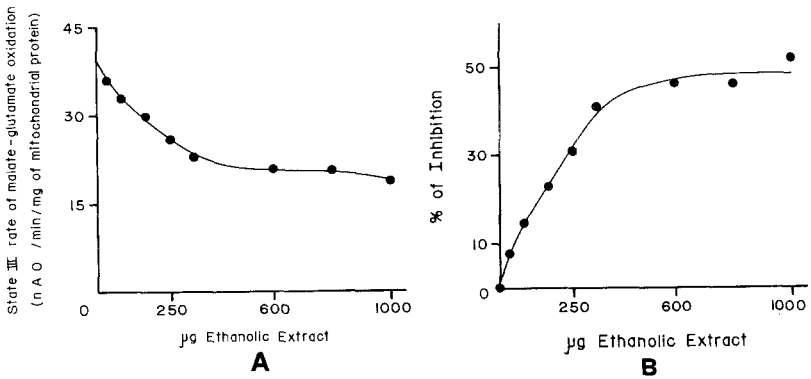

FIG. 6. (A) State III rate of malate-glutamate oxidation by watermelon mitochondria treated with ethanolic extract of corn pollen. (B) Percent of inhibition refers to control of state III rate of malate-glutamate oxidation with the different concentrations of ethanolic extract.

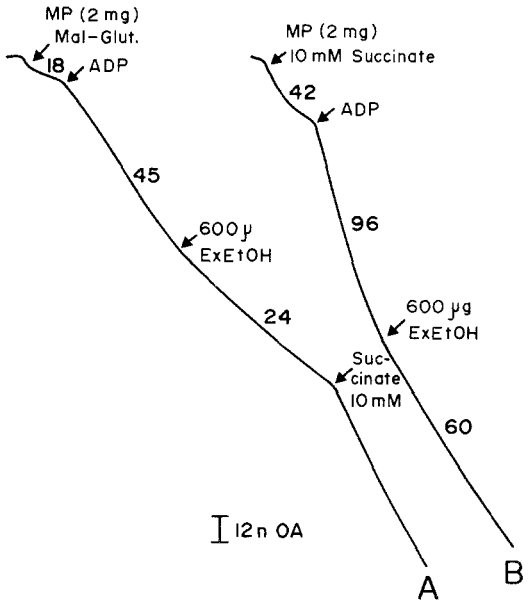

FIG. 7. Oxygen consumption traces illustrating the effect of ethanolic extract of pollen on state III rate of malate-glutamate (trace A) and succinate oxidation (trace B). Numbers given are final concentrations in reaction medium (final volume 3 ml). Numbers on traces are nanoatoms of oxygen consumed per minute per milligram of mitochondrial protein.

ygen consumption decreased from 45 nanoatoms O/min/mg to 24 nanoatoms O/min/mg. Figure 7B shows the rate of succinate oxidation. When 600 μg of the ethanolic extract were added during state III the respiratory rate decreased from 96 to 60 nanoatoms O/min/mg.

Figure 8A and B illustrates the effect of the ethanolic extract on state III succinate oxidation. The respiratory rate decreased with increasing concentration of the extract.

The inhibitory effect on succinate oxidation is smaller than that on malate–glutamate oxidation; however, at the higher concentrations of ethanolic extract, the inhibition was almost the same (Figures 6B and 8B).

In order to locate the probable site of inhibition of the ethanolic extract on the electron pathway, an experiment with menadione or vitamin K_3 (a quinone) was carried out. The oxygen electrode trace shown in Figure 9A and B illustrates that the addition of menadione to mitochondria respiring with malate–glutamate and 600 μg of the ethanolic extract did not neutralize the inhibition. So, we assume that the site of inhibition is not located at the level of coenzyme Q. However, ascorbate–TMPD reversed the inhibitory effect of the ethanolic extract (Figure 9C and D). This substrate releases electrons into the electron pathway at the level of cytochrome *c* facing the outer surface of the inner membrane. Therefore, the ethanolic extract of corn pollen probably inhibits respiration at a site located between coenzyme Q and cytochrome *c* in the electron pathway.

The ethanolic extract had no effect on the oxidation of NADH in submitochondrial particles, probably because it does not cross the inner membrane (Figure 10).

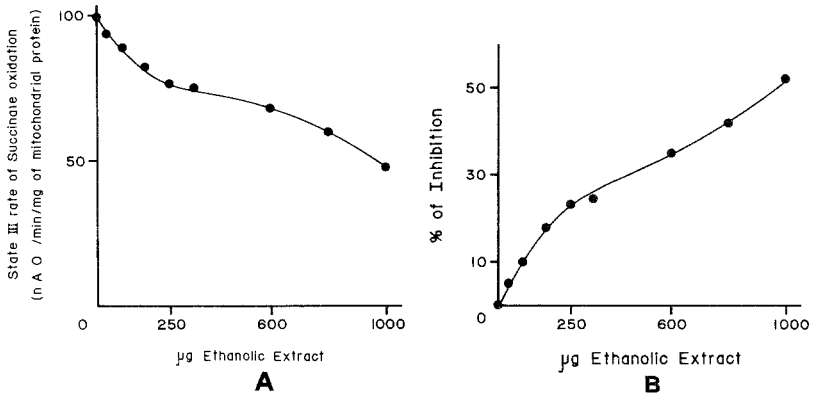

FIG. 8. (A) State III rate of succinate oxidation by watermelon mitochondria treated with ethanolic extract of corn pollen. (B) Percent of inhibition refers to control of state III rate of succinate oxidation with the different concentrations of ethanolic extract.

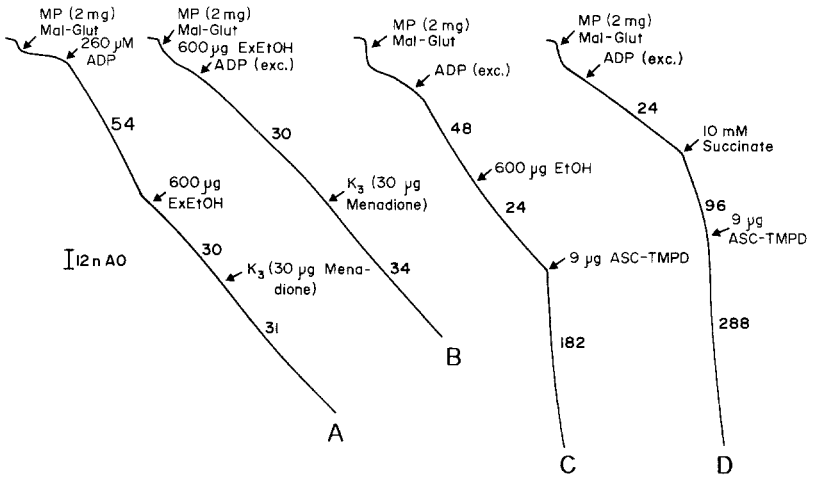

FIG. 9. Effect of ethanolic extract of pollen on state III rate of malate–glutamate, menadione (K_3), and ascorbate–TMPD oxidation by watermelon mitochondria. Numbers given are final concentrations in reaction medium (final volume 3 ml). Numbers on traces refer to nanoatoms of oxygen consumed per minute per milligram of mitochondrial protein.

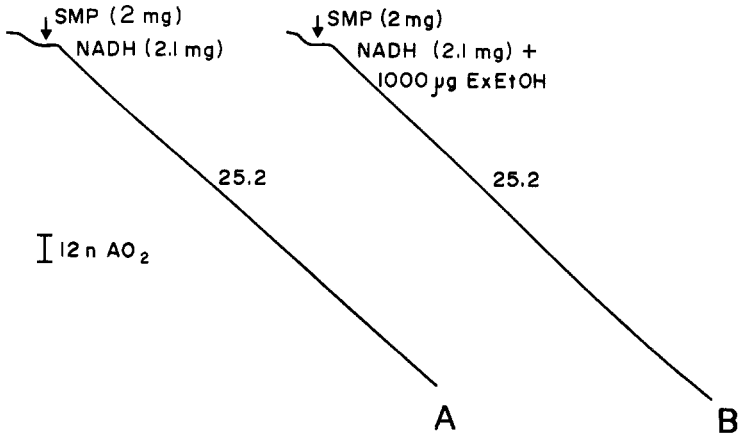

FIG. 10. Oxygen electrode traces illustrating the effect of ethanolic extract on NADH oxidation by watermelon submitochondrial particles. Trace A shows NADH oxidation without extract and trace B with the highest concentrations of ethanolic extract. Numbers given are final concentrations in reaction medium (final volume 3 ml). Numbers on traces refer to nanoatoms of oxygen consumed per minute per milligram of mitochondrial protein.

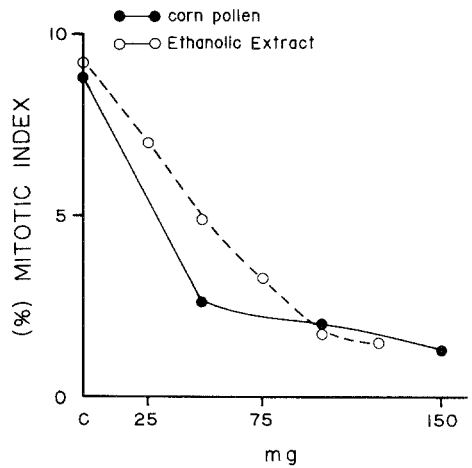

FIG. 11. Mitotic index (%) of meristematic cells of watermelon with corn pollen and with ethanolic extract ($P < 0.01$).

Effects on Cell Division. The mitotic index decreased as the amount of pollen and of ethanolic extract increased (Figure 11). The difference between the control and any of the treatments was significant ($P < 0.01$). But there was no difference among the three corn pollen treatments. Less than 50 mg of ethanolic extract did not produce significant differences ($P < 0.01$). We also observed that the meristematic tissue was destroyed in the treatment with corn pollen and that there were some pycnotic nuclei in the cells treated with the ethanolic extract (Figure 12).

DISCUSSION

The results presented in this study confirm that corn pollen has allelopathic compounds that inhibit plant growth, as Jiménez et al. (1983) reported. The inhibitory effects of the ethanolic extract on the radicle and stem growth of watermelon could be explained by the fact that respiration and energy production were severely inhibited. Inhibition of electron transport was demonstrated in the presence of the ethanolic extract.

Storey (1980) found that plant mitochondria oxidize succinate faster than malate. We confirmed this finding in our study; oxygen consumption at the lower concentrations of ethanolic extract was slower in malate–glutamate than in succinate. Apparently, the ethanolic extract needs a longer period of time to inhibit the specific site in the electron chain, so the inhibitory effect with succinate was not as strong as with malate–glutamate at the lower concentrations.

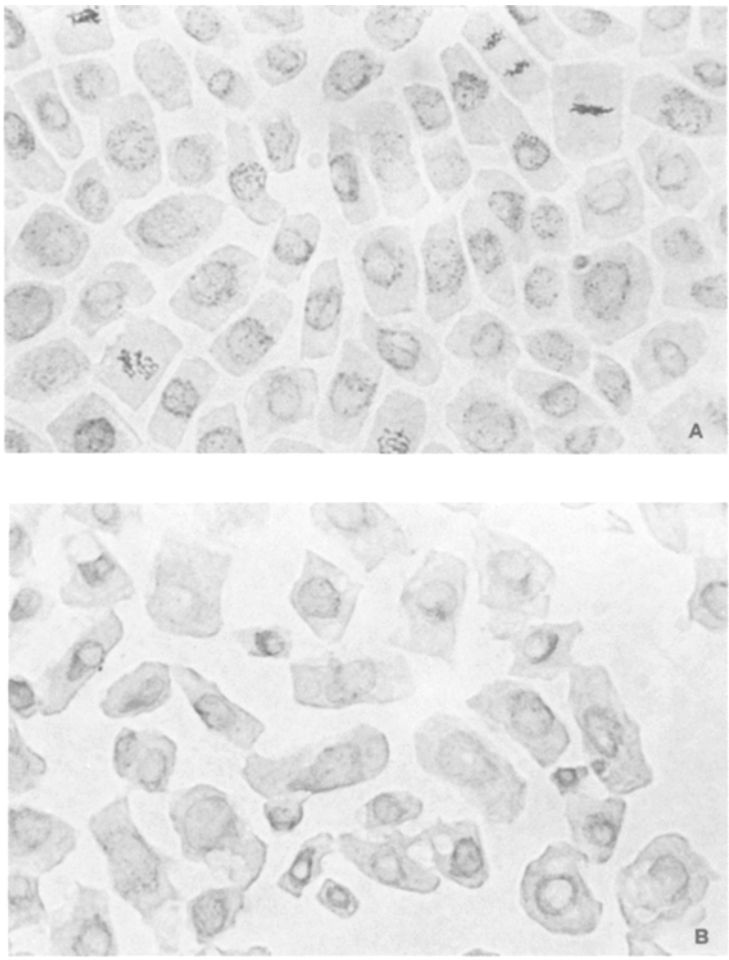

FIG. 12. Appearance of meristematic cells treated with pollen (B, C) and with ethanolic extract (D, E), arrows show the pycnotic nuclei. A is control.

Also, we know that succinate releases electrons at the ubiquinone level and that malate releases electrons at the NADH dehydrogenase level.

The site of action of the ethanolic extract probably is located between the cytochrome *b* and cytochrome *c*. The experiment with synthetic quinone (menadione) showed that the rate of oxygen consumption was not restored as might be expected if the ethanolic extract were acting at the ubiquinone level. However, ascorbate-TMPD reversed the inhibitory effect of the ethanolic extract.

It is well known that ascorbate–TMPD releases electrons at the cytochrome *c* level, which is facing the outer surface of the inner membrane. Douce et al.

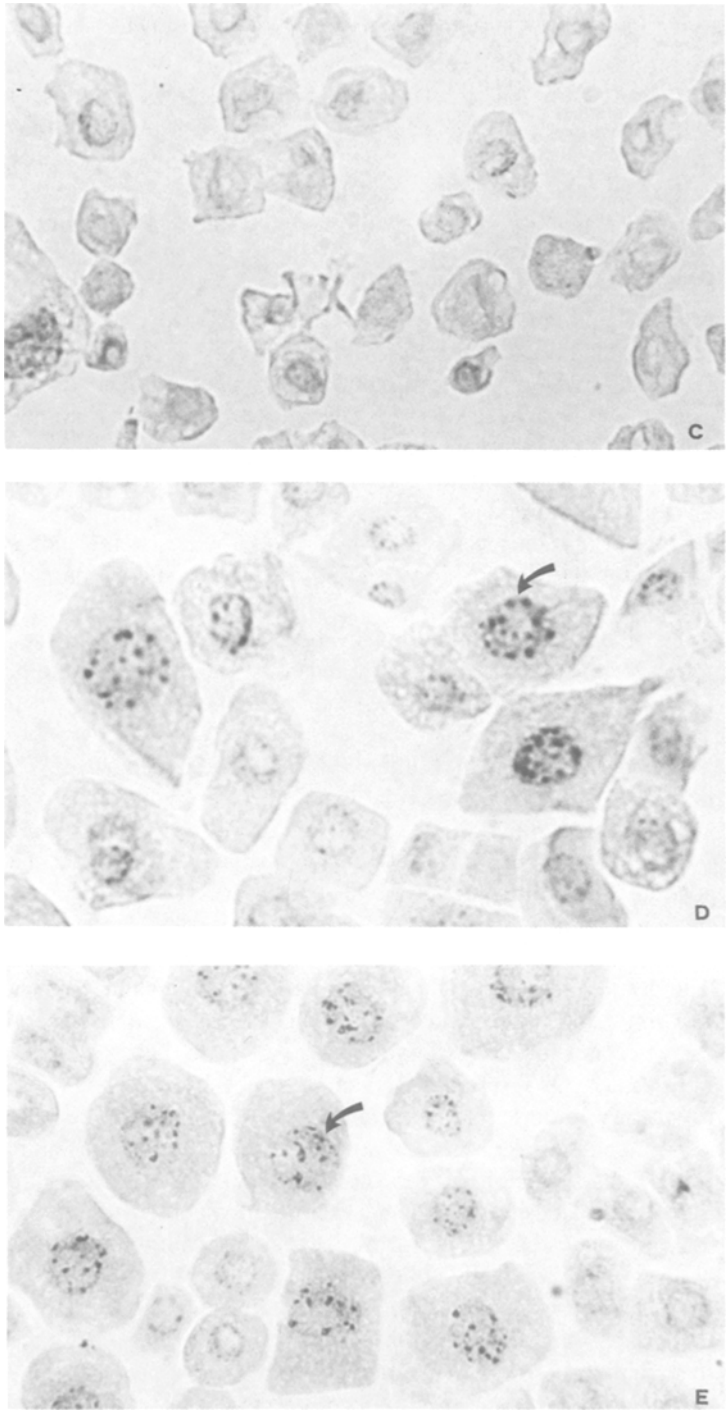

FIG. 12. Continued

(1978) found that β-pinene exerts a strong uncoupling effect at low concentrations, whereas it totally inhibits electron flow at higher concentrations. His work suggests that the inhibition site is probably before cytochrome *c*, at the quinone pool level.

The fact that the ethanolic extract had no effect on the submitochondrial particles suggests that it joins to cytochrome *c*, located inside the submitochondrial particles.

Another process that may be involved in the inhibitory effect of the ethanolic extract is membrane fluidity. Siedow et al. (1978) reported that a quinone analog of dibromothimoquinone decreased the fluidity of the inner mitochondrial membrane and suggested that the inhibition of mitochondrial electron transport might be associated with this effect. They also stated that their results support the concept of Moreland and Huber (1978) in that many lipophilic inhibitors of electron transport may act in a nonspecific fashion by decreasing membrane fluidity. Moreland and Huber (1978) ascribed the effects of some herbicides tested on mung bean mitochondria to a decrease in fluidity and an increase in membrane permeability, and not to a direct interaction with any of the electron-transport compounds.

On the other hand, if inhibition in the respiratory chain exists, oxygen consumption is not stimulated by ADP, and consequently there is no phosphorylation of ADP to ATP. Therefore the production of energy necessary for other processes is decreased.

Muller and his colleagues (Muller, 1965; Muller and Hague, 1967; Muller et al., 1968, 1969) found that the volatile monoterpenes, cineole and camphor, from *Salvia leucophylla* inhibit the growth of *Cucumis sativus* and *Avena fatua*. Oxygen uptake by mitochondria, cell division, and cell elongation in the radicle and hypocotyl were inhibited by these natural products.

Van Sumere et al. (1971) reported that some phenolic compounds, such as aldehydes, benzoic and cinamic acid derivatives, and coumarins reduced the ADP/O ratio and had an uncoupling effect in yeast mitochondria. They concluded that the inhibition of oxidative phosphorylation by these phenolic compounds may account for the regulatory activity of natural growth inhibitors.

Demos et al. (1975) tested the effect of 10 phenolic compounds on hypocotyl growth, respiration and oxidative phosphorylation, and ion transport by mung bean mitochondria (*Phaseolus aureus*). All compounds that inhibited energy metabolism also inhibited hypocotyl growth.

In the present study we also found that cell division is strongly inhibited. Kilham (1966) found that the chemical agents which inhibit cell division can act in two ways: they may affect the synthesis or the structure of DNA–RNA, or they may inhibit energy production necessary for the process of mitosis. Both processes are important for the cell division, and interferences with them generally cause inhibition of the whole process.

Cornman (1946) reported that aqueous solutions of coumarin and para-

scorbic acid block the mitosis in root cell of onion and lily, resulting in an accumulation of metaphases and binucleate cells.

We suggest that interference with respiration and cellular division are not the only physiological mechanisms of inhibition of watermelon seedling growth. There are many other processes that might be affected, including ion transport, membrane transport, and protein synthesis. However, the processes studied are very important for the development and early growth of seedlings. It has been observed (Nawa and Asahi, 1971; Solomos, et al., 1972; Sato and Asahi, 1975), that pea germination is accompanied by a marked increase in respiratory activity of the cotyledons and rapid development of cotyledon mitochondria. This early activity is important because the seedlings are more susceptible to environmental factors, which determine plant establishment and subsequent development. Nevertheless, allelopathic compounds also are active at later stages of growth and, for example, may delay or prevent physiological processes such as flowering or fruiting. Thus, study of the effects of corn pollen allelochemicals on the photophosphorylating chain of chloroplasts would be illuminating.

Our studies of the effects of the allelopathic compounds on respiration and cell division, combined with those of others (Muller et al., 1968, 1969; Koeppe and Miller, 1974; Demos et al., 1975; Van Sumere et al., 1971), allow us to conclude that the effects upon both processes represent an important allelopathic mode of action.

Acknowledgments—We wish to thank Dr. Edmundo Chávez from Instituto de Cardiología, Mexico City, for the laboratory facilities, advice, and revision of this paper; and Dra. Sandra Gómez from Centro de Ciencias de la Atmósfera de la U.N.A.M.

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PHEROMONAL COVARIATION AND KINSHIP IN SOCIAL BEE *Lasioglossum zephyrum* (Hymenoptera: Halictidae)

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Abstract—Both males and females of the primitively eusocial bee *Lasioglossum zephyrum* can distinguish among female conspecifics with regard to genealogical relationship. Closely related females covary with respect to Dufour's gland pheromone products which are believed to function in individual or kin recognition. This is the first report of a population-wide parallel between similarity of communicative glandular product and genetic similarity.

Key Words—Pheromone, correlation, kinship, Dufour's gland, social bee, *Lasioglossum zephyrum*, Hymenoptera, Halictidae, lactones.

INTRODUCTION

The importance of recognizing close kin in selecting mates and in social interactions has been shown experimentally in diverse vertebrate and arthropod taxa (Bateson, 1983; Fletcher and Michener, 1987; Getz et al., 1981; Getz and Smith, 1983; Greenberg, 1979; Hamilton, 1964; Smith, 1983) and is expected theoretically (Bateson, 1983; Maynard Smith, 1982). Applicable models assume that there is a set of heritable phenotypic recognition cues, probably chemical, called "discriminators," which the individual compares to its learned or innately programmed memory of the relevant phenotypic cues (Bateson, 1983; Crozier and Dix, 1979; Getz, 1982; Hölldobler and Michener, 1980; Lacy and Sherman, 1983). While generally accepted as likely, prior to this report there

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was no species in which variation in relevant glandular products was known to correlate with genetic variation.

Overwintered adult females of *Lasioglossum zephyrum* emerge in early spring and mostly build solitary nests into which the first worker brood, most likely full sisters, emerges in early June in Kansas (Batra, 1966). Both males and females discriminate among conspecific females according to genealogical relationship in laboratory studies. Males are preferentially attracted to females unrelated to those the males have previously encountered (Barrows, 1975; Smith, 1983). Females guarding nest entrances admit novel nonnestmate females more often when the entering female is closely related to the guarding female's nestmates (Greenberg, 1979), even when those nestmates are not related to the guarding female herself (Buckle and Greenberg, 1981). Therefore phenotypes used in comparison are learned in the adult stage from nestmates, which in nature are ordinarily relatives. The recognition cues of *L. zephyrum* consist of a pheromone produced by the female (Barrows, 1975; Barrows et al., 1975; Bell, 1974; Kukuk et al., 1977). Living in a nest of unrelated bees does not decrease likelihood of being accepted by a nest of one's kin (Buckle and Greenberg, 1981), and olfactory cues are adequate for males to recognize kin of familiar bees (Smith, 1983). Smith et al. (1985) identified 18 compounds extracted from female bees from three chemical classes: macrocyclic lactones, cuticular hydrocarbons, and isopentenyl esters. The first are products of the sting-associated Dufour's gland, which are used in nest construction and are characteristic for the subfamily Halictinae (Bergström and Tengö, 1979; Cane, 1983; Duffield et al., 1981). Mixtures of the synthetic lactones are attractive to males (Smith et al., 1985). The lactones probably function in sexual communication in *L. zephyrum*, and we believe in communication among female nestmates as well.

METHODS AND MATERIALS

Seven sites of closely aggregated nests of *L. zephyrum* were selected for study during June and July 1983. Aggregations of nests are located along the banks of rivers or streams. The sites sampled were separated by 3 m to 20 km and also were those which provided the background genetic variation for the laboratory breeding program of Greenberg (1979). For each site three to 11 females, mostly workers, were collected from each of several nests between June 30 and July 27 and promptly placed separately in cooled, clean glass vials. Immediately after being brought into the laboratory, the collected females were transferred individually into silanized glass tubes, each just large enough to accommodate an individual female, and kept in the dark for 3–5 hr. The tubes were then cooled to condense the pheromone, after which the females were

removed and the tubes washed with 100 μl of spectral grade hexane. This headspace sample from each female was sealed in a glass ampoule without fractionation or purification and stored at $-10°C$ to $-15°C$ until chemical analysis. The same females used for headspace samples were individually placed in small containers and frozen at $-50°C$ for later allozyme analysis. Detailed analysis of electrophoretic data will be reported elsewhere; only a summary statistic of relatedness calculated by the techniques of Pamilo (1984) is examined here for reference.

Seventy-five headspace samples were analyzed by capillary gas chromatography and later used in the statistical analysis. The samples were analyzed on a Hewlett-Packard 5092 capillary gas chromatograph fitted with a 25-m fused silica SE-30 column. Integration of peak area was performed by an HP 3390 integrator interfaced with the flame ionization detector of the GC. Helium was used as the carrier gas, and all runs were performed splitless for the first 30 sec, followed by a 100:1 split ratio until the end of the run. Temperature was held at 200°C for 5 min, then increased at 5°C/min to 225°C, and held until finished. One microgram of octacosane was coinjected as an internal standard with each sample to allow quantification of the absolute amount of each lactone peak.

All statistical analyses were performed on the untransformed data, using the amount of each lactone released by each bee per minute spent in the glass tube. Stepwise discriminant functions analysis indicated that one site represented by five females was markedly different from the others. If one includes significantly different populations in later analysis, nestmate pairs will, by definition, be more similar than random pairs since the former are always from the same population and the latter include pairs from any of the different populations. We pooled the other six sites and focused analysis on them to examine the type of variation in the largest grouping of individuals who do not differ greatly from each other. The reduced data set of 70 headspace samples was examined by principal components analysis (Dixon, 1981).

RESULTS

Chemical analysis identified octadecanolide, eicosanolide, docosanolide, docosenolide, and tetracosanolide as components of the headspace samples (Figure 1); there was considerable interindividual variation in both amounts and ratios. Minor components were too weakly and inconsistently present, using the headspace extraction technique, to be incorporated into the statistical analysis. Bartlett's sphericity test (Cooley and Lohnes, 1971) applied to Principal Components (PCs) III, IV, and V, indicates a significant deviation from sphericity (chi squared = 89.7, $df = 2$, $P < 0.001$) and justifies inclusion of PC III as an interpretable component.

Octadecanolide and eicosanolide production were highly correlated ($r =$

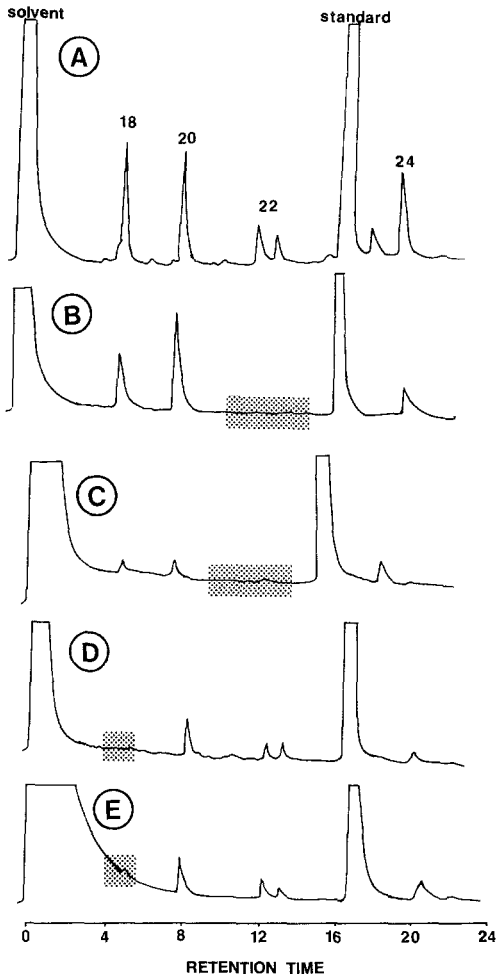

FIG. 1. (A) Capillary gas chromatogram of headspace samples from a female *L. zephyrum*. Peaks from left to right are octadecanolide, eicosanolide, docosanolide, docosenolide, octacosane (internal standard), and tetracosanolide. Numbers above the peaks indicate the numbers of carbons contained in the ring. For further details see Smith et al. (1985). The release rate and standard deviation (parentheses) for each of the lactones in picograms (10^{-12}g) per minute, based on the unreduced data set of 75 females, were as follows, respectively: 0.87 (2.83), 0.92 (9.62), 0.34 (7.37), 0.09 (0.28), 0.54 (0.97). (B and C) Individual chromatograms of a pair of nestmates, lacking 22-carbon lactones (shaded region). (D and E) Chromatograms of a pair of nestmates from a different nest, lacking the 18-carbon lactone (shaded). While the figured pairs of bees demonstrate a striking qualitative difference between family groups, most chromatograms differ largely quantitatively.

0.89, $P < 0.01$), as were both 22-carbon lactones ($r = 0.64$, $P < 0.01$) (Table 1). The first principal component (PC I) can be interpreted as general production rate, which explains 43% of the variance. Accounting for 32% of the variance, PC II appears to be a contrast between bees that produce a great deal of 18- and 20-carbon compounds with little 22-carbon compounds and those bees that produce the opposite mixture. PC III explains 18% of the total variance and appears to contrast 24-carbon lactone production with all others, but maintenance of orthogonality of interpretations is somewhat problematic. Orthogonality (independence of principal components) can be maintained by interpreting PC I as C_{18} and C_{20} production, PC II as C_{22} production, and PC III as C_{24} production. This is similar to the interpretation above, given the preponderance of C_{18} and C_{20} compounds which makes their measure about the same as a "general production" measure. The distinction between the two sets of interpretations is probably of no consequence. This leaves only 8% of the total variance explained by the two remaining PCs.

The 26 pairs of nestmate females are highly correlated with respect to their factor scores on PCs II and III, but not with respect to scores on PC I (Table 1). Neither random pairs of females nor nonnestmate pairs of females from the same aggregation were significantly correlated with respect to scores on any of the first three axes. The mean Pearson product-moment correlation coefficients for 40 sets of 25 randomly paired individuals with the number of significant coefficients in parentheses, were as follows: PC I: $r = -0.001$, (3); PC II: $r = 0.004$, (4); PC III: $r = -0.044$, (4). When 10 different sets of 25 nonnestmates from the same aggregation were paired, the results were as follows: PC

TABLE 1. PRINCIPAL COMPONENT LOADINGS FOR FIVE MACROCYCLIC LACTONES ON FIRST THREE COMPONENTS

	Factor Loadings		
	I	II	III
Octadecanolide	0.809	−0.497	−0.257
Eicosanolide	0.905	−0.345	−0.095
Docosanolide	0.566	0.705	−0.098
Docosenolide	0.310	0.845	−0.221
Tetracosanolide	0.483	0.110	0.866
Variance explained[a]	42.5%	31.5%	18.0%
Nestmate correlation[b]	0.05	0.47*	0.52**

[a] The first three components explain 92% of the total variation.

[b] Pearson product-moment correlation coefficients for the 26 pairs of nestmates included in the PCA. Coefficients for factors II and III are significantly different from zero (*$P < 0.025$; **$P < 0.01$).

I: $r = 0.072$, (0); PC II: $r = 0.14$, (3); PC III: $r = 0.05$, (3). None of the mean coefficients significantly differs from zero, which demonstrates that the correlations among nestmates are not due to either divergence of aggregation means or violations of normality through numerous zero values of production rates for individual lactones.

Allozyme variability allowed calculation of a regression coefficient of the relationship among nestmate females. In all populations of *L. zephyrum* sampled, the nestmates were highly related but were probably not always full sisters ($r = 0.63$–0.95 at $P < 0.05$ with a point estimate for the coefficient of relatedness of $r = 0.79$ v. $r = 0.75$ for full sisters).

DISCUSSION

Earlier laboratory studies demonstrated that both sexes of *L. zephyrum* discriminate among conspecific females by an unknown but presumably chemical mechanism which reveals relatedness between bees. Using passive chemical collection techniques with individual bees from the same wild populations as were examined in the laboratory, we have shown that the relative proportions of the macrocyclic lactones produced in Dufour's gland by the females vary less among nestmates (close relatives) than among random pairs of bees, even within aggregations. Although the observed pattern of chemical variance is expected of any trait for which phenotypic variation is explained largely by genotypic variation, such a relationship has not been previously documented in natural populations for chemical compounds known to play a role in communication.

Divergence of aggregation means does occur, as demonstrated by the distinction of one site in the discriminant functions analysis mentioned in Methods and Materials. This effect alone, however, is not adequate to explain the nestmate correlation, as demonstrated by Monte Carlo-type simulation pairing nonnestmates from the same aggregation, then examining many pairs from within all aggregations.

The similarities found here among nestmates, therefore, do not appear to be due to population structure beyond that of nests. The most likely explanation for similarities among nestmate females is that the composition of the pheromone is largely under genetic control.

The nonsignificant correlation between kin on the primary variance component (PC I) may be due to the effects of age or dominance by which some bees in each nest simply produce more pheromone than others. The correlation between kin on the lesser components (PCs II and III) suggests that regardless of how much pheromone they produce, nestmates are more similar than nonnestmates. The similarity is pronounced as PCs II and III account for half the

total variation. Whether the correlations between the 18- and 20-carbon lactones and between both 22-carbon lactones are due to genetic pleiotropy or another factor is unknown.

A genetic analysis of the same females used in the pheromone study supports the chemical results with regard to nestmate similarity. Allozyme frequencies showing nestmates to be highly correlated but less than full sisters are consistent with Kukuk's (1985) finding that as many as a third of all nests may have a multiply inseminated queen or multiple queens in a New York population. More detailed electrophoretic analysis of relatedness among Kansas bees is considered in another paper (Crozier et al., 1987).

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FACTORS INFLUENCING RELEASE OF HOST-MARKING PHEROMONE BY *Rhagoletis pomonella* FLIES

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Abstract—The effect of fly or fruit treatments on quality and/or quantity of host-marking pheromone (HMP) trail substance released by apple maggot flies (*Rhagoletis pomonella*) following oviposition was evaluated. Among flies, considerable variation existed in the amount of HMP substance deposited, but overall, the amount of substance released on successively offered fruit (over a day or a week) did not change appreciably. Fly diet did not influence pheromone activity. Older flies (28 days) or smaller flies released less or less active HMP trail substance than younger flies (14 days) or larger flies. Females deposited a similar amount of trail substance on large (18–19 mm diam.) or HMP-marked fruit as on small (12–13 mm) or unmarked fruit. Starvation reduced the amount of measurable trail substance deposited but resulted in a more active HMP deposition. Discrepancy between trail measurement and behavioral bioassay results for the starvation treatment indicated that trail measurement results may be misleading under conditions that reduce gut contents of the fly.

Key Words—*Rhagoletis pomonella*, Diptera, Tephritidae, apple maggot fly, oviposition deterrent, bioassay, host-marking pheromone.

INTRODUCTION

Studies of insect recruitment and sex pheromones as well as studies of host-marking pheromones (HMP) have identified numerous factors that influence pheromone release. Production of chemical recruitment trails by the ants *Acanthomyops interjectus* May and *Solenopsis geminata* F. (Hantgartner 1969a,b),

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as well as by eastern tent caterpillars [*Malacosoma americanum* (F.)] (Fitzgerald and Peterson, 1983) is influenced by individual assessment of food quality. These studies showed that ants produced less continuous recruitment trails and that tent caterpillars produced fewer trails following discovery of poor-quality food compared to high-quality feeding sites. Numerous studies have demonstrated that physiological (e.g., age, mating status) and environmental factors (e.g., temperature, light) influence sex pheromone release by moths (Sanders and Lucuik, 1972; Nordlund and Brady, 1974; Baker and Cardé, 1979; Bjostad et al., 1980), dermestid beetles (Hammack et al., 1976), and olive fruit flies (Mazomenos, 1984). Facultative HMP release has been demonstrated in the pheromone deposition behavior of the tephritid fruit fly, *Anastrepha fraterculus* Wiedemann, according to fruit size (Prokopy et al., 1982a), and Zimmerman (1980, 1982) demonstrated that *Hylemya* females can switch HMP release on and off, depending upon the host species being used and apparently in response to the probability of larval competition.

Immediately following egg-laying in a host fruit, a female apple maggot fly, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae), drags her extended ovipositor over the fruit surface and deposits a trail of HMP that deters subsequent females from further oviposition (Prokopy, 1972). Prokopy et al. (1982c) reported that HMP, following apparent production in midgut tissue, is released into the gut contents and accumulates in the hindgut. HMP is released with other gut contents during trail deposition.

We suspected that the behavior of pheromone deposition might represent a fixed action pattern that occurs without alteration (Alcock, 1979). However, whereas dragging the ovipositor following oviposition appears fixed and almost always occurs (Prokopy, 1972), initial lab and field observations revealed considerable variability in time spent dragging and in dragging bout pattern, not only among females, but also among successive dragging bouts by the same female.

To elucidate factors that may influence variability in pheromone release by *R. pomonella* females, we observed pheromone deposition behavior of flies in various behavioral or physiological states and on various fruit treatments. Through measurement of the amount of pheromone trail substance deposited, and through bioassay of female response to deposited pheromone, we evaluated the quantity and quality of pheromone released, usually by individual flies, after a single ovipositional bout. Factors investigated were fly age, fly size, fly diet, starvation, fruit size, and fruit quality.

METHODS AND MATERIALS

Collection and maintenance methods of *Rhagoletis pomonella* are detailed in Prokopy (1981). Unless otherwise stated, all flies were field collected as

larvae from apples and, at time of use, were mature (14–18 days old) and had no previous oviposition experience (naive).

To quantify the amount of trail substance deposited on a fruit, newly marked fruit were dusted with dry magnetic toner, a moisture-sensitive powder used in Olivetti copying machines. Fingerprint and talcum powders were ineffective. The pheromone substance is viscous and is typically a discrete, linear deposition (Figure 1). We measured trail length and area microscopically with an ocular micrometer. The toner technique is most effective for highlighting the HMP trail on very smooth and waxy fruit, such as *Crataegus oxyacantha* Autumn Glory, an introduced, ornamental species of hawthorn that we used in all trail measurement studies. Unfortunately, an insufficient supply of *C. oxyacantha* forced us to use a different species of hawthorn in behavioral bioassays. However, female acceptance of fruit and dragging behavior were equivalent for both hawthorn species.

In the trail measurement studies, a *C. oxyacantha* fruit was attached to a dissecting probe and offered to individually caged flies in a Plexiglas–screen observation cage (15 × 15 × 15 cm). Twenty minutes elapsed between each fruit presentation and, except where indicated, all fruit in a series were offered on the same day.

Time spent during HMP deposition and length and area of trails were quantified for the following categories: (1) *Variability among and within flies:* 14-

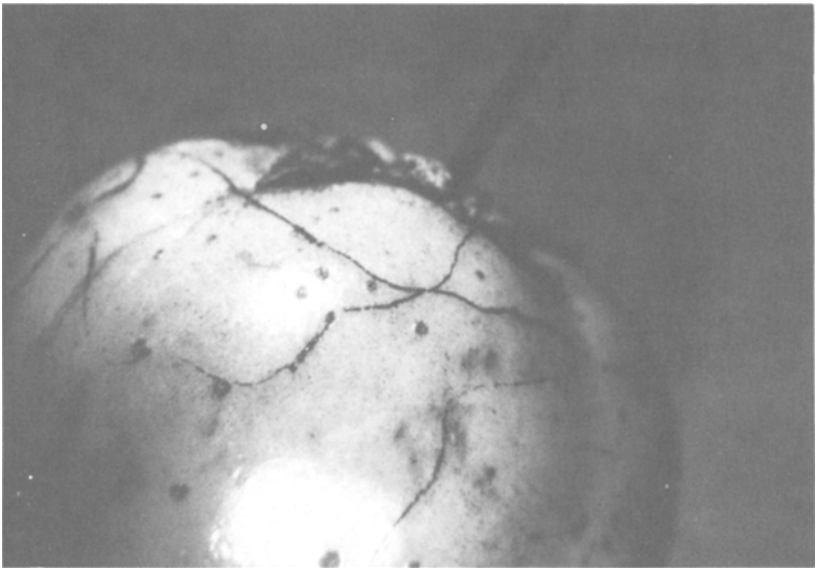

FIG. 1. A HMP trail deposited on a *Crataegus* hawthorn by a *Rhagoletis pomonella* female. The trail has been dusted with Xerox powder.

day-old flies were offered 12 successive 15-mm-diam. fruit. We examined variability among all flies by comparing mean time spent and mean amount of HMP deposited on the 12 fruit. We also considered variability among single bouts by the same fly. (2) *Fly experience over time:* to see if there was a change in deposition over time on the same day, we examined the above data and, for all flies, compared mean time spent and mean HMP released on each fruit (first through twelfth) in the presentation sequence. To see if there was a change in deposition over a week, females (which initially were 14 days old) were offered 12 successive 15-mm-diam. hawthorns on each of seven days. (3) *Fly age:* 14-, 21-, or 28-day-old females were offered 12 successive 14- to 15-mm-diam. hawthorns. (4) *24-hr starvation:* females provided water but no food for 24 hr and females with continuous access to both food and water were offered 12 successive 13-mm-diam. hawthorns. (5) *Fruit size:* females were offered a random series of hawthorns containing six 12- to 13-mm-diam. fruit and six 18 to 19-mm-diam. fruit. (6) *Pheromone-marked and unmarked fruit:* females were offered a random series of 13- to 14-mm-diam. hawthorns containing six clean, unmarked fruit and six pheromone-marked fruit. The pheromone-marked fruit were prepared as follows: pheromone was rinsed with a known volume of distilled water from hawthorns used for oviposition. The amount of pheromone was estimated by counting the number of oviposition punctures in each washed fruit: 1 puncture = 1 dragging bout equivalent (DE). A 20-DE aliquot was applied with a cotton swab onto the assay hawthorns. This amount was known to elicit moderate levels (ca. 47%) of fruit rejection by arriving females (Averill and Prokopy, unpublished data).

For all bioassays of pheromone activity, *Crataegus mollis* (Torr. and Gr.) Scheele (downy hawthorn) were used. Treated and control assay fruit were hung 6–8 cm apart from the ceiling of a Plexiglas–screen (30 × 30 × 30 cm) observation cage. A single mature *R. pomonella* female, which had just begun oviposition in a clean fruit attached to the end of a dissecting probe, was introduced into the assay cage by placing the probe near the cage floor. We allowed the female to fly to an assay fruit overhead and subsequently to visit assay fruit for a maximum of 2 hr. Because females in a physiological state conducive to oviposition accept clean fruit >60% of the time, females were excluded from tests if they rejected several (ca. 6) successive clean fruit. Acceptance (attempting oviposition before leaving) or rejection (leaving without attempting oviposition) was recorded for each visit to a fruit. Immediately following egg deposition, a female was gently transferred to a nonassay fruit, where she commenced and completed ovipositor dragging. In this way, assay fruit were minimally contaminated by pheromone deposited by assay females. For each test, at least 20 females were bioassayed.

The activity of pheromone produced by flies in several treatment categories was bioassayed. (1) *Fly age:* females (10–14, 20–23, or 28–30 days old) were

allowed to oviposit and drag on 15-mm-diam. hawthorns. Because of reduced fly availability, we could not use exactly-aged flies. A fruit marked by a fly from each age category plus two clean fruit were included in each bioassay. (2) *24-hr starvation:* females were starved as in the above starvation tests. During quantitative studies, we noticed that starved females tended to lay a maximum of only five to six eggs (mean $\pm$ SD number of eggs = 3.8 $\pm$ 2.1) when offered 12 successive fruit and tended to deposit more detectable trail substance during their first two dragging bouts than in subsequent bouts. Therefore, to test the effects of starvation on pheromone activity, we ran two bioassay series on 15-mm-diam. fruit. In the first, we collected trails produced during the first or second dragging bouts of starved females and bioassayed them in conjunction with first or second dragging bout trails of unstarved females. Three fruit marked by starved females, three fruit marked by unstarved females, and two clean control fruit were included in a bioassay. In the second series, we collected the third, fourth, or fifth dragging-bout trails of starved and unstarved females. (3) *Fly size:* large and small females selected from a same-age group of flies that originated from hawthorn were allowed to mark 15-mm-diam. *C. mollis* fruit. Four pheromone-marked fruit (two each marked by a small or large fly) plus two clean uninfested fruit were included in a bioassay. Following bioassay, flies were oven dried for 4 hr and weighed. Mean weight ($\pm$ SD) of small flies was 1.49 $\pm$ 0.16 mg and of large flies 3.48 $\pm$ 0.47 mg. (4) *Fly diet:* equal-number cohorts of flies that originated from apple were fed either standard laboratory diet that consisted of a mixture of enzymatic yeast hydrolysate and sugar (Prokopy and Boller, 1970) or aphid honeydew, an important natural food of the apple maggot (Neilson and Wood, 1966; Boush et al., 1969; Dean and Chapman, 1973). Branches of *C. mollis* hawthorn trees containing vigorous colonies of aphids (species unidentified) were collected every few days and held in large buckets in a greenhouse. Honeydew was collected on glass slides under the colonies. Because it was difficult to match the quantity of the two diets, a large excess of both diets was provided from the time of fly emergence until testing. When mature (14–17 days after emergence), individual flies from each group were allowed to oviposit and drag on 16-mm-diam. *C. mollis* fruit. Fruit marked during two dragging bouts were also prepared. Bioassays were run with two clean control fruit plus four marked fruit: two marked during one or two dragging bouts by flies on each diet.

Because several ongoing studies in our lab (e.g., pheromone identification, electrophysiology of pheromone reception) utilized extracts prepared from fruit washings, we ran an additional test wherein pheromone trails were collected (fruit washings) from both of the diet groups and reapplied to fruit as described above. Six, 12, or 23 DE of pheromone produced by flies on either diet were swabbed onto 16-mm-diam. *C. mollis* fruit (treatment). Bioassays were run with six treated fruit (two from each concentration) and two clean control fruit.

RESULTS AND DISCUSSION

Variability in Deposition of Trail Substance Within and Among Flies

Amounts of trail substance produced by 14-day-old flies offered 12 fruit in succession varied substantially, both among flies (Table 1) and among successive dragging bouts by the same fly (Table 2). In the extreme cases, fly 6 dragged for relatively short and consistent periods, but there was less consistency in the amount of substance deposited. Fly 3, on the other hand, exhibited longer (ca. 9×), less consistent dragging times and deposited substantially more (ca. 6×) quantifiable substance.

Variability in Deposition of Trail Substance Over Time

Successive Fruit Marked Over a Day. Mean times spent dragging and pheromone deposition for each of the 12 fruit offered successively to the above females are shown in Table 3. In general, naive flies tended to deposit shorter trails following their initial ovipositional bouts than after succeeding bouts, but this trend was not reflected in time spent dragging or trail area. In subsequent bouts, there was no consistent trend of change in any of the parameters. Thus, no rapid depletion occurs in the amount of quantifiable substance deposited when flies mark a succession of 12 fruit. Because some flies lay up to 30 eggs

TABLE 1. TIME SPENT DURING PHEROMONE DEPOSITION AND LENGTH AND AREA OF HMP TRAIL DEPOSITED BY 14-DAY-OLD *R. pomonella* FEMALES OFFERED 12 SUCCESSIVE *C. oxyacantha* FRUIT ON THE SAME DAY (VALUES ARE MEANS ± SD)[a]

Fly number	Time (sec)	Trail length (mm)	Trail area (mm^2)
1	20.6 ± 5.4ac	29.1 ± 21.8a	2.0 ± 2.2ab
2	37.5 ± 19.5b	42.0 ± 24.5ab	2.2 ± 1.4ab
3	127.1 ± 56.3e	118.9 ± 64.3c	5.9 ± 3.3c
4	29.2 ± 15.4bf	40.4 ± 19.2ad	1.8 ± 0.9b
5	67.3 ± 23.7d	53.9 ± 23.6be	2.5 ± 1.3ad
6	15.2 ± 1.9c	17.3 ± 12.0f	1.1 ± 1.1e
7	34.4 ± 6.3b	62.0 ± 27.5eg	3.1 ± 1.8df
8	28.5 ± 8.1b	33.2 ± 18.4a	2.0 ± 2.3ab
9	102.4 ± 36.6e	101.4 ± 25.5c	5.1 ± 3.7cg
10	23.3 ± 3.7af	49.2 ± 32.8bdg	2.4 ± 1.6abd
11	55.3 ± 22.2d	47.5 ± 26.0bdg	1.9 ± 1.3ab
12	30.2 ± 10.1b	69.2 ± 21.6e	3.8 ± 2.3fg

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls procedure.

TABLE 2. TIME SPENT DURING PHEROMONE DEPOSITION AND LENGTH AND AREA OF HMP DEPOSITED BY TWO 14-DAY-OLD *R. pomonella* FEMALES OFFERED 12 SUCCESSIVE *C. oxyacantha* FRUIT ON THE SAME DAY

	Fly 3			Fly 6		
Fruit sequence	Time (sec)	Trail length (mm)	Trail area (mm^2)	Time (sec)	Trail length (mm)	Trail area (mm^2)
1st	63	41.5	3.8	16	0	0
2nd	137	76.7	4.6	14	13.4	0.4
3rd	121	69.9	5.9	20	38.1	3.3
4th	52	48.8	7.3	14	29.2	2.0
5th	183	133.6	2.9	14	9.5	0.2
6th	113	46.6	2.6	15	8.0	0.2
7th	151	118.0	5.8	13	2.0	0.1
8th	59	169.0	7.1	15	36.6	2.2
9th	212	120.5	2.6	14	23.0	3.2
10th	167	264.0	13.1	16	20.5	1.1
11th	134	183.5	10.7	13	16.5	1.0
12th	—	155.0	4.9	13	11.1	0.9
$\bar{X}$	127.1	118.9	5.9	15.2	17.3	1.1
SD	56.3	64.3	3.3	1.9	12.0	1.1

TABLE 3. TIME SPENT DURING PHEROMONE DEPOSITION AND LENGTH AND AREA OF HMP TRAIL DEPOSITED BY 14-DAY-OLD *R. pomonella* FEMALES ($N = 12$) OFFERED 12 SUCCESSIVE *C. oxyacantha* FRUIT ON THE SAME DAY (VALUES ARE MEANS ± SD)[a]

Fruit sequence	Time (sec)	Trail length (mm)	Trail area (mm^2)
1st	28 ± 15a	24.1 ± 20.6a	1.9 ± 1.7abc
2nd	47 ± 35bc	35.7 ± 33.2ab	2.3 ± 2.0bd
3rd	42 ± 24bc	48.2 ± 25.7bc	2.7 ± 2.1cd
4th	31 ± 14a	34.4 ± 22.3a	2.5 ± 2.7bd
5th	48 ± 48bc	65.2 ± 31.2de	3.8 ± 2.5e
6th	44 ± 26bc	50.5 ± 23.1cd	2.6 ± 1.0d
7th	43 ± 39bc	56.7 ± 35.2cde	2.8 ± 1.7d
8th	47 ± 20b	66.5 ± 41.7de	2.8 ± 1.8d
9th	62 ± 59b	50.9 ± 33.4ce	1.7 ± 1.4ab
10th	56 ± 43b	78.3 ± 67.9e	3.9 ± 3.3e
11th	54 ± 25b	65.7 ± 50.5ce	3.6 ± 3.4e
12th	35 ± 19ac	52.1 ± 40.3ce	2.1 ± 1.2ad

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls procedure.

in one day, it is possible that pheromone depletion may be noted after such numerous dragging bouts. Under laboratory conditions, however, females lay an average of only about eight eggs per day over their lifetime (Averill and Prokopy, 1987).

Successive Fruit Marked Over a Week. Although mean time spent dragging per fruit was fairly consistent among days when flies marked fruit over seven consecutive days, there was some variability in number of fruit accepted for oviposition and amount of quantifiable trail substance deposited (Table 4). For no known reason, significantly fewer eggs were laid on day 3 than on any other days, significantly more trail material was deposited on day 4, and the least material was deposited on day 6. There were, however, no apparent trends from days 1 to 7 for any of the measures.

Thus, the experience of a fly over a day or week does not appreciably influence the amount of pheromonal trail substance released.

Fly Age

Although 28-day-old females spent equal time dragging, they deposited significantly less trail substance in shorter trails than either 14- or 21-day-old females (Table 5). Because the pheromone, along with other gut contents, is released onto the fruit during deposition of the trail, the difference in amount of trail deposition could be due to differential food intake of young vs. old flies. Webster et al. (1979) demonstrated that food (sucrose) intake is considerably greater in 2-week-old females than in 4-week-old females.

The decrease (ca. 40%) in deposition of trail substance with increase in

TABLE 4. DAILY NUMBER OF FRUIT ACCEPTED FOR OVIPOSITION, TIME SPENT DURING PHEROMONE DEPOSITION AND LENGTH AND AREA OF HMP TRAIL DEPOSITED BY *R. pomonella* FEMALES ($N = 11$), INITIALLY 14 DAYS OLD, OFFERED 12 SUCCESSIVE *C. oxyacantha* FRUITS ON EACH OF 7 CONSECUTIVE DAYS (VALUES ARE MEANS ± SD)[a]

Day	Number of fruit accepted	Time (sec)	Trail length (mm)	Trail area (mm^2)
1	10.8 ± 3.0a	27 ± 11ab	43.9 ± 23.1a	2.4 ± 2.2ab
2	8.9 ± 3.9a	29 ± 13a	46.1 ± 29.3a	2.1 ± 1.5a
3	4.6 ± 4.6b	22 ± 9b	43.4 ± 23.3a	2.2 ± 1.4a
4	9.5 ± 2.3a	26 ± 17ab	60.1 ± 39.9b	3.1 ± 2.4c
5	10.1 ± 2.1a	26 ± 16ab	43.3 ± 27.7a	2.8 ± 2.1bc
6	9.4 ± 3.5a	25 ± 11ab	19.6 ± 12.2c	1.2 ± 0.9d
7	10.1 ± 3.2a	27 ± 12ab	37.6 ± 28.3a	2.2 ± 1.9a

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls procedure.

TABLE 5. TIME SPENT DURING PHEROMONE DEPOSITION AND LENGTH AND AREA OF HMP TRAIL DEPOSITED BY DIFFERENT AGE *R. pomonella* FLIES OFFERED A SUCCESSION OF 12 *C. oxyacantha* FRUIT ON THE SAME DAY (VALUES ARE MEANS ± SD)[a]

Fly age (days)	*N*	Time (sec)	Trail length (mm)	Trail area (mm^2)
14	21	32 ± 20a	47.6 ± 26.9a	2.7 ± 2.0a
21	18	29 ± 19a	40.8 ± 23.1a	2.4 ± 2.1a
28	16	31 ± 24a	28.4 ± 23.6b	1.6 ± 1.1b

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls procedure.

fly age (Table 5) parallels results of behavioral bioassays of pheromone activity (Table 6), which show that fruit marked by 10- to 14-day-old females were significantly less acceptable for oviposition than fruit marked by 28- to 30-day-old flies. Fruit marked by 20- to 23-day-old females, which were marked with ca. 30% more trail substance than fruit marked by the 28- to 30-day-old flies (Table 5), were less acceptable for oviposition than fruit marked by the older flies, but this difference was not significant. Further, there was a statistically insignificant increase in acceptability of fruit marked by 20- to 23- vs. 10- to 14-day-old females.

Starvation

Twenty-four-hour starvation severely reduced the mean number of eggs laid as well as the amount of trail substance deposited (Table 7). When offered 12 successive hawthorns, starved females accepted significantly fewer than un-

TABLE 6. PERCENTAGE OF FEMALE *R. pomonella* ACCEPTING *C. mollis* FRUIT MARKED WITH HMP PRODUCED BY DIFFERENT AGE FEMALES ($N = 20$ FOR EACH AGE CATEGORY)[a]

Fruit treatment	Number of female arrivals	Fruit acceptance (%)
Marked by a 10 to 14-day-old female	83	20a
Marked by a 20 to 23-day-old female	105	30ab
Marked by a 28 to 30-day-old female	116	43b
Clean control	156	63c

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to a G test.

TABLE 7. NUMBER OF CLEAN FRUIT ACCEPTED FOR OVIPOSITION, TIME SPENT DURING PHEROMONE DEPOSITION, AND LENGTH AND AREA OF HMP TRAIL DEPOSITED BY STARVED (N = 18) AND UNSTARVED (N = 18) *R. pomonella* FLIES OFFERED 12 SUCCESSIVE *C. oxyacantha* FRUIT (VALUES ARE MEANS ± SD)[a]

Treatment	Number of fruit accepted	Time (sec)	Trail length (mm)	Trail area (mm^2)
Starved	3.8 ± 2.1a	18 ± 10a	10.4 ± 9.4a	0.5 ± 0.6a
Unstarved	10.7 ± 2.6b	17 ± 10a	32.6 ± 20.1b	2.5 ± 1.8b

[a]Values in the same column followed by the same letter are not significantly different at the 1% level according to a t test.

starved females. Although the two groups spent approximately equal times dragging following oviposition, starved flies deposited significantly shorter and smaller area trails. Additionally, unlike successive trails produced by unstarved females, the amount of trail substance, y, deposited by starved females following successive ovipositional bouts, x, decreased rapidly ($y = 23.7 - 5.7x$). Following a fly's two initial dragging bouts, the majority of subsequent trails were very fine and barely perceptible. Dissections of females subjected to 24 hr of food deprivation revealed considerable gut content depletion, which may explain the above observation.

Behavioral bioassays of the pheromone produced by starved and unstarved flies showed poor correspondence to trail measurement results. In the first bioassay series, where we collected pheromone deposited following the first two ovipositional bouts, there was no significant difference in percent acceptance of fruit marked with pheromone drags of starved flies (N = 15) vs. unstarved flies (N = 17) (Table 8), even though our earlier measurements of the trails indicated that starved flies deposited only about half as much trail substance as unstarved flies during these dragging bouts. In the second bioassay series, using pheromone deposited following the third, fourth, or fifth ovipositional bouts, the fruit marked by starved females (N = 22) were significantly less acceptable than fruit marked by unstarved females (N = 19) (Table 8). This is a surprising result because quantitatively, starved flies deposited only ca. $\frac{1}{10}$ as much trail substance during these dragging bouts as unstarved flies. It is possible that by reducing gut contents, the effect of starvation may have been to concentrate the pheromone, resulting in a less dilute, more deterrent deposit that was not assessable using our quantification technique. Starvation causes oocyte resorption in many Diptera (Chapman, 1969), and, if such were the case in *R. pomonella*, it would be advantageous for a starving female to produce a highly deterrent pheromone deposition and, thus, maximally protect each of her few remaining eggs.

TABLE 8. PERCENTAGE OF FEMALE *R. pomonella* ACCEPTING FRUIT MARKED WITH HMP PRODUCED BY STARVED OR UNSTARVED FEMALES FOLLOWING FIRST–SECOND OR THIRD–FIFTH OVIPOSITIONAL BOUTS[a]

	Bouts 1–2		Bouts 3–5	
Fruit treatment	Number of female arrivals	Fruit acceptance (%)	Number of female arrivals	Fruit acceptance (%)
Marked by a starved female	52	50a	127	18a
Marked by an unstarved female	64	45a	138	29b
Clean control	28	75b	96	66c

[a]Values in the same column followed by the same letter are not significantly different at the 5% level according to a G test.

This lack of correspondence between quantitative trail measurements and results of the behavioral bioassays demonstrates that for any fly treatment that reduces gut contents, evaluation of *R. pomonella* pheromone release using a trail measuring technique may be misleading. Therefore, trail measurement must be used in conjunction with other techniques or, owing to labor intensity, be eliminated altogether.

Fly Size

Small females deposited pheromone of either decreased quality or quantity as compared to larger conspecifics (Table 9). A significantly greater proportion of female visits resulted in acceptance of fruit marked once by small females as compared to fruit marked once by large females. This occurred in spite of

TABLE 9. PERCENTAGE OF FEMALE *R. pomonella* ACCEPTING *C. mollis* FRUIT MARKED DURING SINGLE DRAGGING BOUT WITH HMP PRODUCED BY LARGE (N = 24) OR SMALL (N = 23) FEMALE[a]

Fruit treatment	Number of female arrivals	Fruit acceptance (%)
Marked by a large female	167	31a
Marked by a small female	123	50b
Clean control	135	65c

[a]Values in the same column followed by the same letter are not significantly different at the 5% level according to a G test.

the fact that small females spent approximately the same time marking fruit (31 sec) and completed the same number of dragging circles (2.4) per fruit as did large females, which spent 33 sec and completed 2.5 circles. (A dragging circle is a distance dragged by a female that approximates the circumference of the fruit and is estimated by eye.) Because reduction in adult size may result from intraspecific larval competition in small hawthorn hosts (Averill and Prokopy, 1987), these results suggest an intriguing effect of overcrowding on subsequent adult fitness: a small female's decreased ability to pheromonally protect oviposition sites may lead to additional infestation by that same female or subsequently arriving females. As a result, her progeny may more likely suffer reduced larval survivorship or stunted development (Averill and Prokopy, 1987).

Quiring and McNeil (1984) have likewise demonstrated that small female alfalfa blotch leafminers, *Agromyza frontella* (Rondani), produce a HMP that is less effective than that produced by large females.

Fly Diet

Females fed either the laboratory diet or honeydew produced equally active pheromone trails (Table 10). Bioassays wherein aqueous extracts of HMP deposited by flies fed honeydew or laboratory diet were applied to fruit revealed no statistical differences between pairs of any of the concentrations tested (Table 10).

Evaluation of the effect of diet on HMP production was necessary because gut contents appear to comprise most of the material released in the HMP deposition (Prokopy et al., 1982c) and because of the debate on possible effects of diet and host substances on pheromone production (e.g., Hardee, 1970; Hendry, 1976; Miller et al., 1976; Byers, 1983; Wiygul and Wright, 1983.)

Fruit Size

When offered a series of small and large hawthorns, flies spent a significantly longer time marking large vs. small fruit. Flies deposited trails that were of greater length and area on large vs. small fruit, but these differences were not statistically significant (Table 11).

In another study, pheromone-depositing females observed in the lab and field dragged their ovipositors for a significantly longer time and distance on large (20 mm diam.) vs. small (12 mm diam.) hawthorns (Averill and Prokopy, 1987). This difference was more pronounced (ca. 40% greater) in the test series reported in Averill and Prokopy (1987) than in the present study. This may be due to sampling error, although a large number of observations was made, or because the size difference between offered fruit was slightly greater in the former test series. Alternatively, the difference might be due to apple origin flies being used in the present test series vs. hawthorn origin flies in Averill and

TABLE 10. PERCENTAGE OF FEMALE *R. pomonella* ACCEPTING *C. mollis* FRUIT (TEST A) MARKED ONCE OR TWICE WITH HMP PRODUCED BY FEMALES FED LABORATORY OR HONEYDEW DIET OR (TEST B) SWABBED WITH AQUEOUS SOLUTION OF 6, 12, OR 23 DRAGGING BOUT EQUIVALENTS (DE) OF PHEROMONE COLLECTED FROM FLIES FED EITHER DIET[a]

Fruit treatment	Number of female arrivals	Fruit acceptance (%)
Test A		
Marked once by a female fed honeydew diet	128	43a
Marked once by a female fed laboratory diet	136	42a
Marked twice by a female fed honeydew diet	137	32b
Marked twice by a female fed laboratory diet	132	26b
Clean control	129	74c
Test B		
Swabbed with pheromone extract from females fed honeydew diet		
6DE	48	40a
12 DE	45	24a
23 DE	67	22a
Swabbed with pheromone extract from females fed laboratory diet		
6 DE	45	31a
12 DE	60	38 a
23 DE	53	25a
Clean control	88	97b

[a]In test A, values followed by the same letter, and in test B, acceptance values of similar DE treatments of either diet followed by the same letter (e.g., 6 DE of extract from females fed honeydew diet vs. 6 DE of extract from females fed lab. diet), are not significantly different at the 5% level according to a G test.

Prokopy (1987). Recent work by Prokopy et al. (1982b) examining comparative behavioral traits suggests that there may be substantial *R. pomonella* host race differences. It is conceivable that selective pressure for "fine-tuned," flexible dragging behavior may be relaxed in populations developing in apple where the larval carrying capacity may exceed 15 or more per fruit (Prokopy, 1972; Cameron and Morrison, 1974) and the amount of pheromone deposited by a single

TABLE 11. TIME SPENT DURING PHEROMONE DEPOSITION, AND LENGTH AND AREA OF HMP TRAIL SUBSTANCE PRODUCED BY *R. pomonella* FLIES ($N = 18$) WHEN DRAGGING ON LARGE (18–19 mm DIAM.) OR SMALL (12–13 mm DIAM.) *C. oxyacantha* FRUIT (VALUES ARE MEANS ± SD)[a]

Fruit size	Time (sec)	Trail length (mm)	Trail area (mm^2)
Small	17 ± 10a	38.9 ± 25.3a	2.5 ± 1.8a
Large	22 ± 12b	45.9 ± 29.8a	2.9 ± 1.9a

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to a *t* test.

female does not much influence subsequently arriving females (Prokopy, 1972). In contrast, flexible dragging behavior may be adaptively advantageous for populations on hawthorn because: (1) larvae developing in multiple-infested fruit may realize lowered survivorship, (2) the amount of pheromone deposited following a single ovipositional bout is sufficient to deter most females from further egg-laying, and (3) more pheromone is necessary to elicit female deterrence on large (20 mm diam.) fruit vs. small (12 mm diam.) fruit (Averill and Prokopy, 1987).

Pheromone-Marked and Unmarked Fruit

If the amount of pheromone deposited is a flexible trait, flies might deposit less pheromone on fruit that were already pheromone-marked. However, Table 12 shows that females deposited essentially the same amount of trail substance per fruit when offered a series of pheromone-marked or unmarked fruit. Al-

TABLE 12. TIME SPENT DURING PHEROMONE DEPOSITION, AND LENGTH AND AREA OF HMP TRAIL SUBSTANCE PRODUCED BY *R. pomonella* FLIES ($N = 20$) WHEN DRAGGING ON PHEROMONE-MARKED OR CLEAN *C. oxyacantha* FRUIT (VALUES ARE MEANS ± SD)[a]

Fruit treatment	Time (sec)	Trail length (mm)	Trail area (mm^2)
Pheromone-marked	21 ± 14a	30.6 ± 3.6a	1.5 ± 1.4a
Clean	33 ± 21b	23.1 ± 18.3a	1.3 ± 1.4a

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to a *t* test. Pheromone-marked fruits were prepared by applying an aqueous solution of HMP.

though flies deposited a similar quantity of trail substance on both fruit treatments, they spent significantly less time engaged in trail deposition on pheromone-marked vs. unmarked fruit. Females moved more quickly over the fruit surface during preoviposition behavior and dragging.

SUMMARY

When flies were starved, we found that trail substance measurements were not consistent with behavioral bioassay results of pheromone activity, probably because HMP is released along with other gut contents during trail deposition and gut contents appear to comprise most of the released material. Thus, trail measurement results may be a poor indicator of HMP release and must be evaluated in light of behavioral bioassay results.

Overall, our results suggest that numerous factors may affect the quantity or quality of pheromone released not only by different *R. pomonella* females, but also from one dragging bout to the next by the same female. Of the several factors examined, changes in fly quality (i.e., fly age and size) and starvation produced the greatest differences in pheromone deposition, while changes in fly experience, fly diet, or fruit characteristics (size or presence of pheromone mark) produced less pronounced or no differences.

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EFFECT OF VERBENONE ON RESPONSE OF *Dendroctonus brevicomis*[1] TO *exo*-BREVICOMIN, FRONTALIN, AND MYRCENE

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Abstract—*exo*-Brevicomin (E), frontalin (F), and myrcene (M) were released at two rates 10-fold apart with verbenone at four rates 10-fold apart, and without verbenone, in plots with one trap on a vertical cylinder at the pheromone source and one trap on each of four cylinders 5 m away. Catch of the western pine beetle *Dendroctonus brevicomis* decreased with increasing levels of verbenone at both release rates of EFM, but not all differences in catch were statistically significant. Significantly more beetles were caught at the high rate of EFM than at the low rate, combining all rates of verbenone. The percent of total beetles caught at the center trap tended to decrease with increasing rates of verbenone, but the only statistically significant differences were at the low rate of EFM.

Key Words—*Dendroctonus brevicomis*, Coleoptera, Scolytidae, bark beetle, western pine beetle, pheromone, attractant, inhibitor, *exo*-brevicomin, frontalin, myrcene, verbenone.

INTRODUCTION

The colonization of ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., by the western pine beetle, *Dendroctonus brevicomis* LeConte, is mediated by volatile compounds released by the beetles when they bore into the tree (Wood, 1972; Wood and Bedard, 1977). The mixture of *exo*-brevicomin (E) (Silverstein et al., 1968), frontalin (F) (Kinzer et al., 1969), and myrcene (M) (Bedard et al., 1969) was attractive to flying *D. brevicomis* (Bedard et al., 1980a; Wood et

[1] Coleoptera: Scolytidae.

0098-0331/88/0100-0113$06.00/0

al., 1976). The numbers of *D. brevicomis* trapped at and near a source of EFM varied with the relative and absolute release rates of the attractant (Tilden et al., 1983; Tilden and Bedard, 1985). Verbenone (Renwick, 1967) reduced the numbers of *D. brevicomis* trapped at a source of EFM and the natural attractant, and the reduction in catch was greater at a high release rate of verbenone than at a low release rate (Bedard et al., 1980a,b). All four compounds have been isolated from the air around trees under attack by *D. brevicomis* (Browne et al., 1979). Verbenone may be involved in termination of mass attack (Renwick and Vité, 1970) or in regulation of attack density (Byers and Wood, 1980; Byers et al., 1984). Although the roles of verbenone in host colonization are not clear, it may have value as a tree protectant, because it interrupted the response of *D. brevicomis* to bolts cut from a tree under mass attack and appeared to reduce visitation and boring in trees baited with EFM (Bedard et al., 1980b). Verbenone applied to a living tree may prevent initial attacks or attenuate the attack rate so that tree resistance could overcome initial attacks and prevent mass attack and subsequent death of the tree.

Verbenone and other pheromones act as antiattractants for other bark beetles and show promise as agents to prevent attacks or reduce infestations in host trees. A 1 : 1 mixture of verbenone plus *exo-* and *endo*-brevicomin reduced landing of *D. frontalis* Zimm. on previously unattacked trees (Richerson and Payne, 1979), and reduced the density of emerging beetles (Watterson et al., 1982), although trees were successfully attacked. Verbenone reduced the number of *D. adjunctus* Blandford trapped at attractive bolts cut from ponderosa pines attacked by the beetle (Livingston et al., 1983). Verbenone inhibited the response of *Ips paraconfusus* Lanier to natural and synthetic sources of attractant (Byers and Wood, 1980, 1981). MCH (3-methyl-2-cyclohexen-1-one), an antiattractant for *D. pseudotsugae* Hopkins (Rudinsky et al., 1972), reduced attack and brood density in windthrown trees when applied by helicopter in a controlled-release formulation (Furniss et al., 1981; McGregor et al., 1984).

This study is to provide information about the response of *D. brevicomis* to different release rates of EFM and verbenone, a prerequisite to specifying a level of verbenone to protect a tree from successful attack by *D. brevicomis*.

METHODS AND MATERIALS

The study was done July 14, to October 1, 1982. Five plots at least 1.6 km apart were installed on the Bass Lake Ranger District, Sierra National Forest, Madera County, California in 1-hectare or larger openings in stands of ponderosa pine. Five 25.4-cm-diam. × 3-m-long Sonotube cardboard cylinders (Tilden et al., 1983; Tilden and Bedard, 1985) were erected vertically in each plot, with one cylinder at the center of a circle with 5 m radius and the other

four cylinders 90 degrees apart on the circumference of the circle. A cylindrical hardware cloth (0.95-cm mesh) sticky trap 38 cm diam × 30.5 cm high coated with melted Stickem Special was hung around each cardboard cylinder to monitor beetle visitation, with the center of the trap 1.5 m above ground. We used cardboard cylinders to provide the silhouette of a tree and to preclude production of natural pheromones by beetles boring in trees, which would confound our treatments.

E and F, both racemic, and myrcene (purity at least 98%) were evaporated from glass tubes and vials (Tilden et al., 1983; Tilden and Bedard, 1985) at two release rates 10-fold apart and at a ratio that approximated the relative release of the three compounds in nature (Browne et al., 1979; Tilden and Bedard (1985) (Table 1). Verbenone [60:40 of (+) and (−) enantiomers, purity at least 98%] was evaporated at four release rates 10-fold apart from three kinds

TABLE 1. DEVICES USED TO RELEASE *exo*-BREVICOMIN (E), FRONTALIN (F), MYRCENE (M), AND VERBENONE (V), AND RELEASE RATES OF THE COMPOUNDS, SIERRA NATIONAL FOREST, CALIFORNIA, JULY 14, TO OCTOBER 1, 1982

Compound	Release rate (mg/24 hr)[a]	Device
E (1X)	0.7 (0.30)	5 pipets, 0.80 mm ID × 50 mm, flame-sealed one end[b]
F (1X)	0.13 (0.08)	1 pipet, 0.40 mm ID × 32 mm, flame-sealed one end[b]
M (1X)	56.78 (3.16)	3 glass vials, 9 mm ID × 45 mm[c]
E (10X)	8.40 (1.50)	6 tubes, 3.5 mm ID × 52 mm, flame-sealed one end[d]
F (10X)	1.58 (0.25)	1 tube, 2.2 mm ID × 62 mm, flame-sealed one end[d]
M (10X)	700.30 (36.87)	37 vials, 9 mm ID × 45 mm[d]
V (1X)	0.54 (0.19)	one 1.5-ml polyethylene microcentrifuge tube, 10 mm ID × 38 mm, with 4.76-mm-diam. hole in cap[e]
V (10X)	4.74 (1.02)	1 glass cup 9.5 mm ID × 5 mm[e]
V (100X)	61.38 (12.98)	1 metal cap 42 mm ID × 10 mm[f]
V (1000X)	613.77 (91.90)	10 100× metal caps[d]

[a] Mean (standard deviation). Release rates for EFM from Tilden and Bedard (1985). Release rates of verbenone based on measurements made of five of each device on five (1×), nine (10×), or seven (100×) days.
[b] Taped to card and placed inside aluminum teaball.
[c] Inside inverted glass jar with perforated lid, 4 cm ID × 5.8 cm covered with aluminum foil.
[d] Set on hardware cloth shelves inside metal coffee can with perforated bottom and removable aluminum lid, 10 cm ID × 13.5 cm high. EFM were in one container and verbenone was in an adjacent container.
[e] Inside inverted glass saltshaker covered with aluminum foil.
[f] Inside aluminum tea ball.

of devices (Table 1). The devices were put inside containers of various sizes to facilitate handling and to mitigate effects of sun and wind (Table 1) and were hung about 1.3 m above ground at the bottom of the trap on the center cardboard cylinder. The bottoms of the containers were ventilated to permit release of the compounds. Verbenone was put in a container separate from E, F, and M.

Treatments were put out five days per week in the early afternoon (July and August) or late morning (September and October) and picked up the following morning between 0700 and 0830 hr. *D. brevicomis* were picked from traps and placed in labeled vials of kerosene for later counting and sex determination.

The experimental design consisted of two Latin squares (one for each level of EFM) with the factors day group, plot, and verbenone each having five levels (Winer, 1962, pp. 549–554). Fifty days were randomly assigned to 10 day groups so that each group had five days. One of the two levels of EFM and all five of the verbenone treatments (including a control of no verbenone) were presented each day. There were 25 days per level of EFM. Days and day groups were assumed to be random effects, and plots, verbenone, and EFM were assumed to be fixed effects. Data were transformed by $\log_e$ (counts + 1) to equalize variances and examined by analysis of variance. Means of the transformed data were compared by Tukey's honestly significant difference procedure at alpha = 0.05. Means and 95% confidence intervals for the transformed data were back-transformed to median estimates with their associated confidence intervals.

Before the start of the test, release rates of verbenone were measured gravimetrically. Devices were exposed inside a weather shelter near Oakhurst, California (elevation 790 m), where temperatures were assumed to be similar to those in the plots. Weight losses of each device were measured after several 24-hr periods. Release rates of EFM were assumed to be the same as measured the previous year (Tilden and Bedard, 1985).

RESULTS

The numbers of *D. brevicomis* caught at the center and outlying traps appeared to be inversely linearly related, in the log scale, to the log of the verbenone release rate (Figure 1). Analysis of variance of the transformed daily counts of *D. brevicomis* showed a statistically significant effect for verbenone (Table 2). Significantly fewer beetles were caught at the center trap at 10× and greater levels of verbenone for 10× EFM and at 1× and greater levels of verbenone for 1× EFM than were caught when EFM were released alone (Table 3). Significantly fewer beetles were caught at the outlying traps at 100× and

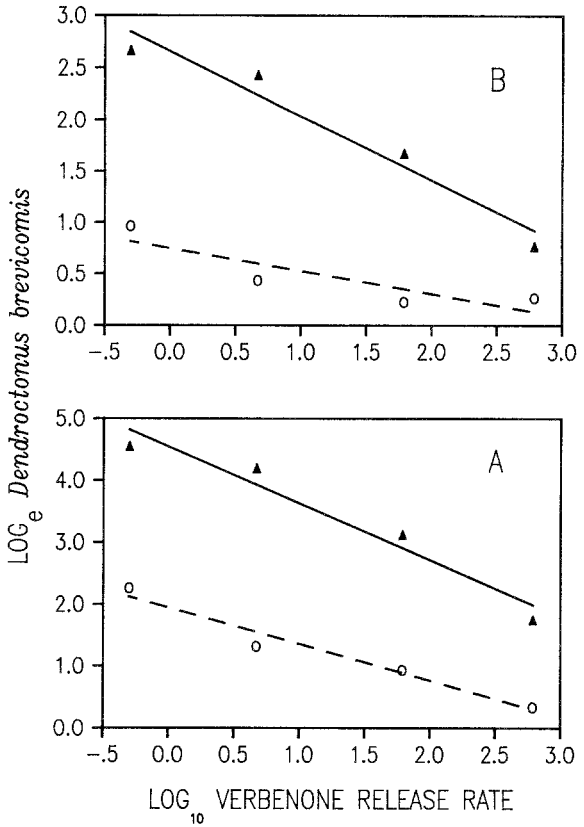

FIG. 1. Mean number [$\log_e$ (counts + 1)] of *Dendroctonus brevicomis* caught per day at one center trap (A) and four outlying traps (B) when *exo*-brevicomin, frontalin, and myrcene were released at two levels and verbenone was released at four levels at center trap. Dark triangles are catch at 10× EFM and open circles are catch at 1× EFM. SE of mean catch at center trap = 0.115 and at outlying traps = 0.114.

1000× levels of verbenone for 10× EFM and at 10× and greater levels of verbenone for 1× EFM than when EFM were released alone. The lowest levels of EFM and verbenone at which significant differences from EFM alone were detected were relatively the same at both release rates of EFM—1× EFM : 1× verbenone and 10× EFM : 10× verbenone (both 1 : 1) at the center trap, and 1× EFM : 10× verbenone and 10× EFM : 100× verbenone (both 1 : 10) at the outlying traps. Reductions in catch at both the center and outlying traps increased with increasing levels of verbenone (Table 3).

TABLE 2. ANALYSIS OF VARIANCE[a] OF *Dendroctonus brevicomis* CAUGHT PER DAY AT AND 5 m AWAY FROM SOURCE OF *exo*-BREVICOMIN (E), FRONTALIN (F), AND MYRCENE (M) RELEASED AT TWO LEVELS ALONE AND WITH VERBENONE RELEASED AT FOUR LEVELS, SIERRA NATIONAL FOREST, JULY 14 TO OCTOBER 1, 1982

Source of variation	Degrees of freedom	Center trap			4 Outlying traps		
		Mean square	F	P^b	Mean square	F	P^b
Between days							
EFM	1	261.98	104.76[c]	**	133.36	135.59[c]	**
Day groups (EFM)	8	2.50	0.71[d]	0.689	0.98	0.70[d]	0.692
Days (day groups)	40	3.51	10.62	**	1.41	4.32	**
Within days							
Plots	4	2.85	8.63	**	1.57	4.80	**
Verbenone	4	77.99	236.20	**	20.67	63.28	**
Plots × EFM	4	2.25	6.82	**	1.36	4.17	**
Verbenone × EFM	4	4.83	14.63	**	4.05	12.40	**
Residual	24	0.43	1.29	0.177	0.21	0.66	0.887
Error	160	0.33			0.33		

[a] Data transformed by $\log_e$ (counts + 1).
[b] P-value of the F ratio. ** denotes $P < 0.01$.
[c] Denominator of F ratio was day groups (EFM) mean square.
[d] Denominator of F ratio was days (day groups) mean square. Denominator of remaining F ratios was error mean square.

The analysis of variance also showed a significant effect for EFM (Table 2). Combining all levels of verbenone, median daily catches of *D. brevicomis* were:

	Center trap	4 outlying traps
10× EFM	40.7	7.1
1× EFM	4.4	0.9

The significant verbenone × EFM interaction at the center and outlying traps indicates that the differences in catch of *D. brevicomis* between the two levels of EFM were not the same at each level of verbenone (Figure 1).

The analysis of variance of percent *D. brevicomis* caught at the center trap showed significant effects due to verbenone and a verbenone × EFM interaction ($P < 0.01$). Within both levels of EFM, percent catch at the center trap tended

TABLE 3. *Dendroctonus brevicomis* CAUGHT PER DAY AT AND 5 m AWAY FROM SOURCE OF *exo*-BREVICOMIN (E), FRONTALIN (F), AND MYRCENE (M) RELEASED AT TWO LEVELS ALONE AND WITH VERBENONE RELEASED AT FOUR LEVELS[a]

	D. brevicomis caught at				
	Center trap			4 Outlying traps	
Verbenone	Median (95% CL)[b]	Reduc.[c]	Percent[d]	Median (95% CL)[b]	Reduc.[c]
EFM = 10×					
0	150.2a (107.9, 208.8)		89.7ab	17.8a (12.6, 25.1)	
1×	94.5ab (67.8, 131.5)	0.37	88.0ab	13.4a (9.4, 19.0)	0.25
10×	65.9b (47.2, 91.8)	0.56	86.7ab	10.4a (7.2, 14.8)	0.42
100×	21.7c (15.4, 30.5)	0.86	83.6ab	4.3b (2.9, 6.4)	0.76
1000×	4.8de (3.2, 7.0)	0.97	81.9ab	1.2cd (0.6, 2.0)	0.93
EFM = 1×					
0	34.5c (24.6, 48.3)		92.9a	2.6bc (1.6, 4.0)	
1×	8.6d (5.9, 12.4)	0.75	79.1ab	1.6c (0.9, 2.6)	0.37
10×	2.7ef (1.7, 4.2)	0.92	77.2b	0.5de (0.1, 1.1)	0.79
100×	1.5f (0.8, 2.5)	0.96	89.0ab	0.3e (0.0, 0.7)	0.90
1000×	0.4g (0.0, 0.9)	0.99	60.0c	0.3de (0.0, 0.8)	0.88

[a] Bass Lake, Sierra National Forest, California, July 14, to October 1, 1982. See Table 1 for release rates of EFM and verbenone.

[b] Median catches and lower and upper simultaneous 95% confidence limits back-transformed from means and confidence limits of 25 daily observations transformed to $\log_e$ (counts + 1). Negative lower limits for outlying traps were set to 0. Medians followed by different letters differ significantly at alpha = 0.05, Tukey's honestly significant difference procedure.

[c] Reduction in median catch = $(C - T)/C$, where C = median catch when verbenone = 0 and T = median catch when verbenone = a treatment.

[d] Mean percent of total catch at center and outlying traps calculated from untransformed counts. Means followed by different letters differ significantly at alpha = 0.05, Tukey's honestly significant difference procedure.

to decrease with increasing levels of verbenone, but the only statistically significant differences occurred at 1× EFM (Table 3).

DISCUSSION

The role verbenone plays in host colonization is uncertain (Browne et al., 1979; Byers and Wood, 1980; Wood, 1982), but we believe that, if released at high enough levels, it could interrupt the natural process of colonization initiated by the production of secondary attractant. We believe this is a reasonable expectation for verbenone because the results from this study and previous ones using natural as well as synthetic attractants (Bedard et al., 1980a,b) indicate

that verbenone can reduce the numbers of *D. brevicomis* trapped at a source of attractant, and that increased levels of verbenone are associated with decreased trap catch (Table 3) (Bedard et al., 1980b).

Relatively high levels of EFM were released in this study to ensure adequate numbers of trapped beetles for comparison of treatments. The 1× level of EFM is usually more than adequate to cause baited trees to be successfully attacked (Bedard et al., 1980b). If verbenone were to successfully protect a tree from attack, it would probably have to either prevent the initial random landing of pioneer beetles, prevent boring and sustained feeding, or interrupt the low level of attractant produced by a few initial attacks. Based on the estimates of Browne et al. (1979), the amounts of E, F, and M released in a 24-hr period by two pairs of beetles would be 85, 58, and 69 times less, respectively, than what we released at the 1× level.

We cannot assume that trap catch is an adequate measure of attack rate on a tree, because trapping prevents too many possible events in host colonization. We believe, however, that the dosage response shown in our results suggests that the higher the release rate of verbenone from a tree, the more likely the tree would be protected from beetle attack.

The plot design in this and previous studies (Tilden and Bedard, 1985; Tilden et al., 1983) provide more information about flight response of bark beetles at and near a pheromone source than would be available from a study using a trap only at the source. Increased levels of verbenone in this study were associated with decreased catch on silhouettes 5 m away from the source of verbenone. Verbenone may have an area effect similar to that demonstrated for MCH (Furniss et al., 1981; McGregor et al., 1984), in which the chemical did not have to be applied directly to the tree to result in reduced attack density and brood production. We infer from the results of our studies that the response of beetles at and near the source varies with the chemical composition of the source and with the absolute and relative release rates of the components.

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BEHAVIORAL RESPONSES OF CRAYFISH (*Orconectes virilis* AND *Orconectes rusticus*) TO CHEMICAL FEEDING STIMULANTS

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Abstract—We conducted two experiments to assess how chemical stimuli affect feeding behavior, grooming, and walking in the crayfishes *Orconectes virilis* and *Orconectes rusticus*. In the first experiment, *O. virilis* was tested with 29 amino acids; in the second experiment, *O. rusticus* was tested with 12 amino acids, 13 additional single compounds, and two six-compound mixtures. In *O. virilis*, the following amino acids, in order of potency, elicited feeding movements: L-isoleucine, glycine, hydroxy-L-proline, L-glutamate, L-valine, and B-alanine. Grooming increased in response to L-phenylalanine, L-tryptophan, L-tyrosine, L-leucine, L-methionine, and D-aspartate. In *O. rusticus*, both mixtures and the following single compounds, in order of potency, elicited feeding movements: cellobiose, sucrose, glycine, maltose, glycogen, nicotinic acid methyl ester, putrescine, and L-glutamate. Grooming increased in response to putrescine only, and walking increased in response to glycogen only. The responsiveness of these crayfishes to a wide variety of chemicals may reflect the omnivorous foraging habits of these crustaceans.

Key Words—Crayfish, *Orconectes virilis*, *Orconectes rusticus*, Crustacea, chemoreception, feeding behavior.

INTRODUCTION

Many behavioral studies have demonstrated that amino acids and related compounds are potent feeding stimulants for marine Crustacea, including lobsters (McLeese, 1970; Mackie, 1973; Carter and Steele, 1982; Zimmer-Faust et al., 1984), crabs (Kay, 1971; Hartman and Hartman, 1977; Field, 1977), and shrimp (Hindley, 1975; Carr, 1978; Johnson and Atema, 1986). Amino acids presum-

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ably provide these flesh-eating species with information about food availability. In contrast, studies of semiterrestrial crabs, which naturally feed on algae and other plant material, demonstrate that feeding is best stimulated by carbohydrates (Robertson et al., 1981; Trott and Robertson, 1984). Chemical feeding stimulants of crayfish are as yet unknown. A purpose of this study was to provide initial information on the chemical response spectrum of two crayfishes and to compare this information to results from similar studies of other crustacea. *Orconectes virilis* and *O. rusticus*, which are common in lakes and streams throughout the midwestern and northeastern United States, are omnivores and consume a great variety of plant and animal material (Lorman and Magnuson, 1978). The flexible feeding habits of crayfish suggest that they might respond to a very broad spectrum of chemicals.

In most cases, feeding is best elicited by specific mixtures of compounds present in prey organisms (Shelton and Mackie, 1971; McLeese, 1970; Mackie, 1973; Carr et al., 1984; Carr and Derby, 1986). However, feeding can also be stimulated by single compounds such as isoleucine (Kay, 1971), ammonium chloride (Borroni et al., 1986), betaine (Carr, 1978), and taurine (Johnson and Atema, 1986). Such single compounds may function as signals for food detection. Johnson and Atema (1986) note that taurine, for example, has excellent signaling properties: it is abundant in prey organisms, but occurs in low concentrations in background seawater. They suggest that crustaceans have consequently evolved chemoreceptors to detect this compound. This idea is supported by the finding that lobsters have large receptor populations narrowly tuned for taurine (Fuzessery et al., 1978; Johnson and Atema, 1983; Johnson et al., 1984).

The dactyl chemoreceptors of crayfish are more broadly tuned than are those of lobsters. One type of receptor responds generally to amino acids (Hatt, 1984); another receptor type responds generally to pyridine compounds (Hatt and Schmiedel-Jakob, 1984). Both receptor types are differentially sensitive to different compounds. Thus, the amino acid receptor is highly sensitive to compounds such as serine, alanine, and histidine and much less sensitive to other amino acids (e.g., glutamate, lysine, tyrosine). Chemicals which very effectively excite receptors may be particularly significant behaviorally, possibly playing a signal role in food detection as suggested for taurine. A second purpose of the present study was to test this hypothesis by assessing the responses of crayfish to single amino acids and comparing this information to the electrophysiologic results of Hatt (1984).

METHODS AND MATERIALS

We collected adult *O. virilis* (27–42 mm carapace length) from Lake Winnipesaukee, Belknap County, New Hampshire. Adult *O. rusticus* (33–40 mm

carapace length) were acquired from a commercial supplier (Connecticut Valley Biological Supply Co., Southampton, Massachusetts). Animals were held individually in plastic basins containing aged tap water (pH 6.9; temperature 20 $\pm$ 2°C). They were fed Purina trout chow three times a week.

During testing, crayfish were isolated in Plexiglas chambers 15 $\times$ 15 $\times$ 7 cm high, filled with 1 liter of aged tap water. A portion of a 1-cc plastic syringe (4.5 cm in length) mounted to the removable top of each chamber formed a delivery tube through which chemicals were injected into the center of the chambers. Prior to testing, animals were placed in the chambers and allowed to acclimate for 72 hr. They were fasted for at least 48 hr before being tested. We conducted experiments between 0800 and 1800 hr under red light (three 40-W incandescent bulbs suspended 40 cm over a row of chambers). When illuminated by red light, the crayfish, which normally were nocturnally active, became more active during the day. The lower light level provided by red bulbs, compared to white bulbs, also reduced visual responsiveness to the observer. Eighteen *O. virilis* (nine males, nine females) and 14 *O. rusticus* (11 males, three females) were tested.

For *O. virilis*, test stimuli were 29 single amino acids; for *O. rusticus*, test stimuli were 25 single compounds and two equimolar mixtures of six compounds (Table 1). The single chemicals were chosen because they have previously been tested as feeding stimulants in other crustaceans and as chemostimulants in electrophysiologic studies of crayfish. The mixtures were tested to determine if combinations of several chemicals would exhibit synergism or antagonism. Each single compound was weighed to prepare a 10^{-2} M solution (for glycogen, a 4 mg/liter solution). To form the amino acid mixture, 50 ml of each 10^{-2} M solution of six amino acids (serine, histidine, glycine, proline, glutamate, lysine) were combined. Likewise, 50 ml of each of two amino acids (serine, glycine) and four additional compounds (glucose, cellobiose, nicotinamide, trimethylamine) were combined to form the general mixture. For both mixtures, the molarity of each single compound was 0.167×10^{-2}; the total mixture molarity was 10^{-2}. Chemicals were dissolved in aged tap water, and the pH of all solutions was adjusted to 6.5 using H_2SO_4 or NaOH. Solutions were placed in 20-ml vials and frozen until the day of use.

In each test, 4 ml of a stimulus solution or control (aged tap water) was injected (over approximately a 10-sec time period) from a 5-ml syringe into the chamber via the delivery tube. Dye experiments indicated that injected solutions diffused to all sides of the chamber within 10 sec and thus contacted the animals within 10 sec, regardless of their positions in the chambers. Since the test chambers contained 1 liter of water, stimulus solutions could be maximally diluted 250 times. Thus, actual concentrations to which animals were exposed were less than the injected concentrations indicated in the results. The test solutions and controls were presented to the crayfish in random order and were blind to the observer. In both experiments, each animal was tested once with each stim-

TABLE 1. TEST STIMULI FOR *O. virilis* AND *O. rusticus*[a]

O. virilis	*O. rusticus*
Alanine	Alanine
β-Alanine	β-Alanine
Arginine	Glutamate
Asparagine	Glycine
Aspartate	Histidine
D-Aspartate	Hydroxyproline
Citrulline	Leucine
Cysteine	Lysine
Cystine	Phenylalanine
Glutamine	Proline
Glutamate	Serine
D-Glutamate	Taurine
Glycine	Betaine
Histidine	Nicotinamide
Hydroxyproline	Nicotinic acid methyl ester
Isoleucine	Putrescine
Leucine	Pyrazinecarboxamide
D-Leucine	Trimethylamine
Lysine	Cellobiose
D-Lysine	Fructose
Methionine	Glucose
Norvaline	Glycogen
Phenylalanine	Maltose
Proline	Mannose
Serine	Sucrose
Threonine	Amino acid mixture
Tryptophan	General mixture
Tyrosine	Control
Valine	
Control	

[a] Except where indicated, L amino acids and D sugars were used.

ulus. Animals were observed for 8 min following stimulus injection, and then the chambers were rinsed and refilled with clean aged tap water. Each crayfish was tested once or twice a day, with at least 3 hr between two tests conducted on the same day.

During the 8 min following stimulus injection, we used stopwatches to record the following four behaviors: total time walking, total time feeding, total time grooming, and the number of times walking legs were touched to the mouth. Time walking was the time animals spent moving across the chamber or climbing the sides. We considered feeding movements to occur when animals

probed or tapped the ground with their first two pairs of walking legs, while opening and closing the dactyls of these appendages. Touching legs to mouth occurred most frequently while animals were performing feeding movements and was considered a feeding response. However, it also occurred during grooming when an animal plucked debris from itself and conveyed it to its mouth. We chose walking, feeding movements and legs touched to mouth as our bioassay because these behaviors were displayed by animals initially presented with a food odor (filtrate from 100 ml of water in which 1 g of trout chow had been soaked for 1 hr). Previous tests also demonstrated that high concentrations of amino acids elicit grooming in *O. virilis* (Tierney and Atema, 1986).

The Friedman two-way analysis of variance by ranks (Siegel, 1956) was used to compare responses to test chemicals and the control for each of the four behaviors recorded. Excepting total time walking in *O. virilis*, significant ($P \leq 0.01$) overall differences occurred in all tests. The Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) was used to compare responses of each test chemical to the control for each of the behaviors shown by the Friedman test to be significantly different overall.

RESULTS

Responses of O. virilis *to chemical stimuli.* Of the 29 amino acids tested six, isoleucine, glycine, hydroxyproline, glutamate, valine, and β-alanine, significantly increased the time spent performing feeding movements, relative to the control (Figure 1A). Two amino acids, tryptophan and tyrosine, significantly reduced the time spent performing feeding movements. Feeding responses to chemicals were also assessed by recording the number of times walking legs were touched to the mouth. Two amino acids, isoleucine and glycine, elicited this behavior significantly ($P \leq 0.05$) more than did the control.

The amount of time spent grooming significantly increased, relative to control tests, in response to six amino acids: phenylalanine, tryptophan, tyrosine, leucine, methionine, and D-aspartate ($P \leq 0.01$, for phenylalanine, tryptophan, tyrosine, and leucine; $P \leq 0.05$ for methionine and D-aspartate).

Responses of O. rusticus *to chemical stimuli.* Eight single compounds and both mixtures significantly increased the time spent performing feeding movements, relative to the control (Figure 1B). The most stimulatory single compounds were the disaccharide sugars cellobiose and sucrose. Maltose and the polysaccharide glycogen were also significantly stimulatory. However, the monosaccharides glucose, mannose, and fructose were not. Cellobiose, sucrose, and maltose were the only chemicals to elicit a feeding response from every animal (Figure 2). The amino acids glycine and glutamate, the amine

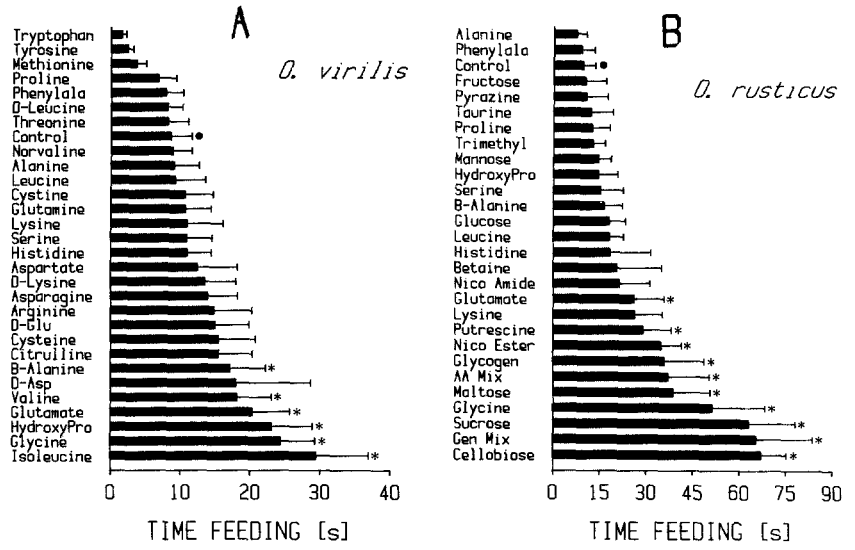

FIG. 1. Feeding responses of *O. virilis* and *O. rusticus* to test stimuli at 10^{-2} M concentration (for glycogen, 4 mg/liter). (A) Bars indicate mean time (seconds) that 18 *O. virilis* spent feeding. (B) Bars indicate mean time (seconds) that 14 *O. rusticus* spent feeding. Horizontal lines indicate SEM. *Time spent feeding is significantly greater in response to test chemicals than in response to control (●), $P \leq 0.05$. Abbreviations: Phenylala, phenylalanine; D-Glu, D-glutamate; D-Asp, D-aspartate; HydroxyPro, hydroxyproline; Pyrazine, pyrazinecarboxamide; Trimethyl, trimethylamine; Nico Amide, nicotinamide; Nico Ester, nicotinic acid methyl ester; B-Alanine, β-alanine; AA Mix, amino acid mixture; Gen Mix, general mixture.

putrescine, and the pyridine compound nicotinic acid methyl ester also significantly stimulated feeding movements. Legs were touched to the mouth significantly more times, relative to the control, in response to the two mixtures and five single compounds (cellobiose, sucrose, glycine, putrescine, and nicotinic acid methyl ester; $P \leq 0.05$).

The amount of time spent walking increased, relative to control tests, in response to only one compound, glycogen ($P \leq 0.05$). Time spent grooming increased in response to putrescine only ($P \leq 0.01$).

In both crayfishes there was marked interanimal variability in general responsiveness to chemical stimuli (illustrated for feeding behavior in *O. rusticus* in Figure 2). In contrast, repeated presentation of the same chemical to crayfish indicated that intraanimal variability was relatively low (Tierney and Atema, personal observation). In our apparatus crayfish were generally consistent in their responsiveness to stimulatory chemicals, with some individuals always highly responsive and others always relatively unresponsive.

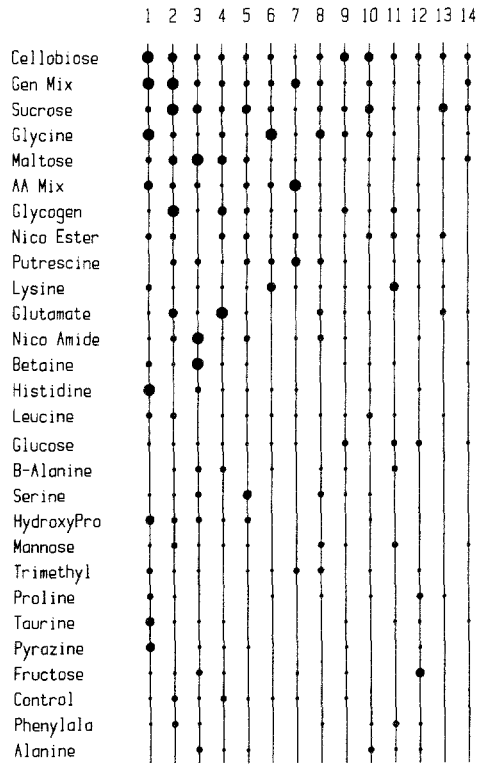

FIG. 2. Interanimal variability: time individual *O. rusticus* spent performing feeding movements in response to chemical stimuli. A continuous line indicates no response, the smallest dots indicate 1–25 sec were spent performing feeding movements, second largest dots indicate 26–75 sec feeding, third largest dots indicate 76–125 sec feeding, and largest dots indicate 126 sec or more feeding. Chemicals are listed in order of potency. Animals are arranged in order of most to least responsive. Abbreviations as in Figure 1.

DISCUSSION

The results demonstrate that even under highly artificial conditions *O. virilis* and *O. rusticus* are stimulated to perform feeding movements by certain single compounds. Cellobiose and sucrose were the most stimulatory compounds tested (Figures 1B and 2), suggesting that carbohydrates are an important feeding stimulus for *O. rusticus*. Additional experiments with *O. virilis* (Tierney and Atema, in preparation) confirm the stimulatory effectiveness of carbohydrates, especially sucrose. Using 20 *O. virilis* and the procedures described above, we found that sucrose was four times more effective at eliciting feeding than were glycine or glutamate. Feeding responses to carbohydrates

have been observed in other crustacean species including porcelain crabs (*Petrolisthes cinctipes*; Hartman and Hartman, 1977), fiddler crabs (*Uca pugilator*; Robertson et al., 1981), and ghost crabs (*Ocypode quadrata*; Trott and Robertson, 1984). Bradycardia following exposure to carbohydrates occurred in crayfish (*Procambarus simulans*; Ashby and Larimer, 1965) and kelp crabs (*Pugettia producta*; Zimmer et al., 1979), indicating the presence of carbohydrate receptors in the branchial chambers of these animals. Like *O. rusticus*, most species were more responsive to disaccharides than to monosaccharides.

Chemosensitivity to specific carbohydrates appears to correlate well with the diets of the animals. Macroalgae, eaten by kelp crabs (Zimmer et al., 1979) and benthic diatoms, a food source for fiddler crabs (Robertson et al., 1981) and ghost crabs (Trott and Robertson, 1984), contain disaccharides as energy storage products. Ghost crabs, which feed on fruit, also responded to fructose and mannose, the major storage products of fresh and decaying fruit. Crayfish are omnivores and consume a wide variety of plant (periphyton and many macrophyte species) and animal (insect larvae, carrion, other crayfish) material (Lorman and Magnuson, 1978; Capelli, 1980). Grazing of macrophytes by *O. rusticus* has been sufficiently extensive to decimate dense stands of water vegetation in Wisconsin lakes (Lorman, 1980). Cellobiose, produced by the partial hydrolysis of cellulose, and sucrose occur abundantly in plants (Davies, 1974) and may provide cues which elicit feeding in natural populations of crayfish.

The amino acids glycine and glutamate elicited feeding movements in both *O. virilis* and *O. rusticus*; isoleucine, hydroxyproline, valine, and β-alanine also elicited feeding movements in *O. virilis*. Excepting D-aspartate, which was more stimulatory than L-aspartate, D isomers of amino acids (D-glutamate, D-lysine, D-leucine) elicited less feeding than did corresponding L isomers. Putrescine, an amine formed from the decarboxylation of ornithine, is found in decaying flesh. In *O. rusticus* it significantly affected feeding behavior and grooming. The increase in time spent grooming when exposed to putrescine suggests that, at test concentrations, this chemical was a general irritant, as well as a feeding stimulant, to the animals. Mixed responses have also been observed in lobsters exposed to ammonia; at low concentrations ammonia is a feeding stimulant, at high concentrations it inhibits feeding (Borroni et al., 1986). Feeding was not associated with the six amino acids that caused increased grooming in *O. virilis*.

In marine crustaceans, amino acids which best elicit feeding include taurine, glycine, β-alanine, glutamate, isoleucine, tyrosine, proline, and methionine (McLeese, 1970; Kay, 1971; Hartman and Hartman, 1977; Hamner and Hamner, 1977; Allison and Dorsett, 1977; Ache, 1982; Johnson and Atema, 1986). These chemicals are abundant in the tissues of marine organisms and probably guide predators and scavengers to food. Amino acids may likewise signal food availability to crayfish. During parts of the year crayfish prey heavily on insect larvae and other crayfish (Capelli, 1980). High concentrations of

free glycine and glutamate occur in the hemolymph of insects (Jeuniaux, 1970), and glycine and glutamate are the most abundant amino acids in the cuticle of crayfish and other crustaceans (Welinder, 1974).

Two amino acids, hydroxyproline and β-alanine, were tested in both species, but elicited feeding in *O. virilis* only. If these compounds are naturally associated with food selection, this difference suggests that the diets of the two species, or the populations sampled, may differ significantly. Compatible with this suggestion is the hypothesis that differences in the natural background concentrations of amino acids may influence responsiveness to food-related chemicals (Johnson and Atema, 1986). The quality and quantity of free amino acids in freshwater is highly variable, and depends on the trophic condition of lakes and the type of vegetation and soil near rivers (Thurman, 1986). This variability may provide a selective pressure that results in differently tuned receptor systems in different species or populations of crayfish.

Bauer et al. (1981) and Hatt (1984) concluded from electrophysiologic studies that the dactyl chemoreceptors of the European crayfish *Austropotamobius torrentium* are sensitive to many amino acids, the most potent being serine, alanine, histidine, β-alanine, ornithine, and proline. Excepting β-alanine, these compounds were not associated with feeding behavior in *O. virilis* or *O. rusticus*. Conversely, the dactyl receptors of *A. torrentium* were relatively insensitive to some compounds (e.g., glutamate, isoleucine) that did stimulate feeding in *O. virilis* and *O. rusticus*. The pyridine compounds pyrazinecarboxamide, nicotinamide, and nicotinic acid methyl ester were also highly stimulatory for *A. torrentium* receptors (Hatt and Schmiedel-Jakob, 1984), but only one of these compounds, nicotinic acid methyl ester, elicited feeding in *O. rusticus*.

Amino acid-sensitive receptors occur on crayfish antennules (Hodgson, 1958), and animals in our behavioral studies may have used these receptors to perceive chemicals that ineffectively stimulate dactyl receptors. Alternatively, differences between physiologic and behavioral responses might result from the testing of single compounds instead of mixtures. Many studies have shown that behavioral responses of crustaceans to mixtures are different from responses to the single components of mixtures (Shelton and Mackie, 1971; Carr, 1978; Robertson et al., 1981; Borroni et al., 1986; Zimmer-Faust et al., 1984). In tests with *O. rusticus*, we found no evidence for synergistic or suppressive interactions among the chemicals in two simple mixtures. This is preliminary evidence that compounds such as serine and histidine, which did not induce feeding when presented singly, also do not inhibit or enhance other compounds in our mixtures. Clearly, however, additional studies, particularly with mixtures that mimic natural foods, are needed to confirm this observation.

Finally, it is possible that the dactyl receptors of *O. virilis* and *O. rusticus* are sensitive to different compounds than are those of *A. torrentium*. The North American species and the European species inhabit different environments, and

chemoreceptors of each species may be tuned in a manner to allow optimal adaptation to local conditions. A more complete understanding of how ecological factors affect the tuning of crustacean chemoreceptors could be gained from studying the ecology, behavior, and chemoreceptor physiology of closely related species adapted to different environments. Crayfish provide ideal subjects for such a comparative study as a great many species are available—approximately 270 in North America—and these animals inhabit virtually every type of freshwater ecosystem.

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AMINO ACID CHEMORECEPTION: Effects of pH on Receptors and Stimuli

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Abstract—A current model of amino acid chemoreception has generated the idea that pH affects the efficiency of stimulus–receptor binding by altering the charge distribution on stimulus molecules. The model suggests that amino acids are maximally stimulatory near their isoelectric points. We point out that, within a broad range of pH values, changes in stimulant amino acids cannot account for altered chemoresponsiveness. We suggest instead that pH-induced changes in chemoreception are a result of changes in charge distribution on the protein receptor.

Key Words—Amino acids, chemoreception, pH, proteins, isoelectric points.

INTRODUCTION

Behavioral and electrophysiologic studies have demonstrated that amino acids are effective chemical stimuli for receptors of a great many species. Single amino acids have been used as model compounds to elucidate structure–activity relationships and thus to infer properties of membrane receptor sites (Hara, 1976a, 1982; Bauer et al., 1981; Caprio, 1982; Hatt, 1984; Valentincic, 1985). Based on studies of rainbow trout olfactory responses, Hara (1976a, 1982) has proposed a hypothetical amino acid receptor site. Hara's model, which is outlined below, has implications for pH effects on chemoreception: amino acids are considered to be maximally stimulating near their isoelectric points. Concern about the acidification of freshwater ecosystems due to acid precipitation has generated a growing interest in how low pH affects chemoreception in aquatic animals (Lemly and Smith, 1985; Jones et al., 1985; Tierney and Atema, 1986; Royce-Malmgren and Watson, 1987). Because Hara's model relates pH to stim-

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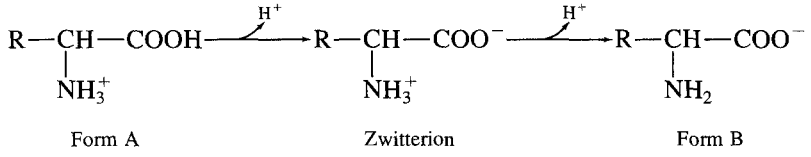
ulus–receptor binding, this model has been cited as a possible explanation for why behavioral responses to chemical stimuli are impaired at low pH levels. Here we point out that neither electrophysiologic data on pH effects on amino acid reception in trout nor observations of impaired behavioral chemoresponsiveness are readily understood in terms of Hara's explanation, i.e., that chemoreception is altered by alteration of the charge distribution on stimulus molecules. We suggest instead that pH-induced changes in chemoreception are a result of changes in charge distribution on the protein receptor.

HARA'S MODEL

Hara (1976a) recorded olfactory responses of trout to amino acids and analogs and determined that, for stimulation to be effective, the following features are essential: (1) ionized α-amino and α-carboxyl groups; (2) a free α-hydrogen; (3) a fourth α-moiety of appropriate size and polar nature; and (4) the L, rather than the D isomer, of the amino acid. From these findings, Hara suggested that an amino acid receptor might consist of two charged subsites, one negative and one positive, which interact with the ionized α-amino and α-carboxyl groups. The two subsites are thought to occur in a particular order around a center which interacts with the α-hydrogen, allowing L isomers to bind more easily than D forms. An additional region of the receptor recognizes and binds with the variable fourth α-moiety and gives the receptor its specificity for particular amino acids. Models basically similar to Hara's have been proposed for chemoreception in humans (Shallenberger et al., 1969), crayfish (Hatt, 1984) and sea stars (Valentincic, 1985).

The presence of ionized α-amino and α-carboxyl groups in stimulus molecules and appropriately charged groups in receptor sites is pH dependent. In neutral aqueous solutions, amino acids exist primarily as dipolar ions (or zwitterions). Addition of an acid to a solution of dipolar molecules will cause the carboxyl group to pick up H^+, forming the undissociated carboxyl group; addition of a base will cause the ammonium group to release an H^+:



Amino acids are positively charged (form A) in acidic solution and negatively charged (form B) in alkaline solutions and migrate accordingly in an electric field. For each amino acid there exists an intermediate pH, called the isoelectric point (pI), where there is no net charge on molecules (most are zwitterions and

the very small balance contains equal numbers of forms A and B) and no net movement in an electric field. For monoamine–monocarboxylic amino acids, the isoelectric point is the arithmetic mean of the dissociation constants of the α-carboxyl (pK_1) and the α-amino (pK_2) groups.

According to Hara, amino acids are most effective as chemostimulants when they are at their isoelectric points where zwitterion forms are maximal. To test this idea, Hara (1976b) studied olfactory responses of trout to amino acids at various pH levels. Responses were indeed pH dependent. When presented at their isoelectric points, some amino acids (e.g., serine and methionine) elicited responses 100% greater than responses elicited at pH levels one pH unit removed from the isoelectric point. Hara notes that pH can influence the ionization of (1) receptors, (2) stimulus molecules, and (3) binding between receptors and stimuli. However, in accounting for altered chemoresponsiveness, he emphasizes the role played by ionization changes of stimulus molecules:

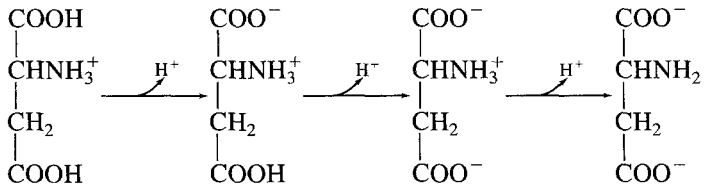
> Most of highly stimulatory amino acids employed had their peak activity somewhere between pH 5.0 and 6.0. The isoelectric points for these amino acids also lie within the same range. This indicates that these amino acids display maximal activity near their isoelectric points at which dipolar ions are at maximum and bearing no net charge. This supports the idea that the binding would take place between ionized amino and carboxyl groups of amino acids and two ionized, one positively and one negatively, subsites of the receptors [Hara, 1976b, p. 38].

EFFECTS OF pH ON AMINO ACIDS AND PROTEINS

As presented by Hara, the concept of an isoelectric "point" where dipolar ions are maximum is misleading. For monoamine–monocarboxylic amino acids there exists, not an isoelectric point, but a broad zone of pH values, delimited by pK_1 and pK_2, where molecules exist almost entirely in the dipolar form. For glycine, for example, the concentration of cationic and anionic molecules is less than one percent of that of zwitterions at all pH values between 4.3 and 7.7 (Edsall, 1943a).

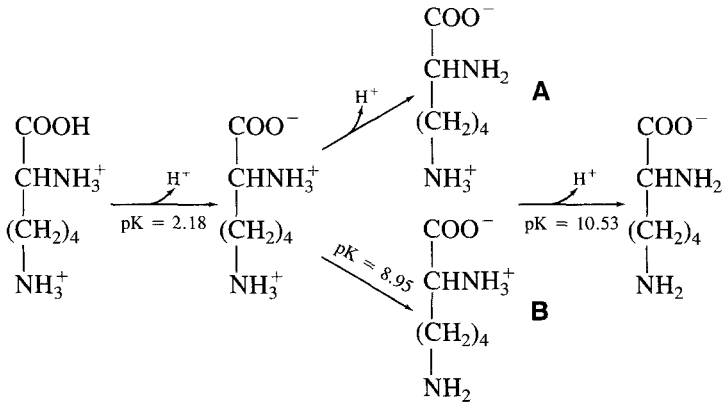
Dicarboxylic amino acids (aspartate, glutamate), diamine amino acids (lysine, arginine, histidine), tyrosine, and cysteine have an additional ionizable group and an additional dissociation constant (pK_R). Consequently, in these amino acids, the presence of ionized α-carboxyl and α-amino groups is not directly correlated with the presence of the zwitterion form of the molecule. This may have implications for the analysis of data in terms of Hara's model. For example, in the aspartate molecule, protons tend to dissociate most readily

from the α-carboxyl group (pK_1 = 1.88 at 25°C; Edsall, 1943a), next from the distal carboxyl group (pK_R = 3.65) and least readily from the α-amino group (pK_2 = 9.60):



The isoelectric point of aspartate is $\frac{1}{2}(pK_1 + pK_2)$ or 2.94, and the compound exists primarily as a zwitterion between pH levels 2.5 and 3.5. However, the great majority of aspartate molecules have the α-carboxyl and α-amino groups ionized in the manner required for Hara's model between pH levels 2.5 and 9.5.

For lysine, a diamino amino acid, protons dissociate in the following manner:



zwitterion

The zwitterion occurs in two forms. The ratio of the highly polar A form to the B form is approximately 1,800,000 to 320,000 (Edsall, 1943a). Thus most zwitterionic lysine molecules do not possess a charged α-amino group. Assuming a charged distal amino group could be accommodated, lysine (and arginine) would, according to Hara's model, be effective chemical stimuli roughly between pH levels 3 and 8, and not at their isoelectric points (9.74 for lysine; 10.76 for arginine).

The above points indicate that Hara's (1976b) data offer no direct support for his idea that the amino acid receptor has two charged subsites that must

interact with ionized amino and carboxyl groups of stimulant amino acids. This idea, in turn, cannot explain the striking differences in responses to amino acids at their isoelectric points and responses one or two pH units removed from the isoelectric points. Alterations in stimulus molecules also cannot account for the pH effects on behavioral responses to chemicals observed in fish (Jones et al., 1985; Lemly and Smith, 1985; Royce-Malmgren and Watson, 1987) and crayfish (Tierney and Atema, 1986). Royce-Malmgren and Watson (1987) found that at pH 7.6 salmon were attracted to glycine and repelled by alanine; at pH 5.1, the salmon were unresponsive to glycine and attracted to alanine. In fathead minnows, reliable responses to chemicals from a food stimulus at pH 7 were reduced at pH 6.5 and eliminated at pH 6.0 (Lemly and Smith, 1985). Likewise, arctic char were attracted to chemicals from food at pH 7.8 and showed reduced attraction at pH levels 5.0 and 4.75 (Jones et al., 1985). Crayfish also showed reduced responses to an amino acid mixture at low pH levels (pH 4.5 and 3.5), relative to responses at pH 5.8 (Tierney and Atema, 1986).

Following Hara (1976b), all of the above reports cite pH-induced changes in stimulus molecules as a possible explanation for altered chemoresponsiveness. Jones et al. (1985, p. 409), for example, suggest that responses of char to chemicals are reduced at low pH because "the structure and charge of stimulatory molecules such as amino acids vary with pH, thereby making each substance, depending on its specific pH curve, more stimulatory at some pHs than at others." However, excepting crayfish tested at pH 3.5, all of the behavioral changes described above occurred at pH levels where alterations in stimulatory amino acids would be negligible. In most of the above studies, reduced responses to chemicals are not readily attributed to malaise induced by lowered pH. The effects of low pH on chemoreception in fish and crayfish appear to be quite specific and, like certain of Hara's (1976b) data, remain to be explained.

The explanation probably involves, not changes in stimulus molecules, but an alternative possibility suggested by Hara (1976b): changes in the state of ionization of the protein receptor. The behavior of protein chemoreceptors may be comparable to that of enzymes. Enzyme proteins typically display an optimum pH where catalytic activity is maximum (Laidler, 1958; Dixon and Webb, 1979). For some enzymes activity drops sharply (within one to two pH units) on either side of the optimum. In all proteins, the α-amino and α-carboxyl groups are tied up in peptide bonds and do not participate in acid and base binding. (Exceptions are the N-terminal and C-terminal groups and cystine, which may possess an α-amino and an α-carboxyl not involved in peptide linkage.) The effects of pH changes on proteins are consequently determined primarily by amino acids with side-chain groups that can ionize (i.e., aspartate, glutamate, histidine, lysine, arginine, tyrosine, cysteine). Although most enzymes contain many such amino acids and many states of ionization, alterations in amino acids at or near the active site are most likely to produce an effect in

binding events. If only one ionic state is catalytically active (as the limited pH range of activity of some enzymes suggests), a significant effect could be caused by the two groups which ionize (or deionize) first as the pH is shifted away from the optimum; additional ionization events would affect only the inactive enzyme (Dixon and Webb, 1979).

Ionizing groups, which in free amino acids dissociate at low or high pH levels, may have pK values that are altered by the proximity of other charged groups in a complex protein molecule (Lehninger, 1975; Dixon and Webb, 1979). Consequently, these groups may in some cases dissociate at pH levels that are closer to neutral in receptor proteins than in free amino acids. Proteins also contain groups (e.g., the imidazolium of histidine and the α-amino of cystine) with pK values in the intermediate (5.5–8.5) pH range (Edsall, 1943b). Thus, it is possible for ionizing groups at a protein receptor site to change charge in response to small pH changes at physiologically appropriate pH levels. Such changes may affect the binding activity of enzymes—and possibly also of chemoreceptors.

If the state of ionization of receptors can indeed affect the efficiency of amino acid–receptor binding, then we can expect different receptors to be affected differently by pH changes. The effects of pH change on chemoreception will depend on which ionizable groups are present at the active site of the receptor, and on the pK values of these groups. Clearly these factors vary among protein receptors. For example, in crayfish (Bauer et al., 1981) and eels (Yoshii et al., 1979), responses to neutral amino acids are not influenced by pH changes between 5 and 9, suggesting that the relevant binding sites on the protein involve groups that do not change charge between these values. Where chemoreception is highly pH dependent, for example in trout (Hara, 1976b) and skates (Novoselov et al., 1980), receptor sites presumably contain groups which change charge at pH levels where marked changes in the efficacy of stimulus–receptor binding are recorded electrophysiologically.

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NOVEL, SPECIES-TYPICAL ESTERS FROM PREPUTIAL GLANDS OF SYMPATRIC VOLES, *Microtus montanus* AND *M. pennsylvanicus*

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Abstract—Olfactory signals may facilitate species recognition between the sympatric voles, *Microtus montanus* and *M. pennsylvanicus*. In an effort to isolate and identify compounds that might contribute to such a chemical communication system, the preputial glands of those voles have been examined. Morphological examinations show both vole species possess preputial glands; however, the glands of *M. montanus* are much larger than those of *M. pennsylvanicus*. Gas chromatographic analysis revealed that the preputial glands of *M. montanus* contain a series of species-typical lipids that are not found in *M. pennsylvanicus*. Using gas chromatography–mass spectrometry and nuclear magnetic resonance spectrometry, the species-typical lipids were identified as esters of branched, saturated, and unsaturated C_5 and C_4 alcohols and straight-chain C_{16}, and "iso" branched C_{17} fatty acids. This is the first description of such esters from mammalian tissues. The results are discussed relative to the possibility that the species-typical esters act as species recognition cues for the sympatric voles.

Key Words—Preputial gland, lipids, esters, *Microtus montanus*, *Microtus pennsylvanicus*, species recognition, pheromone.

INTRODUCTION

The preputial glands are paired accessory sex glands found in all murid and many cricetid rodents. The glands are holocrine and primarily produce lipids;

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some of those lipids possess attractive properties and presumably act as pheromones (Bronson and Caroom, 1971; Brown and Williams, 1972; Gawienowski et al., 1975; Brinck and Hoffmeyer, 1984). The weight and lipid composition of the preputial glands are controlled by androgenic hormones (Sansone and Hamilton, 1967; Spener et al., 1969; Ebling, 1977; Mukherjea, 1977), and the presence of attractive compounds in the glands is also sensitive to the action of gonadal hormones (Gawienowski et al., 1975; Thody and Dijkstra, 1978; Lucas et al., 1982; Brink and Hoffmeyer, 1984). It has been suggested that the glandular products are released in connection with urinary marking (Bronson, 1976; Christiansen et al., 1978), and recent evidence supports that suggestion (Brinck and Hoffmeyer, 1984).

The voles *Microtus montanus* and *M. pennsylvanicus* are sympatric in southwestern Montana (Hodgson, 1972; Douglass, 1976) and are morphologically similar. However, the voles have marked karyotypic differences (Hsu and Bernischke, 1973) and do not hybridize (Gray et al., 1977). For these sympatric voles, the devices that facilitate the discriminations made between hetero- and homospecific mates are critically important. Olfactory signals can direct discriminations between hetero- and homospecifics in voles (Godfrey, 1958), deermice (Moore, 1965), and other rodents (Nevo et al., 1976). The importance of pheromonal communication in the voles *M. montanus* and *M. pennsylvanicus* has been demonstrated by several responses to chemical cues that affect reproductive physiology (Berger and Negus, 1982; Baddaloo and Clulow, 1980; Stehn and Jannett, 1981; Mallory and Clulow, 1977), and it is likely that these voles also use pheromones for species recognition cues.

In this communication we describe investigations of preputial glands as a source of species recognition pheromones. The preputial glands and the preputial gland lipids of adult male *M. montanus* and *M. pennsylvanicus* have been characterized. The compounds judged to be the most probable components of a pheromonal species recognition system have been identified. The chemical characteristics of the compounds identified are discussed with regard to a possible function as species recognition pheromones.

METHODS AND MATERIALS

Animals, Trapping, and Housing. Wild *M. montanus* and *M. pennsylvanicus* were live trapped from two sympatric populations during the summer breeding season from Gallatin and Madison Counties, Montana. The rodents were transported to the laboratory where they were housed one animal per cage under natural lighting conditions and supplied with Nutrena Feeds rabbit chow and water ad libitum. Sawdust shavings and cotton balls were provided for bedding material. A three-day laboratory acclimation period allowed the voles

time to groom themselves and remove any trap-related debris prior to collection of surface lipids. Wild voles classed as "adult" animals met the following criteria: (1) visible tubular development in the caudal epididymis (Boonstra and Youson, 1982; Christiansen et al., 1978); (2) scrotal testes; and (3) body weight greater than 25 g. Laboratory-raised *M. montanus* were used for determination of preputial gland weight in that species. These animals were housed as previously described (Rowsemitt and Berger, 1983) and were a gift from Dr. Carol Rowsemitt (University of Utah, Salt Lake City, Utah).

Lipid Collection, Extraction, and Storage. Voles were sacrificed by an overdose of ether vapor or by cervical dislocation. Preputial glands were excised and frozen in 3 ml water at −20°C until extracted. For lipid extraction the preputial glands were thawed, homogenized, and extracted by the method of Bligh and Dyer (1959). All lipids collected from the extractions were concentrated under a stream of nitrogen and stored in chloroform–methanol (2:1, v/v) at − 20°C, in a nitrogen atomosphere.

Chromatography and Spectra. Preparative thin-layer chromatography (TLC) on 250-μm silica layers (Si250 TLC plates; J.T. Baker Chemical Co., Phillipsburg, New Jersey) provided separation of the major lipids using a hexane–diethylether (95:10, v/v) solvent system. The TLC plates were sprayed with a rhodamine 6G solution (1 mg/ml in H_2O–ETOH; 1:1, v/v), and neutral lipid bands were visualized under UV light. Discrete areas were scraped from the plate, and the lipids were eluted from the adsorbent with hexane.

Gas chromatography (GC) of crude total lipid samples and the lipid fractions collected from TLC plates employed a Varian 3700 gas chromatograph (Varian Associates, Sugar Land, Texas) equipped with a flame ionization detector. Chromatographic separations for routine analysis were accomplished with two columns: (1) a 2-mm-ID × 2-m glass column packed with 3% SE-30 on Supelcoport 100/200; and (2) a 30-m, DB-1 (0.250-μm film; 0.25-mm-ID) capillary column (J and W Scientific, Inc., Rancho Cordova, California). A 30-m, DB-225 (0.250-μm film; 0.25-mm-ID) capillary column (J and W Scientific) was used to investigate the double-bond configuration of certain compounds. Details of GC parameters are given in the figure legends. Separation of neutral esters by preparative gas chromatography was accomplished using a thermal conductivity detector and a 1.25-m × 6-mm-ID, stainless-steel column packed with 5% SE-52 on Chromosorb 100/200 (isothermal, 260°C).

Gas chromatography–mass spectrometry (GC-MS) utilized a VG Instruments 7070E mass spectrometer (VG Instruments, Stamford, Connecticut), operating at 70 eV, which was coupled to a Varian 3700 gas chromatograph equipped with a 30-m DB-5 (0.250-μm film, 0.25-mm-ID) capillary column (J and W Scientific). The column was routinely programmed from 170 to 250°C at 2°C/min to provide resolution of the natural esters prior to introduction into

the ion source. Alcohols investigated by GC-MS were chromatographed (DB-5 column) at 25°C, isothermal for 5 min, then programmed at 5°C/min. A Supelcowax 10 (30-m, 0.25-μm film, 0.25-mm-ID) capillary column (Supelco, Bellefonte, Pennsylvania) was used for certain free alcohol analyses.

Nuclear magnetic resonance (NMR) spectra were obtained in a Fourier transform mode on a Bruker WM-250 MHz NMR spectrometer (Bruker, Billerica, Massachusetts) in $CDCl_3$. Chemical shifts were measured relative to tetramethylsilane.

Ester Synthesis. Hexadecanoic acid (Sigma, St. Louis, Missouri), (*Z*)-9-hexadecenoic acid (Sigma), and 15-methylhexadecanoic acid (Accurate Chemical Co., Westbury, New York) were converted to acid chlorides by reaction with thionyl chloride (50°C, 1 hr) (Aldrich, Milwaukee, Wisconsin). Synthetic esters were prepared by reacting acid chlorides with alcohols (50°C, 45 min) from the following list: 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol (J.T. Baker Chemical Company), and 3-methyl-2-buten-1-ol (Aldrich). 2-Methyl-(*E*)-2-buten-1-ol was synthesized by reducing 2-methyl-(*E*)-2-butenoic acid (Aldrich) with lithium aluminum hydride and esterified as described above. The reaction mixtures were diluted with water, and the synthetic esters were extracted into hexane and purified over a column of Bio-Sil A (Bio-Rad Laboratories, Richmond, California).

Fatty acid methyl esters of the vole lipids were produced by transesterifying natural esters with Meth-Prep II (Applied Science, Inc., Deerfield, Illinois). The methyl ester of (*E*)-9-hexadecenoic acid was purchased from Sigma. Other methyl ester standards were purchased from Sulpelco and Applied Science.

Other Analytical Techniques. For location of double bonds, unsaturated lipids were subjected to ozonolysis (Beroza and Bierl, 1967). Gas chromatography was used to compare the ozonolysis products with aldehyde standards. The chromatography was achieved on a 2-m × 2-mm-ID glass column packed with 3% Silar 5 CP. The column temperature was held at 60°C for 5 min and then programmed to 250°C at 10°C/min and held at 250°C for 21 min.

Free alcohols from the natural esters were analyzed after microsaponification of approximately 20 μg of the natural ester with 10 μl of 5% KOH in methanol. The reaction mixture was heated at 45°C for 30 min, and the free alcohols were extracted into 10 μl of methylene chloride.

Microhydrogenation, over palladium catalyst, of approximately 20 μg of the natural esters was performed as described by Parliment (1973).

Histological Preparations. Tissues were fixed in 4% neutral buffered formalin, dehydrated through graded alcohols (70%, 95%, 100% ethanol), cleared with xylene, and embedded in paraffin. Tissue sections (5 μm) were cut and stained with a routine hematoxylin and eosin procedure.

RESULTS

Preliminary Characterization. Gross morphologic differences between the preputial glands of *M. montanus* and *M. pennsylvanicus* are readily apparent. The glands of the *M. montanus* closely resemble the superficial morphology of lab mouse preputial glands and possess a distinct vesicular appearance with a distal arrangement of bulbar enlargements filled with a clear, amber colored, viscous oil. In contrast, the *M. pennsylvanicus* preputial glands are minute and contain a thick, waxy secretion (*M. montanus* preputial gland weight = 68.8 mg $\pm$ 27.4, $N = 11$; *M. pennsylvanicus* preputial gland weight = 9.8 mg $\pm$ 1.9, $N = 10$; $P < 0.002$ Mann-Whitney U test). Due to the small size and unusual appearance of the preputial glands in *M. pennsylvanicus*, the actual presence of preputial glands in that species was questionable. Subsequent examination of hematoxylin–eosine-stained *M. montanus* preputial glands and the questioned tissues in *M. pennsylvanicus* revealed that the cellular structures of the tissues from both species were identical. The cytologic similarities indicated that the *M. pennsylvanicus* possess preputial glands.

Different lipid profiles for the two species (Figure 1) were revealed by gas chromatography of 10 μg of mature male preputial gland total lipids (3%, SE-30; detection limit = 10 ng). The chromatograms presented are each from the lipids of one individual but are characteristic of chromatograms from the 23 wild, mature male *M. montanus* and the 14 wild, mature male *M. pennsylvanicus* examined. The presence of the species-characteristic peaks at a retention time of about 5 min in *M. montanus* and their absence in *M. pennsylvanicus* was consistent in all individuals examined. The *M. montanus*-specific lipids (Figure 1) were not found in brain, liver, kidney, muscle, or adipose tissues taken from mature male *M. montanus*. Additionally, those same compounds were not present in the preputial gland lipids of mature male laboratory rats (Holtzman albino, $N = 6$) and mice (CD1, $N = 5$).

Defining the general compound class to which the *M. montanus* specific lipids belong was accomplished by TLC. Total preputial gland lipids were fractioned by preparative TLC and specific lipids from discrete TLC adsorbent bands were collected and analyzed by GC. The gas chromatograms showed that the TLC band corresponding to the *M. montanus* typical lipids ($R_f = 0.60$) migrated just below a wax ester standard (hexadecyl stearate, $R_f = 0.68$) but above an alkyl acetate standard (hexadecyl acetate, $R_f = 0.40$). The GC peaks (Figure 1) at 13 min and 15 min ($R_f = 0.68$) cochromatographed with hexadecyl stearate. The gas chromatographic behavior (3% SE-30) of the *M. montanus* typical lipids was compared to that of known standards, and the resulting chromatograms showed those compounds had retention times similar to C_{22} and C_{23} hydrocarbon standards. The *M. montanus* lipids that had retention times of

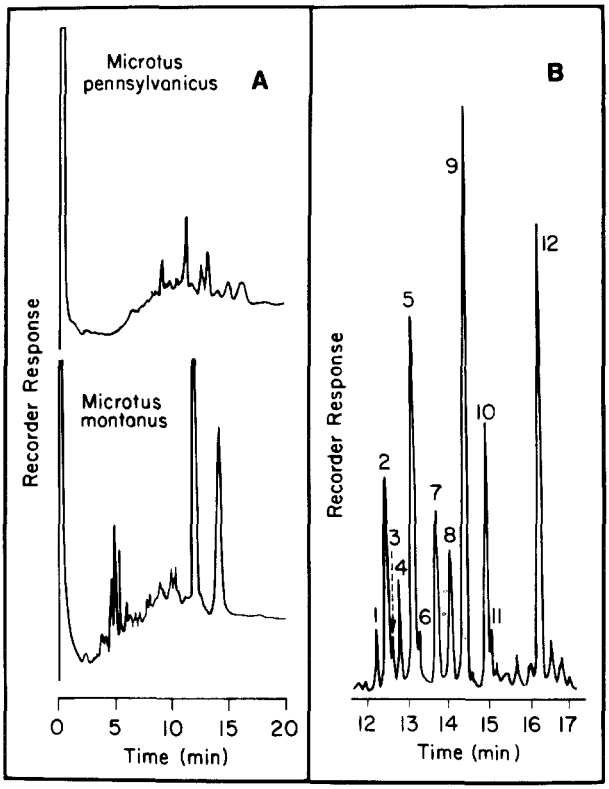

FIG. 1. Gas chromatograms of total preputial gland lipids from *Microtus montanus* and *M. pennsylvanicus* (panel A) and a capillary gas chromatogram of the species-typical lipids from *M. montanus* (panel B). The chromatograms in panel A illustrate the resolution of total preputial gland lipids (10 μg) on a 2-m × 2-mm-ID glass column packed with 3% SE-30; column temperature was programmed from 180° to 295°C at 15°C/min. Panel B shows capillary column resolution of the compounds from *M. montanus* which elute from the packed column (panel A) at a retention time of about 5 min. The chromatography was achieved on a 30-m × 0.25-mm DB-1 column (0.250-μm film) that was temperature programmed from 170° to 250°C at 2°C/min.

approximately 13 and 15 min (Figure 1) eluted from the SE-30 column between C_{32} and C_{34} hydrocarbon standards.

Capillary GC resolved the species-typical lipids as shown in Figure 1. The structures corresponding to the numbered peaks were investigated. The molecular weights of the lipids, determined by mass spectrometry, are listed in Table 1. High-resolution mass spectra indicated each compound contained two oxygen atoms (Table 1), consistent with the ester function. Fragment peaks in the

TABLE 1. STRUCTURAL EVIDENCE BASED ON HIGH-RESOLUTION GC-MS OF SPECIES-TYPICAL LIPIDS FROM PREPUTIAL GLANDS OF ADULT MALE *M. montanus*

GC peak	Molecular weight	Molecular formula	Number of alkyl double bonds
1	326	$C_{21}H_{42}O_2$	0
2	324	$C_{21}H_{40}O_2$	1
3	324	$C_{21}H_{40}O_2$	1
4	324	$C_{21}H_{40}O_2$	1
5	326	$C_{21}H_{42}O_2$	0
6	326	$C_{21}H_{42}O_2$	0
7	322	$C_{21}H_{38}O_2$	2
8	322	$C_{21}H_{38}O_2$	2
9	324	$C_{21}H_{42}O_2$	1
10	340	$C_{22}H_{44}O_2$	0
11	340	$C_{22}H_{44}O_2$	0
12	338	$C_{22}H_{42}2_2$	1

mass spectra suggested the lipids to be esters derived from 16- and 17-carbon acids and 4- and 5-carbon alcohols; either moiety could be saturated or monounsaturated. Each spectrum contained fragment peaks $(M - RCOOH)^+$ (e.g., $m/z = 68$ or 70 in Figure 2), which are characteristic of esters containing long-chain fatty acids. In addition, peaks corresponding to $(RCOOH)^+$ and $(RCOOH + H)^+$ were also seen ($m/z = 256$ and 257 in Figure 2). Finally, acylium ions, resulting from alpha cleavage at the carbonyl, were present in the spectra ($m/z = 239$ in Figure 2).

The species-typical lipids were separated into three fractions by preparative GC. The chromatography resolved peaks 1–6 from peaks 7–11 and isolated peak 12. Hydrolysis of each GC fraction permitted GC and GC-MS analysis of the resulting alcohols. The acyl moieties were analyzed as fatty acid methyl esters.

Alcohol Moieties. The species-typical lipids contain two pairs of isometric C_5 alcohols and one C_4 alcohol (Table 2). The saturated C_5 alcohols eluted from GC before *n*-pentanol and were, therefore, believed to be branched. Mass spectra of the saturated alcohols were diagnostic for 3-methyl- and 2-methyl-1-butanols (Table 2). Mass spectra and GC retentions of the natural alcohols and respective authentic standards were identical. The configuration at the asymmetric center of 2-methyl-1-butanol was not determined.

Mass spectra showed that the remaining C_5 alcohols had molecular weights of 86 (one degree of unsaturation), but analysis by NMR was required to determine their structures. The NMR spectrum of peak 12 (Figure 1), which had

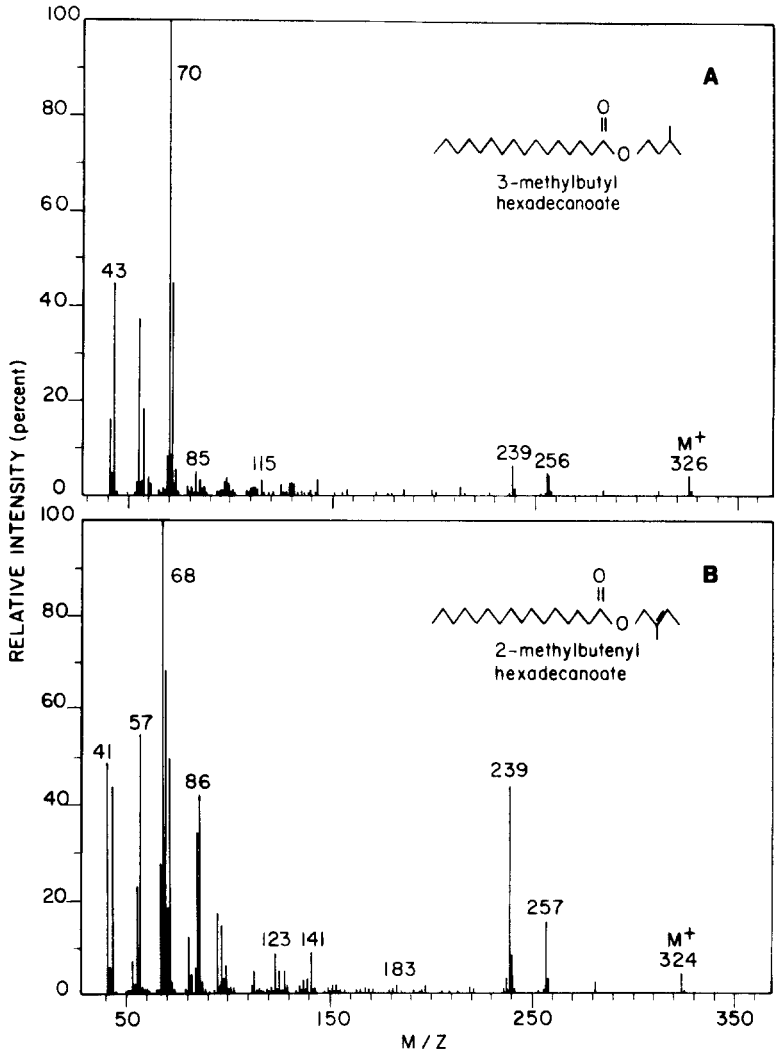

FIG. 2. Mass spectra of peak 5 (panel A) and peak 9 (panel B) of the species-typical esters from the preputial glands of adult male *Microtus montanus*. The spectra are nominal mass enhanced; structure assignments are based on GC, GC-MS, and NMR analyses.

been isolated by preparative GC, indicated the alcohol moieties were 2-methyl- and 3-methyl-2-buten-1-ol. For the ester of 2-methyl-2-buten-1-ol, the definitive NMR resonances were: δ4.44 (2H, s, $-C\underline{H}_2O-$); δ5.54 (1H, q, J = 7 Hz, $CH_3-C\underline{H}=$), and for the ester of 3-methyl-2-buten-1-ol: δ4.49 (2H, d, J

TABLE 2. HYDROLYSIS PRODUCTS OF SPECIES-TYPICAL ESTERS FROM PREPUTIAL GLANDS OF ADULT MALE *Microtus montanus*

Structure assignments	GC fraction[a]			Analytical evidence[b]				
	Peaks 1–6	Peaks 7–11	Peak 12	GC	MS	NMR	O_3	Characteristic MS ions[c]
Alcohols								
2-Methyl-1-propanol[d]	+							
3-Methyl-1-butanol	++	++		+	+			41(89), 42(30), 55(42), 56(90), 57(100), 70(49), M^+ not found
2-Methyl-1-butanol	+	+		+	+			41(73), 42(94), 43(66), 55(100), 57(20), 70(69), M^+ not found
2-Methyl-2-buten-1-ol		++	++	+	+	+		41(44), 43(29), 53(25), 67(15), 68(16), 71(100), M^+ = 86(52)
3-Methyl-2-buten-1-ol[e]			+	+	+	+		41(50), 43(30), 53(24), 67(14), 68(18), 71(100), M^+ = 86(45)
Acids[f]								
(Z)-9-Hexadecenoic acid	+	+		+	+		+	41(100), 55(85), 74(30), 87(21), 143(2), 237(7), M^+ = 268(2)
Hexadecanoic acid		++	++	+	+	+		74(100), 87(58), 143(22), 227(14), 239(7), M^+ = 270(10)
15-Methylhexadecanoic acid	++	++		+	+	+		74(100), 87(69), 143(10), 241(6), 253(2), M^+ = 284(11)

[a] The species-typical esters were resolved by preparative GC as indicated. A + indicates presence in a particular GC fraction; ++ indicates the major alcohols or acids in a given GC fraction.

[b] A + under GC, MS, NMR, and O_3 (ozonolysis) indicates the natural product showed behavior identical to known standards when analyzed by the given techniques.

[c] *m/z* (relative intensity).

[d] 2-Methyl-1-proponal was not recovered from the ester hydrolysis products but was indicated by mass spectral data for peak 1 and was confirmed by GC and GC-MS analyses of peak 1 and known standards.

[e] The mass spectra of 2-methyl-2-buten-1-ol and 3-methyl-2-buten-1-ol were almost identical. NMR analysis provided definitive structure assignments of those alcohols.

[f] The fatty acids were analyzed as fatty acid methyl esters.

= 7 Hz, $-C\underline{H}_2O-$); δ5.32 (1H, t, J = 7, $=C\underline{H}-CH_2O-$). Hexadecanoic esters of synthetic 2-methyl-(*E*)-2-buten-1-ol and 3-methyl-2-buten-1-ol produced NMR resonances identical to those listed above. Peak integrations of the resonances from the natural esters indicated the ester of 2-methyl-2-buten-1-ol to be the predominant isomer (ratio of isomers = 4:1).

The configuration of the double bond in 2-methyl-2-buten-1-ol has not been unequivocally established. However, in the NMR spectra of trisubstituted olefins, the olefinic hydrogens of *E* and *Z* isomers frequently exhibit different chemical shifts (Silverstein et al., 1981). The resonances of the alcohol moiety from the natural ester were identical to those of the hexadecanoic ester of synthetic 2-methyl-(*E*)-2-buten-1-ol, which strongly supports the *E* configuration in the natural ester.

These unsaturated alcohols were not separated by capillary GC, despite concerted efforts, nor were their esters. The natural alcohols did show identical GC retentions with the synthetic standards, and their mass spectra were in agreement also. The mass spectra of the two unsaturated alcohols were nearly identical.

Finally, the mass spectrum of GC peak 1 suggested the presence of a C_4 alcohol (M^+-56 and M^+-57). Based on comparison of GC retention and mass spectra of the natural and synthetic esters, that alcohol was determined to be 2-methyl-1-propanol. The C_4 alcohol was not recovered after hydrolysis of the natural esters.

Acyl Moieties. The species-typical esters contain three major fatty acids (Table 2). Hexadecenoic acid was characterized by GC and GC-MS and was identical to an authentic standard. (*Z*)-9-Hexadecenoic acid is the most abundant unsaturated fatty acid. The structure is supported by GC and GC-MS data. The position of the double bond was determined by analysis of ozonolysis products, and the double-bond configuration, by comparing GC retention (DB-225) of the methyl ester with standard methyl (*Z*)- and (*E*)-9-hexadecenoates. Finally, 15-methylhexadecenoic acid was identified by NMR, GC, and GC-MS. The iso configuration was obvious from the NMR spectrum of peak 12 (δ0.84, 6H, d, J = 7 Hz). This signal collapsed to a singlet upon irradiation at δ1.62. The NMR data eliminated other methyl group positions from consideration. The GC retention and mass spectrum of the natural acid methyl ester were identical to those of the authentic standard.

Ester Structures. The assigned structures of the species-typical esters are listed in Table 3. These were based on the acids and alcohols identified from the hydrolysis of preparative GC fractions. In every case, the natural and synthetic esters had identical GC retentions and mass spectra.

It was learned from the synthetic ester standards that only those with 2-methyl-2-buten-1-ol produced an appreciable mass spectral fragment at m/z = 86 (Figure 2). This fragment had an intensity of less than 0.5% in esters of

TABLE 3. STRUCTURAL ASSIGNMENTS FOR SPECIES-TYPICAL LIPIDS FROM PREPUTIAL GLANDS OF ADULT MALE *Microtus montanus*

GC Peak	Compound name	Analytical evidence[a]				
		GC	NMR	H_2	MS	Characteristic MS ions[b]
1	2-Methylpropyl 15-methylhexadecanoate	+			+	57(100), 70(23), 253(3), 270(15), 271(21), M^+ = 326(6)
2	3-Methylbutyl (*Z*)-9-hexadecenoate	+		+	+	71(100), 236(33), 237(27), 256(2), M^+ = 324(5)
3	2-Methylbutyl (*Z*)-9-hexadecenoate	+		+	+	71(100), 236(23), 237(30), 256(3), M^+ = 324(2)
4						71(100), 236(15), 237(19), 256(2), M^+ = 324(4)
5	3-Methylbutyl hexadecanoate	+			+	70(100), 239(7), 256(4), 257(5), M^+ = 326(3)
6	2-Methylbutyl hexadecanoate	+			+	70(100), 239(16), 256(12), 257(5), M^+ = 326(2)
7	2-Methylbutenyl (*Z*)-9-hexadecenoate[c]	+		+	+	69(100), 86(5), 235(13), 237(4), 253(6), M^+ = 322(0.5)
8						69(100), 86(5), 235(13), 237(3), 253(6), M^+ = 322(0.5)
9	2-Methylbutenyl hexadecanoate[d]	+		+	+	68(100), 86(30), 239(30), 257(10), 258(3), M^+ = 324(5)
10	3-Methylbutyl 15-methylhexadecanoate	+			+	70(100), 253(3), 270(5), 271(4), M^+ = 340(2)
11	2-Methylbutyl 15-methylhexadecanoate					70(100), 253(7), 270(13), 271(8), M^+ = 340(2)
12	2-methylbutenyl 15-methylhexadecanoate[e] and	+	+	+	+	68(100), 86(38), 253(11), 270(2), 271(13), M^+ = 338(6)
	3-methylbutenyl 15-methylhexadecanoate	+	+	+	+	

[a] A + under GC, MS, NMR, and H_2 (catalytic hydrogenation) indicates chromatography, spectra, and hydrogenation products are identical to known standards.

[b] *m/z* (relative intensity).

[c] 3-Methylbutenyl (*Z*)-9-hexadecenoate may also be present.

[d] 3-Methylbutenyl hexadecanoate may also be present.

[e] 2-Methylbutenyl 15-methylhexadecanoate accounts for ~70% of the area under peak 12. Available evidence indicates a *trans* double bond.

3-methyl-2-buten-1-ol. Thus, the predominant isomer of peaks 7 and 9 could be determined from the mass spectra even though the isomers were not separated by GC.

Hydrogenation of the species-typical lipids also supported the assigned structures. Gas chromatograms of the hydrogenation products showed an increase in the quantity of the expected saturated esters and a concomitant loss of the unsaturated compounds. The hydrogenated esters were analyzed by GC-MS, and the spectra of the resulting saturated products were consistent with the assigned structures.

The analytical techniques used could not provide definitive structure assignments for peaks 4 and 8. However, some information was obtained. The peak 4 and peak 8 esters possess an unsaturated C_{16} fatty acid. From GC retention comparisons, the fatty acid is not (*Z*)- or (*E*)-9-hexadecenoic acid. It is probable that the fatty acid of the peak 4 and 8 esters is a double-bond positional isomer of (*Z*)-9-hexadecenoic acid. Hydrogenation of peaks 4 and 8 produces 3-methylbutyl and/or 2-methylbutyl hexadecenoate by GC retention. From the mass spectra and the proportions of the various alcohol moieties in the other esters, peak 4 is believed to contain an ester of 3-methyl-1-butanol and peak 8, an ester of 2-methyl-2-buten-1-ol. The mass spectrum of peak 8 contains the $m/z = 86$ peak which is diagnostic for this alcohol (Table 3).

DISCUSSION

The preputial glands of adult male *M. montanus* are much larger than the glands of adult male *M. pennsylvanicus*. Treatment of *M. montanus* with testosterone propionate increases the weight of the preputial glands (Jannett, 1978). More recently, Rowsemitt et al., 1988 have confirmed the androgen sensitivity of the preputial glands in *M. montanus* and have shown that levels of the species-typical esters described in this report are also androgen-dependent. Similar findings of androgens affecting the size and content of the preputial glands have been reported by other investigators (Sansone and Hamilton, 1967; Spener et al., 1969; Ebling, 1977). It is not known if preputial gland growth in *M. pennsylvanicus* responds to androgens in the same manner as do the preputial glands of *M. montanus*.

The preputial glands of mature male *M. montanus* contain novel esters. Those esters are composed of 2-methyl- and 3-methyl-1-butanols; 2-methyl- and 3-methyl-2-buten-1-ols; and C_{16} and C_{17} fatty acids. This report is the first to describe fatty acid esters containing saturated and unsaturated C_5 alcohols in mammalian tissues. Using GC retention comparison techniques, the presence of methyl branched fatty acids from laboratory mouse preputial glands (Sansone and Hamilton, 1967; Snyder and Blank, 1969) has been suggested. The struc-

ture of the 15-methylhexadecanoic acid reported here has been characterized by GC, GC-MS, and NMR.

Diversity and complexity of scent gland compounds is common in mammalian chemical communication systems (Stoddart et al., 1975; Stoddart, 1974; Epple et al., 1979; see Beauchamp et al., 1976, for a review). If the species-typical esters of *M. montanus* preputial glands are pheromones, they certainly fit that pattern of complex odor formulations. The occurrence of at least 12 compounds that differ by the position or presence of only one carbon is an indication of the complexity of the *M. montanus* species-typical lipids. Finding two compounds under peak 12 demonstrates the similarity of certain compounds. It is apparent that given a few predominant alcohols and fatty acids, the vole preputial glands produce esters in a manner that gives considerable end-product diversity.

The uniqueness and physical properties of the *M. montanus* preputial gland esters suggest they may serve as volatile chemical signals. The molecular weights of the species-typical esters from *M. montanus* range from 322 to 340; all compounds have total carbon numbers of either 21 or 22. Esters of butyric acid ranging from 21 to 28 total carbons (molecular weight range = 310–410) have been described as chemical signals that convey species and subspecies information (Epple et al., 1979). Another study has demonstrated rodent olfactory responses to esters containing 22 carbon atoms (Stacewicz-Sapuntzakis and Gawienowski, 1977). With respect to general compound class, total carbon numbers, and molecular weights, the novel esters from the *M. montanus* preputial glands are similar to previously described odorous compounds that demonstrate attractive properties.

It has been theorized (Wilson and Bossert, 1963) that through evolution, the chemical properties of a pheromone are manipulated to maximize effectiveness for a given function. A hypothetical pheromone should be composed of the most efficient formulation of "persistent" vs. "diffusion" qualities: the relative proportion of those qualities will depend on the pheromone function. It follows that chemosignals mediating short-lived phenomena, for instance alarm signals, should possess a readily diffusible, highly volatile, nature. On the other hand, in order to maximize the communicative efficiency of the sender, home-range marking signals should be more persistent. This persistence allows an animal to advertise a signal over a long distance for a long period of time and requires only minimal redeposition to ensure constant signal emission.

The persistence design feature of pheromones is not without limitations; volatility is correlated with molecular weight in a given homologous series. As molecular weight (and persistence) increases, eventually vapor-phase existence of a molecule becomes almost impossible. The upper practical volatility limit then is an important design factor for pheromones. Wilson and Bossert (1963) suggested that, due to physiochemical characteristics restricting volatility of

large compounds, the upper-limit molecular weights of volatile pheromones would fall in the range of 300–400. The compounds that facilitate species and subspecies recognition in tamarins (Epple et al., 1979) span a molecular weight range of 310–410. The *M. montanus* species-typical preputial gland esters have molecular weights ranging from 322 to 340. The tamarin species recognition cues as well as the identified *M. montanus* preputial gland esters possess molecular weights in the range of what might be predicted for species-recognition pheromones.

This investigation represents the initial work aimed at identifying a rodent species recognition pheromone. Novel, species-typical esters from the preputial glands of adult male *M. montanus* have been identified, and those esters possess molecular characteristics similar to the esters that can function as species-recognition cues (Epple et al., 1979). Although we do not know the function of the species-typical esters, there are no data which argue against those novel esters serving as species-recognition pheromones. We are currently using the compounds isolated for this investigation in bioassays designed to assess the attractive properties of the species-typical esters.

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ELECTROANTENNOGRAM RESPONSES OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*, TO A SPECTRUM OF PLANT VOLATILES

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Abstract—Electroantennograms (EAGs) were recorded from unmated, laboratory-reared, male and female *Ceratitis capitata* (medfly) in response to a range of C_1 and C_2 to C_{12} carbon chain-length aliphatic alcohols, aldehydes, acetates, and acids, and lactones, some of which are known volatiles from leaves and fruits. A large degree of EAG response uniformity between the sexes was observed, with only eight of the 70 compounds tested eliciting significantly larger amplitude EAG responses from female than male antennae. In general, for the five functional-group series tested, aldehydes and alcohols elicited greater responses than acetates, lactones, and acids. The unsaturated alcohols, aldehydes, acetates, and acids elicited equal or larger amplitude EAG responses than their comparable saturated compounds. For four of the functional-group series tested, the EAG response amplitude was significantly greater for a particular carbon chain length, with responsiveness to primary alcohols and aldehydes peaking at C_6, acids peaking at C_{5-6}, and acetates peaking at both C_5 and C_8. The EAG responses to both the 2- and 3-position monoenic alcohols peaked at C_6 and C_8, while the secondary alcohols peaked at C_7. The greatest EAG responses of all compounds tested were elicited by monoenic C_6 alcohols and aldehydes that are constituents of the "general green-leaf odor" that emanates from most plants. The potential adaptive benefit of selective sensitivity to green-leaf volatiles is discussed in regard to foraging behavior of medflies.

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Key Words—Diptera, Tephritidae, Mediterranean fruit fly, *Ceratitis capitata*, plant volatiles, fruit volatiles, green-leaf volatiles, olfaction, electrophysiology, electroantennogram.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (medfly), is a polyphagous pest worldwide, attacking over 250 varieties of fruits, nuts, and vegetables (Hagen et al., 1981). It is widely assumed that medflies and other tephritid fruit flies use olfactory cues, in addition to visual cues (Cytrynowicz et al., 1984; Nakagawa et al., 1978; Prokopy and Roitberg, 1984; Prokopy and Economopoulos, 1976), to seek and assess habitat, food, and ovipositional resources (Prokopy and Roitberg, 1984). Such a phenomenon has been observed in studies of the behavior of the apple maggot, *Rhagoletis pomonella* (Walsh), in response to fruit model traps emanating apple volatiles (Prokopy et al., 1973; Reissig et al., 1982). However, there is little behavioral evidence (Bateman, 1972; Féron, 1962; Keiser et al., 1975) to substantiate this assumption of olfactory orientation to plant volatiles by *C. capitata*.

The olfactory fragrance of a plant and, in particular, a ripening fruit, is a complex blend of usually over a hundred(s) detectable volatile compounds, possessing various functional groups and ranging in structure from simple, short, straight carbon chains to complex multiring sesquiterpenes (e.g., Buttery, 1981; Van Straten and Maarse, 1983) (Table 1). Although the host-plant range of medflies is extremely broad and diverse, most of the host-plant and fruit odors will share some constituents from various classes of volatiles, e.g., aliphatic alcohols, aldehydes, acetates and acids, and monoterpenes, sesquiterpenes, and lactones (Buttery, 1981; Van Straten and Maarse, 1983; Visser et al., 1979) (Table 1).

Little is known about the neurophysiological reception of plant volatiles by Diptera in general, let alone *C. capitata* and other tephritid species. To date, only four electrophysiological studies have been reported on tephritid fruit flies. Recently, Van Der Pers et al. (1984) utilized the electroantennogram (EAG) technique to study the responsiveness and sensitivity of male and female *Dacus oleae* (Gmelin) (olive fruit fly) to six components of its putative pheromones and five chemical analogs, some of which are commonly occurring plant volatiles. An EAG study on tephritids that tested "generally occurring fruit volatiles" was briefly reported by Guerin et al. (1983a). They recorded EAGs from female antennae of three species, *D. oleae, Rhagoletis cerasi* (L.) (cherry fruit fly), and *C. capitata*. Although 30 volatiles were tested (series of aliphatic C_5 to C_{12} terminal position alcohols, aldehydes, and esters), only heptanal, octanal, nonanal, and (*E*)-2-nonenal were reported to elicit the highest amplitude

TABLE 1. SOURCE AND PURITY OF CHEMICALS USED IN ELECTROPHYSIOLOGICAL STUDIES AND THEIR PRESENCE IN A VARIETY OF HOST FRUITS OF *Ceratitis capitata*

Compound	Chemical purity (%)[a]	Source[b]	Presence in fruit of			
			Citrus[c,d]	Papaya[e]	Passion fruit[d]	Peach[d]
Aliphatic alcohols						
Ethanol	100.0	A	+	+	+	+
Propan-1-ol	95.0	B	+	+	+	
2-Propen-1-ol	99.0	C				
Butan-1-ol	100.0	D	+	+	+	+
(*E*)-2-Buten-1-ol	92.3	B				
3-Buten-1-ol	99.1	D				
Pentan-1-ol	99.5	D	+	+	+	+
(*E*)-3-Penten-1-ol	97.0	C				
Hexan-1-ol	100.0	D	+	+	+	+
(*E*)-2-Hexen-1-ol	95.9	D	+	+		+
(*Z*)-2-Hexen-1-ol	99.5	D				
(*E*)-3-Hexen-1-ol	100.0	D			+	
(*Z*)-3-Hexen-1-ol	99.9	D	+	+	+	
Heptan-1-ol	98.0	D	+	+	+	
(±)-Heptan-2-ol	99.2	D			+	
Octan-1-ol	100.0	D	+	+	+	+
(*E*)-2-Octen-1-ol	92.0	C				
(*E*)-3-Octen-1-ol	84.7	C			+	
(±)-1-Octen-3-ol	99.5	D	+			
Nonan-1-ol	100.0	D	+			
(±)-Nonan-2-ol	98.8	D	+		+	
Decan-1-ol	99.0	D	+			
Undecan-1-ol	96.5	B	+			
(±)-Undecan-2-ol	98.0	E				
Dodecan-1-ol	96.9	F	+			+

TABLE 1. (Continued)

Compound	Chemical purity (%)[a]	Source[b]	Presence in fruit of			
			Citrus[c,d]	Papaya[e]	Passion fruit[d]	Peach[d]
Aliphatic aldehydes						
Propanal	54.0	B				
Butanal	67.0	B	+			+
(*E*)-2-Butenal	93.9	F				
Pentanal	99.0	D	+			
Hexanal	98.3	D	+			+
(*E*)-2-Hexenal	98.6	D	+			+
Heptanal	98.1	D	+			+
Octanal	95.5	D	+			
Nonal	91.1	G	+			+
Decanal	97.9	D	+			
Undecanal	91.0	D	+			
Dodecanal	89.5	O	+			
Aliphatic acids						
Formic	88.0	H				+
Acetic	99.9	I	+		+	+
Propanoic	99.8	J	+			
2-Propenoic	95.0	B				
Butanoic	99.0	J	+		+	+
Pentanoic	99+	K	+		+	+
Hexanoic	98.2	D	+		+	+
(*E*)-2-Hexenoic	99.0	D			+	+
Heptanoic	98.2	D	+		+	
Octanoic	100.0	D	+		+	+
Nonanoic	94.1	D	+		+	

Decanoic	99+	D	+		+	+
Dodecanoic	99+	F	+		+	
Aliphatic esters						
Ethyl acetate	99+	H	+	+	+	+
Propyl acetate	98.0	F		+		
Butyl acetate	98.0	B	+	+	+	+
Pentyl acetate	37.0	O	+	+		+
Hexyl acetate	92.0	O		+	+	+
(*E*)-2-Hexenyl acetate	99.7	L				+
Heptyl acetate	97.0	B				
Octyl acetate	98.4	O	+		+	
Nonyl acetate	97.0	C	+			
Decyl acetate	95.0	C	+			
Lactones						
gamma-Butyrolactone	99+	M			+	
gamma-Pentalactone	96.6	D				+
gamma-Hexalactone	98.0	C		+	+	+
gamma-Heptalactone	88.2	M		+	+	+
gamma-Octalactone	95.0	D		+	+	+
gamma-Nonalactone	88.6	N		+	+	+
gamma-Decalactone	97.0	D		+	+	+
gamma-Undecalactone	97.1	N			+	+
mixture of C_8–C_{12} delta-lactones	90+	P		+	+	+

[a] Capillary GLC analysis (12.5-m × 0.2-mm methyl silicone cross-linked column) at USDA-ARS-WRRC, Albany, California.

[b] A, U.S. Industrial Chemicals; B, Eastman Kodak Co.; C, synthesized at USDA-ARS-WRRC, Albany, California; D, Aldrich Chemical Co.; E, Fluka Chemical Co.; F, Chem Service, Inc.; G, Fritzsche, Dodge and Olcott, Inc.; H, Fisher Scientific Co.; I, J.T. Baker Chemical Co.; J, Mallinckrodt Chemical Co.; K, Sigma Chemical Co.; L, CTS Organics; M, K&K Laboratories; N, Norda Chemical Co.; O, source presently unknown, from file at USDA-ARS-WRRC, Albany, California; P, Oril Chemical Co.

[c] Kefford and Chandler (1970).

[d] Van Straten and Maarse (1983).

[e] Flath and Forrey (1977).

EAGs. In addition, Fein et al. (1982) recorded the EAGs of *R. pomenella* to a group of seven esters they identified from apple odor.

The purpose of this initial study was to investigate, by means of EAGs, peripheral olfactory selectivity of adult *C. capitata* to commonly occurring classes of host-plant and fruit volatiles, including the ubiquitous "general green-leaf volatiles" (Visser et al., 1979). The volatiles tested ranged from C_1 or C_2 to C_{12} saturated and unsaturated aliphatic alcohols, aldehydes, acids, acetates, and a series of lactones (Table 1). This research was further intended to provide a basis for studies of single-cell responses by surveying and assessing the degree that the antennal olfactory system of medflies is receptive to classes and/or particular plant/fruit volatiles.

METHODS AND MATERIALS

Insects. Pupae of *C. capitata* were obtained from a laboratory colony, periodically infused with feral flies, maintained at the USDA, Tropical Fruit and Vegetable Research Laboratory, Honolulu, Hawaii. Upon their arrival, pupae were segregated by sex and placed in separate cages. After eclosion, adult flies were provided sucrose cubes, hydrolyzed protein, and water until they were tested two to five days after emergence.

Olfactory Stimuli. Table 1 lists the compounds tested, their supply sources, and purities. The compounds were dissolved in spectrometric grade hexane (that was additionally distilled and treated with an antioxidant) at a rate of one part test compound and nine parts hexane solvent, forming 10% volume per volume solutions. From these solutions, test cartridges were produced for each compound by pipetting 1-μl aliquots onto separate 1 × 2-cm pieces of fluted, glass-fiber filter paper, which were then inserted into individual Pasteur pipets. Before or during each experiment, new cartridges were loaded with compound, individually sealed in zip-lock plastic bags, placed in a freezer (−4°C), and then transferred to a fume hood just prior to testing.

Electrophysiological Recording Technique. Electroantennogram (EAG) techniques used here are a modification of previous techniques utilizing glass capillary Ag–AgCl electrodes filled with insect saline (Schneider, 1957a; Light, 1983). Intact flies were immobilized by a yoke in a Plexiglas block. The recording electrode was inserted into the distal region of the terminal antennal segment or funiculus, while the indifferent electrode was positioned into the hemocoel of the cranial cavity. The signal was amplified 100× by a Grass P-16 microelectrode amplifier (Quincy, Massachusetts) and viewed on either an analog (Tektronix 5113, Beaverton, Oregon) or digital (Nicolet 4094, Madison, Wisconsin) storage oscilloscope. EAG deflections were measured directly from the stored screen image or from either photographs (Tektronix C5-A camera, Polaroid-type 667 film) or digital graphs from an *x–y* plotter (Hewlett Packard 7475A, Sunnyvale, California).

Odor Delivery. The odor delivery system and stimulation technique were essentially the same as that described by Light (1983). A constant flow (1.0 liter/min) of charcoal-filtered and humidified compressed air was passed-over the antenna through a disposable nozzle (automatic pipet tip, Centaur Chemical, Stamford, Connecticut) positioned ca. 1 cm from the antenna. When activated by a timing circuit, a three-way solenoid valve diverted the purified air through the stimulus cartridge where evaporating volatiles were picked up and carried into the nozzle and then onto the antenna. Stimulation duration was 1.0 sec. Because of the variation in volatility of test compounds, only relative comparisons can be made between the odorous stimuli.

Experimental Procedure. For each stimulus, EAGs were recorded from at least five flies of each sex. "Control" stimulations (using filter papers either untreated or impregnated with 1 μl of the hexane solvent) and "standard" stimulations (using fresh cartridges impregnated with 1 μl of 1% hexan-1-ol) were interspersed at approximately every fifth to tenth stimulation.

EAGs to test compounds were evaluated by measuring the maximum amplitude of negative deflection ($-$mV) elicited by a given stimulus and then subtracting the amplitude of the response to the preceding control. The millivolt responses to all compounds were converted to percentage values of the response to the accompanying 1% hexan-1-ol standard, as used in other EAG studies on insect olfaction (Dickens, 1984; Dickens and Boldt, 1985; Light, unpublished). This conversion or normalization of each response to a percentage of standard response allowed for comparison of responses within an individual and among individuals (Payne, 1975). Furthermore, this procedure minimizes and the observed variability in (1) absolute responsiveness among preparations, (2) order of presentation of compounds, and (3) the time-dependent variability in antennal responsiveness (Light, 1983; Dickens, 1984). Mean responses were compared using a t test and the nonparametric Mann-Whitney test (Snedecor and Cochran, 1967). Each stimulation was followed by an interval of ca. 90 sec of clean air. This interval was adequate for recovery of the EAG amplitude, as demonstrated by the measured percent recoveries of both sexes to shorter, 60-sec intervals between two separate 10% stimulations of hexan-1-ol (100.6% $\pm$ 1.8% for males and 99.3% $\pm$ 2.1% for females).

RESULTS

Selectivity

In general, the following EAG results suggest significant differences in the size of acceptor populations for the various odorants and/or odorant classes examined. Although slight differences between males and females in the magnitude of their EAG responses to each test odorant was found, in only a few cases were the differences significant.

The mean responses of *C. capitata* antennae to the hexan-1-ol standard (1 μl of 1% v/v hexane) were not significantly different between males and females, with mean EAGs of -1.09 mV (SE = 0.08 mV) and -1.14 mV (SE = 0.11 mV) for 15 males and 13 females, respectively.

Aliphatic Alcohols. For the series of saturated primary alcohols tested, responses of both male and female medflies peaked at hexan-1-ol and declined as carbon chain lengths either increased and decreased from six (Figure 1). Only for undecan-1-ol were EAG magnitudes found to be significantly different between the sexes, with female antennae more responsive than male antennae ($P < 0.05$).

In general, for both of the limited series of *E* geometric configurations of the 2- and 3-unsaturated primary alcohols tested, antennal responsiveness of both sexes increased as chain length increased from three to eight carbons (Figure 2). The only exception found was of female antennae being slightly, but not significantly, more responsive to (*E*)-2-hexen-1-ol than to (*E*)-2-octen-1-ol. (*E*)-2-Buten-1-ol elicited significantly greater ($P < 0.05$) EAGs from females than males.

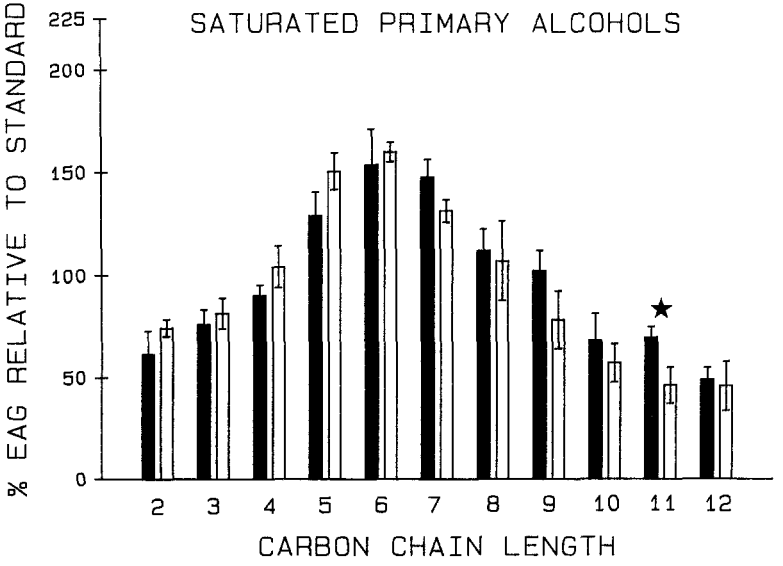

FIG. 1. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of saturated primary alcohols of various carbon chain lengths. Vertical lines represent standard errors, $N = 5$, a 100% response is approx. -1.1 mV, and stars represent significant differences in responsiveness between the sexes.

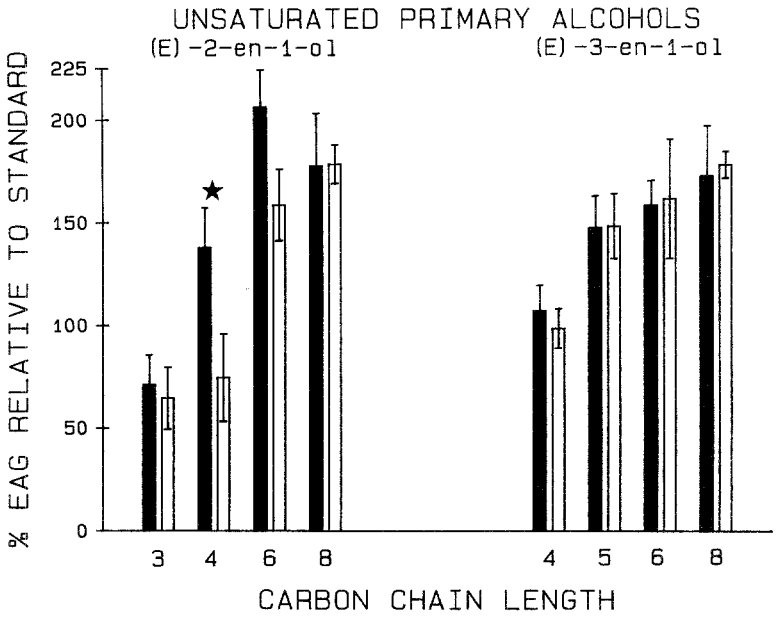

FIG. 2. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of unsaturated (*E*)-2- and (*E*)-3- primary alcohols of various carbon chain lengths. See Figure 1 legend for further information.

In two tests where *E* and *Z* geometric isomers of both hex-2-en-1-ol and hex-3-en-1-ol were compared, no significant differences were found between either of the hexenol isomers (Figure 3). Furthermore, there were no significant differences for either sex between the commonly tested 2- and 3-position unsaturated alcohols (i.e., the butenols and octenols, Figure 2) except for the hexenols (Figure 3). For males and females, both the (*Z*)- and (*E*)-hex-2-en-1-ol isomers elicited significantly ($P < 0.05$) greater EAGs than their hex-3-en-1-ol isomeric counterparts.

For the limited series of racemic saturated secondary alcohols tested, both male and female antennae were significantly less stimulated as chain length increased from seven to 11 carbons (Figure 4). EAGs of females to the secondary alcohols were slightly greater than males and were significantly greater ($P < 0.05$) for (±)-heptan-2-ol. The responses to the only 3-position and unsaturated secondary alcohol tested, (±)-1-octen-3-ol, were intermediate to the responses elicited by (±)-heptan-2-ol and (±)-nonan-2-ol. As with (±)-hepan-2-ol, female antennae were more responsive than male antennae to (±)-1-octen-3-ol.

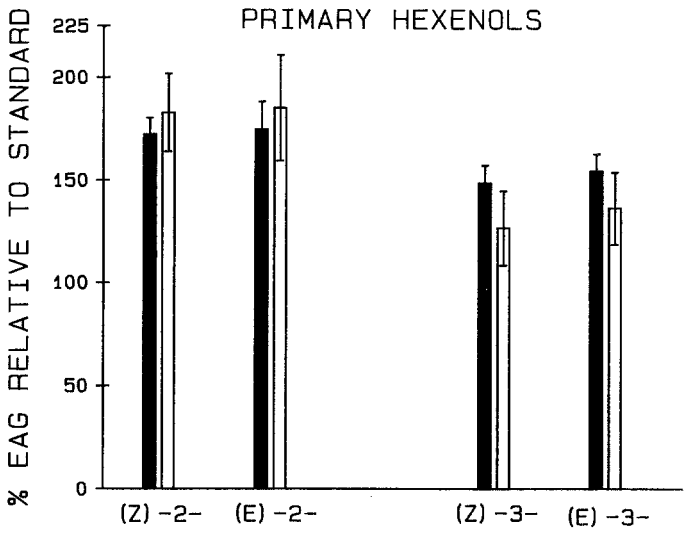

FIG. 3. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of primary hexenols. See Figure 1 legend for further information.

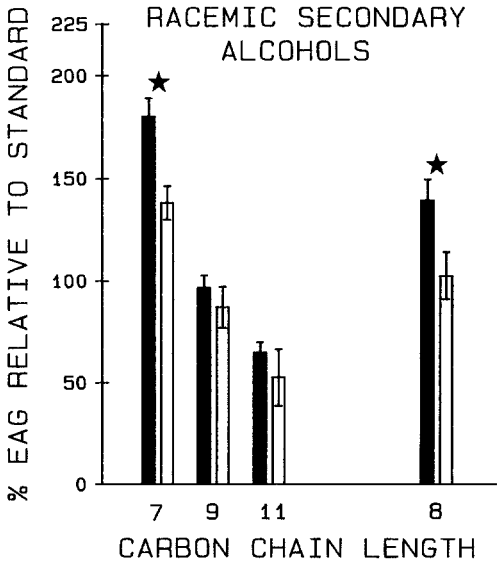

FIG. 4. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of racemic secondary alcohols of various carbon chain lengths, either saturated [(±)-heptan-2-ol, (±)-nonan-2-ol, and (±)-undecan-2-ol] or unsaturated [(±)-1-octen-3-ol]. See Figure 1 legend for further information.

Aliphatic Aldehydes. Both male and female antennae were significantly more responsive to the six- to 10-carbon chain saturated aldehydes than to either the lower or higher chain-length aldehydes (with the exception of dodecanal for females) (Figure 5). There was generally a stepwise progressive decrease in antennal responsiveness to aldehydes as chain length increased from hexanal to undecanal, but there was a significant upturn in responsiveness from undecanal to dodecanal. Among the saturated aldehydes tested, only hexanal elicited significantly different responses ($P < 0.05$) between the sexes, with female antennae more responsive than male antennae. Both of the unsaturated aldehydes tested, (*E*)-2-butenal and (*E*)-2-hexanal, elicited significantly greater ($P < 0.01$) EAGs than their saturated aldehyde counterparts (Figure 5).

Aliphatic Acids. The response magnitude rose significantly for both pentanoic and hexanoic acids over the lower, relatively level responses to both increasing and decreasing chain lengths of the other saturated acids (Figure 6). The inclusion of a double bond slightly, but not significantly, increased EAG responsiveness to both 2-propenoic and (*E*)-2-hexenoic acids over their respective saturated acids (Table 2 and Figure 6).

Aliphatic Acetates. The series of saturated acetates elicited EAGs that

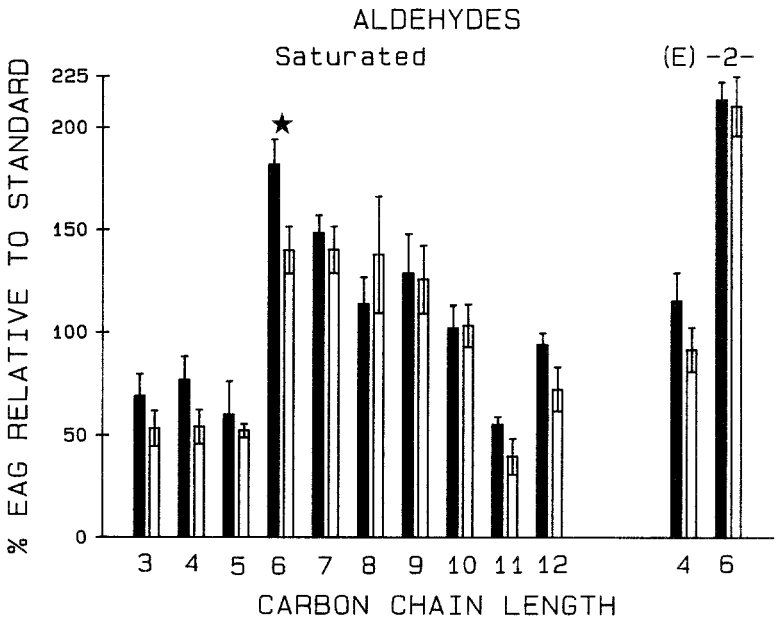

FIG. 5. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of saturated and (*E*)-2-unsaturated aldehydes of various carbon chain lengths. See Figure 1 for further information.

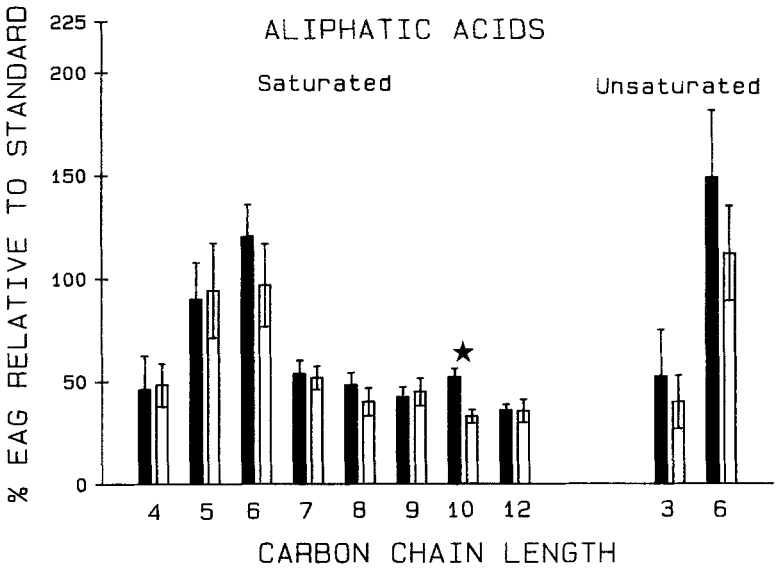

FIG. 6. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of aliphatic acids of various carbon chain lengths and 2-propenoic and (*E*)-2-hexenoic acids. See Figure 1 for further information.

TABLE 2. OCCURRENCE AND MILLIVOLT MAGNITUDE OF NEGATIVE/POSITIVE POLARITY EAGS OBSERVED IN *Ceratitis capitata*

Compound	Sex	Number of positive/negative polarity responses out of five replications[a]			Magnitude of polarity phases[b]	
		ER	−/+	+	Negative ($X \pm$ SE)	Positive ($X \pm$ SE)
Acetic acid	M	0	5	0	−0.46 ± 0.09	+0.93 ± 0.14
	F	1	2	0	−0.19 ± 0.09	+1.48 ± 0.30
Propanoic acid	M	0	3	2	−0.29 ± 0.14	+1.31 ± 0.28
	F	0	2	2	−0.12 ± 0.16	+1.74 ± 0.25
Butanoic acid	M	1	4	0	−0.61 ± 0.08	+1.46 ± 0.25
	F	0	4	0	−0.39 ± 0.28	+1.31 ± 0.27
2-Propenoic acid	M	0	4	0	−0.63 ± 0.28	+1.58 ± 0.17
	F	0	4	0	−0.50 + 0.17	+1.55 ± 0.23
Pentanoic acid	M	2	0	0		
	F	0	0	0		
Hexanoic acid	M	2	0	0		
	F	0	0	0		

[a] ER, early recovery; −/+, negative/positive polarity response patterns; +, positive polarity response patterns; see text for definitions.
[b] Mean millivolt responses for −/+ and + data, exclusive of ER data.

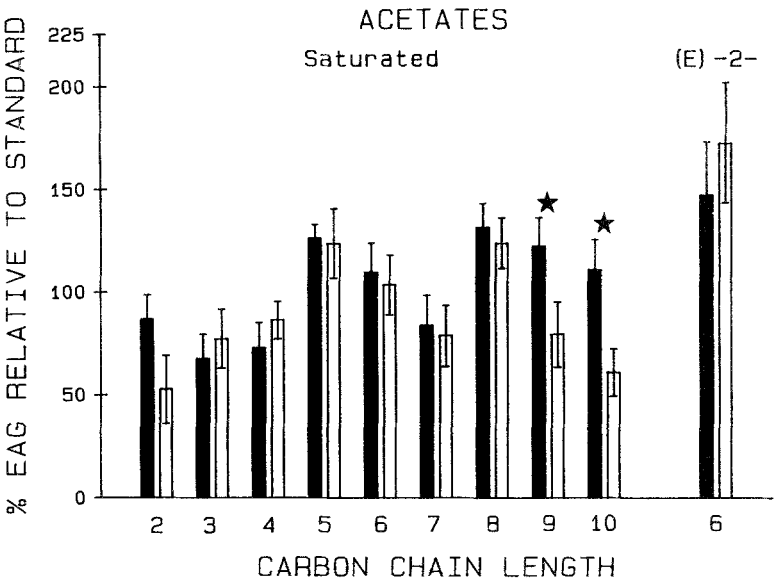

FIG. 7. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of saturated acetates of various carbon chain lengths and the unsaturated (*E*)-2-hexyl acetate. See Figure 1 for further information.

peaked at both C_5 and C_8 (Figure 7). Female antennae were significantly more responsive ($P < 0.05$) than male antennae to both nonyl- and decyl-acetates.

Lactones. The EAGs to a series of lactones did not significantly vary as either (1) side-chain length of the gamma-lactones increased from zero (gamma-butyrolactone) to seven carbons (gamma-undecalactone) except for the greater female responsiveness to gamma-nonalactone (i.e., five-carbon side-chain) or (2) as the lactone ring increased from four (gamma) to five (delta) carbons (Figure 8). Except for gamma-nonalactone, for each lactone tested, male EAGs were slightly, but not significantly, greater than female EAGs.

EAG Response Polarity

The EAGs recorded to the majority of the volatiles tested had purely negative voltage polarities and time-course shapes. Upon odor stimulation, the majority of the recordings typically consisted of a rapid negative voltage deflection that was more or less maintained throughout the odor stimulation duration followed, upon termination of the odor stimulation, by a slower, more gradual (positive polarity) approach or recovery to (but not exceeding) the prestimulation background potential (Figure 9A). However, to specific short chain-length acids alone, a range in degree of biphasic, "negative, then positive polarity" EAGS during odor stimulation was observed (Figure 9B–D). These biphasic

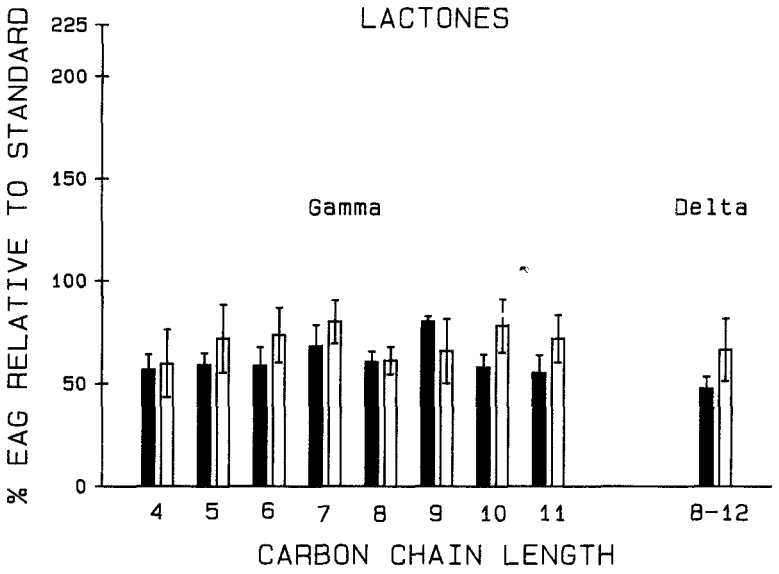

FIG. 8. Mean EAG responses of female (filled bars) and male (blank bars) *C. capitata* to 1-μl doses of 10% solutions (v/v) of gamma-lactones of various carbon chain lengths and a mixture of C_8–C_{12} delta-lactones. See Figure 1 for further information.

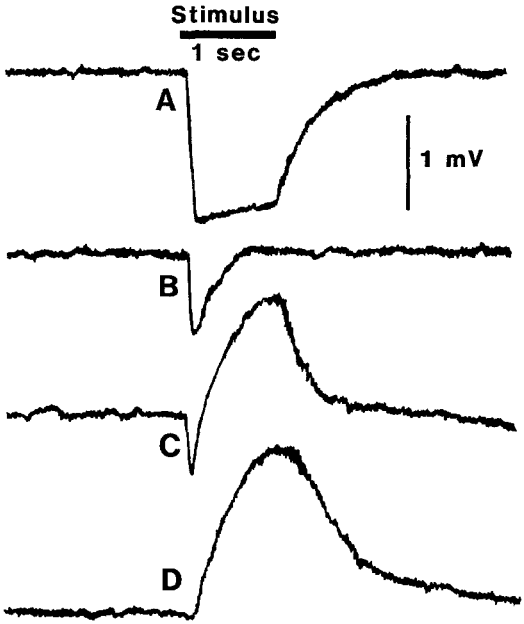

FIG. 9. Reproduction of the four types of EAG polarity and time-course shapes recorded from a single male medfly: (A) typical "negative-polarity" EAG, in this case to hexan-1-ol, (B) "early recovery" EAG to pentanoic acid; (C) biphasic "negative-positive" EAG to butanoic acid; and (D) "positive polarity" EAG to propanoic acid.

EAGs ranged from: (1) "early recovery"; (2) "negative positive" polarity; to (3) an apparently "positive polarity." The "early recovery" was typified by an initial, negative, stimulus-onset response that became spikelike in shape, its recovery to baseline starting immediately during the odor stimulation period and not after its cessation (e.g., 40% of the male's responses to pentanoic and hexanoic acids, Table 2). The "negative/positive" EAG was characterized by the initial negative deflection being reversed by a two to seven times greater positive deflection that greatly overshot the prestimulus baseline during the remainder of the stimulus duration, then, after stimulus termination, the response potential returned to the baseline level [e.g., stimulations by acetic, propanoic, 2-propenoic, and butanoic acids; Table 2]. In the apparently "positive polarity," the response potential never dipped, but rose slowly during stimulation and then dropped to baseline potential after stimulation cessation (e.g., responses of certain individuals to propanoic acid, Table 2).

DISCUSSION

The EAG response is thought to be the expression of generator potentials of many simultaneously stimulated receptor cells with potentially different acceptor specificities (Boeckh et al., 1965; Kaissling, 1971; Schneider, 1969). Further, the negative amplitude of the EAG deflection as been interpreted to be a measure of the relative number of acceptors responding to an odor stimulus (Payne, 1975; Dickens and Payne, 1977). Thus, the EAG results presented here, in general, suggest significant differences in size of acceptor populations for the various odorants and/or odorant classes examined.

Polarity of EAG Responses

Positive polarity and biphasic EAG potentials have been observed before (e.g., Schneider, 1957a, b). Positive polarity EAG responses were elicited by a puff of air during deep ether narcosis in male *Bombyx mori* (L.), while cycloheptanone elicited biphasic "early recovery" waveforms, and air puffs during the beginning phase of ether or chloroform narcosis evoked biphasic negative/positive potentials (Schneider 1957a, b). At the receptor level, Schneider (1969) and others (Boeckh et al., 1965; Kaissling, 1971) have correlated extracellularly recorded, negative-polarity generator potentials (termed depolarizations) with "excitation" (i.e., increased action potential frequency) in receptor neurons, and positive-polarity receptor potentials (termed hyperpolarizations) with "inhibition" (i.e., decreased action potential frequency) in receptor neurons. Boeckh (1962, 1967) and Kaissling (1971) observed positive extracellular DC potentials that they termed "inhibitory potentials" when sensilla basiconica of both the blowfly, *Calliphora erythrocephala*, and the carrion beetles, *Thanatophilus rugosus* and *Necrophorus humator*, were stimulated with propanoic,

butanoic, or pentanoic acids. We found that these same short-chain acids also elicited positive and biphasic EAGs in medfly antennae. However, whether the polarity of EAGs is an accurate representation of receptor potential depolarizations, hyperpolarizations, or the subtractive interaction of the two polarizations must be questioned because of the extreme attenuation inherent in EAG recordings. Further, Kafka (personal communication) and others have suggested that biphasic and positive-polarity EAGs might be recording artifacts attributable to electrode potentials.

Effects on EAG Responsiveness

Sexuality. Although slight differences between males and females in the magnitude of their EAGs to each test odorant were found, in only a few cases were the differences significant. Among the 70 compounds tested, only eight compounds, undecan-1-ol, (*E*)-2-buten-1-ol, (±)-heptan-2-ol, (±)-1-octen-3-ol, hexanal, nonyl acetate, decyl acetate, and decanoic acid, elicited significantly larger EAGs in female antennae than in male antennae. On the other hand, the general similarity in antennal responses between the sexes suggests a common ecological need for host-plant and/or habitat recognition by both sexes. Once alighting on the plant, the sexes might utilize the resource in a similar manner: for example, attaining food (plant sap, nectar, honeydew, protein, etc.); or in a dissimilar manner: for instance, for males as a lekking and courtship site or for females as an ovipositional site.

No sexual dimorphism in EAGs to plant volatiles has been reported for other tephritid fruit flies, e.g., the apple maggot (Fein et al., 1982) and the olive fruit fly (Van Der Pers et al., 1984), or for many other insect species, e.g., *Leptinotarsa decemlineata* (Visser, 1979), *Yponomeuta* species and *Adoxophyes orana* (Van Der Pers, 1981), *Rhynchaenus quercus* (Kozlowski and Visser, 1981), and *Oulema melanopus* and *Pseudaletia unipuncta* (Wellso et al., 1984).

Carbon Chain Length. For four of the five functional-group series tested, both definitive EAG amplitude maxima and antennal responsiveness profiles were affected by variation in carbon-chain length. Overall, it appears that medfly antennae were selectively more responsive to six-carbon and, to a lesser extent, five-, seven-, and eight-carbon, chain lengths for these functional-groups, which are ubiquitous in fruit and leaf volatiles.

The present EAG study is the first to have systematically assessed carbon-chain series on each of these fundamental functional-group classes, while for the most part limited series of only aldehydes and primary alcohols have been tested on other species. Similar to the medfly, the Colorado potato beetle (Visser, 1979) and the oak flea weevil (Kozlowski and Visser, 1981) were found to have peak EAG responses to both C_6 aldehydes and alcohols. Antennal respon-

siveness of the cotton boll weevil, *Anthonomus grandis* (Dickens, 1984), and the cereal aphid, *Sitobion avenae* (Yan and Visser, 1982), peaked at C_6 for the primary alcohol series. However, for aldehydes, the majority of the EAG plant-volatile studies have found that responsiveness peaks at heptanal, with a ranking for most species of C_7, C_8, C_9, and then C_6 [for Diptera: *C. capitata*, *D. oleae*, and *R. cerasi* (Guerin et al., 1983a); *P. rosae* (Guerin and Städler, 1982; Guerin et al., 1983b); and *Delia antiqua* (Guerin and Städler, 1982); for Lepidoptera: *Yponomeuta* spp. (Van Der Pers, 1981) and for Coleoptera: *A. grandis* (Dickens, 1984); and *Trirhabda bacharides* (Dickens and Boldt, 1985)].

Unsaturation. For the limited number of monoenic alcohols, aldehydes, acids, and acetates tested, the unsaturated monoenes elicited equal or larger EAGs than their comparable saturated compounds. Significantly greater responses for both medfly sexes were observed for both (*E*)-2- and (*E*)-3-octen-1-ol over octan-1-ol, (*E*)-2-hexenal and (*E*)-2-butenal over their saturated analogs, and (*E*)-2-hexenyl acetate over hexyl acetate. In addition, female antennae responded significantly greater to (*E*)-2-buten-1-ol than butan-1-ol. In all but one study on EAG response selectivity, the EAG responses to monoenic alcohols, aldehydes, and acetates exceeded those to saturated analogs (see Visser, 1983, 1986). The exception is a study of *D. antigua*, where hexan-1-ol elicited a greater response than the unsaturated isomeric analogs tested (Guerin and Städler, 1982).

Functional Groups. Under our experimental conditions, the greatest antennal responses generally were elicited by aldehyde and alcohol moieties, followed by acetate, lactone, and acid functional groups, as has been found in most species (see above references and Visser 1983, 1986). Furthermore, the monoenic aldehyde, (*E*)-2-hexenal, elicited greater EAGs in medflies than the 2- and 3-position (*E*)- and (*Z*)-hexen-1-ols and other monoenic alcohols, which in turn exceeded the saturated aldehydes and alcohols. The saturated secondary alcohols elicited responses slightly greater than or equal to the saturated primary alcohols.

Thus, carbon-chain length, unsaturation, and type and position of functional groups all have significant effects on the magnitude of the EAG response (i.e., the relative numbers of acceptors responding throughout the antennal olfactory organ) of medflies to odor molecules. The specificities and affinities of the presumed acceptor classes that are present on the medfly antennae are still unknown. However, our preliminary single-cell recordings from various trichoid and basiconic antennal hairs and pegs indicate that receptors are present that are narrowly and specifically tuned to chain length, functional group(s), and chirality of the odor molecules (Dickens et al., unpublished). Of the ca. 30 recordings to date, we have not discovered any receptors with broad responsiveness to various chain lengths of a functional group. However, receptors have been found that are selectively "tuned" to virtually a "single" chain

length for a particular functional group, while responding differentially over a broader range of chain lengths to another functional group. These observations will be explored through further selectivity, sensitivity, and differential adaptability experiments on single receptors.

Green-Leaf Volatiles. The top 16 compounds eliciting the greatest antennal responses of male and female medflies can be ranked as follows: (*E*)-2-hexenal ≥ (*E*)-2-hexen-1-ol ≥ (*Z*)-2-hexen-1-ol ≥ (*E*)-2-octen-1-ol ≥ (*E*)-3-octen-1-ol ≥ (*E*)-2-hexenyl acetate ≥ hexanal > heptan-2-ol ≥ (*E*)-3-hexen-1-ol ≈ (*Z*)-3-hexen-1-ol ≈ hexan-1-ol ≈ heptanal ≈ heptan-1-ol ≈ (*E*)-3-penten-1-ol ≈ (*E*)-2-hexenoic acid ≈ octanal. This ranking is dominated (five of the top 11 compounds) by what have been termed "green odors" or "general green-leaf volatiles" because of the ubiquitous production of these compounds by either intact or, more often, damaged plant leaf parenchyma (see Visser et al., 1979; Buttery, 1981). Produced through the oxidative fragmentation of the plant fatty acids, linoleic and linolenic acids, the following green-leaf volatiles are commonly identified in various ratios, depending on the plant species: hexanal, hexan-1-ol, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, and (*E*)-2-hexen-1-ol.

Along with plant species-specific blends of important discriminatory "key compounds" (often terpenes and their analogs), the green-leaf volatiles dominate the selective EAG responsiveness of insect antennae over aldehydes and alcohols with shorter or longer carbon chain lengths [e.g., carrot fly (Guerin and Visser, 1980); onion and cabbage root flies (Guerin and Städler, 1982); the syrphid fly, *Metasyrphus venablesi* (Hood Henderson and Wellington, 1982); Colorado potato beetle (Visser, 1979); oak flea weevil (Kozlowski and Visser, 1981); cotton boll weevil (Dickens, 1984); the chrysomelid, *T. bacharides* (Dickens and Boldt, 1985); and *Yponomeuta* spp. and *A. orana* (Van Der Pers 1981)]. Thus, as with most species studied electrophysiologically (Visser, 1983, 1986), medflies have antennae "selectively tuned" for reception of green-leaf volatiles. This suggests that medfly antennae have greater populations of acceptors for these six-carbon volatiles. The general receptivity for leaf volatiles by phytophagous insects appears to be comparable, apparently, regardless of the ecological host range of the herbivorous insect, ranging from the near monophagy by *T. bacharides* (a biological control candidate for the weed, *Baccharis halimifolia* L.), to polyphagy by medflies.

Most detailed EAG studies to date have utilized oligophagous insects. In the few studies where kairomonal attractants have been identified or suggested, these are "key compounds" that are relatively unique to the host species or host genus. Such compounds often elicit large EAG responses that significantly exceed the responses to green-leaf volatiles. A well-studied example is the carrot fly where (*E*)-asarone and (*E*)-methylisoeugenol elicit large EAG responses and are attractive in the field (Guerin et al., 1983b). Further, Guerin et al.

(1983b) found that the attraction of *P. rosae* to (*E*)-asarone was synergized by (*E*)-2-hexenal. But in only a few cases have general green leaf volatiles been attractive along (see Visser, 1986; Visser and Ave, 1978). Whether single consitituents of the general green-leaf odor or other fruit odors attract medflies is uncertain. In field tests in Sardinia, Guerin et al. (1983a) found that heptanal attracted predominantly female medflies to sticky traps. However, in field tests in Hawaii, we found no attraction to a total of 21 individual saturated and unsaturated primary alcohols, aldehydes, and acetates (Cunningham et al., unpublished). Thus, questions remain about the effectiveness of the individual components of the green-leaf odor as well as the entire and properly proportioned blend emitted by the plant.

Because of the widespread occurrence of general green-leaf volatiles throughout plants, it is unlikely that their qualitative presence alone could serve to discriminate between host and nonhost plants. On the other hand, the relatively high selectivity and responsiveness of *C. capitata* antennae, and other insect antennae, to the general green-leaf volatiles suggests a large sensory investment in the reception of these compounds that may be adaptive. The reception of green-leaf volatiles may be fundamental to such short- and/or long-range appetitive behaviors that occur on foliage, such as foraging for water, food, and shelter, and the establishment and subsequent attractiveness of lek sites. Because these foraging and lekking behaviors of medflies are readily observed on both host and nonhost plants (Christenson and Foote, 1960; Prokopy and Roitberg, 1984; Prokopy et al., 1986), it is possible that the more universal plant olfactory cues, i.e., the general green-leaf volatiles, might influence these discriminative searching behaviors. The perception of the complex or specific host-plant odors may result in the initiation and maintenance of various searching or foraging behaviors that are mechanistically based on visual optomotor anemotactic and/or phototelotactic orientation (Light, 1986). Such has been hypothesized for the cherry fruit fly (Levinson and Haisch, 1984), the apple maggot (Prokopy, 1986), and the medfly (Féron, 1962; Nakagawa et al., 1978).

A number of additional experiments must be undertaken before determining the applicability of the EAG technique to screen a series of plant volatiles for their potential semiochemical activity with medflies. These additional experiments are presently under investigation (Jang et al., unpublished) and include laboratory and field bioassays, along with comparative EAG studies utilizing known potent male lures (i.e., trimedlure and α-copaene), putative male pheromones (Baker et al., 1985), and the range of volatiles present in specific host fruits.

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BIOASSAYS FOR ALLELOPATHY: Measuring Treatment Responses with Independent Controls

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Abstract—In bioassays for allelopathy, where responses to treatments are determined in conjunction with responses to independent controls, statistical comparisons among treatments require an index which measures each treatment response (T) in relation to its control response (C). The most commonly used index, the treatment-control ratio (T/C), exhibits two analytical problems. First, means of T/C values are distorted upward when any of the individual values is greater than one, i.e., when stimulation occurs. Second, the distribution of T/C values may not be normal and homoscedastic. We provide two alternative indices of response whose means do not exhibit the upward distortion of T/C means. Then, the two indices are compared to T/C values in an empirical test for normality and homoscedasticity on a large bioassay data set. Results indicate that for this data set, one of the alternatives, RI, is clearly superior. RI is defined as $1 - (C/T)$ if $T \geq C$ and as $(T/C) - 1$ if $T < C$.

Key Words—Bioassay, response index, allelopathy, normality, homoscedasticity, parametric analysis.

INTRODUCTION

One problem apparent in the allelopathic literature is how to analyze inhibition and stimulation responses across a series of bioassays, each of which has its own control. In experiments with only one control, statistical analysis is relatively standard because absolute responses from treatments are compared to those from the control. However, with multiple experiments where different

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treatments are conducted with independent controls, comparisons are often made with the ratio of each treatment response to its control response (e.g., Newman, 1978; AlSaadawi and AlRubeaa, 1985; AlSaadawi et al., 1985; Gilmore, 1985; Stevens and Tang, 1985; Carter and Grace, 1986).

The treatment/control ratios (T/C) present two analytical problems. First, averaging ratios across several experiments may produce an upward distortion in the mean if any of the T/C values is greater than one. For example, suppose bioassays of a source plant leachate were conducted in two different months with separate controls. Such an experiment would have two purposes: to determine monthly variation in effects and to determine the effect over all months. If T/C is 0.5 the first month and 2.0 the second month, then the arithmetic mean of the ratios is 1.25, which would suggest stimulation overall. But clearly, the treatment response is double the control in the first month and the control response is double the treatment response in the second month, so the net effect over both months logically is neither inhibition or stimulation. Therefore, the arithmetic mean of T/C values produces an upward distortion in the mean effect of the two treatments.

The second analytical problem with ratios is that they commonly are not distributed normally, so parametric statistical tests may be inappropriate. The arcsine of the square root of ratios often is recommended to normalize them (Sokal and Rohlf, 1969). However, this transformation is useful only when the ratios are true proportions, i.e., when they range from zero to one, because the arcsine of a variable is defined positively only when the variable ranges from zero to one. With bioassays, T/C values may be greater than one if stimulation occurs, so the transformation is impossible.

Here, we suggest alternative response indices which do not exhibit an upward distortion in their arithmetic means and which may be useful when researchers wish to apply parametric statistics to determine differences in mean effects of treatments.

METHODS AND MATERIALS

Choosing an Index of Response. If we define T/C as the ratio of a single treatment response to its control response, the mean of the ratios of N treatments is given by

$$\overline{T/C} = [\sum (T/C)]/N \tag{1.0}$$

Then, the problem is to define an index, whose mean does not exhibit the upward distortion associated with $\overline{T/C}$.

Logarithms of T/C values can be averaged arithmetically across N independently derived ratios as follows, where LI refers to the "log index":

$$\overline{LI} = \left(\sum LI\right)/N = [\sum \log (T/C)]/N \tag{2.0}$$

LI has the advantage of avoiding the upward distortion associated with averaging the untransformed ratios, but it has the disadvantage that logarithmically transformed ratios are difficult to interpret on visual inspection. Use of logarithms to the base 2 at least permits recognition of halvings of effects (i.e., an *LI* of 0 indicates a mean treatment response equal to the control response, and an *LI* of -1 indicates a mean treatment response one half of the control, etc.). Theoretically, *LI* can range from $-\infty$ to $+\infty$. Positive values of *LI* indicate stimulation by treatments and negative values indicate inhibition.

One way to avoid the difficulty of inspection of logarithmically transformed ratios is to take the inverse (antilogarithm) of $\overline{LI}$ after it has been computed. However, presentation of the inverse of $\overline{LI}$ has the complication that standard errors about it are asymmetrical. The inverse of $\overline{LI}$ is the same as the geometric mean (*GM*) of the *T*/*C* values:

$$\text{antilog}\left\{\left[\sum \log\,(T/C)\right]/N\right\} = \left(\prod\,(T/C)\right)^{1/N} = GM \tag{2.1}$$

A second index that does not exhibit the upward distortion associated with averaging *T*/*C* values is *RI*, the "response index," determined as follows:

$$\text{If } T > C, \text{ then } RI = 1 - (C/T) \tag{3.0}$$

$$\text{If } T < C \text{ then } RI = (T/C) - 1 \tag{3.1}$$

Then, *RI* values may be averaged arithmetically as follows:

$$\overline{LI} = \left[\sum\,(RI)\right]/N \tag{3.2}$$

RI ranges from -1 to $+1$, with positive values indicating stimulation by the treatments and negative values indicating inhibition by them, relative to the controls. *RI* is easy to interpret on visual inspection because it is simply the proportional reduction of the treatment relative to the control when stimulation occurs (a negative *RI*) or of the control relative to the treatment when inhibition occurs (a positive *RI*).

To illustrate the use of *LI* and *RI* in comparison to *T*/*C*, we have selected a large set of bioassays conducted to test for allelopathic effects. Ten source leachates were prepared from fresh leaf and litter samples, collected monthly from plants in Florida's sand pine scrub community. Four target seeds—three native grasses and commercial lettuce—were placed in the leachates and in controls of distilled water to determine germination rates and radicle growth. Each month's bioassays had an independent control. [Details of the bioassays and raw data are given by Richardson (1985)].

The three variables, *T*/*C*, *LI* and *RI*, were calculated for each combination of source leachate, control, month, and target species. Means and standard deviations were then determined for each target species for both germination and radicle length of seedlings. For comparisons with $\overline{T/C}$, $\overline{LI}$ was reconverted to a ratio through its inverse function (antilog $\overline{LI}$) and $\overline{RI}$ was reconverted through

its inverse function ($1 + \overline{RI}$ if $\overline{RI} \leq 1.0$ and $1/(1 - \overline{RI})$ if $\overline{RI} > 1.0$); note that these inverse functions were applied to the means ($\overline{LI}$ and $\overline{RI}$), not to the individual values (*LI* and *RI*). The results (Table 1) showed that $\overline{T/C}$ behaved similarly to the inverse of $\overline{LI}$ (Pearson's $r = 0.979$, $P = 0.0001$) and to the inverse of $\overline{RI}$ ($r = 0.981$, $P = 0.0001$), and the two inverses behaved even more similarly to each other ($r = 0.997$, $P = 0.0001$). However, for each species' germination or radicle length, the inverse of $\overline{LI}$ and the inverse of $\overline{RI}$ were less than or equal to $\overline{T/C}$ (Table 1)—a result illustrating the upward distortion of the arithmetic mean of *T*/*C* values.

The difference between $\overline{T/C}$ and the inverse of $\overline{LI}$ was highly correlated with the standard deviation about $\overline{T/C}$ ($r = 0.972$, $P = 0.0001$), because the geometric mean is less than the arithmetic mean by a function of the standard deviation around the arithmetic mean (Beckenbach and Bellman, 1961). In contrast, the inverse of $\overline{RI}$ differs from $\overline{T/C}$ only to the degree that some of the *T*/*C* values are greater than one. For example, the difference between $\overline{T/C}$ and the inverse of $\overline{RI}$ is correlated strongly with $\overline{T/C}$ plus one standard deviation ($r = 0.983$, $P = 0.0001$). In fact, if all *T*/*C* values are less than one, then the inverse of $\overline{RI}$ equals $\overline{T/C}$.

Normality and Homoscedasticity of Indices of Response. Given the three indices of response, *T*/*C*, *LI*, and *RI*, application of parametric statistics to determine differences in mean responses of various treatment groups requires that the distributions of the indices be normal with equal (homoscedastic) variances.

TABLE 1. $\overline{T/C}$, $\overline{LI}$, AND $\overline{RI}$ VALUES FROM DETERMINED BIOASSAYS FOR GERMINATION AND RADICLE LENGTH OF THREE GRASSES (*Andropogon gyrans*, *Leptochloa dubia*, AND *Schizachyrium scoparium*) AND LETTUCE (*Lactuca sativa*)[a]

Species	*N*	$\overline{T/C}$	Inverse of $\overline{LI}$	Inverse of $\overline{RI}$
Germination				
Andropogon gyrans	77	0.91	0.86	0.88
Leptochloa dubia	80	0.79	0.76	0.79
Schizachyrium scoparium	90	0.82	0.75	0.81
Lactuca sativa	150	0.90	0.85	0.90
Radicle length				
Andropogon gyrans	77	1.10	0.93	0.95
Leptochloa dubia	80	0.96	0.95	0.95
Schizachyrium scoparium	90	1.00	0.95	0.97
Lactuca sativa	150	1.51	1.41	1.36

[a] The *N* bioassays result from 10 different species leachates applied monthly to the test seeds.

A review of the sample distributions of *T/C*, *LI*, and *RI* from the bioassays described above (Richardson, 1985) provides empirical data on normality and homoscedasticity.

The distributions of *T/C*, *LI*, and *RI* for germination and for radicle length of each target species were tested for differences from normality with the Kolomogorov *D* statistic. For a given sample size, a larger *D* value indicates a greater deviation from normality. For eight such tests, distributions different from normal were statistically significant ($P \leq 0.05$) in four tests for *T/C*, in six test for *LI*, and in three tests for *RI* (Table 2). If we chose the best index as the lowest *D* value from each of the eight cases and we arbitrarily scored indices as ties when *D* values are within 0.01 of each other, then both *T/C* and *RI* were best in seven of eight cases, whereas *LI* was best in only two of eight cases. Thus, although no index provided normal distributions in all cases, both *RI* and *T/C* performed much better than *LI*.

To compare homoscedasticity of variances, the standard deviations (sd_1) about $\overline{T/C}$, $\overline{LI}$, and $\overline{RI}$ for germination and radicle length of each of the four target species were determined (Table 3). Next, the four standard deviations were averaged to yield a mean ($\overline{sd}_1$) and standard deviation (sd_2) of the standard deviations. Then, the coefficient of variation was calculated from the mean and standard deviation ($CV = 100^* (sd_2/\overline{sd}_1)$. For germination and for radicle length, the *CV* of the standard deviations of $\overline{RI}$ was lower than the *CV* for $\overline{LI}$ or for $\overline{T/C}$, so $\overline{RI}$ provides the most homoscedastic index of the three (Table 3).

TABLE 2. OBSERVED *D* VALUES FROM KOLOMOGOROV TEST FOR DIFFERENCES FROM NORMALITY OF DISTRIBUTIONS OF THREE INDICES OF RESPONSE: *T/C*, *LI*, AND *RI*[a]

Species	*N*	*T/C*	*LI*	*RI*
Germination				
Andropogon gyrans	77	0.09	0.08	0.08
Leptochloa dubia	80	0.09	0.11**	0.09
Schizachyrium scoparium	90	0.11**	0.19**	0.10*
Lactuca sativa	150	0.29**	0.40**	0.30**
Radicle length				
Andropogon gyrans	77	0.16**	0.07	0.06
Leptochloa dubia	80	0.08	0.11*	0.08
Schizachyrium scoparium	90	0.09	0.13**	0.08
Lactuca sativa	150	0.08*	0.11**	0.15**

[a] Smaller *D* values indicate less difference from normality for comparisons of a given sample size. Probabilities of significant difference from normality of the observed *D* (or larger) are indicated by * for $P \leq 0.05$ and by ** for $P \leq 0.01$.

TABLE 3. STANDARD DEVIATIONS ABOUT $\overline{T/C}$, $\overline{LI}$, AND $\overline{RI}$ FOR FOUR TARGET SPECIES' GERMINATION AND RADICLE LENGTH, MEAN AND STANDARD DEVIATION ($\overline{sd}_1 \pm sd_2$) OF STANDARD DEVIATIONS, AND COEFFICIENT OF VARIATION OF MEAN AND STANDARD DEVIATION

Species	*N*	*T/C*	*LI*	*RI*
Germination				
Andropogon gyrans	77	0.31	0.52	0.27
Leptochloa dubia	80	0.17	0.34	0.17
Schizachyrium scoparium	90	0.30	0.70	0.27
Lactuca sativa	150	0.19	0.70	0.19
$\overline{sd}_1 \pm sd_2$	4	0.24 ± 0.07	0.56 ± 0.17	0.23 ± 0.05
CV (%)		30	30	23
Radicle length				
Andropogon gyrans	77	0.71	0.82	0.38
Leptochloa dubia	80	0.15	0.23	0.14
Schizachyrium scoparium	90	0.27	0.45	0.23
Lactuca sativa	150	0.51	0.57	0.27
$\overline{sd}_1 \pm sd_2$	4	0.41 ± 0.25	0.52 ± 0.24	0.26 ± 0.10
CV (%)		61	47	39

DISCUSSION

Calculating a mean effect over many treatments with independent controls is valid only if the treatments, which are different at the treatment level, represent true replicates at a higher level. Bioassays for allelopathy often involve treatments with independent controls under many circumstances: (1) to determine seasonal variation, (2) to provide pH or osmotically adjusted controls, (3) to determine effects of temperature on activity, and (4) to determine field location effects on treatments in blocked or split-plot designs. In most of the above cases, determination of overall effects may be as important as investigation of differences among treatments, and experiments are designed so treatments represent true replicates for determination of overall effects. Generally, as specific treatment effects become known, more complicated designs with independent controls are necessary in order to assess treatment effects under more realistic conditions. Thus, as investigations become more sophisticated, the alternative indices, *RI* and *LI*, may become more appropriate than simple *T/C* ratios.

In addition to avoiding the upward distortion of mean effects associated with the arithmetic mean of *T/C* values, it is necessary to use an index that tends to exhibit normality and homoscedasticity. For the data set used here, *RI*

and *T/C* were superior to LI in regard to normality. RI was superior to both *T/C* and *LI* in regard to homoscedasticity.

With other data sets, we have found that *RI* is usually, but not always, superior to *LI* and *T/C*. We do not expect universal acceptance of *RI* as some innately superior index of response, but we recommend testing indices for normality and homoscedasticity before applying parametric statistical tests for treatment differences.

The statistical considerations recommended here are not a substitute for proper experimental design and biological interpretation. In some cases, treatment differences are so extreme that statistical considerations may be preempted. For example, an unmentioned, minor problem with averaging *LI* values is that *LI* is undefined when $T/C = 0$. However, when the treatment completely suppresses germination, statistical tests may be unwarranted. Similarly, we have found that *RI* usually proves superior to *LI* and to *T/C* in not deviating from normality only when treatment effects are moderate. With extremely large inhibitory or stimulatory effects, *RI* often deviates more from normality than *LI* and *T/C* (i.e., see lettuce radicle length in Table 2), but where effects are larger, statistical considerations are less necessary for biological interpretation.

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UPWIND FLIGHT ORIENTATION TO PHEROMONE IN WESTERN PINE BEETLE TESTED WITH ROTATING WINDVANE TRAPS[1,2]

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Abstract—In the first trap design, a rotating windvane was connected to a 30 × 30 × 30-cm "square box" sticky trap enclosing a synthetic pheromone source (*exo*-brevicomin, frontalin, and myrcene) at the windvane's rotation axis. A second design used the windvane attached to two tubular (19-cm-diam. × 30-cm) sticky traps each suspended 120 cm from the same pheromone source and opposingly aligned "downwind" and "upwind" of the windvane. Significantly more beetles of each sex of *Dendroctonus brevicomis* LeC. (Coleoptera: Scolytidae) were caught on the downwind side compared to the upwind side of the square-box design. Even larger differences in catch, four times more males and 3.4 times more females, were found on the downwind tubular trap compared to the upwind one. The windvane trap design provides rigorous evidence that insects, especially bark beetles, orient upwind to pheromone sources (from at least 1.2 m downwind until reaching the source).

Key Words—*Dendroctonous brevicomis*, Coleoptera, Scolytidae, pheromone, anemotaxis, upwind orientation, *exo*-brevicomin, frontalin, myrcene.

INTRODUCTION

Upwind flight orientation by insects to pheromone has become a well-known fact or paradigm. This knowledge is based largely on correlative evidence be-

[1] *Dendroctonus brevicomis* LeC. (Coleoptera: Scolytidae).

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tween wind direction and observations of flying insects or trap catches in the field. There are many studies on Lepidoptera and other insects orienting to pheromone sources in wind tunnels (Kellogg et al., 1962; Farkas and Shorey, 1974; Kennedy, 1977, 1983; Kennedy et al., 1980, 1981; Cardé, 1984). In some larger insects it is possible to correlate wind direction measurements using windvanes and simple observations of wing fanning (Elkinton et al., 1984). With many smaller insects, such as bark beetles, it is difficult to document their upwind flight orientation response to pheromone. Consequently, "very little (olfactory) research has investigated orientation by flying beetles, nor has it addressed the intricacies of orientation behaviour" (Borden, et al., 1985).

One reason this is so is that bark beetles have been difficult to fly in wind tunnels. Flight exercise is required in at least some bark beetles before they will take off flying upwind in pheromone-bearing wind (*Scolytus multistriatus:* Choudhury and Kennedy, 1980) or before they will ignore light and respond instead to attractant odors (*Trypodendron lineatum:* Graham, 1959). Even with flight exercise, preliminary studies (Byers, Schlyter, and Löfqvist, unpublished) on *Ips typographus* in a wind tunnel have been largely unsuccessful. The beetles fly towards lights and seem to have difficulty orienting upwind without leaving the plume and striking the side of the tunnel. The possibility that bark beetles are "less accurate" than moths when orienting to a pheromone source may be explained by differences in their requirements: male moths need to find females while bark beetles must find only an aggregation spread widely over a tree. Thus, it is possible that orientation mechanisms in flying bark beetles may be different from those in better-understood insects.

Due to the inherent difficulties of wind-tunnel experiments and flight observations on small beetles, close-range orientation studies have been done in the laboratory on walking bark beetles. The open arena bioassay has indicated they walk upwind and chemotactically orient to pheromone sources (Wood and Bushing, 1963; Birch and Wood, 1975; Byers and Wood, 1981) or to host odors (Byers et al., 1985). The beetles could also do so under natural conditions, although we have little or no evidence of this.

In the field, there are several studies on bark beetles that indicate, with various degrees of credibility, that beetles fly upwind to pheromone sources, presumably when stimulated by pheromone molecules within a plume. However, these studies were not truely rigorous, in that they relied on descriptive observation or they utilized correlations between wind direction and trap catches. For instance, McMullen and Atkins (1962) and Rudinsky (1963) observed that Douglas-fir beetles flew "upwind" and directly to a pheromone source although some "flew by and circled back before landing." Gara (1963) stated *Ips paraconfusus* take off in all directions, but usually with the wind. Upon encountering pheromone-laden air, they oriented against the wind and flew back toward the source of attraction.

Later studies have attempted to quantify the orientation behavior by catch-

ing beetles in traps placed at or near a pheromone source (Chapman, 1962; Gara, 1963; Coster and Gara, 1968; Seybert and Gara, 1970; Gray et al., 1972; Tilden et al., 1979; Byers, 1983; Helland et al., 1984; Schlyter et al., 1987). The conclusions of these studies, however, were all dependent on correlations of trap catch with wind direction measured nearby (usually at one place) and time-averaged. Thus, sometimes the wind direction may not have been aligned with the "prevailing wind direction," and it was not known precisely what the relative frequencies of beetle attraction/wind direction were over certain periods during the test. Therefore, the objective of this study was to obtain direct evidence of upwind orientation to a pheromone source which was both quantitative and not dependent on correlative wind data. A pair of rotating traps on a windvane which aligns with the wind provides a novel design for testing anemotactic orientation to odor sources in insects. The hypothesis is that the "downwind" trapping surfaces will intercept and catch significantly more beetles than the control traps placed "upwind."

METHODS AND MATERIALS

A windvane was constructed that held either a box-type sticky trap or two tubular sticky traps suspended on opposing arms. A 5-mm metal shaft served as the axle which rotated inside two ball bearings fixed near the top of a 1.5-m metal pole that was driven vertically into the ground. A polycarbonate vane was attached to a support about 16 cm from the axle and counterbalanced by metal weights (Figure 1). The dimensions of the vane were approximately 2 mm thick $\times$ 15 cm top $\times$ 38 cm bottom $\times$ 39 cm and 45 cm sides. In the first trap design, a square box was constructed from four, 30 $\times$ 30-cm, 6.3-mm wire-mesh screens coated with Stikem Special,® Seabright Enterprises, Emeryville, California. The box rested on 4-mm fiber board and was aligned so that the downwind side was perpendicular to the vane (Figure 1).

In the second trap design, 19-cm-diam. $\times$ 30-cm-high tubular sticky traps were constructed from the same materials and suspended equidistant from the axle (120 cm) on the ends of a 2 $\times$ 2-cm wooden beam (Figure 2). The two opposing traps were aligned with the vane so that one trap would be consistently downwind while the other was upwind of the pheromone source. The pheromone components of *D. brevicomis*, *exo*-brevicomin and frontalin (both >96%), and the host component, myrcene (>98%), all from Chem. Samples Co., Cleveland, Ohio, were released individually from glass tubes (two for each component) inside a dispenser (Byers and Wood, 1980; Tilden and Bedard, 1985). The release rates varied significantly with temperature but were estimated to be about 3 mg/day of each component.

Beetles were collected from the sides of the box trap once or twice daily (seven replicates) during its placement in the Sierra National Forest near Bass

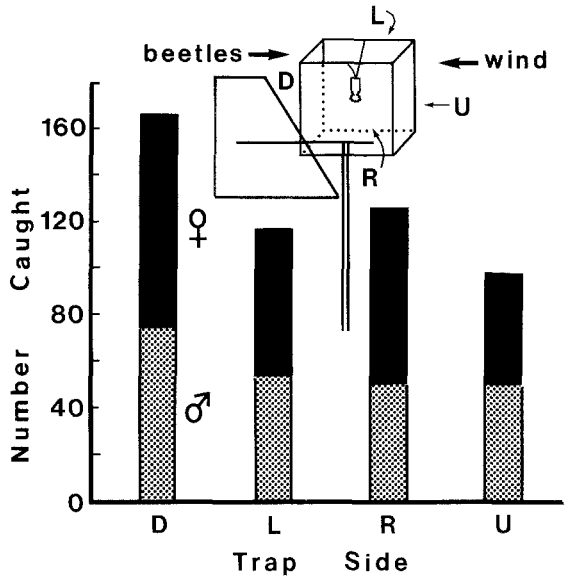

FIG. 1. Upwind orientation of *D. brevicomis* to a pheromone source within a "box" sticky trap (30 cm on a side) placed upon a windvane at 1.4 m height. The pheromone components, *exo*-brevicomin, frontalin and myrcene, were each released at 3 mg/day from within the box trap. Letters designate trap sides orientation: D = downwind, R = right and L = left of downwind, and U = upwind.

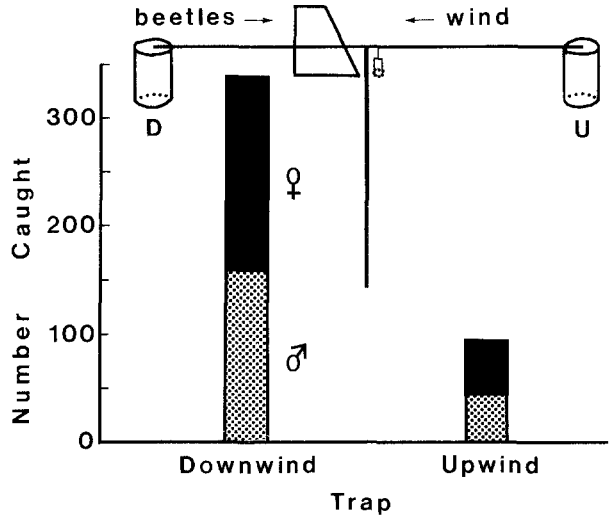

FIG. 2. Upwind orientation of *D. brevicomis* to a pheromone source placed at the rotation axis of a windvane (1.4 m height). Opposing tubular sticky traps (30 cm × 19 cm diam.) were aligned with the wind direction and each was 1.2 m from the rotation axis. The pheromone components, *exo*-brevicomin, frontalin, and myrcene, were each released at 3 mg/day in the forest.

Lake, Madera County, California, at 1000 m elevation (August 19–23, 1985). The opposing traps of the second design were similarly picked of beetles (six replicates) during testing in the same area (August 24–27, 1985). Wind speeds were measured with a fan anemometer for several 1-min periods during general alignment with the windvane in order to obtain an estimate of environmental conditions. Effects of position on the distributions of catch between the downwind side and the other three sides of the box trap and also between the opposing traps were determined with the Wilcoxon matched-pair test. Binomial confidence limits for sex ratios were calculated (Byers and Wood, 1980) and compared for significant differences with chi square.

RESULTS

The catches of *D. brevicomis* on the downwind, left and right of downwind, and upwind sides of the box sticky trap situated on the windvane are shown in Figure 1. As might be expected, if the beetles were flying upwind just prior to landing, the downwind side caught more beetles (32.3%) than either the left (22.8%) or right sides (25.7%) or the upwind side (19.3%), and the upwind side caught the fewest beetles. The downwind side caught significantly more males and females than did the upwind side (1.5 and 1.9 times more, respectively, ($P < 0.05$), and the sex ratios caught on the various sides were not significantly different (Table 1). It is apparent, however, that each of the four sides caught a significant proportion of the total catch.

TABLE 1. NUMBER OF *D. brevicomis* CAUGHT AND RATIOS (WITH 95% BINOMIAL CONFIDENCE LIMITS) ON WINDVANE TRAP WITH BOX TRAP OR OPPOSING TRAPS (SEE FIGURES 1 AND 2)

	Number caught		Sex ratio
	Male	Female	M/F[a] 95% BCL
Box trap			
Downwind side	74	92	0.80(0.59–1.09)
Left side[b]	53	64[c]	0.83(0.58–1.19)
Right side[b]	56[c]	76	0.74(0.52–1.04)
Upwind side	50[c]	49[c]	1.02(0.69–1.51)
Opposing traps			
Downwind trap	156	182	0.86(0.69–1.06)
Upwind trap	39[c]	53[c]	0.74(0.49–1.11)

[a] Sex ratios were not significantly different ($P > 0.05$, χ^2).
[b] When one is facing upwind.
[c] Wilcoxon matched-pair test showed trap surfaces had significantly different distributions of catch for a particular sex compared to the corresponding downwind side or downwind trap ($P < 0.05$).

In order to further test the anemotactic orientation hypothesis, two opposing traps were placed 120 cm from the pheromone source such that one trap was always aligned downwind while the other was upwind (Figure 2). Here it can be seen that the trap catches were even more different since four times more males and 3.4 times more females were caught on the downwind trap compared to the upwind trap (Figure 2). The differences in catch were statistically significant for both males and females ($P < 0.05$), while the sex ratios caught on each trap were not significantly different (Table 1). The wind was observed to change direction rather frequently (about once every minute) by as much as 180°. This was apparently more variable than observed earlier (Byers, 1983). Wind speeds varied between 0.5 and 1.5 m/sec with brief periods of calm (wind changing). All tests were conducted during favorable flight weather (warm and sunny, midday temperature 25–32°C).

DISCUSSION

The catches of *D. brevicomis* on the respective sides of the box sticky trap on the windvane indicate that both sexes orient upwind to pheromone sources, at least in the final landing stage. The fact that significant proportions apparently were caught on sides other than the downwind side can be explained in several ways. One possibility is that the wind direction changed more abruptly than could be followed by the windvane so that anemotactic beetles were caught by inappropriate sides. It is almost certain that the zigzag flight path of bark beetles as they traverse a narrowing plume (as in other insects, Kellogg et al., 1962; Traynier, 1968) would cause significant catches on the sides. The observation of earlier scientists also indicate that beetles that fly out of the odorous air attempt to turn and even circle back to the source, resulting in catches on the upwind side. After the experiments were concluded, I became aware of another example where a similar windvane trap (as in Figure 1) indicated that onion flies oriented upwind to host-plant odors, at least within 20 cm of the source (Dindonis and Miller, 1980). A box trap of much larger dimensions would have probably given much larger differences in catch among the sides.

Tests of anemotaxis on a larger dimension were attempted by separating the trapping surfaces about 120 cm from the source in both the downwind and upwind directions. The higher catches on the downwind trap indicated that beetles were responding anemotactically at least 1.2 m from the pheromone source. The fact that some beetles were caught on the upwind trap can be explained by: (1) the windvane may have sometimes lagged behind the change in wind direction and (2) not all anemotactic beetles were caught by the downwind trap and, when they were not trapped at the source, they dispersed at random on even continued more or less upwind until they were caught by the upwind trap. It

would have been interesting to have compared these results with a test where a box trap was also placed over the source in order to reduce the possibility of the second explanation above. Of course, many trap configurations are possible in combination with the windvane concept.

Lindelöw and Weslien (1986) sum up our knowledge of *I. typographus* flight behavior during the dispersal flight: "when released, beetles always flew downwind for at least 10–15 m, after which time they were impossible to observe." However, it is sometimes possible to observe beetles a few meters before landing at a pheromone source during the late afternoon as sunlight is transmitted through their wings. Others have used white sheets to offer a contrast (Chapman, 1962; Seybert and Gara, 1970). These observations are not quantitative and are only correlated with wind direction, which is often imperfectly known.

Several quantitative studies have provided evidence for upwind orientation to attractive sources in bark beetles but one or more drawbacks are apparent: (1) the tests used marked beetles, often selected, that were subsequently released (unnaturally), (2) the tests were performed in unnatural settings (cages, grassland), (3) the tests used fixed-position traps that may not have been optimally aligned with the prevailing wind direction, (4) the wind direction was averaged and could only be correlated on a gross scale since instantaneous catch and wind direction were not determined, and (5) the wind measurements were usually only at one location and somewhat distant from the traps, and in some cases measurements were from weather stations several kilometers away.

Chapman (1962) found that more *Trypodendron lineatum* were caught on the downwind sides of sticky traps placed over attractive logs (drawbacks 3–5 above). Gara (1963) performed an interesting experiment in which a line of nine traps, placed parallel to the wind direction, released pheromones from each in increasing and then decreasing amounts. Marked *Ips paraconfusus* were then released at either the upwind or downwind areas of the line. The suggestion was that since the percent recoveries on the traps were virtually identical, this must mean that beetles released upwind flew with the wind until turning upwind in response to pheromone, while beetles released downwind flew immediately upwind in response to pheromone. Ignoring anemotactic theories and personal observation of flying beetles, however, one could argue that there is a more simple explanation of Gara's results. This is that beetles do not utilize wind direction when orienting to pheromone as their release either upwind or downwind made no difference on their distribution of recapture along the trap line. However, the windvane trap results presented here allow us to believe more strongly in Gara's explanation.

Coster and Gara (1968) released marked *D. frontalis* in a grassland area from four cardinal directions that were 30.5 m from a pheromone source. The beetles released from the downwind direction were recaptured in the highest

proportion, but the numbers were probably too low for reliable statistics (drawbacks 1–5). Seybert and Gara (1970) found that caged *I. pini*, artificially induced to fly, flew proportionately more to the upwind wall (75%) when the cage was placed 6 m downwind of a pheromone source (no statistics, drawbacks 2–4). In a similar experiment with caged *D. ponderosae*, Gray et al. (1972) showed a statistically significant preference for upwind flight to attractants (drawbacks 2–4). Tilden et al. (1979) used sticky traps in four cardinal directions at 1.5- and 4.5-m distances from a pheromone source to monitor the attraction of *D. brevicomis*. They found that the downwind trap consistently caught more beetles than the upwind trap (drawbacks 3–5). Byers (1983) used three trap rows, each 21 m long of 15 sticky traps, placed perpendicular to the prevailing wind and each succeeding row was 4.6 m further downwind from a pheromone source in order to observe the upwind orientation of *I. paraconfusus*. Both sexes were caught in a narrowing and concentrating pattern as they approached the source, but the male catch at the source was significantly reduced compared to female catch (also compared to the sex ratios on the rows of traps), indicating that male orientation to pheromone is less focused than that of females (drawbacks 3–5).

Helland et al. (1984) released marked *I. typographus* in grassland from four cardinal directions either 12 or 20 m from a central pheromone source. Beetle catch was monitored in 64 traps, each capable of catching beetles from four cardinal directions with respect to the source, stationed in eight 24-m-long "spokes" radiating from the source. The pattern of catch was consistent with an upwind orientation to pheromone, for beetles released downwind, and there was evidence that beetles "swarmed" around the source and did not always move straight into the trap (drawbacks 1–5). In many respects their results are reminiscent of those obtained by Gara (1963) above. Schlyter et al. (1987) placed barrier traps in four cardinal directions each 3 m from a central pheromone source and found that *I. typographus* was again caught in a pattern consistent with upwind anemotaxis (although the wind measurements were taken several kilometers away from the experiment, drawbacks 3–5).

The present study provides a method for continuously integrating wind direction and flight intensity in a natural setting with naturally responding beetles. Thus, the method of coupling a windvane with beetle traps circumvents the five experimental drawbacks encountered in the past. However, much more remains to be understood about close-range flight orientation (observed here) and as well longer-range orientation that often was probed in the experiments discussed above.

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NOVEL DIFFUSION–DILUTION METHOD FOR RELEASE OF SEMIOCHEMICALS: Testing Pheromone Component Ratios on Western Pine Beetle

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Abstract—Each of the pheromone components of the Western pine beetle, *Dendroctonus brevicomis* LeC. (Coleoptera: Scolytidae), *exo*-brevicomin (E) and frontalin (F), were released in the forest at various ratios 0.01 : 1, 0.1 : 1, or 1 : 1 to a constant dose of the opposite component (E or F) plus the host monoterpene myrcene (M), which were each released at 1.5 mg/day. The components were released by a new method that combines the principles of chemical diffusion through a tube with mole percentage dilution of the chemical. Both sexes of *D. brevicomis* were attracted similarly at comparable ratios (and release rates) of E or F and showed similar logarithmic relationships (r^2 = 0.92–0.99). The bark beetle predator, *Temnochila chlorodia* (Mannerheim) (Coleoptera: Trogositidae) was apparently less sensitive to E than *D. brevicomis*, being relatively less attracted to amounts of E equivalent to that released by 70 females, while none were attracted to that from seven females (while this rate still attracted significant numbers of conspecifics). The apparent insensitivity of bark beetles to extreme ratios between pheromone components in contrast to moths is discussed. The advantages of the diffusion–dilution method of releasing semiochemicals compared to previous methods of absorbents, wicks, capillary tubes, and semipermeable plastic membranes are also discussed.

Key Words—*Dendroctonus brevicomis*, Coleoptera, Scolytidae, *Temnochila chlorodia*, Trogositidae, pheromone, frontalin, *exo*-brevicomin, myrcene, controlled release.

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INTRODUCTION

Knowledge of qualitative and quantitative release of synthetic semiochemicals from dispensers is of major importance to the understanding of the chemical ecology of an organism. During isolation of relevant semiochemicals, one must usually dispense volatile compounds in order to evoke certain behaviors which can then be compared to the natural ecology. Thus, scientists are interested in simulating and experimenting with these natural processes by varying the release rates of semiochemicals and quantifying the behavioral reactions. Generally, with a laboratory or field bioassay, the experimenter wants to know (1) the purity of the semiochemical and whether it remains pure during the test and (2) the semiochemical's rate of release and whether it remains constant. One usually wants to release a very pure semiochemical whose purity remains constant and to know that the exact release rate remains constant during the test. Ideally, one should be able to adjust the release rates to any specified amount conveniently and within a range of several orders of magnitude. To date I know of no release methods which allow experimenters this degree of power—but such a method is presented here.

Several methods have been used in varying degrees with advantages and disadvantages for the experimenter. Some of these methods include: (1) release from wicks in bottles containing pure semiochemicals or solvent mixtures, (2) release from rubber septa that have absorbed semiochemical, (3) release from semipermeable plastic bags or vials containing semiochemical, and (4) release from open-ended tubes of glass or other inert material containing semiochemical. These methods, discussed in more detail later, all have one or more disadvantages, such as uneven release rates over time, possible reactive effects on the semiochemicals, or they are inconvenient to specify or vary the release rate (which is often practically limited to a narrow range). In this paper, a new method for releasing semiochemicals, which combines the principles of volatile gas diffusion through a tube (Fick's first law) with Raoult's law of vapor pressures for mixtures of volatile liquids, is presented.

In bark beetles (as in most other previous insect studies), the release of synthetic pheromone components has usually been done at only one level. The level was not necessarily similar to the natural release rate, and often the experimenter had only a rough estimate of the degree of correspondence (Roelofs, 1979). In fact, a compound cannot be properly termed a semiochemical until the synthetic release and behavioral response is determined at levels that correspond to the natural biologic condition. This is a major problem in chemical ecology and, until quite recently, has generally been ignored because of practical difficulties. Natural release rates of pheromone from bark beetles, which must feed in order to produce it, have been especially difficult to measure because of the low amounts relative to host volatiles (Schlyter et al., 1987a).

Browne et al. (1979) and Schlyter et al. (1987a) are among the few who have determined the average release rate of pheromone from bark beetles feeding in a host tree. The former estimated that a female Western pine beetle (*Dendroctonus brevicomis* LeC. (released her pheromone component, (+)-*exo*-brevicomin (E), at 4.1 μg/day, while the male released his component, (−)-frontalin (F), at 0.86 μg/day. Together these two components with the host monoterpene myrcene (M), released by boring activity at 410 μg/day/entrance hole (Browne et al., 1979), are equally attractive to both sexes (Wood et al., 1976). Tilden and Bedard (1985) have used these component ratios to test three release rates over two orders of magnitude (1×, 10×, and 100×) compared to similar levels of a 1 : 1 : 1 (E : F : M) ratio. Their release rates of the active enantiomers of the 1 : 1 : 1 mixture at the 1× level began at amounts equivalent to that released by 52 females and 220 males according to calculations based on the results of Browne et al. (1979), assuming beetles release pure enantiomers (Stewart et al., 1977). The highest level (100×) was equivalent to 5200 females and 25,000 males. Tilden and Bedard (1985) also tested a 5 : 1 : 400 (E : F : M) ratio at 1×, 10×, and 100× (i.e., 87 females and 76 males at 1×), but since the ratios of more than one component were varied simultaneously and the levels of all components were different from the 1 : 1 : 1 ratio and level, they could not easily determine the effects of various component ratios. Very few studies, in fact, have investigated the behavioral effects of pheromone component ratios in Scolytidae (Schlyter et al., 1987b).

Therefore, the purpose of this study was to test the effects of changing the ratio of either E or F relative to a standard 1 : 1 : 1 (E : F : M) bait on the attractive response of both sexes of *D. brevicomis*. Since Tilden and Bedard (1985) had investigated the effects of release rates equivalent to large beetle aggregations, it was decided to decrease the amounts released of either component from about 3.5 × (183 females and 872 males) down to .035 × of their lowest rate or about two females or nine males. In order to obtain these low release rates, a new semiochemical release method, termed "diffusion–dilution," was developed and applied.

METHODS AND MATERIALS

Diffusion–Dilution Method of Semiochemical Release. Fick's differential equations describing diffusion can serve to determine the instantaneous rate of release of a semiochemical from a capillary tube:

$$\text{release rate} = -\pi * r^2 * D * (C_2 - C_1)/x \qquad (1)$$

Where: r = radius of the tube, D = diffusion coefficient, C_2 = liquid concentration, C_1 = 0 (assuming convection carries vapor away), and x = distance between tube opening and meniscus level of liquid (Villars and Be-

nedek, 1974) (* = BASIC symbol for multiply). More complicated equations (Brooks, 1980) are needed to describe the release over time as the level of liquid decreases in the tube. In practice, however, it is usually more accurate to measure the release rate over the expected experimental period because one does not know precisely the diffusion coefficient (D) and other contributing factors (e.g., meniscus curvature, surface tension, and temperature effects). Tilden and Bedard (1985) and others (Browne, 1978; Byers and Wood, 1980; Byers, 1982; Tilden et al., 1983) have used 52-mm-long × 3.5-mm-ID glass tubes sealed at the bottom to dispense *exo*-brevicomin (E) and myrcene (M) by filling the tubes to a level about 40 mm below the opening. Similarly, they have used 62-mm-long × 2.2-mm-ID tubes to dispense frontalin (F) by filling to a level about 50 mm below the opening. At these distances, diffusion is rather constant over time (pseudo zero order; Brooks, 1980), and so the measured release rate of each has been considered constant at about 1.5 mg/day (Tilden and Bedard, 1985).

The concentration (C_2) is actually the vapor pressure of the semiochemical, and this can be varied according to Raoult's law, which states that the vapor pressure (release rate) of a volatile substance (semiochemical) is proportional to its mole fraction in a solvent. The following equation can then be derived for purposes of diluting semiochemicals with solvent in order to obtain a specific semiochemical release rate:

$$mls = fws * (gsem/fwsem - fsem * \text{gsem/fwsem})/\text{fsem/gs} \qquad (2)$$

Where: *mls* = milliliters of solvent; *fws* = formula weight (= molecular weight) of solvent; *gs* = grams solvent per milliliter (density); *gsem* = grams of semiochemical; *fwsem* = formula weight of semiochemical; and *fsem* = mole fraction of semiochemical ($0 < fsem \leq 1$). For example, a stock solution of E in ethanol that would yield a release rate 10% that of a neat solution and that is to be made using 0.5 g of E would require 0.52 ml of E (0.5 g * ml/0.96 g) and 1.6 ml ethanol ([46 g/mole * (0.5 g/156 g/mole − 0.1 * 0.5 g/156 g/mole)/0.1] * ml/0.828 g). Stock solutions of E in ethanol that should release about 1%, 0.1%, 0.01%, and so forth, that of a neat solution can then be made simply from the 10% solution by serial 1:10 (1 + 9) dilutions.

Testing Pheromone Component Release Rates on Beetles. Field tests of *D. brevicomis* pheromone component ratios were conducted in the Sierra National Forest near Bass Lake, California, at 900 m elevation, August 20–30, 1985. Various release ratios of E to F + M or F to E + M were composed, based on the formula above, using ethanol as the diluting solvent and the appropriate glass tubes (each component alone) of Tilden and Bedard (1985) described above (Table 1, Figure 1A). Tubes with fresh solution were replaced each morning in order to minimize release rate errors due to differential loss of the ethanol and the consequent effect on concentration of the semiochemical. Two groups (about 100 m apart) of eight sticky traps each (6-mm mesh metal-screen cylinder, 19

TABLE 1. CATCHES OF MALE AND FEMALE *Dendroctonus brevicomis* (AND SEX RATIOS) AND *Temnochila chlorodia* ON STICKY TRAPS RELEASING VARIOUS RATIOS OF ETHANOL (A), FRONTALIN (F), *exo*-BREVICOMIN (E), AND MYRCENE (M)

Component release rates[a]	*D. brevicomis*			*T. chlorodia*	
	Male	Female	M/F sex ratio (95% BCL)[b]	Male	Female
A[c]	0	0		0	0
F + M + A[c]	6	10	0.60 (0.23–1.59)	0	0
E + M + A[c]	8	3	2.67 (0.77–9.26)	4	2
0.01 F (in A[d]) + E + M	53**[e]	57*	0.93 (0.64–1.35)	5	4
0.01 E (in A[c]) + F + M	60**	68**	0.88 (0.62–1.25)	0	0
0.1 F (in A[d]) + E + M	98	107**	0.92 (0.70–1.20)	4	7
0.1 E (in A[c]) + F + M	124**	158**	0.78 (0.62–0.99)	2	1
E + F + M + A[c]	155	170	0.91 (0.73–1.13)	5	4

[a] E, F and M were each released at approximately 1.5 mg/day or fraction thereof.
[b] Sex ratios were not significantly different ($P > 0.05$, χ^2) 95% BCL = 95% binomial confidence limits.
[c] A (ethanol) was released from E-tubes at about 66 mg/day.
[d] A (ethanol) was released from F-tubes at 21 mg/day.
[e] Wilcoxon matched-pair tests indicated catch was significantly different from the catch on the corresponding 10% serial rate (or control for the 0.01 rate) within the same sex at $P < 0.05$ (*) or $P < 0.01$ (**).

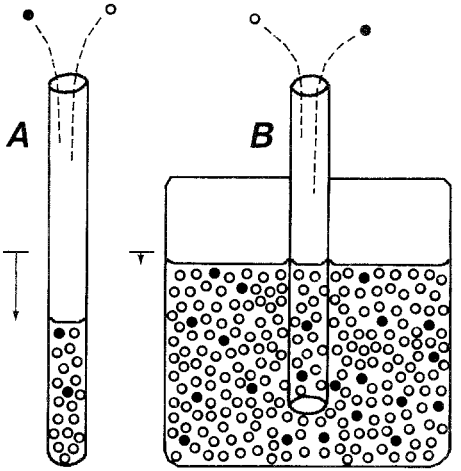

FIG. 1. Cross-sections of two possible dispensers for semiochemical release that demonstrate Raoult's law of solute–solvent volatization. The mole percentages of semiochemical (●) and solvent (○), e.g., 10% and 90% as shown, determine the corresponding release rates of each compared to respective neat solutions as controlled by the diffusion rate through the tube. Dispenser B is preferable to dispenser A because the larger reservoir remains practically constant, both in concentration and level, as does the release rate, during prolonged periods of release.

cm diam. × 30.5 cm high, coated with Stikem Special placed at 1.2 m height; Bedard and Browne, 1969) contained the respective baits (Table 1) in sun/wind protective dispensers (Byers and Wood, 1980). The traps were randomized each day (six, 1-day replicates/trap) within a group and were spaced 25 m apart on a line. *D. brevicomis* and *Temnochila chlorodia* (Mannerheim) were collected each day and their sex was determined.

Wilcoxon matched-pair tests were used to compare the male and female catches on the 1:1:1 (E:F:M) ratio to their corresponding catches on the 0.1 E or 0.1 F ratios, and these ratios were compared to corresponding 0.01 dilutions, while the 0.01 dilutions were compared to each of the components alone. Binomial confidence limits for sex ratios were calculated (Byers and Wood, 1980) and compared for significant differences with chi square. The relationships of E or F release (mg/day) with trap catch (E:F:M = 100%) were determined using several regression equations [power, exponential, logarithmic, linear, $Y = a + bX^2$, and $Y = a + b/X$].

RESULTS

The alcohol-diluted E and F tube dispensers seemed to yield the predicted release rates based on the logarithmic relationship with the catch of *D. brevi-*

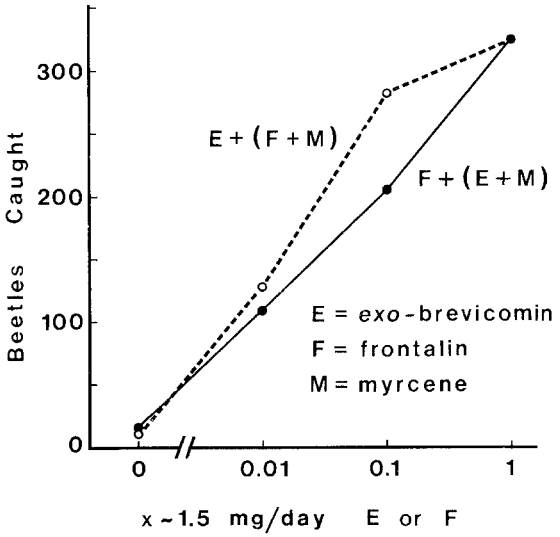

FIG. 2. Attraction of *D. brevicomis* to pheromone component ratios released from sticky traps. Various rates of E (*exo*-brevicomin) plus constant rates of F (frontalin) and M (myrcene), each at 1.5 mg/day, or various rates of F plus constant rates of E and M (each point represents mean of 12 one-day replicates) were released in the Sierra National Forest, August 20–30, 1985.

comis (Figure 2). The increases in catch of each sex to increases in either component were in most cases statistically significant for each 10-fold increase in release (Table 1). However, the catch on the 1:1:1 ratio at 1.5 mg each/day was not significantly different from that on the 0.1 E or 0.1 F releases. The sex ratios of catch on all pheromone component ratios and rates were comparable and not significantly different based on chi-square analysis (Table 1). However, on the E or F components alone there is an indication of sexual preferences, but the numbers are too low for reliable statistics. It is interesting to note that the total catches for each of the component ratios at a particular release rate were similar, as were the catches of each sex (Figure 2, Table 1). Ethanol (A in Table 1) was perhaps not the most appropriate choice for solvent as some "less aggressive" bark beetles use it to locate hosts (Moeck, 1970; Klimetzek et al., 1986). However, ethanol did not appear to be attractive to *D. brevicomis* when presented alone, although the possibility remains that it might have enhanced the attraction to the pheromone components.

Logarithmic relationships (linear increase in attraction with 10-fold increases in pheromone release) are usually expected for dosage–response curves. Several curvilinear regressions of *D. brevicomis* component release (X), but not including $X = 0$ since this value is infinitely smaller than 0.01, against percent catch (Y), E:F:M catch = 100%, confirmed that logarithmic curves had the highest coefficients of determination. The logarithmic relationship of male response to F (+ E + M) from 0.01 to 1 had an $r^2 = 0.99$, $Y(\%) = 92.9 + 14.3 \ln X$, and female response to F (+ E + M) over the same range had an $r^2 = 0.99$, $Y(\%) = 92.9 + 14.4 \ln X$. The male response to E (+ F + M) from 0.01 to 1 had a $r^2 = 0.96$, $Y(\%) = 98.2 + 13.3 \ln X$, and female response an $r^2 = 0.84$, $Y(\%) = 102.4 + 13.0 \ln X$.

T. chlorodia was caught in a pattern consistent with an attraction to E only, as noted previously (Bedard et al., 1980). However, both sexes seem equally responsive to release rates of E, and they seem less sensitive than *D. brevicomis* because proportionately less were caught on the traps with the 0.1 and 0.01 E releases (Table 1).

DISCUSSION

Diffusion–Dilution Release of Semiochemicals. Let us first consider diffusion from the glass tube with a neat solution of semiochemical in which the release rate is inversely proportional to the depth of the meniscus level in the tube (equation 1). If we assume an average release rate of F of 1.5 mg/day over 10 days, according to Tilden and Bedard (1985), when the meniscus changed from 53 below the top (as it was similar to this, see Methods and Materials) to a level 4 mm lower after 10 days (about 14.5 mg loss), then the beginning instantaneous rate on the first day should have been 1.56 mg/day and the ending rate 1.45 mg/day (equation 1). Another viewpoint is that the change in meniscus

level is inversely proportional to the square root of time (Brooks, 1980; Kydonieus, 1980). Thus, if we begin with a release of 12.4 mg/day at $t = 1$, solved by successive approximation by computer, with a nearly full tube of F, then after 63 days of elution we would expect a rate of 1.56 mg/day and, 10 days later, about 1.45 mg/day (as above). The assumed total release $[\Sigma_{t=1}^{732} 12.4/(t/10)^{-1/2}]$ would equal 206.5 mg, which corresponds to a meniscus level change in the F tube of 57.1 mm, close to our 57-mm ending level assumed above.

These calculations indicate that for all practical purposes the release rate is constant (pseudo zero order) for a particular test period. This is true as long as the meniscus is relatively far from the tube opening and the level does not change much, such as when low amounts are released from a not-too-volatile substance (pheromone). The advantage of tube dispensers is that the release rate can be immediately adjusted to the pseudo constant rate by placing a small amount at a level several centimeters from the opening. The weight and volume changes can also be easily determined by weighing and by measuring meniscus level changes.

Larger release rate discrepencies should be expected when using the above diffusion method with diluted semiochemical (diffusion–dilution), although they should be much less than an order of magnitude used here. The solvent used was ethanol, which is not too volatile for a solvent (bp 78.5°C, vapor pressure 61–122 mm at 26–36°C) but much more so than M (2.06–3.42 mm at 26–36°C, which, based on the neat release rate, is similar to E). However, the higher volatility of ethanol meant that proportionately more was released than E or F over time, which would cause the mole percentage of these compounds to increase during the day. Thus, at most we would expect an increase of E or F release of 43% during the 10-h flight period (0900 to 1900 hr) based on the measured loss of ethanol in the tube (30% of total). Since some E or F also was released, the actual increase was less than 43%.

A compensatory phenomenon was simultaneously occurring since the meniscus level also fell about 30%; this would cause the reverse effect and diminish the rate of release (at most 9%). These partially compensating effects occurred throughout the day, so the error in release rate became larger with time. However, variation in temperature probably had effects at least as, or more, severe (up to 100% as seen from the vapor pressures over the daily temperature range, above). Absolute errors on the order of 10% could be possible if calibration had been at sea level but testing was at higher altitudes (L.E. Browne, personal communication). In any case, these errors during the course of the day were on the order of less than 50–100%, insignificant compared to the 1000% differences between each serial 1 : 10 dilution.

The tube-type release device is adequate for most experiments, but if tests for longer periods and constant rates are desired, a similar device with a larger reservoir is preferred (Figure 1B). Both dispensers shown in Figure 1 would

have the same release rate initially since their openings and diffusion distances are identical. However, as noted above, after a time both the concentration of semiochemical and diffusion distance change in the tube, while a similar release from the larger reservoir has little effect (Figure 1). Many other solvents could be used. Theoretically, it is desirable to have a solvent that has chemical properties and polarity similar to the released semiochemical. Work is now underway to determine the release rates of various semiochemicals in different solvents by means of porapak Q volatile collections and quantitative gas–liquid chromatography.

Comparison with Other Controlled Release Methods. The first dispensers that released synthetic bark beetle pheromones used filter paper (Wood et al., 1967) or metal tubes containing gas–liquid chromatographic packing to hold the compounds until they evaporated (Wood et al., 1968). Since then, experimental elution devices have included four major types. The first type uses various absorbent materials, such as rubber septa, and is widely used with moth pheromones. Depending on the absorbent, this type generally has release curves that decrease exponentially or geometrically, thus being rather constant after several days of elution. I believe this method is less desirable because the rates change dramatically soon after application, and it is difficult to specify and vary the release without much laborious measurement. A second method has used wicks dipped in semiochemical (Tilden et al., 1979; Bedard et al., 1980), but the inexact surface area and physical properties of wicks make it difficult to either specify or vary the release accurately. A third method uses semipermeable plastic bags or vials to enclose semiochemical (Vité et al., 1986; Klimetzek et al., 1986; Schlyter et al., 1987b). This results in a constant release rate that is proportional to the thickness of the plastic and its surface area (Kydonieus, 1980). However, it is easy to see that release rates over several orders of magnitude are impractical due to the limitation of dispenser sizes. Furthermore, much testing is needed for each particular vial or bag to determine the release rate, so rates cannot be specified over a broad range. In all these methods, the absorbent, wick, or semipermeable plastic could have degrading effects on a particular semiochemical which would not be expected if inert materials are used.

The "test-tube" type dispenser used here has many advantages over the other three methods in that glass will not affect the semiochemical, and the rate can be specified depending on the area of the opening and the level of the liquid. The criticism of Kydonieus (1980) that capillary reservoir systems have parabolic curves of release (large amounts initially) can be easily overcome by using tubes with liquid levels that are several centimeters from the top opening. The major problem has been that a large array of tube sizes is needed (which cannot be varied freely) to encompass several orders of magnitude in release, the same problem as with the plastic bags. However, by mole percentage dilution, one

can specify any desired rate that is less than a known rate of a neat semiochemical. A mixture of semiochemicals probably could be released from one dispenser solution if their mole percentages were relatively small (< 10% of neat). These small mole percentages would tend to minimize any unpredicted effects on both vapor pressures and possible physical interactions between semiochemical molecules.

Attraction to Pheromone Component Ratios. Both male and female *D. brevicomis* had similar attraction levels over the concentration range of either E or F (with constant release rates of M and the opposite component). In fact, one may find it somewhat surprising that all the logarithmic relationships were very similar (see Results, Figure 2) because males seem to prefer E alone while females prefer F alone (Bedard et al., 1969; Vité and Pitman, 1969; Pitman and Vité, 1971; Hughes and Pitman, 1970; Byers, 1987) (Table 1). On the other hand, Byers and Wood (1981) found that both sexes were equally responsive (logarithmic relationship) at each step in a concentration range of E : F : M covering five orders of magnitude in the laboratory. Similarly, regression analysis of the results of Tilden and Bedard (1985) shows that *D. brevicomis* males and females have logarithmic attraction levels to increasing E : F : M release rates (r^2 = 0.82–0.99) at a center trap and traps 5 m away. Furthermore, the separation of E and F components either horizontally or vertically from 0 to 3 m (perpendicular to the mean wind) decreased the catch on each component but more or less equally for both sexes (Byers, 1987). Thus, except for the components alone, the sexes seem to respond equally to all comparable ratios and concentrations of E : F. This suggests that each component is equally important and has a similar function in the synergism and in the orientation to sources.

In contrast, Schlyter et al. (1987b) varied the ratios and rates of release of (−)-*cis*-verbenol (cV) and 2-methyl-3-buten-2-ol (MB), keeping one or the other constant, and found differences in the response of *I. typographus* to each component. They suggested that MB was more important for close-range orientation, while cV was more important further downwind from the source. Their theory was based largely on differences between catches on traps placed at the source and those placed a few meters away. However, regression analysis of their results indicates both MB and cV have logarithmic relationships (r^2 = 0.88–0.98) of concentration ratio and catch on both "close and long-range traps." Further work on component release ratios with traps at varying distances from the source is needed for both bark beetle species before we can form definitive theories on the function of the synergistic components during the upwind orientation to pheromone.

The release of various extreme component ratios is rather unnatural. A 1 : 100 ratio of F : E (0.01 F) at 1.5 mg E/day would simulate nine males with 183 females, which probably never occurs in nature. Despite these extreme sex ratios of simulated feeding beetles, the responses of *D. brevicomis* to the above

ratio (0.01 F) or to 872 males with two females (0.01 E) were still significant, catching about 34% and 39%, respectively, as many beetles as that on E:F (872 males and 183 females).

In contrast, some moths can be exceptionally sensitive to component ratio variations of as little as 10% or less when the synergists are *E* and *Z* isomers of long-chain monounsaturated acetates (Roelofs and Cardé, 1977; Cardé and Baker, 1984). As shown here and by Schlyter et al., (1987b), one reason bark beetles may be less sensitive to component ratios than moths could be because they must respond to ratios from various groups of beetles and not to a "preferred" individual with a consistent ratio. Initially the beetle aggregation would be composed of small numbers so the variation in semiochemical ratios would be expected to be rather large until later when a statistical mass-average would result. However, male moths would be better adapted if they were able to respond to the individual variation in ratios of many potential female mates—this variation is theoretically larger than the variation between small populations, all other things being equal. Another possible reason is that bark beetles often synthesize pheromone components from monoterpene precursors in the tree, where we expect a large variation (Sturgeon 1979), whereas moths synthesize their components de novo (Bjostad and Roelofs, 1981). This could explain the insensitivity of *I. typographus* for cV:MB ratios since cV is made from α-pinene in Norway spruce (Klimetzek and Francke, 1980). In *D. brevicomis*, biosynthesis of F does not seem to be affected by the host, while E is only produced by the female during feeding (Byers, 1983a), although we do not know if a host precursor is involved. Finally, both *I. typographus* and *D. brevicomis* are faced with a changing ratio between pheromone components during the colonization of a tree, introducing even more potential variation (Birgersson et al., 1984; Byers et al., 1984).

Pheromone Qualification. The term "pheromone" was defined by Nordlund (1981) in his semiochemical glossary as "a substance that is secreted by an organism to the outside that causes a specific reaction in a receiving organism of the same species." Using this definition without the implicit quantitative qualification, it is possible to believe erroneously that one has identified a "pheromone" when in fact the substance is irrelevant. This is because many secreted substances, if presented in unnaturally high concentrations, could cause a specific reaction (death or repellency and possibly inhibition, interruption, or disruption of other behaviors). Furthermore, many substances are believed to be active only in small amounts relative to other pheromones, e.g., verbenone in *D. frontalis* (Rudinsky 1973), 2,3-MCH in *D. pseudotsugae* (Rudinsky and Ryker, 1980), or ipsdienol in *I. typographus* (Schlyter et al., 1987c). Some bark beetle pheromones are attractive at lower release rates but become progressively less attractive to the male sex only, e.g., *I. paraconfusus* (Byers, 1983b) and *I. typographus* (Schlyter et al., 1987b,c). Many moth species are

known to be disrupted by higher, and unnatural, concentrations of their pheromone (cf. Cardé, 1984). Therefore, in order to properly define an isolated substance as a certain type of semiochemical, one should have a knowledge of the natural secretion rate compared to the effects of release of synthetic substances on the receiving organism in relation to its ecology. It is hoped that the diffusion–dilution method presented here will aid chemical ecologists in answering the second question concerning release rates of synthetic compounds.

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CHEMICAL CORRELATES OF RHESUS MONKEY FOOD CHOICE:
The Influence of Hydrolyzable Tannins

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Abstract—Feeding behavior was quantified of free-ranging adult rhesus monkeys in the Himalayan foothills of northern Pakistan. Twenty-eight food and 63 nonfood plant samples were collected and analyzed for tannins, alkaloids, fiber, lignin, protein, amino acids, total nonstructural carbohydrates, and free glucose. Most major foods of the rhesus were low in tannins and high in nutrients. The rhesus ate mainly parts of herbaceous plants, although woody plants were more abundant, possibly because herbs were lower in condensed tannins than woody plants. Feeding was negatively correlated with the astringency of tannins, ellagitannins, and total phenolics among all samples. Among leaves of both herbs and woody plants, feeding was negatively correlated with measures of hydrolyzable tannins. Total protein, total nonstructural carbohydrates, and water were not related to food choice and may not have been limiting at the site.

Key Words—*Macaca mulatta*, rhesus monkeys, food choice, tannins, nutrients.

INTRODUCTION

Generalist herbivores such as rhesus monkeys must make complex decisions in order to select a nutritionally balanced, nontoxic diet from among available plant parts and species that vary greatly in chemical composition (Boyd, 1971;

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Glander, 1981; Milton, 1979). Mathematical foraging models predict that food choice should be related to the rate of nutrient acquisition (e.g., energy, protein, etc.) and to the costs of foraging and food acquisition (Belovsky, 1984; Emlen, 1966; MacArthur and Pianka, 1966; Pyke et al., 1977). Herbivore food choice may also be influenced by the presence of plant defense compounds such as alkaloids, tannins, nonprotein amino acids, and cyanogenic glycosides (Harborne, 1982; Swain, 1977; Rosenthal and Janzen, 1979).

In this study we investigated the relationship of plant chemistry to the feeding behavior of free-ranging rhesus monkeys (*Macaca mulatta*) in the Himalayan foothills of northern Pakistan. The selection by the rhesus of different plant species and parts was quantified, and food and nonfood plant samples were collected. These samples were analyzed for nutrient content (total protein, free amino acids, glucose, and total nonstructural carbohydrates) and plant defensive substances (tannins, alkaloids, fiber, and lignin) that might influence food selection. The main goal of this project was to determine which of these factors were important in influencing the diet of the animals under study. The results of the study were tested against the prediction that the rhesus should select for limiting nutrients and avoid overingestion of toxins or digestibility-reducing compounds.

The primates most extensively studied in regard to the chemistry of their foods are the Old World monkey subfamily Colobinae. Colobines have a ruminant-like modified forestomach in which microbial fermentation occurs, and are thus capable of greater digestion of plant fiber and probably detoxification of plant defense chemicals than animals with hindgut (monogastric) fermentation (Freeland and Janzen, 1974; Parra, 1978). Colobines generally choose as their main foods items low in fiber and tannins (McKey et al., 1981; Oates et al. 1977, 1980), but because their diets usually include more leaves and unripe fruits than monogastric primates such as rhesus monkeys, colobines probably ingest higher levels of tannins and plant cell wall components (fiber) than other primates.

Of the monogastric primates, the best studied in terms of food chemistry is the New World mantled howler monkey (*Alouatta palliata*) which consumes mainly tree leaves and fruits (Milton, 1979; Glander, 1981). In Costa Rica, howlers select mainly leaves low in condensed tannins and high in protein and balanced amino acids (Glander, 1981). Among the Old World primates, a comparison of high ($N = 5$) and low preference ($N = 8$) food plants of western gorillas (Pongidae) showed that preferred plants were lower in lignin than less preferred plants (Calvert, 1985). Within the Old World subfamily Cercopithecinae (the subfamily that includes rhesus monkeys), food selection by vervet monkeys (*Cercopithecus aethiops*) from two species of acacia is negatively correlated with condensed tannin contents (Wrangham and Waterman, 1981). The rhesus troop studied in Pakistan differs from the above-mentioned primate species in the temperate rather than tropical nature of its habitat. The diet of these

rhesus differs from those of most primates in the greater consumption of herbaceous leaves and stems and may be most similar to that of baboon species (Richard, 1985). Unlike most previous studies of primate food chemistry, in the present work we chemically analyzed many common plant items that were never eaten as well as items that were eaten.

Tannins have been related to decreased food consumption, growth, and survival in studies with captive rodents (Glick and Joslyn, 1970a; Lindroth and Batzli, 1984; Tamir and Alumot, 1970). In field studies tannins have been negatively related to food choice of ruminants (Provenza and Malechek, 1984) and primates (see above). The antifeedant effect of tannins in vertebrates may be due to a "puckery" or astringent mouth feel caused by the binding of tannins to salivary proteins (Bate-Smith, 1972a). Inhibition of growth in mammals by tannins has been linked to decreased protein digestibility in vivo in rats (Tamir and Alumot, 1970) and voles (Lindroth and Batzli, 1984; Lindroth et al., 1986), increased excretion of endogenous nitrogen (Glick and Joslyn, 1970b), as well as direct toxic effects (Lindroth and Batzli, 1984). In contrast, inhibition of protein digestibility by tannins has not been demonstrated in insects in vivo, and it has been suggested that many insects have digestive adaptations (e.g., high gut pH, presence of surfactants, binding of tannins to peritrophic membrane) which allow them to minimize the effects of tannins (Bernays et al., 1980, 1981; Martin and Martin, 1984; Martin et al., 1985).

Tannins fall into two main structural classes of polyphenolics: condensed tannins (proanthocyanidins), which are composed of two or more flavanol units bound together by carbon–carbon linkages, and hydrolyzable tannins, which are esters of sugars and gallic acid (gallotannins) or hexahydroxydiphenic acid or related phenolics (ellagitannins). The distribution of hydrolyzable tannins is restricted to some families of dicots, whereas condensed tannins occur in most plant families (Swain, 1979). Condensed tannins inhibit feeding in several primate species including *Colobus guereza* (Oates et al., 1977), *C. satanas* (McKey et al., 1981), *Presbytis johnii* (Oates et al., 1980), and *Cercopithecus aethiops* (Wrangham and Waterman, 1981). Hydrolyzable tannins have been identified in some plants in these studies but have not been specifically quantified. Their impact on primate feeding behavior is therefore little explored. In the present study we separately quantified condensed tannins, gallotannins, ellagitannins, total phenolics, and total astringency of plant samples to determine which of these aspects of tannin content were most related to food choice by rhesus monkeys.

METHODS AND MATERIALS

Study Site and Field Methods. All results of the feeding study and descriptions of the field site are presented in detail elsewhere (Goldstein, 1984). The study site was a forest area of approximately 7 km^2 (encompassing most of the

home range of the focal animal troop) in the Murree Hills of northwestern Pakistan near the village of Dunga Gali (73°22'E, 34°3'N) at an elevation of 2000–2750 m. Climate at the site was temperate, with a monsoon season from July through August and 5–8 m of snow each winter. The boundaries of the site were for the most part naturally occurring delimiters such as ridges and streams. The site was divided into six elevation zones (150 m each) starting at 2000 m and up to 2750 m. Transects (100 m) were laid out along contour lines placed midway through each of the six elevation zones. The transects were marked off across the entire width of the site in each elevation zone. The abundance of tree and shrub species was measured by counting all woody plants (dbh > 5 cm) within areas 5 m to each side of the first 20 m of each 100 m transect. A total of 279 transects was sampled. The dominant tree species at the site were *Abies pindrow* and *Pinus wallichiana* with *Taxus baccata*, *Cedrus deodara*, and *Picea smithiana* also occurring commonly. The major hardwood trees found in the area included *Quercus dilatata*, *Prunus cornuta*, *Populus ciliata*, and *Juglans regia*. In disturbed sites, which made up 30% of the study area, grasses, *Trifolium* (clover), and *Viburnum* shrubs were common. The area has been protected by the government against uncontrolled use, but portions of the forest have been periodically disturbed by human habitation and agriculture.

During the period from September 1978 through August 1979, the feeding behavior of nine adults (two males and seven females) in one rhesus troop was quantified using instantaneous focal animal sampling (Altmann, 1974). Data on feeding and other activities were recorded at the beginning of each minute during 10- or 20-min observation periods. Theese periods were spread evenly throughout the day from dawn to dusk. A total of 3646 of the minute feeding records were collected. The results of the feeding study are expressed as the percent of the total number of feeding records that each item was recorded as eaten. These values represent an estimate of the proportion of the total time allotted to feeding that was spent on each item. The troop was difficult or impossible to locate during some months in the winter and monsoon, and so these seasons are underrepresented in the feeding data set. Feeding accounted for 45% of all activity records of the rhesus. Consumption of insects and mushrooms was negligible (0.06% combined). Fully 84% of the feeding records were of vegetative plant parts while only 12% were of fruits, flowers, and seeds.

Ninety-one plant samples were collected for chemical analysis. These included samples of 28 different items that were seen to be eaten by the rhesus. In collecting these items, we chose samples that were as similar as possible to those eaten by the study animals (i.e., same tree, same patch of herbs, same stage of maturity, etc.). Sixty-three very common items that were never seen to be eaten and thus could be inferred to be low in preference were also sampled. Most of the food and nonfood samples consisted of 10 or more individual items (leaves, fruits, etc.) which were later combined for analysis. Thirty-seven

of the plant samples (mostly fruits and flowers) were weighed and stored fresh in ethanol in an attempt to better preserve phenolic contents (Swain, 1979) and to prevent molding of water-rich samples that would have required a very long drying period. All other samples were weighed, air dried, reweighed, and stored dry. No samples were collected at the site of several important foods (grasses and needles of *Abies* and *Pinus*) because we could not precisely identify the items being eaten in these cases (e.g., young vs. mature needles, leaf tips vs. leaf bases, species of grasses, etc.). Samples of needles from *Pinus wallichiana* and *Abies concolor* (no samples of *Abies pindrow* were available) were collected from the Arnold Arboretum, Boston, Massachusetts, on Nov. 2, 1986, in order to provide approximate chemical values for conifer needles.

Chemical Methods. The dried samples were ground to pass a 60-mesh sieve and extracted in replicate three times with hot 50% aqueous methanol. The ethanol-preserved samples were cut up with scissors or crushed with a glass rod, heated to boiling, filtered, and the residue extracted two times with 50% methanol. For all samples, the three extracts were combined, filtered, brought up to a known volume, and stored at 4°C. All phenolic and tannin analyses were performed on these extracts.

Total extractable phenolic contents were measured using the Folin-Denis method with tannic acid as a standard (Singleton and Rossi, 1965). Condensed tannins and ellagitannins were quantified using standard methods, and percent dry weight values were calculated from E 1%, 1 cm values (Bate-Smith, 1972b, 1975). Gallotannins were quantified by reaction of the plant extracts with 5% potassium iodate at 0°C for 50 min and measurement of the pink color produced at 550 nm using tannic acid (supplied from Fisher Scientific) as a standard (modified from Bate-Smith, 1977). For samples that showed increased absorbance with the condensed, gallo-, or ellagitannin assays, an absorption spectrum was prepared to determine if the sample had a spectral peak appropriate for that assay (540–560, 550 and 600, respectively). Samples that did not have such a peak were considered negative for that analysis. The protein-precipitating capacity (astringency) of extractable tannins was assayed using dilute blood protein and expressed as dry weight tannic acid equivalents as described in Bate-Smith (1973a) except that instead of fresh human finger blood, diluted defibrinated rabbit blood (Gibco) was used (Marks, 1985). Because condensed tannins are not always totally extractable (Bate-Smith, 1973b, 1975), the unextracted powder and/or the residue (after extraction) of each sample were also analyzed for astringency and condensed tannins. Total astringency (TAE; tannic acid equivalents) and CT (total condensed tannin) values represent the sum of values for extracts plus residues for ethanol-preserved samples, or the higher of the values for unextracted powders or extracts plus residues in the case of dried samples (Marks, 1985).

The gallotannin and ellagitannin methods have not been tested with such

a diverse set of plant materials before, but they have been used to quantify tannins in maples (Bate-Smith, 1977, 1978), geraniums (Bate-Smith, 1981), and *Prunus* (Schroeder, 1986). The gallotannin (iodate) method reacts somewhat with hexahydroxydiphenic acid-type residues of ellagitannins as well as galloyl residues, whereas the ellagitannin method is specific for ellagitannins (Bate-Smith, 1977). These two methods were significantly correlated with each other and with measures of astringency and total phenolics for the 91 plant samples in this study (Marks, 1985). Significant concentrations (>1%) of gallo- and ellagitannins were found almost exclusively in plant families (e.g., Fagaceae, Geraniaceae, Hippocastanaceae, Polygonaceae, Rosaceae, Saxifragaceae, etc., see Appendix 1) known to contain these compounds (Bate-Smith, 1972c, 1984), thus confirming the specificity of these assays.

Total nonstructural carbohydrates of the aqueous methanol extracts were quantified using the phenol–sulfuric method (Strickland and Parsons, 1972). Free glucose in these extracts was measured with the glucose oxidase–peroxidase method (Raabo and Terkildsen, 1960). Fiber (crude normal fiber) was prepared by a modification of the method of Morrison (1972). The powder or residue of each sample was extracted sequentially with aqueous methanol, ether, dichloromethane, and hot 3% sulfuric acid. The residue (fiber) was dried and weighed. Lignin in this fiber fraction was determined by the acetyl bromide method of Morrison (1972).

All samples were spot tested for the presence of alkaloids by recording the amount of precipitation with Dragendorff's reagent on a 0–3 visual scale after appropriate solvent extraction (Marks et al., 1985). Free amino acids were measured in aqueous methanol extracts using the ninhydrin method (Spies, 1957). Total protein was quantified by hydrolyzing plant material with 3% sulfuric acid at 100°C for 24 hr and using ninhydrin to measure the amino acids released (Marks et al., 1985).

Statistical Analyses. Because plant parts may differ greatly in chemical composition and because herbs may differ from woody plants in their defensive chemistry, the plant samples were divided into subsets by part (leaves, flowers, fruits, stems, etc.) and by habit (herbaceous vs. woody). Herb leaves and herb whole plants were not found to be significantly different in two-tailed t tests and thus were combined into one subset (referred to hereafter as herb leaves). Woody plants and herbs were compared by part (leaves, flowers, and stems) using two-tailed t tests). Other part types were insufficient in sample size for statistical analysis. Spearman correlations between feeding and the chemical variables were calculated for leaves of herbs and woody plants separately and for the whole data set ($N = 91$). Spearman rather than Pearson correlations were used because the feeding data were not normally distributed (most plants had 0 feeding observations). All statistics were done using SAS on an IBM mainframe computer.

RESULTS

Major Foods of Rhesus Monkeys. The plants eaten most often by the rhesus on a yearly basis are listed in Table 1 along with data on chemical composition and percent of total feeding records. By far the most important food sources were *Trifolium* sp. (30% of total feeding observations), unidentified herbs and grasses, and *Abies pindrow*. Other items were important foods during certain seasons. For example, roots of *Oenothera rosea* were staple items during winter, and *Viburnum* fruits and flowers were readily consumed when available in the spring.

Two species of clover occurred at the study site, *Trifolium repens* and *Trifolium praetense*, the former being much more common. Because these species were indistinguishable in the field except during flowering stages, the two species were treated as a single item in the feeding study and sample analyses. Tannin contents in clover were negligible as measured by hemanalysis and chemical methods, and protein content was 16.5%, similar to the mean for all 91 samples (16.2%). Some populations of *Trifolium repens* are known to be cyanogenic (Conn, 1979). All six *Trifolium* samples gave negative results when tested using picric acid paper (Marks, 1985); however, tests for cyanogenesis with dried sample are inconclusive because of possible destruction of cyanogenic glycosides upon drying (Conn, 1979).

Major foods were generally low in astringency (the mean TAE value for the most eaten 10 items sampled of those analyzed was 2.7 as compared to 4.6 for all 91 samples; data from Tables 1 and 2) and high in protein (*Populus ciliata* and *Prunus cornuta* leaves and *Oenothera rosea* roots) or total nonstructural carbohydrates (*Viburnun foetens* flowers and fruits). The conifer needles collected at the Arnold Arboretum were high in tannins and low in protein; the pine and fir needles eaten by the rhesus were probably similar (see Feeding and Plant Abundance, below). Analysis of *Trifolium*, *Juglans* leaves, *Prunus* leaves, and *Oenothera* roots for individual amino acid contents showed that sufficient amounts of most essential amino acids are available to the rhesus in these foods (Marks, 1985).

Relationships Between Feeding and Chemistry. The results of the chemical analysis for all 91 different plant samples are presented in Appendix 1 alphabetically by plant family. Two or more replicate samples were collected and analyzed for *Trifolium* sp. whole plants, *Rumex nepalensis* leaves and seeds, and *Viburnum foetens* flowers. Only the averages of the replicate values are included in Appendix 1 because the results were very similar between replicates (Marks, 1985). Mean chemical contents of herbaceous and woody plant parts are compared in Table 2. Among leaves and flowers, condensed tannins were lower in herbs than in woody plants. Flowers and stems of herbs were lower in fiber than those of woody plants.

Correlations between feeding and chemical contents among leaves of woody plants and herbs and for all samples combined are shown in Table 3. Other subsets were too small (Table 2) or contained too few eaten items for correlational analyses. Total astringency, phenolics, and ellagitannins were significantly negatively correlated with feeding for all 91 samples. Feeding was negatively correlated with gallotannins among leaves of woody plants and with both gallo- and ellagitannins among leaves of herbs. All other chemical variables showed no significant relationships with feeding.

Feeding and Plant Abundance. Selection by the rhesus among tree and shrub species from which parts were eaten can be explained partially on the basis of plant abundance alone. A plot of abundance vs. percent of total feeding records (Figure 1) suggests a linear relationship between the two variables with the exception of one point (*Pinus wallichiana*). The Pearson correlation coefficient between feeding and abundance for the eight trees is insignificant (r = 0.274), but when the outlying point (*Pinus*) is excluded, the correlation coefficient is highly significant (r = 0.942, $P < 0.01$). The samples of *Pinus wallichianum* collected at the Arnold Arboretum contain 5.3% condensed tannins and 11% protein (Table 1). Pine species also contain monoterpenes (e.g., α-pinene) which deter feeding by mammals (Farentinos et al., 1981). Thus pine may be chosen less than other items due to high secondary compound and low

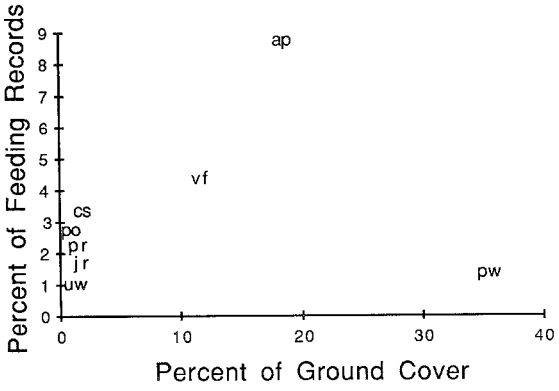

FIG. 1. Percent of plant species abundance vs. percent of feeding records. Tree and shrub abundance expressed as percent ground cover vs. percent of rhesus monkey feeding records. All data are from Goldstein (1984). ap = *Abies pindrow*, cs = *Cedrela serrata*, jr = *Juglans regia*, po = *Populus ciliata*, pr = *Prunus cornuta*, pw = *Pinus wallichiana*, uw = *Ulmus wallichiana*, vf = *Viburnum foetens*.

nutrient contents. Fir needles (Table 1) contain similar levels of tannins and nutrients to pine. The large numbers of feeding records for both pine and fir were most likely due to the extremely great abundance of these trees. Possible chemical factors involved in the greater use of fir needles over those of pine cannot be examined because of lack of samples of these items from the study site.

DISCUSSION

For all 91 samples and among vegetative parts of herbs and woody plants, feeding was significantly negatively correlated with hydrolyzable tannins (gallo- or ellagitannins) but not with condensed tannins. Previous studies have shown that primate feeding behavior is negatively related to condensed tannin content (Glander, 1981; McKey et al., 1981; Oates et al., 1977; Wrangham and Waterman, 1981), but this is the first demonstration of inhibition of primate feeding by hydrolyzable tannins. Using thin-layer chromatography, Gartlan et al. (1980) detected hydrolyzable tannins in 27 and 31% of the samples from Kibale and Doula-Edea, respectively. By our methods (potassium iodate for gallotannins and nitrous acid for ellagitannins), the samples from Pakistan appear to contain more hydrolyzable tannins than those examined by Gartlan et al. (1980), hydrolyzable tannins occurring at levels of 0.2% and above (potassium iodate method) in 50% of the samples (data from Appendix 1). Other northern temperate forests contain many of the same genera as those occurring at the Pakistan site and are therefore likely to have similar levels of hydrolyzable tannins to those reported here. Further research is required to test the hypothesis that hydrolyzable tannins may be more important as feeding deterrents in temperate forests than in tropical forests.

More than 50% of all feeding records were of herbaceous plants located in disturbance zones, even though these zones made up less than 30% of the study site area (Goldstein, 1984). Herb leaves and flowers were lower in condensed tannins than those of woody plants (Table 2). Selection for parts of herbaceous plants over those of woody plants, even though woody plants are more abundant, shows an apparent preference for herbs, which in part may be due to lower condensed tannin contents in herbs than woody plants (Table 2) (Bate-Smith, 1972a). Pine and fir needles (not included in the means in Table 2) were likely to be high in condensed tannins (Table 1), whereas grasses and other herbaceous plants that were not sampled were probably low in condensed tannins (Bate-Smith, 1972a; Capinera et al., 1983; Rice and Pancholy, 1973). The lack of negative correlation between condensed tannins and feeding (Table 3) may be due to incomplete sampling of food items and to the confounding

TABLE 1. PERCENT OF FEEDING RECORDS AND CHEMICAL COMPOSITION OF FAVORED FOOD PLANTS

Plant item	% of feeding records	Percent dry weight				
		Tannins[a]	Fiber	Protein	Free amino acids	TNC[b]
Herbs						
Trifolium sp.	30.00	1.2	56.4	16.5	1.9	5.1
Grasses (unidentified)	12.80	not sampled				
Herbs (unidentified)	19.09	not sampled				
Oenothera rosea root	1.38	2.3	63.0	18.5	4.4	5.8
Trees and Shrubs						
Abies pindrow needles	8.85	not sampled				
Viburnum foetens total	4.43					
Flowers	2.66	1.3	42.9	14.1	2.1	19.6
Fruits	1.72	1.1	39.7	8.5	1.2	16.2
Cedrela serrata young leaves	3.32	4.4	51.6	18.4	0.5	6.8
Populus ciliata total	2.85					
Young catkins	2.07	4.0	53.8	22.8	2.0	0.5
Mature catkins	0.77	1.6	53.3	16.0	1.2	6.8
Prunus cornuta total	2.21					
Young leaves	1.76	7.8	41.4	20.6	3.6	7.1
Mature leaves	0.41	1.4	42.6	18.3	1.0	3.7
Juglans regia total	1.94					
Young leaves	1.66	6.1	67.8	25.8	2.1	5.1
Petioles	0.19	8.1	63.9	13.4	0.9	9.9
Young leaves and stems	0.08	9.3	50.4	18.1	2.2	9.3
Pinus wallichiana needles	1.85	not sampled				
Ulmus wallichiana total	1.02					
Flowers	0.36	1.8	48.8	19.7	0.9	6.9

[a]Tannins = total astringency (TAE) as measured by hemanalysis.
[b]TNC = total nonstructural carbohydrates.

TABLE 2. CORRELATIONS BETWEEN FEEDING AND CHEMICAL CONTENTS AMONG PLANT PARTS AND FOR ALL SAMPLES COMBINED

	Spearman Correlation Coefficients with Percent of Feeding Records				
	All samples	Herbs		Woody Plants	
	n = 91 except n of water = 83	Vegetative parts; n = 34 except n of water = 30	Reproductive parts; n = 13	Vegetative parts; n = 35 except n of water = 31	Reproductive parts; n = 7
TAE[a]	−0.24*[b]	−0.35*	0.14	−0.08	−0.28
CT	0.10	−0.16	0.40	0.07	0.50
ET	−0.24*	−0.37*	0.76*	−0.26	NA[c]
GA	−0.08	−0.23	0.59*	−0.35*	−0.27
TP	−0.26*	−0.26	0.07	−0.09	−0.21
Fiber	0.11	0.32	0.10	0.10	0.43
Lignin	−0.14	−0.15	0.16	−0.02	−0.67
Protein	0.11	0.10	−0.33	0.17	−0.39
AA	0.00	0.42*	−0.11	−0.07	0.95*
TNC	−0.08	−0.18	−0.10	−0.22	0.14
Glucose	0.05	−0.18	−0.05	0.12	−0.25
Water	0.09	0.24	−0.10	0.22	0.43
Alkaloids	0.08	0.28	−0.07	0.05	−0.36

[a] TAE = total astringency, CT = total condensed tannins, ET = ellagitannins, GT = gallotannins, TP = total phenolics, AA = amino acids, TNC = total nonstructural carbohydrates.
[b] * indicates Spearman correlations coefficients were significant.
[c] NA = not applicable because ellagitannin contents = 0.0.

TABLE 3. MEANS OF CHEMICAL CONTENTS FOR ALL SAMPLES AND BY PLANT PART[a]

	All samples		Herbs				Woody plants			
	n = 91 except n of water = 83		Vegetative parts; n = 34 except n of water = 30		Reproductive parts; n = 13		Vegetative parts; n = 35 except n of water = 31		Reproductive parts; n = 7	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TAE[b]	4.6	3.3	4.9	3.4	3.6	3.1	5.4	3.1	2.1	1.0
CT	1.9	3.2	1.5	1.6a[c]	1.0	0.9a	2.9	3.0b	1.7	1.0
ET	0.6	1.4	0.9	1.6a	0.2	0.4b	0.7	1.4a	0.0	0.0
GT	1.3	2.5	1.1	2.0	1.3	3.6	1.6	2.7	0.6	0.5
TP	4.2	4.2	3.9	3.7	4.5	3.7	5.2	5.0	1.4	0.8
Fiber	48.1	10.4	49.7	6.2a	39.2	11.5b	50.2	10.7a	43.8	8.7
Lignin	3.8	2.4	4.2	2.0	2.9	2.0	3.9	1.9	2.2	0.5
Protein	16.2	6.7	17.2	6.3a	12.5	4.4b	15.4	5.0	16.9	4.8
AA	1.8	2.3	2.2	2.9	1.7	1.8	1.4	1.0	1.2	0.6
TNC	9.5	6.8	.7.0	4.0a	15.9	10.0b	8.7	4.5a	12.8	12.0
Glucose	0.9	1.7	0.3	0.7a	2.1	3.2b	0.5	0.7a,b	2.0	2.2b,c
Water	73.5	15.3	76.5	14.4	75.1	20.3	69.0	12.5	73.9	19.2

[a]All means are percent dry weight except water contents.
[b]TAE = total astringency, CT = total condensed tannins, ET = ellagitannins, GT = gallotannins, TP = total phenolics, AA = amino acids, TNC = total nonstructural carbohydrates, SD = standard deviation.
[c]a,b,c = Means with different letters are significantly different ($P < 0.05$) in Tukey multiple comparison tests.

factor of plant abundance. Neither gymnosperms nor monocots contain hydrolyzable tannins (Swain, 1979). Therefore the negative correlations between feeding and hydrolyzable tannin contents would not have been diminished had samples of grasses and conifer needles been included in the chemical and statistical analyses.

The lack of selection for protein and the relatively high average protein contents of both the eaten (17.5%) and uneaten plant samples (15.9%) as compared to the amount of protein used to maintain captive rhesus (16.4%; Greenberg, 1970) indicates that the rhesus may not be protein limited, except possibly in winter when all foods are minimally available (Marks, 1985). In contrast, Glander (1981) found that mean protein content in tree leaves in Costa Rica was only 11% and that leaves eaten by howler monkeys were significantly higher in protein than uneaten leaves. Thus animals may only select for protein when it is limiting in their environment. Water content also showed no relationship to rhesus monkey food choice and was probably not limiting due to abundant precipitation throughout most of the year.

Our results show that food selection by rhesus monkeys in Pakistan is influenced by the presence of hydrolyzable tannins in leaves of both herbs and woody plants. Feeding was negatively correlated with hydrolyzable tannins, total phenolics, and astringency among all samples, but in no cases were condensed tannins related to food choice. These results indicate the importance of measurement of hydrolyzable tannins, astringency, and total phenolics in addition to condensed tannins in future studies of the effects of tannins on food choice by animals feeding on dicotyledonous plants. Although nutrients may have influenced the selection of individual items (e.g., in important foods, see Table 1), nutrients showed no overall relationship to feeding by rhesus monkeys and may not have been limiting in the environment. This premise was not fully tested, however, because several important food items were not collected and because the abundance of food items was not sufficiently quantified (only the abundance of common trees was measured, and the abundance of individual plant parts was not quantified). We suggest that in future studies of this type, collection of samples of all major food items and more complete measures of food abundance will facilitate a more accurate characterization of the important factors in herbivore food choice.

APPENDIX 1.

Chemical Composition and Time Spent Feeding of Plants from Pakistan

Plant Family Species and Part	Sample Number	Chemical Composition (Percent Dry Wt., except Water)													
		Tannins													
		TAE	CT	ET	GT	TP	Fiber	Lignin	Water	Protein	AA	Alk	TNC	Glu	% Tim Feeding
Araceae															
Arisaema jacquemontii															
flowers	032	12.3	0.1	0.0	0.0	1.2	34.1	0.7	92.1	19.7	5.1	0	42.0	8.6	0.00
mature leaves	074	3.5	0.6	0.4	0.2	3.4	44.8	5.6	87.0	36.5	5.6	0	7.7	0.0	0.00
Sauromatum venosum															
mature leaves	056	3.8	0.6	0.7	0.2	2.2	41.6	3.2	53.3	23.7	13.4	0	2.8	0.2	0.00
Asclepiadaceae															
Cynanchum auriculatum															
young stems	041	3.7	0.4	0.0	0.0	0.5	47.7	2.2	83.9	27.9	3.8	1	8.9	0.1	0.00
Asteraceae															
Chrysanthemum leucanthemum															
flowers	037	0.0	0.0	0.0	0.0	6.1	40.3	1.5	85.0	15.8	1.5	1	16.0	0.4	0.00
mature leaves	047	1.9	0.4	0.0	0.8	1.3	45.8	3.2	77.0	12.3	1.0	0	6.9	0.1	0.00
Senecio chrysanthemoides															
stems	031	5.5	0.2	0.0	0.0	0.5	52.9	2.4	87.8	11.7	1.5	0	6.6	0.8	0.00
mature leaves	046	5.9	0.6	0.0	0.0	3.0	24.2	2.7	77.0	12.3	0.2	0	6.9	0.1	0.03
Berberidaceae															
Berberis lycium															
young leaves	015	9.8	0.3	0.0	1.2	5.8	37.5	2.9	76.5	17.3	1.3	0	3.7	0.1	0.00
Buxaceae															
Sarcococca saligna															
fruits	001	2.9	0.3	0.0	0.6	0.7	29.6	2.6	70.9	16.5	0.7	3	35.4	5.5	0.42
mature leaves	081	1.7	0.4	0.0	0.0	2.7	42.0	2.2	73.0	19.3	1.9	2	10.5	0.6	0.00

Appendix 1 continued.

Plant Family Species and Part	Sample Number	Chemical Composition (Percent Dry Wt., except Water)													
		TAE	CT	ET	GT	TP	Fiber	Lignin	Water	Protein	AA	Alk	TNC	Glu	% Tim Feeding
Caprifoliaceae															
Lonicera quinqueloculariis															
mature leaves	050	3.8	0.5	0.0	0.0	2.7	51.2	4.8	76.7	09.4	0.5	0	10.8	0.4	0.06
Viburnum cotinifolium															
mature leaves	098	4.3	5.3	0.5	0.9	1.9	44.0	2.5	70.0	13.5	0.6	0	8.9	0.3	0.00
Viburnum foetens															
flowers	*210	0.7	2.0	0.0	0.5	1.9	42.9	1.2	86.3	14.1	2.1	0	19.6	1.8	2.66
fruits	020	1.1	1.7	0.0	0.0	0.2	39.7	2.0	85.8	8.5	1.3	0	16.2	4.2	1.72
mature leaves	051	4.2	6.0	0.0	0.0	3.3	58.8	2.9	66.7	10.2	0.6	0	4.5	0.2	0.05
Cornaceae															
Cornus macrophylla															
sap	005	0.7	0.0	0.2	0.0	0.0	37.3	2.4	94.1	51.6	11.8	0	16.0	7.2	0.08
mature leaves	053	11.5	0.9	1.3	4.8	10.9	46.0	6.4	66.7	17.7	0.5	0	9.9	0.1	0.00
Euphorbiaceae															
Euphorbia wallichiana															
mature leaves	030	3.1	0.2	0.2	2.2	20.6	35.9	1.3	63.0	13.7	1.3	0	22.4	0.3	0.00
Fabaceae															
Indigofera heterantha															
mature leaves	060	6.0	6.0	0.4	4.8	11.2	64.2	3.0	66.0	19.7	0.9	0	5.1	0.0	0.00
Trifolium sp.															
whole plant	*277	1.2	0.3	0.0	0.0	0.3	56.4	4.4	82.0	16.5	1.9	0	5.1	0.0	30.0
Fagaceae															
Quercus dilatata															
mature leaves	003	15.1	13.7	3.6	12.0	20.6	54.4	5.4	.	8.2	0.9	0	21.0	0.7	0.00
mature leaves	054	8.2	2.0	0.6	5.8	4.6	67.8	6.3	53.3	12.1	0.9	0	5.6	0.3	0.00
Q. incana															
mature leaves	067	4.3	2.8	1.4	2.6	5.4	54.4	7.1	40.0	11.8	0.7	0	5.0	0.5	0.06
Fumariaceae															
Corydalis diphylla															
whole plant	029	4.0	0.7	0.5	0.6	3.3	51.0	2.4	.	27.4	9.5	2	3.9	0.0	0.14

Appendix 1 continued.

Plant Family Species and Part	Sample Number	Chemical Composition (Percent Dry Wt., except Water)													
		Tannins													
		TAE	CT	ET	GT	TP	Fiber	Lignin	Water	Protein	AA	Alk	TNC	Glu	% Tim Feeding
Geraniaceae															
Geranium wallichianum															
stems	038	3.0	1.1	0.2	0.9	5.5	47.7	5.5	84.2	6.1	0.4	1	4.0	0.2	0.00
mature leaves	049	17.6	0.5	1.6	2.2	8.5	41.6	3.2	77.0	19.8	0.0	0	6.9	0.5	0.00
G. nepalense															
mature leaves	052	7.9	1.9	6.0	0.7	11.4	46.4	5.8	80.0	16.2	0.0	0	5.5	0.3	0.00
Gentianaceae															
Gentiana argentia															
whole plant	021	3.1	0.4	0.0	0.0	1.2	52.6	3.7	.	13.0	1.1	0	8.4	0.3	0.08
Hippocastanaceae															
Aesculus indica															
young leaves	008	7.9	1.9	0.0	0.0	6.7	56.2	1.9	80.8	29.8	2.0	0	8.4	2.0	0.00
mature leaves	096	8.2	4.3	0.5	3.2	6.7	48.4	4.3	70.0	9.2	0.4	0	9.7	0.3	0.00
leaf stems	014	5.3	0.7	0.0	0.0	0.2	68.2	1.2	89.5	20.0	1.5	1	10.7	3.8	0.00
Juglandaceae															
Juglans regia															
young leaves (spring)	013	9.3	1.7	0.1	0.4	4.0	50.4	2.3	84.7	18.1	2.2	1	9.3	0.5	0.08
young leaves (summer)	025	6.1	2.7	0.4	0.1	6.0	67.8	6.3	.	25.8	2.1	0	5.1	1.5	1.66
petioles of young leaves (summer)	026	8.1	0.5	0.0	0.0	0.3	63.9	6.4	82.6	13.4	0.9	0	9.9	1.6	0.19
mature leaves	062	2.7	2.0	0.4	0.4	2.4	59.2	5.0	73.0	15.4	0.0	0	7.0	0.9	0.00
Labiatae															
Origanum vulgare															
mature leaves	048	5.1	1.2	0.4	0.0	1.7	53.2	4.6	73.0	12.3	0.2	0	4.9	0.8	0.00
Plectranthus rugosus															
mature leaves	071	2.1	0.5	0.3	0.1	2.9	52.0	3.9	77.0	17.0	1.5	0	4.8	0.3	0.00
Prunella vulgaris															
flowers	044	4.6	0.4	0.0	13.1	10.6	44.5	3.7	79.6	5.1	0.9	0	9.4	0.3	0.00
mature leaves	084	3.1	1.0	0.3	0.8	2.1	56.4	5.0	83.0	12.3	2.1	0	4.5	0.0	0.00

Appendix 1 continued.

Plant Family Species and Part	Sample Number	Chemical Composition (Percent Dry Wt., except Water)													
		Tannins													
		TAE	CT	ET	GT	TP	Fiber	Lignin	Water	Protein	AA	Alk	TNC	Glu	% Tim Feeding
Meliaceae															
Cedrela serrata															
young leaves	018	4.4	0.4	0.0	0.0	5.6	51.6	1.4	79.2	18.4	0.5	0	12.9	0.7	3.32
Onagraceae															
Oenothera rosea															
root	016	2.3	0.3	0.0	1.4	4.2	63.0	11.0	.	18.5	4.4	1	5.8	0.2	1.38
Oxalidaceae															
Oxalis corniculata															
whole plant	069	2.0	3.1	0.1	0.3	1.4	50.4	3.9	83.0	13.2	0.9	0	3.7	0.0	0.00
Plantaginaceae															
Plantago sp.															
whole plant	087	3.8	1.1	0.1	0.5	1.2	57.4	4.7	87.0	22.0	1.0	1	3.7	0.0	0.00
Polygonaceae															
Polygonum amplexicaule															
mature leaves	082	5.5	4.1	0.3	0.2	2.3	49.6	5.8	80.0	15.4	0.3	0	6.0	0.2	0.00
Rumex nepalensis															
mature leaves	*278	3.5	2.0	0.2	0.2	0.1	58.1	3.2	86.2	19.5	2.0	0	4.5	0.1	0.03
fruits	*234	2.0	2.3	0.2	2.4	1.6	54.0	7.4	13.0	06.7	0.1	1	3.3	0.1	0.55
Ranunculaceae															
Anemone obtusiloba															
flowers	023	5.1	0.4	0.0	0.0	2.6	45.0	3.8	83.4	17.6	4.5	1	18.7	2.3	0.00
Aquilegia pubiflora															
flowers	045	3.5	2.5	0.0	0.5	2.9	35.4	2.9	80.6	16.1	3.0	0	17.6	0.3	0.00
mature leaves	085	7.2	0.5	0.3	0.0	8.6	46.8	5.9	87.0	19.4	0.6	0	5.3	0.0	0.00
Rhamnaceae															
Rhamnus virgata															
mature leaves	068	3.7	3.7	0.4	0.3	4.6	41.2	4.5	66.0	12.4	2.1	0	11.2	0.0	0.00

Appendix 1 continued.

Plant Family Species and Part	Sample Number	Chemical Composition (Percent Dry Wt., except Water) Tannins TAE	CT	ET	GT	TP	Fiber	Lignin	Water	Protein	AA	Alk	TNC	Glu	% Tim Feeding
Rosaceae															
Duchesnia indica															
mature leaves	093	7.5	1.0	4.6	8.1	2.7	46.4	7.0	66.0	20.3	1.6	0	7.3	0.4	0.00
Fragaria nubicola															
mature leaves	066	6.6	2.2	3.4	3.7	11.8	52.0	6.4	70.0	15.8	0.5	0	8.2	0.6	0.00
Geum urbanum															
mature leaves	073	10.9	0.7	5.9	7.0	14.4	40.8	3.0	72.0	19.0	1.2	0	8.6	0.3	0.00
Prunus cornuta															
young leaves minus midvein	027	3.8	7.8	0.1	0.8	4.1	41.4	3.1	.	20.6	3.6	2	7.1	0.1	1.76
petiole and midvein	028	3.5	1.9	0.0	0.0	0.6	42.4	1.0	.	16.7	1.3	2	10.4	0.0	0.00
mature leaves	102	1.4	3.8	0.2	0.0	1.6	42.6	2.3	60.0	18.3	1.0	0	3.7	0.0	0.41
Pyrus lanata															
mature leaves	095	2.0	5.0	0.4	0.7	2.9	54.0	4.0	70.0	12.2	1.5	0	5.2	0.3	0.19
Rosa brunonii															
young stems	040	4.3	0.1	0.9	1.9	3.4	67.9	6.6	77.1	13.9	0.5	0	16.5	1.0	0.00
mature leaves	090	6.9	3.4	3.5	6.8	2.9	50.0	6.7	66.0	21.0	2.7	0	6.7	0.1	0.00
R. macilentus															
mature leaves	070	13.4	0.3	7.5	8.0	18.2	34.8	6.8	66.0	14.3	2.3	0	7.6	0.0	0.00
Rubus pedunculosis															
mature leaves	094	4.3	1.7	1.3	2.3	5.2	43.2	3.9	53.0	08.9	1.6	0	6.7	0.1	0.00
Sorbaria tomentosa															
mature leaves	063	3.3	9.1	0.1	0.4	3.9	56.4	1.9	73.0	18.8	2.4	0	6.6	0.2	0.00
Spirea vaccinifolia															
mature leaves	058	4.1	6.6	0.6	1.4	5.0	40.4	5.3	33.0	10.8	1.7	0	11.1	0.4	0.00
Rutaceae															
Boenninghausemia albiflora															
mature leaves	061	1.8	0.5	0.0	0.3	2.5	38.4	3.3	33.0	21.2	0.0	0	6.1	0.4	0.00
Skimmea laureola															
mature leaves	072	5.4	0.3	0.2	0.0	1.9	44.4	4.0	66.0	13.1	4.8	0	7.2	0.0	0.00

Appendix 1 continued.

Plant Family Species and Part	Sample Number	Chemical Composition (Percent Dry Wt., except Water) Tannins TAE	CT	ET	GT	TP	Fiber	Lignin	Water	Protein	AA	Alk	TNC	Glu	% Tim Feeding
Salicaceae															
Populus ciliata															
young catkins	012	4.0	2.6	0.0	0.9	1.1	53.8	2.1	82.0	22.8	2.0	0	0.5	0.0	2.07
mature catkins	017	1.7	2.5	0.0	1.5	2.4	53.3	2.6	32.4	16.0	1.2	2	6.8	0.0	0.77
mature leaves	065	3.3	1.2	0.2	0.9	3.0	57.0	7.2	60.0	09.7	1.2	0	6.6	0.2	0.00
Saxifragaceae															
Bergenia ciliata															
flowers	009	6.1	1.7	1.5	1.0	12.2	31.2	0.9	89.5	11.7	1.7	0	24.2	9.4	0.41
stems	057	3.2	1.0	0.8	0.2	2.1	38.3	1.2	90.3	10.7	6.6	0	21.0	0.4	0.21
mature leaves	086	5.7	2.0	1.2	3.1	8.6	50.8	6.4	88.0	10.1	1.6	0	4.4	0.0	0.00
Scrophulariaceae															
Scrophularia decomposita															
seeds	039	2.6	1.1	0.0	0.0	2.3	55.1	6.1	65.2	5.7	0.0	1	10.1	0.1	0.00
Veronica melissafolia															
flowers and seeds	033	0.7	0.4	0.0	0.0	4.1	41.6	2.1	71.6	12.4	0.5	1	10.7	0.5	0.00
mature leaves	055	8.6	0.7	0.5	0.1	2.4	49.2	1.6	35.0	18.9	1.8	0	6.6	0.2	0.00
Wulfenia amherstiana															
flowers	043	1.5	2.4	0.0	0.0	8.7	39.6	2.5	80.0	09.2	0.1	0	18.7	0.2	0.00
mature flowers	059	1.2	0.5	0.5	0.1	1.3	30.5	2.6	68.1	07.8	1.7	0	11.1	0.2	0.00
mature leaves	083	3.1	1.7	0.6	3.0	5.8	45.6	2.5	70.0	8.6	0.5	0	14.7	0.1	0.00
Thymelaceae															
Daphne papyraceae															
mature fruits	002	2.0	0.2	0.0	0.0	2.3	38.9	2.4	84.9	20.9	0.5	0	4.3	2.3	0.00
mature leaves	075	4.8	0.8	0.1	0.5	2.4	45.2	3.6	73.0	10.5	0.9	1	7.0	0.3	0.00
Ulmaceae															
Ulmus wallichianum															
flowers	011	1.8	2.4	0.0	0.5	1.3	48.8	2.3	74.8	19.7	0.9	1	6.9	0.3	0.36

Appendix 1 continued.

Plant Family Species and Part	Sample Number	Chemical Composition (Percent Dry Wt., except Water)													
		Tannins													
		TAE	CT	ET	GT	TP	Fiber	Lignin	Water	Protein	AA	Alk	TNC	Glu	% Tim Feeding
Valerianaceae															
Valeriana jatmansi															
flowers	024	2.5	0.9	0.0	0.0	2.5	47.4	2.7	82.1	16.7	1.0	2	13.6	3.0	0.00
mature leaves	076	3.8	2.0	0.0	0.0	2.6	52.8	3.9	87.0	14.7	3.7	1	6.2	0.0	0.00
V. stracheyi															
whole plant	022	3.3	1.5	0.0	0.0	2.6	45.6	2.4	.	15.9	3.6	1	1.6	0.1	0.22
Violaceae															
Viola repens															
flowers	006	3.9	0.1	0.0	0.0	2.6	10.9	0.5	86.8	10.9	3.5	2	18.6	2.2	0.00
Nonflowering plants															
Unidentified fern															
stems	007	3.1	2.3	0.0	0.0	1.8	49.4	1.8	89.2	14.2	2.0	0	18.3	4.2	0.03
Onychium contiguum															
young stems	036	5.0	0.3	0.0	0.0	3.7	50.2	3.8	86.6	29.0	1.3	0	8.2	0.3	0.00
Adiantum capillus veneris															
leaves	064	3.0	8.0	0.0	0.0	4.4	58.0	3.4	73.0	12.4	0.6	0	7.8	0.5	0.00
Pteridium aquilinum															
mature leaves	079	1.7	5.5	0.0	0.0	1.8	62.8	3.8	77.0	14.6	0.7	0	5.5	0.3	0.00
Unidentified lichen															
whole	004	0.0	0.0	0.0	0.0	0.0	79.6	16.8	82.8	12.1	0.0	0	4.1	0.0	0.00
Taxus baccata															
needles	042	4.5	3.5	0.0	0.0	5.2	42.6	1.5	65.7	14.3	0.2	3	14.0	0.8	0.00

TAE= tannic acid equivalent, CT= proanthocyanidins, ET= ellagitannins, GT= gallotannins, Pro= protein, AA= free amino acids, Alk= alkaloids, TNC= total nonstructural carbohydrates, Glu= glucose.

* Sample numbers with a 2 as the first digit are average values for 2 or more replicate samples.

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OVIPOSITIONAL BEHAVIOR OF LESSER PEACHTREE BORER IN PRESENCE OF HOST-PLANT VOLATILES[1]

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Abstract—Reactions of lesser peachtree borer [*Synanthedon pictipes* (G&R)] to volatiles of peach wood, either natural or chemically fractionated, were observed. Mated females were stimulated by and responsive to such materials and deposited significantly more eggs on substrates, including unnatural hosts, that had been treated with aqueous mixtures of bark-canker materials. Stimulation to oviposit occurred even when the female was blinded, indicating the presence of chemical cues. Natural canker–bark extracts immediately stimulated ovipostion and for a few hours significantly increased the number of eggs laid. However, average fecundity was not increased. Antennectomy did not significantly decrease response to volatiles by gravid females, and alternate sites of such chemoreception were not located. Complex mixtures derived by solvent extraction, steam distillation, and volatiles trapping from bark, canker, and gum all had activity. Observations of insect behavior in outdoor cages and also in the laboratory indicated that visual, chemosensory, and mechanosensory receptors are involved in host finding and oviposition.

Key Words—Attractants, extracted volatile compounds, *Synanthedon pictipes*, Lepidoptera, Sesiidae, chemosensory, insect behavior, oviposition, mechanosensory, host-insect interactions, olfactory receptors, oviposition behavior, apple, peach, pear, plant odors, *Prunus* spp.

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INTRODUCTION

In recent years, research on elucidation of insect–host interactions has included ovipositional responses of gravid females to their hosts. This relationship is crucial in most insect–plant interactions, but unfortunately it is sometimes difficult to establish and is the least understood relationship involved in such interactions (Stadler, 1983). It is generally accepted that chemical cues may invoke both long- and short-range orientation by the ovipositing female to the host. The distinction between feeding and ovipositional stimulation is often obscure, as exemplified by the dipteran pests (*Liriomyea* spp.). Fortunately, in studying nonfeeding lepidopterous females this problem is not encountered. However, with such females, moisture may become extremely important in their host-seeking (Saxena and Goyal, 1978). Another issue that needs clarification is the distinction between ovipositional stimulation and ovipositional attraction. Although these functions are not necessarily mutually exclusive (Brown et al., 1970), a chemical attractant may not stimulate oviposition, and, conversely, a stimulant may not attract.

The lesser peachtree borer, *Synanthedon pictipes* (G&R) (LPTB), is an important pest of peaches and cherries in most production areas. The host range of LPTB consists of the genus *Prunus*; it may therefore be considered a restricted feeder, thus requiring fairly specific host-finding mechanisms. Females tend to oviposit on roughened bark of healthy trees near wounds where fresh gum is present (Bobb, 1959). Neonate larvae utilize light, chemotaxes, and thigmotaxes as cues in an active search for entry into the bark (Wiener and Norris, 1982). The reproductive behavior of adult Sesiidae females has been reported by Girault (1907), King (1917), Bobb (1959), and Cleveland et al. (1968), but the chemical relationships between the mated female and her host plants have not been defined. Observations have generally concluded that females are markedly attracted to the gummy deposits on peach wood that originate from cankers formed by cold damage, *Cytospora* sp. fungi, or mechanical injuries. A chemical isolated from peach bark, larval frass, and gum mixtures was reported by Gentry and Wells (1982) to be attractive to gravid females of peachtree borer, *S. exitiosa* (Say). Since *S. exitiosa* and *S. pictipes* are congeneric species, some commonality in such behavior might be expected.

Our research was conducted to quantify the attractancy of gravid LPTB females to components of peach bark and to determine if such components stimulate oviposition or increase fecundity. This paper presents the results of our attempts to isolate and identify such components and to record the ovipositional behavior of LPTB in the laboratory and field at Vincennes, Indiana, Peoria, Illinois, and Delaware, Ohio.

METHODS AND MATERIALS

LPTB was reared using the methods of Cleveland et al. (1968). Newly mated females were maintained in screen cages on damp sand at 10°C until used in experiments, either the same or the next day. Various parameters of response were determined in preliminary experiments. For experiments utilizing natural canker and gum, the cankerous areas on either Cresthaven or Redskin peach trees were removed by cutting both sides and ends of the canker and scraping bark, canker, gum and debris from the entire area. Gum was isolated by carefully selecting debris-free material. Healthy bark used as check material was peeled from 10- to 15-cm-diam. limbs from the same trees. All test materials were maintained in sealed plastic bags at 4 ± 1°C until used. Materials for scanning electron microscopy were mounted on aluminum stubs with conductive cement and sputter-coated (model Hummer V, Technics, Springfield, Virginia) with 500 Å of gold. SEM observations were performed with a Hitachi model S-500 (Mountainview, California) at 20 KV accelerating voltage.

Chemical Cues Experiment. Small branches (2.5 × 25 cm), cut from pear trees (a LPTB nonhost) and from peach trees (a host) were presented to LPTB adult females in 50 × 50 × 50-cm screen cages in an air-conditioned greenhouse. In test 1, branch ends were impaled by nails to a 35 × 35-cm board and exposed to 10 gravid females. Pear branches were coated with aqueous canker–gum mixtures and tested in comparison to nontreated (clean) pear branches and water wicks. In test 2, peach wood was similarily treated and exposed to LPTB. The board was rotated periodically to ensure equal environmental exposure of each treatment. The test was replicated six times and data were submitted to analysis of variance.

Visual Cues Experiments. In test 1, to determine the role that sight may play in host response, latex paint was applied to both compound eyes of 10 mated females. No adverse effects of the paint treatment upon the insects was noted. These treated insects were placed into a 30 × 30 × 30-cm screen cage which contained one peach branch (10 × 2 cm), treated with an aqueous canker–gum–bark mixture from Redskin peach trees, and one pear branch the same size. A duplicate cage was used with 10 control (nonpainted) females. Behavior and egg deposition were observed in both cages for 6 hr.

In Test 2, females, either normal or blinded with latex paint, were caged with apple, pear, and peach branches (10 × 2 cm). The canker–gum–bark mixture was layered onto a moist cotton pad placed beneath the branches but inaccessible to the insects. The cages consisted of 3.78-liter ice-cream cartons (20 × 18 cm) with the middle of the carton removed and replaced with screenwire. A screen bottom separated the cotton pad 5 cm from the rest of the cage.

Treated cages as well as controls without the canker material consisted of three replications of three insects. After log transformation, 24-hr egg counts were submitted to analysis of variance and Duncan's (1955) multiple-range test.

Ovipositional Stimulation Experiments. Two other types of tests were conducted to determine if peachwood canker or gum might actively stimulate oviposition or increase fecundity. In the first experiment, air from the exhaust of an air compressor was passed through flasks containing aqueous suspensions of either canker material, gum, healthy bark, or distilled water. The volatile-laden air immediately flowed into four 13.5 × 10.5 × 3.5-cm plastic sweater boxes containing exhaust ports in the opposite ends. The boxes were layered with 2-cm-thick cotton batting saturated with distilled water. Twelve females, eclosed and mated the same day, were introduced into each box, and the eggs that were deposited on either the cotton or the box were counted after 24 hr in one series of tests and 48 hr in a second series of tests.

Another experiment was conducted in individual plastic 100-ml cups (6.5 × 6 cm) with copper screen covers. Aqueous canker material (0.5 ml) was applied to the center of a 1-cm layer of moist cotton either on top of the cotton so that the insects could contact it or inverted so that the material was not accessible either tactilely or visually to the insects although they were exposed to volatiles. Each treatment was replicated 25 times; moist cotton pads were used as controls. Surgically altered LPTB females were used as subtreatments consisting of five replicate Latin-square arrangements as follows: (1) normal mated female, (2) mated female with one antenna removed at base of pedicel, (3) mated female with both antennae removed, (4) mated female with both antennae three-fourths removed, and (5) mated female with tip of hypopharnyx removed. Cups were maintained in an air-conditioned greenhouse at 85 ± 5°C and 75 ± 10% relative humidity. Eggs were counted at 24-hr intervals for 72 hr. The entire test was replicated on each of five dates, and the mean number of eggs per test was submitted to analysis of variance after log transformation using the individual tests as replicates. A similar test was conducted and 80% confidence levels were computed for egg production at 5-, 24-, and 48-hr intervals to determine if the canker extracts might cause more immediate effects.

Behavior in Outdoor Cages. A 1.8 × 1.8 × 2.4-m screen cage was constructed for outdoor observations of LPTB behavior. Peach branches of various sizes and shapes, with and without foliage, were suspended at a height of 1.5-m in the four quadrants of the cage into which 10 mated females were released. Observations were made only on sunny, warm days since LPTB females were found to be unresponsive under other conditions. Females were observed for ovipositional movements and egg deposition.

A large 6 × 2.5 × 2.5-m cage was constructed over two peach trees (var. Cresthaven) in a peach orchard located at Southwestern Indiana Purdue Agri-

cultural Center, north of Vincennes. One tree was spot treated with 2-chloroethylphosphonic acid (Union Carbide Corp.) to promote gum formation. After two weeks, copious gum production around the treated portions of the trunk and limbs and a general weakening of the tree was apparent with some shedding of foliage. Varying numbers of gravid LPTB females were released from the middle of the cage, and the females were observed for periods varying from 1 to 6 hr. Behavior and egg deposition were observed.

A replicated experiment was conducted in which a 2-cm-thick peach branch was divided into four 8-cm sections and placed in the four quadrants of the small cage containing 12 females which were replaced on three consecutive days. Treatments consisted of (1) a smooth branch section, (2) an identical section with canker–gum mixture applied, (3) a section with one small (0.5 cm) new canker, and (4) a section with one large (1.5 × 0.5 cm) older canker. After log transformation, total egg numbers were submitted to analysis of variance and Duncan's (1955) multiple-range test.

Olfactometer Experiments with Natural Canker Materials. To quantify LPTB ovipositional behavior, we devised an olfactometer from a 50 × 5.5-cm glass tube. One end was closed with a cork containing a length of 0.5-cm latex tubing leading to a vacuum pump which was exhausted outside. The other end of the glass tube was corked with a rubber stopper modified to hold a screen cage (8.5 × 4 cm) in which the test insect was confined inside the tube during testing. Air from outside the laboratory was drawn through a charcoal filter into a glass sample chamber and finally into the olfactometer. The sample chamber contained a filter paper strip (2 × 0.5 cm) on which a drop (generally 0.01–0.02 ml) of test material was placed. Air flow was adjusted to 0.1–0.3 m/sec and the temperature was maintained at 18 ± 2°C. A fluorescent desk lamp was positioned directly over the insect holding cage of the olfactometer. A single insect was placed in the cage which was then attached to the front rubber stopper and inserted into the chamber. The insect was allowed to acclimate to the air flow for 30 sec prior to inserting the treated filter paper strip into the air flow. Reactions were observed and recorded for at least 1 min. Strong ovipositional thrusts with the ovipositor were considered positive responses. Five insects were used for each observation. Insects were maintained overnight on damp sand at 10°C and were used only one or two times during one days' observations.

In one experiment, females, surgically altered in a manner similar to those in the ovipositional stimulation experiment, were exposed to natural volatiles from canker material in the olfactometer in an attempt to determine the location of chemoreceptors. Response was tabulated according to abdominal movement, and subsequent egg deposition in plastic cups layered in cotton (Cleveland et al., 1968) was recorded for each insect.

Olfactometer Experiments with Materials Isolated from Canker and Bark.

To collect volatiles from bark, peachwood bark (var. Redskin and Bisco) was collected in the spring of 1982 and frozen dry at −18°C. These materials (ca. 1.0 kg from each variety) were ground in a Wiley mill; the ground bark was placed in a 40 × 60-cm plastic bag that had been outfitted for collection of volatiles. Nitrogen, at a flow rate of 1 liter/min, was used to sweep volatiles onto 1 × 8-cm Tenax columns. Collections proceeded for 4.5 days, but were interrupted each night, when the bark was refrozen. A new trap with fresh Tenax was used each day. The traps were eluted with 10 ml of acetone to remove condensed moisture and then with 50 ml of diethyl ether. Washings were combined, and the solvent was concentrated by distillation through a 1 × 30-cm Vigreux column. Residual acetone was codistilled with pentane, leaving the sample in 10 ml of pentane (fraction 1). Portions (200 g) of the ground Redskin and Bisco peach bark after volatile collection were stirred for 2 hr each with three 500- to 600-ml portions of distilled water at room temperature. After a brief settling period, the water was decanted through glass wool to remove most suspended materials. The fine particles remaining were allowed to settle out overnight under refrigeration. The decanted aqueous extract was subjected to a continuous extraction with diethyl ether for 18 hr, and the ether extract was concentrated by distillation through a 1 × 30-cm Vigreux column. Residual ether was codistilled with pentane to provide 10 ml of fraction 2. A portion of the ether-extracted material was insoluble in pentane, so it was dissolved in 10 ml of acetone (fraction 3).

For fractionation of gum and canker, samples (ca. 3–4 kg total) were collected from Redskin peach trees on July 29, 1982, at Vincennes, placed together in a carboy and immediately covered with dichloromethane. The carboy and contents were transported to Peoria and stored at 4°C. After a month, the contents of the carboy were mixed thoroughly, and a 5-ml sample of the dischloromethane solution was removed and served as fraction 4. Gum and cankers (21 g) were cut into 2- to 5-mm sections and then Soxhlet extracted for 3 hr with acetone. The residue was air-dried, ground finely in a mortar, and reextracted with acetone for 7 hr. The combined extracts were concentrated to ca. 20 ml on a rotary evaporator at 40°C, and the concentrate was divided into two equal portions (one portion = fraction 5). The remaining portion was subjected to azeotropic distillation with absolute ethanol on a rotary evaporator at 40°C to remove water, then the ethanol was removed with dichloromethane, and the sample (fraction 6) was redissolved in 10 ml of acetone.

A second portion of gum and canker material (20 g) was chopped and then stirred with 500 ml of distilled water at room temperature for 4 hr. The water extract was decanted and subjected to a 3-hr simultaneous steam distillation–extraction using a Likens-Nickerson apparatus with dichloromethane as the extracting solvent at ambient pressure (Likens and Nickerson, 1964). Dichloromethane was codistilled with pentane, thus giving fraction 7 for assay.

Finally, a third portion of gum and canker material (20 g) was chopped and then stirred with 200 ml of distilled water at room temperature for 4 hr. The total slurry was extracted in a separatory funnel with diethyl ether–ethyl acetate (1 : 1) to give fraction 8 after distillation of the solvent. Samples were sealed in ampules until assayed against gravid LPTB females.

Cytospora *Experiment.* Cankerous lesions on peach trees, which are known to be attractive to ovipositing LPTB, are usually associated with fungal pathogens. Swift (1986) reported that cankers associated with *Cytospora* were infested with LPTB earlier and to a greater extent than uninfected wounds. He suggested that the *Cytospora* may have caused a physical alteration of the wounds making them more suitable for egg deposition and larval invasion. The most important organism causing such cankers, *Cytospora cincta* Sacc., was cultured on potato–dextrose–agar, and aqueous washes of the cultures were exposed to LPTB females in the olfactometer to determine whether it played a role in host specificity of LPTB.

RESULTS AND DISCUSSION

Chemical Cue Experiment. Observations of LPTB behavior in cages showed that more eggs were deposited on branches that were coated with extracts of gummy, cankerous peachwood bark. Pearwood, a nonhost of LPTB, became attractive when peach canker material was applied to it. In a 6-hr observation of branches impaled on nails exposed to LPTB females (test 1), significant differences ($P = 0.01$) were observed. Pearwood alone received 11% (SE = 1.6) of total eggs, while identical pearwood with canker material on it received 82% (SE = 10.7), and a water wick control had 7% (SE = 1.2) of total eggs. When peach wood was exposed in a similar test (test 2), wood with canker material received 82% (SE = 16.1), while wood without such material had only 14% (SE = 5.9) of total eggs, and a water wick check had 4% (SE = 1.3). In these experiments, some eggs were always deposited on unnatural hosts such as the pear wood or moist cotton. This may have been due to a concentration of volatile molecules from the treated ar.. causing stimulation of females resting on these sites. However, some indiscriminate egg-laying may be expected from laboratory-reared LPTB, since they normally oviposit on moist cotton without the presence of host stimuli.

Visual Cue Experiments. In test 1, blinded females immediately responded and began ovipositional movements in the presence of peach and pear wood. They moved about the cage and, upon encountering the branches, would oviposit. After 6 hr, an average of 13.9 eggs per blind female had been laid, with 70% on peach and 30% on pear. Normal females had laid 21.0 eggs per female with 98% on peach and only 2% on pear.

Results of test 2, in which normal and blind females were exposed to three different types of wood with and without stimulation by canker extracts, are shown in Table 1. When no canker was present, normal females oviposited almost exclusively on peach wood. However, in the presence of strongly attractive canker material, peach was not preferred over apple but was preferred over pear. In this experiment, the numbers of eggs deposited by normal females were greater where additional canker material was not present, although the difference was not significant. It is not known whether the greater egg deposition was due to a few particularly fecund individuals or to the presence of inhibitory volatiles in high concentrations of canker material. Blind females in canker-saturated environments indiscriminately desposited eggs on all three substrates, but apple apparently corresponded more closely to peach than pear in stimulating tactile response from LPTB. It was observed that blind females were strongly responsive to the canker material. When blind females were stimulated, then placed upon an apple branch, they immediately began probing with their ovipositor. Unstimulated blind females ordinarily remained docile. Since eggs are desposited on such nonhosts by blind insects, it would seem logical that the sensory hairs present on the ovipositor (Figure 1) were mechanoreceptors rather than chemoreceptors. Since no transmission electron microscopic examinations were made, it is unknown how either the long (± 100 μm) or the short (1.0–1.5 μm) structures are innervated. In these observations, there was never any evidence of chemoreception by the ovipositor. However, in the presence of a strong enough stimulus, even a normal female will oviposit on apple or pear. Normal females lay a much higher percentage of eggs on the wood than on cotton (75:25), than blind females do (16:84). This is an indication that sight does have some significance, as well as that blind females may ovi-

TABLE 1. HOST SELECTION BY GRAVID LPTB FEMALES WITH OR WITHOUT VISION IN PRESENCE OR ABSENCE OF STIMULATION BY CANKER MATERIAL

	Percentage of total eggs[a] deposited when			
	Canker material absent		Canker material present	
Offered host	Normal female	Blind female	Normal female	Blind female
Peach wood	92.3a[b]	41.0a	62.7a	42.3a
Apple wood	5.0b	20.3a	32.7a	38.7a
Pear wood	2.7b	38.7a	4.7b	19.0a

[a] Three females used in each test.
[b] Means within columns followed by the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

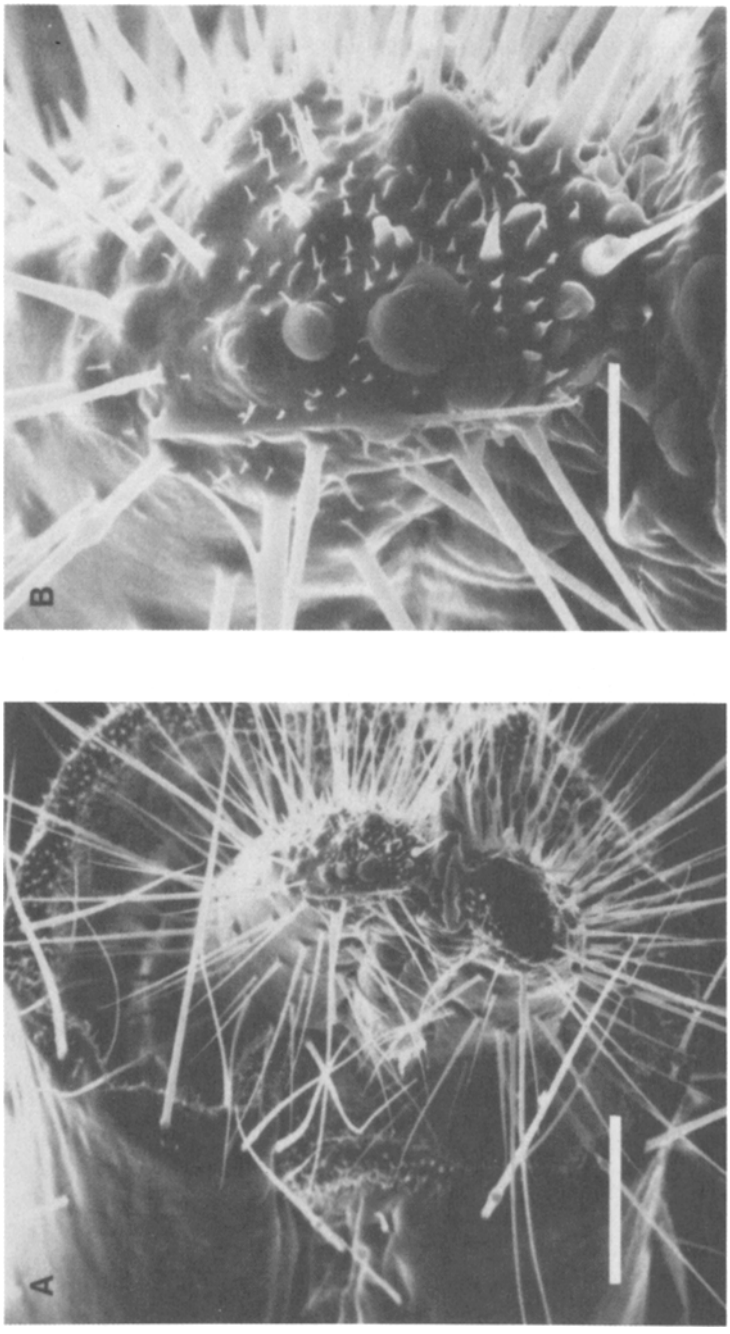

FIG. 1. Scanning electron micrograph of the tip of the ovipositor of LPTB: (A) bar = 50 μm; (B) bar = 10 μm.

posit indiscriminately in the presence of stimuli. Another indication of the importance of sight is the observation that while normal females are stimulated and will follow a canker source in still air, blind females, although stimulated, will not follow such a source.

Ovipositional Stimulation Experiments. Preliminary observations showed that females placed in closed Petri dishes were inactive and did not oviposit in the presence of peach canker material. However, after removal of the tops and replacement with screen covers, abdominal responses began immediately. It is unknown whether the observed lack of response in closed dishes was due to habituation, to a repellent in the chemical, or to other causes. After exposure to air, females in the treated dish laid 166 eggs compared with only 72 in the untreated dish (18 hr). This led to the hypothesis that fecundity of LPTB increased in the presence of canker material. This hypothesis, however, was rejected after examination of the data from the experiments in which ovipositing LPTB females were exposed to volatiles from canker, gum, and bark of peach. In the first experiment, in which air was bubbled through aqueous substrates into a plastic oviposition chamber, mean numbers of eggs per female were lowest in the control chamber, both after 24- and 48-hr observations. However, no significant differences existed among the treatment means.

Plastic cups with screen tops were used to provide a free exchange of air in the second test. A complicating factor in using bioassays such as these with LPTB is the propensity of the colonized female to oviposit on cotton or other substrates where sufficient moisture is present. For this reason, the canker was placed on the under side of the cotton in one set of tests to detect quantitative differences caused by volatile fractions. Results of this test also indicated that no significant differences in egg production occurred either in the presence or absence of canker material or its volatiles. This was true whether the female had been surgically altered or was normal.

Another experiment, again in plastic cups, in which counts of eggs from five females were made at shorter intervals (Figure 2), indicated that a significant effect was produced by the canker material but also that it was short-lived. The canker apparently stimulated early egg production, but total fecundity was not statistically increased. This observation is in line with other observations in the laboratory and field and indicates that the canker material not only attracts mated female LPTB, but also stimulates oviposition to some degree. However, the fact that unstimulated insects will oviposit in the presence of moisture complicates interpretation of these data.

Behavior in Outdoor Cages. The behavior of gravid LPTB females was observed inside a small cage in the presence of various peachwood substrates. Several interesting observations were made which may aid in understanding ovipositional behavior in the field: (1) There was no attraction to peach foliage even on hot, sunny days. (2) Most oviposition occurred on rough areas of the

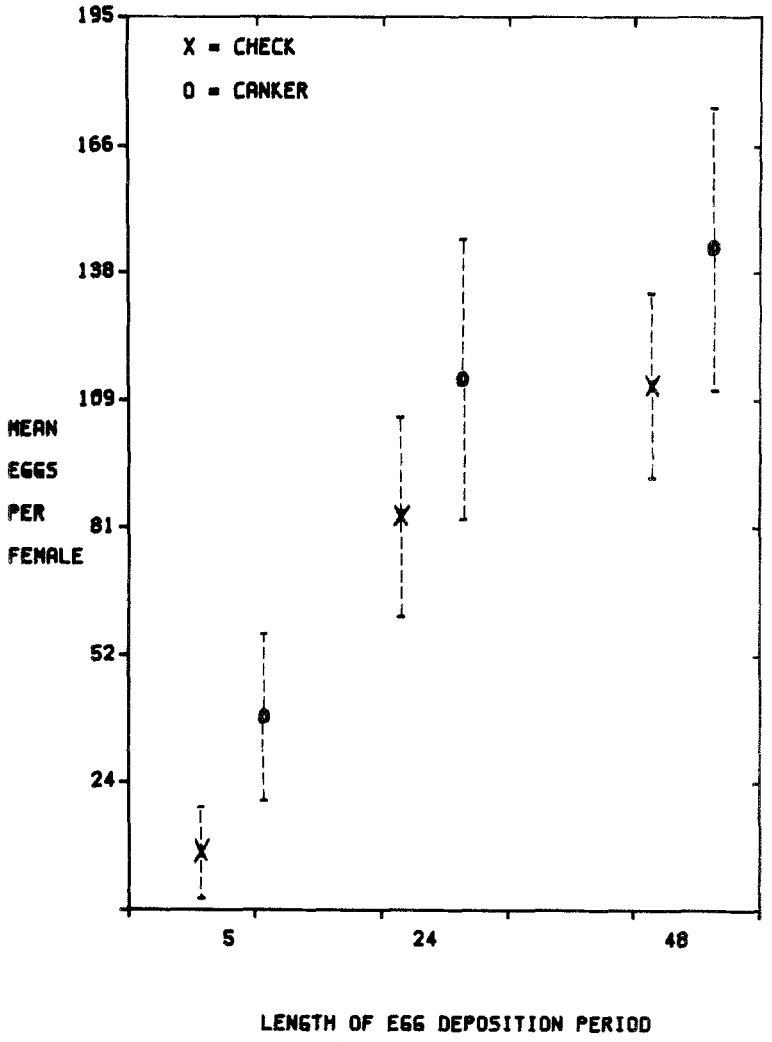

FIG. 2. Egg production of mated LPTB in the presence of host volatiles over time after mating.

bark. (3) Branches less than ca. 1 cm, even with canker extract on them, were not attractive for oviposition. (4) Branches with natural cankers were extremely attractive. (5) Although excised canker material would attract females to a substrate, oviposition upon that substrate seemed to depend on the texture. Thus, in the field, chemosensory and mechanosensory receptors as well as vision, function in the act of oviposition.

Observations in the large field cage were made on warm, sunny days because females would rarely fly on overcast days, and on windy days, they became lost in the grass. Females exhibited a high degree of attraction to sunny areas of the cage, preferring to rest on the side of the cage or tops of the trees in direct sunlight. When released in the center of the cage between the treated and untreated tree, they would usually orient toward the treated tree with the copious gum deposits, although the attraction to light seemed to be greater than attraction to either tree. When greater numbers of females (>50) were released, some could readily be located in the treated tree and, more rarely, in the untreated tree. The untreated tree had more foliage, which may have provided more hiding places, but ovipositing females were found much more often on the treated tree. Once the females alighted on the tree, they were immediately stimulated and walked around on the branches (usually the larger branches and trunk) with their abdomens dragging until they located a site that was sufficiently rough to oviposit. The rougher bark and canker areas invariably received more eggs than smoother areas. Much exploring of the cracks and crevices was made with the ovipositor prior to oviposition. The setae present on this organ (Figure 1) must function to locate the most strategic position for eggs after chemoreceptors have led the female to the proper site. These observations indicate that olfaction, as with many other insect species, is important for close-range identification of a suitable host by LPTB.

Results of the replicated experiment using a segment branch are presented in Table 2. The branch section containing a large, older canker received significantly more eggs ($P = 0.05$) than one with canker material applied, and also had significantly more eggs ($P = 0.01$) deposited on it than sections with no added material or with only a small canker.

Olfactometer Experiments with Natural Canker Materials. LPBT females exhibit a definite typical response pattern when stimulated by volatiles from natural canker material. This includes antennal movement, followed by a downward arching of the posterior abdomen with associated ovipositional thrusts of

TABLE 2. EGG DEPOSITION BY LPTB FEMALES IN OUTDOOR SCREEN CAGE (12 FEMALES REPLACED DAILY FOR 3 DAYS)

Peachwood with	Mean No. of eggs per branch[a]
No canker	44a
Small canker	68a
Added canker material	109c
Large canker	163d

[a] Means within the column followed by the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

the ovipositor. This behavior does not occur when either virgin females or males are exposed to such volatiles. Results of the test to attempt to determine the location of chemosensory structures on the LPTB are shown in Table 3. Unfortunately, we were unable to determine sensory sites since olfactory-mediated excitement was obtained no matter which type of surgery was applied. Even complete antennectomy failed to suppress ovipositional movements in the presence of stimulatory materials. Since the antennectomy retained the scape of the antennae, we thought that enough sensory receptors may have been present there to invoke response. However, coating these structures with fingernail polish did not cause further suppression of response. Using another lepidopterous insect, *Plodia interpunctella* HBN., Deseo (1976) found that, following 50% antennectomy, olfactory stimuli which ordinarily increased fecundity lost their effect, and fecundity became the same as if there were no stimulation. This indicated that the chemoreceptors involved in oviposition were located on the distal half of the antennae. Similarly, Yamamoto and Fraenkel (1960) found that oviposition in the tobacco hornworm appeared to be initiated by olfactory stimuli which were completely suppressed by amputation of the antennae.

It is evident from our research that the sensory receptors mediating oviposition only function after mating. However, experiments involving ablation of various parts of the LPTB body reported here, as well as other observations, including tarsal amputation and ablation of tarsal sensilla with acids, failed to determine the sites of such receptors. Stadler (1976) stated that negative results in ablation experiments may not only be the effect of the removed sensory input, but could also be a kind of irritation or reduction in general activity. In our observations, ablation obviously had a detrimental effect upon the insect, but, contrary to expectations, positive rather than negative results were obtained. Whether this may be a result of elimination of inhibitory influences has yet to be determined. In any event, the location of chemoreceptors functioning in ovi-

TABLE 3. RESPONSE OF LPTB WITH VARIOUS DEGREES OF ANTENNECTOMY TO NATURAL PEACH CANKER VOLATILES IN OLFACTOMETER AND SUBSEQUENT FECUNDITY

Treatment	Response (%)[a]	Fecundity[b]
None, control, mated	100	42.2(24.6)
Single complete antennectomy	100	33.2(19.6)
Double complete antennectomy	85	31.1(52.1)
Double 3/4 antennectomy	100	36.8(42.1)
Virgin female	0	0 (0)
Virgin male	0	

[a] Response as indicated by ovipositional movement.
[b] Mean number of eggs per female per day (standard deviation of mean of total eggs).

positional response of LPTB is still unknown, and although the majority of the receptors may reside on the antennae, those present on other parts of the body apparently function in the absence of antennal receptors.

Olfactometer Experiments with Materials Isolated from Canker and Bark. Assays of mixtures obtained by solvent extraction, steam distillation, and volatiles trapping from peach wood and canker–gum materials indicated the extreme difficulty of isolation of the component(s) responsible for ovipositional response. Of the three fractions obtained by steam distillation of peach bark, there was little difference in response to Redskin or Bisco fractions (Table 4). Fractions 1 and 3 both elicited a good ovipositional response, while fraction 2 elicited only a 10% response to Redskin and 20% to Bisco; this was the only variation between the two. Apparently, most of the activity was concentrated in the pentane-insoluble fractions. The Soxhlet-extracted fractions from canker and gum were all active, except fraction 4 which elicited no response. The dichloromethane-extracted fractions showed the least activity, although enough active volatiles were present for stimulation.

Other fractionation procedures utilizing various solvents, collection columns, and distillation procedures were attempted, but all failed to isolate the activity into a recognizable fraction. One problem is that the active molecules apparently excite the LPTB at extremely low concentrations. Behavioral responses such as those employed in these experiments are apparently not suitable for quantitatively distinguishing between fractions when all or most of the fractions contain volatiles that elicit response. For this reason, some of these same materials were assayed with the electroantennogram (EAG) method. Results of these studies (Anderson et al., 1987) indicate that this technique may be more discriminating and probably should be used for primary screening prior to un-

TABLE 4. OVIPOSITIONAL RESPONSE TO MIXTURES OBTAINED BY SOLVENT EXTRACTION, STEAM DISTILLATION, AND VOLATILES TRAPPING FROM PEACH WOOD AND CANKER–GUM MATERIAL

Fraction No.	*N*	Response (%)[a]
1	10	70
2	10	10
3	10	90
4	5	0
5	45	44
6	40	75
7	15	33
8	10	60

[a] No response was elicited by females exposed to solvent alone (pentane).

dertaking behavioral assays. Habituation by such active volatiles, as well as repellent or inhibitory effects due to hypernormal concentrations within the bioassay arena, are possibilities, among others, that need exploring.

Cytospora *Experiment.* When extracts of *Cytospora cincta* cultures were presented to gravid LPTB females in the olfactometer, no response was evident. Although LPTB larvae aid in invasion by this organism by producing new openings in host tissue previously sealed off by callus tissue (Hildebrand, 1947), *Cytospora* apparently has no chemical function in attraction of females to host plants or to stimulation of oviposition.

In finding and consuming host plants, insects may draw upon olfaction, vision, mechanoreception, gustation, and perhaps additional signals. The lesser peachtree borer, according to our observations, uses at least the first three signals in varying degrees. The LPTB has a survival advantage in that it may oviposit in the same host tree whence it encloses. However, earlier authors (Girault, 1907; King, 1917) reported that mated females flew from tree to tree, and subsequently observations have confirmed this, but reports of distances flown are sketchy. To some degree, either sight or olfaction or both must be involved in host-finding. Wind-tunnel observations (Reed, unpublished data) show that mated female LPTB will orient upwind and fly 3 m to a source of canker material, indicating that olfaction may be important at some distance. Observations in the greenhouse (Reed, unpublished data) indicate that the female in flight can follow a hand-held source of canker volatiles, attempt to land on it, and oviposit. These observations plus results of the studies reported here indicate that LPBT females rely upon olfaction in host feeding. More research is needed on the aspect of discrimination between olfaction and sight and on determination of distances involved in such relationships. Electrophysiologic procedures may be helpful in elucidation of these relationships. However, it must be recognized that chemoreception of plant chemicals is far more complex than the perception of pheromones (Stadler, 1980).

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VARIABILITY OF CHEMOSENSORY STIMULI WITHIN HONEYBEE (*Apis mellifera*) COLONIES: Differential Conditioning Assay for Discrimination Cues

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Abstract—Differential training of honeybee workers using the proboscis extension reflex is applied to the problem of evaluating compounds that may potentially provide cues for kin recognition in the honeybee *Apis mellifera*. These cues were obtained by contaminating glass rods and steel needles with different materials found in the hive. In particular it is shown that workers discriminate between: cuticular waxes from different adult workers; eggs from the same and different hives; similar aged larvae within the same hive; and needles contaminated with the Nasonov gland secretions of different adult workers. It appears that some of these differences are due to phenotypic variation among individuals that cannot be directly attributed to environmental factors.

Key Words—Chemosensory cues, olfaction, kin recognition, honeybees, *Apis mellifera*, Hymenoptera, Apidae, differential conditioning, proboscis extension reflex, learning.

INTRODUCTION

Chemicals play a role in mediating kinship interactions in several social insect species (for reviews, see: Breed and Bennett, 1987; Gadagkar, 1985; Gamboa et al., 1986a). Honeybees, for example, are polyandrous and can discriminate within the hive between those individuals that are their full sisters and those that are their maternal half sisters (Frumhoff and Schneider, 1987). The recognition cues appear to have a genetic component (Breed et al., 1985; Getz and

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Smith, 1983, 1986). It has also been shown that there is sufficient genetic variability in the volatile odors emanating from adult workers so that, using the proboscis extension reflex, workers can be differentially trained to discriminate between groups of adult workers from different patrilines within the same hive (Getz et al., 1986). Preferential rearing results, obtained by Noonan (1985) and Page and Erickson (1984), suggest that nurse bees can assess the relatedness of larvae to themselves. There is even evidence that workers are able to assess the relatedness of eggs or, at least, very young larvae to themselves (Visscher, 1986).

In several other species of social insects, kin and nestmate recognition, mediated by olfactory cues, is also a well-established phenomenon. Questions relating to the origin of these cues—specifically, the relative contributions of environmental sources, gestalt sources (chemicals transferred between individuals), and individual genetic variation—have been addressed in carpenter ants (Carlin and Hölldobler, 1986, 1987; Carlin et al., 1987), fire ants (Obin, 1986), paper wasps (Gamboa et al., 1986a, b), and sweat bees (Buckle and Greenberg, 1981; Smith and Wenzel, 1987). Although we have previously demonstrated that volatile odors contain information that potentially could be used by workers to assess their kinship to other workers (Getz et al., 1986), cues sensed through contact chemoreception appear to be more likely as candidates for recognition of kin (for a discussion of this see Obin, 1986). Inside a crowded hive, volatiles are constantly mingling, and the origin of a particular odor may be masked. On the other hand, it is possible that a worker can readily determine the relative fraction of certain chemicals present in the epicuticle of another individual by placing its antennae on that individual. Evidence suggests that genetic variation in the composition of cuticular waxes of worker honeybees exists (Carlson and Bolten, 1984). In other species of social insects, especially several species of ants (Clement et al., 1987; Morel and Vander Meer, 1987; Obin, 1986), the existence of variation in the cuticular wax components of individuals is well established.

It has been reported that workers use "the colony odour, which adheres to their body" to recognize individuals that belong to the same honeybee colony (Renner, 1960). It has also been demonstrated, using differential conditioning of the proboscis extension reflex, that the olfactory system of worker honeybees is sensitive enough to discriminate between mixtures containing different proportions of the following compounds occurring in the epicuticle of workers: the two fatty acids, un- and dodecanoic acids; and mixtures containing different proportions of the two *n*-alkanes, tri- and pentacosane (Getz and Smith, 1987). Many other chemicals may be implicated in recognition including secretions from the Nasonov and mandibular glands (Breed, 1981; Crewe, 1982; Free and Winder, 1983). Thus the picture is complex, and the role of recognition cues could be context-dependent.

A first step towards understanding the problem of kin recognition within

honeybee colonies is to identify sources of variation in the chemosensory labels of individuals. In the experiments described here, we show how the proboscis extension reflex in honeybees can be used to identify and assay the discrimination of such chemosensory cues. Exactly how an individual can use these cues to assess its relatedness to other individuals, however, is a question that involves some understanding of the honeybee olfactory system and, more generally, how olfactory signals are stored and processed by individuals (for further discussion on this point, see Getz and Chapman, 1986).

METHODS AND MATERIALS

In all experiments the proboscis extension reflex in honeybees was used to differentially condition workers to discriminate between two glass rods or two stainless-steel needles contaminated with various worker secretions and hive products. A total of three hives was used. Each hive was obtained by instrumentally inseminating a dark queen with semen from one dark and one light drone to yield two visually distinguishable worker patrilines: a light patriline and a dark patriline (for more details see Getz et al., 1986). The differential conditioning methodology is modified from Bitterman et al. (1983) and reported in detail in Getz et al. (1986). For the sake of completeness, we summarize the methodology below.

In the late afternoon on the day preceding an experiment, 40–50 workers (primarily foragers) were removed from the hive and harnessed in small brass tubes with their mouthparts, antennae, and legs free to move. They were then fed a 1.5 M sucrose solution until satiated and left overnight in the dark, at room temperature (around 18°C). The following morning, one half to one hour prior to training, each bee was fed several droplets of sucrose solution.

As elucidated in the experiments discussed below, pairs of 50-mm-long, 1-mm-OD, hollow glass rods or pairs of thin steel dissecting needles were used in the differential training experiments. These rods and needles were prepared within an hour of the start of each replicate of an experiment. Depending on the experiment, glass rods were contaminated by rubbing them on various parts of workers of different ages and life stages or areas of comb. Also, the tips of some glass rods were dipped in albumen which then acted as a "glue" to extract eggs from cells. The needles were used in experiments involving Nasonov gland secretions. Each needle was contaminated by rotating its tip in the area of the opening to the Nasonov gland located between the 6th and 7th abdominal tergites of an adult worker. This area was exposed by stretching the abdomen with forceps (for more details see Pickett et al., 1980). With the exception of experiment 7 described below, care was taken that the needles did not touch any part of the bee other than Nasonov gland area.

Individual bees were differentially conditioned to respond (by extending their proboscides; see Bitterman et al., 1983) to one of two contaminated rods

or needles. Stimulation was achieved by touching a rod to both antennae. The positive conditioned stimulus (CS+) was rewarded with 1.5 M sucrose solution as a positive unconditioned stimulus (US+). The latter was given as a droplet to the bee to drink immediately after a 3-sec application of the CS+ (if the proboscis had not been extended, extension was elicited by touching the antenna with the same solution). The negative conditioned stimulus (CS−) was unrewarded and, if the proboscis was extended upon stimulation, a drop of 1.0 M solution of sodium chloride was placed on the proboscis as a negative unconditioned stimulus (US−) (for more details see Getz et al., 1986).

In each experiment, approximately 30 bees were differentially conditioned to two different rods by presenting one or other of these rods in a sequence of 16 trials. This conditioning was carried out by placing each test bee in turn on a platform below an air exhaust system approximately every 10 min and stimulating it with the CS+ (using the appropriate rod or needle) and the associated US+ or the CS− and, when necessary, the associated US−. The stimuli were presented in the following order (the indicated division into two eight-trial groups is only for the purposes of data analysis):

16 trial sequence

$$= \begin{cases} \text{Trial (training):} & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 \\ \text{CS:} & + & - & - & + & - & + & + & - \\ \\ \text{Trial (evaluation):} & 9 & 10 & 11 & 12 & 13 & 14 & 15 & 16 \\ \text{CS:} & + & - & - & + & - & + & + & - \end{cases}$$

Data were obtained by scoring the number of errors that an individual bee made during trials 9–16 (evaluation sequence). Thus, responses (extension of the proboscis) to any of trials 10, 11, 13, or 16, or nonresponses to any of trials 9, 12, 14, or 15 were scored as errors. Each individual could thus make 0–8 errors and an error histogram and average error $\bar{e}$ could be calculated for each group. The distribution of errors in these histograms can be compared using an $n \times 2$ chi-squared contingency table analysis, where the value of n depends on whether the tail categories of the histograms need to be combined to meet minimum expected frequency criteria (for more details see Getz et al., 1986).

The following experiments were conducted using pairs of glass rods. The number of times each experiment was repeated is noted in parentheses.

Experiment 1. (A) Each rod was rubbed on the cuticle of the dorsal surface of the thorax of a different forager from the same hive (not repeated). (B) As a no-discrimination control for the above experiment, each rod was rubbed on the cuticle of the dorsal surface of the thorax of the same forager (two replicates). (C) As a discrimination control for the above experiment, one rod was uncontaminated and the other rod was rubbed on the cuticle of the dorsal surface of the thorax of a forager (not repeated).

Experiment 2. (A) Each rod was rubbed on the cuticle of the dorsal surface of the thorax of a different newly eclosed worker (both workers were removed from cells just prior to eclosion—see Getz and Smith, 1986 for details) (two replicates). (B) As a control for the above experiment, each rod was rubbed around the inner surface of a different cell (two replicates).

Experiment 3. (A) Each rod was pushed into the center of a different cell containing a curled 5-day-old larva (avoiding any type of injury to the larva) (two replicates). (B) As a control for the above experiment, each rod was dipped into a different cell containing a globule of food provisioned by the workers (two replicates).

Experiment 4. (A) Each rod was tipped with wetted albumen (as a glue) and was used to remove an egg from a cell, where both eggs came from the same hive (four replicates). Note that, as far as was possible, eggs were selected from contiguous cells to increase the probability that they were of similar age. (B) As a control for the above experiment, the same procedure was repeated except the eggs were from different hives (three replicates).

Experiment 5. To obtain a baseline error level for the above discrimination experiments, one rod was contaminated with paraffin wax and the other with beeswax (not repeated).

The workers and material used to contaminate rods in the above experiments all came from the same hive, except for one of the rods in each replicate of experiment 4B which required the use of a second hive. A third hive set up from the same genetic stock as the first two provided bees for the following Nasonov gland secretion experiments which used pairs of steel needles rather than glass rods.

Experiment 6. (A) Each needle was rotated in the exposed Nasonov gland area of a different individual forager, where both individuals were from the same hive (two replicates). (B) As a discrimination control for the above experiment, one needle was contaminated with Nasonov gland secretion while the other was not (cf. experiment 1C) (not repeated).

Experiment 7. As in 6A, each needle was first rotated in the exposed Nasonov gland area of a different individual, but then was also rubbed on the upper thorax of that same worker. Both individuals were from the same hive (cf. experiment 1A) (not repeated).

Note that the designations FS and HS are used in the Results section below to indicate whether a particular replicate of one of the experiments 1A, 2A or 6A involved full or half sisters, respectively.

RESULTS

The sample size and average error over the eight-trial evaluation sequence (see Methods and Materials) are given in Table 1 for each replicate of each experiment. The largest values for the average error $\bar{e}$ were obtained for the no-

TABLE 1. SUMMARY OF RESULTS[a]

Expt. No.	Brief description	Rep. No.	CS+	CS−	Test group size (N)	Average number of errors ($\bar{e}$)
1A	Different foragers	i	Thorax (FS[b])	Thorax	25	3.2
		ii	Thorax (HS[b])	Thorax	32	3.4
B	Same forager	i		Thorax	29	4.6
		ii		Thorax	29	4.4
C	Control	i	Clean rod	Thorax	16	2.1
		ii	Thorax	Clean rod	17	1.5
2A	Different eclosing workers	i	Thorax (HS)	Thorax	35	3.5
		ii	Thorax (FS)	Thorax	31	3.9
B	Different cells	i	Cell 1	Cell 2	35	4.2
		ii	Cell 1	Cell 2	39	4.1
3A	Different larvae	i	Larva 1	Larva 2	35	1.9
		ii	Larva 1	Larva 2	32	2.8

B	Different larval food	i	Cell 1	Cell 2	35	3.9
		ii	Cell 1	Cell 2	38	3.9
4A	Eggs from same hive	i	Egg 1	Egg 2	22	3.1
		ii	Egg 1	Egg 2	21	3.0
		iii	Egg 1	Egg 2	24	4.0
		iv	Egg 1	Egg 2	21	3.9
B	Eggs from different hives	i	Egg 1	Egg 2	26	3.8
		ii	Egg 1	Egg 2	23	3.6
		iii	Egg 1	Egg 2	19	3.4
5	Control[c]	i	Paraffin	Beeswax	12	1.7
		ii	Beeswax	Paraffin	13	0.4
6A	Different workers	i	Nasonov (HS)	Nasonov	29	4.2
		ii	Nasonov (HS)	Nasonov	30	3.7
B	Control		Nasonov	Clean needle	26	0.5
7	Combination		Nasonov plus thorax (HS)	Nasonov plus thorax	30	3.5

[a]The significance of these results is discussed in the text.
[b]FS and HS denote whether the two individuals are full sisters or maternal half sisters, respectively.
[c]Both replicates are actually the same run of an experiment in which the test bees have been split in two groups, each receiving the mirror image treatment with regard to the CS+ and CS− stimuli.

discrimination control experiment (1B), where the error combined across both replicates is $\bar{e} = 4.5$ ($n = 58$). The lowest values for $\bar{e}$ were obtained for the discrimination control experiments (1C, 5, 6B), especially for the runs beeswax vs. paraffin ($\bar{e} = 0.4$) and Nasonov vs. clean needle ($\bar{e} = 0.5$). Note from experiments 1C and 5 that there appears to be an asymmetry in the results based on which of the stimuli are used as the CS+ and CS−. In experiment 1C, discrimination is stronger (that is, $\bar{e}$ is smaller) when the contaminated rather than the blank rod is used as the CS+ ($P < 0.01$, 2×2 chi-squared analysis). In experiment 5, discrimination is stronger when the more natural beeswax stimulus is used as the CS+ ($P < 0.05$, 2×2 chi-squared analysis). Thus, in conjunction with our experience in using odor stimuli (unpublished results), it appears that workers are more easily trained to discriminate between two chemosensory stimuli if they are positively conditioned to the stimulus that is either stronger or contextually the more natural of the two than vice versa.

In experiments 1A and 1B, the results from replicates within experiments are very similar, but across experiments the differences between any two replicates are significant, at least at the $P < 0.01$ level (2×4 chi-squared analysis). Furthermore, the results obtained from either replicate of experiment 1A are significantly different from either replicate of experiment 1C, at least at the $P < 0.05$ level (2×3 chi-squared analysis). Thus it follows that workers can perceive differences between rods rubbed on the backs of different foragers but discrimination is not as strong as exhibited by the discrimination control.

Since the replicates in experiment 1B are so similar ($P > 0.95$, 4×2 chi-squared analysis), they can be combined and used as a no-discrimination control for experiment 2A, as well as for the other experiments discussed below. In this case, both replicates exhibit that discrimination has taken place (for example, replicate ii of experiment 2A is significantly different from the no-discrimination control at the level $P < 0.01$, 3×2 chi-squared analysis), albeit not as strongly as obtained in the different foragers experiment 1A. Only the results from replicates i of experiment 1A and ii of experiment 2A are significantly different (in this case $P < 0.01$, 3×2 chi-squared analysis). The results from experiment 2B can be used to assess whether differences in the cuticular waxes of eclosing workers could be due to contaminants picked up from the inside surfaces of cells. Only replicate ii of this experiment differed significantly from the combined no-learning control ($P < 0.05$, 4×2 chi-squared analysis), while only replicate i of experiment 2A differed significantly from both replicates of experiment 2B (in both cases $P < 0.01$, 4×2 chi-squared analysis).

Among all experiments, the level of discrimination between pairs of natural cues was strongest in the different larvae experiment ($P < 0.001$, 4×2 chi-squared analysis for both replicates of experiment 3A when compared with the no-learning control). The results of experiments 3B can be used to assess whether food contaminants influence the larval discrimination results. Although

both replicates of experiment 3B indicate that some level of discrimination is evident with respect to the no-learning control experiment 1B ($P < 0.05$ for replicate i and $P < 0.01$ for replicate ii, 4×2 chi-squared analysis), the level of discrimination is much weaker than in the larval experiment 3A (for any two replicates between experiments 3A and 3B, we have $P < 0.001$, 4×2 chi-squared analysis).

The results across replicates of the discrimination between eggs experiments 4A and 4B are quite variable. Furthermore, there is no evidence for increased discrimination in the case of eggs obtained from different hives compared with the case of eggs obtained from the same hive. In replicates iii and iv of experiment 4A, discrimination is weak but evident when compared with no-learning control experiment 1B ($P < 0.05$ for both replicates, 3×2 chi-squared analysis). However, replicates i and ii of experiment 4A exhibit stronger levels of discrimination than do replicates iii and iv (for example, the results of replicates i and iv are significantly different at $P < 0.05$ while the results of replicates ii and iii are significantly different at $P < 0.001$, 2×2 chi-squared analysis).

From experiments 6A and 6B it is evident that, although honeybee workers experience no difficulties learning to discriminate the Nasonov gland secretion, they do experience some difficulties discriminating between secretions from half sisters in the same hive. A stronger level of discrimination is exhibited in replicate ii when compared with replicate i ($P < 0.05$, 3×2 chi-squared analysis) but, interestingly, little improvement in the level of discrimination is obtained through the addition of cues from the thorax in experiment 7. Note that it is difficult to compare the results from experiments 6A and 7 with 1A since the bees are from different hives, which may affect the level of cue variability among individuals (even though the hives are from the same genetic stock).

DISCUSSION

The results indicate that workers can discriminate between glass rods rubbed over the epicuticle of different foragers irrespective of whether the individuals are full or half sisters. They appear to be able to do the same if the individuals are newly eclosed half sister workers, but possibly to a lesser extent if the newly eclosed workers are full sisters. The difference between cues in the cuticle of eclosing individuals cannot be attributed to contaminants picked up in the cells. One could speculate that newly eclosed full sisters are more similar than half sisters in terms of recognition cues, but they become more distinct through environmental odors acquired as adults. However, the results presented here are too cursory to come to any firm conclusions. A definitive study of this and some of the other questions discussed below would involve testing several

hives, preferably set up with specific genealogies. It would also involve more replicates per hive and perhaps the presentation of stimuli obtained from groups containing different numbers of individuals, as was done in our volatile odor recognition cue study (Getz et al., 1986).

The Nasonov gland secretion also exhibits some variability between individuals, and this variability may be enhanced through additional cuticular wax discrimination. As mentioned in the previous section, the results of experiments 7 and 1A cannot be compared since different hives were used in these experiments. Thus one cannot come to any conclusions relating to additive or synergistic effects of cues. Again, a definitive study involves an extensive amount of experimentation and is required before any concrete conclusions can be reached.

The relatively strong differences in cues produced by different larvae raises some interesting questions. First, these differences cannot be attributed to contamination from the food provisioned for those larvae. The cues, however, may have nothing to do with individual or kin recognition, but may reflect the cues (pheromones) that larvae use to communicate their physiologic state (for example, need for food) to the nurse bees (see Jaycox, 1970; Free and Winder, 1983).

Prior to the experiment, we had anticipated that if workers could discriminate between eggs, then the level of discrimination would be stronger if the eggs come from different hives rather than the same hive. This would be the case, as discussed by Visscher (1986) in interpreting the results of his experiments, if queen pheromone or cues from an environmental source played an important role in labeling eggs. Although discrimination is evident in all replicates of within- and between-hive experiments, the strongest level came from the within-hive replicates. Thus it would appear that some other factors, such as the age of the egg (developmental stage), provide the strongest cues for discrimination. That is not to say that other cues do not exist or, if cues do exist, that they cannot be perceived by the nurse bees.

This raises the general question of what we are able to infer about an individual's ability to discriminate cues by observing its behavior. In the context of discrimination training, failure to observe discrimination does not imply that the test individuals are not detecting differences between two stimuli. This includes the proboscis extension reflex; individuals that are particularly hungry, regardless of any other considerations, may extend their proboscides in the hope of being fed, or some individuals may be able to detect differences but are poor learners. Also, it may be easier to train bees using the proboscis extension reflex if the cues occur naturally in a nectar-gathering context, for example certain floral odors. Finally, if one conducted experiments, such as 7, to assess the combined effects of two sets of cues, then the occurrence of thresholds, saturation effects, hierarchical ordering of information (see Carlin and Hölldobler, 1986), to mention just a few, would obscure what the insect is actually sensing.

CONCLUSION

From the results presented here, it is clear that the proboscis extension reflex can be used to assess the potential of different secretions as a source of labels for kin recognition. The situation is not always straightforward since time-dependent differences in the physiology of individuals, especially in preadult stages, may influence the results. Once a promising systems, such as cuticular waxes in adults, has been identified, then more extensive studies, including the heritability aspects of the labeling system, need to be undertaken.

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INFLUENCE OF *Heliothis virescens*[1] SEX PHEROMONE DISPENSERS ON CAPTURES OF *H. zea*[1] MALES IN PHEROMONE TRAPS RELATIVE TO DISTANCE AND WIND DIRECTION[2]

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Abstract—Evaluations conducted by placing *Heliothis virescens* (F.) sex pheromone (virelure) dispensers at different distances in the predominant downwind and upwind directions from *Heliothis zea* (Boddie) pheromone traps indicated that reductions in *H. zea* male captures were greatest relative to distance when the *H. zea* traps were located downwind from the virelure dispensers than when the traps were located upwind. When operating traps for both species at the same site, the influence of virelure dispensers on captures in *H. zea* pheromone traps would be minimized by placing the *H. zea* traps upwind of the *H. virescens* traps and, if wind direction is variable, the traps should be spaced at least 75 m apart.

Key Words—Tobacco budworm, cotton bollworm, *Heliothis virescens*, *Heliothis zea*, Lepidoptera, Noctuidae, sex pheromone, virelure, trapping, pheromone interaction.

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[2] In cooperation with the Texas Agricultural Experiment Station, Texas A&M University, College Station, Texas 77843. This paper reports the results of research only. Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by USDA.

INTRODUCTION

The identification of the sex pheromone components for the cotton bollworm (BW), *Heliothis zea* (Boddie) (Klun et al., 1980a) and the tobacco budworm (TBW), *Heliothis virescens* (F.) (Klun et al., 1980b) in conjunction with the development of a sex pheromone trap (Hartstack et al., 1979, 1980) for these two important pest species has created greater opportunities for the development of trapping systems for surveillance and prediction as well as for studying the movement of the adults. Since these two pests are frequently found in the same fields, the ideal trapping system would be one in which the same trap could be used for both species; however, considerable research has shown that the pheromones are not compatible for use in the same trap.

Haile et al. (1973) reported that the baiting of electric grid traps with virgin TBW and BW females simultaneously reduced the number of males captured by 24.2% and 77.5%, respectively, when compared with traps baited with one or the other species. At lower densities, the reductions in captures of TBW were not evident, while the reductions in captures of BW were consistently high. As such, there was a stronger and more consistent inhibitory effect of TBW females on BW male captures than vice versa. Roach (1975) reported that baiting pheromone traps with both BW and TBW virgin females resulted in a reduction in the capture of both species as compared to traps baited with only one species. Roach (1975) suggested that some form of behavioral or chemical antagonism existed between the moths or their pheromones when the species were combined. Carpenter et al. (1984) reported that trap captures of BW males were significantly reduced when BW and TBW pheromone or virgin female baits were used together in the same trap but that the presence of BW pheromone in the same trap did not significantly reduce male entrapment of TBW. These studies indicate that when attempting to trap both *Heliothis* species in the same pheromone trap, BW captures were reduced, but TBW captures were not reduced.

Research conducted indicates the cause for this effect. Klun et al. (1980a,b) reported that all four components identified from heptane washes of ovipositors of BW also were found in TBW, but that three additional components were identified in TBW. Klun et al. (1980b) reported that one of these components, (*Z*)-9-tetradecenal (Z9-TDAL), appeared to be a deterrent to BW attraction and that field data indicated that Z9-TDAL had a role in the maintenance of pheromonal specificity in the two species. Shaver et al. (1982) found that very small amounts (0.2–0.37%) of Z9-TDAL reduced captures of BW when mixed with the four components of the BW pheromone in dental rolls. Significant reductions were also observed when Z9-TDAL was formulated in rubber septa with the BW pheromone mixture even at quantities as low as 25 μg/septum when the original pheromone load was 2.5 mg. Shaver et al. (1982) also reported that

(Z)-11-hexadecen-1-ol, another of the components identified from heptane washes of TBW ovipositors but not found in BW, reduced captures of BW, but the amounts needed were larger (1.85–7.50%).

These studies show that one or two components found in TBW and not in BW which contribute to species specificity are responsible for the incompatibility of the two pheromones in the same trap and that the major effect expected would be on BW.

Ample evidence has been cited for the reduction of BW captures in traps baited with both species. However, because of the large effects on BW captures of very small quantities of at least one of the TBW pheromone components, a concern when operating traps baited with sex pheromone of only one species, but when both species are being trapped in the same area, is the distance between traps of the two species needed to minimize the interaction between the two. Since pheromone is windborne, another concern was the influence of wind direction. The results of a study conducted during the 1983 and 1984 growing seasons on the influence of wind direction and distance on the interaction between BW traps and TBW sex pheromone dispensers are reported here.

METHODS AND MATERIALS

The studies were conducted in a corn field located in Burleson County, Texas. A 75–50 wire cone trap (Hartstack et al., 1979) was used with its base maintained at tassel level. Laminated plastic dispensers manufactured by the Hercon Division (Health-Chem Corporation, South Plainfield, New Jersey, which contained all four components in the ratios initially identified (Klun et al., 1980a) at a dose of 1.25 mg of total sex pheromone in a 3.2-cm^2 (2.54 × 1.27 cm) dispenser, were used to bait the BW traps during both years of the study. Two different TBW sex pheromone (virelure) dispensers were used during the two years. In 1983, laminated plastic dispensers, also manufactured by Hercon, containing the two major virelure components at a 16 : 1 ratio at a dose of 20 mg in a 1.6 cm^2 (1.27 × 1.27 cm) dispenser were used. In 1984, black PVC plastic dispensers containing the two major virelure components at a ratio of 14 : 1 at a dose of ca. 6 mg of total pheromone (Hendricks, 1983; Hendricks et al., 1987) were used. The dispensers for both species were replaced every two weeks.

Because alignment of the BW pheromone traps and the virelure dispensers was difficult relative to wind direction, the prevailing wind direction for the time of the growing season when the studies were conducted was determined by compass reading, and this orientation was 168° (SSE) magnetic compass reading for the upwind direction.

To evaluate the downwind effects of virelure dispensers on their captures,

the BW traps were installed 5 m from the edge of the corn field and virelure dispensers were placed initially at 5, 15, and 30 m upwind of them. (Figure 1). Some studies were conducted up to 60 m in 1983. The virelure dispensers were attached to the top of steel reinforcement bars that were 2 m high. The bars were placed in 1.5 m pieces of electrical conduit driven into the ground. The evaluations consisted of four treatments: three different intervals between BW traps and the virelure dispensers and one treatment without virelure dispensers which served as the check. A line of 16 traps with 100 m between traps was used in 1983. The experimental design was a Latin-square in time with the treatments being rotated each night so that each treatment was at each location in each replicate during a four-day period. The rotation involved moving the virelure dispensers to the appropriate distance each night while the traps were kept at the same location. Edge traps baited with BW pheromone dispensers were installed at both ends of the trap line to minimize the edge effects. The same experimental design was used in 1984, except for the 200 m between traps to minimize the possibility of interaction between treatments in adjacent traps and there were no edge traps in the trap line because of insufficient space in the field. The distances studied in 1984 were 5, 50, and 75 m and the check with no virelure dispensers.

The interaction when the BW traps were located upwind of the virelure dispensers was evaluated only in 1983. The BW traps were installed 35 m from the edge of the field, and the distances between the trap and the virelure dispensers were 5, 15, and 30 m. There was a check treatment with no virelure

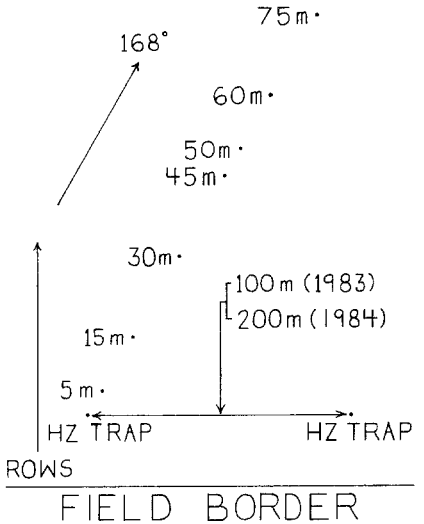

FIG. 1. Downwind array of traps, 1983–1984.

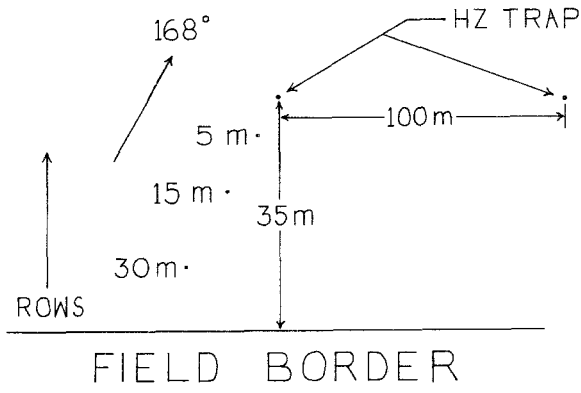

FIG. 2. Upwind array of traps, 1983.

dispensers (Figure 2). The experimental design was the same as for the downwind evaluations. The line of 16 traps spaced 100 m apart was a continuation of the trap line used for the evaluation of downwind effects, and it also had edge traps.

To evaluate the effect of variability in wind direction and speed on the results of the experiments, hourly wind direction and speed readings were obtained from the FAA Flight Service Station at Easterwood Airport, College Station, Texas, located ca. 13.4 km from the field where experiments were conducted. The readings for the period between 10 PM and 1 AM (CST) were used. The wind direction was categorized as being $\pm 90°$, $\pm 45°$, or $\pm 22.5°$ deviation from the 168° wind direction used in the experiments. Wind direction categories were assigned only when there were at least two hourly readings with wind speeds >0 m/sec. Specific categories of wind direction were assigned when at least two readings fell within the smallest wind direction interval. When wind velocity was >0 m/sec only once on any one night, the wind velocity was considered 0 and no wind direction was assigned for that night. Wind direction was not specified when it was not in any of the three categories.

The data were analyzed in four-day intervals necessary to complete the rotations. Analysis of variance was used and the means were compared using Duncan's multiple-range test with $\alpha = 0.05$ through use of the General Linear Models Procedure (SAS Institute, 1982).

RESULTS AND DISCUSSION

Table 1 shows the mean captures of BW males per trap per night during each four-day interval when the BW traps were located 5, 15, and 30 m downwind from the virelure dispensers as compared to captures in check traps with

TABLE 1. CAPTURE OF *Heliothis zea* MALES IN PHEROMONE TRAPS LOCATED AT VARIOUS DISTANCES DOWNWIND FROM *Heliothis virescens* Dispensers (1983, 1984)

Distance (m)	$\bar{X}$ No. males ± SD/trap/night for indicated test period[a]			
	1983			
	7/7–7/10	7/11–7/14	7/15–7/18	7/7–7/18
Check	251.6 ± 185.5 A	101.8 ± 49.8 A	85.4 ± 34.5 A	146.3 ± 133.7 A
30	225.8 ± 158. 4 A	82.2 ± 40.3 B	54.9 ± 25.6 B	121.0 ± 120.3 B
15	235.3 ± 146.8 A	78.2 ± 34.6 B	52.9 ± 22.8 B	122.1 ± 118.6 B
5	106.5 ± 111.0 B	54.8 ± 26.5 C	39.9 ± 15.6 C	85.1 ± 84.9 C
	7/9–7/12	7/13–7/16	7/17–7/20	7/9–7/20
Check	128.7 ± 34.2 A	81.1 ± 38.3 A	65.8 ± 31.7 A	91.9 ± 43.5 A
30	108.8 ± 42.7 B	62.4 ± 31.3 BC	43.0 ± 23.6 B	71.4 ± 43.0 B
15	98.1 ± 38.5 B	65.1 ± 33.7 B	37.9 ± 23.7 B	67.0 ± 40.4 B
5	66.2 ± 27.6 C	48.6 ± 28.9 C	25.8 ± 18.2 C	46.9 ± 29.9 C

	7/21–7/24		7/25–7/29[b]		7/21–7/29		7/30–8/2	
Check	47.6 ± 23.2	A	47.6 ± 26.8	A	47.6 ± 24.7	A	18.4 ± 11.0	A
60							16.6 ± 9.5	AB
45	32.6 ± 19.3	B	22.0 ± 10.6	B	27.3 ± 16.2	B	16.3 ± 7.3	AB
30	32.5 ± 15.9	B	20.4 ± 12.6	B	26.4 ± 15.4	B	13.1 ± 9.2	B
15	23.0 ± 13.7	B	10.6 ± 5.8	C	16.8 ± 12.1	C		

1984

	6/23–6/26		6/27–6/30		7/1–7/4		6/23–7/4	
Check	301.6 ± 91.0	A	312.8 ± 174.4	A	242.8 ± 62.4	A	285.7 ± 120.7	A
75	302.7 ± 107.1	A	284.6 ± 142.2	A	221.8 ± 96.0	A	268.5 ± 116.1	A
50	284.7 ± 94.3	A	274.9 ± 156.9	A	218.3 ± 79.6	A	263.1 ± 116.4	A
5	163.6 ± 51.7	B	101.3 ± 41.6	B	151.1 ± 74.6	B	140.2 ± 64.1	B

[a] Means in the same column for each test period followed by the same letter are not significantly different [$\alpha = 0.05$, Duncan's multiple-range test].

[b] Data for 7/28 were used in the analysis because there was an error in the rotation of treatments.

no virelure dispensers. Captures were extremely high the first few days of the test and tended to be more stable thereafter. In the first interval for the period July 7–10, 1983, only the 5-m treatment was significantly different from the others. In the next two intervals for the period July 11–18, significant differences in captures were observed between the check, the 30- and 15-m treatments, and the 5-m treatments. When the data for all three intervals were analyzed together, there were significant differences between the check, the 30- and 15-m treatments, and the 5-m treatment. A very large reduction was observed with the 5-m distance. Categorization of wind velocity and direction during the test period (Table 2) indicated that wind direction was within $\pm 22.5°$ of the SSE prevailing wind direction three nights, within $\pm 45°$ on three nights, within $\pm 90°$ on three nights, and from an unspecified direction on three nights.

Since the categorization of wind speed and direction could not be specified for at least the first two nights when BW captures were very high, data from the first two days were dropped and data from two days at the end of the evaluation period were used. For the two nights that were added, the wind direction was within $\pm 22.5°$ of the SSE prevailing wind. Analysis of these data indicated that the differences between treatments were more consistent throughout the evaluation period, and it showed a reduction between the 30-m and the 15-m treatments. Overall, the 5-m treatment caught only about half the number of males captured in the check.

Two intervals in which the 5-m treatment was replaced with a 45-m distance and that were conducted between July 21 and 29, 1983, indicated a significant difference in the captures between the check and the 45- and 30-m treatments. The 15-m treatment was significantly different from the others. During this test period, wind conditions were very good for the evaluation since wind direction was within $\pm 22.5°$ of the SSE prevailing wind direction seven of the eight nights.

Although captures had declined considerably by the time of the last interval (July 30–August 2), in which the 15-m treatment was substituted with a 60-m treatment, the only significant difference observed was between the check and the 30-m treatment, but there was still a trend for a reduction due to the presence of the virelure dispensers. During this period, wind conditions were suitable during two nights and there were two nights when there was no wind.

In 1984, the studies were designed to identify the limits of the interaction when the traps were located downwind from virelure dispensers. The results in each of the three intervals from June 23 to July 4 were consistent with no significant differences observed between the check, 75-m, and 50-m treatments, although all these were significantly different from the 5-m treatment. Analysis of data from all three intervals combined indicated that there was still a tendency for reductions in the mean number of males captured at the 75-m and 50-m distances; however, the reductions were relatively small compared to the 5-m

TABLE 2. CATEGORIZATION OF WIND SPEED AND DIRECTION CONDITIONS DURING JULY 7–AUGUST 2, 1983[a]

	Wind direction[b]				Wind speed[b]
Date	Unspecified	±90°	±45°	±22.5°	0[c]
July 7	X				
8	X				
9	X				
10					X
11				X	
12				X	
13			X		
14		X			
15			X		
16		X			
17		X			
18				X	
19				X	
20				X	
21					X
22					X
23				X	
24				X	
25				X	
26				X	
27				X	
29[d]				X	
30			X		
31					X
August 1				X	
2					X

[a] Data are based on hourly readings made between 10 PM and 1 AM each night at the FAA Flight Service Station at Easterwood Airport, College Station, Texas.
[b] Categories of wind direction were assigned only when there were at least two hourly readings with wind speeds >0 m/sec. Specific categories of wind direction were assigned when at least two hourly readings were within the smallest wind direction interval.
[c] If there was only one hourly reading with wind speeds >0 m/sec during any one night, the wind speed was considered to be 0 and no wind direction category was assigned.
[d] Data collected on July 28, 1983, were not used in the analysis because the treatment rotations were made improperly.

treatment which was only about half as large as the check. Although there was one night when the wind direction was beyond the 90° limit and two nights when the wind speed was 0 during the evaluations, wind conditions were suitable with wind direction within ±45° of the established SSE direction on eight of the 12 nights (Table 3).

TABLE 3. CATEGORIZATION OF WIND SPEED AND DIRECTION CONDITIONS DURING PERIOD JUNE 23–JULY 4, 1984[a]

	Wind direction[b]				Wind speed
Date	Unspecified	± 90°	± 45°	± 22.5°	0[c]
June 23				X	
24					X
25	X				
26				X	
27				X	
28		X			
29					X
30				X	
July 1			X		
2				X	
3				X	
4				X	

[a] Data are based on hourly readings made between 10 PM and 1 AM each night at the FAA Flight Service Station at Easterwood Airport, College Station, Texas.
[b] Categories of wind direction were assigned only when there were at least two hourly readings with wind speeds >0 m/sec. Specific categories of wind direction were assigned when at least two hourly readings were within the smallest wind direction interval.
[c] If there was only one hourly reading with wind speeds >0 m/sec during any one night, the wind speed was considered to be 0 and no wind direction category was assigned.

When the BW traps were located at various distances upwind from virelure dispensers, the effects were less than when the BW traps were located downwind (Table 4). In the first two intervals for the period July 7–14, 1983, no significant differences were observed among the check, 30-m, and 15-m treatments. In the two intervals between July 19 and 26, the only significant difference was between the 5-m treatment and the other treatments. There was a trend for the effect of the virelure dispensers to decrease as the distance increased. The overall results for all five intervals indicated that the only significant difference was between the 5-m distance and the other treatments and showed decreasing effect with increased distance, with both the check and the 30-m treatments being very similar. For the 20-day period of the evaluation, there were three nights when the wind direction exceeded the 90° deviation, three nights with no wind, and nine, two, and three nights, when the wind was within ±22.5°, ±45°, and ±90° of the SSE wind direction, respectively (Table 2).

Reduction of BW captures when the virelure dispensers were located upwind was expected since the wind conveys the the virelure pheromone com-

TABLE 4. CAPTURE OF *Heliothis zea* MALES IN PHEROMONE TRAPS LOCATED AT VARIOUS DISTANCES UPWIND FROM *Heliothis virescens* DISPENSERS (1983)

Distance (M)	$\bar{X}$ No. males ± SD/trap/night for indicated test periods[a]		
	7/7–7/10	7/11–7/14	7/15–7/18
Check	108.1 ± 76.3 A	54.8 ± 33.6 A	55.1 ± 28.7 A
30	108.4 ± 76.3 A	49.1 ± 29.8 AB	59.3 ± 40.4 A
15	87.0 ± 70.3 AB	45.4 ± 29.2 AB	63.9 ± 41.8 A
5	58.7 ± 43.5 B	35.9 ± 23.5 B	48.8 ± 26.1 A
	7/19–7/22	7/23–7/26	7/7–7/26
Check	37.3 ± 25.2 A	41.7 ± 21.0 A	59.4 ± 48.3 A
30	33.9 ± 25.9 A	42.6 ± 17.8 A	58.6 ± 49.5 A
15	30.6 ± 17.8 A	37.6 ± 19.5 A	52.9 ± 44.5 A
5	19.2 ± 13.4 B	21.3 ± 12.8 B	36.8 ± 29.9 B

[a] Means in same column for the same test period followed by the same letter are not significantly different [$\alpha = 0.05$, Duncan's multiple-range test].

ponents downwind and into the area where the BW would be responding to its pheromone, thus disrupting or repelling the males. The virelure components may not have to overlap the pheromone plume from the BW sex pheromone trap to have an effect on reducing captures since the presence of the virelure components may be sufficient to disrupt the BW male searching behavior prior to locating the sex pheromone plume from the trap. When the BW traps were located upwind of the virelure dispensers, the influence of the pheromone components from the dispensers was reduced because the exposure of the BW males to virelure in the trap area would be minimized. In this case, the trap area includes the BW sex pheromone plume as well as the area that searching BW males would have to cross to find the plume from the pheromone trap.

Although these data do not provide an absolute measure of the interaction between BW traps and virelure dispensers relative to wind direction and distance, it does provide some guidelines from which to make initial recommendations relative to the minimum spacing between BW and TBW traps. Since the greatest effects were observed when BW traps were located downwind from virelure dispensers, and the least effect was when they were located upwind, the interaction between the two, at least relative to captures in BW traps, should be minimized by installing the BW traps in a direction upwind from *H. virescens* traps. If wind direction is expected to be variable, the traps should be spaced at least 75 m apart.

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RESPONSE OF MOUNTAIN PINE BEETLE, *Dendroctonus ponderosae* HOPKINS, AND PINE ENGRAVER, *Ips pini* (SAY), TO IPSDIENOL IN SOUTHWESTERN BRITISH COLUMBIA[1]

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Abstract—In bioassays conducted with walking beetles in the laboratory (*S*)-(+)-, (*R*)-(−)-, and (±)-ipsdienol were attractive alone, but reduced the attraction of both sexes of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, to extracts of female frass. Field trapping studies indicated that attraction of *D. ponderosae* to a stimulus composed of myrcene + *trans*-verbenol + *exo*-brevicomin was significantly reduced with the addition of (±)- and sometimes (*S*)-(+)-ipsdienol. Thus, (*S*)-(+)-ipsdienol produced by males of this species may act as an antiaggregation pheromone. (*S*)-(+)-Ipsdienol is thought to function as a repellent allomone against the pine engraver, *Ips pini* (Say), in regions where *I. pini* utilizes (*R*)-(−)-ipsdienol as an aggregation pheromone. However, in southwestern British Columbia *I. pini* was attracted to the (±)-ipsdienol used in field bioassays of *D. ponderosae*, a finding consistent with the production of both enantiomers by *I. pini* in this region. When presented with the ternary semiochemical bait for *D. ponderosae*, (±)-ipsdienol was not attractive to *I. pini*. Thus, the activity of (*S*)-(+)-ipsdienol as a repellent allomone against *I. pini* seems to be replaced in southwestern British Columbia by the inhibitory effects of myrcene, *trans*-verbenol, *exo*-brevicomin, or some combination thereof.

Key Words—*Dendroctonus ponderosae, Ips pini*, Coleoptera, Scolytidae, pheromone, bark beetle, ipsdienol.

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INTRODUCTION

The mountain pine beetle, *Dendroctonus ponderosae* Hopkins, is a destructive forest pest which attacks several species of pine in western North America. These bark beetles mass attack host trees in response to aggregation pheromones and host tree kairomones which are released as the beetles bore into the tree. The female-produced terpene alcohol *trans*-verbenol, in combination with host tree monoterpenes, is partially responsible for the aggregation of *D. ponderosae* (Pitman et al., 1968), while low concentrations of *exo*-brevicomin, produced by males, are also involved (Rudinsky et al., 1974; McKnight, 1979; Conn et al., 1983; Borden et al., 1983).

Once a certain attack density is reached, the attack switches to adjacent trees (McCambridge, 1967; Geiszler and Gara, 1978; Geiszler et al., 1980), preventing the increased competition within hosts and reduced brood survival which occur at overly high attack densities (Reid, 1963). This switching was hypothesized to be due to a decrease in resin flow (Renwick and Vité, 1970), and a decrease in aggregation pheromone production (Pitman and Vité, 1969; Borden et al., 1987), but more likely results from the production of antiaggregation pheromones (Rudinsky et al., 1974; Borden et al., 1987). Several compounds that are produced by *D. ponderosae* have been found to inhibit attraction in laboratory and field tests, and this has led to speculation that they may act as antiaggregation pheromones. These compounds include frontalin (Ryker and Libbey, 1982), *exo*- and *endo*-brevicomin (Ryker and Rudinsky, 1982), verbenone (Ryker and Yandell, 1983), and pinocarvone (Libbey et al., 1985).

Hunt et al. (1986) have shown that male *D. ponderosae* exposed to myrcene vapors produce large quantities of ipsdienol, a compound which Byers (1982) reported as a possible antiaggregation pheromone for the western pine beetle, *Dendroctonus brevicomis* LeConte. Ipsdienol is also the aggregation pheromone of the pine engraver, *Ips pini* (Say) (Birch et al., 1980; Lanier et al., 1980). Moreover, there is pronounced geographic variation in the response of *I. pini* to the enantiomers of ipsdienol (Birch et al., 1980; Lanier et al., 1980). The production of ipsdienol by both *D. ponderosae* and *I. pini* and the sympatric distribution of these species in southwestern British Columbia suggests that this compound may be used in interspecific communication. We report the responses of walking *D. ponderosae* in the laboratory, as well as flying *D. ponderosa* and *I. pini* in the field, to (*S*)-(+)-, (*R*)-(−)-, and (±)-ipsdienol alone and in combination with *trans*-verbenol, *exo*-brevicomin, and the host tree monoterpene myrcene.

METHODS AND MATERIALS

Laboratory Bioassays. Bioassays were performed in a draftfree, darkened room using an open arena olfactometer similar to that described by Borden et

al. (1968). Groups of 10 beetles were released from overturned, 60 × 20-mm disposable Petri dishes, on a 24-cm-diam. filter paper. The release point was 8 cm lateral to and 12 cm downwind of the outlet of an airstream with a flow rate of 1400 ml/min. During the summer of 1984, four or five groups of 10 beetles were used for each treatment, while in 1985 the number was expanded to 100 beetles in groups of 10. A 6-V, 25-W microscope light was placed at the opposite end of the filter paper to draw the photopositive beetles across the airstream. Twenty microliters of a pentane dilution of each test stimulus was deposited on a rolled filter paper (4.25 cm diam.) inside a glass tube (1 cm ID). The glass tube was placed at the outlet of the airstream, which then flowed across the center of the arena perpendicular to the path of the walking beetles. Each group of beetles was allowed 2 min to respond to the test material. A positive response was scored when a beetle walked to within 1 cm of the stimulus source.

The experimental stimuli used in the bioassays were (*S*)-(+)-, (*R*)-(−)-, and (±)-ipsdienol (sources and purities listed in Table 1), each tested at concentrations ranging between 4 and 400,000 ng/20 μl pentane. These same dilutions of (*S*)-(+)-, (*R*)-(−)-, and (±)-ipsdienol were also tested in combination with pentane extracts of frass produced by female *D. ponderosae* boring in lodgepole pine bolts for 24 hr. Control stimuli were pentane and the pentane extract of frass produced by females. The frass extract was tested at a concentration of 0.02 g equivalents when used as a standard or combined with ipsdienol dilutions. The frass standard was used whenever bioassays were being performed to verify that the test beetles were responsive. Tests were not run on days with low barometric pressure as beetles responded poorly on these days. Beetles were kept in disposable Petri dishes at 2–4°C between bioassays but were rewarmed to room temperature 10 min prior to use. Each time a new stimulus was tested, the filter paper arena was changed and the bioassay room was aired out.

Percent positive response data were transformed using the arcsine transformation (Sokal and Rohlf, 1981), and then analyzed by ANOVA and the Newman-Keuls test using SPSS^x (SPSS, 1983).

(*S*)-(+)-Ipsdienol (95% optical purity) and (*R*)-(−)-ipsdienol (98% optical purity) were obtained by kinetic resolution of racemic ipsdienol (Sharpless et al., 1983) utilizing the Sharpless asymmetric epoxidation (Katsuki and Sharpless, 1980) to the extent of 80%. The enantiomeric excesses of the isolated products were determined by capillary gas chromatography of the acetyl-(*S*)-lactyl derivatives as reported by Slessor et al. (1985).

Field Trapping Experiments. All field trapping experiments were done in 1984 and 1985 in lodgepole pine forests near Shinish Cr. Road, approximately 30 km northeast of Princeton, British Columbia. All compounds tested in the field are listed in Table 1, along with the sources, purities, release devices, and release rates.

TABLE 1. SOURCE, PURITY, RELEASE DEVICES, AND RELEASE RATES (AT 22°C IN LABORATORY) FOR MONOTERPENES AND TERPENE ALCOHOLS TESTED FOR ATTRACTANCY TO *D. ponderosae* IN THE FIELD

Compound	Source	Purity[a] (%)	Release device	Approximate release rate (mg/24 hr)
β-Myrcene (M)	Sigma Chem. Co. St. Louis, Missouri	98	Traps: 25 ml polyethylene snap cap vial (closed)	150.00
		85	Trees: two 1.8-ml polyethylene centrifuge tubes (closed)	20.00
(±)-*exo*-Brevicomin (eB)	Albany Int'l Co. Columbus, Ohio	98	Traps: Conrel fibre (Albany Int'l Co.) 0.2 mm ID, one end open	0.05
		98	Trees: Glass capillary tube, 1.1–1.6 mm ID, one end open	0.50
trans-Verbenol [75%(−); 25%(+)] (tV)	Phero Tech Vancouver, British Columbia (12% *cis*)	88	1.8 ml polyethylene centrifuge tube (open), containing 30 μl on cotton plug	1.00
Ipsdienol[b] (I)	(±): Borregard A.S., Sarpsborg, Norway	91	1984 high: 10 glass capillary tubes, 0.8–1.1 mm ID, one end open	0.50
	(*S*)-(+): chemical purity		1984 low: 1 glass capillary	0.05
	optical purity	91	tube, 0.8–1.1 mm ID, one	
	(*R*)-(−): chemical purity	95	end open	
	optical purity	93	1985: five 2-μl microcaps (Drummond Scientific Co., Broomall, Pennsylvania)	0.15
		98		

[a] As determined by gas chromatography.
[b] (*S*)-(+)- and (*R*)-(−)-ipsdienol resolved from (±) by E. Czyewska, Department of Chemistry, Simon Fraser University.

Trapping experiments were done in a randomized block design using eight-funnel, multiple funnel traps (Lindgren, 1983) placed in lines 25 m apart with 25 m between each trap. Baits were suspended from a wire attached to the third funnel from the bottom of the trap such that the bait was positioned about 1 cm below that funnel. Jars at the bottoms of the traps contained an aqueous solution of approximately 2.5 ml/liter of Liqui-nox detergent (Alconox Inc., New York, New York) to decrease surface tension and kill trapped insects, and 40 mg/liter of sodium azide to inhibit microbial growth; beetles were collected from these jars and preserved in 90% ethanol prior to being counted and their sex determined.

In 1984, eight replicates were performed of the following bait treatments: (1) myrcene + *trans*-verbenol + *exo*-brevicomin + high release rate (±)-ipsdienol; (2) myrcene + *trans*-verbenol + *exo*-brevicomin + low release rate (±)-ipsdienol; (3) myrcene + *trans*-verbenol + *exo*-brevicomin; (4) high release rate (±)-ipsdienol; (5) low release rate (±)-ipsdienol; and (6) unbaited control.

In 1985, 12 replicates were performed of the following bait treatments: (1) myrcene + *trans*-verbenol + *exo*-brevicomin + (±)-ipsdienol; (2) myrcene + *trans*-verbenol + *exo*-brevicomin + (*S*)-(+)-ipsdienol; (3) myrcene + *trans*-verbenol + *exo*-brevicomin + (*R*)-(−)-ipsdienol; (4) myrcene + *trans*-verbenol + *exo*-brevicomin; (5) (±)-ipsdienol; (6) (*S*)-(+)-ipsdienol; (7) (*R*)-(−)-ipsdienol; and (8) unbaited control.

One experiment of two days duration was conducted in 1984 and, in 1985, three experiments each of three days duration were conducted with baits re-randomized after each experiment.

Tests for homogeneity of variances were performed on the numbers of beetles caught using Cochran's *C* test as well as the Bartlett-Box *F* test using SPSS^x (1983). The data were heteroscedastic; therefore, they were analyzed using the Kruskal-Wallis test (Sokal and Rohlf, 1981) followed by a nonparametric multiple comparisons test (Conover, 1980, p. 231). The data on sex ratios were homoscedastic, so they were analyzed by ANOVA and the Newman-Keuls test using SPSS^x (1983).

Baited-Tree Experiments. Two baited-tree experiments were laid out in a randomized block design at the same location as the trapping experiment. The baits were contained in waterproofed, cardboard receptacles (Phero Tech Inc., Vancouver, British Columbia) stapled onto mature lodgepole pine trees of diameter at breast height (dbh) 22–34 cm, which were a minimum of 15 m apart. In 1984, 10 replicates were performed of the following bait treatments: (1) myrcene + *trans*-verbenol + *exo*-brevicomin + high release rate (±)-ipsdienol; (2) myrcene + *trans*-verbenol + *exo*-brevicomin; (3) high release rate (±)-ipsdienol; and (4) unbaited control.

In 1985, 11 replicates were performed of the following bait treatments: (1)

myrcene + *trans*-verbenol + *exo*-brevicomin + (±)-ipsdienol; (2) myrcene + *trans*-verbenol + *exo*-brevicomin + (*S*)-(+)-ipsdienol; (3) myrcene + *trans*-verbenol + *exo*-brevicomin + (*R*)-(−)-ipsdienol; (4) myrcene + *trans*-verbenol + *exo*-brevicomin; (5) (±)-ipsdienol; (6) (*S*)-(+)-ipsdienol; (7) (*R*)-(−)-ipsdienol; and (8) unbaited control.

Following the flight period, attack densities were assessed on all of the trees in the experiments by counting the entrance holes within two, 20 × 40-cm frames held at eye level on opposite sides of the trees at 90° from the bait station.

The data were homoscedastic (Cochran's *C* and Bartlett-Box *F* tests), so mean attack densities were analyzed by ANOVA and the Newman-Keuls test using SPSS^x (1983).

RESULTS

Table 2 shows the responses of both sexes of *D. ponderosae* in laboratory bioassays to dilutions of (*S*)-(+)-, (*R*)-(−)-, and (±)-ipsdienol. In most of these experiments (±)-, (*S*)-(+)-, or (*R*)-(−)-ipsdienol at one or more concentrations elicited a significantly higher percentage of positive responses than pentane controls, particularly at higher concentrations of ipsdienol. Ipsdienol at the two highest concentrations (400,000 ng/20 μl pentane and 40,000 ng/20 μl pentane) often elicited positive responses that were not significantly different from responses to female frass extracts.

Table 3 shows the responses of both sexes of *D. ponderosae* in laboratory bioassays to female frass extracts combined with either (*S*)-(+)-, (*R*)-(−)-, or (±)-ipsdienol at various concentrations. In most of these experiments the ipsdienol inhibited responses to the attractive frass extract, although the effect was significant only at low concentrations of ipsdienol.

Field trapping studies in 1984 (Figure 1A) indicated that traps baited with (±)-ipsdienol at high or low release rates were not significantly more attractive to *D. ponderosae* than unbaited control traps. When the attractive bait composed of myrcene + *trans*-verbenol + *exo*-brevicomin was combined with (±)-ipsdienol at high or low release rates, trap catch was significantly lower than for myrcene + *trans*-verbenol + *exo*-brevicomin alone, indicating that (±)-ipsdienol inhibits response of *D. ponderosae* to these attractive semiochemicals.

Field trapping studies in 1985 (Figure 1B–D) generally supported the results obtained in 1984. In all three experiments, traps baited with (±)-, (*S*)-(+)-, or (*R*)-(−)-ipsdienol were not significantly more attractive to *D. ponderosae* than the unbaited control traps. In two of three experiments, (±)-ipsdienol significantly inhibited response to myrcene + *trans*-verbenol + *exo*-brevicomin. (*S*)-(+)-Ipsdienol significantly inhibited the attractiveness of myr-

TABLE 2. POSITIVE RESPONSES OF WALKING *D. ponderosae* TO (+)-, (−)-, AND (±)-IPSDIENOL

Exp.	Stimulus	Percent response ($\bar{X}$ ± SE)[a]					
		Females			Males		
		A	B	C	D	E	F
1	pentane, 20 μl	4 ± 2 a	5 ± 2 a	4 ± 2 a	4 ± 2 a		
	(+)-ipsdienol, 4 ng	2 ± 2 a	10 ± 3 a	2 ± 1 a	6 ± 4 a		
	(+)-ipsdienol, 40 ng	12 ± 4 a	8 ± 3 a	3 ± 2 a	8 ± 4 a		
	(+)-ipsdienol, 400 ng	28 ± 2 b	22 ± 4 b	3 ± 2 a	20 ± 4 ab		
	(+)-ipsdienol, 4,000 ng	30 ± 3 b	27 ± 4 b	4 ± 2 a	26 ± 2 bc		
	(+)-ipsdienol, 40,000 ng	30 ± 4 b	26 ± 4 b	9 ± 3 a	30 ± 3 bc		
	(+)-ipsdienol, 400,000 ng		40 ± 5 c				
	♀ frass extract, 0.02 g equiv.	38 ± 7 b	38 ± 5 c	30 ± 4 b	38 ± 7 c		
2	pentane, 20 μl	4 ± 2 a	5 ± 2 a		4 ± 2 a		
	(−)-ipsdienol, 4 ng	4 ± 2 a	5 ± 2 a		6 ± 2 a		
	(−)-ipsdienol, 40 ng	8 ± 2 ab	11 ± 3 a		8 ± 2 a		
	(−)-ipsdienol, 400 ng	12 ± 2 ab	5 ± 2 a		10 ± 3 a		
	(−)-ipsdienol, 4,000 ng	8 ± 2 ab	7 ± 2 a		8 ± 4 a		
	(−)-ipsdienol, 40,000 ng	16 ± 5 ab	9 ± 2 a		14 ± 2 ab		
	(−)-ipsdienol, 400,000 ng	22 ± 4 b	27 ± 3 b		26 ± 7 bc		
	♀ frass extract, 0.02 g equiv.	38 ± 7 c	37 ± 4 c		38 ± 7 c		

TABLE 2. Continued

Exp.	Stimulus	Percent response ($\bar{X} \pm SE$)[a]					
		Females			Males		
		A	B	C	D	E	F
3	pentane, 20 μl	5 ± 3 a	8 ± 3 a	6 ± 4 a	5 ± 3 a	10 ± 4 a	10 ± 4 ab
	(±)-ipsdienol, 4 ng	3 ± 3 a	23 ± 6 ab	10 ± 4 ab	3 ± 3 a	28 ± 6 ab	20 ± 4 ab
	(±)-ipsdienol, 40 ng	13 ± 3 a	18 ± 5 ab	16 ± 3 ab	10 ± 0 a	25 ± 3 ab	10 ± 0 ab
	(±)-ipsdienol, 400 ng	13 ± 3 a	18 ± 9 ab	8 ± 4 a	13 ± 3 a	25 ± 6 ab	14 ± 2 ab
	(±)-ipsdienol, 4,000 ng	15 ± 3 a	8 ± 8 a	8 ± 4 a	15 ± 3 a	18 ± 3 a	4 ± 4 a
	(±)-ipsdienol, 40,000 ng	13 ± 3 a	33 ± 5 b	6 ± 3 a	5 ± 3 a	40 ± 8 b	10 ± 4 ab
	(±)-ipsdienol, 400,000 ng	65 ± 3 b		22 ± 4 b	45 ± 3 b		20 ± 3 b
	♀ frass extract 0.02 g equiv.	45 ± 6 b	40 ± 4 b	36 ± 4 c	40 ± 0 b	43 ± 3 b	36 ± 4 c

[a] Letters A–F refer to separate series of bioassays. Means in a column within an experiment followed by the same letter are not significantly different ($P > 0.05$), Newman-Keuls test. Forty beetles tested/treatment in experiments 3A, 3B, 3D, and 3E; 50 beetles tested/treatment in experiments 1A, 1D, 2A, 2D, 3C, and 3F; 100 beetles tested/treatment in experiments 1B, 1C, and 2B.

TABLE 3. POSITIVE RESPONSES OF WALKING *D. ponderosae* TO (+)-, (−)-, AND (±)-IPSDIENOL IN COMBINATION WITH FEMALE FRASS EXTRACTS

		Percent response ($\bar{X} \pm SE$)[a]		
		Females		Males
Exp.	Stimulus	A	B	C
4	pentane, 20 μl	4 ± 2 a	10 ± 3 a	10 ± 7 a
	♀ frass extract, 0.02 g equiv., with			
	(+)-ipsdienol (ng) 4	13 ± 5 ab	40 ± 3 b	30 ± 4 b
	40	20 ± 6 abc	41 ± 5 b	40 ± 4 bc
	400	19 ± 3 abc	36 ± 5 b	46 ± 4 c
	4,000	17 ± 4 abc	42 ± 4 b	53 ± 3 c
	40,000	18 ± 3 abc	49 ± 5 b	56 ± 6 c
	400,000	24 ± 3 bc	45 ± 5 b	
	♀ frass extract, 0.02 g equiv.	30 ± 4 c	51 ± 6 b	73 ± 2 d
5	pentane, 20 μl	4 ± 2 a		9 ± 3 a
	♀ frass extract, 0.02 g equiv., with			
	(−)-ipsdienol (ng) 4	11 ± 3 ab		
	40	20 ± 4 bc		29 ± 4 b
	400	25 ± 5 c		35 ± 3 b
	4,000	28 ± 5 c		34 ± 5 b
	40,000	34 ± 4 c		41 ± 6 b
	400,000	30 ± 4 c		48 ± 3 b
	♀ frass extract, 0.02 g equiv.	30 ± 4 c		39 ± 7 b
6	pentane, 20 μl	6 ± 2 a		10 ± 3 a
	♀ frass extract, 0.02 g equiv., with			
	(±)-ipsdienol (ng) 4	14 ± 2 ab		16 ± 2 a
	40	20 ± 4 b		22 ± 4 abc
	400	20 ± 4 b		20 ± 3 ab
	4,000	22 ± 4 b		30 ± 3 bc
	40,000	28 ± 4 bc		34 ± 5 bc
	400,000	40 ± 3 c		34 ± 2 c
	♀ frass extract, 0.02 g equiv.	36 ± 2 c		36 ± 4 c

[a] Letters A–C refer to separate series of bioassays. Means in a column within an experiment followed by same letter are not significantly different ($P > 0.05$), Newman-Keuls test. Fifty beetles tested/treatment in experiments 4A and 4C; 100 beetles tested/treatment in experiments 4B, 5A, 5C, 6A and 6C.

cene + *trans*-verbenol + *exo*-brevicomin in one of three experiments. (*R*)-(−)-Ipsdienol did not induce a significant inhibition in any of the experiments.

Because of the large numbers of beetles caught in traps in the 1985 experiments, sex was only determined for the beetles caught in the July 26–29 ex-

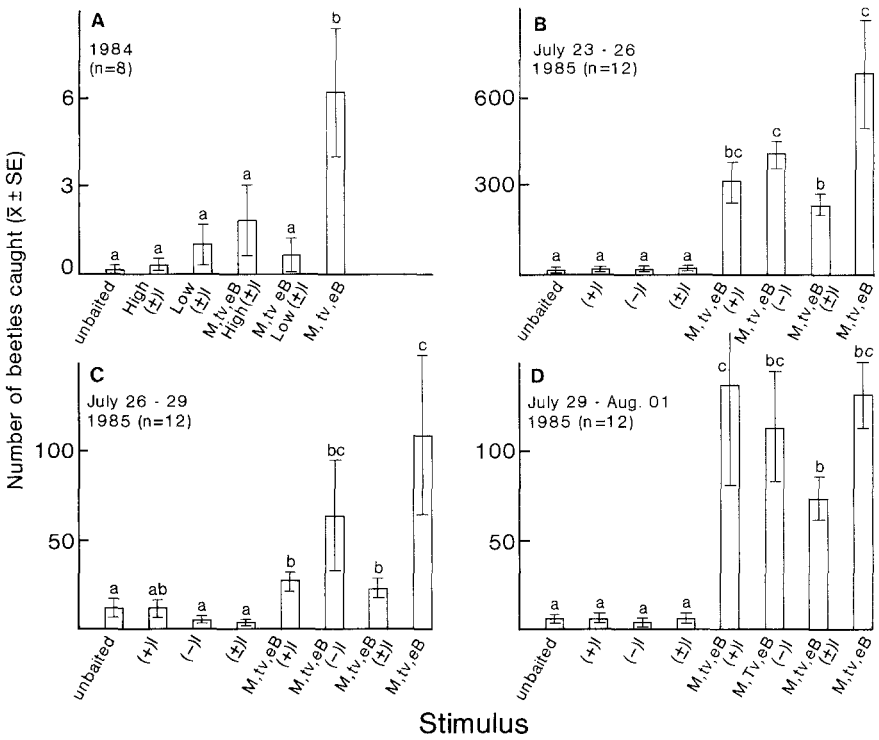

FIG. 1A–D. Number of *D. ponderosae* caught (±SE) in multiple-funnel traps baited with beetle-produced volatiles and host monoterpenes. Experiments conducted in 1984 and 1985 at Shinish Cr. Road, 30 km northeast of Princeton, British Columbia. In each experiment values with the same letter are not significantly different ($P > 0.05$), nonparametric multiple comparisons test (Conover, 1980, p. 231).

periment. The ratios of males to females caught were not significantly different (Newman-Keuls test, $P > 0.05$) for all baits containing myrcene + *trans*-verbenol + *exo*-brevicomin. However, these ratios (range 59–70% male) were all significantly higher (Newman-Keuls test, $P < 0.05$) than those for baits that contained only ipsdienol, or for unbaited traps (range 22–34% male). This result may indicate that traps baited with the standard semiochemical bait composed of myrcene + *trans*-verbenol + *exo*-brevicomin were more attractive to males than females.

In 1984 and 1985 the mean attack densities on trees baited with myrcene + *trans*-verbenol + *exo*-brevicomin were higher than on trees that were also baited with (±)-, (*S*)-(+), or (*R*)-(−)-ipsdienol (Table 4), although the effect was never statistically significant. Neither (±)-, (*S*)-(+)-, nor (*R*)-(−)-ipsdienol alone induced attack at levels above control trees by any scolytid species.

TABLE 4. RANKED ATTACK DENSITIES OF *D. ponderosae* ON LODGEPOLE PINES (*Pinus contorta*) BAITED WITH HOST MONOTERPENES AND BEETLE-PRODUCED VOLATILES. SHINISH CR. ROAD, 30 KM NORTHEAST OF PRINCETON, B.C.

Year and No. of replicates	Treatment	dbh ($\bar{X}$ ± SE)[a]	Trees attacked		Attack density / m^2 ($\bar{X}$ ± SE)[a]
			No.	%	
1984	M,tV,eB,High(±)I	24.6 ± 1.5 a	5	50	17 ± 12 a
(N = 10)	Blank	25.0 ± 1.7 a	2	20	18 ± 9 a
	High (±)I	24.3 ± 1.1 a	5	50	23 ± 7 a
	M,tV,eB	23.0 ± 0.6 a	6	60	24 ± 18 a
1985	Blank	25.7 ± 0.6 a	1	9	2 ± 2 a
(N = 11)	(+)I	25.3 ± 0.9 a	1	9	3 ± 3 a
	(−)I	25.6 ± 0.8 a	1	9	6 ± 6 a
	(±)I	26.0 ± 0.8 a	2	18	7 ± 6 a
	M,tV,eB,(+)I	25.8 ± 0.6 a	9	82	40 ± 9 b
	M,tV,eB,(±)I	25.7 ± 1.0 a	10	91	43 ± 13 b
	M,tV,eB,(−)I	26.9 ± 1.0 a	9	82	44 ± 8 b
	M,tV,eB	28.1 ± 1.0 a	10	91	55 ± 10 b

[a]Values for treatments in each experiment followed by the same letter are not significantly different ($P > 0.05$), Newman-Keuls test.

Although the field trapping studies were designed to test the responses of *D. ponderosae*, the traps also caught numerous pine engravers, *I. pini* (Figure 2A–D). The 1984 data indicated that *I. pini* were strongly attracted to (±)-ipsdienol, with the high release rate being significantly more attractive than the low release rate (Figure 2A). Traps baited with myrcene + *trans*-verbenol + *exo*-brevicomin were not significantly more attractive than the unbaited control traps. When this three-component mountain pine beetle bait was added to (±)-ipsdienol, the attractiveness of (±)-ipsdienol to *I. pini* was significantly reduced. Thus, either myrcene, *trans*-verbenol, *exo*-brevicomin, or some combination of these compounds inhibits the response of *I. pini* to its aggregation pheromone.

The 1985 data for *I. pini* supported the 1984 data, although in the July 29–August 1 experiment, there were no significant differences in response (Figure 2B–D). In addition, neither (*S*)-(+)- nor (*R*)-(−)-ipsdienol were more attractive than an unbaited control, indicating that the two enantiomers were synergistic in attracting *I. pini* in this region. In both 1984 and 1985 the sex ratios of trapped *I. pini* did not appear to be affected by the stimulus present in the traps, although too few beetles were captured to perform statistical analyses on sex ratios.

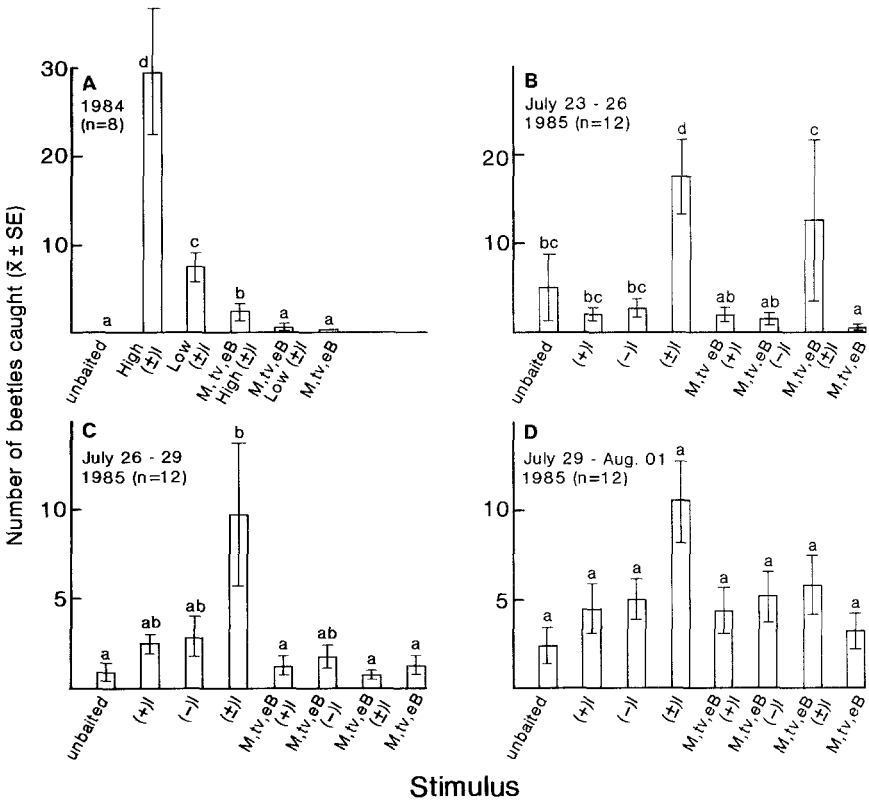

FIG. 2A–D. Number of *I. pini* caught (±SE) in multiple-funnel traps baited with beetle-produced volatiles and host monoterpenes. Experiments conducted in 1984 and 1985 at Shinish Cr. Road, 30 km northeast of Princeton, British Columbia. In each experiment values with the same letter are not significantly different ($P > 0.05$), nonparametric multiple comparisons test (Conover, 1980, p. 231).

DISCUSSION

Bioassays show that (*S*)-(+)-ipsdienol may weakly inhibit the attraction of both sexes of *D. ponderosae* to the attractive semiochemicals present in extracts of female frass in the laboratory (Table 3), as well as inhibiting attraction to a blend of myrcene + *trans*-verbenol + *exo*-brevicomin in the field (Figure 1A–D, Table 4). Thus, the 97% (*S*)-(+)-ipsdienol produced in large quantities by male *D. ponderosae* (Hunt et al., 1986) may function as an antiaggregation pheromone. In this way male beetles could contribute to the regulation of attack density, spacing, and termination of the attack on host trees. This could help

to prevent overly high attack densities that result in increased competition within hosts and reduced brood survival (Reid, 1963). When the (*S*)-(+)-ipsdienol concentration reached a high level, incoming beetles would avoid that host and attack other trees. The role of (*S*)-(+)-ipsdienol as an epideictic or spacing pheromone would facilitate a more efficient partitioning of the phloem tissue resource. A similar epideictic role is hypothesized for *trans*-verbenol, (+)-ipsdienol, and verbenone in *D. brevicomis* on ponderosa pine (Byers, 1983; Byers et al., 1984).

The reduction in trap catch due to ipsdienol (Figure 1A–D) is similar in magnitude to the reductions caused by other compounds that have been reported as antiaggregation pheromones of *D. ponderosae* (Ryker and Libbey, 1982; Ryker and Rudinsky, 1982; Ryker and Yandell, 1983; Libbey et al., 1985). Further study is required to determine how any or all of these compounds interact to modify the behavior of *D. ponderosae* in natural systems, as well as whether these compounds have any potential as pest-management tools.

Although male *D. ponderosae* produce large quantities of (*S*)-(+)-ipsdienol (Hunt et al., 1986), (±)-ipsdienol has a much stronger inhibitory effect than (*S*)-(+)-ipsdienol on the attraction of *D. ponderosae* to its aggregation pheromones (Figure 1A–D, Tables 3 and 4). This strongly inhibitory activity of (±)-ipsdienol suggests that the beetles in this geographic region may be exposed to an approximately racemic mixture of ipsdienol in nature. Although *I. pini* from California, Idaho, and southeastern British Columbia produce predominantly (*R*)-(−)-ipsdienol (Birch et al., 1980; Plummer et al., 1976; Slessor et al., 1985), *I. pini* from Manning Park, about 65 km from our study site, produce an average of 34% (*R*)-(−)-ipsdienol with tremendous variation between individuals (Miller et al., unpublished). Possibly the strong inhibitory effect of (±)-ipsdienol on *D. ponderosae* in our experiments represents an adaptation to avoid competition from a sympatric population of *I. pini* that produces both enantiomers of ipsdienol in substantial amounts. This avoidance response would be particularly advantageous to *D. ponderosae*, as *I. pini* develop more rapidly and may outcompete *D. ponderosae* within host trees (authors' unpublished observations).

It would be interesting to test the inhibitory effects of (*S*)-(+)-, (*R*)-(−)-, and (±)-ipsdienol on *D. ponderosae* in California, Idaho, and southeastern British Columbia where *I. pini* produce predominantly (*R*)-(−)-ipsdienol (Birch et al., 1980; Plummer et al., 1976; Slessor et al., 1985). It is possible that selection pressure due to competition with *I. pini* in these areas would favor *D. ponderosae* which were more strongly inhibited by (*R*)-(−) than by (±)-ipsdienol.

It is not known why the inhibitory effect of ipsdienol on the attraction of *D. ponderosae* to extracts of female frass in the laboratory was most pronounced at low concentrations (Table 3). The decreased inhibition at higher

concentrations may be due to the slight attractive effect of ipsdienol at higher concentrations (Table 2).

The attractiveness of ipsdienol in the laboratory at the highest concentration tested (400,000 ng/20 μl pentane and 40,000 ng/20 μl pentane) (Table 2) is somewhat surprising because of the inhibitory effects of low concentrations of (*S*)-(+)-, (*R*)-(−)- and (±)-ipsdienol on the response of *D. ponderosae* to female frass extracts (Table 3). However, the concentrations that are significantly more attractive than the pentane controls are probably higher than any ipsdienol concentrations likely to occur in nature. Although it is possible that ipsdienol could be a multifunctional pheromone in *D. ponderosae* and that it has different effects depending on the concentration, it is more likely that the attraction was an artifact of using artificially high concentrations or that a minor contaminant in the ipsdienol was causing the attraction.

Although Pitman et al. (1966) cautioned that variation in olfactory behavior between laboratory and field bioassays greatly limits the usefulness of laboratory bioassays, their reservations have proven to be without foundation for many species. However, with *D. ponderosae*, bioassay results are often very different depending on whether the study is conducted in the field or with walking beetles in a laboratory olfactometer. While myrcenol was attractive to *D. ponderosae* of both sexes in laboratory bioassays (Conn, 1981), it slightly inhibited the response to other attractants in the field (Conn et al., 1983). Similarly, 3-caren-10-ol was attractive in the laboratory (Conn, 1981), but this compound did not effect total trap catch in the field (Conn et al., 1983). Our laboratory and field results for ipsdienol are also contradictory. The divergence of laboratory and field results may be a result of differences in the responses to natural frass in the laboratory and the three-component semiochemical bait in the field. Alternatively, these differences may be due to concentration effects or to the test compounds having different effects on flying and walking insects. Until proven reliable, bioassays conducted with walking *D. ponderosae* in the laboratory should not be used as anything more than a preliminary test of compounds that should subsequently be field tested.

The response of *I. pini* to multiple funnel traps baited with (±)-ipsdienol, as well as the lack of a response to (*S*)-(+)- or (*R*)-(−)-ipsdienol (Figures 2A–D), indicates that *I. pini* in this region require both enantiomers to induce a response. This result is in agreement with the findings of Miller et al. (in press) that *I. pini* from nearby Manning Park produce an approximately 34:66 ratio of (*R*)-(−)- to (*S*)-(+)-ipsdienol on the average. This ratio of ipsdienol enantiomers suggests that *I. pini* from southwestern British Columbia are genetically close to New York populations, which also produce both enantiomers in a similar ratio, and for which both enantiomers act synergistically to produce a response (Lanier et al., 1980). These two populations are clearly distinct from populations from California (Birch et al., 1980), Idaho (Plummer et al., 1976), and the Kootenay region of southeastern British Columbia (Slessor et al., 1985).

The inhibitory effect of the ternary mountain pine beetle bait on the response of *I. pini* to (±)-ipsdienol indicates that either myrcene, *trans*-verbenol, *exo*-brevicomin, or some combination of these compounds inhibits the response of *I. pini* to its aggregation pheromone. Birch et al. (1980) have shown that *I. pini* from California are attracted to (*R*)-(−)-ipsdienol, the naturally predominating enantiomer, while (*S*)-(+)-ipsdienol interrrupted the response of *I. pini* to an attractive source in field tests. Notably (*S*)-(+)-ipsdienol is produced by *I. paraconfusus* (Silverstein et al., 1966) and *D. brevicomis* (Byers, 1982), both of which are sympatric competitors of *I. pini* in California. Thus, in regions where *I. pini* is repelled by (*S*)-(+)-ipsdienol, it could act as a repellent allomone to keep *I. pini* out of trees attacked by *I. paraconfusus* (Birch et al., 1980), *D. brevicomis* (Byers, 1982), or *D. ponderosae* (Hunt et al., 1986). It should be noted that Byers (1982) suggested that ipsdienol may be involved in regulating the colony density of *D. brevicomis* without testing the effect of (*S*)-(+)-ipsdienol, the enantiomer produced by this species. Our data on the inhibitory effects of (*S*)-(+)- and (±)-ipsdienol for *D. ponderosae* suggests that Byers may not have been justified in assuming that the inhibitory activity that he found in (±)-ipsdienol would necessarily be present with (*S*)-(+)-ipsdienol, the enantiomer produced by *D. brevicomis*.

If *trans*-verbenol or *exo*-brevicomin, which are both produced by *D. ponderosae*, are involved in inhibiting the attraction of *I. pini* to (±)-ipsdienol, then they may be functioning as repellent allomones to reduce interspecific competition for the same hosts. In this way the activity of (*S*)-(+)-ipsdienol as a repellent allomone against *I. pini* may be replaced in southwestern British Columbia by the inhibitory activity of one of these compounds. Alternatively, *I. pini* may simply be avoiding high concentrations of myrcene due to their preference for dead and dying trees which probably have more tolerable levels of monoterpenes.

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CARDENOLIDE CONTENT AND THIN-LAYER CHROMATOGRAPHY PROFILES OF MONARCH BUTTERFLIES, *Danaus plexippus* L.,[1] AND THEIR LARVAL HOST-PLANT MILKWEED, *Asclepias asperula* subsp. *capricornu* (WOODS.) WOODS.,[2] IN NORTH CENTRAL TEXAS

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Abstract—This paper is the second in a series on cardenolide fingerprinting of monarch butterflies and their host-plant milkweeds in the eastern United States. Spectrophotometric determinations of the gross cardenolide content of *Asclepias asperula* plants in north central Texas indicated wide variation ranging from 341 to 1616 μg/0.1 g dry weight. The mean plant cardenolide concentration (886 μg/0.1 g) is the highest for any milkweed species on which monarch cardenolide profiles have been produced. Forty-one butterflies reared individually on these plants contained a skewed distribution of cardenolide concentrations ranging from 231 to 515 μg/0.1 g dry weight with a mean of 363 μg/0.1 g. The uptake of cardenolide by the butterflies was independent of plant concentration, suggesting that saturation occurs in cardenolide sequestration by monarchs when feeding on cardenolide-rich host-plants. Female monarchs contained significantly greater mean cardenolide concentrations (339 μg/0.1 g) than did males (320 μg/0.1 g). The mean dry weight of the male butterflies (0.211 g) was significantly greater than the female mean (0.191) so that the mean total cardenolide contents of males (675 μg) and females (754 μg) were not significantly different. Butterfly size was not significantly correlated to butterfly cardenolide concentration when differences due to sex and individual host-plant concentration were removed. Thin-layer chromatograms of 24 individual plant–butterfly pairs developed in two sol-

[1] Lepidoptera: Danaidae.
[2] Apocynales: Asclepiadaceae.

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vent systems resolved 22 individual spots in the plants and 15 in the butterflies. *A. asperula* plants appear to contain several relatively nonpolar cardenolides of the calotropagenin series which are metabolized to more polar derivatives in the butterflies. Quantitative evaluation of the R_f values, spot intensities, and probabilities of occurrence in the chloroform–methanol–formamide TLC system produced a cardenolide fingerprint clearly distinct from those previously established for monarchs reared on other *Asclepias* species. Our data support the use of fingerprints to make ecological predictions concerning larval host-plant utilization. *A. asperula* subsp. *capricornu* and *A. viridis* Walt. are the predominant early spring milkweeds throughout most of the south central United States. Cardenolide-rich monarchs reared on these two species may be instrumental in establishing and reinforcing visual avoidance of adults by naive predators throughout their spring and summer breeding cycle in eastern North America.

Key Words—*Asclepias asperula*, milkweed, Asclepiadaceae, *Danaus plexippus*, monarch butterfly, Lepidoptera, Danaidae, Oncopeltus, Hemiptera, Lygaeidae, cardenolide, cardiac glycoside, digitoxin, chemical ecology, chemotaxonomy, chemical defense, ecological chemistry, thin-layer chromatography, plant–insect interactions, coevolution.

INTRODUCTION

In this paper we present the second analysis in a series of investigations of monarch remigration and recolonization along the western margins of their distribution in the eastern United States. Our purposes are to establish the timing and extent of host-plant utilization by monarchs as they remigrate from Mexican overwintering sites into temperate North America, to establish the quantitative variation in cardenolide contents of butterflies as they relate to their host plants, and to develop thin-layer chromatography cardenolide fingerprints of adult monarchs for milkweed species commonly utilized during recolonization.

In our first paper (Lynch and Martin, 1987), we reported on the cardenolide characteristics of monarchs reared as larvae on *Asclepias viridis* Walt. in northwestern Louisiana. We now report on cardenolides in monarch butterflies collected as larvae or chrysalids in populations of *A. asperula* (Dcne.) Woods. in north central Texas. For those monarchs remigrating through Texas and Louisiana, *Asclepias viridis* and *A. asperula* represent the first common and widespread milkweeds they encounter. In Florida, *A. viridis* is more localized and *A. humistrata* is more heavily utilized for oviposition by monarchs (Brower, 1961, 1962; Cohen and Brower, 1982). Clearly, these three species are the principal milkweeds that migrant monarchs encounter as they reach the temperate United States and serve as larval host-plants for many, if not most of the first spring generation of monarch larvae.

METHODS AND MATERIALS

Geographic and Ecological Distribution of Asclepias asperula. *Asclepias asperula* Dcne., a common milkweed of the subgenus Asclepiodora, has a widespread distribution in the southwestern United States in two distinct subspecies (Woodson, 1954). *A. asperula* subsp. *asperula* (Dcne.) Woods. ranges from eastern California across Arizona and New Mexico into the westernmost counties of Texas, northward throughout Colorado and Utah into southern Idaho, and southward through northern Mexico to the states of Durango and Tamaulipas. *A. asperula* subsp. *capricornu* (Woods.)Woods. occurs in a wide vertical band from south central Texas through central Oklahoma and into north central Kansas. Both subspecies are largely absent throughout most of western Texas, western Oklahoma, and western Kansas, and remain both geographically and morphologically distinct.

Recaptures of autumnal migrating monarchs (Urquhart and Urquhart, 1978) and numerous personal observations in Texas, the Great Plains, and the southwestern United States (Lynch, unpublished field catalog) all suggest *A. a. asperula* occurs to the west of the eastern monarch's migration routes and does not play a significant role as a monarch host-plant. Therefore, no pairwise samples were collected from the typical subspecies for our analysis. In contrast, *A. a. capricornu* often occurs abundantly within the region of monarch remigration and appears to serve as a major host plant for first and second-generation monarchs in temperate North America. Figure 1 illustrates the known distribution of *A. asperula* subsp. *capricornu*. Along the eastern boundary of its distribution, this milkweed is often abundant in open fields and pastures and often occurs in close sympatry with *A. viridis*, a morphologically similar milkweed of the same subgenus. Although they largely remain distinct, individuals with intermediate vegetative and floral morphology may occur, suggesting occasional hybridization between the two species (Lynch, personal observation). Other milkweed species do occur in this region but are only occasional and produce less biomass.

Location, Methods, and Dates of Sample Collections. During May 1983, immature monarchs (*Danaus plexippus* L.) were collected from milkweeds in open pastures and roadsides in northcentral Texas. Collections of individually matched plant–butterfly pairs generally followed the procedures established in Brower et al. (1982, 1984a,b) and Lynch and Martin (1987). Fifth-instar larvae were collected along with a sample of leaves from their individual host-plant. A portion of this material was placed with the larva into an 8-oz. clear plastic food container (Sweetheart Plastics No. 40, Wilmington, Massachusetts), and the remaining leaves were placed in one-pint freezer bags and frozen for later chemical analysis. In addition to searching for and collecting wild fifth-instar

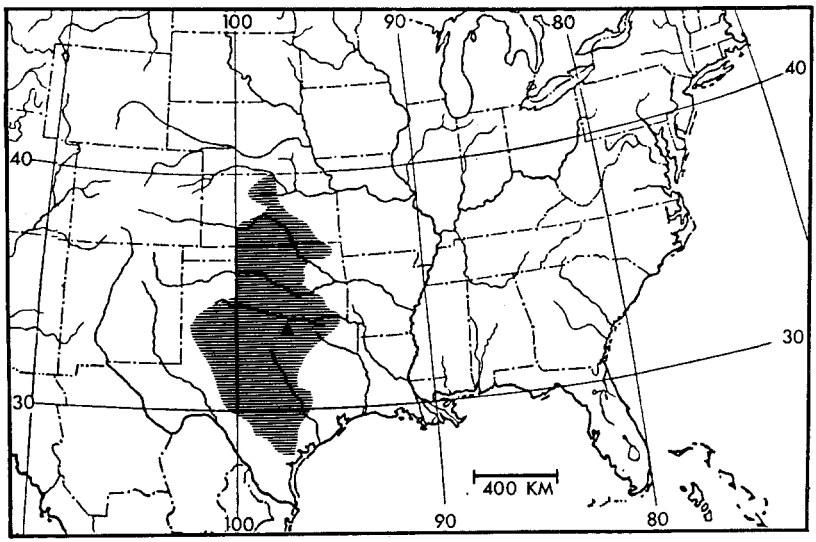

FIG. 1. The known geographic distribution of the milkweed *Asclepias asperula* subsp. *capricornu* (Woods.) in the United States (after Woodson, 1954). The triangle locates the northcentral Texas population analyzed in this study. See text for discussion of relative abundance.

larvae, small larvae were bagged with nylon net bags on individual host-plants and later collected as chrysalids or as late fifth-instar larvae.

The rearing of the butterflies and the treatment of plant material following collection was identical for both bagged and unbagged material. Any chrysalids collected from bags were removed along with their silken pads which were then taped to the lid of the container. Within three days each fifth-instar larva had spun a silken pad on the lid of the container and hung upside down to shed its last larval skin. After pupation, the remaining leaves in the container were discarded, and a Whatman No. 1 filter paper was placed on the bottom to reduce humidity and to soak up fluids evacuated during emergence. The containers were stored at room temperature until emergence. Upon darkening of the chrysalids (usually occurring the evening before emergence), small cylinders of metal window screen were sandwiched between the lid and bottom of the container creating an open air cage in which the emerging adults could hang to expand and dry their wings. Approximately 12 hr after emergence, each butterfly was placed in a glassine envelope and frozen for later measurement and chemical analysis. Each plant–butterfly pair was provided with a collection number (S.P. Lynch, unpublished field catalog). A single-digit alphabetical suffix was used to identify more than one butterfly collected from the same host plant.

In all, 41 pairwise butterflies (22 females and 19 males) were analyzed from 31 individual plants. Wild collections included Nos. 6665, 6666, 6668, and 6669B collected May 7, near Denton; No. 6670A collected May 7, near Decatur; Nos. 6701A and 6701B collected May 8, near Mineral Wells, and No. 6702 collected May 8, near Granbury. Thirty-five *A. asperula* plants were each bagged with two first- or second-instar larvae in an open pasture in Denton on May 7. Eighteen chrysalids and 15 fifth instars were collected from the bags on May 21. These were Nos. 6983B, 6984A, 6984B, 6985–6987, 6988A, 6988B, 6989, 6990A 6990B, 6991, 6992A, 6992B, 6993–6998, 6999A, 6999B, 7000, 7001, 7002A, 7002B, 7003, 7004, 7005A, 7005B, 7006A, 7006B, and 7007B.

Determination of Total Cardenolide Content. The individual plant and butterfly samples were prepared and extracted with 95% ethanol as described by Brower et al. (1972). Spectrophotometric determinations were performed at 626 nm with a Gilford 280 single-beam UV–visible spectrophotometer at 20.0°C following the 2,2′,4,4′-tetranitrodiphenyl (TNDP) method of Brower et al. (1972, 1975), Brower and Moffitt (1974), and Lynch and Martin (1987), except for slightly different cuvette dilutions. Freshly prepared TNDP solutions were used and stored in a refrigerator for no longer than 10 days. In the absence of the molar absorptivities of the TNDP complex of the *A. asperula* cardenolides, estimates were obtained using a digitoxin standard and reported in micrograms (equivalent to digitoxin) per 0.1 g dry weight (Roeske et al., 1976; Brower et al., 1982).

Thin-Layer Chromatography (TLC). Pigments and other interfering substances were removed in a lead acetate precipitation procedure (Nelson et al., 1981; Brower et al., 1982) which utilized 6 ml of the *A. asperula* or butterfly extract. After cleanup, the evaporated residue was dissolved in a calculated amount of chloroform in order to deliver 75 or 100 μg/20 μl of solution.

Twenty microliters of the plant and butterfly samples were spotted with Drummond Wiretrol 5-μl pipet onto a heat-activated 20 × 20-cm TLC plate (0.25 mm, EM Science 5765, Silica Gel 60 F-254 or EM Science 11845, Silica Gel 60 with a preconcentrating zone) providing six plant–butterfly pairs and three digitoxin and digitoxigenin (Sigma Chemical Co., St. Louis, Missouri) standards per plate. Development was in preequilibrated filter paper-lined glass chambers in either chloroform–methanol-formamide (CMF) (90 : 6 : 1 by volume) four times or in ethyl acetate–methanol (EM) (97 : 3 by volume) two times (Brower et al., 1982). The plates were visualized by spraying with 20 ml of 0.4% TNDP in toluene followed by 20 ml of 10% potassium hydroxide in 50% aqueous methanol. The plates were photographed within 1 min. using Kodachrome 25 film and a Pentax Spotmatic 35 mm camera equipped with a Pentax ringstrobe.

Four plates were run in each of the two solvent systems. Each plate contained six plant–butterfly pairs of mixed sexes, two pairs each selected from the

low, middle, and high ranges of plant cardenolide concentration based on spectroassay results. Similar concentration ranges were intentionally placed in different positions on each plate to reduce or eliminate error due to plate position. Pooled plant and butterfly extracts were prepared and spotted on the plates. Three of the four TLC plates developed in the EM solvent system contained a preconcentrating zone of kieselguhr and were not spotted with any pooled butterfly sample. Two additional plates containing pooled *A. asperula* and *A. viridis* extracts, respective pooled butterfly extracts, and the cardenolides, desglucosyrioside, labriformin, and labriformidin were developed, one in CMF and one in EM.

All plates contained the two digitoxin and digitoxigenin standards spotted as a mixture in the outermost positions and the center. The distance of the concentrated centers of each sample spot from the origin was measured to the nearest 0.5 mm and later converted to an $R_{digitoxin}$ (R_d) value by drawing a line from the outer digitoxin spots to the central spot and dividing the spot migration distance by the calculated digitoxin migration distance. Comparisons of TLC patterns were also based on the mean and standard deviations of each spot's intensity value (SI) as well as each spot's probability of occurrence (PO). Spot intensity was determined using a subjective but consistent visual 1–5 scale with 1 being the lightest and 5 the darkest.

Statistical Analyses. Statistical analyses were made on an IBM 3330 computer housed at Louisiana State University in Baton Rouge utilizing SAS statistical software, release 82.2 (SAS, 1982). Statistical procedures generally followed those in Lynch and Martin (1987). We analyzed dry weights, wing and body measurements, cardenolide concentrations, and total cardenolide content of the butterflies, as well as the cardenolide concentrations of the plants. Using PROC UNIVARIATE and FREQ PLOT NORMAL tests to examine frequency distributions and to test for normality, all data except cardenolide concentrations in the butterflies were determined to be within normal ranges.

A two-step regression was carried out on the spectrophotometric data, the first to consider the overall relation of cardenolides in the butterflies (Y, the dependent variable) to their corresponding host-plant cardenolides (X, the independent variable), the calculated r^2, the slope, and the intercept; and the second to analyze the dependence of the butterfly cardenolide concentration, butterfly dry weight, and the total cardenolide per butterfly on the cardenolide concentration of the plants (total and by sex). Unless otherwise indicated in the text, type IV sums of squares statistics were used if empty cells were present in the matrix or there was one or more significant interactions in the model, whereas type II sums of squares were used if there was none.

Two-way analyses of variance (ANOVA) were also carried out on the TLC data to determine the influence of sex and plate number and their interaction on both R_d values and on SI values. Although we realize that our TLC spot com-

parison methodology largely predetermines the correspondence of the respective plant and butterfly spots for each plant–butterfly pair, the SAS linear regression program allows for the simultaneous testing of the dependence of the R_d values or the SI values for all butterfly spots on the corresponding values of their respective plant spots as affected by plate variation or sex in the entire sample.

RESULTS

Measurements of Gross Cardenolide Content. We found *A. asperula* host plants to be rich in cardenolides. Each of the 31 plants used in this study contained high amounts of cardenolides, with concentrations ranging from 341 to 1616 μg/0.1 g dry weight. The mean concentration for all plants measured was 886 μg/0.1 g dry weight with a standard deviation of 254.5 μg (Table 1). The pattern of variation is shown in Figure 2. The curves over the histograms for both plants and butterflies display the limits of the expected normal distributions calculated by the z statistic (Steel and Torrie, 1960). The concentration distribution for the plants, although negatively kurtotic (moment of kurtosis = −0.33), was approximately normal (moment of skewness = 0.07; mean and median, respectively, 886 and 903 μg; $W = 0.973$, $P < 0.54$).

Butterflies reared on *A. asperula* were also found to be cardenolide-rich with a mean of 363 μg cardenolide/0.1 g dry weight. The range of cardenolide concentrations in the butterflies, 231–515 μg/0.1 g dry weight, was narrower than the range in the plant samples (Table 1). The concentration distribution in the butterflies was not normally distributed (moment of skewness = 0.34; mean and median, 362.9 and 346.1 μg, respectively; $W = 0.936$, $P < 0.037$). The $\log_{10}$ transformed data, however, did not depart significantly from normality (moment of skewness = 0.04; mean and median, respectively 2.55 and 2.54; $W = 0.947$; $P < 0.085$). Further statistical analyses involving butterfly concentrations are therefore based on the $\log_{10}$ transformed data.

TABLE 1. CARDENOLIDE CONCENTRATIONS OF MONARCH BUTTERFLIES, *Danaus plexippus* L., AND THEIR HOST-PLANT MILKWEED, *A. asperula* subsp. *capricornu* (Woods.) Woods.[a]

		Plant material			Butterfly material		
	N	Mean	SD	Range	Mean	SD	Range
Males	19	847.16	228.37	341–1274	320.97	54.69	231–431
Females	22	919.68	275.96	341–1616	399.12	75.85	285–515
Total	41	886.07	254.54	341–1616	362.90	76.95	231–515

[a] Data are μg (equivalent to digitoxin) per 0.1 g dry wt. of butterfly or plant material.

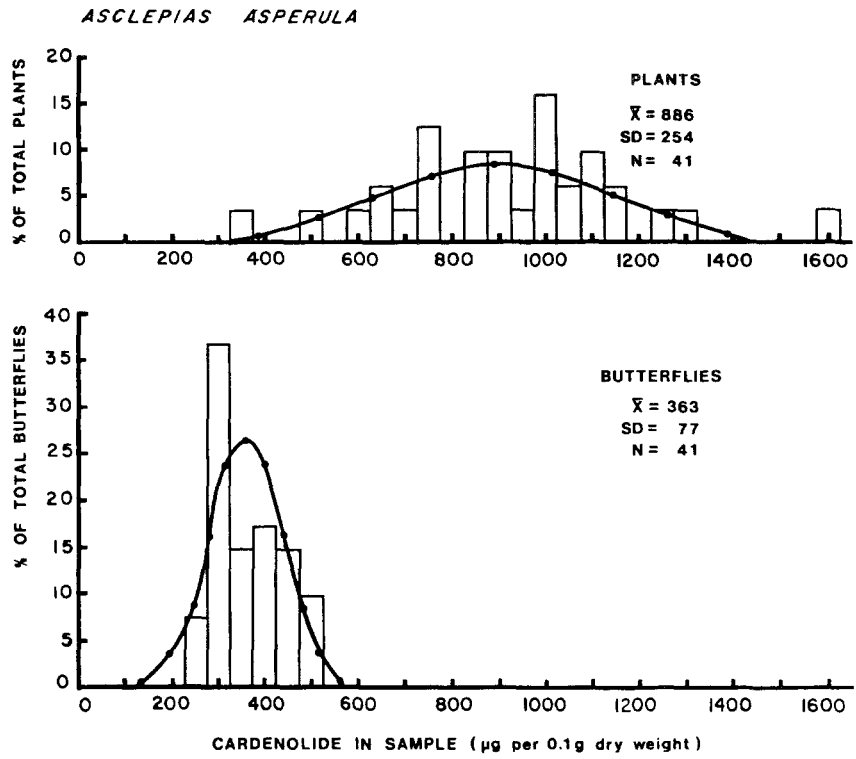

FIG. 2. Frequency distributions of the gross cardenolide content of 41 *Asclepias asperula* plants and 41 monarch butterflies reared thereon. Concentrations are expressed as µg per 0.1 g dry weight, equivalent to digitoxin. The width of each bar is 50 µg. The curves above the histograms are the normal distrubutions calculated by the z statistic (Steel and Torrie, 1960).

Table 1 shows differences in the mean cardenolide concentrations of plants fed on by the male vs. the female butterflies. An analysis of variance indicated that the difference in the plants according to the sex of the butterfly is not significant ($F = 0.82$, $P < 0.37$). However, differences in the means for log cardenolide concentrations in male and female butterflies were significant ($F = 13.61$, $P < 0.0007$; $r^2 = 0.26$). The grand variances of the cardenolide concentrations of the plants upon which male and female butterflies were reared were not significantly different (for the s^2 male/s^2 female, $F = 1.46$ with 18 and 21 *df*, $P < 0.42$) nor were the grand variances of the log cardenolide concentrations between the male and female butterflies ($F = 1.36$ with 18 and 21 *df*, $P < 0.51$).

The mean cardenolide concentration in the plants was 523 μg (2.4 times) greater than that of their respective butterflies, and the overall range of variation in the plants (SD = 254.5) was also greater than that in the butterflies (SD = 77.0). Therefore, the regression of butterfly concentrations on their respective plant concentrations is not a significant fit to either a linear or log plot (Figure 3, Table 2A). For graphic simplicity, we have plotted the "best fit" linear model $Y = 0.003X + 360$ in Figure 3. The calculated regression model, $\log_{10} Y = 0.00001X + 2.539$ (Table 2A) was not a significantly better fit. In the latter regression, which utilizes the log transformed butterfly concentrations, $r = 0.036$ ($P < 0.82$), the intercept was significantly different from 0 ($P < 0.0001$), and the slope was not ($P < 0.82$).

In a second set of regressions comparing plant and butterfly cardenolide concentrations by the sex of the butterfly (Table 2B), we found that the overall model was highly significant ($P < 0.0012$) and that plant concentration, sex, and their interaction were all significant using type IV SS statistics. By running separate regressions on each sex, we found that there was no significant correlation between butterfly concentration and plant concentration (females $P < 0.166$; males $P < 0.099$).

Table 3 lists the dry weights and total cardenolide contents of the butterflies. The dry weights were normally distributed (mean and median = 0.201 and 0.205 g, respectively; $W = 0.967$, $P < 0.401$) with the average male butterfly being slightly heavier (0.211 g) than the female (0.191 g). The total cardenolide contents were also normally distributed (mean and median = 717 and 692 μg, respectively; $W = 0.974$, $P < 0.573$). The variances for the two sexes did not differ significantly for either dry weight ($F = 1.67$; $df = 18, 21$; $P > 0.26$) or total cardenolide ($F = 1.07$; $df = 18, 21$; $P > 0.88$). Analyses of the variances indicated that the mean dry weights of the two sexes were significantly different ($F = 4.46$, $P < 0.0412$) but that the mean total cardenolide content in the female butterflies (754 μg) was not significantly greater ($F = 3.63$, $P < 0.0642$) than in the males (675 μg).

A regression analysis was run to determine the relationship of the butterfly dry weights (Y) to the cardenolide concentrations of the plants (X). The overall model indicated no significant correlation ($r^2 = 0.005$; $F = 0.185$, $P < 0.67$) as did models with either sex. The dry weights of the butterflies were similarly compared to the log cardenolide concentrations of the butterflies. The main regression model indicated a significant negative correlation between butterfly dry weight and cardenolide concentration ($\log_{10} Y = -1547.1X + 5952.4$; $r^2 = 0.203$; $P < 0.0031$). However, the second regression model, which also included sex and the plant concentration as covariates, indicated no overall significant correlation ($R^2 = 0.273$; $F = 1.77$, $P < 0.1268$) and that, when using type II SS statistics, none of the variables or their interactions were significant predictors of butterfly dry weight (all P values > 0.22).

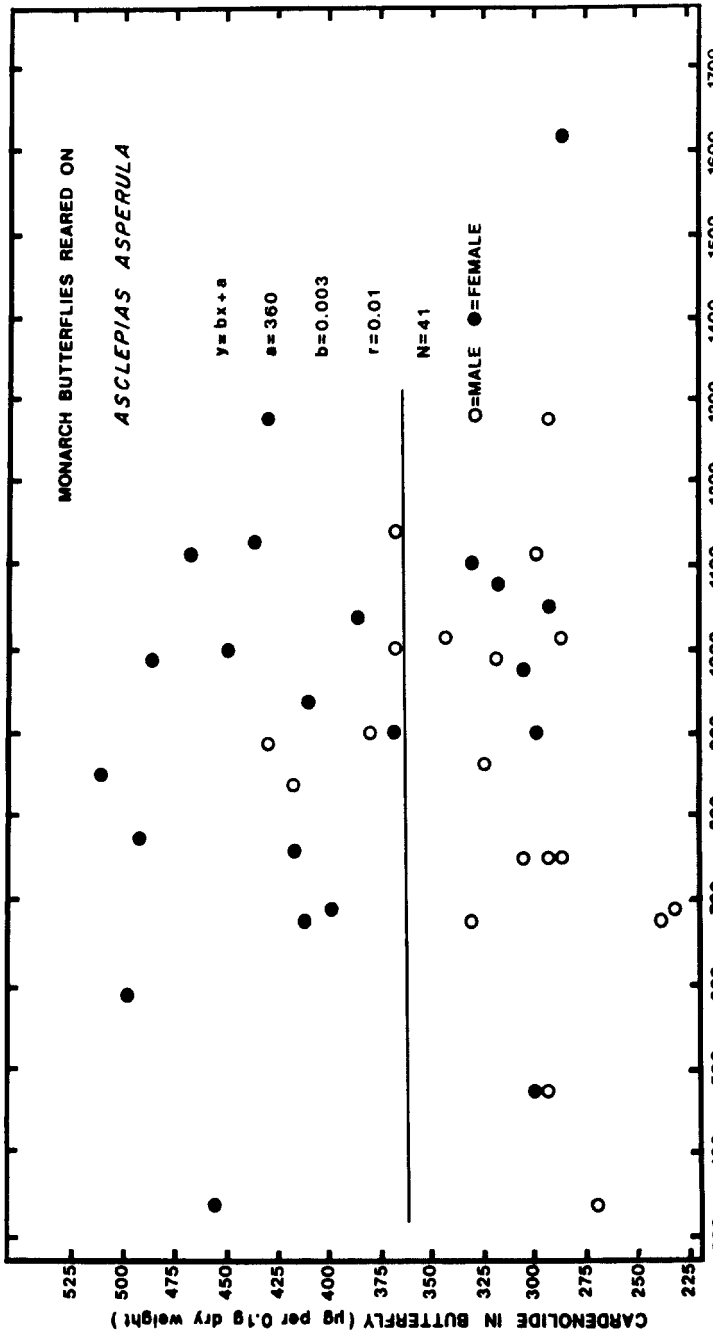

FIG. 3. Cardenolide concentrations of monarch butterflies (*Y* axis) as a function of the cardenolide concentrations of their larval food plant, *Asclepias asperula* (*X* axis). Each of the 41 data points represents one plant–butterfly pair. Open circles are males and solid circles are females. The line is derived from the regression equation $Y = bX + a$.

TABLE 2. LINEAR REGRESSION ANALYSES OF CARDENOLIDE CONCENTRATIONS (LOG_{10} μg/0.1 g DRY WT) IN BUTTERFLIES (Y = DEPENDENT VARIABLE) VS. CARDENOLIDE CONCENTRATIONS (μg/0.1 g DRY WT) IN THEIR RESPECTIVE PLANTS, ACCORDING TO FUNCTION LOG_{10} $Y = bX + a$.

Source of variation	*df*	SS	MS	*F*	*P*
A. Overall regression ($r^2 = 0.0013$)					
Plant concentration	1	0.00045	0.00045	0.052	<0.8203
Error	39	0.33797	0.00867		
Corrected total	40	0.33843			
			SE	*T* for H = 0	*P*
Estimated value of parameters					
a = y intercept = 2.538583			0.053261	47.663	<0.0001
b = slope = 0.000013			0.000058	0.229	<0.8203
Equation for the line: $\log_{10} Y = 0.000013X) + 2.538583$					
B. Butterfly concentrations on plant concentrations by sex					
Model ($R^2 = 0.0012$)	3	0.1166	0.0389	6.49	<0.0012
Error	37	0.2218	0.0060		
Corrected total	40	0.3384			
Plant conc	1	0.2780		4.64	<0.0379
Sex	1	0.0587		9.80	<0.0034
Plant × sex	1	0.0286		4.78	<0.0353
Females ($r^2 = 0.093$)	1	0.0144	0.0144	2.06	<0.1666
Error	20	0.1397	0.0070		
Corrected total	21	0.1541			
Males ($r^2 = 0.152$)	1	0.0147	0.0147	3.04	<0.0991
Error	17	0.0821	0.0483		
Corrected total	18	0.0968			

TABLE 3. SUMMARY OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT IN BUTTERFLIES REARED ON *A. asperula* subsp. *capricornu* (Woods.) Woods.

		Dry weight (g)			Total cardenolide (μg)		
	N	Mean	SD	Range	Mean	SD	Range
Males	19	0.211	0.034	0.134–0.262	675	134	324–859
Females	22	0.191	0.026	0.139–0.242	754	130	551–988
Both	41	0.201	0.032	0.134–0.262	717	136	324–988

The same linear regression model used to compare butterfly cardenolide concentrations and dry weights was also run to relate total cardenolide in the butterflies to the plant concentrations. We found no significant dependence of butterfly total cardenolide on the plant concentration ($r^2 = 0.006$; $F = 0.241$, $P < 0.626$) and that both sexes similarly showed no dependence. Based on the equation $Y = 0.0419X + 680.18$, the overall model predicts that butterflies which fed on plants containing 341 μg/0.1 g cardenolide (the lowest plant concentration in our study) would contain approximately 694.5 μg, those which fed on 886-μg plants (the mean plant concentration) would contain 717.3 μg, and those which fed on 1616-μg plants (the highest plant concentration in our study) would contain 747.9 μg. The second regression for total cardenolide indicated that neither the log plant concentration nor the sex of the butterflies nor their interaction were significant predictors when using type II SS statistics.

TLC Cardenolide Profiles of Plants and Butterflies. Although TLC plates were run in two separate solvent systems, only those plates run in the chloroform–methanol–formamide (CMF) system were analyzed quantitatively. As found by Brower et al. (1982, 1984a,b) with three California milkweeds and by Lynch and Martin (1987) with *A. viridis* in Louisiana, the ethyl acetate–methanol (EM) system resolved fewer cardenolides in *A. asperula* than did the CMF system and the individual spots were larger and less well defined. While the EM system may eventually provide information on the identity of individual cardenolide compounds by confirmation with known standards and may prove useful in their isolation and purification, the CMF system has proven more valuable in the quantitative analyses of individual cardenolides and in the establishment of TLC "fingerprint" profiles. For these reasons the remaining discussion will be concerned entirely with the CMF profiles.

Figure 4 is a photograph of portions of two plates run in the CMF system. Represented are six butterfly–plant pairs including four female butterflies (Nos. 7005, 6994, 7001, and 6666) and two male butterflies (Nos. 6983B and 6669B). The number sequence of the individual spots is in the channel between the first plant–butterfly pair shown on plate 15 and between the first plant–butterfly pair shown on plate 14. Additional spot numbers have been added where spots are particularly clear, and the standards are identified along the right and left margins. Headings for each pair include the research number (top), identity as either plant (P) or butterfly (B), and the approximate amount of cardenolide spotted, in micrograms (lowest figures).

Table 4 summarizes the means and standard deviations for both $R_{digitoxin}$ (R_d) and spot intensity (SI) values for all the spots in the 24 plant and butterfly samples analyzed by TLC. Also included in Table 4 are the probability of occurrence of each spot (PO) based on the proportion of plants or butterflies in which each spot occurred and the subsample size or number of times each spot was detected. The mean migration distance for the 12 digitoxin references (three

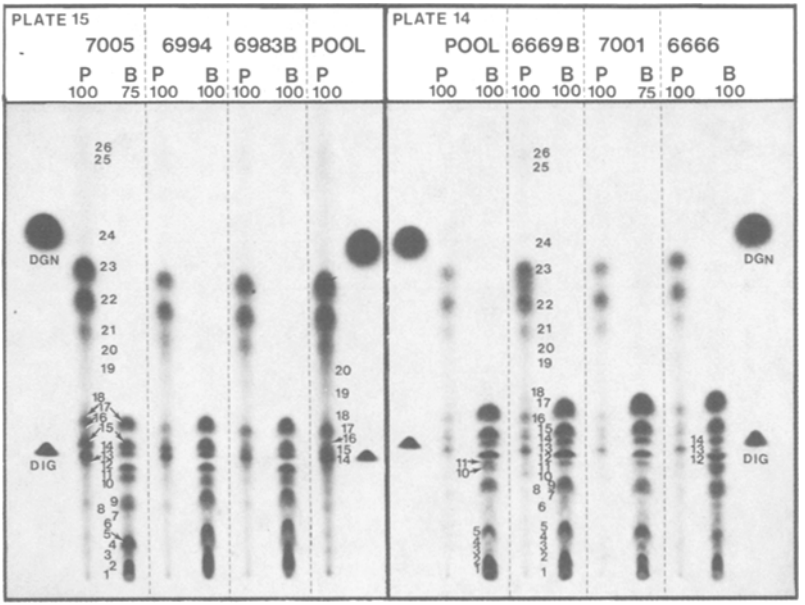

FIG. 4. Photographic reproduction of the thin-layer chromatographic profiles of the cardenolides present in monarch butterflies and their host plant, *Asclepias asperula*. Represented are six plant–butterfly pairs run in the chloroform–methanol–formamide system including two males (No. 6983B and 6669B) and four females (Nos. 7005, 6994, 7001, and 6666). Twenty-six individual spots were resolved including 22 in the plants (all except spots 2, 4, 7, and 9) and 15 in the butterflies (all except spots 8, 16, and 18–26). Also included are two standards, digitoxin (DIG) and digitoxigenin (DGN), which were run on each plate. Several spots with low color intensity are visualized poorly through photographic reproduction (but see the generalized drawing in Figure 5).

on each plate) was 32.1 mm with a range of 29.0–38.0 mm and a SD of 5.7 mm. The 12 corresponding digitoxigenin references had a mean migration distance of 87.8 mm with a range of 82.0–90.5 mm and a SD of 3.6. The mean R_d value for digitoxigenin in the 12 reference channels was 2.75 with a range of 2.5–2.9 and a SD of 0.15.

Relatively weak spots were occasionally absent or difficult to discern in individual plants or butterflies, yet the standard deviations of the R_d values are low, indicating the resolution of the cardenolides is good. Figure 5 represents the mean cardenolide profile for the 24 plants and butterflies utilized in the TLC analysis. The relative positions and darkness of the spots are calculated from the mean R_d and SI values presented in Table 4. The shapes and sizes of the spots are drawn from a representative TLC plate and then generalized in the

TABLE 4. SUMMARY OF MEANS AND STANDARD DEVIATIONS FOR $R_{digitoxin}$ (R_d) VALUES AND SPOT INTENSITIES (SIs) FOR 26 CARDENOLIDES AND PROBABILITY OF THEIR OCCURRENCE IN 24 PAIRED PLANTS AND BUTTERFLIES[a]

Spot No.	Means				Standard deviations				Probability of spot		Subsample sizes	
	R_d		SI		R_d		SI					
	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly
26	3.46		1.00		0.34		0.00		0.29		7	
25	3.29		1.00		0.24		0.00		0.29		7	
24	2.65		1.00		0.12		0.00		0.38		9	
23	2.39		3.75		0.07		0.85		1.00		24	
22	2.13		3.83		0.05		0.82		1.00		24	
21	1.91		2.58		0.04		0.78		1.00		24	
20	1.73		1.32		0.08		0.48		0.79		19	
19	1.60		1.15		0.04		0.38		0.54		13	
18	1.32		1.17		0.04		0.39		0.50		12	
17	1.21	1.26	2.50	4.96	0.03	0.03	0.51	0.20	1.00	1.00	24	24
16	1.10		1.68		0.03		0.48		0.92		22	
15	1.04	1.08	2.63	4.63	0.02	0.03	0.58	0.50	1.00	1.00	24	24
14	0.99	0.99	2.50	3.83	0.02	0.01	1.00	0.48	0.17	1.00	4	24
13	0.94	0.95	3.04	1.31	0.05	0.02	0.55	0.48	1.00	0.67	24	16
12	0.88	0.88	1.50	4.00	0.02	0.03	0.51	0.51	0.83	1.00	20	24
11	0.79	0.81	1.00	2.71	0.04	0.03	0.00	0.46	0.67	1.00	16	24
10	0.72	0.76	1.15	2.59	0.05	0.04	0.38	0.73	0.54	0.92	13	22
9		0.63		3.92		0.05		0.28		1.00		24
8	0.58		1.42		0.05		0.50		1.00		24	
7		0.53		1.81		0.06		0.54		0.67		16
6	0.41	0.43	1.00	1.47	0.00	0.05	0.00	0.77	0.04	0.79	1	19
5	0.34	0.34	1.00	3.29	0.04	0.05	0.00	0.62	0.92	1.00	22	24
4		0.30		2.28		0.05		0.75		0.75		18
3	0.19	0.22	1.00	2.13	0.04	0.03	0.00	0.62	0.92	0.67	22	16
2		0.13		3.46		0.03		0.51		1.00		24
1	0.06	0.07	1.00	2.92	0.01	0.02	0.00	0.65	0.25	1.00	6	24

[a]Data are based on chromatograms of 24 butterflies and the corresponding plants on which they were reared (chloroform–methanol–formamide system).

figure. The mean R_d values for the 12 digitoxin and digitoxigenin standards are also indicated as dotted lines across the profile. In all, twenty-six spots were resolved in the CMF system. Twenty-two spots were discerned in the plants and 15 in the butterflies. Spots 2, 4, 7, and 9 were not present in any of the plants, while spots 8, 16, and 18–26 were absent in the butterflies.

In the plants there were six spots (Nos. 1, 6, 14, and 24–26) which were

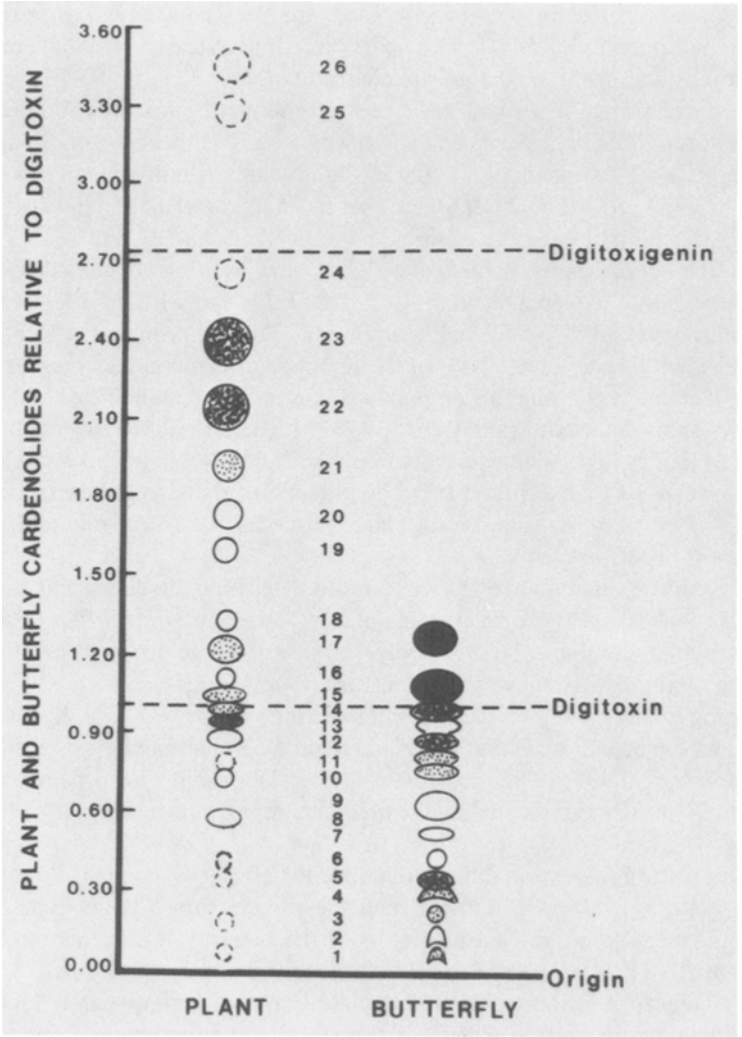

FIG. 5. The mean cardenolide profiles for 24 monarch butterflies and their individual *Asclepias asperula* host plants based on the chloroform–methanol–formamide TLC system. Shown are the mean R_d and spot intensity values for 22 plant and 15 butterfly cardenolide spots. Spot intensities are represented as follows: $1.00 < 1.25$, dotted circle; $1.25 < 2.00$ closed circle; $2.00 < 3.00$, light stipple; $3.00 < 4.00$, dark stipple; 4.00–5.00 black circle. The mean R_d value for digitoxigenin was 2.75.

detected in less than 50% of the samples ($PO < 0.50$), although we found these spots consistently present in pooled samples spotted with an estimated 100 μg of cardenolide. This left 16 spots present in at least 50% of the individual profiles and useful as diagnostic characters (Nos. 3, 5, 8, 10–13, and 15–23). Twelve spots (55%) had an $R_d > 1.0$ (above digitoxin) while 10 spots (45%) had an $R_d < 1.0$. Spots 25 and 26 were consistently above digitoxigenin, although spot 22 occasionally migrated slightly above digitoxigenin's position. Spots 8, 13, 15, 17, and 21–23 were present in all 24 plant channels and generally had the highest spot intensities.

In the butterflies, no spots had a $PO < 0.50$, leaving all spots detected as diagnostic. Only two spots had an $R_d > 1.0$ (13%, spots 15 and 17), while the remaining spots (87%) were below digitoxin. The butterflies only stored cardenolides within the lowest 36% of the R_d range of cardenolides present in the plants. The mean R_d value for all plant spots was 1.29 compared to 0.64 for all butterfly spots detected. Spots 1, 2, 5, 9, 11, 12, 14, 15, and 17 were observed in all 24 butterflies. Where in common, the butterfly spots had significantly greater mean SIs than did their corresponding plant spots with the exception of spot 13. The mean SI value for all plant spots was 1.96 compared to 3.15 for all butterfly spots.

Several regression analyses were run to determine the dependence of butterfly R_ds and SIs on the corresponding plant R_ds. Because of low POs of several of the spots in common R_f range, only 160 of a possible 408 spot pairs (24 × 17 plant–butterfly pairs) were present and used in this analysis. Our initial regression model examined the dependency of the butterfly R_ds on the plant R_ds alone. As expected, we found a high degree of correlation ($F = 16639$, $P < 0.0001$; $r^2 = 0.991$). This near one-to-one relationship of the paired plant–butterfly R_d values is indicated by the predicted regression equation $Y = 1.018X + 0.005$.

Our second regression model contained the effects of the plant R_ds, the sex of the butterfly, the plate number, and their interactions. Using type IV SS statistics, the only significant predictors of the butterfly R_ds in this model are the plant R_ds ($F = 15{,}695$, $P < 0.0001$) and plate number ($F = 3.73$, $P < 0.013$). Duncan's option indicated that plate 1 contained significantly higher R_d values than the other three plates while plates 2 and 3 and plates 3 and 4 were not significantly different. The experimental variable created by differential migration rates on the plates for separate TLC runs is difficult to control, but fortunately, although statistically significant, its magnitude is not great. We strongly suggest that both sexes be run on each plate as we have done so that possible differences attributable to sex and plates can be separated.

Similar regression analyses were run for the spot intensities. Again, our initial regression model examined the dependency of the butterfly SIs on the plant SIs alone. This regression indicated a weak but significant correlation (F

$= 11.27$, $P < 0.0010$; $r^2 = 0.0667$) and produced the equation for the line $Y = 0.373X + 2.725$. To remain consistent with our treatment of the R_d values, our second regression contained the effects of the plant SIs, sex, plate, and their interactions. The overall model was not significant ($F = 1.37$, $P < 0.1674$). Again with type IV SS statistics, only the plant SIs contributed significantly to the model ($F = 9.47$, $P < 0.0025$), and the R^2 (0.125) was only slightly greater than the r^2 (0.0667) of the plant SIs alone. Duncan's option indicated that none of the plates or their combinations were significantly different in spot intensity.

DISCUSSION

General Field Observations During Sample Collections. Each spring millions of monarch butterflies return to temperate North America through Texas and Louisiana from their overwintering sites in Michoacan, Mexico. Whether monarchs reaching the Atlantic Coastal Plain pass up the Florida peninsula or remigrate into Texas and Louisiana and then travel across the Gulf Coast has not yet been elucidated (Brower, personal communication). The overwintering colonies begin to break up in early to mid-March and by late March the first remigrants can be seen across the southern United States (Lynch and Martin, Brower and Malcolm, personal observations). In Texas and Louisiana, *A. asperula* subsp. *capricornu* and *A. viridis* represent the first abundant and widespread milkweeds they encounter.

We have found numerous early instars on *A. asperula* subsp. *capricornu* in central and eastern Texas by early April. First-, second-, and early third-instar larvae usually feed on floral parts, remaining hidden in the more compact preanthesis inflorescences. Later instar larvae usually feed on the leaves. While only a small percentage of larvae reach the fourth- and fifth-instar stages (Lynch and Martin, personal observations), the abundance of *A. asperula* in central to eastern Texas suggests a great number of larvae survive to pupate and emerge as adults. By early May the remigrants, faded and battered by the journey, largely disappear, and freshly emerged first generation adults can be seen in increasing numbers. In Texas, adult monarchs are seldom seen after late May, and immature stages are generally absent even though many plants remain green and healthy through most of June. We have observed freshly emerged monarchs in central Oklahoma in late May searching and mating in fields of *A. viridis* and *A. asperula* and in central Kansas during June laying eggs on *A. speciosa* and *A. syriaca*.

For the majority of our pairwise samples, we circumvented the problem of low survivorship by removing crab spiders from the plants and bagging two first- or second-instar larvae on randomly selected plants. This virtually eliminated spider predation and tachinid parasitism, a common problem in rearing

wild-found larvae. Bagging provided the further advantage of knowing without question that larvae ate only on the individual plants from which they were collected. In contrast to western U.S. monarchs, which nearly always form their chrysalids upon their host plant (Brower et al., 1982, 1984a, b), no chrysalids were found on unbagged plants. Even bagged monarchs tended to spin their pads on the nylon net bags rather than on the leaves of the plant.

Quantitative Variation of Plant and Butterfly Cardenolides. A. asperula subsp. *capricornu* plants contain a higher mean cardenolide concentration than other *Asclepias* species examined to date, with the possible exception of three closely related, leafless desert species of the series roseae (*A. albicans*, *A. subaphylla*, and *A. masonii*) that have not been reported as host-plants for monarchs (Brower et al., 1982, 1984a, b; Roeske et al., 1976; Seiber et al., 1983, 1984; Lynch and Martin, 1987; Lynch, personal observations). While wide variation occurred in the cardenolide concentrations of the *A. asperula* plants, a much narrower range of variation was found in the respective butterflies. The mean cardonolide concentration in the butterflies was also much lower than in the plants. In *A. eriocarpa*, another cardenolide-rich milkweed, Brower et al. (1982) also found the butterflies had a lower mean concentration and a narrower concentration range than their host plants. The opposite was observed in *A. viridis*, where both the mean and range of cardenolide concentration in the butterflies was greater than in the plants (Lynch and Martin, 1987). Similar patterns were found in *A. californica* and *A. speciosa* butterfly–plant pairs (Brower et al., 1984a, b). Both species have a lower mean cardenolide concentration than *A. viridis*.

These data indicate there is a limit to the amount of cardenolide the butterflies can absorb and that saturation occurs. Seiber et al. (1980) suggested that monarchs sequester cardenolides more efficiently when present in low concentrations, with uptake generally conforming to an inverse logarithmic relationship with increasing concentrations. Butterflies reared on host-plants rich in cardenolides would sequester these compounds at or near maximum (or optimum) concentrations and butterflies reared on low cardenolide host-plants would significantly reflect host-plant concentrations up to maximum (or optimum) levels. Therefore, the regression equation $Y = a(\log_{10} X) + b$ should fit the overall relationship between monarch and host-plant concentrations. Work with *A. californica* (Brower et al., 1984a), *A. speciosa* (Brower et al., 1984b), and *A. viridis* (Lynch and Martin, 1987) confirm this relationship.

We were unable statistically to confirm any relationship between cardenolide concentrations in the butterflies and their respective host-plants in this study. All *A. asperula* plants examined contained high levels of cardenolide, and our samples represented only the saturation portion of the curve. The calculated slopes were so small that concentrations and total cardenolide in the butterflies approximated the intercept. Although we chose to log transform the

butterfly concentration data, so that we could assume normality in our statistical analyses (e.g., Table 2), and plotted the linear regression of butterfly concentrations on their respective plant concentrations in Figure 3, we recognize that neither may reflect the overall cardenolide concentration relationships between monarchs and their various host-plants.

Duffey et al. (1978) and Scudder et al. (1986) suggest that cardenolides in *Oncopeltus*, a common milkweed bug, are sequestered in a specialized inner epithelium (the dorsolateral space) through a nonsaturable, passive diffusion process in proportion to the cardenolide content in the food source and the hemolymph. While the mechanisms for cardenolide uptake in monarchs are unknown, patterns observed in all monarch–milkweed pairwise analyses to date could fit a physical uptake model. Larvae feeding on *A. asperula* and other cardenolide-rich host plants would ingest a total amount of cardenolides approaching or exceeding their physical limits of absorption and/or storage. A relatively narrow range of cardenolide concentrations would therefore be expected in the adults, largely restricted to the physical variation among individual butterflies and not significantly correlated to host-plant concentrations. On milkweeds containing low to moderate amounts of cardenolides, the total cardenolides ingested by individual larvae would be less than that imposed by their physical limitations. Variations in host plant concentrations would compound the physical variation among the butterflies so that a wide range of variation would be expected. Diffusion rates into available larval tissues would decelerate as tissue concentration increased, producing the logarithmic relationship seen in *A. speciosa* (Brower, et al., 1984b), *A. californica* (Brower et al., 1984a), and *A. viridis* (Lynch and Martin, 1987). That saturation does not occur in *Oncopeltus* may simply reflect the presence and function of its specialized dorsolateral space.

The higher mean concentration of cardenolide we observed in female butterflies compared to male butterflies reared on *A. asperula* is consistent with data on butterflies reared on several other *Asclepias* species (Brower and Glazier, 1975; Brower et al., 1975, 1982, 1984b; Lynch and Martin, 1987) and in wild summer-breeding populations in the eastern United States (Brower et al. 1972, Brower and Moffitt, 1974). These data may simply reflect differences in the reproductive morphology between males and females. Brower (1984) cites work with Thomashow in which eggs produced by butterflies reared on *A. curassavica* were found to contain higher levels of cardenolide (0.6% by dry weight) than adults (0.4%). A female may lay as 100 (Erickson, 1973) to 400 eggs (Urquhart, 1960), and as much as 97–388 μg of cardenolide could be present in the female reproductive tract. Dixon et al. (1978) found that monarch females that had laid all their eggs were less emetic to pigeons than freshly eclosed females.

Brower et al. (1972) generated considerable controversy when they first

suggested that monarchs incur a physiologic cost in sequestering cardenolides. More recent work by Brower et al. (1982, 1984a, b) and Lynch and Martin (1987) do not directly support this hypothesis. Vaughan and Jungreis (1977) indicated the presence of cardenolide-resistant Na, K-ATPases in the monarch which may reduce or eliminate physiologic costs of cardenolide sequestration in monarchs. Moore and Scudder (1985) report similar ouabain-resistant Na, K-ATPases in *Oncopeltus*, which similarly does not appear to incur physiologic cost from the sequestration of cardenolides.

If size reduction is used as a potential indicator of metabolic cost in monarchs, higher levels of sequestration in females in comparison to the significantly larger males confuse the issue. Female monarchs also tend to feed on host-plants with higher cardenolide concentrations (Brower et al., 1982, 1984a,b; Lynch and Martin, 1987). Although not statistically significant, *A. asperula*-reared female butterflies fed on host-plants with a mean cardenolide concentration of 920 μg/0.1 g while males fed on host plants with a mean of 847 μg/0.1 g. We ran several regressions on *A. asperula* butterflies and their host plants to examine the relationship between size and cardenolide content. There was a significant negative correlation between all 41 butterfly dry weights and cardenolide concentration ($F = 9.925$, $P < 0.0031$). Female dry weights alone were also negatively correlated with their cardenolide concentrations ($F = 8.26$, $P < 0.0094$), while males were not ($F = 0.505$, $P < 0.487$). In a multiple regression model including butterfly concentration, host-plant concentration, and the sex of the butterflies, none of the variables or their interactions were significantly correlated to butterfly dry weight using type II SS statistics.

Qualitative Analyses of Plant and Butterfly Cardenolides. Comparisons of the standards, desglucosyrioside, labriformin, and labriformidin, with pools of plants and butterflies spotted on the same plate and developed in the EM system clearly indicated the absence of these three compounds in the butterflies and only their possible occurrence in the plants. The match of the particularly high R_f values of spots 26 and 25 in the CMF system suggests they may be the epoxy cardenolides labriformin and labriformidin, respectively. Both compounds are characterized by having unusually high oxygen–carbon ratios and are highly nonpolar. Plant spot 16 in the CMF system could be desglucosyrioside, a 3′-OH derivative of labriformin and labriformidin. However, it has been established (Seiber et al., 1980; Nelson et al., 1981; Brower et al., 1982, 1984b) that monarchs metabolize labriformidin to labriformin and labriformidin to desglucosyrioside when obtained from their hosts-plants. Thus the absence of these compounds in monarchs obtained from *A. asperula* plants strongly suggests the absence of desglucosyrioside, labriformin, and labriformidin in these plants. Carolyn Nelson (personal communication) has suggested that acetate derivatives of various cardenolides also have high R_f values and could be mistaken for labriformin and/or labriformidin. As no additional standards were present

on our plates, we chose not to speculate further on the identity of the cardenolides in *A. asperula.* No specific cardenolides have been isolated and identified from the *Asclepias* subgenus Asclepiodora, and both *A. viridis* and *A. asperula* may well contain previously unidentified cardenolides.

Although unique, the general TLC patterns of *A. asperula* plant and butterfly cardenolides are similar to those found in *A. viridis* and also conform to those observed by Brower et al. (1982, 1984a, b) in *A. eriocarpa*, *A. speciosa*, and *A. californica.* Larval metabolites are more polar than their parent cardenolides, providing a pattern of cardenolides with lower R_f values in the CMF system. Relatively few milkweed cardenolide structures have been established, and most spots found in established TLC fingerprint profiles remain unknown. In addition, different compounds may have similar polarities and fail to separate in a particular system (e.g., calactin and calotropin in the CMF system and desglucosyrioside and labriformin in the EM system). Nevertheless, the general storage pattern appears consistent for those milkweed species examined so far and similarities in certain cardenolides or even entire series do not preclude the possibility of distinguishing among cardenolide patterns for butterflies reared on different milkweeds.

Cardenolide Profiles and Monarch Migration. Although we have observed large instar larvae in April on *A. amplexicaulis*, *A. longifolia*, and *A. viridiflora*, these milkweed species are less common and produce considerably less biomass than either *A. asperula* subsp. *capricornu* or *A. viridis*. Brower (1961, 1962) and Cohen and Brower (1982) report that the common sandhill milkweed, *A. humistrata* Walt., is heavily utilized by monarch remigrants in western and central Florida. If remigrating monarchs do in fact oviposit predominantly on these three southern milkweeds as they reenter the temperate United States, the majority of late spring and early summer breeding monarchs seen in the central and northeastern United States and Canada would possess their characteristic TLC profiles. All three milkweeds are relatively rich in cardenolides and produce adult monarchs that contain high levels of these compounds. These toxic first generation monarchs could be instrumental in educating naive predators as they continue northward, establishing visual rejection by would-be predators of later generation monarchs regardless of taste or toxicity due to cardenolide content (Lynch and Martin, 1987; Brower and Malcolm, in preparation).

Seiber et al. (1986) have shown that 85% of all monarchs sampled from the overwintering sites in Mexico contain similar patterns of epoxy cardenolide glycosides, which suggests that *A. syriaca* and *A. speciosa* are the predominant late summer food plants of monarchs in eastern North America. Both species produce monarchs relatively poor in cardenolides (Brower et al., 1984b; Seiber et al., 1986), and the predominant glycosides in each are the polar syrioside and aspecioside which appear to have a significantly lower emetic potency than cardenolides of intermediate polarity often sequestered by monarchs in Califor-

nia (Brower et al., 1982, 1984b). Therefore, the continued presence of highly toxic, first spring generation adults during the summer breeding cycle in eastern North America would be particularly critical in reinforcing visual avoidance of monarchs by potential predators.

More extensive avian and rodent predation occurs at the Mexican overwintering site than occurs in overwintering sites along the California coast (Calvert et al., 1979; Fink and Brower, 1981; Fink et al., 1983; Brower et al., 1984b; Brower and Calvert, 1985). Brower and Moffitt (1974) showed that 49% of the overwintering adults in California contained sufficient cardenolides to individually produce emesis in blue jays. The cardenolide-rich *A. eriocarpa* and *A. erosa* both are utilized heavily by late summer breeding monarchs in California (Brower et al., 1982; Lynch, personal observations). Brower and Fink (1981) calculated that only 10% of the butterflies sampled at the Michoacan overwintering site were individually emetic. In contrast, Brower and Moffitt (1974) showed that nearly 55% of their September sample population of Massachusetts monarch adults were emetic. Brower et al. (1972) earlier found that approximately 40% of fall migrating monarchs in Ontario and Massachusetts contained emetic concentrations of cardenolide but that migrating monarchs in Maryland and Florida contained significantly less cardenolide.

Brower (personal communication) suggests these geographic differences in emetic potencies of late summer monarchs in eastern North America may reflect cardenolide loss in metabolism and excretion as the butterflies migrate southward. Alternately, we suggest that large numbers of adults reared as larvae on *A. viridis*, *A. asperula*, and *A. humistrata* are still present in the mid and late summer northern populations but that progressively fewer of these early generation butterflies survive as monarchs emigrate southward in the fall. This hypothesis can be verified by matching cardenolide fingerprint profiles of wild-caught northern butterflies against established cardenolide fingerprints of butterflies reared on the predominant southern milkweeds.

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RESPONSE OF GENERALIST AND SPECIALIST INSECTS TO QUALITATIVE ALLELOCHEMICAL VARIATION

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Abstract—We examined the effects of a set of four biosynthetically related iridoid glycosides, aucubin, catalpol, loganin, and asperuloside, on larvae of a generalist, *Lymantria dispar* (Lymantriidae), the gypsy moth, and an adapted specialist, the buckeye, *Junonia coenia* (Nymphalidae). In general, *L. dispar* grew and survived significantly less well on artificial diets containing iridoid glycoside, compared to a control diet without iridoid glycosides. In choice tests, previous exposure to a diet containing iridoid glycosides caused larvae subsequently to prefer iridoid glycoside-containing diets even though they were detrimental to growth and survival. In contrast, *J. coenia* larvae grew and survived better on diets with aucubin and catalpol, the two iridoid glycosides found in the host plant *Plantago lanceolata* (Plantaginaceae), than on diets with no iridoid glycoside or with loganin and asperuloside. The results of choice tests of diets with and without iridoid glycosides and between diets with different iridoid glycosides reflected these differences as well. These results are discussed in terms of (1) differences between generalists and specialists in their response to qualitative variation in plant allelochemical content, (2) the induction of feeding preferences, and (3) the evolution of qualitative allelochemical variation as a plant defense.

Key Words—Iridoid glycoside, *Junonia coenia*, *Lymantria dispar*, Lepidoptera, Nymphalidae, Lymantriidae, induction, insect–plant interaction, generalist herbivore, specialist herbivore.

INTRODUCTION

Plant families are often characterized by the presence of a particular group of allelochemicals. For example, the Cruciferae contain glucosinolates (Hegnauer,

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1973; Rodman and Chew, 1980), the Asclepiadaceae contain cardenolides (Hegnauer, 1973; Gibbs, 1974), and the Scrophulariaceae contain iridoid glycosides (Jirawongse, 1964; Kooiman, 1970; Jensen et al., 1975). Plant species within such families usually contain some, but not all, of the taxon-specific array of biosynthetically related compounds, and closely related species may have different subsets of the array. However, there may be variation among populations of a single species, among individuals within a population, and among different parts of an individual plant (Dolinger et al., 1973; McKey, 1979; Brower et al., 1982; Roby and Stermitz, 1984a; Louda and Rodman, 1983a,b) in what particular compounds are present and in what amounts they occur. This pattern may also change through the season (Brower et al., 1982; Mooney and Chu, 1974) and the conditions under which the plant is growing (Lincoln and Mooney, 1984; Rhoades, 1979; Cooper-Driver et al., 1977; McKey et al., 1978). Thus the spectrum of chemical compounds to which an insect herbivore is exposed may vary with the individual plant or plant population, the part of the plant eaten, and the phenology of the plant and insect, as well as among species or genera.

Many insect species specialize on a particular set of plant species characterized by a distinctive group of allelochemicals (Ehrlich and Raven, 1964), such as pierid butterflies on the glucosinolate-containing crucifers (Rodman and Chew, 1980) and checkerspot butterflies in the genus *Euphydryas* on plants containing iridoid glycosides (Bowers, 1983). In contrast, generalist insect species are predicted to be repelled or poisoned by such qualitative chemical defenses, and indeed, often are (Feeny, 1976).

Qualitative allelochemical variation may be an important component of plant defense. Different individual compounds of the same chemical class may have very different effects on insect herbivores and occur within a single plant; thus the chemical composition of an individual plant may be important in whether or not it is a suitable hostplant (Chambliss and Jones, 1966; Metcalf et al., 1980; Miller and Feeny, 1983; Bentley et al., 1984a,b; Berenbaum, et al., 1986). In addition, the combination of two or more related compounds or types of compounds may have positive or negative synergistic effects on insects feeding on those plants (Nielsen et al., 1977; Berenbaum, 1985). Therefore, qualitative or quantitative variation in the chemical make-up of host plants may be critical determinants of fitness for individual insects.

The experiments presented in this paper examine the responses of specialist and generalist insect herbivores to an array of biosynthetically related plant allelochemicals, the iridoid glycosides. The iridoid glycosides are a group of monoterpene-derived compounds found in over 50 families of plants (Bobbitt and Segebarth, 1969; Jensen et al., 1975, Hegnauer and Kooiman, 1978; El-Naggar and Beal, 1980; Dahlgren et al., 1981; Gershenzon and Mabry, 1983). Larvae of butterflies in the genus *Euphydryas* use iridoid glycosides as feeding stimulants and attractants (Bowers, 1983), as do larvae of the buckeye, *Junonia*

coenia Hbn. (Nymphalidae), another specialist on iridoid glycoside-containing plants (Bowers, 1984). In addition, butterflies in the genus *Euphydryas* sequester iridoid glycosides to use in their own defense against predators (Bowers, 1980, 1981; Bowers and Puttick, 1986; Stermitz et al., 1986a). Two iridoid glycosides, ipolamiide, found in plants of the genus *Stachytarpheta* (Verbenaceae), and specioside, from *Catalpa speciosa* (Bignoniaceae), are deterrent to generalist insects (El-Naggar and Doskotch, 1980; Bernays and DeLuca, 1980).

To compare the response of a generalist and a specialist insect to a natural array of different iridoid glycosides, we chose the generalist species, *Lymantria dispar* (L.) (Lymantriidae), the gypsy moth, and the specialist, *J. coenia*. *J. coenia* feeds on a variety of plants that contain iridoid glycosides (Bowers, 1984). The experiments were designed: first, to compare the response of generalist and specialist insects to the iridoid glycosides; second, to determine how qualitative variation in allelochemical content affects these insects; and third, to compare the effectiveness of iridoid glycosides in inducing feeding preferences in a generalist and a specialist insect.

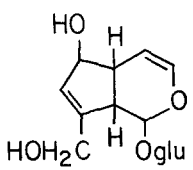
METHODS AND MATERIALS

Organisms and Culture Conditions. Cultures of the test species were maintained in environmental chambers under conditions of 14 : 10 hr light–dark photoperiod, and temperatures of 25°C day, 20°C night.

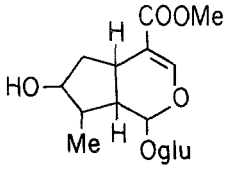
Lymantria dispar is a generalist feeder and has been recorded feeding on over 450 species of plants (Leonard, 1974). Egg masses were obtained from the Gypsy Moth Rearing Facility at Otis Air Force Base, Massachusetts, where they are raised on an artificial diet (J. Baker, personal communication). Our culture was maintained on an artificial diet with no additives (see below).

Junonia coenia is a specialist on iridoid glycoside-containing plants and has been recorded in the wild on host-plant species from four families: Acanthaceae, Plantaginaceae, Scrophulariaceae, and Verbenaceae (Bowers, 1984). Larvae use iridoid glycosides as feeding stimulants (Bowers, 1984), and adult females use these compounds as oviposition cues (Pereyra and Bowers, unpublished). *Plantago lanceolata* L. (Plantaginaceae) is a commonly utilized host plant (Bowers, 1984), even though it is an introduced species. The *J. coenia* culture was started from eggs obtained from females collected in Columbia, South Carolina, and maintained on leaves of *P. lanceolata* collected from plants grown in an experimental garden in Cambridge, Massachusetts.

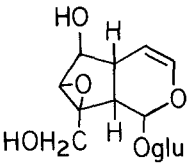
Iridoid Glycosides and Artificial Diets. The four iridoid glycosides used in this study, aucubin, catalpol, loganin, and asperuloside (Figure 1), are biosynthetically related (Inouye, 1971) and occur in a variety of angiosperm families (Table 1). Aucubin is a precursor of catalpol, and these two are perhaps the most ubiquitous of all iridoid glycosides, occurring together in many plant spe-



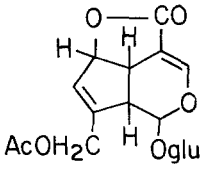
AUCUBIN



LOGANIN



CATALPOL



ASPERULOSIDE

FIG. 1. Iridoid glycosides used in this study.

TABLE 1. PLANT FAMILIES CONTAINING THE FOUR IRIDOID GLYCOSIDES USED IN THIS STUDY[a]

Plant Family	Iridoid glycosides
Apocynaceae	aucubin, loganin
Bignoniaceae	catalpol
Buddleiaceae	aucubin, catalpol
Callitrichaceae	aucubin, catalpol
Caprifoliaceae	loganin
Cornaceae	aucubin, loganin
Daphniphyllaceae	asperuloside
Ericaceae	asperuloside
Eucommiaceae	aucubin, asperuloside
Globulariaceae	aucubin, catalpol, asperuloside
Hammamelidaceae	asperuloside
Hippuridaceae	aucubin, catalpol
Lentibulariaceae	aucubin, catalpol
Loganiaceae	aucubin, loganin
Orobanchaceae	aucubin
Plantaginaceae	aucubin, catalpol
Rubiaceae	asperuloside
Scrophulariaceae	aucubin, catalpol
Verbenaceae	aucubin

[a] Sources for these data were Bobbitt and Segebarth (1969) and El-Naggar and Beal (1980).

cies (El-Naggar and Beal, 1980; Kaplan and Gottlieb, 1982), although they may also occur independently of each other (El-Naggar and Beal, 1980). Loganin is quite prevalent in the Loganiaceae and Caprifoliaceae, and it may occasionally be found in the same plant species as aucubin (El-Naggar and Beal, 1980). Asperuloside has been particularly well-documented as characteristic of the Rubiaceae (e.g., Kaplan and Gottlieb, 1982, and references therein). It only rarely occurs with the other three iridoid glycosides used in this study (Bobbitt and Segebarth, 1969) (Table 1). Aucubin and catalpol are characteristic of many *J. coenia* host plants (Bowers, 1984), while loganin and asperuloside are not.

Both for rearing of larvae and choice experiments, artificial diets were made according to the basic recipe of Lincoln et al. (1982). A control diet (AD) contained no additional components. The experimental diets contained 0.05 g iridoid glycoside per 28.4 g dry weight diet, thus presenting larvae with a diet with 0.18% dry weight iridoid glycoside, well within the range of iridoid glycoside content normally found in plants (Bobbitt et al., 1961; Roby and Stermitz, 1984b). Four iridoid glycosides were used for these experimental diets: asperuloside (AD + asp), aucubin (AD + auc), catalpol (AD + cat), and loganin (AD + log). All four iridoid samples were pure by high-performance liquid chromatography (HPLC) analysis. A sixth diet contained 0.09% each aucubin and catalpol (AD + a&c), thus the total amount of iridoid glycoside in this diet was equivalent to the total amount in the other diets. A seventh diet contained dried, finely ground leaves of *P. lanceolata*, comprising 3.5% dry weight of the diet (AD + P.l.).

Growth Rate and Survival. Larvae were reared in Petri dishes with damp paper towel taped to the lid to maintain humidity. To compare growth and survival of *J. coenia* and *L. dispar* in response to an array of iridoid glycosides, larvae were reared on six different diets: AD, AD + auc, AD + cat, AD + log, AD + asp, and AD + P.l. In addition, *J. coenia* larvae were also reared on AD + a&c. Newly hatched, unfed larvae of each species were put onto each of the artificial diets in groups of 10. For each species there were five replicates in each treatment, with one exception: there were only four replicates of *L. dispar* larvae on AD + asp. Larvae were weighed as a group at hatching and weighed and counted every five days thereafter for 15 days. One-way analyses of variance were performed on the $\log_{10}$-transformed weights, and arcsin-transformed percentage survival, and followed by Student-Newman-Keuls (SNK) range tests to test for differences among the means.

Choice Tests. To determine whether larvae could distinguish between diets with and without iridoid glycosides and between diets with different iridoid glycosides, and to determine the effect of previous rearing experience (induction), choice tests were conducted in Petri dishes with individual larvae in the last instar. Prior to testing, larvae had been reared from hatching on one of the seven test diets described above. Larvae were starved for approximately 12 hr

and then an individual larva was put into a Petri dish and offered cubes (approximately 8 × 6 × 6 mm) of two artificial diets placed about 1.5 cm apart in the middle of the dish. They were allowed to feed ad libitum for 48 hr. The sample sizes ranged from six to 10 larvae for each test, depending on the number of larvae available. The dry weight of each diet eaten was calculated using standard gravimetric methods (Waldbauer, 1969; Bowers, 1983, 1984). In a few cases, if larvae did not eat any diet, the amount eaten was a negative number (see Bowers, 1983, 1984), which was assumed to be 0 for analysis. The amounts eaten of the two diets were compared by two-way ANOVA with diet and larva as the main effects.

RESULTS

Growth and Survival. The incorporation of iridoid glycosides into the artificial diets clearly had a detrimental effect on the growth of *L. dispar* larvae; they weighed 1.5–3 times less after 15 days on the artificial diets with iridoid glycosides than on the diet without iridoid glycosides (Table 2). Diets could be grouped according to their effect on larval weights: (1) larvae reared on AD were the heaviest; (2) those reared on the diets with aucubin, catalpol, and *P. lanceolata* leaves were intermediate; and (3) those on the diets with loganin and asperuloside grew the least (Table 2). Although the specific ranks of the diets within these groups changed over the course of the experiment (Table 2), the weights of larvae fed on the diet with *P. lanceolata* leaves, which contain aucubin and catalpol [in amounts ranging from 0.05 to 1.0% dry weight (Fajer, unpublished data)], were always very similar to those of larvae on the diets with aucubin and catalpol. Survival of larvae was not significantly different on the different diets ($F = 2.40$, $df = 4, 19$, $0.05 < P < 0.10$) (Table 2).

Aucubin and catalpol were necessary for successful larval feeding and growth of *J. coenia* (Table 3). Again, the diets fell into three groups: (1) larvae grew best on the diets with aucubin and catalpol (AD + auc, AD + cat, AD + P.l., and AD + a&c), (2) larvae grew about half as well on AD, and (3) larvae weighed one quarter to one fifth less on the diets with loganin and asperuloside as on those with aucubin and catalpol (Table 3). Interestingly, the diets containing loganin and asperuloside supported the poorest growth in both *L. dispar* and *J. coenia*. The similarity of larval weights on AD + a&c with those on the other three diets containing aucubin and catalpol was not surprising. On day 5, *J. coenia* larvae were heaviest on the diet with catalpol and were still heaviest at the end of the experiment on day 15. Although there was a marginally significant effect of diet on survival ($P = 0.05$), and SNK analysis did not detect differences among the diets, larval survival loosely corresponded to larval weights (Table 3).

TABLE 2. MEAN WET WEIGHT (±SE) PER LARVA IN MILLIGRAMS AND MEAN PERCENTAGE (±SE) SURVIAL OF *Lymantria dispar* LARVAE REARED ON ARTIFICIAL DIETS WITH AND WITHOUT IRIDOID GLYCOSIDES[a]

	AD	AD + auc	AD + cat	AD + log	AD + asp	AD + P.l.	*P*
Weight							
Day 5	5.67(0.16) a	4.22(0.42) ab	5.01(0.21) a	3.29(0.46) b	3.65(0.40) ab	3.92(0.53) ab	<0.01
Day 10	20.87(0.81) a	10.95(0.60) bc	13.98(0.61) bc	8.59(1.57) c	10.46(1.16) bc	15.79(2.63) b	<0.001
Day 15	53.67(2.45) a	26.73(2.47) b	34.28(3.24) b	17.62(3.22) c	23.11(3.48) c	32.13(2.69) b	<0.001
Survival							
Day 15	76.0(6.8)	60.0(4.5)	72.0(9.2)	80.0(4.5)	50.0(10.2)	62.0(8.6)	$0.10 < P < 0.25$

[a]Weights were compared by one-way ANOVA followed by the Student-Newman-Keuls range test. The *P* value indicates the results of the ANOVA, and the same letters indicate mean weights that were significantly different across diets at $P < 0.05$ by the SNK range test (Sokal and Rohlf, 1969). Mean weight per larva at hatching is 0.48 mg (±.01 SE).

TABLE 3. MEAN WET WEIGHT (±SE) PER LARVA IN MILLIGRAMS AND MEAN PERCENTAGE SURVIVAL (±SE) OF *Junonia coenia* LARVAE REARED ON ARTIFICIAL DIETS WITH AND WITHOUT IRIDOID GLYCOSIDES[a].

	AD	AD + auc	AD + cat	AD + log	AD + asp	AD + P.l.	AD + a&c	*P*
Weight								
Day 5	0.71(0.11) d	1.00(0.07) bcd	1.68(0.20) a	0.79(0.04) cd	0.78(0.09) cd	1.31(0.19) ab	1.12(0.10) bc	<0.001
Day 10	2.86(0.47) b	5.51(0.41) ab	7.84(1.30) a	2.21(0.33) b	2.28(0.41) b	7.54(1.10) a	6.50(1.14) a	<0.001
Day 15	16.37(2.54) b	33.08(3.27) a	42.67(5.98) a	7.50(1.05) c	8.47(2.10) c	34.57(5.87) a	42.31(10.53) a	<0.001
Survival								
Day 15	40.0(13.7)	62.0(5.8)	68.0(8.6)	48.0(3.8)	40.0(7.1)	58.0(3.8)	66.0(5.1)	= 0.05

[a]Weights were compared by one-way ANOVA followed by the Student-Newman-Keuls range test. The *P* value indicates the results of the ANOVA, and the same letters indicate mean weights that were not significantly different across diets at $P < 0.05$ by the Student-Neuman-Keuls range test (Sokal and Rohlf, 1969). Although there was a significant difference among the percent of larvae surviving on the various diets, SNK did not distinguish among the means.

Choice Tests. Larvae of *L. dispar* that had been reared on artificial diet with no additives and were then given a choice of diets with and without iridoid glycosides showed no preference for AD over AD + cat or AD + log (Figure 2). Surprisingly, they significantly preferred the diet AD + asp over AD even though this diet reduced growth (Figure 2). When *L. dispar* larvae were reared on diets that contained iridoid glycosides, a preference for iridoid glycosides was induced (Jermy et al., 1968), and they significantly preferred those diets (Figure 2), even though iridoid glycosides depressed growth when added to the diet (Figure 2). The choice tests between two different iridoid glycoside-containing diets showed that a preference was induced by the presence of loganin, and larvae preferred it over the diet with catalpol, but no preference was induced by aucubin when larvae were given a choice with aucubin and catalpol.

Larvae of *J. coenia* will not feed and survive well enough to use in choice tests if reared on an artificial diet without either iridoid glycosides or plant material that contains iridoid glycosides. Larvae that had been reared on artificial diet with 3.5% *P. lanceolata* leaves significantly preferred diets with leaf material or iridoid glycosides over the diet with no additives (Figure 3). They did not distinguish between the diet with *P. lanceolata* leaf material, which contains aucubin and catalpol, and diets with pure aucubin and catalpol (Figure 3). Results with *J. coenia* from California were the same (Bowers, 1984). Larvae that had fed on iridoid glycoside-containing diets significantly preferred those diets to diets without iridoid glycosides (Figure 3), even though some of these diets contained iridoids that depressed growth (Figure 3). One exception was in larvae offered a choice of the diet with catalpol and the control diet. Although the mean amounts eaten are very different for the two diets, the differences were not statistically significant due to one caterpillar which ate equal amounts of the two diets, while others all preferred AD + cat.

Interestingly, *J. coenia* larvae that had been reared on AD + a&c preferred this diet over AD + P.l. Larvae given the same choice but which had been reared on AD + P.l., ate similar amounts of the two diets (Figure 3). However, a comparison of the proportion of AD + P.l. eaten by larvae that had been reared on each of these two diets showed no significant difference (one-way ANOVA after arcsin transformation, $F = 3.71$, $df = 1, 16$, $0.05 < P < 0.10$). *J. coenia* larvae also preferred AD + log over AD + cat when they had been reared on AD + log. If reared on AD + cat and given this same choice, the mean amounts eaten are again quite different, but this is not statistically significant because two of nine larvae preferred AD + cat over AD + log, while the rest preferred AD + log (Figure 3). A comparison of the proportion of AD + log eaten by these two groups of larvae showed that larvae ate a significantly higher proportion of AD + log when they were reared on that diet (one-way ANOVA after arcsin transformation, $F = 5.59$, $df = 1, 16$, $P < 0.05$). This comparison suggests that larvae may have an innate preference for the iridoid

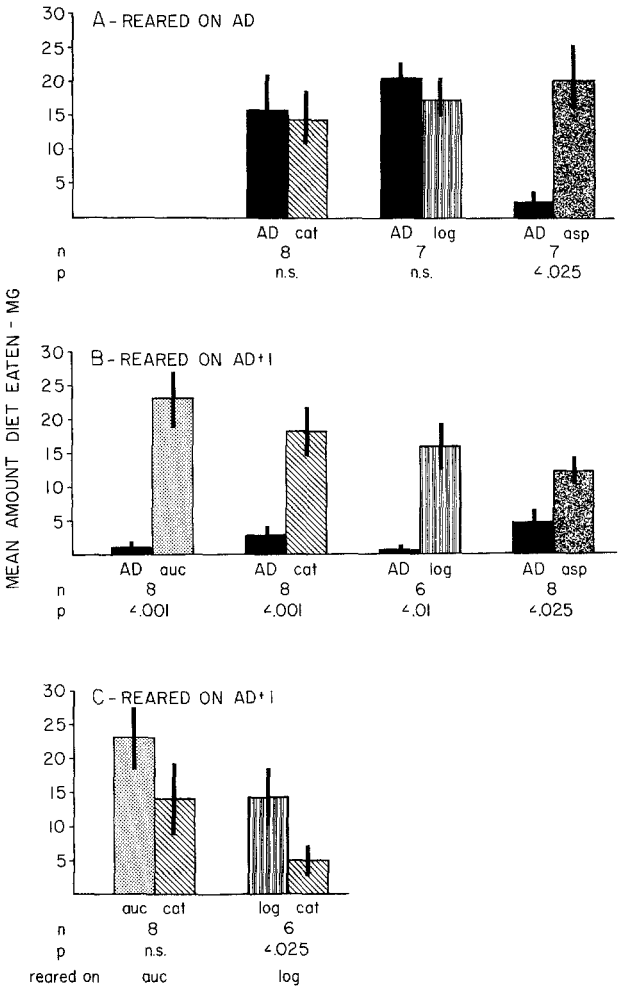


FIG. 2. Choice test results for *Lymantria dispar*. (A) Larvae reared on artificial diet with no additives and then given a choice of diets with and without iridoid glycosides. Choice tests were not run for AD + auc because of low numbers of larvae. (B) Larvae reared on an artificial diet with an iridoid glycoside and then given a choice of that diet and a control diet with no iridoid glycosides. (C) Larvae reared on a diet with an iridoid glycoside and then given a choice between that diet and another diet with a different iridoid glycoside. The vertical bars on each column indicate one standard error. *P* values are for the diet effect in a two-way ANOVA with larva as the other main effect. AD = artificial diet with no iridoid glycosides (control); auc = artificial diet with 0.18% aucubin; cat = artificial diet with 0.18% catalpol; log = artificial diet with 0.18% loganin; asp = artificial diet with 0.18% asperuloside.

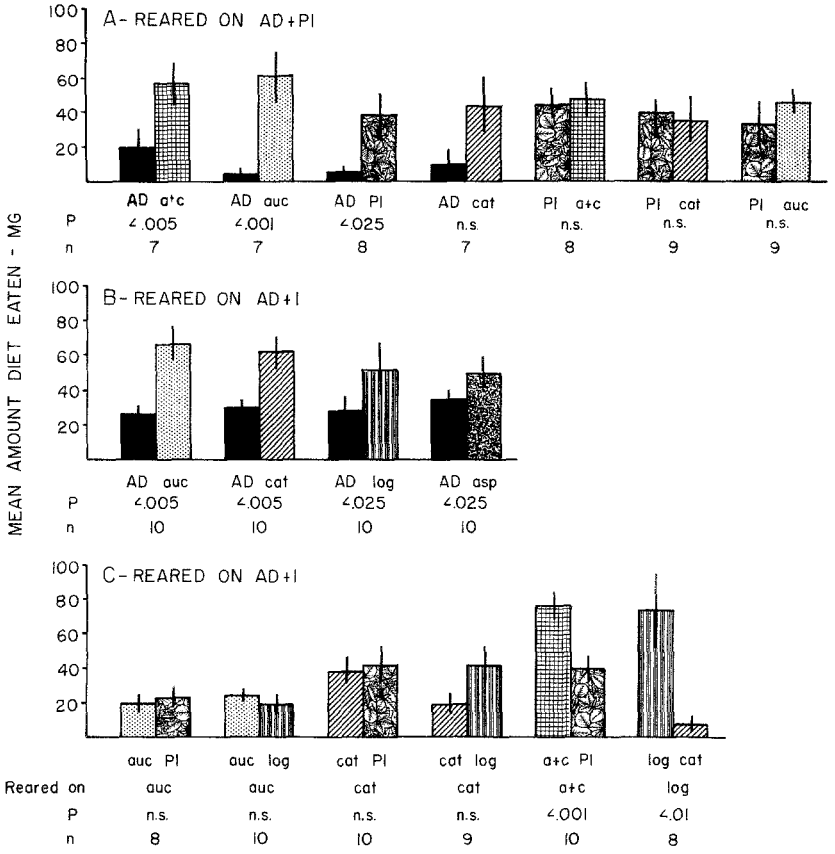


FIG. 3. Choice test results for *Junonia coenia*. (A) Larvae reared on an artificial diet with 3.5% dry weight ground leaves of *Plantago lanceolata* and then given a choice of diets with and without iridoid glycosides. (B) Larvae reared on an artificial diet containing 0.18% of an iridoid glycoside and then given a choice of that diet and a control diet containing no iridoid glycosides. (C) Larvae reared on an artificial diet containing 0.18% of an iridoid glycoside and given a choice of that diet and another diet with either another iridoid glycoside, 3.5% dry weight ground leaves of *P. lanceolata*, or 0.09% aucubin + 0.09 catalpol. The vertical bars on each column indicate one standard error. *P* values are for the diet effect in a two-way ANOVA with larva as the other main effect. AD = artificial diet with no iridoid glycosides (control); auc = artificial diet with 0.18% aucubin; cat = artificial diet with 0.18% catalpol; log = artificial diet with 0.18% loganin; asp = artificial diet with 0.18% asperuloside; P.l. = artificial diet with 3.5% dry weight *P. lanceolata* leaves; a&c = artificial diet with 0.09% aucubin + 0.09% catalpol.

glycoside loganin and that being reared on that diet strengthens that preference. This behavior was not expected since AD + log reduces growth (Table 3).

DISCUSSION

The generalist, *L. dispar* and the adapted specialist, *J. coenia*, responded differently to the presence of iridoid glycosides in their food. In general, *L. dispar* grew and survived less well on diets with iridoids, while *J. coenia* performed best on diets with the two iridoid glycosides typical of its primary host plant *P. lanceolata*. We did not distinguish between deterrent or toxic effects (Blau et al., 1978; Miller and Feeny, 1983; Brower, 1984) of the various iridoid glycosides in these experiments as the basis for differential growth on diets with and without these compounds (Puttick and Bowers, 1988). For both insect species, catalpol and aucubin supported better growth of larvae than loganin and asperuloside (Tables 2 and 3).

The results with *J. coenia* suggest that loganin and asperuloside may be toxic rather than deterrent to these larvae: larvae prefer diets containing these two iridoid glycosides in choice tests (Figure 3), but grow very poorly on those diets. A toxic effect might be expected since loganin and asperuloside are not found in the host plants of *J. coenia*, which are in the Plantaginaceae, Scrophulariaceae, Verbenaceae, and Acanthaceae (Table 1). An alternative explanation may be that loganin and asperuloside are more toxic or deterrent to first-instar larvae than to later instar larvae. Early instar toxicity may be due to a lack of detoxifying enzymes in the gut of early instars (Gould, 1984; Cohen, 1983). Growth might be slowed in the early instars, leading to differences in the total growth of larvae on loganin and asperuloside compared to the diets with other iridoid glycosides. Thus *J. coenia* appears to be physiologically adapted to the specific iridoid glycosides, catalpol and aucubin, which are typical of its host plants, and not to iridoid glycosides as a group. However, in choice tests, *J. coenia* larvae chose diets with iridoid glycosides that decrease growth rate (loganin and asperuloside) over a diet without these compounds. From a chemical perspective, this behavior suggests that for specialists such as *J. coenia*, the basic iridoid skeleton may be important in eliciting attraction, but that physiological tolerance to particular iridoids depends on the specific chemical structure.

L. dispar generally grew poorly on diets with iridoid glycosides, but showed an induced preference for these compounds in the diets. De Boer and Hanson (1984) found an induction of feeding preference for non-hostplant species in *Manduca sexta* (Sphingidae), but these plants were not toxic to the larvae. Induction of preference for artificial diets has been shown previously (Stadler and Hanson, 1978) but not for diets which were deleterious to larvae, as we found. One explanation for this apparently maladaptive behavior is provided by

work on other lepidopterous larvae which showed that hostplant switching may cause reduction in growth rate and less efficient conversion of food (Schoonhoven and Meerman, 1978; Scriber, 1979, 1982; Barbosa et al., 1986). In other words, since hostplant switching may be metabolically costly, induction of feeding preference could still be adaptive overall.

These results have important implications in considering patterns of herbivory in plant populations. Most plants do not contain a single defensive compound, but contain a suite of biosynthetically related compounds (for iridoid glycosides, see Stermitz et al., 1986a). Individual plants or populations may vary in which particular compounds are present and in what amounts (e.g., Dolinger et al. 1973; Berenbaum et al., 1986). The work of Stermitz et al. (1986b) on *Castilleja sulphurea* (Scrophulariaceae) provides a case in point. They found that some populations of *C. sulphurea* have as many as eight iridoid glycosides. The relative amounts of these different iridoid glycosides vary substantially from one population to another. One population was found to contain almost solely (95%) catalpol; in contrast, other populations sampled contained catalpol in amounts ranging from 21% to 46% of the total iridoid glycoside content.

Individual compounds may have particular effects on the insect herbivores, such as we have shown with iridoid glycosides and *J. coenia* and *L. dispar*. These compounds may also interact to produce synergistic effects. Mutations leading to variations in enzyme kinetics or blockage of certain biosynthetic steps may cause variation in the relative amounts of chemical constituents in different individual plants, or to the accumulation of some compounds and not others. Because different but biosynthetically related compounds may have very different effects on both generalists and specialists, such alteration in chemical composition may protect individual plants with such mutations. For example, in *P. lanceolata*, a mutation may cause a modification of the biosynthetic pathway leading to the accumulation of a precursor of aucubin and catalpol which is not normally found in *P. lanceolata*. Individuals (or populations) with this altered iridoid glycoside composition may be protected from herbivory by specialists, such as *J. coenia*, which our results suggest is not physiologically adapted to iridoid glycosides as a group but only to the specific iridoid glycosides characteristic of its host plants. Such qualitative and quantitative variation in allelochemical profile of plants must be an important determinant of patterns of herbivory in plant populations.

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EFFECT OF QUALITATIVE AND QUANTITATIVE VARIATION IN ALLELOCHEMICALS ON A GENERALIST INSECT: Iridoid Glycosides and the Southern Armyworm

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Abstract—The behavioral and physiological effects of plant allelochemicals have been difficult to demonstrate; it is not often clear whether the compounds are deterrent, toxic, or both. In this study, we compared the qualitative and quantitative effects of several iridoid glycosides on a generalist lepidopteran herbivore, *Spodoptera eridania* (Noctuidae). Larval growth and survivorship and larval preference or avoidance were measured on artificial diets containing different iridoid glycosides at different concentrations. We also tested the toxicity/deterrence of these compounds. We found that iridoid glycosides retarded larval growth significantly at relatively low concentrations and that they were usually avoided in preference tests. The toxicity/deterrence test did not always reflect the results of these other tests. The merits of using a variety of methods for determining deterrence and/or toxicity of plant allelochemicals are discussed.

Key Words—Iridoid glycosides, *Spodoptera eridania*, Lepidoptera, Noctuidae, allelochemical variation, toxicity/deterrence, plant–insect interaction.

INTRODUCTION

Plant allelochemicals such as alkaloids, coumarins, terpenoids, and others may function as deterrents and/or toxins to generalist and nonadapted specialist insect species (e.g., Burnett et al., 1974; Berenbaum, 1978; Blau et al., 1978;

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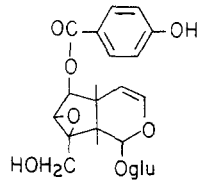
Miller and Feeny, 1983), while adapted specialists may be unaffected by the same allelochemicals and may use them as feeding or oviposition cues (e.g., Chambliss and Jones, 1966; Schoonhoven, 1972; Bowers, 1983, 1984). Iridoid glycosides (Figure 1) are typical of over 50 plant families including the Scrophulariaceae, Bignoniaceae, Verbenaceae, Acanthaceae, Plantaginaceae, Caprifoliaceae, and Oleaceae (Jirawongse, 1964; Kooiman, 1972; Jensen et al., 1975). Several species of Lepidoptera, including those in the genus *Euphydryas* (Nymphalidae) (Bowers, 1983), *Ceratomia catalpae* (Sphingidae) (Nayar and Fraenkel, 1963), and *Junonia coenia* (Nymphalidae) (Bowers, 1984), specialize on plants that contain iridoid glycosides, and the larvae use these compounds as feeding attractants and stimulants. On the other hand, iridoid glycosides appear to deter generalist insects. For example, ipolamiide was a feeding deterrent for three generalist insect species: *Spodoptera littoralis* (Lepidoptera: Noctuidae) and *Schistocerca gregaria* and *Locusta migratoria* (Orthoptera: Acrididae) (Bernays and DeLuca, 1981); specioside from *Catalpa speciosa* (Bignoniaceae) was deterrent to generalist insects (Chang and Nakanishi, 1983). In addition,



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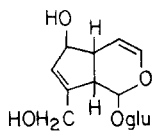
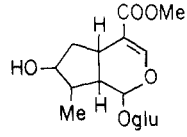


AUCUBIN



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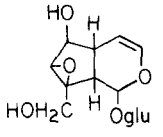

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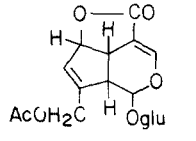

ASPERULOSIDE

FIG. 1. Structures of the iridoid glycosides used in this study.

growth rates of gypsy moth larvae *Lymantria dispar* (Lymantriidae) were depressed on artificial diets containing iridoid glycosides (Bowers and Puttick, 1988).

The effects of plant allelochemicals on insect behavior and/or physiology have been difficult to demonstrate (Schoonhoven, 1972; Feeny, 1975; Rhoades, 1979). It is often not clear whether the compounds deter feeding, whether they have a toxic effect once they have already been ingested (Waldbauer, 1962), or both. Blau et al. (1978) developed an assay for toxicity which "permitted a clearer distinction between feeding inhibition and toxicity". Using this method, Blau et al. (1978) found that allylglucosinolate, found in many cruciferous plants, was acutely toxic to larvae of *Papilio polyxenes* (Papilionidae), which do not normally feed on crucifers, while larvae of *Pieris rapae* (Pieridae), which are crucifer specialists, were not affected. Larval growth of the generalist, *Spodoptera eridania* (Cram.) (Noctuidae), was inhibited by high but not by low concentrations of the compound. Usher and Feeny (1983) also used this method and found that atypical secondary compounds occurring in the family Cruciferae, such as alkaloids, cucurbitacins, and cardenolides, were not toxic to *P. rapae*. Similarly, Miller and Feeney (1983) found that benzylisoquinoline alkaloids were toxic to, and/or feeding inhibitors of, three generalist Lepidoptera species: *Hyphantria cunea* (Arctiidae), *L. dispar*, and *S. eridania*.

In this study, we compared the qualitative and quantitative effects of several iridoid glycosides on a generalist lepidopteran herbivore, the southern army worm, *S. eridania*. First, in a series of experiments to compare the qualitative effects of different iridoid glycosides, we compared the growth rates and survivorship of larvae on artificial diets containing each of the four iridoid glycosides aucubin, catalpol, loganin, and asperuloside, with those on a control diet. Then, by means of choice tests, we determined larval preference for or avoidance of diets containing these four compounds. Finally, we tested whether these four compounds were toxic to *S. eridania* larvae using the method of Blau et al. (1978). Second, we determined the quantitative effects of another iridoid glycoside, catalposide, by comparing growth rates and survivorship of larvae reared on artificial diets containing different amounts of this compound. We also measured larval preference for or avoidance of diets containing different amounts of catalposide and determined whether catalposide was toxic or deterrent to larvae and in what amounts.

METHODS AND MATERIALS

Southern Armyworm S. eridania. *Spodoptera eridania* is widely distributed across the southern United States. Larvae feed on a wide variety of herbaceous plants including agricultural crops (Metcalf et al., 1962; Soo Hoo and Fraenkel,

1966; Tietz, 1972). They are easy to maintain in the laboratory and have been used in several studies on the effect of different allelochemicals on generalist insect herbivores (e.g., Blau et al., 1978, Scriber and Feeny, 1979, Miller and Feeny, 1983). *Spodoptera eridania* larvae used in our experiments were reared from eggs from laboratory strains obtained from M. Evans at the University of Wisconsin and from D. Lincoln at the University of South Carolina. Larvae were reared in Petri dishes and maintained in the laboratory under controlled conditions (25°C 16 hr light–20°C 8 hr dark) on artificial diet (Bowers, 1983).

Qualitative Effects of Iridoid Glycosides. To study the effects of different iridoid glycosides on larval growth and survival, larvae were fed artificial diets containing purified catalpol (AD + cat), aucubin (AD + auc), loganin (AD + log), or asperuloside (AD + asp) at levels of 0.18% dry weight of the diets or on a control diet with no iridoid glycosides. This amount is well within the range of iridoid glycoside levels normally found in plants (Bobbitt et al., 1961; Roby and Stermitz, 1984). Growth rates of larvae were measured by rearing five groups of 10 larvae on each of these experimental diets and on the control diet. Hatching was synchronized by refrigeration. Newly hatched larvae from several egg masses were mixed, weighed in groups of 10, placed on diet, and reared for 15 days, by which time larvae were usually in the fifth instar. Larvae were counted and weighed as a group every five days. Larval weights were log-transformed and compared by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) range test (Sokal and Rohlf, 1969). The number of larvae surviving was compared by one-way ANOVA followed by the SNK range test.

After the growth experiment, the surviving larvae were reared on the same diets for one to two days until they reached the last instar, when surviving larvae were put into choice tests. Larvae were starved for 18 hr and then individually given a choice between the diet they had been reared on and one of the other diets. They were allowed to feed ad libitum for 48 hr, and then the dry weight of each diet eaten was calculated using standard gravimetric methods (Waldbauer, 1968).

The method of Blau et al. (1978) was used to test whether iridoid glycosides were toxic and/or deterrent to *S. eridania*, using newly molted final instar larvae. Determination of the calibration line and the feeding assays were carried out concurrently using freshly made artificial diets. To calculate the calibration line, the control diet (AD) was fed to 40 individual larvae in separate Petri dishes for 24 hr in amounts ranging from none to *ad libitum*. Relative growth rate (RGR) and relative consumption rate (RCR) were calculated for each larva as follows: RGR = biomass gained/mean larval weight (mg/mg/day), and RCR = food ingested/mean larval weight (mg/mg/day).

Mean larval weight was calculated as the average of the weight at the

beginning and the end of the experiment. Feeding assays to determine the deterrent and/or toxic effects of iridoid glycosides were run for 24 hr. Twenty individual larvae in Petri dishes were fed on each of the four artificial diets containing iridoid glycosides, or on a control diet with no additives. Larvae and remaining food were frozen at the end of the experiment, then dried at 50°C for 96 hours. Final dry weights of food remaining and of larvae were determined directly. Starting weights of larvae and diets were converted from fresh to dry weights using conversion factors obtained from additional larvae and diet set aside at the beginning of these arrays.

Quantitative Effects of Iridoid Glycosides. An analogous set of experiments to those described above (growth and survival, diet choice, and deterrence and/or toxicity) were carried out to determine whether iridoid glycosides had a dose-dependent effect on *S. eridania*. Larvae were reared on artificial diets containing the iridoid glycoside catalposide (Figure 1), comprising 0, 0.36, 0.72, 1.8, 3.6, and 7.2% dry weight of the diet, for 15 days. Growth and survivorship were determined as described above. Surviving larvae from this experiment were then reared to the final instar when they were given a choice test. The diets used for the choice tests were: a control with no iridoid glycoside (AD), AD + 1.8% catalposide and AD + 7.2% catalposide.

The toxic and/or deterrent effects of increasing doses of catalposide were determined by the method of Blau et al. (1978), using two strains of *S. eridania*. The first strain was the same as that used for the experiments described above. A calibration line using 40 larvae fed varying amounts of the control diet was obtained (see above), and concurrently, 20 larvae were tested on diets containing 0.18, 0.36, 0.72, 1.8, 3.6, or 7.2% catalposide. This experiment was repeated using a second strain obtained from D. Lincoln at the University of South Carolina, and adding an additional experimental diet containing 5.4% catalposide.

RESULTS

Qualitative Effects of Iridoid Glycosides. After 15 days, the weights of *S. eridania* larvae on artificial diets with different iridoid glycosides were significantly different (Table 1). Larvae grew as well on the diet containing asperuloside as on the control diet, while growth rate was reduced almost by half on diets containing aucubin and loganin, and larvae grew least on the diet containing catalpol (Table 1). Survivorship of larvae was significantly lower on diets containing catalpol, aucubin, and loganin than on the control diet or AD + asp, on which larval survivorship was similar (Table 1).

To compare the effects of iridoid glycosides on young and old larvae, we

TABLE 1. MEAN WEIGHT (MG) AND SURVIVORSHIP OF *S. eridania* LARVAE AFTER 15 DAYS, REARED ON ARTIFICIAL DIETS CONTAINING 0.18% OF EACH OF FOUR DIFFERENT IRIDOID GLYCOSIDES[a]

	AD	AD + auc	AD + cat	AD + log	AD + asp	*P*
Mean weight	325.43a	184.06b	110.38c	173.43b	313.17a	<0.001
	(123.35)	(58.43)	(50.73)	(45.01)	(122.38)	
Survivorship	0.84ab	0.64abc	0.54bc	0.50c	0.94a	<0.01
	(0.25)	(0.18)	(0.15)	(0.25)	(0.06)	

[a] SD given in parenthesis. Results were compared by a one-way analysis of variance and differences among means by a Student-Newman-Keuls (SNK) range test. AD = artificial diet; auc = aucubin; cat = catalpol; log = loganin; asp = asperuloside. Values followed by the same letter in the same row are not significantly different at the 0.05 level by the SNK range test.

compared the RGR and mortality of larvae on the various diets during the first and the last five days of the 15-day experiment by a two-way ANOVA. RGR was significantly higher in the younger larvae (Table 2) and, although differences in RGR on the diets were small, there was a significant effect of diet on

TABLE 2. COMPARISON OF RELATIVE GROWTH RATE (RGR) AND MORTALITY OF *S. eridania* LARVAE ON DIFFERENT IRIDOID GLYCOSIDES BETWEEN 0–5 DAYS AND 10–15 DAYS OF AGE BY MEANS OF TWO-WAY ANALYSIS OF VARIANCE WITH REPLICATES[a]

	AD	AD + cat	AD + log	AD + auc	AD + asp
RGR					
Day 0–5	0.37	0.36	0.37	0.38	0.38
	(0.004)	(0.030)	(0.008)	(0.005)	(0.001)
Day 10–15	0.34	0.28	0.32	0.32	0.31
	(0.015)	(0.022)	(0.027)	(0.013)	(0.012)
Diet effect, *P*	<0.001				
Age effect, *P*	<0.001				
Diet × Age, *P*	<0.05				
Mortality					
Day 0–5	0.14	0.36	0.42	0.30	0.04
	(0.26)	(0.20)	(0.22)	(0.19)	(0.06)
Day 10–15	0.02	0.04	0.04	0.04	0.02
	(0.05)	(0.09)	(0.09)	(0.09)	(0.05)
Diet effect, *P*	<0.05				
Age effect, *P*	<0.001				
Diet × age, *P*	NS				

[a] SD given in parenthesis. AD = artificial diet; auc = aucubin; cat = catalpol; log = loganin; asp = asperuloside.

TABLE 3. MEAN AMOUNTS (MG ± SD) OF ARTIFICIAL DIETS EATEN BY LAST-INSTAR LARVAE OF *S. eridania in* CHOICE TESTS[a]

Diet prior to test	Choice		Mean amount eaten		N	P
	Diet 1	Diet 2	Diet 1	Diet 2		
AD	AD	AD + log	81.12 ± 33.08	51.02 ± 16.71	10	<0.05
	AD	AD + auc	105.31 ± 17.13	49.98 ± 20.69	10	<0.001
	AD	AD + cat	99.12 ± 27.63	38.55 ± 21.36	10	<0.001
	AD + cat	AD + log	26.42 ± 11.20	98.26 ± 52.56	7	<0.05
AD + cat	AD + cat	AD	12.35 ± 11.23	28.53 ± 14.73	7	NS
	AD + cat	AD + log	26.57 ± 13.35	24.05 ± 10.51	8	NS
	AD + cat	AD + auc	17.63 ± 14.17	17.53 ± 11.26	8	NS
AD + auc	AD + auc	AD	20.45 ± 12.36	25.38 ± 20.81	10	NS
	AD + auc	AD + cat	42.58 ± 17.20	35.08 ± 14.24	9	NS
	AD + auc	AD + log	26.73 ± 9.98	30.36 ± 11.26	9	NS
AD + log	AD + log	AD	29.86 ± 11.46	55.42 ± 11.94	10	<0.001
	AD + log	AD + cat	39.97 ± 25.67	41.36 ± 15.37	9	NS
AD + asp	AD + asp	AD	66.55 ± 26.42	69.03 ± 24.88	10	NS
	AD + asp	AD + cat	56.17 ± 30.12	86.62 ± 22.25	10	NS
	AD + asp	AD + log	78.04 ± 29.92	52.04 ± 27.30	10	NS
	AD + cat	AD + log	42.00 ± 28.29	71.94 ± 26.58	10	<0.05

[a]Results within each choice test were compared by means of paired *t* tests. All diets contained 0.18% of the iridoid glycosides. AD = artificial diet, auc = aucubin, cat = catalpol, log = loganin, asp = asperuloside.

RGR in the older larvae. There was a significant interaction between diet and age, indicating that the effects of the diets on growth changed with the age of the larvae (Table 2). Mortality was two to ten times higher in the younger larvae, but there was no significant interaction between diet and age in their effect on mortality (Table 2).

Results from the choice tests showed that larvae that were reared on AD without iridoid glycosides preferred this control diet to diets with iridoid glycosides added (Table 3). However, if larvae were reared on diets containing any one of the four iridoid glycosides and then given a choice of that diet and the control, they showed no preference for the control diet but ate equal amounts of the control diet and the diet with iridoid glycosides (Table 3), with the exception of larvae reared on AD + log which preferred the control diet. If larvae were reared on a diet with iridoid glycosides and then given a choice of two diets each with a different iridoid glycoside, they showed no preference with one exception: larvae reared on AD + asp and given a choice of AD + cat and AD + log preferred AD + cat. Larvae reared on all diets except AD + auc were offered the choice of AD + cat and AD + log. Larvae reared on the control diet (AD) and on AD + asp preferred AD + log, while those reared on AD + cat and AD + log showed no preference (Table 3).

Neither catalpol nor loganin appear to have had a toxic or a deterrent effect as measured by the method of Blau et al. (1978), since the data points for these diets fell just above the calibration line but inside the 95% confidence limits (Figure 2). However, aucubin and asperuloside did have a toxic effect, since data points for these two diets fell below the calibration line and outside of the 95% confidence limits.

Quantitative Effects of Iridoid Glycosides. Weight of larvae after 15 days was significantly lower in those fed diets containing 0.36, 0.72, and 7.2% catalposide than in larvae reared on AD only (Table 4); larval weights on diets with 1.8% and 3.6% catalposide were lower than those on AD but not significantly so. RGR was higher for younger larvae in both control and experimental treatments (Table 5), as we found for larvae on the other four iridoid glycosides (Table 2). Mortality was also significantly higher for the younger larvae (Table 5). There was a significant interaction between age and diet for both RGR and mortality, indicating that the response of larvae to increasing doses of catalposide changed with age.

Larvae showed significant differences in diet preferences (Table 6). Larvae reared on AD without iridoid glycosides showed a preference for AD when the choice was AD or AD + 7.2% catalposide, but larvae reared on AD + 1.8% catalposide showed no preference for AD over this diet. However, if larvae were reared on AD + 1.8% catalposide, they preferred it to AD + 7.2% catalposide. Larvae reared on AD + 7.2% catalposide preferred the other diets

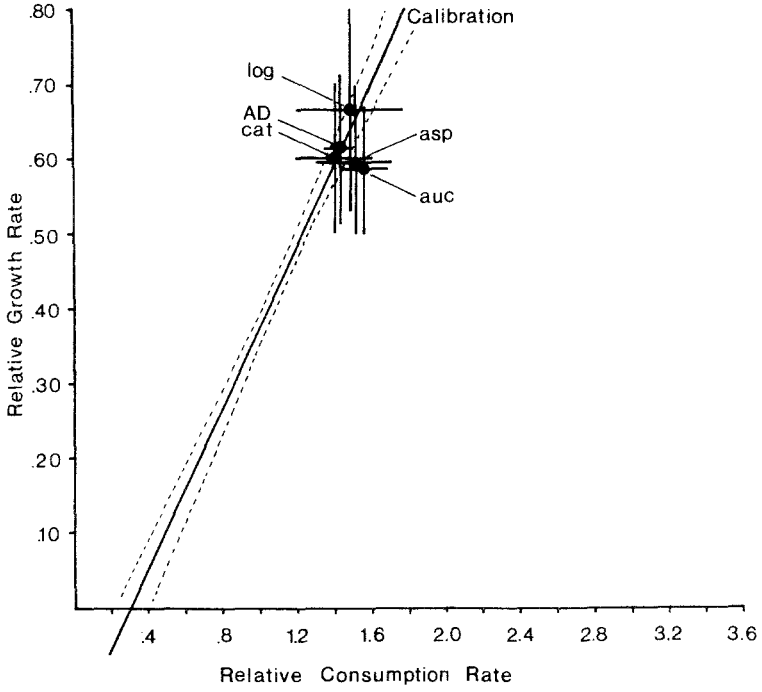

FIG. 2. Relative growth rates (RGR) (mg/mg/day) and relative consumption rates (RCR) (mg/mg/day) of *Spodoptera eridania* larvae fed artificial diets containing 0.18% of the iridoid glycosides catalpol (cat), aucubin (auc), loganin (log), and asperuloside (asp) for 24 hr during the final instar. Each data point represents the mean and range of values for 20 larvae. (Calibration line: $y = -0.19x + 0.55$, $N = 34$, $r = 0.96$).

TABLE 4. MEAN WEIGHT (MG) AND SURVIVORSHIP OF *S. eridania* LARVAE AFTER 15 DAYS, REARED ON ARTIFICIAL DIETS (AD) CONTAINING DIFFERENT AMOUNTS OF CATALPOSIDE (C'SIDE).

		C'side (%)					
	AD	0.36	0.72	1.8	3.6	7.2	*P*
Mean weight	73.34a	24.64c	38.78bc	53.36ab	60.08ab	26.15c	<0.001
	(19.79)	(7.44)	(16.22)	(22.67)	(5.69)	(4.86)	
Survivorship	0.58ab	0.54ab	0.46b	0.72a	0.76a	0.68ab	<0.05
	(0.11)	(0.11)	(0.06)	(0.30)	(0.11)	(0.08)	

[a] SD given in parenthesis. Results were compared by a one-way analysis of variance and differences among the means by a Student-Newman-Keuls (SNK) range test. Values followed by the same letter in the same row are not significantly different at the 0.05 level by the SNK range test.

TABLE 5. COMPARISON OF RELATIVE GROWTH RATE (RGR) AND MORTALITY OF *S. eridania* LARVAE ON ARTIFICIAL DIETS CONTAINING DIFFERENT CONCENTRATIONS OF IRIDOID GLYCOSIDE CATALPOSIDE (C'SIDE) BETWEEN 0–5 DAYS AND 10–15 DAYS OF AGE BY TWO-WAY ANALYSIS OF VARIANCE WITH REPLICATES[a]

		C'side (%)				
	AD	0.18	0.36	1.8	3.6	7.2
RGR						
Day 0–5	0.36	0.35	0.35	0.35	0.35	0.33
	(0.004)	(0.011)	(0.007)	(0.011)	(0.008)	(0.011)
Day 10–15	0.31	0.27	0.31	0.30	0.33	0.28
	(0.013)	(0.026)	(0.016)	(0.036)	(0.009)	(0.016)
Diet effect, *P*	<0.001					
Age effect, *P*	<0.001					
Diet × age, *P*	<0.05					
Mortality						
Day 0–5	0.34	0.36	0.50	0.16	0.18	0.16
	(0.09)	(0.05)	(0.07)	(0.15)	(0.08)	(0.06)
Day 10–15	0.08	0.10	0.03	0.17	0.006	0.16
	(0.05)	(0.07)	(0.05)	(0.21)	(0.09)	(0.06)
Diet effect, *P*	<0.05					
Age effect, *P*	<0.001					
Diet × age, *P*	<0.001					

[a] SD given in parenthesis.

to this diet. In short, larvae avoided the diet with the highest concentration of catalposide, even when they had been reared on it.

The results from the toxicity/deterrence test were in general agreement with the results from the growth experiment. Catalposide appeared to have a toxic effect on both strains of larvae at all the concentrations tested (Figure 3A and B), since all the data points fell below the calibration one and outside the 95% confidence limits of the calibration line. However, there were some differences in the response of the two strains to catalposide. Larvae of the first strain responded to the highest concentration (7.2%) by an extremely high consumption rate (Figure 3A), whereas consumption rates of larvae of the second strain were in the same range at all catalposide concentrations (Figure 3B). Larvae of the second strain were more efficient at converting food into body mass, achieving higher RGRs for the same range of RCRs than the first strain did (Figure 3A and B).

TABLE 6. MEAN AMOUNTS (MG ± SD) OF ARTIFICIAL DIETS EATEN BY LAST-INSTAR LARVAE OF *S. eridania* IN CHOICE TESTS[a]

Diet prior to test	Choice		Mean amount eaten		N	P
	Diet 1	Diet 2	Diet 1	Diet 2		
AD	AD	AD + 7.2% C'side	19.6 ± 9.9	5.7 ± 9.4	10	<0.005
	AD	AD + 1.8% C'side	9.1 ± 7.2	8.9 ± 6.8	9	NS
AD + 1.8% C'side	AD + 1.8% C'side	AD	11.7 ± 9.7	8.6 ± 5.7	10	NS
	AD + 1.8% C'side	AD + 7.2% C'side	23.4 ± 36.4	2.1 ± 3.5	10	<0.05
AD + 7.2% C'side	AD + 7.2% C'side	AD	0.9 ± 1.0	10.1 ± 9.2	10	<0.025
	AD + 7.2% C'side	AD + 1.8% C'side	3.2 ± 4.7	8.9 ± 6.4	10	<0.05

[a] Results within each choice test were compared by means of paired t tests. AD = artificial diet, C'side = catalposide.

DISCUSSION

In this study, we used three methods to examine the response of the generalist herbivore, *Spodoptera eridania*, to iridoid glycosides: (1) We determined how the growth and survival of *S. eridania* were affected by qualitative and quantitative variation in the iridoid glycoside content of their diet. (2) We tested larval preference for or avoidance of diets containing different iridoid glycosides. (3) We tested whether the compounds were toxic or deterrent to the larvae. The results suggested that all three methods should be used in order to assess how a particular group of plant allelochemicals may or may not affect a particular insect species.

Qualitative Effects of Iridoid Glycosides. The growth of armyworm larvae was significantly depressed by three of the four iridoid glycosides tested (catalpol, aucubin, and loganin). Larvae grew slowest on catalpol, but growth was also depressed by aucubin and loganin. However, larvae grew almost as well on the diet containing asperuloside as on the control diet, and even for young larvae, mortality when fed on the diet with asperuloside was quite low (Table 2). These results suggested that at least three of the iridoids tested are either deterrent or toxic to armyworm larvae. However, the method of Blau et al. (1978) showed aucubin and asperuloside were toxic to armyworm larvae, while

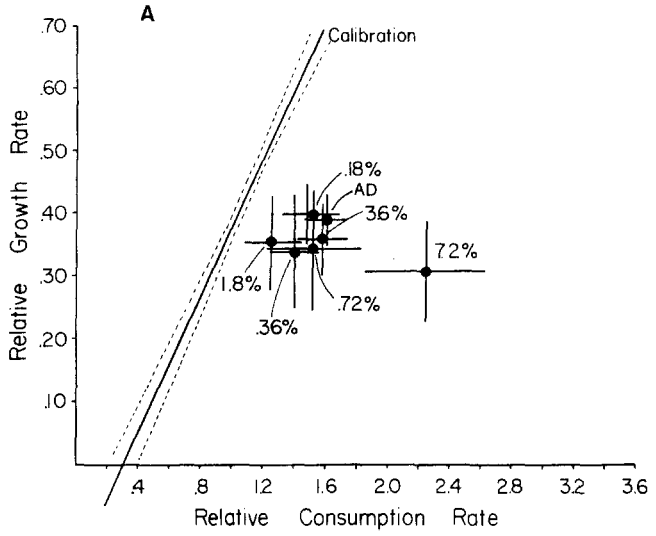

FIG. 3. (A) Relative growth rate (RGR) (mg/mg/day) and relative consumption rates (RCR) (mg/mg/day) of *Spodoptera eridania* larvae of the first strain (from Wisconsin) fed artificial diets containing different amounts of the iridoid glycoside catalposide. Each data point represents the mean and range of values for 20 larvae. (Calibration line: $y = -0.19x + 0.55$, $N = 34$, $r = 0.96$) (B) Relative growth rates (RGR) (mg/mg/day) and relative consumption rates (RCR) (mg/mg/day) of *Spodoptera eridania* larvae of the second strain (from South Carolina) fed artificial diets containing different amounts of the iridoid glycoside catalposide. Each data point represents the mean and range of values for 20 larvae. Means and ranges of diets with 0.36, 0.72, and 7.2%; and 3.6, and 5.4% catalposide, respectively, had values that were not different by more than 0.01 mg/mg/day; consequently, these values are shown as two single points to avoid confusion. (Calibration line: $y = 0.01x + 0.44$, $N = 40$, $r = 0.96$).

loganin and catalpol were not. This is in contradiction to the results from the longer-term growth experiment (Table 1). Short-term tests such as the toxicity/deterrence test of Blau et al. (1978) are often revealing and are very convenient, and it is important to be able to distinguish between deterrence and toxicity. However, longer-term tests such as the measurement of growth rates may yield results that reflect more accurately the effects of allelochemicals, even though long-term growth rates do not directly test whether the compounds are deterrent or toxic to larvae.

Larval age at which such tests measuring growth, consumption, and preference or avoidance are conducted may be important in the interpretation of the results, since developmental differences have been shown to exist among insects both in food preference and larval performance (Slansky and Scriber, 1985). A

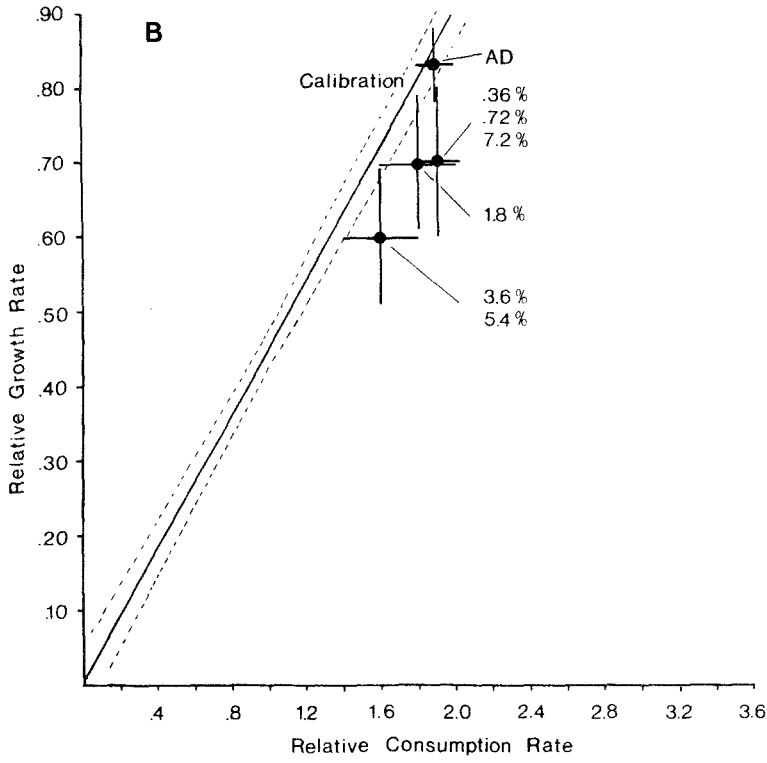

FIG. 3. Continued.

24- or 48-hr test on older larvae may not be a good indicator of the overall effects of a particular compound or group of compounds.

In general, one might expect that the RGR of younger larvae would be higher than that of older larvae (Slansky and Scriber, 1985), as we found. Although we expected the effect of different iridoids on RGR to be more apparent in earlier rather than in later instars, we found that the growth rates of smaller larvae on the different diets were not depressed relative to the control diet (Table 2). The very small differences among RGRs at 0–5 days may, in part, be due to the technical difficulties involved in quantifying small amounts of growth in very small larvae. Our experiments showed that mortality of young *S. eridania* on diets with iridoid glycosides was much higher than that of older larvae which had been feeding on these diets for 10–15 days, and also that the effects of different iridoids on mortality of young larvae were significant. In other words, the younger larvae were more susceptible to the effects of iridoid glycosides. One possible reason for this may be the lack of detoxification enzymes in the early stages. In particular, the mixed-function oxidase (MFO) system has been

implicated in protecting herbivorous insects from plant allelochemicals (Krieger et al., 1971; Brattsten et al., 1977; Ahmad, 1982). There have been no successful demonstrations of MFO activity in first- and second-instar larvae of any lepidopteran, implying that younger larvae may lack the enzymatic machinery necessary to detoxify certain compounds (Gould, 1984).

The results of the choice tests indicated that all four iridoid glycosides were deterrent to *S. eridania* larvae if they had not previously fed on diets with iridoid glycosides. However, the results of the toxicity/deterrence tests did not indicate that these compounds were deterrent. Interpreting the results of these two different tests is complicated by the fact that the presence of a choice situation appears to be critical: if larvae are not given a choice they will eat diets with iridoid glycosides, but they will avoid these compounds if given an alternative. The choice tests suggest that plants containing iridoid glycosides would be avoided by these larvae if alternative hosts were present. On the other hand, if larvae were first reared on diets containing iridoids, subsequent choice tests indicated no preference for diets either with or without iridoids, except in the case of loganin where larvae preferred the control diet (Table 3). Although a preference for iridoid glycosides was not induced (Jermy et al., 1968; De Boer and Hanson, 1984) by feeding on diets containing these compounds, neither did larvae avoid iridoid glycosides (with the exception of loganin). In a similar experiment using the same array of diets fed to another generalist lepidopteran, *L. dispar*, larvae initially made no distinction between diets with and without iridoid glycosides when previously reared on the control diet. However, *L. dispar* larvae significantly preferred diets with iridoid glycosides when they had previously been reared on these diets, even though iridoids decreased their growth and increased their mortality (Bowers and Puttick, 1988). The effects of iridoid glycosides may thus be quite different for different generalist species.

Quantitative Effects of Iridoid Glycosides. In general, the growth rate of larvae was depressed by the presence of the iridoid glycoside catalposide in the diet, and larvae avoided diets with the highest concentration of catalposide in the choice tests. There was not, however, a clear dosage-dependent response in the growth of larvae to increasing amounts of catalposide (Table 4). Low levels decreased growth, intermediate levels showed gradually increasing growth with increasing dosage, and the highest dosage (7.2%) dramatically reduced growth. The toxicity/deterrence test on the same strain used for the above growth and choice tests showed a greatly increased consumption rate at the highest dosage, while results at the lower dosages were closely clustered (Figure 3A). The higher consumption rate of larvae on the highest dosage may have occurred in an effort to maintain growth rate by increasing throughput or favoring rate over efficiency (Slansky and Feeny, 1977).

We are unable to explain why the results for larvae from this first strain fed on the control diet did not fall on the calibration line that was produced

using larvae fed on this same control diet. However, this necessitated our repeating the experiment with another group of larvae, which we obtained from another source. The two strains of armyworm performed quite differently in the toxicity/deterrence test. The second strain maintained a growth efficiency for a given consumption rate that was almost double that of the first strain of larvae, and the very high consumption rate observed for larvae of the first strain on 7.2% catalposide was not observed in the second strain. One implication of this for plant chemical defense is that plants may be subjected to a variable array of responses on the part of herbivores. Such variability may induce plants to change levels of defensive compounds in different parts of their range, depending on the particular herbivores present.

In summary, the results of these experiments suggest several important points regarding the experimental study of the effects of plant allelochemicals on insect herbivores. First, different species of generalists (and specialists), as well as different populations or strains of the same species, may be quite different in their response to a particular group of chemical compounds; such differences may confound our attempts to generalize about how plant allelochemicals might affect different groups of insects. Second, relatively small changes in chemical structure may have large effects on insect feeding responses. Aucubin and catalpol, for example, are very closely related biosynthetically (Inouye, 1971) and have very similar structures, yet these two compounds had very different effects on *S. eridania*. Finally, assessment of the effects of plant allelochemicals on herbivorous insects should involve not only tests of toxicity or deterrence, growth and mortality, or food choice alone, but a combination of these. Such a multifaceted approach should provide a more integrated view of how insects respond to plant allelochemicals.

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OXYGENATED MONOTERPENES PRODUCED BY YEASTS, ISOLATED FROM *Ips typographus* (COLEOPTERA: SCOLYTIDAE) AND GROWN IN PHLOEM MEDIUM[1]

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Abstract—When yeasts associated with *Ips typographus* beetles were grown in an aqueous phloem medium for two days, the main oxygenated monoterpenes produced were α-terpineol and borneol. Terpinene-4-ol, myrtenol, and *trans*-pinocarveol were also found but in lesser amounts. Of the six strains used in this study, *Hansenula capsulata* and *Candida nitratophila* produced the largest amounts of oxygenated monoterpenes. Addition of α-pinene to the phloem medium generally reduced the amounts of oxygenated monoterpenes, probably because this substance is toxic to all tested yeast species. Our *Candida diddensii* strain seemed to be particularly sensitive to α-pinene. None of the yeast strains produced *cis*-verbenol, *trans*-verbenol, or verbenone from the medium or from added α-pinene.

Key Words—Microbial transformations, microorganisms, yeasts, bark beetle, Coleoptera, Scolytidae, *Ips typographus*, oxygenated monoterpenes, α-terpineol, borneol.

INTRODUCTION

Yeasts are commonly associated with bark beetles (Shifrine and Phaff, 1956; Callaham and Shifrine, 1960; Whitney, 1971), but it is uncertain what role they play in the attack sequence of tree-killing bark beetles (Bridges et al., 1984). It

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is generally supposed that beetles inoculate a tree with different fungi in order to overcome the tree's resistance to attack (Whitney, 1982). One line of defense that the trees have against a bark beetle–fungus attack is a resin containing toxic terpenoids; the beetles have adapted to this by detoxifying oxygenation of some of the compounds (Hughes, 1973). In view of the general occurrence of mixed function oxidases (White et al., 1980), it is reasonable to believe that fungi and bacteria possess a similar detoxification system. A *Bacillus cereus* strain isolated from the North American bark beetle *Ips paraconfusus*, could convert α-pinene to *trans*-verbenol, *cis*-verbenol, and probably myrtenol (Brand et al., 1975). The verbenols are among those substances known as attractants for many bark beetle species, but in view of other findings (Byers and Wood, 1981; Conn et al., 1984), it is still uncertain how microorganisms are involved in the production of pheromones.

In our work with the spruce bark beetles, *Ips typographus* (L.), and its associated yeasts, we found that some of the yeasts could convert the beetles' aggregative pheromone component *cis*-verbenol to verbenone (Leufvén et al., 1984), a substance suspected to have antiaggregative properties (Bakke, 1981). The oxygenated monoterpenes found by Birgersson et al. (1984) in guts of *I. typographus* beetles attacking a Norway spruce (*Picea abies*, Karst.), were both qualitatively and proportionally different from those found in the gallery walls surrounding the beetles (Leufvén and Birgersson, 1987). There is a striking similarity between the shape of the curves depicting the varying amounts of certain oxygenated monoterpenes found in phloem surrounding *I. typographus* beetles from different attack phases (Leufvén and Birgersson, 1987) and the curve showing the total number of colony-forming units (produced on Sabouraud medium) originating from beetles in different attack phases (Leufvén and Nehls, 1986).

In the present study, we have investigated the ability of *I. typographus* associated yeasts to produce, from a water extract of spruce phloem, some of the oxygenated monoterpenes found in phloem from the walls of bark beetle galleries. To see which oxygenated monoterpenes, if any, the isolated yeast strains could produce from the pheromone precursor α-pinene under our *in vitro* conditions, we added this substance to some of the incubations.

METHODS AND MATERIALS

The yeast strains used in this study were *Candida diddensii* (CCUG 11142), *Candida nitratophila* (CCUG 11139), *Cryptococcus albidus* var. *diffluens* (CCUG 11141), *Cryptococcus laurentii* var. *magnus* (CCUG 11125, now reclassified as *Cryptococcus heveanensis*), *Hansenula capsulata* (CCUG 11140),

Hansenula holstii (CCUG 11128) and *Pichia pinus* (CCUG 13846). All of these strains were isolated from natural Swedish populations of *Ips typographus* bark beetles. The strains, which are deposited with the Culture Collection University of Göteborg (CCUG), have also been used in previous studies (Leufvén et al., 1984; Leufvén and Nehls, 1986).

All incubations were made in aqeuous phloem and bark medium prepared in the following way. Phloem and bark of Norway spruce were cut in centimeter-size pieces and extracted for 4 hr in tap water at room temperature; approximately 250 g phloem and bark were used per liter of water. After removal of the solid material by filtration through coarse paper, the medium was frozen in 0.5-liter bottles and stored until used. After thawing and autoclaving, the phloem medium had a pH just below 6.

Cotton-wool-stoppered, 250-ml Erlenmeyer flasks with 50 ml of phloem medium were used for all incubations. After being heavily inoculated with yeasts from Sabouraud's solid medium, the culture flasks were kept at room temperature and continually agitated for two days. At the end of the incubation period, two 4-ml aliquots were transfered to screw-capped centrifugation tubes. To facilitate extraction, yeast cells were removed by centrifugation; in a preliminary experiment, only very small amounts of extractable oxygenated monoterpenes were found in the yeast pellet. Each of the two supernatants were extracted three times with a total of 2.5 ml of a mixture of 10% diethyl ether in pentane. The extracts of the two supernatants were combined, and 2.5 μg of heptyl acetate in hexane were added as a standard.

In some experiments, racemic α-pinene (EGA-Chemie) dissolved in ethanol was added during the incubation. At the time of inoculation, 5 mg of α-pinene was added, followed by another 10 mg after one day of incubation. The total volume of added ethanol was 300 μl. Prior to its use, the α-pinene was passed through a short, heat-activated, silica column in order to remove oxygenated monoterpenes. Camphene and β-pinene, 0.56% and 1.22%, respectively, were present as impurities in the α-pinene used. Phloem medium without inoculated yeasts was used as a control in all incubation experiments.

The ether–pentane extracts were concentrated by evaporation and analyzed on a Finnigan 4021 GC-MS system equipped with a 25-m fused silica column, ID 0.15 mm, coated at the Department of Chemical Ecology with a 0.30-μm-thick film of Superox FA (RSL). The initial temperature in the gas chromatograph was 50°C for 4 min, followed by an increase of 8°C/min up to 200°C and then kept isothermal. Compounds were quantified by measuring the area of prominent and/or typical mass spectral fragments and comparing the calculated total peak area with that of added heptyl acetate (cf. Leufvén and Birgersson, 1987). The mass fragments used for each compound are shown in Figures 1 to 3.

RESULTS

The major oxygenated monoterpenes found in the phloem medium after the two-day incubation period were α-terpineol, borneol, terpinene-4-ol, *trans*-pinocarveol, myrtenol, and an unidentified alcohol. The amounts of these major components, per 50 ml of medium, are presented in Figures 1 and 2. The bars represent the means from three different incubations, except the controls in which five (no α-pinene added) or six (α-pinene added) incubations were carried out.

Under the in vitro conditions used in this study, our *Candida nitratophila* and *Hansenula capsulata* strains produced the largest amounts of oxygenated monoterpenes. *Candida diddensii*, *Hansenula holstii*, and *Pichia pinus* also produced relatively large amounts of certain of these monoterpenes, whereas *Cryptococcus albidus* var. *diffluens* and *Cryptococcus laurentii* var. *magnus* usually only produced minor amounts.

The main product after incubation in the phloem medium was α-terpineol (Figure 1), especially when the *C. nitratophila* strain was used. However, if α-pinene was not added, the amounts produced by this strain, per 50 ml medium, varied greatly: i.e., from 60 to 250 μg. Large amounts of α-terpineol were also produced by *H. capsulata* but quantities varied largely for this strain as well.

The compound found in the second largest amounts was borneol (Figure 1), the largest amounts of which were produced by *H. capsulata* followed by *C. nitratophila* and *C. diddensii*. The amounts of borneol produced by the different yeast strains was partly paralleled, but on a smaller scale, by the amounts of *trans*-pinocarveol produced (Figure 2). Terpinene-4-ol (Figure 2) was found as a major oxygenated monoterpene, especially after incubations with *H. capsulata*, but was also present in relatively large amounts in control incubations without yeasts. Myrtenol and an unidentified monoterpene alcohol (having 79 and 93, respectively, as characteristic mass spectral fragments) were produced in incubations with *H. capsulata*, in amounts of 5–10 μg/50 ml of medium. The unidentified terpene was also produced in incubations with *C. nitratophila* (Figure 2).

None of the yeast strains used could produce *cis*-verbenol, *trans*-verbenol, and verbenone, substances which participate in regulation of bark beetle behavior, from compounds present in the phloem medium or from the added α-pinene. Verbenone was detected in amounts of up to 2 μg/50 ml medium, but there were no differences between incubations with and without yeasts (Figure 3).

Small amounts of a nonterpenoid compound, 2-phenyl-ethanol, were produced by some of the yeast strains, particularly *H. holstii*, *H. capsulata*, and *C. nitratophila* (Figure 3).

In addition to the above-mentioned compounds, minor amounts of several

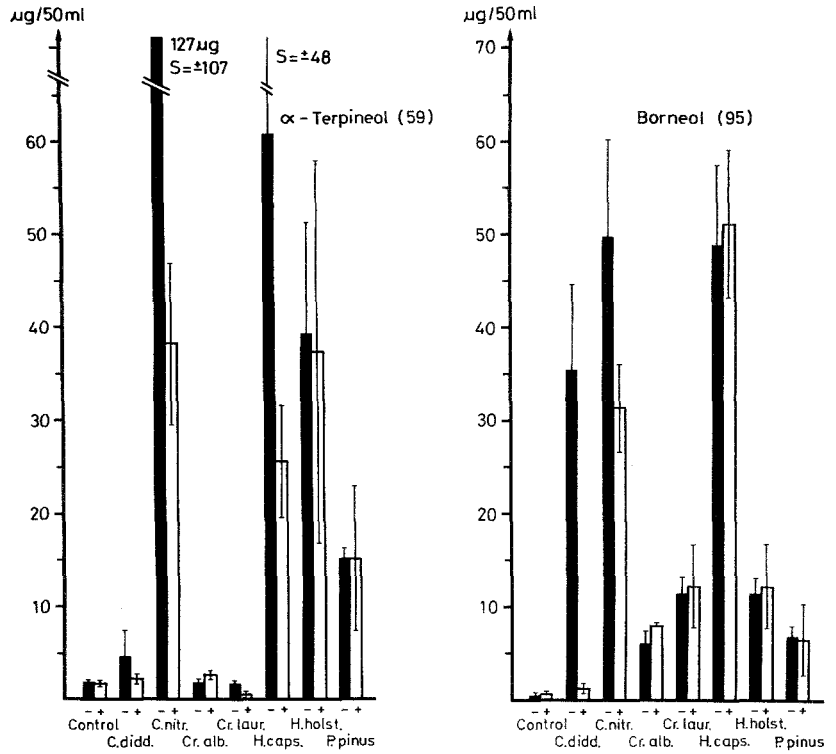

FIG. 1. Amounts of α-terpineol and borneol extracted from 50 ml of phloem medium inoculated with different strains of yeasts and incubated for two days with (solid bars) or without (open bars) added α-pinene. Thin vertical bars represent standard deviation. The mass fragment used for quantification is shown in parentheses after the compound name.

other substances were detected in the extracts from some of the inoculated incubations. Myrtenal and perilla alcohol were among those compounds, as was an unidentified substance with a mass spectrum closely resembling borneol (large *m/z* 95 fragment) but with a retention time similar to that of myrtenol. The two unidentified compounds reported from *I. typographus* gallery phloem, called monoterpene alcohol A and B (*m/z* 71 and 93) by Leufvén and Birgersson (1987), were also detected in the phloem medium after incubation with several of the yeast strains, i.e., *C. nitratophila*, *H. capsulata*, *H. holstii*, and *P. pinus*.

In general, the amounts of oxygenated monoterpenes produced in incubations to which racemic α-pinene was added (open bars in the figures) were lower than those produced in incubations without α-pinene. The most obvious examples are the production of borneol (Figure 1), *trans*-pinocarveol, myrtenol,

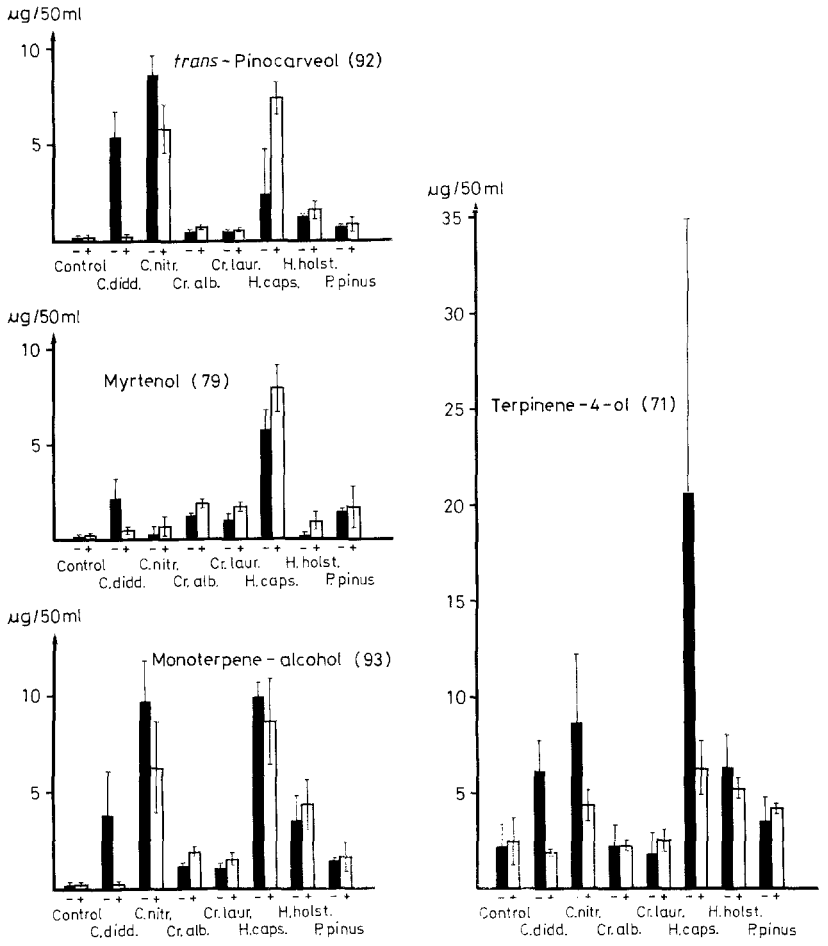

FIG. 2. Amounts of terpinene-4-ol, *trans*-pinocarveol, myrtenol, and an unidentified monoterpene alcohol extracted from 50 ml of phloem medium inoculated with different strains of yeasts and incubated for two days with (solid bars) or without (open bars) added α-pinene. Thin vertical bars represent standard deviation. The mass fragment used for quantification is shown in parentheses after the compound name.

and the unidentified monoterpene alcohol, quantified on mass spectral fragment *m/z* 93 (Figure 2), by *C. diddensii*. Contrary to this general trend, larger amounts of *trans*-pinocarveol and myrtenol were found after incubation of *H. capsulata* in phloem medium in which α-pinene was added than were found after incubation of this yeast strain in medium to which α-pinene had not been added.

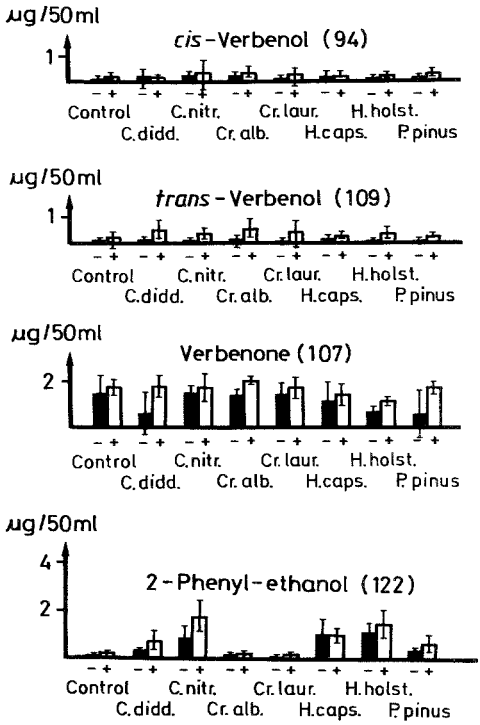

FIG. 3. Amounts of *cis*-verbenol, *trans*-verbenol, verbenone, and 2-phenylethanol extracted from 50 ml of phloem medium inoculated with different strains of yeasts and incubated for two days with (solid bars) or without (open bars) added α-pinene. Thin vertical bars represent standard deviation. The mass fragment used for quantification is shown in parentheses after the compound name.

DISCUSSION

The main oxygenated monoterpenes found in phloem medium after incubation with yeasts isolated from the spruce bark beetle were α-terpineol and borneol. These two compounds were also found as the major oxygenated monoterpenes in phloem surrounding spruce bark beetle galleries in an attacked Norway spruce (Leufvén and Birgersson, 1987). Terpinene-4-ol, *trans*-pinocarveol, myrtenol, *trans*-verbenol, and verbenone were also found in relatively large amounts in the phloem walls and, with the exception of *trans*-verbenol and verbenone, these compounds were also produced by some of the yeasts used in the present study. The failure of our strains to produce the verbenols and verbenone could, of course, be explained by incorrect in vitro conditions,

but we assume that the isolated yeast strains do not produce the verbenols, at least not from compounds present in, or added to, the incubation medium. The small amounts of verbenols and verbenone actually detected were probably autoxidation products from terpene hydrocarbons. This is well in accordance with the similarity in the amounts of verbenone found in all yeast incubations and controls (Figure 3). The inability of our beetle-associated yeast strains to produce verbenols and/or their previously reported ability to convert verbenol to verbenone (Leufvén et al., 1984) agrees with the findings of Conn et al. (1984), who demonstrated that axenic beetles exposed to α-pinene vapors had more verbenols in their guts than septic beetles treated in the same way. On the other hand, a bacterial strain isolated from bark beetles has been used to produce both *cis*- and *trans*-verbenol from α-pinene (Brand et al., 1975).

The origin of the oxygenated monoterpenes found in the phloem medium after incubation is still unclear. In this study, preparation of the medium by water extraction and subsequent sterilization by autoclaving probably excluded all monoterpene hydrocarbons from the incubation medium. Preliminary chromatographic investigations of the medium did not indicate the presence of any monoterpene hydrocarbons. Larger terpenoids that may have been present in the medium could have been precursors of the oxygenated monoterpenes, but this possibility has not been investigated.

Addition of α-pinene to the incubation medium usually failed to increase the amounts of oxygenated monoterpenes present after incubation. The production of *trans*-pinocarveol, and possibly myrtenol, by *H. capsulata* are exceptions. However, in most cases, the addition of α-pinene probably had a more or less toxic effect, especially in some of the incubations with *C. diddensii*. The decrease in production is not surprising, since it is known that monoterpene hydrocarbons are somewhat toxic, even to fungi associated with bark beetles (Raffa et al., 1985; DeGroot, 1972). In our experiments with the addition of α-pinene to *H. capsulata* incubations, the production of *trans*-pinocarveol, and possibly myrtenol, from α-pinene increased, while the production of α-terpineol and terpinene-4-ol from unknown sources decreased. This change in priority of production makes it very tempting to suggest that in order to detoxify α-pinene, *H. capsulata* converts it to *trans*-pinocarveol and possibly myrtenol. In view of the fact that *H. holstii* and *C. diddensii* were the yeasts isolated in the largest numbers from *I. typographus* beetles (Leufvén and Nehls, 1986), it is surprising that *C. diddensii* seems to be the yeast strain less suited to cope with the toxic properties of α-pinene. The major producers of oxygenated monoterpenes, *H. capsulata* and *C. nitratophila* were both bark beetle associates isolated in lesser numbers by Leufvén and Nehls (1986). The reason for this discrepancy concerning production ability and numbers isolated is not clear, but one has to bear in mind that conditions in the natural environment of the yeasts and during *in vitro* incubations are inevitably different and that the yeast

strains isolated by Leufvén and Nehls (1986) came from crushed beetles, not from gallery wall phloem.

The nonterpenoid compound 2-phenylethanol was not detected in gallery wall phloem (Leufvén and Birgersson, 1987), but it was found in bark beetle guts (Birgersson et al., 1984) and was found to be produced by yeasts, especially *H. holstii*, growing in synthetic Sabouraud medium (Leufvén et al., 1984), and in a phloem medium from pine (Brand et al., 1977). It is not yet evident whether or not this compound plays a part in the "attack dynamics" of the bark beetle–microorganism complex, although this has been suggested (Renwick et al., 1976).

Despite the fact that *in vitro* incubations have been used, this study shows that some of the compounds found in phloem from gallery walls of *I. typographus* beetles attacking Norway spruce can be produced by yeasts associated with the bark beetle. The actual precursors of the oxygenated monoterpenes are, however, not revealed by this study.

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USE OF PREDATOR ODORS AS REPELLENTS TO REDUCE FEEDING DAMAGE BY HERBIVORES. III. Montane and Meadow Voles (*Microtus montanus* and *Microtus pennsylvanicus*)

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Abstract—This study investigated the influence of the major anal-gland compounds from the stoat (*Mustela erminea*) and fecal and urine compounds from the red fox (*Vulpes vulpes*) in generating an avoidance response by montane voles (*Microtus montanus*), as well as suppressing feeding by montane and meadow (*M. pennsylvanicus*) voles on apple trees in orchards. In trap bioassays, a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane significantly reduced vole captures. Other mixtures of stoat compounds reduced the number of new voles captured but not total individuals. 2,5-Dihydro-2,4,5-trimethylthiazoline, a component of fox feces, significantly reduced vole captures in one of two bioassays. Deer mice (*Peromyscus maniculatus*) did not show a negative response to any predator odor. In overwinter field bioassays, mixtures of 2-propylthietane and 3-propyl-1,2-dithiolane clearly reduced vole feeding on apple trees in four test blocks. 2,5-Dihydro-2,4,5-trimethylthiazoline and a synthetic fox urine mixture also significantly reduced vole attack in respective orchard blocks. Similarly, the intensity of vole feeding, in terms of amount of bark and vascular tissues removed from trees, was reduced by 60% to 97% in predator odor treatments compared with the control. Our study reports the first long-term (four to five months) use of synthetic semiochemicals as area repellents for crop protection from vole feeding damage.

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Key Words—Anal-gland compounds, 2-propylthietane, 3-propyl-1,2-dithiolane, stoat, vole, red fox urine, feces, 2,5-dihydro-2,4,5-trimethylthiazoline, crop protection, feeding suppression, orchard, interspecific communication, kairomones, predator odors, *Microtus* spp., *Vulpes vulpes*, mustelids.

INTRODUCTION

The use of chemical signals may have several applications in wildlife management and crop protection. In particular, preparations of predator odors have significantly reduced feeding by herbivore species such as snowshoe hares (*Lepus americanus*) (Sullivan et al., 1985a; Sullivan, 1986) and black-tailed deer (*Odocoileus hemionus columbianus*) (Müller-Schwarze 1972, 1983; Melchiors and Leslie, 1984; Sullivan et al., 1985b). The synthetically produced active components of certain predator odors have successfully reduced feeding damage to forest seedlings by snowshoe hares (Sullivan and Crump, 1984, 1986a) and generated an avoidance response by northern pocket gophers (*Thomomys talpoides*) in an agricultural environment (Sullivan and Crump, 1986b). Similarly, several stress-inducing odorants from fox (*Vulpes vulpes*) feces have been reported for the rat (Vernet-Maury et al., 1984).

Several species of voles (genus *Microtus*) inflict feeding damage to agricultural and forest crops in various parts of the world. These herbivorous rodents feed on the bark, vascular tissues, and sometimes roots of trees in fruit orchards and coniferous and deciduous forest plantations as well as vegetable, grain, and forage crops in cultivated fields (see reviews by Hansson and Nilsson, 1975; Green, 1978; Byers, 1984, 1985; Hansson, 1985). The use of toxicants has been the major method to control voles. However, the advent of predator odors as area repellents may provide effective protection for forest and agricultural crops from vole depredations.

Avoidance responses of voles (*M. agrestis*) to predator odors, particularly those of the weasel (*Mustela nivalis*) and stoat (*M. erminea*), were reported in several studies (Stoddart, 1976, 1980; Gorman, 1984). These responses are innate and presumably adaptive since the chances of an individual vole suffering predation would be reduced (Gorman, 1984). Stoats (also called ermine or short-tailed weasels) and related mustelids prey on voles throughout their ranges in north temperate and boreal latitudes. In addition, the red fox is another principal predator of voles and related rodents.

The chemical composition of anal gland secretions has been reported for the stoat (Crump 1978, 1980; Schildknecht and Birkner, 1983; Crump and Moors, 1985). The major sulfur-containing compounds identified in these studies were 2-propylthietane, 2-pentylthietane, and 3-propyl-1,2-dithiolane. Similarly, the most active volatile component of fox feces was identified as 2,5-dihydro-2,4,5-trimethylthiazoline (Vernet-Maury, 1980; Vernet-Maury et

al., 1984), and in fox urine was 3-methyl-3-butenyl methyl sulfide (Wilson et al., 1978; Whitten et al., 1980; Sullivan and Crump, 1986a).

This paper reports on the influence of the major anal-gland compounds from the stoat and the fecal and urine compounds from the fox in generating an avoidance response by montane voles (*M. montanus*), as well as suppressing feeding by montane and meadow (*M. pennsylvanicus*) voles on apple trees in orchards.

METHODS AND MATERIALS

Predator Odor Compounds. Anal-gland compounds from the stoat, 2-propylthietane, 2-pentylthietane, and 3-propyl-1,2-dithiolane, were prepared according to Crump (1978, 1980, 1982). Two other constituents, indole and *o*-aminoacetophenone, were available commercially. In an attempt to simulate the anal-gland secretion of the stoat, one group of compounds (2-propylthietane, 3-propyl-1,2-dithiolane, and indole) was mixed with squalene (on a 1:1 basis) in a 16:1:4 ratio (mixture *X*). A second group of compounds (2-propylthietane, 3-propyl-1,2-dithiolane, indole, and *o*-aminoacetophenone) was mixed in a 7:1:0.1:0.6 ratio (mixture *Y*). These two ratios approximate those found in stoats in the wild. A third group of compounds (2-propylthietane, 2-pentylthietane, and 3-propyl-1,2-dithiolane) was mixed in a 4.5:1:1 ratio (mixture *Z*). In addition, 2-propylthietane and 3-propyl-1,2-dithiolane were mixed in a 1:1 ratio.

The principal active component from fox feces, 2,5-dihydro-2,4,5-trimethylthiazoline, was prepared by bubbling ammonia (gas) into a stirred mixture of 3-mercaptobutan-2-one (Hromatka and Haberl, 1954) (7.8 g), acetaldehyde (11 g), and anhydrous sodium sulfate (8 g) in ether (50 ml) for 20 min at room temperature and then for a further 10 min with heating under reflux (Dubs and Pesaro, 1974). The mixture was cooled, filtered, and concentrated. Distillation of the residue gave *cis*- and *trans*-2,5-dihydro-2,4,5-trimethylthiazoline, 5 g (57%), bp 69-72°/18 mm. Mass spectrum: 129 (52 114 (30) 88 (100) 69 (65) 55 (53) 42 (85). PMR ($CDCl_3$) 1.5 (m, 6, C_2 and C_5 methyls), 2.1 (2s, 3, C_4 methyls), 4.3 (1, m, H_5), 5.5 (1, m, H_2). The NMR spectrum was identical with that of authentic material (Mussinan et al., 1977).

The principal volatile component from red fox urine, 3-methyl-3-butenyl methyl sulfide, was prepared according to Wilson et al. (1978). 2-Phenylethyl methyl sulfide was prepared by alkylation of the corresponding thiol (von Braun et al., 1929), which was obtained from 2-phenylethanol via the Bunte salt method of El-Hewehi and Taeger (1958). An undiluted synthetic mixture of these latter compounds and the commercially available 4-heptanone, 6-methyl-5-hepten-2-one, benzaldehyde, acetophenone, 2-methylquinoline, and gerany-

lacetone was prepared according to the composition of compounds reported by Wilson et al. (1978) and Whitten et al. (1980). This mixture was composed of pure compounds only with no odor fixative. Purity of synthesized compounds ranged from 95% to 98%.

All compounds and mixtures were dispensed in 140-μl capillary tubes (75 mm in length). The capillary tubes protected the compounds from adverse weather conditions and, for optimum evaluation purposes, maintained the respective odor around the entrance to live-traps in the trap bioassays and around the base of apple trees in the tree bioassays. In the trap bioassays, three or four tubes were anchored in the ground near the entrance to a trap. In the tree bioassays, one capillary tube was attached to young (1-year-old) trees and two tubes were attached to large older (15–30 years) trees by plastic twist ties. Approximately 30 mg of each compound (neat) or test mixture was placed in a given capillary tube using a 1-ml microsyringe with 20-gauge needle. In all bioassays, empty capillary tubes were available as controls to complete the design of a given experiment.

Trap Bioassays. Bioassays were conducted at the Agriculture Canada Research Station, Summerland, British Columbia, Canada, during fall periods from September 1983 to October 1985. The study area was located in old field grassland habitat composed mainly of crested wheatgrass (*Agropyron cristatum*) with several herbaceous annuals in minor abundance. The climate is semiarid and so the study area was irrigated at three-week intervals during summer months to maintain a suitable habitat for voles. Deer mice (*Peromyscus maniculatus*) also occupied this habitat in numbers substantial enough to warrant recording in the bioassays. Yellow pine chipmunks (*Eutamias amoenus*) and Great Basin pocket mice (*Perognathus parvus*) also were present but in minor abundance and were rarely captured during bioassays.

Montane voles were live-trapped on checkerboard grids with Longworth live-traps. Separate control and treatment grids were used in each of three experiments. In experiment A, each grid had 15 (3 $\times$ 5) trap stations, and in experiments B and C, each grid had 18 (3 $\times$ 6) stations, located at 15.2-m intervals. Two live-traps were set at each station near recently used vole runways. Traps were baited with whole oats, and coarse brown cotton was supplied as bedding. In all experiments, traps were set on the afternoon of day 1, checked on the mornings of days 2 and 3, and then removed from the grids. Trapping periods were conducted at three- or four-week intervals in experiment A on September 26–28, October 22–24, and November 7–9, 1983; in experiment B on September 3–5, 25–27, and October 22–24, 1984; in experiment C on September 8–10, 28–30, and October 19–21, 1985.

Traps were cleaned thoroughly before trapping commenced, and there were no periods of prebaiting (i.e., traps locked open and set in field) during these experiments. For control periods prior to experiments, locked traps were set in

the field to prebait with subsequent trapping to monitor vole populations on respective control and treatment grids. All voles captured were ear-tagged with serially numbered tags, sex and breeding condition noted, weighed on Pesola spring balances, and point of capture recorded.

The following compounds were tested: a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane in experiment A; mixture Y of stoat anal-gland compounds, and 2,5-dihydro-2,4,5-trimethylthiazoline in experiment B; mixture Z of stoat anal-gland compounds, and 2,5-dihydro-2,4,5-trimethylthiazoline in experiment C.

Tree Bioassays. Each of two blocks (each 560 m^2 in area and separated by 100 m) was planted with 48 (6 × 8) 1-year-old crabapple (*Malus* spp.) trees on November 11–12, 1983 in the grassland study area at Summerland Research Station. One block served as a control and the other was treated with a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane. Comparable densities (110 per hectare) of voles were present in each block. Vole feeding damage to trees (proportion girdled) was checked on December 1, 1983, February 2, and finally on March 2, 1984, when the experiment was terminated. Voles were no longer feeding on the apple trees as preferred alternative vegetation had begun to appear.

In a second experiment, a 1-hectare block of Spartan apple orchard (30-year-old trees with interplanted rows of 15-year-old trees at an overall spacing of 5.6 × 4.6 m) at the Coldstream Ranch, 12 km east of Vernon, British Columbia, was assigned treatments as follows: 100 (10 × 10) trees with one tube of 2-propylthietane and one tube of 3-propyl-1,2-dithiolane attached to opposite sides of the base of each tree; 50 (5 × 10) trees with the mixture X of stoat anal-gland compounds; 100 (10 × 10) trees with 2,5-dihydro-2,4,5-trimethylthiazoline on every second tree; 50 (5 × 10) trees with the synthetic fox urine mixture on every second tree; 40 (4 × 10) trees with 3-methyl-3-butenyl methyl sulfide on every second tree; and 50 (5 × 10) trees as controls. This block had an average density of 80–90 voles per hectare. Treatments were dispensed on November 11, 1983, and feeding damage (proportion of trees attacked) by montane and meadow voles was checked on February 4 and March 20, 1984. The experiment was then terminated. The area of bark and vascular tissues removed by vole attack may be considered as a measure of feeding intensity. The height and width of each damage wound were recorded to give an approximate measurement of the amount of bark and vascular tissues removed from a given tree.

In the third experiment, each of three blocks (each 560 m^2 in area and separated by 50 m) was planted with 48 (6 × 8) 2-year-old crabapple trees on October 25, 1985, in old field grassland habitat adjacent to the Coldstream Ranch orchard at Vernon, British Columbia. One block served as a control, the second block had a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane,

and the third block had 2,5-dihydro-2,4,5-trimethylthiazoline. This block had an average density of 60–70 voles per hectare. Feeding damage to trees (proportion attacked) was checked on March 31, 1986, at which time the experiment was terminated.

Statistical Analysis. Comparisons of the number of individuals and total captures of montane voles and deer mice, and number of trees surviving (Summerland) and attacked (Vernon), on control and treatment areas during each experiment were analyzed by chi-square with significance levels of $P < 0.05$ and $P < 0.01$.

RESULTS

Trap Bioassays. The 1:1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane significantly reduced the number of voles captured in traps in experiment A (Table 1). This reduction was evident in both number of individuals caught and total captures. In terms of individuals on the treatment grid, 77.4% (31 to 7) fewer voles were captured in the trapping period with the anal-gland mixture compared with the preceding control period. In addition, vole numbers rebounded dramatically to 33 animals during the subsequent control period. In experiment B, the number of voles captured was reduced by the presence of the stoat anal-gland compounds, but these differences were not statistically significant (Table 1). However, the presence of 2,5-dihydro-2,4,5-trimethylthiazoline at trap entrances did result in significant avoidance of traps by voles during the first night and for total captures. There was little difference between control and treatment grids in vole response to traps for 2,5-dihydro-2,4,5-trimethylthiazoline and mixture Z in experiment C.

There were no significant differences between the proportions of male and female voles captured on control and treatment grids in these three experiments (Table 2). Similarly, the proportion of breeding male and female voles differed little between control and treatment areas. Significantly more males were in reproductive condition on the treatment than control grid in the control period of experiment B. However, this difference was not related to any of the predator odor treatments. The number of voles captured for the very first time (i.e., previously untagged) was significantly reduced on the treatment grid with mixtures of stoat anal-gland compounds in experiments B and C. There was no difference in proportion of new voles between control and treatment for the 1:1 ratio of 2-propylthietane and 3-propyl-1,2-dithiolane.

Table 3 lists the number of deer mice captured during the trapping periods within each experiment. Deer mice did not avoid any of the predator odors when comparing control and treatment captures. In addition, there were no differences between the proportions of male and female deer mice captured, those in breeding condition, or those newly caught on control and treatment grids.

TABLE 1. NUMBER OF MONTANE VOLES CAPTURED ON CONTROL AND TREATMENT GRIDS FOR EACH OF TWO CONSECUTIVE NIGHTS DURING EACH OF THREE TRAPPING PERIODS WITHIN A GIVEN EXPERIMENT[a]

Experiment + trapping period	Control					Treatment				
	1st night	2nd night			Total indiv. (total caps)	1st night	2nd night			Total indiv. (total caps)
		Non-recap		Recap			Non-recap		Recap	
Experiment A										
Control	19	3	+	15	22(37)	25	6	+	8	31(39)
2-PT + 3-PDIT	17b	4	+	16	21c(37)d	3b	4	+	0	7c(7)d
Control	21	2	+	13	23(36)	24	9	+	6	33(39)
Experiment B										
Control	20	5	+	9	25(34)	15	7	+	11	22(33)
Mixture Y	21	17	+	9	38(47)	16	12	+	8	28(36)
TTH	17e	16	+	12	33(45)a	4e	17	+	4	21(25)a
Experiment C										
Control	10	5	+	5	15(20)	10	5	+	9	15(24)
TTH	8	3	+	6	11(17)	6	5	+	4	11(15)
Mixture Z	8	6	+	6	14(20)	9	2	+	5	11(16)

[a] a-a, $P < 0.05$; b-b, c-c, d-d, e-e, $P < 0.01$; significant difference by chi-square. 2-Propylthietane (2-PT), 3-propyl-1,2-dithiolane (3-PDIT), mixture Y (7:1:0.1:0.6), mixture Z (4.5:1:1), 2,5-dihydro-2,4,5-trimethylthiazoline (TTH).

TABLE 2. NUMBER OF INDIVIDUAL MALE AND FEMALE MONTANE VOLES, PROPORTION BREEDING, AND PROPORTION OF NEW VOLES (BOTH MALES AND FEMALES) IN PARENTHESES, CAPTURED ON CONTROL AND TREATMENT GRIDS FOR EACH TRAPPING PERIOD WITHIN A GIVEN EXPERIMENT[a]

Experiment + trapping period	Control				Treatment			
	Males	Prop. breed.	Females	Prop. breed	Males	Prop. breed.	Females	Prop. breed.
Experiment A								
Control	8	0.75	14	0.43 (0.18)	7	0.71	24	0.25 (0.29)
2-PT + 3-PDIT	5	0.80	16	0.38 (0.14)	2	0.00	5	0.00 (0.14)
Control	9	0.33	14	0.07 (0.22)	11	0.09	22	0.00 (0.30)
Experiment B								
Control	9	0.44b	16	0.44 (0.28)	11	0.91b	10	0.40 (0.38)
Mixture Y	19	0.53	19	0.58 (0.58)c	12	0.75	16	0.81 (0.25)c
TTH	10	0.30	22	0.05 (0.34)	12	0.33	9	0.00 (0.29)
Experiment C								
Control	7	1.00	8	0.63 (0.27)	5	1.00	10	0.60 (0.20)
TTH	4	1.00	7	0.86 (0.18)	3	1.00	7	0.86 (0.10)
Mixture Z	5	0.60	9	0.78 (0.29)a	3	1.00	8	0.88 (0.00)a

[a] a-a, $P = 0.05$; b-b, $P < 0.05$; c-c, $P < 0.01$; significant difference by chi-square. 2-Propylthietane (2-PT), 3-propyl-1,2-dithiolane (3-PDIT), mixture Y (7:1:0.1:0.6), mixture Z (4.5:1:1), 2,5-dihydro-2,4,5-trimethylthiazoline (TTH).

Tree Bioassays. Results of the bioassay with young apple trees at Summerland during the 1983–1984 winter are illustrated in Figure 1A. The 1:1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane clearly reduced vole feeding on trees to a significant degree in the treatment block. Snow cover from December 15 to January 20 may have reduced the effectiveness of the odor as voles, via snow tunnels, were able to feed above the protective zone of the stoat odor. However, as of March 1984, only 16.7% of control trees had not been girdled by voles compared with 80.4% of treatment trees.

The results of experiments conducted at Vernon during the 1983–1984 winter are illustrated in Figures 1B–D. The stoat anal gland mixture X was the most effective in suppressing vole feeding. The compounds 2-propylthietane

TABLE 3. NUMBER OF DEER MICE CAPTURED ON CONTROL AND TREATMENT GRIDS FOR EACH OF TWO CONSECUTIVE NIGHTS DURING EACH OF THREE TRAPPING PERIODS WITHIN A GIVEN EXPERIMENT[a]

Experiment + trapping period	Control					Treatment				
	1st night	2nd night			Total indiv. (total caps)	1st night	2nd night			Total indiv. (total caps)
		Non-recap		Recap			Non-recap		Recap	
Experiment A										
Control	6	3	+	5	9(14)	7	6	+	4	13(17)
2-PT + 3-PDIT	10	0	+	5	10(15)	9	5	+	7	14(21)
Control	9	1	+	7	10(17)	9	4	+	3	13(16)
Experiment B										
Control	12	6	+	6	18(24)	11	2	+	6	13(19)
Mixture Y	8	2	+	5	10(15)	9	1	+	8	10(18)
TTH	5	3	+	4	8(12)	6	1	+	2	7(9)
Experiment C										
Control	9	4	+	5	13(18)	5	1	+	2	6(8)
TTH	7	5	+	6	12(18)	7	2	+	7	9(16)
Mixture Z	15	4	+	9	19(28)	7	4	+	7	11(18)

[a]2-Propylthietane (2-PT), 3-propyl-1,2-dithiolane (3-PDIT), mixture Y (7:1:0.1:0.6), mixture Z (4.5:1:1), 2,5-dihydro-2,4,5-trimethylthiazoline (TTH).

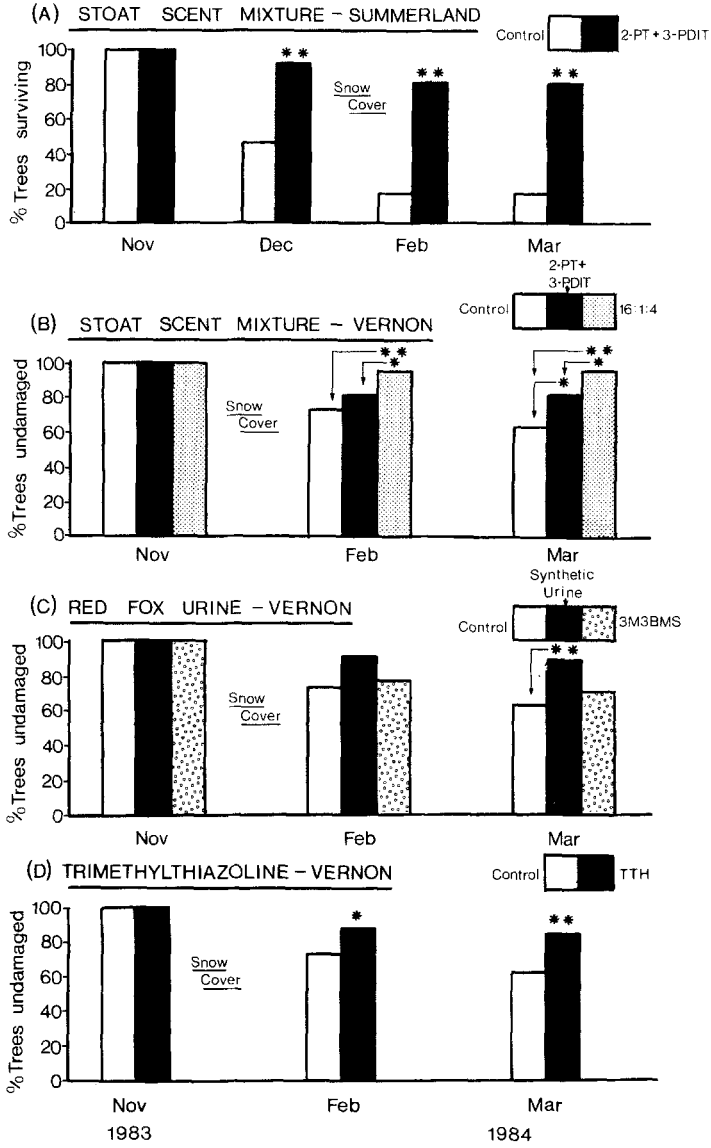

FIG. 1. (A) Percentage of 1-year-old apple trees that survived feeding damage by voles in control (n = 48) and treatment (n = 46) blocks at Summerland Research Station. Treatment consisted of stoat anal gland compounds (stoat scent mixture): 2-propylthietane (2-PT) and 3-propyl-1,2-dithiolane (3-PDIT) (1 : 1 ratio). Percentage of apple trees damaged by voles in control and treatment blocks at Coldstream Ranch, Vernon. Treatments consisted of (B) stoat scent: 2-PT and 3-PDIT (1 : 1 ratio) and the stoat anal gland mixture X (16 : 1 : 4); (C) synthetic red fox urine and 3-methyl-3-butenyl methyl sulfide (3M3BMS); and (D) 2,5-dihydro-2,4,5-trimethylthiazoline. Trials were started on November 11–12, 1983 and terminated in March 1984. $*P < 0.05$, $**P < 0.01$; significant difference by chi-square.

and 3-propyl-1,2-dithiolane, attached separately to opposite sides of each tree in that block, also had significantly less damage than control trees (Figure 1B). 3-Methyl-3-butenyl methyl sulfide, the highly volatile and characteristic skunky odor of fox urine, did not suppress damage. However, only 13 of the 36 capillary tubes still had active compound at the end of the trial. The synthetic fox urine mixture did suppress feeding damage (11.6% compared with the control level of 37.5%), possibly because the volatility of 3-methyl-3-butenyl methyl sulfide was retarded by the mixture of seven other compounds (Figure 1C). 2,5-Dihydro-2,4,5-trimethylthiazoline also significantly reduced the number of trees damaged by voles compared with the control (Figure 1D).

The amounts of bark and vascular tissues removed in vole feeding attacks at Vernon during the 1983–1984 winter for various treatments and the control are listed in Table 4. Voles clearly fed more (3.8–5.0 times) intensively on control trees than on those with the mixtures of stoat anal-gland compounds. This pattern was also evident in the blocks (2,5-dihydro-2,4,5-trimethylthiazoline and the fox urine mixture) that had a treatment tube attached to every second tree. The most dramatic difference in feeding was recorded in the block with 2,5-dihydro-2,4,5-trimethylthiazoline where, on treated trees, removal of bark and vascular tissues was 3.1% of that on control trees. The 3-methyl-3-butenyl methyl sulfide treatment did not follow this pattern with comparable amounts of bark and vascular tissues removed from trees with the odor (10.5 cm^2) and those without (16.1 cm^2).

The bioassay with young apple trees at Vernon during the 1985–1986 winter resulted in 81.3% of control trees undamaged by voles compared with 97.9% for the trees with a 1:1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane. The 2,5-dihydro-2,4,5-trimethylthiazoline treatment

TABLE 4. AVERAGE AMOUNTS OF BARK AND VASCULAR TISSUES REMOVED IN VOLE FEEDING ATTACKS IN VERNON APPLE ORCHARD DURING 1983–1984 WINTER (Sample size in parentheses)

	Amount of bark and vascular tissues (cm^2) removed per tree	
	Average	95% confidence limits
Control	16.1(48)	6.9–25.3
2-Propylthietane + 3-propyl-1,2-dithiolane	4.2(96)	1.3–7.1
Stoat anal-gland mixture X (16:1:4)	3.2(47)	−3.1–9.5
2,5-Dihydro-2,4,5-trimethylthiazoline	0.5(47)	−0.1–1.1
Fox urine mixture	6.5(21)	−2.3–15.3
3-Methyl-3-butenyl methyl sulfide	10.5(17)	−0.1–21.1

(81.3%) did not differ from the control in terms of undamaged trees. In general, vole attack of these 2-year-old trees was considerably less than that recorded in the similar experiment at Summerland during the 1983–1984 winter.

DISCUSSION

This study has clearly demonstrated that voles will avoid traps treated with certain anal-gland compounds of the stoat as well as the principal component of fox feces, 2,5-dihydro-2,4,5-trimethylthiazoline. Our technique using trap bioassays was similar to that used by Stoddart (1976, 1980) and Gorman (1984), who also reported that voles avoided traps scented with weasel and stoat anal-gland secretions, respectively. Both of these latter studies reported that a certain proportion of the vole population did enter live-traps treated with weasel or stoat odor as was also found in our study. Clearly, there is a certain level of behavioral heterogeneity within these natural populations as outlined by Stoddart (1983) in his discussion regarding the use of live-traps as a tool for olfactory bioassays. This heterogeneity may also partly explain the lack of avoidance by marked voles recorded for the mixtures of anal-gland components in experiments B and C and for the 2,5-dihydro-2,4,5-trimethylthiazoline in experiment C. Further evidence for this heterogeneity was provided by the significantly fewer new voles captured in the stoat anal-gland treatment than control traps in experiments B and C (see Table 2). This trend suggests that significant results may have been obtained if these experiments had been conducted in areas where the vole population was not dominated by marked individuals who had some previous experience with live-traps. It should also be noted that, on average, there were 35–50% fewer voles in 1985 (experiment C) than 1983 (experiment A), which reflects the multiannual cycle of *M. montanus* (Sullivan and Sullivan, in preparation), another potential source of heterogeneity in trap responses.

Neither mixture of stoat anal-gland compounds (Y nor Z, see Table 1) generated a significant avoidance of traps by voles compared with the 1 : 1 ratio of 2-propylthietane and 3-propyl-1,2-dithiolane in experiment A. These results suggest that a lower concentration of 2-propylthietane or addition of a substantial medium such as squalene or petroleum jelly to retard the volatility of this compound may generate a consistent avoidance response by voles. Similarly, the responses of voles to traps treated with 2,5-dihydro-2,4,5-trimethylthiazoline was inconsistent, which could be related to variable release rates of the compound in the two trials.

In terms of crop protection, consistent overwinter avoidance of apple trees with various predator odors has been demonstrated. The 1 : 1 ratio of 2-propylthietane and 3-propyl-1,2-dithiolane in three test blocks and the mixture X in one test block effectively suppressed feeding on trees by voles compared with a control. The synthetic fox urine mixture and 2,5-dihydro-

2,4,5-trimethylthiazoline also significantly reduced feeding by voles but the latter compound did not differ from the control in a second overwinter experiment. Other than variable release rates as discussed for the trap bioassays, we do not have an explanation for this variability in vole response to 2,5-dihydro-2,4,5-trimethylthiazoline. Of all compounds tested in the Vernon orchard block during the 1983–1984 winter, 2,5-dihydro-2,4,5-trimethylthiazoline reduced the average amount of bark and vascular tissues removed to 0.5 cm^2 per tree, the lowest value of all predator odors tested (Table 4).

The lack of response of deer mice to the stoat anal-gland compounds and 2,5-dihydro- 2,4,5-trimethylthiazoline was clearly a paradox. Similar results were obtained for the Eurasian counterpart, *Apodemus sylvaticus*, of the North American deer mouse for both weasel (Stoddart, 1976, 1980) and stoat (Gorman, 1984) scent. We do not have a logical explanation for the lack of response of deer mice to predator odors. However, a speculative argument might lie in the opportunistic and inquisitive nature of this rodent species which lives in many different habitats. Thus, the species is much more dispersed over the landscape than *Microtus* spp., for example, which have well-defined habitat preferences. Because of this widespread distribution, deer mice may persist as a species despite an apparent ambivalent attitude towards predator odors and the presumed greater likelihood of being preyed upon.

The use of semiochemicals as area repellents for crop protection and wildlife management has been advocated by Shumake (1977) and Müller-Schwarze (1983). In terms of pheromones, avoidance and reduced activity responses to intraspecific odors have been reported in fossorial yellow voles (*Lagurus luteus*) (Fan, 1983), bank voles (*Clethrionomys glareolus*) (Brinck and Hoffmeyer, 1984), and other rodents such as muskrats (*Ondatra zibethicus*) (Van Den Berk and Müller-Schwarze, 1984) and beaver (*Castor canadensis*) (Müller-Schwarze and Heckman, 1980; Müller-Schwarze et al., 1983). However, there have been no long-term (e.g., overwinter) field tests of synthetic pheromones as area repellents. To date, only synthetic predator odors or kairomones have been field tested and found to significantly suppress feeding by snowshoe hares (Sullivan and Crump, 1984, 1986a) and generate an avoidance response in pocket gophers (Sullivan and Crump, 1986b). The present study has assessed the overwinter (four to five months) efficacy of several synthetic predator odors designed as area repellents for crop protection from vole attack. When encapsulated in controlled-release devices, these compounds have considerable potential for providing long-term protection for forest and agricultural crops that experience vole feeding damage. Additional work is required to determine the optimum concentrations, mixtures, and release systems for these compounds when formulated for long-term field use.

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USE OF PREDATOR ODORS AS REPELLENTS TO REDUCE FEEDING DAMAGE BY HERBIVORES. IV. Northern Pocket Gophers (*Thomomys talpoides*)

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Abstract—This study investigated the influence of the major anal-gland compounds from the stoat (*Mustela erminea*) and ferret (*M. putorius*) in generating an avoidance response by northern pocket gophers (*Thomomys talpoides*) in tree fruit orchards in the Okanagan Valley of British Columbia, Canada. A secondary objective assessed the impact of additional predator odors on gopher avoidance behavior in laboratory bioassays. In field bioassays, a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane, as well as 3,3-dimethyl-1,2-dithiolane, placed in gopher burrows did not reduce the number of gophers colonizing treatment versus control grids in orchard blocks. However, these predator gophers did dramatically alter the distribution of gophers. Significantly more gophers were captured at perimeter than nonperimeter trap stations on treatment versus control grids in two of three orchards. In all orchards, significantly more gophers were captured at perimeter stations after the predator odors had been placed in burrows than prior to the start of the experiment. Gophers clearly avoided 2,5-dihydro-2,4,5-trimethylthiazoline, a component of fox (*Vulpes vulpes*) feces, but did not avoid 2,2-dimethylthietane from the mink (*M. vison*) or 3-methyl-3-butenyl methyl sulfide from fox urine in laboratory bioassays. Poor avoidance was also recorded for 3,3-dimethyl-1,2-dithiolane, although this may be due to the state of polymerization of this compound. An improved formulation is required to dispense these semiochemicals in controlled-release devices within orchards and other forest–agricultural areas.

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Key Words—Predator odors, mustelids, anal-gland compounds, pocket gopher, avoidance response, thietanes, dithiolanes, crop protection, orchard, kairomones, 2,5-dihydro-2,4,5-trimethylthiazoline, fox urine and feces, *Vulpes vulpes*, *Mustela* spp., *Thomomys talpoides*.

INTRODUCTION

Interspecific chemical communication between predator and prey may be manipulated to manage mammalian herbivore pests for forest and agricultural crop protection. Sullivan et al. (1988) have outlined several studies using preparations and synthetic components of predator odors to generate avoidance responses and suppress feeding in various species of herbivores. In particular, Sullivan and Crump (1986a) reported on the avoidance response of northern pocket gophers (*Thomomys talpoides*) to mustelid anal-gland compounds.

Pocket gophers are a persistent problem in the fruit-growing regions of British Columbia, Canada (Anderson and Kluge, 1986; Sullivan et al., 1987) and Washington and Oregon in the United States. The fossorial vole, *Microtus pinetorum*, occurs in orchards of the eastern United States and Canada (Byers, 1984, 1985). These fossorial rodents feed on the bark, vascular tissues, and roots of orchard trees. Pocket gophers also inflict similar feeding damage to coniferous seedlings in reforestation areas of western forests (Barnes, 1973; Barnes et al., 1982; Crouch, 1982). Feeding damage by these rodents may result in reduced growth and yield or direct mortality from girdling.

The influence of the major anal-gland compounds from the stoat (*Mustela erminea*) and ferret (*M. putorius*) in generating an avoidance response by pocket gophers was conducted in laboratory bioassays and field bioassays in natural (grassland) habitats (Sullivan and Crump, 1986a). The effective compounds tested were 2-propylthietane, 3-propyl-1,2-dithiolane, a mixture of stoat compounds, and 3,3-dimethyl-1,2-dithiolane. Additional sulfur-containing compounds which may be relevant predator odors for pocket gophers include 2,2-dimethylthietane from anal-gland secretion of the mink (*M. vison*) (Schildknecht et al., 1976, 1981; Brinck et al., 1978; Anderson and Bernstein, 1980; Sokolov et al., 1980), 2,5-dihydro-2,4,5-trimethylthiazoline from fox (*Vulpes vulpes*) feces (Vernet-Maury et al., 1984), and 3-methyl-3-butenyl methyl sulfide from fox urine (Wilson et al. 1978; Sullivan and Crump, 1986b).

This paper reports on (1) the influence of these additional compounds plus 3,3-dimethyl-1,2-dithiolane in generating an avoidance response by pocket gophers in laboratory bioassays, and (2) an assessment of the effectiveness of the mustelid compounds reported by Sullivan and Crump (1986a) in acting as area repellents to pocket gophers in tree fruit orchards.

METHODS AND MATERIALS

Predator Odor Compounds. Anal-gland compounds from the genus *Mustela* (2-propylthietane, 3-propyl-1,2-dithiolane, 2,2-dimethylthietane, and 3,3-dimethyl-1,2-dithiolane) (also in fox feces, see Vernet-Maury et al., 1984) were prepared according to Crump (1978, 1980a,b, 1982). 2-Propylthietane and 3-propyl-1,2-dithiolane were mixed in a 1 : 1 ratio. 2,5-Dihydro-2,4,5-trimethylthiazoline and 3-methyl-3-butenyl methyl sulfide were prepared according to the procedures outlined in Sullivan et al. (1988).

All compounds were dispensed in 140-μl capillary tubes (75 mm in length). The capillary tubes provided a point source of odor and prevented contamination of the arena or pocket gophers, themselves, in the laboratory bioassays. This encapsulation also protected the compounds from adverse environmental conditions in the field bioassays. Approximately 30 mg of each compound or mixture was placed in a given capillary tube using a 1-ml microsyringe with a 20-gauge needle. An empty capillary tube represented the control to complete the design of a given bioassay.

Laboratory Bioassays. The avoidance behavior of pocket gophers to the various predator odors was assessed by tallying the number of "captures" in the control and treatment rooms of a bioassay arena (see Sullivan and Crump, 1986a, for details of arena and bioassay procedure). In general, the arena, with a Longworth live-trap extending into an opening in each of the control and treatment rooms, simulated a burrow system for a gopher to enter. One capillary tube with a given compound was attached with adhesive tape to the floor of the tunnel of the live-trap in the treatment room. An empty tube was attached in a similar manner to the trap in the control room.

The number of gophers captured in each room was tallied to assess the impact of each compound on avoidance behavior. The control and treatment rooms were alternated for each trial, with individual gophers being tested only once in a given trial. Only one trial was conducted per given day with a new compound tested on each consecutive day. Five groups of gophers were tested. Each group was tested initially with both arena rooms acting as controls to determine if there was a preference by individual gophers to enter and be "captured" in one room or the other. Depending on availability of compounds, 2,5-dihydro-2,4,5-trimethylthiazoline was tested on all five groups, 2,2-dimethylthietane and 3,3-dimethyl-1,2-dithiolane were each tested on two groups, and 3-methyl-3-butenyl methyl sulfide was tested on one group. Sample sizes of each group varied depending on number surviving throughout the series of trials and exclusion from results of those individuals responding to neither control nor treatment conditions. All gophers were collected from intensive study areas

at Summerland and Vernon, British Columbia, Canada. Male and female gophers were pooled for all trials.

Field Bioassays. Pocket gopher populations were monitored on control and treatment grids in a Bartlett and d'Anjou pear (*Pyrus domestica*) orchard at the Agriculture Canada Research Station and a private Spartan apple orchard (*Malus domestica*), both in Summerland, British Columbia, Canada, and in a MacIntosh apple orchard at the Coldstream Ranch, 12 km east of Vernon, British Columbia. Age and spacing of trees in each orchard was: Summerland pear—15 years at 4.6 × 6 m; Summerland apple—27 years at 6 m × 6 m; Vernon apple—30 years at 4.7 × 6.2 m interspersed with 4.7 × 9.2 m. All orchard populations were monitored at three-week intervals from July to October 1985 and subsequently in April (Vernon), July (pears), and April to July 1986 at three-week intervals (Summerland apple). Gopher populations could not be monitored over the winter because of frozen ground.

Gophers were live-trapped on 0.41-hectare (pears), 0.42-hectare (Summerland apple), and 1-hectare (Vernon apple) checkerboard grids with Longworth live-traps. The pear and Summerland apple orchard grids were irregularly shaped with 24 and 27 trap stations, respectively; the Vernon grids had 49 (7 × 7) stations. All stations were located at 15.2-m intervals. Live-traps were set within recently used tunnels or burrows indicated by fresh excavations of soil. Thus, traps did not usually have permanent stations but were moved in response to gopher activity. Traps were baited with whole oats, peanut butter, and carrot; coarse brown cotton was supplied as bedding. Traps were set on day 1, checked on the morning and afternoon of day 2 and morning of day 3, and then locked open between trapping periods. Each of the Summerland pear and apple control and treatment grids had 15–20 traps set in a given trapping period. An average of 20–25 traps was set on each of the Vernon grids during a trapping period. All gophers captured were ear-tagged with serially numbered tags, breeding condition noted, weighed on Pesola spring balances, and point of capture recorded.

To further assess the field efficacy of the mustelid anal-gland compounds that gophers avoided to a significant degree in the laboratory tests of Sullivan and Crump (1986a), capillary tubes containing the mixture of 2-propylthietane and 3-propyl-1,2-dithiolane and tubes containing 3,3-dimethyl-1,2-dithiolane were attached to the inside of 500-ml (73 × 110 mm) cans (open at both ends) by plastic twist-ties and placed in gopher burrows. Prior to these field experiments in August 1985, all resident gophers were permanently removed during two consecutive trapping periods from the control and treatment grids in each orchard block. This removal allowed the odor of the anal-gland compounds to fumigate the burrow system of the treatment grid without disturbance from the digging activities of resident gophers.

The mixture of two compounds (two tubes per can with one tube directed each way and with two cans per each of the squares on a matrix grid) was placed in the burrows of the treatment grids of the Vernon and Summerland apple orchards on August 24–25 and August 29–30, 1985, respectively. 3,3-Dimethyl-1,2-dithiolane was dispensed in an identical manner on the treatment grid of the Summerland pear orchard on August 24, 1985. Cans with empty tubes were placed in burrows at the same density and systematic distribution on each control grid in a given orchard. These experiments commenced immediately after completion of the second removal trapping period in each orchard. The experiments tested whether or not new gophers would colonize or avoid the treatment area that smelled of a mustelid predator. In general, gophers that colonize a new area usually inhabit the already existing burrow systems.

Statistical Analysis. Comparisons of the number of gophers captured in control and treatment rooms in the pen bioassays as well as number of gophers and location of captures on control and treatment grids in the field bioassays were analyzed by chi-square with significance levels of $P < 0.05$ and $P < 0.01$.

RESULTS

Laboratory Bioassays. The responses of gophers to the various predator odor compounds are illustrated in Figure 1. There was no difference between males and females in these trials. Gophers avoided the 2,5-dihydro-2,4,5-trimethylthiazoline from fox feces with significant differences between control and treatment in two trials and near significance ($P = 0.058$) in a third. They did not avoid the 3,3-dimethyl-1,2-dithiolane from the ferret, 2,2-dimethylthietane from the mink, or 3-methyl-3-butenyl methyl sulfide from fox urine. Similarly, the control trials showed no difference in gopher preference for either arena room.

Field Bioassays. The responses of gophers to mustelid anal-gland compounds in the field are illustrated in Figure 2. Comparable numbers of gophers colonized both control and treatment grids in all orchard blocks following the two removal periods and placement of the repellent compounds in the ground. At the Vernon apple orchard, 2.5 times as many new gophers appeared on the control compared with the treatment grid during the first trapping period (September). However, this difference was not evident in subsequent trapping periods in October. There was no difference between any of the control and treatment grids in number of gophers in July 1986, 11 months after the experiment began.

At first, these results were quite discouraging, particularly in light of

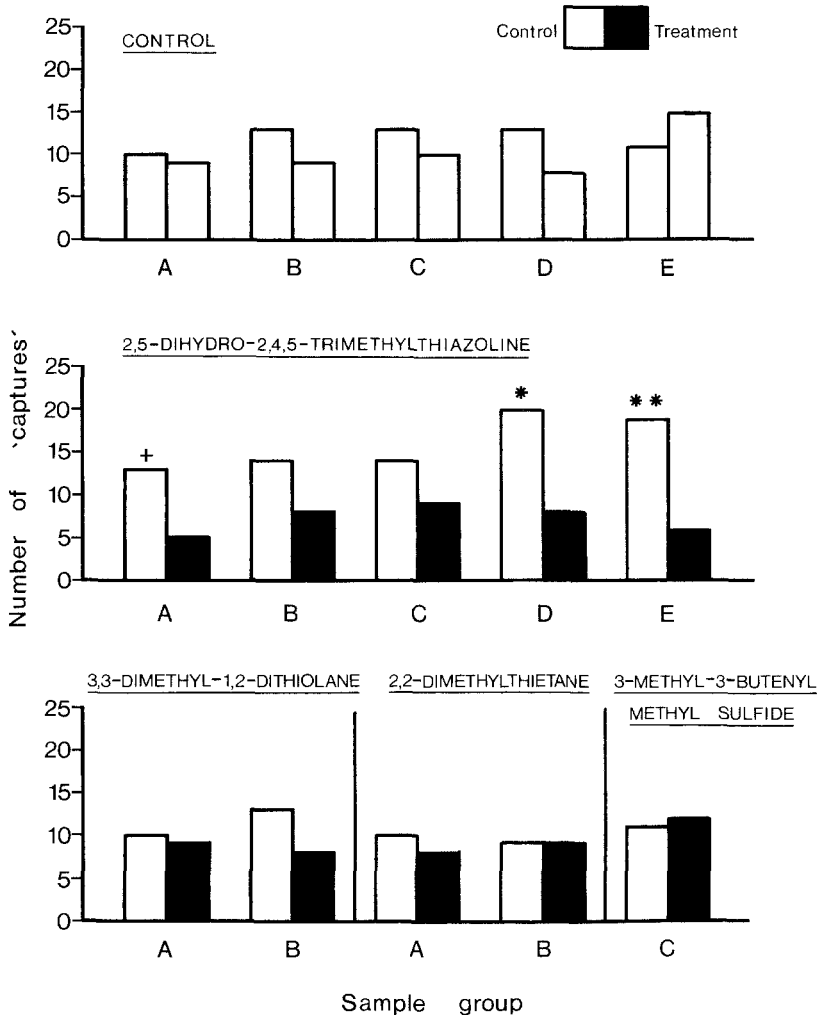

FIG. 1. Avoidance behavior responses ("capture" in live-traps) of pocket gophers to predator odors in the laboratory bioassay arena. $*P < 0.05$; $**P < 0.01$; significant difference by chi-square. $^{+}P = 0.058$.

the significant laboratory and field bioassay results reported by Sullivan and Crump (1986a). Although the anal-gland compounds did not alter the number of gophers on treatment compared with control grids, these predator odors did dramatically alter the distribution of gophers (Figure 3). In both Summerland orchards, significantly more gophers were captured at perimeter than nonperimeter trap stations on the treatment versus control grids. In addition, this sig-

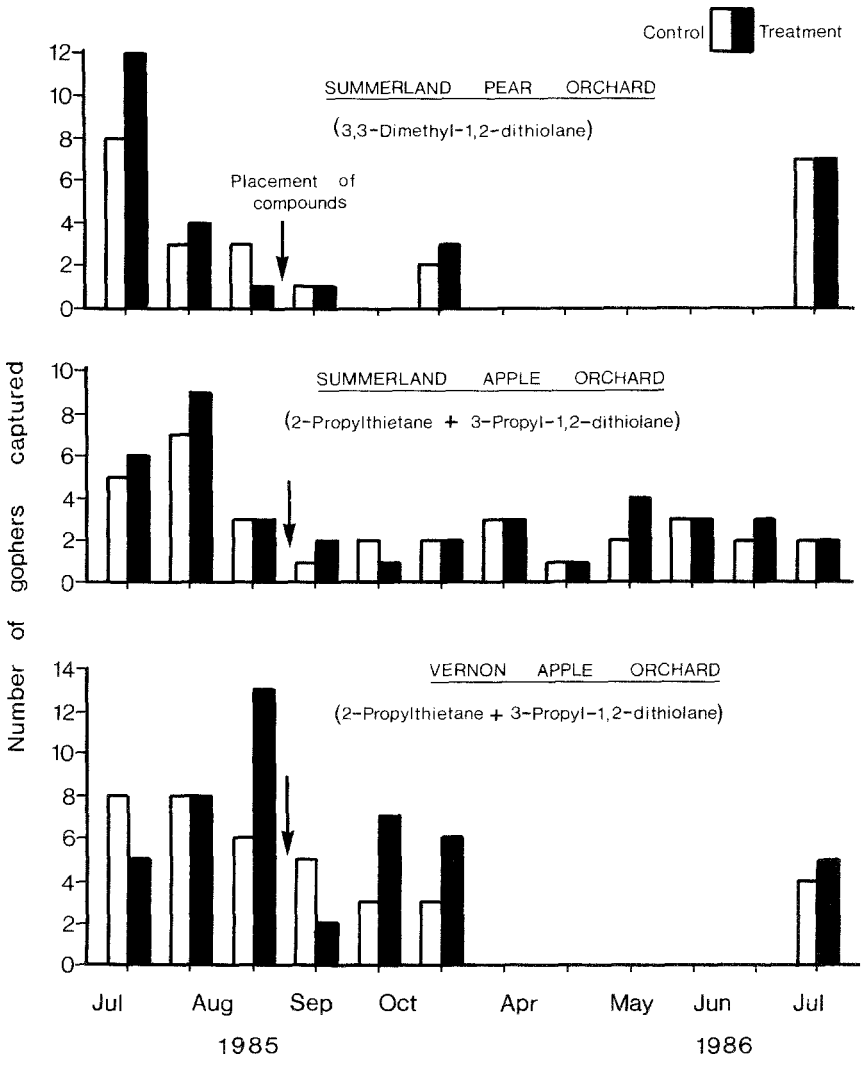

FIG. 2. Number of gophers captured in field bioassays on control and treatment grids in the Summerland pear and apple orchards and Vernon apple orchard. All gophers were removed from grids in two consecutive trapping periods in August 1985 prior to placement of mustelid anal-gland compounds in burrow systems of treatment grids.

nificant pattern was also evident when comparing location of gopher captures before and after the placement of repellent in the ground on treatment grids at the Summerland pear and Vernon apple orchards. In this regard, the results from the Summerland apple orchard approached significance ($P = 0.08$). There

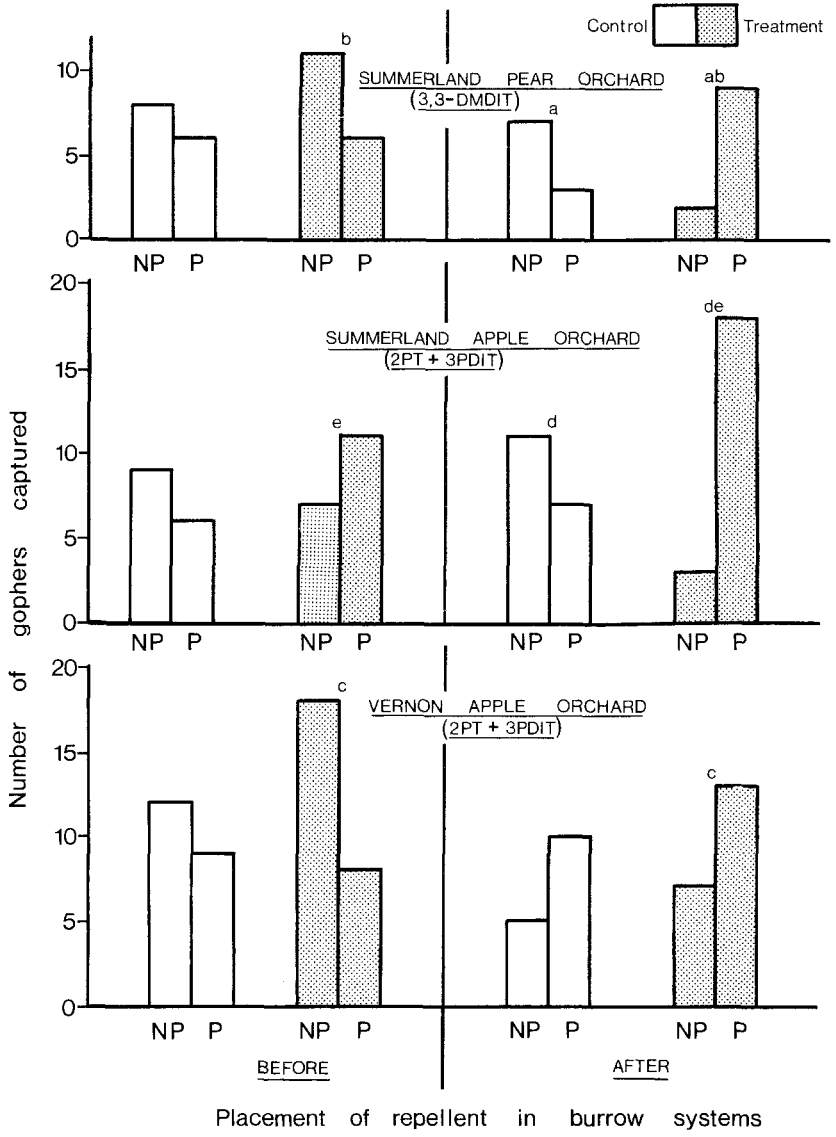

FIG. 3. Number of gophers captured in perimeter and nonperimeter trap stations on control and treatment grids in the Summerland pear and apple orchards and Vernon apple orchard. All gophers were removed from grids in two consecutive trapping periods in August 1985 prior to placement of mustelid anal-gland compounds in burrow systems of treatment grids. a–a, b–b, c–c, $P < 0.05$; d–d, $P < 0.01$; significant difference by chi-square. e–e, $P = 0.08$. P = perimeter, NP = nonperimeter. 3,3-Dimethyl-1,2-dithiolane (3,3-DMDIT), 2-propylthietane (2PT), 3-propyl-1,2-dithiolane (3PDIT).

were no significant differences between the control and treatment grids at Vernon with respect to perimeter and nonperimeter captures of gophers.

DISCUSSION

The results of laboratory bioassays in this study indicate that 2,5-dihydro-2,4,5-trimethylthiazoline will produce an avoidance response by pocket gophers. This compound should clearly be tested in the field as a potential gopher repellent, particularly in light of the significant results obtained with voles (*Microtus* spp.) reported by Sullivan et al. (1988). The other component of fox feces (and ferret anal-gland secretion), 3,3-dimethyl-1,2-dithiolane, was not effective in laboratory bioassays. This compound was somewhat effective in earlier bioassays with results from three trials (control vs. treatment): 14–9; 14–9; 8–1 (Sullivan and Crump, 1986a). In addition, 3,3-dimethyl-1,2-dithiolane, in association with 3-propyl-1,2-dithiolane (both polymerized) produced a significant overwinter reduction in number of gophers (Sullivan and Crump, 1986a). This former compound also significantly altered the distribution of gophers in a pear orchard in this study. Again, additional work is warranted to determine the optimum efficacy of 3,3-dimethyl-1,2-dithiolane in repelling gophers from crop protection areas. Since dithiolanes readily polymerize when exposed to ambient conditions, their effectiveness and durability may be a function of this change. A similar polymerization occurred with 3-propyl-1,2-dithiolane in another field study, and this may have contributed to its longevity (Sullivan and Crump, 1984).

The major compound of the mink, 2,2-dimethylthietane, and one from red fox urine, 3-methyl-3-butenyl methyl sulfide, did not affect the avoidance behavior of gophers in the laboratory. These results are somewhat surprising even though the mink is not a major predator of pocket gophers. As outlined by Sullivan and Crump (1986a), weasels and snakes are the most important predators, in terms of body size, of gophers because they are capable of entering subterranean burrows. The red fox will excavate burrow systems to prey on gophers, and this may help explain the significant avoidance response generated by 2,5-dihydro-2,4,5-trimethylthiazoline. The lack of response to the fox urine component, 3-methyl-3-butenyl methyl sulfide, is in accord with the results of Vernet-Maury et al. (1984) in their experiments with stress-inducing odorants in the rat. Interestingly, this fox urine component is effective in suppressing feeding in the snowshoe hare (*Lepus americanus*) as shown by Sullivan and Crump (1986b).

Although the number of gophers captured on treatment grids in our orchards did not differ from the controls, the distribution of gophers within the treatment grid area was significantly affected. The majority of gophers did not

enter beyond the perimeter of our treatment grids. This result, along with those of Sullivan and Crump (1986a), provides strong conclusive evidence that gophers will avoid predator odor (at least certain mustelid anal-gland compounds) that has been placed in their burrow systems in a field situation. Our technique of dispensing odors clearly needs improvement. The majority of cans used in the trials could not be relocated after the 1985–1986 overwinter period. Thus, there was no measure of how many cans were still functional. A technique is required to dispense the compounds in controlled-release devices at a multitude of sources within burrows in a given orchard or other forest–agricultural area. Once the resident gophers are removed, as many burrows systems as possible should be "fumigated" with one or more predator odors. Formulation of such devices is clearly a critical next step to effective use of these semiochemicals for crop protection from pocket gophers.

The rapid colonization of removal areas by gophers was an indication of the intense social pressure which forces the annual surplus of juvenile as well as adult gophers into any available "open habitat" (Sullivan and Crump, 1986a; Sullivan and Sullivan, in preparation). Thus, to keep gophers completely out of an orchard block, a buffer strip of at least 25 m should be added to the area treated with predator odors. Gophers may then colonize and reside on the outside of an orchard without causing any feeding damage within the orchard itself. An additional technique could utilize a mechanical burrow builder (Anderson and Kluge, 1986) to make artificial burrows within the buffer strip around an orchard. These burrows could then be fumigated with predator odors as a line of defense against colonist gophers. Similar strategies could be adapted to other forest and agricultural situations.

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COMPONENTS OF FEMALE SEX PHEROMONE OF *Eoreuma loftini* DYAR[1]

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Abstract—Three chemicals isolated from ovipositor rinses of virgin female *Eoreuma loftini* Dyar (Mexican rice borer) moths were identified as (Z)-13-octadecenyl acetate (I), (Z)-11-hexadecenyl acetate (II), and (Z)-13-octadecenal (III) in the approximate ratio 8:1:1.3, respectively. Preliminary tests indicate that in laboratory bioassays, these chemicals in the above ratio induce the same activity response as heptane extracts of rice borer ovipositors. Only one combination of two chemicals (I and III, 6.2:1 ratio) elicited a similar response. Individual chemicals elicited no response. In field tests, the average number of male rice borer moths caught per night over a five-night period was the same in traps baited with 200–600-μg quantities of the 8:1:1.3 ratio of chemicals dispensed from strips of filter paper as with traps baited with virgin females. The two-component mixture (I and III, 6.2:1 ratio) was not active in the field.

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Key Words—Mexican rice borer, *Eoreuma loftini*, Lepidoptera, Pyralidae, sugarcane, (Z)-13-octadecenal, (Z)-13-ocadecenyl acetate, (Z)-11-hexadecenyl acetate.

INTRODUCTION

Mexican rice borers (MRB), *Eoreuma loftini* Dyar (Lepidoptera: Pyralidae) (previously *Chilo loftini* Dyar and *Acigona loftini* Dyar) were first found in sugarcane fields in the lower Rio Grande Valley in Texas in the summer of 1980 (Fors, 1981; Fors and Abarca, 1983; Johnson and Van Leerdam, 1981). Its previously recorded range was Arizona, California, and Mexico. MRB caused yield losses in sugarcane in the 1980–1981 season (Fors, 1981) and were found in several alternate hosts (Johnson and Van Leerdam, 1981). Since the MRB could potentially become the primary pest of sugarcane in the lower Rio Grande Valley of Texas, research was initiated to develop technology to manage MRB populations to reduce sugarcane damage. Female sex pheromones have been identified for species related to MRB, including *Chilo zacconius* (Zagatti et al., 1983), *Chilo suppressalis* (Nesbitt et al., 1975), *Chilo partellus* (Nesbitt et al., 1979), and *Chilo sascariphagus* (Nesbitt et al., 1980). The presence of a female sex pheromone in MRB was demonstrated by Brown et al. (1988). The research reported herein was designed to identify sex pheromone components of this rice borer and to verify their activity in preliminary laboratory and field evaluations.

METHODS AND MATERIALS

Insects. Insects used in this study were obtained from a colony maintained by USDA, Animal and Plant Health Inspection Service (APHIS) at rearing facilities in San Benito, Texas, and Moore Air Base, Mission, Texas. Larvae were reared on a modified soy flour–corn grits diet at 29 ± 1°C. The insects were sexed as pupae, and males and females were kept separate to ensure availability of virgin adults. All adults were kept at 26 ± 1°C on a 14:10-hr light–dark photoperiod. The photoperiod was adjusted in insects used for laboratory bioassay so that the dark phase coincided with normal working hours.

Preparation of Ovipositor Extracts. Ovipositor extracts were prepared by excising ovipositors from virgin MRB females (24–48 hr old) during the period in which they were actively calling, normally about 5 hr after start of scotophase. Ovipositors were obtained by gently squeezing the abdomen of suitable females to cause extension of the ovipositor, which was excised with fine dissecting scissors. Excess fat tissue was trimmed with dissecting scissors, and the cut surface was touched to paper tissue to remove contaminating hemolymph. Excised ovipositors were then soaked in heptane for 2 hr; normally 50 ovipos-

itors were soaked in ca. 1 ml of heptane, giving 50 female equivalents (FE)/ml. The ovipositors were removed from the heptane, and the extracts were tested in the laboratory bioassay to verify activity. The laboratory bioassay was reported by Brown et al. (1988) and consisted of suspending test materials in glass jars containing five male MRB and observing the males for 1 min to determine individual responses. A numerical rating was assigned for each male depending on the type and intensity of response as follows: 1 = no response; 2 = intermittent crawling; 3 = continuous crawling; 4 = crawling with intermittent flying or fluttering of wings; 5 = continuous flying or fluttering of wings (including attempted mating with test material). Each material was tested with four different groups of males giving a total of 20 observations per test material.

Fractionation of Ovipositor Extract. The crude ovipositor extract was fractionated on an 80–120 mesh silica gel column (2.2 × 22.0 cm) by eluting with 100-ml portions each of hexane; 1, 2, 5, 10, and 25% ether in hexane; and then with ether and methylene chloride. Fractions (50 ml) collected were monitored by the laboratory bioassay and were analyzed by capillary gas chromotography (GC) on the 50-m OV-1701 column.

GC and GC-MS Analyses. Gas chromatography was performed on a Varian 3700 gas chromatograph equipped with either: (1) 50 m × 0.32 mm ID vitreous silica OV-1701 column (SGE, Austin, Texas), (2) 50 m × 0.25 mm ID vitreous silica Silar 10C (Alltech Associates, Deerfield, Illinois), or (3) 25 m × 0.32 mm ID vitreous silica BP-1 column (SGE, Austin, Texas) using a Grob splitless injection system and a flame ionization detector. N_2 pressure was 22 psig. Retention times and temperature programs are shown in Table 1. Mass spectral studies were performed on a Finnigan 1020 OWA mass spectrometer equipped

TABLE 1. RETENTION TIMES AND RELATIVE AMOUNTS OF INDIVIDUAL COMPONENTS IN OVIPOSITOR WASHES OF VIRGIN FEMALE MEXICAN RICE BORERS

	Retention time (min)							
	OV-1701[a]			BP-1[b]		Silar 10-C[c]		
Compound	Standard	Isolated	ng/ovipositor	Standard	Isolated	Standard	Isolated	*E* isomer
Z13-C_{18}Ac	35.39	35.36	43.6 (±8.3)	31.68	31.70	22.40	22.39	21.78
Z11-C_{16}Ac	24.35	24.37	5.5 (±2.1)	24.66	24.68	18.45	18.47	18.13
Z13-C_{18}Al	26.24	26.24	7.2 (±2.4)	24.91	24.94	20.94	20.93	

[a] OV-1701, 50 m × 0.32 mm vitreous silica (SGE, Austin, Texas), 60°C for 1.0 min followed by 15°C/min heating to 225°C, N_2 flow 1.8 cc/min.

[b] BP-1, 25 m × 0.32 mm vitreous silica (SGE, Austin, Texas), 60°C for 1.0 min followed by 7°C/min heating to 200°C, N_2 flow 2.0 cc/min.

[c] Silar 10C, 50 m × 0.25 mm vitreous silica (Alltech Associates, Deerfield, Illinois), 60°C for 1.0 min followed by 10°cc/min heating to 180°C, N_2 flow 1.7 cc/min.

with a 1.83 m × 2 mm ID glass column packed with OV-101 on Chromosorb 750, 100–125 mesh, pressure 20 psig, temperature programmed from 80 to 230°C at 20°/min. Compounds present were tentatively identified by comparison of their spectra with those of the EPA-NIH Mass Spectral Data Base (Heller and Milne, 1978). Standard compounds were purchased or prepared by published methods and were subjected to GC and mass spectral analyses for comparison of retention times and mass spectra.

Individual components of 100 FE of MRB ovipositor heptane rinse were separated by preparative GC on a modified Tracor 550 chromatograph using a 2 m × 4 mm ID 1.5% SP-2250/1.95% SP-2401 on 100/120 Supelcoport column at 160°C and collected in 1.7-mm glass capillary tubes using a Brownlee-Silverstein thermal gradient collector (Brownlee and Silverstein, 1968). The double-bond positions were established by microozonalysis. These components, along with commercial or synthesized standards, were then ozonized in methylene chloride (Beroza and Bierl, 1967), treated with triphenyl phosphine, and analyzed by capillary GC on a 50-m OV-1701 column at 50°C.

The concentration of each of the three identified chemicals in ovipositors of virgin MRB females was determined by GC analysis of heptane extracts from 10 groups of 50 ovipositors. Analyses were performed on the 50-m OV-1701 capillary column at a temperature program of 60°C for 1.0 min followed by 15°C/min heating to 225°C with a N_2 flow of 1.8 cc/min. Quantitation was made by comparing with standards of the identified compounds.

Laboratory and Field Tests. Activity of combinations of (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal was tested in the laboratory by the method described by Brown et al. (1988). (Z)-11-Hexadecenyl acetate was prepared for these tests by acetylating (Z)-11-hexadecen-l-ol with acetic anhydride and pyridine. (Z)-13-Octadecenal was obtained commercially, and (Z)-13-octadecenyl acetate was prepared by first reducing (Z)-13-octadecenal to (Z)-13-octadecen-l-ol using excess sodium borohydride in methanol at 0°C and then acetylating with acetic anhydride and pyridine. Purity of the three compounds was determined by capillary GC on a 50-m OV-1701 column and isomeric purity was determined on a 50-m Silar 10C column. A stock solution in hexane was prepared containing pheromone components in the ratio of 8:1:1.3 of (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal, respectively. Dilutions were made to determine the limits of sensitivity in laboratory tests. Also 100 ng of the pheromone blend, single compounds, and all possible combinations of two compounds in the same ratios as they occur in the blend identified from ovipositor extracts were compared with freshly prepared ovipositor extracts for activity in the laboratory bioassay.

All blends and extracts were dispensed from ca. 2-cm^2 strips of filter paper in the laboratory bioassay. The effectiveness of the 8:1:1.3 ratio of the three

chemicals in capturing male MRB in field trapping studies was determined by comparison of catches of male rice borers in traps baited with three virgin females and in traps baited with different amounts of the pheromone blend. The pheromone blend (200, 400, or 600 μg) was dispensed onto ca. 2-cm^2 strips of filter paper, and traps were baited late each afternoon to ensure that the chemicals would be emitted during the period of sexual activity of the male MRB moths. Pherocon IC (Zoecon Corporation, Palo Alto, California 94304) traps were used in all field studies, and the traps were placed 50 m apart and 5 m inside a sugarcane field. Tests were run three times with four replicates per run. Traps were randomized nightly. Pheromone blend of the same ratio was impregnated in black PVC plastic dispensers (Hendricks, 1983) at a rate of 5 mg pheromone per lure and was compared with similar PVC lures containing 5 mg total concentration of all possible combinations of two compounds in the ratios identified from ovipositor extracts. Male MRB catch in traps baited with the synthetic chemicals was compared with catches in traps baited with three virgin females. The data were analyzed using ANOVA and comparison of the means was made with Duncan's new multiple-range test.

RESULTS AND DISCUSSION

Fractionation and GC-MS Analyses. Three components were present in all ovipositor extracts examined. GC-MS analyses revealed that several samples also contained varying amounts of straight-chain C_{21}, C_{23}, and C_{25} hydrocarbons and an unidentified phthalate. However, these hydrocarbons and the phthalate were not present in some of the active extracts, nor were they present in active fractions obtained from silica gel columns. Activity in laboratory bioassays was obtained with fractions eluted with 5% and 10% ether in hexane from silica gel columns. Each of the active fractions contained the three components detected in all ovipositor extracts. These three components were tentatively identified by comparison with a mass spectral library as octadecenal, hexadecenyl acetate, and octadecenyl acetate. Related species are known to use 16-, and 18-carbon alcohols, aldehydes, and acetates with a double bond in the 11 or 13 position, respectively, as pheromonal components.

Previously reported sex pheromone components for related *Chilo* species include: (1) (*Z*)-11-hexadecenal and (*Z*)-13-octadecenal from *C. suppressalis* (Nesbitt et al., 1975); (2) (*Z*)-11-hexadecenal and (*Z*)-11-hecadecen-1-ol from *C. pastellus* (Nesbitt et al., 1979); (3) (*Z*)-13-octadecenyl acetate and (*Z*)-13-octadecen-1-ol from *C. sacchariphagus* (Nesbitt et al., 1980); and (4) hexadecan-1-ol and (*Z*)-11-hexadecen-1-ol from *C. zacconius* (Zagatti et al., 1983). Several of these compounds were prepared for comparison, and it was found that (*Z*)-13-octadecenal, (*Z*)-11-hexadecenyl acetate, and (*Z*)-13-octadecenyl acetate had the same retention times on the 50-m OV-1701, BP-1, and Silar

10C capillary columns as the three compounds isolated from ovipositor extracts and also produced identical mass spectra. Retention times and relative amounts of these components in ovipositor extracts are shown in Table 1.

The concentration of the compounds identified as (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal was determined to be 56.3 ng per ovipositor in a ratio of 8 : 1 : 1.3, respectively, from ovipositors of 24 to 48-hr-old virgin MRB females. Both the *E* and *Z* isomers of 11-hexadecenyl acetate and 13-octadecenyl acetate were available for comparative purposes, and the *E* and *Z* isomers had retention times of 18.13 min and 18.45 min for the 11-hexadecenyl acetate isomers and 21.78 and 22.40 min for the 13-octadecenyl acetate isomers, respectively, on the Silar 10C capillary columns. Samples of the three standards and of individual compounds isolated from the ovipositor extracts were epoxidized using *m*-chloroperbenzoic acid in methylene chloride at 0°C. Retention times on capillary columns were identical and peaks were sharp and symmetrical, indicating isomers are as stated.

Ozonolysis of each isolated compound and its commercial or synthesized standard yielded an aldehyde with the same retention time (11.68 min) as *n*-valeraldehyde by capillary GC analysis on OV-1701 at 50°C, indicating that the double bond in each compound is between carbon 5 and carbon 6 from the hydrocarbon end. For comparison, (Z)-7-, (Z)-9-, and (Z)-11-hexadecenyl acetate were subjected to GC analysis under the conditions given in Table 1. Retention times were 23.86, 24.01, and 24.37 min for the (Z)-7, (Z)-9, and (Z)-11 isomers, respectively, on the OV-1701 column; and 18.03, 18.17, and 18.45 min, respectively, on the Silar 10C column. Silar 10C is equivalent to SP2340, which has been used to provide good resolution of positional isomers of monounsaturated alcohol, aldehyde, and acetate pheromonal blends found in lepidopteran insects (Heath and Tumlinson, 1984).

Preliminary Laboratory and Field Tests. In laboratory bioassays, response of male rice borer moths to a blend of 8 : 1 : 1.3 (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal, respectively, was similar in intensity to response of the males to heptane extracts of ovipositors obtained from virgin females (Table 2), with relative responses of 3.5 for the synthetic chemicals and 4.1 for the ovipositor extracts. The deletion of (Z)-11-hexadecenyl acetate from the mixture of synthetic chemicals caused a significant decrease in the response of males, although laboratory response to the mixture of (Z)-13-octadecenyl acetate and (Z)-13-octadecenal was significantly greater than response to the solvent blank. However, none of the chemicals induced a response in males in the laboratory when tested either alone or in pairs lacking in either (Z)-13-octadecenyl acetate or (Z)-13-octadecenal.

Trap catch data for virgin females and pheromone blend [8 : 1 : 1.3 ratio of (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate and (Z)-13-octadecenal, respectively] showed no significant difference in average number of male

TABLE 2. LABORATORY ACTIVITY AND TRAP CATCHES ON MALE RICE BORER MOTHS EXPOSED TO (Z)-11-HEXADECENYL ACETATE (Z11–C_{16}Ac), (Z)-13-OCTADECENAL (Z13–C_{18}Al) AND (Z)-13-OCTADECENYL ACETATE (Z13–C_{18}Ac) IN VARIOUS BLENDS

Component blend	Laboratory response rating[a]	Trap Catch (males/trap/night)[b]
Z13–C_{18}Ac (I)	1.6 c[c]	[d]
Z11–C_{16}Ac (II)	1.4 c	
Z13–C_{16}Al (III)	1.8 c	
I + II (8.0:1.0)	1.6 c	0.9 c
II + III (1.0:1.3)	1.7 c	0 d
I + III (6.2:1.0)	2.5 b	0.6 c
I + II + III (8.0:1.0:1.3)	3.5 a	3.8 b
Females[e]	4.1 a	6.1 a
blank	1.4 c	0 d

[a] 100 ng of blend or chemical was dispensed onto 2-cm^2 filter paper strips.
[b] Pheromone blends were formulated in black polyvinyl chloride.
[c] Numbers followed by the same letter are not significantly different, $P = 0.05$, Duncan's new multiple-range test.
[d] Individual compounds were not tested for trap catch in field tests.
[e] In laboratory tests, 1.0 FE of heptane ovipositor extract was used as a control; in field tests, control traps were baited with three virgin females/trap.

MRB moths caught per trap night in the traps baited with females or with 200, 400, or 600 μg of pheromone. In a test to evaluate the various combinations of two chemicals formulated in PVC as lures, virgin females did catch significantly more moths than the three-component mixture (8:1:1.3 ratio) formulated in a PVC slow-release lure (Table 2). Of the combinations of two chemicals, an 8:1 mixture of (Z)-13-octadecenyl acetate plus (Z)-11-hexadecenyl acetate and a 6.2:1 mixture of (Z)-13-octadecdenyl acetate plus (Z)-13-octadecenal caught some moths, although considerably less than either the females or the three-component blend.

Results from our tests indicate that all three components of the MRB sex pheromone identified in this study are necessary for a complete response by MRB males. Although there was a definite response in laboratory studies to a two-component blend consisting of (Z)-13-octadecenyl acetate and (Z)-13-octadecenal, the response was limited to wing fluttering and movement toward the source, with no attempt to land on paper strips containing the chemicals. However, males responding to the three-component blend frequently landed on the treated paper and exhibited precopulatory mating behavior, including curvature of the abdomen (Brown et al., 1988). Likewise, when the three-component blend was dispensed on small strips of filter paper attached to dowel

rods placed in sugarcane fields, rice borer males were observed through night vision equipment to land on the strip and exhibit precopulatory mating behavior. Some of the moths responding in such a manner were captured to confirm that they were male MRB.

When the various binary blends of pheromone components were placed in sugarcane fields in the same manner, a few male rice borers were detected hovering and orienting toward binary mixtures of (Z)-13-octadecenyl acetate combined with either (Z)-11-hexadecenyl acetate or (Z)-13-octadecenal, but none were observed landing on baits of these binary mixtures. No response was detected toward filters treated with a binary mixture of (Z)-11-hexadecenyl acetate and (Z)-13-octadecenal. This is in agreement with the trap catch data in which trap catches were greatest in traps baited with the three-component mixture, followed by binary mixtures containing (Z)-13-octadecenyl acetate, and none caught in traps baited with (Z)-11-hexadecenyl acetate plus (Z)-13-octadecenal. The fact that all the MRB caught in traps baited with the synthetic chemical blend were males and that males contacting the chemical blends in sugarcane fields exhibited typical precopulatory mating behavior supports the conclusion that these chemicals are components of the pheromone system in Mexican rice borers.

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EVIDENCE FOR OCCURRENCE OF MOUNTING SEX PHEROMONE ON BODY SURFACE OF FEMALE *Dermacentor variabilis* (SAY) AND *Dermacentor andersoni* (STILES) (ACARI: IXODIDAE)[1]

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Abstract—Male *Dermacentor variabilis* and *D. andersoni* respond to an unknown chemical or chemicals present on the body surfaces of partially engorged conspecific female ticks. Following contact, the males mount the females and apply their mouthparts and legs against the female dorsal body surface. Then, the males turn with these appendages still in close contact and crawl to the female's venter, whereupon they locate the gonopore, probe the vulva, and copulate. Similar responses are elicited by heterospecific as well as conspecific females. However, the response is lost when the female cuticle is cleaned (delipidized) with organic solvents. It can be restored by applying hexane extracts prepared from female cuticle to the previously cleaned females. Males do not use surface texture as the primary stimulus for mate recognition. Male ticks also respond to hexane extracts applied to spherical inanimate objects, ("dummy" female), suggesting that a chemical or chemicals soluble in organic solvents has been transferred to these objects. These findings suggest the existence of a previously undescribed pheromone, the mounting sex pheromone (MSP). This contact sex pheromone enables males excited and attracted by 2,6-dichlorophenol to identify the female as a potential mating partner. The MSP is the second in the series of three sex pheromones guiding the hierarchy of behavioral responses which constitute tick courtship behavior.

Key Words—*Dermacentor variabilis*, *Dermacentor* andersoni, acarina, acari, Ixodidae, mounting sex pheromone, ticks, American dog tick, Rocky Mountain wood tick.

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INTRODUCTION

In ticks, as in insects, mating is regulated by pheromones, primarily sex pheromones. The best known sex pheromones are the volatile phenolic compounds, e.g., 2,6-dichlorophenol (2,6-DCP). These compounds, which have been reported in 14 species of ixodid ticks, are secreted by sexually mature females to attract mate seeking males (Sonenshine, 1985). However, recognition of the volatile pheromone does not guarantee mating. In some species, the male will copulate only if it recognizes a species-specific pheromone in the vulva (Sonenshine et al., 1982). In others, mounting of heterospecific females may be aborted before the male reaches the gonopore (Khalil et al., 1983). Males attracted to 2,6-DCP placed on inanimate objects fail to recognize these objects as potential mates and leave quickly even if they make physical contact with them (Sonenshine, unpublished). These observations suggest that some stimulus provided by the body surface of the sexually mature female tick may be required to induce mounting and further exploration of these mating partners by the males. This stimulus may act as a contact sex pheromone. Alternatively, the characteristic shape and texture of the cuticular ridges may provide the recognition signal.

Cuticular contact sex pheromones occur in several insects, e.g., tsetse flies (Carlson et al., 1978). In arachnids, e.g., Tetranychidae (Cone, 1979) and spiders (Watson, 1986), they act as arrestants, with the male "guarding" the immature female until she molts to the adult stage. However, no contact sex pheromone of cuticular origin has been described in ticks.

We report the results of studies to establish the role of cuticular compounds and physical features of female ticks in mediating the mating behavior of male ticks.

METHODS AND MATERIALS

The American dog tick, *Dermacentor variabilis*, was colonized from wild-caught ticks collected near Richmond, Virginia. The Rocky Mountain wood tick, *Dermacentor andersoni*, was colonized from a population obtained from the U.S. Public Health Service, Rocky Mountain Laboratory, Hamilton, Montana. Ticks were reared and stored for experimentation as described previously by Sonenshine et al. (1977).

Newly emerged male and female adult ticks of both species were allowed to feed on laboratory rabbits (*Oryctalagus cuniculus*), immunologically naive to ticks, and were removed from the host six to seven days postattachment. They were used either in bioassays or to prepare cuticular extract.

Preparation of Cuticular Extracts. Cuticular extract (extract) was prepared from 100 engorging female ticks of each species, which were removed from the host rabbit after feeding for six to seven days. The ticks were frozen to facilitate removal of the cuticle (−20°C). Once frozen, the females were cut in two along the anterior–posterior midline and the body contents removed. The tick cuticle was placed in precleaned screw-top vials containing Shen's solution (Oliver, 1972). The cuticle was shaken briefly in the Shen's solution, and the solution then discarded. This cleaning process was repeated three to five times until all the visible blood and other contaminants had been removed from the cuticle. The cuticle was then dried on tissue paper and transferred to precleaned vials containing GC spectrophotometric grade hexane (2× distilled, Burdick and Jackson Labs., Inc., Muskegon, Michigan). The cuticle and solvent were vigorously shaken together, allowed to settle (5 min), the solvent decanted into another precleaned vial, and the extraction process repeated (three times). The combined solvent extract was reduced to 0.3 ml (N_2) in a precleaned microvial and stored at −20°C until required for bioassay. This extraction process is similar to one described by Gilby (1980). Male cuticular extract was prepared from fed (seven days) adults. These were frozen to −20°C. Once frozen, they were washed in hexane (100 ml) three times and the combined solvent extract reduced to 0.2 ml (N_2).

Bioassays. Three types of bioassay were carried out: on conspecifics, on heterospecifics, and on inanimate objects that could be coated with extract. All male ticks were prescreened to ensure that only those that were responding to females were used in the bioassays. Females were also prescreened to ensure that they were capable of exciting a mating response by the male before being cleaned. The females were cleaned as described below and assayed to ensure that they would no longer attract males before the extract was applied. Bioassays were performed in the laboratory at approximately 25°C on filter paper in a 9-cm-diam. Petri dish. The response of the male when presented to the immobilized female was scored by a system based on the mating behavior sequence described by Sonenshine (1985). The female was immobilized by attaching its anterior end to adhesive tape. The mating behavior sequence used in these experiments was as follows: (1) orientation of the male towards the female or "dummy" female (2,6-DCP source); (2) awareness of the female (touching with appendages); (3) mounting of the female; (4) turning on the dorsal surface of the female; (5) movement of the male to the ventral surface; and (6) locating and probing the gonopore.

Each stage was given one point when the male had achieved that stage. The number of points gained by each male was recorded. The number of times that the males successfully located and probed the gonopore were also recorded. The first two stages were not included in the final analysis as they are a measure

of the attractiveness of the 2,6-DCP, which has already been established for *D. andersoni* and *D. variabilis* (Sonenshine et al., 1977). Thus a completely successful male would gain four points in locating and probing the gonopore. The number of points gained by the males in their response to females or extracts was totaled and compared to the control using a one-tailed *t* test for comparing percentages (Sokal and Rohlf, 1969).

Chemical Removal of Female Cuticle Compounds. D. variabilis and *D. andersoni* females were cleaned in hexane after preliminary trials had demonstrated that it was as effective in removing the cuticular lipids as a combination of hexane, chloroform, and acetone. Females were cleaned by immersing them in the solvent and "scrubbing" them with a fine, stiff-bristle brush. The detached, partially fed female ticks were cleaned in this way for 10 min, which invariably resulted in the death of the female tick (hereafter termed "cleaned" female). Following surface cleaning, the females were tested for their ability to excite male mating response. This was done by adding 6 ng of 2,6-DCP (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) with a microcapillary pipet to the foveal gland area of the dorsal surface. Male responses were scored as previously described. Controls for these experiments were unmodified females tested with the same males challenged previously with the surface-cleaned females.

Extract-Coated Females. Cleaned females of both species were coated with either conspecific or heterospecific extract and then were bioassayed with conspecific and heterospecific males. Only those females that had been solvent cleaned and that had failed to excite the male mating response beyond stage three were treated and used in these bioassays.

In the conspecific bioassays, test females were coated with 6 μl of female extract containing ca. 1.5–2.0 female equivalents. Following coating, the females were maintained at host skin temperature (30°C) in sealed vials in an incubator to allow complete solvent evaporation. Control females were treated in exactly the same way, but only hexane was added. 2,6-DCP was added to both test and control females before being tested with the males. To perform the assay, a male was placed alongside the female (<2 cm), and its responses were observed and recorded. The same males were used in the test as in the controls, e.g., male 1 was used with both test female 1 and control female 1 and then discarded.

In the heterospecific assays, the cleaning and scoring schedule was the same as used for the conspecifics (see above). Cleaned *D. variabilis* females were coated with *D. andersoni* female extract and were tested with *D. variabilis* and *D. andersoni* males. Cleaned *D. andersoni* females were coated with *D. variabilis* female extract and tested with *D. variabilis* and *D. andersoni* males. Controls were cleaned females with hexane only. Cleaned females were coated with conspecific male extract (approximately 1.5–2.0 male equivalents). Sol-

vent was allowed to evaporate. Extract-coated females were then tested with conspecific males as described above.

Physical Disruption of Female Cuticle. The surface of the female was abraded with fine sandpaper to completely remove the cuticular ridges which the male may be using as orientation cues to locate the gonopore. No solvents were applied and no chemicals were removed. 2,6-DCP was added, and the male response observed as described above.

Inanimate Objects ("Dummy" Female Ticks). Males prescreened with conspecific females were used for this test. Smooth, inert spherical plastic beads (18 mm diam.) were prepared by roughening the surface with sandpaper and attaching them with fine copper wire to a cardboard support. A sample of extract (16 μl containing ca. 4–5 FE) was added incrementally to the entire surface and the hexane solvent allowed to volatilize while the treated specimen was held in an incubator (30°C). Each species extract was tested with both *D. variabilis* and *D. andersoni* males. Controls were plastic beads with hexane only added. The time spent by the male in contact with extract-coated dummy ticks was recorded. The same male was used for both the test and control parts of the experiment. The data were analyzed using a paired one-tailed t test, SAS Statistical Package (SAS Institute Inc., Cary, North Carolina).

RESULTS

The results of these experiments are summarized in three tables: Table 1, Conspecific Assays; Table 2, Heterospecific Assays; and Table 3, Inanimate Objects Assays.

The first column of the tables, "Extract Added," specifies which extract (*D. andersoni* female or *D. variabilis* female), if any, was added to either the cleaned female or inanimate objects. The second column, "Species of Female Treated," shows the species of female, if any, to which the extract was added (this column is not included in Table 3 as extract was added to inanimate objects only). This column also gives other relevant information on any other modification, i.e., if the female tick in column one was cleaned (clean) or not cleaned (n/clean) or if it was abraded (abraded) or not abraded (n/abraded). The third column, "Species of Male Used," shows which males were used in the bioassays.

Conspecific Assays. The male mating response was significantly reduced ($P < 0.01$) when solvent cleaned *D. andersoni* and *D. variabilis* females were tested with the males of both species (Table 1, experiments 1 and 2). When conspecific cuticular extract was applied to the prescreened, cleaned females, the male response was shown to be significantly greater than when it was absent ($P < 0.01$) (Table 1, experiments 3 and 4). In the case of *D. variabilis*, the

TABLE 1. REMOVAL AND RESTORATION OF MALE MOUNTING RESPONSES TO FEMALE TICKS IN *D. andersoni* (DA) AND *D. variabilis* (DV) AS DETERMINED BY BIOASSAY WITH CONSPECIFIC PARTNERS[a]

Exp. No.	Extract added	Species of female treated	Species of male used	T/C	*N*	Complete Test		Score	
						No.	Percent	Total	Percent[b]
1	None	DA clean	DA	T	20	0	0	24	30.0**
	None	DA n/clean	DA	C	20	20	100	80	100.0
2	None	DV clean	DV	T	20	0	0	17	21.3**
	None	DV n/clean	DV	C	20	20	100	80	100.0
3	DA	DA	DA	T	20	16	80	69	86.3**
	Hexane	DA	DA	C	20	0	0	11	13.8
4	DV	DV	DV	T	20	20	100	80	100.0**
	Hexane	DV	DV	C	20	0	0	13	13.8
5	None	DV abraded	DV	T	40	13	32.5	65	40.6**
	None	DV n/abraded	DV	C	40	40	100	160	100.0
6	None	DA abraded	DA	T	20	2	10	11	13.8**
	None	DA n/abraded	DA	C	20	20	100	80	100.0

[a] T = test; C = control; N = number of repetitions; Complete test, No. = total number of males reaching the gonopore, stage 6; Complete Test, % = % of all males tested that reached the gonopore; Score, total = the sum of points gained by males completing part or all of the test; Score, % = (total points gained by males completing part or all of the test/total points possible) × 100.

[b] $*P < 0.05$;$** P < 0.01$.

mating response was 100% restored, with *D. andersoni*, it was 86.3% restored. When the surface of the *D. andersoni* and *D. variabilis* females was abraded with find sandpaper, the male mating response was again greatly reduced ($P < 0.01$) (Table 1, experiments 5 and 6). Males did not respond to extracts made from the body surfaces of other conspecific males at the same concentration that was found to be effective for female extracts.

Heterospecific Assays. D. variabilis males showed a significant response to *D. andersoni* female extract when placed on cleaned *D. variabilis* females ($P < 0.01$) (Table 2, experiment 1). *D. andersoni* males also showed a significant response to *D. andersoni* female extract when placed on cleaned *D. variabilis* females ($P < 0.01$) (Table 2, experiment 2). *D. variabilis* males showed a significant response to *D. variabilis* female extract placed on *D. andersoni* females ($P < 0.01$) (Table 2, experiment 3). *D. andersoni* males also showed a significant response to *D. variabilis* female extract placed on *D. andersoni* females ($P < 0.05$) (Table 2, experiment 4). *D. variabilis* males showed a significantly higher response to conspecific female extract (100%) than to het-

TABLE 2. REMOVAL AND RESTORATION OF MALE MOUNTING RESPONSES TO FEMALE TICKS IN *D. andersoni* (DA) AND *D. variabilis* (DV) AS DETERMINED BY BIOASSAY WITH HETEROSPECIFIC PARTNERS[a]

Exp. No.	Extract added	Species of female treated	Species of male used	T/C	*N*	Complete test		Total score	
						No.	Percent	No.	Percent[b]
1	DA	DV	DV	T	20	16	80	67	83.8**
	Hexane	DV	DV	C	20	0	0	11	13.8
2	DA	DV	DA	T	20	14	70	70	87.5**
	Hexane	DV	DA	C	20	0	0	17	21.3
3	DV	DA	DV	T	20	17	85	74	92.5**
	Hexane	DA	DV	C	20	0	0	9	11.3
4	DV	DA	DA	T	20	8	40	49	61.3*
	Hexane	DA	DA	C	20	0	0	24	30.0

[a] See footnote[a] in Table 1 for explanation of terms.
[b] $*P < 0.05$; $**P < 0.01$.

erospecific female extract (83.8%) ($P < 0.05$). *D. andersoni* males showed a significantly higher response to conspecific female extract (86.3%) than to heterospecific female extract (61.3%) ($P < 0.05$). *D. variabilis* males were more responsive to conspecific female extract (100%) than *D. andersoni* males (86.3%) ($P < 0.05$). *D. andersoni* males and *D. variabilis* males were equally responsive to heterospecific extract (no significant difference between percentage scores).

Inanimate Objects (Dummy Ticks). *D. andersoni* males responding to conspecific extract-coated dummy female ticks showed a significantly higher percentage score than those males presented with controls ($P < 0.05$). *D. andersoni* female extract induced *D. andersoni* males to attempt the complete mating sequence (stage 6) in four cases (Table 3, experiment 1). However, the *D. andersoni* male response to dummy ticks coated with *D. variabilis* female extract was not significant (Table 3, experiment 2). *D. variabilis* males responding to conspecific extract-coated dummy female ticks showed a highly significantly increased percentage score compared to those males responding to controls ($P < 0.01$) (Table 3, experiment 3). *D. variabilis* males also showed a significantly increased response to *D. andersoni* female extract when compared with the controls ($P < 0.05$) (Table 3, experiment 4). Male *D. variabilis* ticks reached stage 6 seven times when responding to *D. variabilis* female extract and three times when responding to *D. andersoni* extract.

An analysis of the total time spent by each male tick in contact with the dummy tick revealed that *D. andersoni* males spent a highly significantly in-

TABLE 3. EXCITATION OF MALE MOUNTING PHEROMONE RESPONSES BY FEMALE TICK EXTRACTS DEPOSITED ON INANIMATE OBJECTS (DUMMY TICKS)[a]

Exp. No.	Extract added	Species of males used	T/C	N	Complete test		Total score		Time analysis[c]
					No.	Percent	No.	Percent[b]	
0	none	DA	C	20	0	0	22	27.8	
1	DA	DA	T	20	4	20	48	60.0*	$P = 0.0031$
2	DV	DA	T	20	1	5	30	37.5[ns]	$P = 0.2742$
	none	DV	C	20	0	0	16	20.0	
3	DV	DV	T	20	7	35	53	66.3**	$P = 0.0047$
4	DA	DV	T	20	3	15	41	51.3*	$P = 0.0001$

[a] See footnote[a] in Table 1 for an explanation of terms.
[b] $*P < 0.05$; $**P < 0.01$.
[c] Time analysis: The Student's t test was used to compare the time male ticks spent on the treated dummy ticks vs. the time ticks spent on untreated dummy ticks.

creased amount of time on *D. andersoni* female extract-coated ticks ($P = 0.0031$). The amount of time spent by *D. andersoni* males in contact with *D. variabilis* females extract-coated dummy ticks was not significantly different from the time spent by the *D. andersoni* male on the control dummy tick. The amount of time spent by male *D. variabilis* ticks in contact with female *D. variabilis* extract-coated dummy ticks was highly significant ($P = 0.0001$). Male *D. variabilis* also spent a highly significant amount of time in contact with *D. andersoni* female extract-coated dummy ticks ($P = 0.0047$). The same statistical analysis demonstrated that there was no significant difference in the amount of time spent on each of the control dummy ticks.

DISCUSSION

The results of the bioassays clearly indicate the presence of a cuticular sex pheromone, the mounting sex pheromone (MSP), produced by *D. andersoni* and *D. variabilis* females. Following their attraction by 2,6-DCP, males encountering this pheromone mount the females and apply their legs and mouthparts against the female body surface so that the male body is in direct contact with the female. Then, while still in close contact, they move to the female's venter and probe until they locate the gonopore. Thus, this sex pheromone mediates the mounting phase of ixodid tick courtship behavior, as described by Sonenshine (1985). This cuticular sex pheromone is essential for recognition of the female as a potential mating partner. Males are unable to recognize females

lacking this cuticular pheromone as potential mates, and these males terminate their courtship activities. However, the males' mating response can be restored by depositing the extract onto the bodies of surface-delipidized females or even transferred onto inanimate objects (dummy ticks), evidence which supports the hypothesis that this represents pheromone-mediated behavior.

Our evidence indicates that male *D. andersoni* and *D. variabilis* have a similar response to both heterospecific and conspecific females. There was no significant difference between the mounting responses of the males of either species to heterospecific vs. conspecific extracts when the extracts were applied to cleaned female ticks. However, male *D. andersoni* did not respond to female *D. variabilis* extract applied to dummy ticks, even though they exhibited strong, positive responses to the same extract applied to cleaned females.

Cuticular lipids are known to serve as sex pheromones in several species of insects where they act as contact aphrodisiacs. In the housefly, three compounds occur in the cuticular lipid fraction of sexually mature females which attract sexually active males. The most active component is an unbranched C_{23} alkene, (*Z*)-9-tricosene; the C_{28}–C_{30} methylalkanes, (*Z*)-9,10-epoxytricosane and (*Z*)-14-tricosen-10-one, enhance its activity (Blomquist et al., 1984). In the tsetse fly, *Glossina morsitans*, three paraffins which occur on the surface of the females, 15,19-dimethylheptatriacontane, 17,21-dimethylheptatriacontane, and 15,19,23-trimethylheptatriacontane, induce mating attempts by male flies upon contact with the females (Carlson et al., 1978). In both the housefly and tsetse fly examples, the compounds that act as cuticular sex pheromones are nonvolatile, requiring physical contact by the male before they can be detected. Following recognition of the pheromone in these insects, the males respond with mating attempts usually resulting in copulation.

Aside from the fact that male ticks also appear to require recognition of some lipid or lipids on the cuticle in order to mate, there is little similarity with the insect examples described above. In the case of ticks, the males have already been excited to begin mate seeking behavior by 2,6-DCP. The cuticular lipids act as a "mounting sex pheromone," enabling the male to recognize the female as a suitable mating partner and aid its search for the gonopore. Thus, the tick mounting sex pheromone is part of a multicomponent pheromone system that directs a sequence of behavioral events, i.e., mate seeking, mounting, location of the gonopore, and copulation. In contrast, the different hydrocarbons that comprise the dipteran pheromones all excite a single behavioral response, i.e., release of copulatory activity. The cuticular mounting sex pheromone is the third sex pheromone described in *D. variabilis* and *D. andersoni*, distinctly different from the general attractant sex pheromone and the highly species-specific genital sex pheromone. The mounting sex pheromone mediates a specific series of events in a complex hierarchy of stimulus and response events that constitutes courtship in these species.

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DIHYDROMATRICARIATE-BASED TRIGLYCERIDES, GLYCERIDE ETHERS, AND WAXES IN THE AUSTRALIAN SOLDIER BEETLE, *Chauliognathus lugubris* (COLEOPTERA: CANTHARIDAE)

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Abstract—The soldier beetle *Chauliognathus lugubris* is shown to contain triglycerides and glyceride ethers of 8-dihydromatricaria acid, and waxes of the C_{12} homolog of this acid, as well as the previously reported free acid. The triglycerides contain one, two, or three dihydromatricariate moieties, with any remaining positions esterified with normal fatty acids. The glyceride ethers were monostearyl ethers of glycerol esterified with dihydromatricaria acid and oleic or linoleic acid. The waxes, which also include a dihydromatricaria chromophore in the alcohol moiety, occur only in the females and are present in paired accessory glands in the abdomen. The ethers are restricted to females and appear to be associated with developing eggs. The triglycerides are much more abundant in females than males. Triglycerides, glyceride ethers, and waxes represent about 95% of the dihydromatricariate moiety (average, ca. 590 μg) in females with free acid the remainder; in males free acid is present to over 50% (ca. 22 μg) and the remainder is triglyceride (ca. 15 μg). Larvae contain mainly tridihydromatricariate-substituted triglyceride and a smaller quantity of the free acid.

Key Words—Dihydromatricaria acid, triglyceride, (Z)-dec-8-ene-4,6-diynoic acid, glyceride ethers, waxes, antifeedant, *Chauliognathus lugubris*, Coleoptera, Cantharidae, accessory glands, defense.

INTRODUCTION

The presence of (Z)-8-dihydromatricaria acid [(Z)-dec-8-ene-4,6-diynoic acid] in soldier beetles was first reported by Meinwald et al. (1968) for *Chauliog-*

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nathus lecontei. These authors showed that the acid was produced as a milky secretion in a series of defensive glands in the prothorax and the first eight abdominal segments. The same acid, along with precoccinelline and related alkaloids, was also found in the Australian species *C. lugubris* (F.) [=*C. pulchellus* (Macleay)] (Moore and Brown, 1978). Subsequently Eisner et al. (1981) reported dihydromatricaria acid in glands and hemolymph of *C. pennsylvanicus* and demonstrated its feeding deterrency to jumping spiders (*Phidippus* spp.). In the present paper we report the additional presence in *C. lugubris* of triglycerides and glyceride ethers of dihydromatricaria acid, and waxes of the C_{12} homolog of this acid with C_{12} and C_{14} alcohols also including the dihydromatricaria chromophore. The waxes and ethers occur in females only.

Nomenclature. The term "dihydromatricaria chromophore" is used to describe the chromophore associated with the conjugated ene-diyne system present in dihydromatricaria acid (Figure 1), which gives rise to the UV spectrum described by Eisner et al. (1981) [233 nm, (log ξ = 3.03), 240 (3.45), 254 (3.78) 267 (3.99) and 282 (3.90)]. The term "total dihydromatricariate" is used for all material bearing the above chromophore regardless of the chemical nature or chain length. It is normally expressed quantitatively as the corresponding amount of free dihydromatricaria acid.

METHODS AND MATERIALS

Gas Chromatography. Gas chromatography (GC) was conducted on a Varian 2100 instrument with a Varian CDS 111 data processor, and with flame ionization detectors and a nitrogen flow of 25 ml/min. The following glass columns were used: column 1, 2 m × 2 mm ID of 3% (w/w) OV-101 on Chromosorb W; column 2, 2 m × 3 mm ID of 5% (w/w) OV-101 on Gas-Chrom Q; column 3, 4 m × 3 mm ID of 5% (w/w) Carbowax 20 M on Gas-Chrom Z; column 4, 2 m × 3 mm ID of Porapak P; and column 5, 2 m × 3 mm ID of 1.5% (w/w) OV-17 on Gas-Chrom Q.

Samples for mass spectra or other purposes were collected from the gas chromatograph in capillary tubes cooled, when necessary, by carbon dioxide snow. Kovats' indices (I) were measured isothermally using saturated alkanes as standards. Percentage compositions, when quoted, are uncorrected for the response factors of the individual components.

Thin-Layer Chromatography. Thin-layer chromatography (TLC) was performed using two main systems: system 1, 200-mm glass plates coated with silica gel HR with zinc sulfide fluor and developed with dichloromethane; and system 2, 200-mm glass plates coated with aluminum oxide G with zinc sulfide fluor developed with hexane–ether 2:1. Visualization was by ultraviolet (UV) irradiation (254 nm) or iodine staining. When required, the components were

recovered from the plates by excision of the spot or band, extraction of the material with dichloromethane–methanol 9 : 1, filtration of the extract, and concentration to the desired volume in a stream of nitrogen.

High-Performance Liquid Chromatography. High-performance liquid chromatography (HPLC) was performed on a Waters Associates instrument with two M6000 pumps, a model 720 system controller, and a model 450 variable wavelength UV detector. The column was a Regis Workhorse octadecylsilyl 300 × 4.6 mm ID reversed phase. Gradient programmed elution (system A) was used with two solvents: water (A) and methanol–2-propanol 7 : 3 (B). The stages used were: initially 25% A, 75% B; at 8 min, 10% A, 90%B; at 23 min, 100% B, at 30 min, 100% B; at 31 min, 25% A, 75% B. All gradients were linear, the flow rate was 2 ml/min, and the wavelength was set to 267 nm. Alternatively, an isocratic system (system B), with methanol–dichloromethane 85 : 15, a flow rate of 2 ml/min, and simultaneous UV and refractive index (RI) detection, was used. Samples for mass spectroscopy and other purposes were obtained by collection and evaporation of the eluant.

The quantities quoted in Tables 1 and 2 (except for the free acid) are the HPLC peak height at 267 nm for a 50-μl injection at sensitivity ×1.0. The solvent gradient was such that, within experimental error and except for the free acid, the peak widths at half height were constant. For the free acid, the width was only one third that of the other components and its quantity was calculated accordingly.

Mass Spectrometry. Mass spectra were determined on a VG Micromass 70-70 mass spectrometer interfaced to a Hewlett Packard 5790A gas chromatograph and a VG 11-250 data system. Volatile samples were introduced via GC with a 25-m bonded-phase 5% phenyl-, methyl-silicone capillary column (SGE, BP5, 0.5 μm film thickness). Components of low volatility, after separation by TLC or HPLC, were analyzed by flash volatilization from an extended gold support (Brown et al., 1985), or a polyimide probe tip, with rapid data acquisition. This method markedly increased the relative abundance of molecular parent ions compared with conventional probe techniques, simplified the fragmentation patterns, and facilitated the analysis of unresolved mixtures. Electron ionization (EI) mass spectra were determined with an ionization energy of 70 eV and trap current of 100 μA. Chemical ionization (positive ion, PCI, and negative ion, NCI) mass spectra were obtained at 50 eV ionization energy and 200 μA emission current. Methane, isobutane, and ammonia were used as reagent gases at estimated source pressures of 100, 80, and 60 Pa, respectively. Source temperatures were 180–210°C.

Ultraviolet Spectroscopy. Ultraviolet spectroscopy was performed on a Pye Unicam SP 8000 recording spectrophotometer. Dichloromethane and methanol were the solvents in 1-mm or 10-mm quartz cells.

Insects. Batches of *C. lugubris* beetles were obtained from beneath flow-

ering *Eucalyptus* spp. trees according to season in the Canberra area. Larvae were obtained from soil and litter samples also collected in the Canberra area.

Treatment of C. lugubris. The secretion from the gland was obtained as described previously (Moore and Brown, 1978).

Total extraction, *C. lugubris* beetles, or parts thereof, were crushed in dichloromethane and left in this solvent at 5°C for 24 hr. The dichloromethane extract was separated by filtration, the residue reextracted with dichloromethane and filtered again, and the filtrates combined. The dichloromethane solution was diluted to 25 ml for UV spectroscopy or concentrated for TLC analysis. Estimates of total dihydromatricariate were made by measurements of the UV absorbance of the untreated dichloromethane extracts using the extinction coefficient given by Eisner et al. (1981). The relative abundances of the different components were determined by a TLC separation followed by UV absorbance measurements. Samples for HPLC were purified by chromatography on a silica Sep-Pak eluting with dichloromethane–methanol 9:1. The solution was then evaporated, the residue taken up in methanol–2-propanol 7:3 (500 μl), and the resulting solution filtered through a nylon membrane (pore size 0.45 μm).

Transesterification. Methanolysis reactions were carried out by heating in 1% sulfuric acid in methanol (50 μl) in a Reacti-vial at 100°C for 15 min. The product was used directly for GC or TLC analysis. Acetic acid transesterifications were carried out similarly using 1% sulfuric acid in acetic acid at 90°C for 2 hr.

Hydrolysis of Glyceride Ethers. A sample of the glyceride ether was treated with methanolic sodium hydroxide (1%, 50 μl) at 70°C for 30 min in a sealed Reacti-vial. The resulting solution was extracted with hexane and the hexane extract treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (25 μl) at 70°C for 30 min. The hexane solution was analyzed directly by GC.

Hydrogenation. Hydrogenations were carried out in septum-sealed Reacti-vials (with a vent) with a trace of Adams platinic oxide catalyst and methanol (20–30 μl) as solvent. The hydrogen was introduced by means of a syringe and bubbled through the mixture to provde agitation.

Ozonolysis. Microzonolyses were conducted in carbon disulfide solution in a Reacti-vial cooled by carbon dioxide snow. Ozonized oxygen was bubbled through using a 10-ml syringe, which previously had been charged from the ozonizer, till a starch iodide paper turned blue. The ozonide was then decomposed with a trace of triphenylphosphine and the resulting solution analyzed directly by GC (column 3 at 70°C and column 4 at 120°C).

Reference Compounds. (*Z*)-8-Dihydromatricaria acid and its methyl ester used as reference compounds were from samples previously characterized (Moore and Brown, 1978) from *C. lugubris* glands. Tripalmitin was obtained from Calbiochem (La Jolla, California) and batyl alcohol from Sigma Chemical Company (St. Louis, Missouri).

RESULTS

Chromatographic Separations. Previous work on various species of *Chauliognathus* involved either analysis of secretion obtained directly from the defensive glands or chemical extractions, specifically aimed at isolating only free acids. However, TLC analysis (on silica with dichloromethane–methanol 9:1) of a dichloromethane extract of whole *C. lugubris* beetles showed, in addition to the free acid, a great deal of UV-absorbing material of high R_f. Subsequently TLC (system 1) showed the free dihydromatricaria acid at the origin, a major UV absorbing spot (fraction A) at R_f 0.70, and a partially resolved series of components from R_f 0.60 to 0.30. Exposure to iodine vapor gave rise to strong staining at R_f 0.55 probably due to the normal fats (tripalmitin had R_f of 0.55). Methyl dihydromatricariate had R_f of 0.64 but was not present in the extract. The UV spectrum of fraction A (in methanol) and that of the partially resolved band showed the characteristic dihydromatricaria chromophore (cf. Eisner et al., 1981). Absorbance measurements showed that the free acid represented about 5% of total dihydromatricariate, fraction A about 45%, and the other band about 50%.

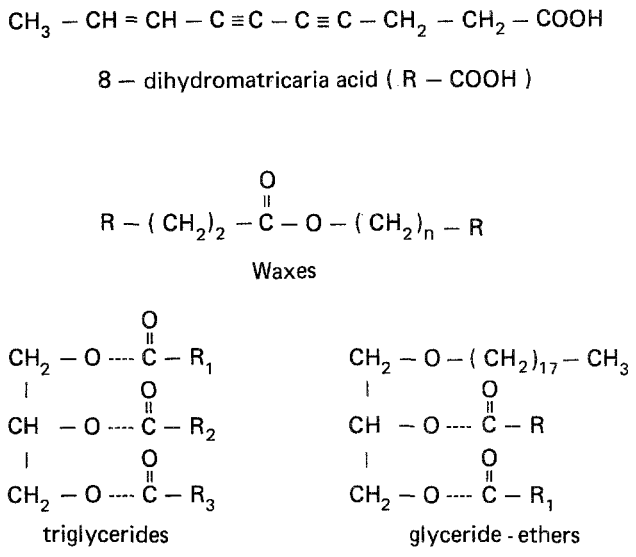

FIG. 1. Formulas for *C. lugubris* components. A dotted line indicates that the position of the substituent may be undetermined. Waxes: A1, $n = 3$; *A2*, $n = 5$. Glyceride ethers: B1, R_1 = linoleyl, B2, R_1 = oleyl. Triglycerides: C, $R_1 = R$, $R_2 = R_3$ = palmityl, stearyl, oleyl or linoleyl; D1, $R_1 = R_2 = R$, R_3 = linoleyl; D2, $R_1 = R_2 = R$, R_3 = palmityl or oleyl; D3, $R_1 = R_2 = R$, R_3 = stearyl; E, $R_1 = R_2 = R_3 = R$.

TLC with system 2 gave better resolution and produced four clearly resolved spots. These had R_f values of 0.74 (ca. 60% of the total absorbance), 0.6 (7%), 0.43 (15%), and 0.23 (15%), and there was a little free acid at the origin. All of these fractions showed the dihydromatricaria chromophore. The strong iodine-staining area coincided with the spot at R_f 0.74. Subsequent TLC of the components from these spots on system 1 showed that the R_f 0.74 spot comprised fraction A and another fraction (B) with R_f 0.53; the 0.6 material had R_f 0.47 (fraction C); the 0.43 material had R_f 0.43 (fraction D); and the 0.23 material had R_f 0.35 (fraction E).

Reversed-phase HPLC of the *C. lugubris* mixture achieved greater resolution of the components and the observed peaks have been correlated with HPLC analyses of the separate TLC fractions A–E (Table 1). Normal-phase HPLC added little to the information derived from silica TLC and was not continued.

Identification of Fraction A. GC analysis of TLC fraction A (column 1, 250°C) showed four major peaks with retention times 7.95 min (15%, I = 2903), 9.01 min (5%, I = 2951), 13.50 min (51%, I = 3106), and 15.26 min (28%, I = 3155). These indices suggested that the components were two pairs of homologs with a chain-length difference of two carbon atoms. Separate GC analyses of HPLC peaks A1 and A2 showed that the first two GC components

TABLE 1. HPLC OF *C. lugubris* DICHLOROMETHANE EXTRACT

R_t (min)	Peak height, cm (%)[a]	Fraction	Mol wt[b]
1.4	3.0 (3.8)	free acid	162
6.4	12.3 (15.7)	E	524
7.7	9.0 (11.5)	A1	348
9.4	22.5 (28.7)	A2	376
10.3	1.2 (1.5)	A3	
11.0	0.8 (1.0)	A4	
13.05	2.1 (2.7)	D1	642
13.95	14.6 (18.6)	D2	618 + 644
15.4	1.0 (1.3)	D3	646
17.2	0.5 (0.6)	C	
20.0	0.2 (0.3)	C	
21.4	0.4 (0.5)	C	
22.45	1.5 (1.9)	C	
23.25	0.3 (0.4)	C	
24.1	0.2 (0.3)	C	
24.6	1.7 (2.2)	B1	750
25.6	7.2 (9.2)	B2	752

[a] The peak height (cm) represents the total dihydromatricariate content of the component.
[b] From mass spectroscopic measurements.

were derived from A1 and the second pair from A2. The mass spectra of the material from peaks A1 and A2 (PCI, methane) showed the presence of components with molecular weights of 348 (MH^+ m/z 349) and 376 (MH^+ m/z 377), respectively.

Fraction A1 was methanolyzed and the product analyzed by TLC (system 1). Two UV-absorbing spots were resolved at R_f 0.59 [cf. methyl (Z)-8-dihydromatricariate at 0.60] and at R_f 0.23. Both of these components showed the characteristic dihydromatricaria UV spectrum. GC analysis (column 2, 150°C) of the R_f 0.59 band from methanolysis of A1 showed two peaks; the major one had R_t of 7.16 min (90%, I = 1698) and the minor had R_t 8.68 min (10%, I = 1747). After catalytic reduction, these two peaks collapsed to one, which was identified by GC R_ts (columns 2 and 3) as being due to methyl dodecanoate. GC-MS (EI) of the unreduced methyl ester fraction showed that both components had molecular weights of 204 and similar fragmentation patterns. The mass spectra were strongly reminiscent of that of methyl (Z)-8-dihydromatricariate and thus these components are almost certainly the Z and E isomers of the C_{12} homologue of methyl 8-dihydromatricariate. Ozonolysis of the major methyl ester component gave only acetaldehyde as a detectable product. The major component would be thus methyl (Z)-dodec-10-ene-6,8-diynoate and the minor component probably the E isomer. The stereochemistry was assigned by analogy with the C_{10} homologs, where Meinwald et al. (1968) have shown the Z isomer to predominate.

Material from the R_f 0.23 fraction from methanolysis of A1 was analyzed by GC on column 1 at 150°C and two components were detected; these had retention times of 5.87 min (76%, I = 1647), and 7.15 min (24%, I = 1698). After catalytic reduction only one component was detected and this was identified by GC R_ts (columns 2 and 3, 150°C) as dodecanol. GC-MS of the unreduced material showed molecular weight of 176 for the first eluting material. These components were therefore, presumably, the Z and E isomers of a C_{12} alcohol containing the dihydromatricaria chromophore. Ozonolysis led to the detection of only acetaldehyde showing the components of this methanolysis fraction to be (Z)- and (E)-dodec-10-ene-6,8-diynol. There was a greater proportion of the E isomer than is the case with the methyl esters. A1 therefore consisted of waxes derived from the above acids and alcohols.

The main component of fraction A1 was (Z)-dodec-10-ene-6,8-diynyl (Z)-dodec-10-ene-6,8-diynoate. E isomers (particularly in the alcohol moiety) were represented in less abundant waxes of A1. Similarly A2 was shown to contain waxes of the homologous (Z)- and (E)-tetradec-12-ene-8,10-diynol and (Z)- and (E)-dodec-10-ene-6,8-diynoic acid. The minor wax fractions, A3 and A4, appeared to be mixtures and were not studied further.

Identification of Fraction B. Fraction B was obtained by successive TLC on systems 1 and 2. However, the strong iodine staining, concurrent with the

UV absorption, indicated that the dihydromatricariate-containing components were cochromatographing with the ordinary fats. HPLC (system B) of fraction B showed two UV-absorbing peaks with retention times of 5.66 min (B1) and 6.52 min (B2). In addition to these components, the RI detector showed several other components, presumably fats, at longer retention times.

The mass spectrum of B2 (PCI, NH_3) showed an $(M + NH_4)^+$ ion at *m/z* 770, consistent with a molecular weight of 752, and major fragments at *m/z* 591 (M-161, loss of dihydromatricariate), and *m/z* 471 (M-281, loss of oleate). The negative ion spectrum (NCI, isobutane) showed a small ion at *m/z* 751 $[(M - H)^-]$ and major ions at *m/z* 281 (oleate) and *m/z* 161 (dihydromatricariate). These spectra were consistent with a monostearyl ether of glycerol esterified with dihydromatricaria acid and oleic acid.

The mass spectrum (PCI, NH_3) of B1 was rather similar and showed an ion at *m/z* 768, indicating a molecular weight of 750, and major ions at *m/z* 589 (M-161, loss of dihydromatricariate) and *m/z* 471 (M-279, loss of linoleate). Thus B1 was very similar to B2 except that the oleate moiety had been replaced by linoleate. This slightly more polar component would be expected to elute before B2 on reversed-phase HPLC.

Methanolysis of B2 followed by GC analysis (column 3 at 200°C) showed that the major methyl esters produced were in fact methyl oleate and methyl dihydromatricariate. The small quantities of other esters produced probably arose because the sample was not completely clear of the normal fats. There was some evidence of the latter in the form of small peaks at higher mass in the mass spectrum and in the fact that the ratio of dihydromatricariate to fatty ester was lower than expected. Methanolysis of B1 was not very helpful because of even greater interference from these fats. However, the abundance of methyl linoleate was much higher than in any of the other methanolysis reactions.

Transesterification of B2 with acetic acid–sulfuric acid followed by GC (column 1, 180°C programmed at 4°C/min) gave two major components with R_t 7.48 min and 18.64 min, respectively. The mass spectrum of the short R_t material (PCI, NH_3) showed a probable molecular weight of 368, consistent with replacement of one of the ester groups by acetate and elimination of the other. The mass spectrum of the long R_t material (PCI, NH_3) showed a probable molecular weight of 428 and was consistent with replacement of both the original ester moieties in the molecule by acetate.

Hydrolysis followed by silylation of B1 and B2 gave a product with an R_t (columns 1 and 5) identical with that of the trimethylsilyl ether of batyl alcohol (racemic 3-octadecyloxy-1,2-propanediol). This supported the presence of a stearyl ether moiety, probably in a primary position, in B1 and B2.

Identification of Fraction C. The mass spectrum of TLC fraction C (PCI, NH_3) showed prominent $(M + NH_4)^+$ ions at *m/z* 782 (mol wt 764) and 756 (mol wt 738) consistent with triglycerides with one dihydromatricariate and two

oleate moieties; and one dihydromatricariate, one oleate, and one palmitate, respectively. There were additional less intense ions representing glycerides with other possible combinations of fatty acids.

Methanolysis of fraction C followed by GC analysis (column 3 at 200°C) led to the detection of the following methyl esters: methyl palmitate (17%), methyl (*Z*)-8-dihydromatricariate (16%), methyl (*E*)-8-dihydromatricariate (5%), methyl stearate (7%), methyl oleate (51%), and methyl linoleate (3%). The ratio of dihydromatricariate to the normal fatty esters (allowing for chain length) was consistent with a 1 : 2 ratio of dihydromatricariate to fatty esters.

HPLC showed that fraction C was composed of a number of different components most of which were minor. Because of the small amounts of the components, individual mass spectra were not recorded. This greater nonhomogeneity was consistent with random distribution of fatty acid moieties in two positions of glycerol.

Identification of Fraction D. The mass spectrum (PCI, NH_3) of D2, the major HPLC peak of fraction D, indicated that at least two compounds were present. The major one had a molecular weight of 644 [$(M + NH_4)^+$ *m/z* 662] and fragments at *m/z* 483, 363, and 103; the minor component had a molecular weight of 618 [$(M + NH_4)^+$ *m/z* 636] and had fragments at *m/z* 457, 363, and 103. These mass spectra indicated that the major component was a triglyceride with two dihydromatricariate moieties and one oleate group (ions of *m/z* 483 represented M-dihydromatricariate and *m/z* 363 M-oleate). The minor component would be similar but with a palmitate group instead of the oleate.

The minor components of the fraction D (D1 and D3) showed molecular weights of 642 and 646, respectively, and were therefore considered to be triglycerides with linoleate and stearate in addition to the two dihydromatricariate groups.

Methanolysis of fraction D followed by GC analysis (columns 2 and 3) led to the detection of methyl (*Z*)-8-dihydromatricariate (45%), the *E* isomer (4%), methyl palmitate (10%), methyl oleate (34%), methyl stearate (5%), and methyl linoleate (1.5%). These data supported the mass spectral findings since the peak area ratio of the normal fatty esters to dihydromatricariate (ca. 1 : 1), after allowing for differences in molar responses to the detector because of chain length, was close to that expected for a triglyceride with two dihydromatricariate moieties and one fatty ester moiety.

Identification of Fraction E. The mass spectrum (PCI, NH_3) of fraction E showed a molecular weight of 524 [$(M + NH_4)^+$ *m/z* 542] and also major fragment ions at *m/z* 363 (M-161, loss of dihydromatricariate) and *m/z* 103. All these data indicated that fraction E was a triglyceride with dihydromatricariate in all three positions. This was confirmed by methanolysis of fraction E followed by GC analysis (column 2 at 150°C programmed at 4°C/min to 250°C and column 3 at 150°C) when only methyl (*Z*)-dihydromatricariate and about

10% of the *E* isomer were detected. Fraction E was therefore largely tri-(*Z*)-8-dihydromatricarin.

Biological Distribution of Components. To appreciate better the role of the various dihydromatricaria-containing components, an attempt was made to locate precisely their occurrence in the beetles. Earlier work had shown that the defensive glands contained little but the free acid. Males (3, average weight 40 mg) and females (3, average weight 60 mg) were assayed individually by UV spectroscopy. Males contained an average of 37 μg of total dihydromatricariate but females averaged much higher at 590 μg. TLC separation (system 1) followed by UV estimation of the resulting fractions showed that the males contained 22 μg of free acid and the remaining material was in the band representing fraction B–E. Females, however, contained only 24 μg of free acid; ca. 44% (260 μg) of the total dihydromatricariate was fraction A (waxes) and the remainder (306 μg) was in the band due to fractions B–E.

The above findings were subsequently supported and expanded by HPLC analyses of individual males and females. These results are summarized in Table 2. Males again were shown to contain much less dihydromatricariate material than females, and most of theirs was free acid; the remainder was triglyceride. Males did not appear to contain any waxes or ethers, and the small quantities of these components detected in some specimens could well have been acquired adventitiously through contact with females. It was again noticeable that the free acid content of both sexes was similar, but females had very much more

TABLE 2. HPLC ANALYSIS OF INDIVIDUAL BEETLES

			Fraction					
Beetle	Wt (mg)	Acid	A	B	C	D	E	Total
Females								
1	48.7	8.3	46.3	15.7	12.1	31.1	25.2	139
2	47.5	8.7	12.5		6.8	1.3	4.7	34
3	46.9	3.3	32.4	1.2	12.1	3.5	5.4	58
4	65.4	6.0	27.6	9.8	4.9	22.6	14.0	85
5	55.5	2.8	20.6	5.4	2.7	13.6	9.2	54
Males								
1	43.0	2.6			1.4	0.1	0.8	4.9
2	43.4	3.4	0.3	0.1	1.4		0.3	5.6
3	35.4	5.0	0.3		0.9	1.4		7.6
4	36.4	1.9		0.05	0.6	0.1	0.04	2.7
5	38.7	4.8	0.1		0.9		0.3	6.2

[a]The values quoted are the peak heights (cm) at 267 nm and $\times 1.0$ sensitivity for a 50-μl injection from 500 μl of solution.

of the bound dihydromatricariate. There was considerable variation in the proportions of the various components between individuals. This may have reflected the level of maturity as well as individual variation.

Since individual males were very low in dihydromatricariate, an extract of 40 males was analyzed by TLC (system 1). The bands corresponding to the free acid and triglyceride–glyceride ether were removed (no waxes were present) and, after extraction, the UV spectra of the resulting solutions were recorded. These showed that free acid represented ca. 55% of the total dihydromatricariate. Subsequently HPLC analysis of the triglyceride–glyceride ether band extract showed that it contained mainly triglycerides with a single dihydromatricariate moiety (C, 64%) with smaller quantities of disubstituted (D, 8.5%) and trisubstituted (E, 27%) triglycerides. No glyceride ethers were detected. These data were consistent with those for individual males (Table 2) and confirmed the absence of waxes and glyceride ethers in males.

The following parts of beetles were then assayed separately for dihydromatricariate: (1) Elytra, from males or females contained very little dihydromatricariate material. (2) The area from around the defensive gland of several combined males and females contained mainly free acid but with small quantities of the other components. (3) Eggs, removed by dissection, contained triglycerides (mainly with two or three dihydromatricariate moieties) and ethers (37%). No waxes or free acid were detected. (4) Viscera from males contained mainly triglycerides with a smaller quantity of the free acid.

Since the wax was present only in the females and was absent from the eggs, it seemed likely that it would occur in abdominal accessory glands and be used for coating the eggs immediately prior to oviposition. This was confirmed upon dissection when large paired colleteral glands were discovered. These glands, which were replete with a white wax like substance, filled much of the sixth and seventh abdominal segments and opened, via individual ducts, into the vagina. GC analysis of their contents revealed only the components of the wax fraction A, of the total extracts.

The very high level of glyceride ethers in eggs suggested that, in the extracts studied, these components were largely derived from eggs and hence would be absent from males. Also it might be expected that nongravid females would be low in ethers. To confirm this supposition, an extract of a number of females of low gravidity was examined. This sample showed a high level of wax (60%) but only about 6% of glyceride ethers (16%) after allowance for waxes and free acid; most of the remainder was triglyceride. This value for ethers was lower than for the bulk sample (21%), despite the fact that the latter contained males as well as females. These data indicated that the glyceride ethers were associated with eggs or possibly the ovarian system as a whole. Thus the level of glyceride ethers in individual females reflects the state of ovarian development.

Several batches of larvae of *Chauliognathus* spp., after extraction with dichloromethane, showed the characteristic dihydromatricariate UV spectrum. TLC and HPLC of this solution showed that the major UV-absorbing material was fraction E (trisubstituted triglyceride); there was a small quantity of free acid present, but none of the other components was detected.

Preliminary investigation of a female of *Chauliognathus nobilitatus* (Erichson) by HPLC showed a very similar distribution of dihydromatricaria-containing compounds. However, in this species, there was very little, if any, free acid, and the wax fraction (A) showed greater diversity in chain length.

DISCUSSION

The detection, in *C. lugubris*, of glycerides and waxes based on the dihydromatricariate moiety contributes to our knowledge of the defensive chemistry of these beetles. The results also demonstrate a significant difference between the sexes, which was not apparent in the case of the defensive glands. In males the free acid accounts for much of the total dihydromatricariate content, but in the females it constitutes only a very small proportion of the total.

The dihydromatricariate-containing triglycerides are probably associated with the fat body and would provide a further dose of antifeedant material to increase the discomfort of any predator naive enough to consume one of these beetles; they may also act as a reserve of the acetylenic moiety from which depleted defensive glands are replenished. The greater abundance of dihydromatricariate-substituted material in females than in males presumably reflects the larger bulk of fat body and the presence of accessory glands and developing eggs in the former sex.

An interesting discovery reported in this paper concerns the wax-containing accessory glands present only in females. The waxes have the dihydromatricaria chromophore incorporated into both their alcoholic and acidic components; they are present in very substantial quantities (ca. 300 μg/female or about 0.5% of body weight). The location of the glands and the nature of their secretion suggest that they dispense an egg protectant. The waxes contained in these glands would be deposited on the eggs as they are laid providing, possibly, both a bacteriostatic and antifeedant protective coating. It is noteworthy that the chain length of the acidic component of the wax is increased to C_{12} and that the alcoholic moiety to mainly C_{14} but with a smaller proportion of C_{12} material. This increase in chain length presumably enhances the properties of the wax in its role as a protective surface lipid.

We have no knowledge of the specific role of the glyceride ethers in eggs but they are presumably part of the protective mechanism. As far as we are aware, this is the first report of glyceride ethers from insects, although they

have been reported previously from marine sources, especially shark liver oil, and from mammals and microorganisms (see Downing, 1976).

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BENEFICIAL ARTHROPOD BEHAVIOR MEDIATED BY AIRBORNE SEMIOCHEMICALS. II. Olfactometric Studies of Host Location by the Parasitoid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae)[1]

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Abstract—The response of *Microplitis croceipes* (Cresson) to odors from a plant–host complex was investigated using a four-choice olfactometer. Female, but not male, parasitoids responded in a dose-dependent fashion to volatiles from the plant–host complex and oviposition experience enhanced this response. Female age had no apparent effect on the response. Both artificially damaged leaves and frass elicited positive responses but of lower magnitude than those elicited by the plant–host complex. Volatiles collected from the plant–host complex placed on filter paper also elicited positive responses by female parasitoids.

Key Words—Hymenoptera, Braconidae, *Microplitis croceipes*, *Heliothis zea*, parasitoid, host selection, volatile attractant, olfactometer.

INTRODUCTION

Host location, as defined by Lewis et al. (1976), is often mediated by chemical cues (Vinson, 1976). These chemical cues are defined as long- and short-range, depending on whether they act over a distance that precludes gustation (i.e., are olfactory) or act only by gustation (Vinson, 1976; Weseloh, 1981). Only a few long-range chemical cues used by parasitoids during their host-location

[1] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

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process have been identified. A few examples are the plant odors allyl isothiocyanate by *Diaeretiella rapae* (Hymenoptera: Ichneumonidae) (Read et al., 1970), α-pinene by *Heydenia unica* (Hymenoptera: Pteromalidae) (Camors and Payne, 1972), and several sesquiterpenes by *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) (Elzen et al., 1984).

Although 13-methylhentriacontane has been isolated from *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) and shown to elicit the short-range behaviors of antennation and ovipositor probing in the parasitoid *Microplitis croceipes* (Cresson) (Jones et al., 1971), no long-range chemical cues have been identified for female *M. croceipes*. There is evidence that *M. croceipes* does use olfactory cues during its host-location process; Drost et al. (1986) reported on the response of *M. croceipes* in a wind tunnel to olfactory cues. This paper reports olfactometric studies of the response of *M. croceipes* to olfactory cues and factors that might affect its host-location behavior.

METHODS AND MATERIALS

Insects. Microplitis croceipes, reared on *H. zea*, were obtained as pupae from the Insect Biology and Population Management Research Laboratory (Tifton, Georgia). The pupae were held for emergence in an environmental chamber maintained at 26°C, a relative humidity of ca. 70%, and a photocycle of 15:9 hr light–dark. Male and female parasitoids were caged together to allow mating and were provided with water and honey.

Heliothis zea eggs were obtain from the Insect Attractants, Behavior, and Basic Biology Research Laboratory (Gainesville, Florida). After hatching, larvae were fed pink-eye, purple-hull cowpea [*Vigna unguiculata* (L.)] seedlings.

Olfactometer. The response of *M. croceipes* to volatile chemicals was examined in a four-choice olfactometer (Pettersson, 1970) with dimensions as described by Vet et al. (1981) (Figure 1). Differences are as follows: internal chamber height was 16 mm; inlet port inside diameter was 9.5 mm; and insect trap, odor chamber, and humidifying vials were of a slightly different design (Figure 1). In addition, illumination was provided by a 20-W circular fluorescent lamp 20 cm above the olfactometer. The complete olfactometer system was housed in a room maintained at 27–28°C.

A vacuum pump, placed outside the room, drew air through the olfactometer at a rate of 1200 ml/min (300 ml/min through each of the four quadrants). Odor fields were visualized using ventilation smoke tubes (Mine Safety Appliances Co., Pittsburgh, Pennsylvania). The pressure inside the olfactometer was never more than 4 cm of water (ca. 2.9 mm Hg) below atmospheric pressure when measured with an open-end, water-filled manometer connected to the olfactometer at the center extractor tube.

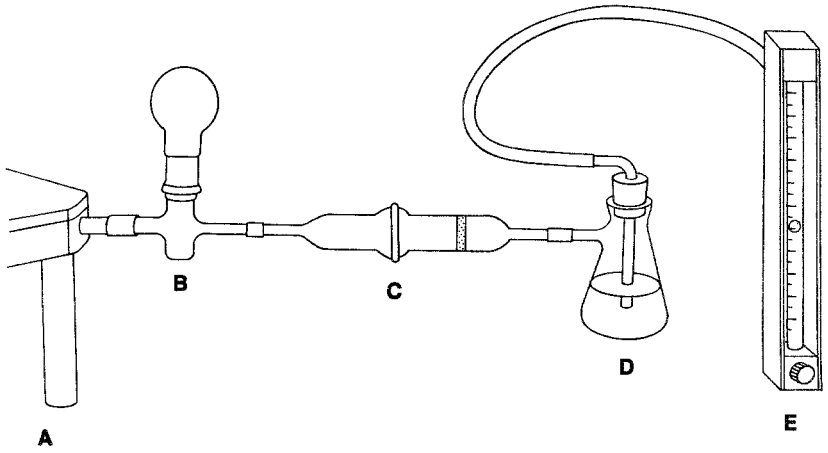

FIG. 1. Schematic diagram of air inlet system for each arm of four-choice olfactometer: (A) olfactometer; (B) parasitoid trap vial; (C) odor chamber; (D) humidifying vial; and (E) flowmeter.

General Experimental Procedure. All experiments were conducted 3–6 hr after parasitoids had experienced "lights-on." Parasitoids were tested singly by introducing them through the disconnected extractor tube. The relative time spent in each quadrant and the time of final choice were recorded with an Epson HX-20 portable computer with optional expansion unit. A final choice was defined as the entrance of a parasitoid into an insect trap vial. Time of final choice was defined as time between introduction into the olfactometer and entrance into an insect trap vial. Parasitoids were given a maximum of 10 min to make a final choice. The percent time per quadrant was calculated based on total time spent in each of the four quadrants before a final choice was made. Each insect was tested only once and, after ca. 10–20 insects were tested, the olfactometer, trap vials, and odor chamber vials were dismantled, rinsed with absolute ethanol, and washed in hot detergent.

System Bias. The response of inexperienced female *M. croceipes M. croceipes* in the olfactometer when no odor source was present was examined to determine if females exhibited preferences toward any quadrant(s). Twenty-eight 3-day-old female *M. croceipes* were tested to humidified air only (controls) in all four quadrants.

Effect of Oviposition Experience and Odor Concentration. Three-day-old females were used to study the effects of oviposition experience and odor concentration on female response. Inexperienced females had no oviposition experience or any previous exposure to larvae, frass, or foliage. Experienced females had searched larval-damaged foliage with frass present and had oviposited once

in an *H. zea* larva just prior to being tested. The odor source consisted of a third-instar *H. zea* larva feeding on a 4-day-old (ca. 6-cm-tall) cowpea seedling with the accompanying foliage damage and frass. This odor source was placed in the odor chamber (Figure 1C) and was termed one plant–host complex equivalent (PHCE). Four concentrations of PHCE odor were tested (1.00, 0.75, 0.50, and 0.25 PHCE). PHCEs of less than one were produced by splitting the odor flow and adding back humidified air to regain the original 300 ml/min flow rate. The odor was introduced into only one quadrant, the other three quadrants contained humidified air only (controls). Each replicate consisted of an inexperienced and an experienced female (random order) being tested to each of the four odor concentrations (in order of increasing concentration) in each of the four quadrants (in random order) (32 females per replication). Seven such replications were made.

Male Response. The response of male *M. croceipes* to one PHCE was examined using 3-day-old males with no previous exposure to larvae, frass, or foliage. Seven males were tested to the odor source at each of the four quadrants (in random order) for a total of 28 males.

Effect of Female Age. The effects of age on female response were studied using 0.75 PHCE as the odor source. Four age classes were used: 1, 3–4, 6–7, and 10–12 days after adult emergence. Each replicate consisted of an inexperienced female in each of the four age classes (in random order) being tested at each of the four quadrants (in random order) (16 females per replication). Seven such replications were made. Inexperienced females were used in this and the following experiments because we were interested in the innate response of females.

Source of Attraction. To determine the origin of attractive volatile chemicals, three possible sources were compared to the PHC for activity. These were damaged leaves, *H. zea* larvae, and frass. Damaged leaf odor was produced by using a wire to make four 2-cm-long scratches on a 4-day-old cowpea seedling just prior to testing. Larval odor was from a third-instar *H. zea* larva which had fed on cowpeas for two days preceding testing. The larva was placed in the odor source chamber without foliage or frass. Frass odor was obtained from ca. 10 fresh (less than 1-hr-old) pellets of frass from third-instar *H. zea* larvae feeding on cowpea seedlings. Each replicate consisted of an inexperienced female being tested to each of the four treatments (in random order) in each of the four quadrants (16 females per replicate). Seven such replicates were made.

Collection and Assay of Attractive Volatiles. Volatiles were collected from the plant–host complex to determine if such attractants could retain their bioactivity. Volatiles were collected from third-instar *H. zea* larvae actively feeding on cowpea seedlings. This odor source was contained in a glass chamber (5.0 cm ID × 20.0 cm long), consisting of two halves connected by a 50/50 ground-glass joint. The male half held a coarse-glass frit to provide laminar flow through

the chamber. Both halves had a 24/40 ground-glass joint, opposite the 50/50 joint, to connect the chamber to the rest of the volatile collection system. Humidified and prefiltered (activated charcoal) air was blown at a rate of 300 ml/min over 3–5 PHCE for 2–4 hr. Plant–host-complex volatiles were collected on activated charcoal (ca. 1.5 mm thick and 4 mm diam.) and subsequently extracted with three 20-μl volumes of methylene chloride and two 20-μl volumes of pentane. The methylene chloride–pentane extract was then tested at the following concentrations: 0.1, 0.5, 1.0, 5.0, and 10.0 plant–host complex hour equivalents (PHCHE). Fifty microliters of each extract was placed on filter paper (4.25 cm diam.), which was then placed in the odor chamber after the solvent had dried. One replicate consisted of an inexperienced female being tested to each of the five concentrations, in order of increasing concentration, in each of the four quadrants (random order), 20 females per replication. Eight such replicates were made.

Statistical Analyses. Friedman rank sums (FRS) (Conover, 1980), based on percent times per quadrant, were used to test for quadrant preferences when all four odor fields contained humid air only (controls).

In all other experiments, each treatment was tested against three controls, and preference for the treatment odor field over control odor fields was tested using FRS. To compare percent times per odor field between treatments, Duncan's new multiple-range test (DNMRT) (Steel and Torrie, 1960) was used after analysis of variance (ANOVA) of percents transformed by angular transformation. Percent making final choices were compared with DNMRT after ANOVA of percent making final choices after angular transformation. For all dose–response data, the concentration sum of squares were divided into polynomial components and the reported equations represent the polynomials in X using significant components. Times of final choice were compared by DNMRT after ANOVA. Significance levels were 0.05 in all tests.

RESULTS

General Behavioral Observations. After being released into the olfactometer, the parasitoids were observed to walk on the bottom, top, and sides of the olfactometer, occasionally stopping to groom their antennae, legs, wings, or abdomens. Parasitoids also made occasional flight attempts, defecated, and stood motionless.

When female parasitoids crossed from a control field into a sample field, they frequently began more intensive antennation and also walked more slowly, although these behaviors were not quantified. Females also were observed to follow the border between sample and control fields and to make klinotactic turns after leaving a sample field.

Bias. Female parasitoids spent an averge of 29, 28, 16, and 27% of their time in quadrants 1, 2, 3, and 4, respectively. There was no significant preference for any quadrant ($P > 0.25$), and no female made a final choice to humid air only.

Experience and Concentration. The effects of oviposition experience and odor concentration on female response are shown in Table 1. Both inexperienced and experienced females spent significantly more time in the test odor field than control odor fields at all odor concentrations. Experienced females spent a greater percent of their time in the odor field than inexperienced females at all concentrations tested ($P << 0.005$). Although mean percent time spent in the odor field increased with odor concentration for both types of females, the ANOVA indicated a significant linear effect of PHC odor concentration (x) on percent time in the test odor field (y) only for experienced females ($y = 30.4x + 57.0$; $N = 112$; $r = 0.37$; $P < 0.01$).

There were significant effects of both experience and concentration on percent making final choice. Experienced females were more likely to make final choices than inexperienced females and there were significant linear effects of odor concentration (x) on percent making final choice (y) for both inexperienced females ($y = 68.6x + 26.8$; $N = 28$; $r = 0.69$; $P < 0.01$) and for experienced females ($y = 50.0x + 55.4$; $N = 28$; $r = 0.62$; $P < 0.01$).

TABLE 1. RESPONSE OF INEXPERIENCED AND EXPERIENCED FEMALE *M. croceipes* TO FOUR CONCENTRATIONS OF PLANT–HOST COMPLEX ODOR[a]

Type of female / Odor conc. (PHCE)	Mean % time in odor field[b]	Mean % making final choices	Mean time of final choice (sec)
Inexperienced			
0.25	54.8	46.4	233.8
0.50	60.2	57.1	195.2
0.75	64.8	78.6	146.5
1.00	67.4	96.4	155.9
Experienced			
0.25	64.8	64.3	258.0
0.50	75.4	82.1	211.2
0.75	81.7	100.0	117.0
1.00	84.8	100.0	133.6

[a] Twenty-eight females were tested at each experience–concentration combination.
[b] Both types of females spent significantly ($P < 0.005$) more time in the treatment odor fields than control odor fields at all concentrations tested using Friedman rank sums.

The ANOVA of times of final choice revealed a significant effect only for odor concentration, and no significant effect of experience on time of final choice ($P > 0.25$). ANOVA revealed a significant decrease in time of final choice (y) with increased odor concentration (x) for both inexperienced females ($y = -93.1x + 238.0$; $N = 78$, $r = 0.25$; $P < 0.05$) and experienced females ($y = -181.3x + 291.8$; $N = 97$; $r = 0.36$; $P < 0.01$).

Males. Male parasitoids did not spend significantly more time in the plant–host complex odor field than control odor fields ($P > 0.25$), nor did they make any final choices to this odor source.

Age. All age classes tested spent significantly more time ($P < 0.005$) in the treatment odor fields than in control odor fields; however, there were no differences in percent time in odor field between any age classes ($P > 0.1$). The number of females ($N = 28$) making final choices were 20, 20, 18, and 17 for the 1, 3- to 4-, 6- to 7-, and 10- to 12-day-old parasitoids, respectively, and were not significantly different ($P > 0.25$). There also was no significant effect of age on time of final choice ($P > 0.25$).

Individual Components. The responses of inexperienced female parasitoids to individual components of the plant–host complex are shown in Table 2. Female parasitoids spent significantly more time in the odor fields that contained damaged-leaf odor and frass odor than in control odor fields. However, females spent significantly more time in odor fields containing plant–host complex odor than in odor fields that contained damaged-leaf or frass odor. Female parasitoids did not spend more time in the odor field containing *H. zea* larval

TABLE 2. RESPONSE OF INEXPERIENCED FEMALE *M. croceipes* TO INDIVIDUAL COMPONENTS OF PLANT–HOST COMPLEX[a]

Odor source	Mean % time odor field[b]	Mean % making final choice[c]	Mean time of final choice (sec)[d]
Plant–host complex	68.7**,c	82.1a	143.5
Artificially damaged leaves	50.7**,b	32.1b	160.4
Frass	46.9**,b	32.1b	161.5
H. zea larvae	34.5NS,a	3.6c	287.5

[a] Twenty-eight females were tested to each odor source.

[b] **Denotes females spent significantly ($P < 0.005$) more time in treatment odor fields than in control odor fields, NS denotes no significant difference ($P = 0.05$) from control (P calculated using Friedman rank sums). Percents with letters in common do not differ significantly ($P = 0.05$) using Duncan's new multiple-range test (DNMRT).

[c] Column means with letters in common do not differ significantly ($P = 0.05$) using DNMRT.

[d] There were no significant differences among the components in time of final choice (F test P value > 0.25).

odor than in control odor fields. Female parasitoids also made significantly more final choices to damaged leaves and frass than to *H. zea* larva, but fewer than to the plant–host complex. The ANOVA on the times of final choice did not reveal any significant effect of odor source ($P > 0.25$).

Collected Volatiles. The responses of inexperienced females to the collected plant–host complex volatiles are shown in Table 3. Female parasitoids neither spent significantly more time in the odor fields that contained the two lowest concentrations (0.1 and 0.5 PHCHE) than in control odor fields, nor did they make any final choices to these concentrations. Female parasitoids did, however, spend more time in the odor fields that contained 1.0, 5.0, and 10.0 PHCHE than in control odor fields. ANOVA revealed significant linear and quadratic components of collected volatile concentration (x) on percent time in the odor field (y) ($y = 21.7 + 16.6x - 1.2x^2$; $N = 160$; $r = 0.51$; $P < 0.01$). ANOVA also revealed significant linear and quadratic components of collected volatile concentration (x) on percent making final choice (y) ($y = -3.2 + 14.5x - 0.9x^2$; $N = 80$; $r = 0.73$; $P < 0.01$). The ANOVA on the times of final choice did not reveal any significant effect of concentration ($P > 0.1$).

DISCUSSION

The results demonstrate that female, but not male, *M. croceipes* respond in a dose-dependent fashion to volatiles from the *H. zea* larva–cowpea plant–host complex. Although females without any experience responded (i.e.,

TABLE 3. RESPONSE OF INEXPERIENCED FEMALE *M. croceipes* TO FIVE CONCENTRATIONS OF COLLECTED PLANT–HOST COMPLEX VOLATILES PLACED ON FILTER PAPER[a]

Concentration of collected volatiles (PHCHE)	Mean % time odor field[b]	Mean % making final choices	Mean time of final choice (sec)[c]
0.1	29.0 NS	0.0	
0.5	33.4 NS	0.0	
1.0	47.7 *	12.5	177.3
5.0	71.7 **	46.9	192.5
10.0	67.2 **	53.1	187.4

[a] Thirty-two females were tested at each concentration level.

[b] * and ** denote significantly ($P < 0.05$ and $P < 0.005$, respectively) more time spent in treatment odor fields than control odor fields, NS denotes no significant ($P = 0.05$) difference from controls (P calculated using Friedman rank sums).

[c] There were no significant differences between any concentrations in time of final choice (F test P value > 0.1).

innately), there was a clear effect of oviposition experience on female response. It is not clear whether this is a case of associative learning (olfactory cues with oviposition) or a case of a change in state of motivation, although learning appears to be an important part of host selection by parasitoids (Arthur, 1981). The effect of the "oviposition experience" may not be the oviposition per se but rather the exposure to damaged leaves, frass, and/or salivary secretions, etc. In fact, Drost et al. (1986) demonstrated that the wind-tunnel response of female *M. croceipes* exposed only to frass was no different than that of females exposed to the plant–host complex including oviposition. This type of experience might be used to increase the efficiency of parasitoids released into the field (Gross et al., 1975).

Age of inexperienced female parasitoids had no apparent effect on their response in the olfactometer. Drost et al. (1986) reported similar results for experienced females in wind-tunnel studies.

The results of the individual component experiments suggest that no single component elicits a response equal to the PHC, but that some combination, acting either additively or synergistically, is required. Preliminary findings suggest that the sum of all components may not give the complete activity either and that some interaction such as larval feeding or salivary secretions on the leaf tissues might be required. These effects will be investigated in future studies.

Although female parasitoids spent approximately an equal amount of time in the odor field that contained either the plant–host complex odor or the collected volatiles, fewer final choices were made to the collected volatiles than to the plant–host complex. This is probably due to the extreme volatility of the collected volatiles, which were never active for more than 10 min, and possibly to differences in the ratios of chemicals released by the plant–host complex and the collected volatiles.

In conclusion, our data clearly indicate that *M. croceipes* females respond to olfactory cues from the plant–host complex. The fact that females are attracted to leaves artificially damaged and to frass suggests that *M. croceipes* may use volatile chemicals from these sources as synomones and kairomones (as defined by Nordlund and Lewis, 1976), respectively, during its host-location process. In addition, we feel that this olfactometer can be used to identify volatile attractants for *M. croceipes* and that these chemicals might be used to improve the effectiveness of *M. croceipes* in the field.

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IRIDOID GLYCOSIDE SEQUESTRATION BY TWO APOSEMATIC *Penstemon*-FEEDING GEOMETRID LARVAE[1]

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Abstract—The iridoid glycoside catalpol was found to be sequestered by larvae of *Meris alticola* feeding on *Penstemon virgatus* and by larvae of *Neoterpes graefiaria* which utilize *P. barbatus*. The strikingly similar larval patterns of these two ennomine geometrids were previously considered to be disruptive, but predator-based Müllerian mimicry is equally likely to be involved. The cryptic adult moths generally contain only small amounts of catalpol, having left most of the bitter iridoid in the pupal case and in the meconium excreted after eclosion. One *Neoterpes* female did contain considerable catalpol in the abdomen, presumably in the eggs.

Key Words—*Meris alticola*, *Neoterpes graefiaria*, Lepidoptera, Geometridae, *Penstemon virgatus*, *Penstemon barbatus*, Scrophulariaceae, iridoid glycosides, catalpol, Müllerian mimicry, sequestration.

INTRODUCTION

The remarkable similarity between last-instar larval color patterns of *Meris alticola* and *Neoterpes graefiaria* in Arizona has been described and pictured (Poole, 1970). Genesis of the black-and-white striped and yellow–orange spot-

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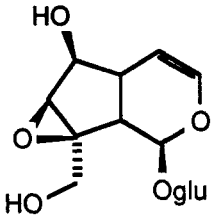
ted patterns was discussed, assessed as being disruptive, and attributed to adaptation based on an unusual grazing behavior. Müllerian mimicry based on distastefulness was not ruled out, but was considered unlikely since the *Penstemon* host plants were not known to contain poisonous secondary metabolites. It has recently been established, however (Bowers and Puttick, 1986; Stermitz, et al., 1986), that aposematic *Euphydryas* (checkerspot butterfly) species sequester bitter iridoid glycosides, such as catalpol, from larval host plants.



catalpol

It was suggested (Stermitz et al., 1986) that all aposematic insects monophagous on iridoid-containing plants would sequester these bitter substances. Since *Penstemon* species are now well-known to contain iridoid glycosides, an investigation of *Meris–Neoterpes–Penstemon* chemistry was undertaken.

METHODS AND MATERIALS

Collections and Treatment of Organisms. Six late instar larvae of *Meris alticola* Hulst and rosette stage *Penstemon virgatus* A. Gray were collected May 31, 1986, on the Fort Valley Experimental Station grounds, 7.5 miles northwest of Flagstaff, Arizona. This was the same location previously described (Poole, 1970). One larva was found on the host plant and the others were found basking longitudinally on grass stalks. No other larvae were encountered in several hours of searching. Larvae were fed in the laboratory on *P. virgatus* until cocoon spinning began. After completion of pupation, the silk cocoons were removed from the pupae and the pupae transferred to vials containing filter paper on the bottom and sides. Eclosion occurred approximately two weeks later. Adult moths were removed from the vials two days after eclosion and dried prior to analysis. The filter papers were extracted with methanol and the solution evaporated to dryness to obtain the meconium extract.

One last-instar larva of *Meris alticola* was found consuming *Besseya plantaginea* (James) Rydb. in Greta Valley west of Guffey, Colorado, on June 11,

1985, and was transferred to the laboratory on a leaf where cocoon formation and pupation took place. Several days after eclosion, the pupal case and cocoon silk were separated and the adult was pinned. Identification of the adult was by D. Ferguson, Smithsonian Institution, and it was then returned for analysis.

Several rosettes of fresh Arizona *P. virgatus* were held in the refrigerator for larval feeding in Colorado and were analyzed when all larvae had pupated. Collections of *P. virgatus* were also made in Park County, Colorado near Jefferson where it is host to the checkerspot butterfly *Poladryas arachne*.

One female adult *Neoterpes graefiaria* (Hulst) was taken at a UV light on August 4, 1986, 0.5 mi. west of Highway 92, on Lot 4, Ash Canyon Road, in the Southeast Huachuca Mountains, Cochise County, Arizona (elevation 5100 ft). It oviposited in a small jar, and the larvae (McFarland rearing No. R8612), which hatched out 11 days later, were reared on leaves of *P. barbatus* (Cav.) Roth. Feeding continued on refrigerator-held leaves in Colorado until September 8, when several larvae began to form cocoons. Frass and small bits of leaves or dirt were incorporated into the cocoons. Pinching of the last-instar larvae with tweezers elicited reflex bleeding from the intersegmental membrane area. The clear liquid was collected with a micropipet and washed from the pipet with methanol into vials where the methanol was evaporated. Two last-instar larvae were crushed into methanol and the methanol filtrate used for analysis. Two adults eclosed 21 days after pupation. These were immediately removed to vials containing filter paper on the bottom. Meconium material was obtained as above, and the adults were killed and allowed to dry at room temperature before analysis. The webbing material was highly contaminated with frass and host-plant parts and was not analyzed. The webbing was removed from the pupal case before analysis. Leaves of *P. barbatus* that remained after larval feeding were used for iridoid analysis.

Iridoid Glycoside Analyses. Qualitative iridoid analyses were by thin-layer chromatography (TLC) and quantitative analyses were by gas chromatography (GC) of the trimethylsilyl derivatives, as previously described in detail (Stermitz et al., 1986). A detailed investigation of the host plants for the present study was not necessary. In the case of *P. barbatus*, recent reports (Chaudhuri et al., 1981; Junior, 1982) characterized the iridoid content. Comparative TLC, GC, and [^{1}H]NMR spectroscopy on the crude iridoid fraction from our collection showed catalpol and globularin (10-cinnamoylcatalpol) to be the major components as expected from the literature data. No literature data was available on *P. virgatus*, but an analysis (L'Empereur and Stermitz, unpublished results) of Colorado collections, which are host to *Poladryas arachne*, showed that catalpol and globularin are also the major iridoids of this *Penstemon*. The same iridoids were shown by comparative TLC and GC to also be the major iridoids of Arizona *P. virgatus*.

RESULTS

The numbers of larvae available, as well as losses and other difficulties in rearing, did not allow us to get the most complete set of analytical data desirable for all insect stages, but the analyses we did obtain clearly show sequestration of relatively large amounts of catalpol by the larvae. Specific data for four *M. alticola* are given in Table 1 and for four *N. graefiaria* in Table 2.

In the case of the *Meris*, we did not directly analyze larvae, but assumed that the total catalpol content of pupal case, cocoon silk, meconium, and mature adult combined would reflect the catalpol present in the original larva, presuming no pupal metabolism of the iridoid. One reason for this approach was that our first (rather incomplete) analysis was on the insect raised on *Besseya plantaginea* (fourth entry of Table 1). This had shown a remarkable catalpol content (19% of the dry weight) in the cocoon silk. An alternative explanation was that the adult had released meconium on the silk after eclosion. The Arizona *Meris* larvae were all raised through to adulthood, so that meconium and cocoon silk could be checked separately. The data for males 1 and 2 suggests that this interpretation may be correct, since high meconium and low silk catalpol content was found. This is somewhat clouded by the fact that all the Arizona *Meris* produced relatively poor cocoons as compared to the cocoon of the Colorado specimen. The low dry weights for the Arizona silk are shown in Table 1. The reason for this is not known, but these and similar geometrids sometimes do form poor cocoons if the proper substrate or arrangement of substrate is not available for the construction of these rather elaborate structures.

The *Meris* larvae and adults are close in size to those of *Euphydryas anicia*, which we have studied more extensively (Stermitz et al., 1986; Gardner and Stermitz, in preparation). The total iridoid load is comparable as well, with

TABLE 1. *Meris alticola* ANALYSES

Insect	Total weight,[a] mg, and (catalpol content, mg)				
	Larva	Adult	Pupal case	Cocoon silk	Meconium[b]
Female 1[c]	203	45 (0.28)[e]	3.4 (0.04)	1.2 (0.05)	(0.09)
Male 1[c]	130	20 (0.02)	4.2 (0.25)	1.4 (0.08)	(0.51)
Male 2[c]	138	22 (0.05)	4.0 (0.53)	0.9 (0.005)	(0.44)
Unknown 1[d]	unknown	27 (none)	5.0 (0.22)	4.3 (0.82)	unknown

[a] Larva wet weight; others dry weight.
[b] Total meconium weight not known.
[c] Raised on *Penstemon virgatus*.
[d] Raised on *Besseya plantaginea*.
[e] Abdomen: 0.27 mg, all other parts combined: 0.01 mg.

TABLE 2. *Neoterpes graefiaria* ANALYSES

Insect	Total weight, mg, and (catalpol content, mg)				
	Larva	Adult[a]	Pupal case[a]	Meconium	Reflex emission
Male 1	unknown[b]	10 (0.05)	3.2 (0.71)	(0.60)	unknown
Male 2	unknown[b]	11 (0.11)	2.9 (0.51)	(0.35)	unknown
Male 3	unknown[b] (2.0)				(28 μg/μl[c])
Male 4	unknown[b] (1.7)				(21 μg/μl[d])

[a] Dry weight.
[b] Larval weight was not recorded. Visually, the larvae are similar in size or slightly smaller than *Meris alticola* larvae (see Table 1).
[c] Four microliters analyzed from one emission.
[d] Eight microliters analyzed from one emission.

entries 1–4 of Table 1 showing 1.0%, 3.8%, 3.1%, and 3.8% total catalpol calculated as percent dry weight of the adult butterfly. In the *Meris* case, the catalpol has generally been eliminated after the larval stage, while *Euphydryas* keep appreciable amounts of catalpol in the adult. In the case of the one female *Meris* available, little catalpol was found in the meconium, cocoon silk, and pupal case. The adult abdomen was therefore separated from the remainder of the insect prior to analysis. The abdomen section, presumably with eggs, was indeed high in catalpol content.

A direct analysis was done on two *Neoterpes* larvae (Table 2). These were kept without food for about 48 h so that much of the gut could be emptied. The resulting catalpol content, about 2 mg per larva, was comparable to that found in the *Meris*. The pupal case and meconium yielded large quantities of catalpol (Table 2), with relatively little in one adult and somewhat more in the second.

Hemolymph obtained from reflex bleeding of the disturbed *Neoterpes* larvae was analyzed for catalpol content. A single emitted drop (4 μl) was obtained from one larva, while the second emitted more material (8 μl). The total catalpol content of these emissions were 0.11 and 0.17 mg, respectively, from which the entries of Table 2 were calculated. The genesis of the material is not known, although it appears to arise from the intersegmental membrane area.

DISCUSSION

The finding of high catalpol content in immature stages, pupal cases, cocoon silk, and meconium of *Meris* and *Neoterpes*, as well as in the liquid emitted by *Neoterpes* reflex bleeding, suggests that Müllerian mimicry could be

a basis for the strikingly similar larval patterns. Since it has been shown (Bowers, 1981) that *Euphydryas phaeton* butterflies are unpalatable to bluejays if their larvae feed on catalpol-containing plants (including *Penstemon antirrhinoides*), bird predation might be responsible for development of this mimicry. Other predators that depend on visual cues could also be involved. Bitter iridoid glycoside sequestration has been suggested to account for other Lepidopteran mimicry as well. Thus, it was suggested (Bowers, 1981) that adults of western U.S. *Euphydryas* butterflies may be members of a Müllerian mimicry ring. Batesian mimicry could account for the adult resemblances between palatable *Chlosyne harrisii* and unpalatable *E. phaeton* and, particularly, for their "virtually indistinguishable" larvae as suggested by Bowers (1983).

We have not observed any direct predator pressures on *Meris* and *Neoterpes*. It was suggested (Poole, 1970) that the *Meris–Neoterpes* resemblance was not likely to be a predation-based mimicry because of the relatively low population densities of these organisms, since a critical density (Holling, 1965) was probably not present. It has, however, been pointed out (Endler, 1986) that, in the absence of direct observation of predator pressures, one can use mimicry as a basis for suggesting that such pressures had indeed occurred. In fact, it has been stated (Endler, 1986) that "mimicry can be one of the strongest lines of evidence for natural selection in the wild." The development of the *Meris–Neoterpes* resemblance may have been based on predation pressures when populations of the organisms were much higher. Final proof that we are observing mimicry must await experimental demonstration of *Meris* and *Neoterpes* larval distastefulness as well as predator avoidance of one organism after encountering the other.

Finally, the present work also extends the variety of defensive strategies employed by insects utilizing iridoid glycoside-containing plants (Bowers and Puttick, 1986). We can add deposition of these bitter substances in cocoon silk and in an emission from reflex bleeding when larvae are disturbed. It seems likely that we are only beginning to appreciate the breadth and depth of iridoid glycoside utilization by insects.

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EVALUATION OF SOME WEED EXTRACTS AGAINST FIELD DODDER ON ALFALFA (*Medicago sativa*)

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Abstract—Experiments were conducted to evaluate the effectiveness of some weed extracts against field dodder *Cuscuta campestris* Yunck. on alfalfa (*Medicago stiva* L.) and to identify and quantify the phytotoxic agents of these extracts. All concentrations of aqueous extract of every weed showed significant effectiveness on dodder when compared to the untreated plant under lath house and field conditions. Control percentages of the (0.5 g) of Bermuda grass (*Cynodon dactylon*) and wall goosefoot (*Chenopodium murale*) ranged between 83 and 96, and the same concentration caused injury to alfalfa foliage up to 43% when applied in the field. Phytotoxic agents were identified as phenolic compounds such as chlorogenic, isochlorogenic, *p*-coumaric acids, and scopoletin. Their quantities varied with species; Bermuda grass had the highest content (32.2 μg/g dry weight) followed by Johnson grass (*Sorghum halepense*), well goosefoot, and tumble pigweed (*Amaranthus albus*). These results might aid in screening for effective alternate approaches for controlling dodder on alfalfa planted for seeds.

Key Words—Extracts, phytotoxicity, *Medicago sativa*, dodder, *Cuscuta campestris, Amaranthus albus, Chenopodium murale, Cynodon dactylon, Sorghum halepense.*

INTRODUCTION

Dodder, *Cuscuta campestris*, is considered one of the most important parasitic weeds that infest a broad spectrum of plants in the world. It is especially detrimental to alfalfa (*Medicago sativa* L.) (Dawson, 1970, 1978). Lee and Timmons (1956) reported that field dodder causes great losses to alfalfa planted for

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seed production through direct seed reduction, increased harvest and seed-cleaning costs, and lower quality and sale value of seeds.

Many approaches have been studied, such as mechanical, cultural, or chemical, to control dodder on alfalfa (Ashton, 1976). Of these, herbicides are the most promising but their use depends on whether alfalfa is cultivated to produce seeds or animal feed. One of the recent alternatives to herbicides is allelopathy as an aid for weed control. Several researchers have begun to evaluate crop plants and weeds for their allelopathic potential for weed control or as sources of naturally occurring herbicides (Massantini et al., 1977; Putnam and Duke, 1974; Al-Saadawi, 1985). Fay and Duke (1977) found that some of the *Avena* spp. accessions contained scopoletin (6-methoxy-7-hydroxycoumarin) an allelopathic agent that reduced annual weed growth and caused chlorosis, stunting, and twisting when planted in close association. Leather (1983) reported that the aqueous extracts of sunflower (*Helianthus annuus*) cultivars inhibited germination and suppressed 75% of wild mustard (*Brassica Kaber*) growth. Rhizobitoxin, another allelopathic agent, produced by *Rhizobium japonicum*, was found to have the same phytotoxic effects as the herbicide amitrol (IH-1-2,4-triazol-3-amine) on some weed species (Owens, 1973).

Relatively few studies have dealt with the effect of weed plant extract on growth of other weeds. Our tests, therefore, were developed to evaluate the phytotoxic potential of some weed extracts on field dodder (C. campestris) infesting alfalfa. Another goal was to isolate and/or identify the phytotoxic compounds present in those weed extracts.

METHODS AND MATERIALS

Plant Materials and Extraction. Four weeds were collected during July and August of 1984 from the Agricultural and Water Resources Center Research Station in Fudhailyia, 25 km northeast of Baghdad. The weeds were tumble pigweed (*Amaranthus albus* L.), wall goosefoot (Chenopodium murale L.), Bermuda grass (Cynodon dactylon L. Pers.) and Johnson grass (*Sorghum halepense* L.). Procedures used for extraction were basically those of Wang and Francis (1978). All parts of each weed were cut into 2 to 3-cm pieces and left to dry at room temperature for three days, then macerated in a blender with 80% methyl alcohol in a ratio of 1:2.5 w/v. The mixture was allowed to stand overnight under refrigeration. After filtration, extracts were evaporated to dryness under pressure. The residues were dissolved in water to the desired concentrations: 0, 0.05 0.25, and 0.5 g dry weight material per milliliter of water.

Lath-House Experiment. Alfalfa seeds var. Local were planted in clay loam soil in 10-cm-diam. plastic pots in August 1984 under lath-house conditions. Dodder seeds were treated with concentrated sulfuric acid for 20 min to enhance

germination, and 50 seeds were planted in each pot with alfalfa. Alfalfa was thinned to one plant per pot when the plants reached 5 cm in height. The experiment was set in a split-plot design with three replications. The main plots represented weed extracts, while the subplots represented extract concentrations. Thirty days after planting, dodder stems and tendrils started to cover the alfalfa plants. At this stage, extracts were sprayed on the dodder and alfalfa using a 1-liter pot-sprayer and at 20°C and 70% relative humidity. A single application of each concentration of each extract was applied to cover both alfalfa and dodder foliage. Two weeks later, treatments were evaluated. Dodder control was visually estimated on a scale of 0 (for no effect) to 10 (for 100% effectiveness). The same scale was used to evaluate treatment injury to alfalfa foliage. After two additional weeks, dodder and alfalfa dry weights were taken for each treatment. All statistical analyses and tests were conducted according to the methods designated by Steele and Torrie (1980).

Field Experiment. A uniform alfalfa field was chosen to conduct the field experiment at the Agricultural and Water Resources Center Station, Fudhailyia. The experimental setting was a split-plot design with three replications. Plots of 50 × 50 cm were prepared in which the alfalfa stand was cut to 5 cm height in the spring of 1985. Small plots were chosen to achieve uniformity in the alfalfa stand and to minimize the amount of extract needed for experimentation. Field dodder infestation was initiated by placing infected alfalfa seedlings in each plot to allow contact with healing foliage. The dodder branched, then almost covered, all the alfalfa, and at this stage treatments were applied. Evaluation of the treatments was made according to the same scale used in the lathhouse experiment. The dry weights of both alfalfa and dodder were taken at random and for each plot.

Identification of Phytotoxins. Since many phytotoxic substances in plants are phenolic in nature (Chou and Young, 1975), an attempt was made to isolate toxic phenolic compounds from various parts of the weeds. Each aqueous weed extract was concentrated by vacuum to 10 ml and further extracted with petroleum ether three times to remove chlorophyll and fatty compounds. The extract was then chromatographed on Whatman No. 1 paper in one dimension with BAW (*n*-butanol–acetic acid–water) 63:10:27. The chromatograms were examined with short (2537 Å) and long (3360 Å) UV light. The distinctive bands were cut from the chromatograms and eluted with 50% aqueous methanol. Eluates of bands were reduced to dryness, taken up in 5 ml absolute methanol, and chromatographed on Whatman No. 1 paper in two different solvent systems: 6% AA (6% aqueous acetic acid) and BAW (63:10:27), respectively. The spots were detected under long- and shortwave UV light with and without ammonium gas (NH_3) and the chromatograms sprayed with diazotized *p*-nitroaniline (Bray et al., 1950) and diazotized sulfanilic acid reagents (Smith, 1960). Phytotoxic phenolics appeared as absorbing or fluorescing spots under

UV light with distinguishable colors after being sprayed with reagents. Quantitative estimation of total phenolic compounds was done by using Folin deines pigment according to Pearson (1970).

RESULTS

Lath-House Experiment. Treatments of tumble pigweed, wall goosefoot, and Bermuda grass extracts killed almost all the exposed stems and tendrils of field dodder one week after application. Control of dodder, measured as a percentage of the control treatment, dodder, and alfalfa foliage dry weights, and foliage injuries were summarized in Table 1.

Results showed highly significant differences between treated and untreated dodder. Control of dodder was increased with higher extract concentrations except for that of wall goosefoot at 0.25 g/ml water, which was less effective than the 0.05 concentration. For all weed extracts, no significant differences

TABLE 1. EFFECTS OF EXTRACTS ON FIELD DODDER AND ALFALFA UNDER LATH-HOUSE CONDITIONS

Weed extract sources	Concentrations (g/ml water)	Dodder control (%)	Dodder dry weight (g/plant)	Alfalfa injury (%)	Alfalfa weight (g/plant)
Tumble pigweed	0.00	0.00	0.38	0.0	0.203
	0.05	40.0	0.033	3.3	0.224
	0.25	80.0	0.06	10.0	0.654
	0.5	80.0	0.04	10.0	0.653
Wall goosefoot	0.00	0.0	0.334	0.0	0.326
	0.05	50.0	0.049	10.0	0.405
	0.25	13.3	0.079	10.0	0.653
	0.50	83.3	0.021	13.3	0.724
Bermuda grass	0.00	0.00	0.351	0.0	0.267
	0.05	10.0	0.065	6.6	0.170
	0.25	88.0	0.046	10.0	0.606
	0.50	90.0	0.054	10.0	0.80
Johnson grass	0.00	0.0	0.343	0.00	0.179
	0.05	70.0	0.041	10.0	0.235
	0.25	76.6	0.017	6.66	0.552
	0.50	83.3	0.093	10.00	0.745
LSD ($P < 0.05$)		9.091	0.105	8.3	0.065

were found between their mode of control at 0.25- and 0.5-g concentration levels, as these gave an average control of 80–90%. The highest control was obtained with the 0.5- and 0.25- concentrations of Bermuda grass.

Treated dodder dry weights were significantly lower than untreated. Although no significant differences were found between the concentrations of the same weed extract, higher reduction in dodder weights with higher concentrations was noticeable. An exception was that of 0.25 g/ml water of wall goosefoot, as mentioned before. This was consistent with the percentages of dodder control obtained. An average of 80–90% reduction in dodder foliage dry weight was obtained from extract treatments when compared to the untreated control. This indicates the high phytotoxic characteristics of these extracts.

Alfalfa foliage injury was rated on the whole appearance of plants compared to those not treated. The highest percent of injury (13.3%) was caused by the application of 0.5 g/ml of goosefoot although statistically the precent injury was not different from that of lower concentrations. Most injured plant foliage did recover.

All treatments caused an increase in alfalfa dry weights except with 0.05 g of Bermuda grass, which reduced it to 0.17 g/plant compared to 0.267 g/plant for those not treated. However, all other treatments raised alfalfa weights in inverse proportion to their inhibitory effectiveness on dodder. In all treatments and for all concentrations, wall goosefoot extracts stimulated alfalfa growth the most, resulting in 0.53 g/plant compared to 0.43 g/plant for Johnson grass and tumble pigweed and to 0.46 g/plant for Bermuda grass.

Field Experiment. Dodder control (percentage) and its dry weight and alfalfa foliage injury and its dry weight results were recorded (Table 2). Comparing weed extracts, and for all concentrations used, wall goosefoot gave the highest percentage of dodder control (73%) followed by Bermuda grass and tumble pigweed (64 and 55%, respectively). Highly significant differences were found between treated and untreated plots. The differences between weed extract concentrations were also significant, and that was obvious for wall goosefoot: 0.05, 0.25, and 0.5 g/ml water which resulted in 50, 73.3, and 96.6% dodder control, respectively.

Reduction in dodder dry weights was inversely proportional to concentration and higher percentages of control. Significant differences were also found between dodder dry weights of the treated and untreated plots. Higher concentrations of all plant extracts (0.25 and 0.5 g) caused a higher percentage of injury to alfalfa. However, no significant differences were found between Johnson grass and tumble pigweed concentrations in alfalfa foliage injury. Bermuda grass and wall goosefoot extracts at 0.5 g caused the highest percentage injury to alfalfa foliage (43.3%). In general, higher extract concentrations caused noticeable injuries to alfalfa foliage in the field as compared to pot-planted

TABLE 2. EFFECT OF WEED EXTRACTS ON FIELD DODDER AND ALFALFA UNDER FIELD CONDITIONS

Weed extracts sources	Concentrations (g/ml water)	Dodder control (%)	Dodder dry weight (g/plant)	Alfalfa injury (%)	Alfalfa dry weight (g/plant)
Tumble pigweed	0.00	0.00	0.437	0.00	0.256
	0.05	23.3	0.1	0.00	0.542
	0.25	60.00	0.059	3.30	0.369
	0.50	83.30	0.015	6.60	0.885
Wall goosefoot	0.00	0.00	0.413	0.00	0.212
	0.05	50.00	0.07	20.00	0.365
	0.25	73.3	0.05	20.00	0.674
	0.50	96.60	0.013	43.3	0.937
Bermuda grass	0.00	0.00	0.416	0.00	0.237
	0.05	23.30	0.072	00.00	0.234
	0.25	73.30	0.034	35.00	0.527
	0.50	96.60	0.014	43.30	0.898
Johnson grass	0.00	0.00	0.408	0.00	0.208
	0.05	26.60	0.074	10.0	0.335
	0.25	36.60	0.051	10.0	0.687
	0.50	76.60	0.016	10.0	0.797
LSD ($P < 0.05$)		6.9	0.036	4.07	0.051

alfalfa. However, this injury did not affect foliage dry weights. Even though higher extract concentrations resulted in higher injury to foliage, weights increased with higher concentrations and consequently higher dodder control.

Identification of Phytotoxins. Phytotoxins identified were phenolic compounds found in weed extracts (Tables 3 and 4). Chlorogenic and isochlorogenic acids were found in all extracts. Tumble pigweed extract contained *p*-coumaric acid and scopoletin, while wall goosefoot extract contained scopoletin and chlorogenic and isochlorogenic acids. All phenolic acids, except *p*-hydroxybenzoic acid and scopoletin, were found in Bermuda grass extract. Chlorogenic, isochlorogenic, *p*-coumaric, and *p*-hydroxybenzoic acids were identified in Johnson grass extract. Unknown compounds were also found but not identified. Their R_fs and colors under UV light suggest a flavonoid nature (Table 4).

Quantitative analyses showed that Bermuda grass, Johnson grass, wall goosefoot, and tumble pigweed contained up to 32.2, 21.4, 21.1, and 9.3 μg/g dry weight of phenolic compounds, respectively (Table 4). The high percent

TABLE 3. CHROMATOGRAPHY OF WEED EXTRACTS

Compounds	R_f of Whatman No. 1[a]			Fluorescence[b]				Reagent colors[c]	
	BAW	6% AA	IBW	Long UV	Long UV + NH_3	Short UV	Short UV + NH_3	p-Nit	Sulfan. acid
Caffeic acid	0.83	0.65	0.72	bl	bl	bl	bl	f bn bl	—
Chlorogenic acid	0.58	0.65	0.59	l bl	yel-gr	l bl	yel-gr	bn	tan
Isochlorogenic acid	0.76	0.26	0.7	l bl	yel-gr	l bl	yel-gr	bn	tan
Ferulic acid	0.90	0.64	0.76	bl	l bl	bl	l bl	f bn bl	tan
O-Coumaric acid	0.91	0.87	0.96	l bl	w l bl	l bl	w l bl	bn	or red
p-Coumaric acid	0.95	0.74	0.93	bl	l bl	bl	l bl	dk-gray-viol	red-br
p-OH-Benzoic acid	0.92	0.82	0.65	f abs	bl	f abs	f bl	f wine	or red
Scopoletin	0.76	0.45	0.78	bl	l bl	bl	l bl	bl black	f br rose
Scopolin	0.41	0.81	0.55	bl	l bl	bl	l bl	—	—
Vanilic	0.76	0.37	0.75	ab	abs	ab	ab	viol	or red tan
Unknown(a)	0.30	0.14	0.07	yel	l yel	yel	l yel	bn	yel-tan
Unknown(b)	0.40	0.07	0.48	br	yel	br	yel	bl bn	tan
Unknown(c)	0.16	0.76	0.83	l bl	l bl	l bl	l bl	bn	or red

[a] R_fs are averages of three runs. 6% AA = 6% acetic acid, BAW = 63:10:27 butanol–acetic acid–water, IBW = 140:20:60 isopropanol–*n*-butanol–water.
[b] bl = blue, bn = brown, f = faint, abs = absorption, yel = yellow, viol = violet, dk = dark, gr = green, w = white, l = light.
[c] Diazotized and diazotized sulfanilic acid *p*-nitroaniline.

TABLE 4. DISTRIBUTION AND QUANTITIES OF PHENOLIC COMPOUNDS IN WEED EXTRACTS

Toxins	Weed species			
	Tumble pigweed	Wall goosefoot	Bermuda grass	Johnson grass
Caleicacid			+	
Chlorogenic acid	+	+	+	+
Isochlorogenic acid	+	+	+	+
Ferulic acid			+	
O-coumaric acid			+	
p-Coumaric acid	+		+	+
p-Hydroxybenzoic acid				+
Scopoletin	+	+	+	
Scopolin				
Vanillic acid	+			
Unknown a	+	+	+	
Unknown b			+	
Unknown c			+	
Quantity (μg/g)	9.3	21.1	32.2	21.4

of dodder control and alfalfa injury caused by 0.5 g of Bermuda grass might be explained by the higher (32.2 μg/g) concentration of phenolic compounds.

DISCUSSION

Results of both pot and field experiments suggested that toxicity of weed extracts on field dodder had increased when higher concentrations were used, when 0.25 g of wall goosefoot was used. However, considerable injury to alfalfa foliage occurred, especially when 0.25 and 0.5 g of both wall goosefoot and Bermuda grass were applied under field conditions. The difference in alfalfa injuries between pot and field results might be explained by the higher temperature in the field (32 $\pm$ 2°C) compared to 20 $\pm$ 2°C under lath-house conditions. Considering alfalfa injury for both experiments; 0.25 g of tumble pigweed and 0.05 and 0.5 g of Johnson grass were the best treatments. However, Bermuda grass at 0.25 g gave the highest dodder control but in the field resulted in 35% injury to alfalfa.

Many reports have shown the inhibitory activity of phenolic compounds. Rasmussen and Rice (1971) identified ferulic and *p*-coumaric acids from *Sporobolus pyramidatus* and found allelopathic effects on associated species, resulting in either reduced growth or eliminating them from the stand. Langdale and

Ciddens (1967) reported that small quantities of the same acid were effective in inhibiting IAA activity in *Avena* spp. coleoptiles. More detailed study was conducted by Zenk and Muller (1963) and showed that these aicds increase IAA decarboxylation in *Amaranthus retroflexus* seedlings. Chlorogenic acid was found to inhibit many enzymes such as photophosphorylase (Sondheimer, 1964). Lodhi (1975) tested the biological activity of ferulic, caffeic, and *p*-coumaric acids isolated from hackberry (*Celtis laevigata*) leaves and found that they reduced germination and growth of associated herbaceous species. Al-Algawi (1980) found that Bermuda grass eliminated growth of associated species and the same plant leaf and rhizome inhibited seed germination and seedling growth of cotton and some weeds (Abdul-Rhaman, 1983). All of these studies, as well as others, support the assumption that extract phytotoxicity of the weeds in this study might be due to the phenolic compounds.

Results of this study showed strong evidence that plant parts of these weed species contain phenolic compounds that have phytotoxic activity against dodder. With respect to the possible practical use of these extracts as an approach for dodder control, these rates might be too high to be of commercial and practical importance. This is supported by Shettel and Balke (1983), who concluded that relatively higher concentrations were required to reduce plant growth. However, other detailed studies should be concentrated on screening for naturally occurring chemicals in a wide spectrum of wild plants. It would also be useful to determine which of these phenolic compounds have phytotoxic activity and the mechanisms of such phytotoxicity.

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EXTRACTION OF TANNIN FROM FRESH AND PRESERVED LEAVES

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Abstract—The extractability of tannin from fresh, lyophilized, and dried leaves collected at various times in the growing season was determined using the radial diffusion assay for protein-precipitating phenolics. The amount of tannin extracted depended on the method of leaf preservation and on the maturity of the leaf. Early in the season, more tannin was extracted from lyophilized leaves than from fresh leaves, but late in the season more tannin was extracted from fresh leaves. At all times, more tannin was extracted with aqueous acetone than with aqueous or acidic methanol.

Key Words—Leaf preservation, tannin extraction, phenolic analysis, protein precipitation.

INTRODUCTION

Although tannin is frequently cited as an example of a plant defensive chemical (Swain, 1979), experimental documentation of a defensive role for tannin is limited. Clear demonstration of a defensive function for tannin depends on accurate quantitation of tannin. When adequate analytic methods are available, it will become possible to determine whether there is any correlation between tannin content and patterns of herbivory.

The accuracy with which a chemical component in a biological matrix, such as tannin in a plant leaf, can be determined depends on several factors. The tissue of interest must be collected and preserved so that the component is not altered or destroyed. The component must be extracted from the tissue in high yield. The method of assaying the component must be free of interferences from other materials present in the extract. However, there is no consensus on

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the best method for preserving leaves or the most efficient solvents for extracting tannins (Swain, 1979). Definitive studies of the role of tannin have been hampered by the limited knowledge of appropriate methods for sample collection and tannin extraction.

Although extraction and analysis of fresh tissues would probably minimize changes to the tannin, most studies of the ecological role of tannin are conducted under conditions where immediate analysis is impossible. Instead, tissues are collected and preserved for later extraction and analysis. Lyophilization is thought to be the gentlest method of preservation, and lyophilized leaves may be equivalent to fresh leaves (Martin and Martin, 1983). However, diminished extractability of tannin after lyophilization has been noted in some cases (Price et al., 1979). Drying the tissue at ambient or at elevated temperature is more convenient than lyophilization, although little tannin can be extracted from samples dried at elevated temperatures (Bate-Smith, 1975; Price et al., 1979). Lower temperature drying is preferable (Swain, 1979), but even drying at room temperature may alter the chemical nature of tannin (Butler, 1982).

Three solvents are commonly used to extract tannin from plant samples: boiling aqueous methanol, aqueous acetone, or acidic methanol. Boiling aqueous methanol is thought to be the most effective solvent for condensed tannin (Bate-Smith, 1975), but the recovery of tannin is estimated to be as low as 30% for some tissues (Bate-Smith, 1973a; Swain, 1979). Since aromatic ester (depside) bonds are hydrolyzed by aqueous alcohols, they are thought to be unsuitable for extraction of hydrolyzable tannin (Haslam et al., 1961; Swain, 1979).

Aqueous acetone is routinely used to extract hydrolyzable tannin (Jones et al., 1976; Foo and Porter, 1980), but no quantitative estimates of recovery are available. Some authors believe condensed tannin is extracted quite efficiently with aqueous acetone (Feeny and Bostock, 1968; Fletcher et al., 1977; Lane and Schuster, 1981), but others have found that condensed tannin is recovered in low yields when aqueous acetone is employed (Stafford and Cheng, 1980; Martin and Martin, 1984).

Acidic methanol is the best solvent for extracting the condensed tannin from some varieties of sorghum (Price et al., 1978). It is hypothesized that the tannin in those varieties is chemically unique, perhaps covalently attached by an acid-labile bond to some component of the grain. Although aqueous methanol (pH 5–6) causes methanolysis of depside bonds in hydrolyzable tannins, at more acidic pH values (pH < 3) methanolysis does not occur (Haslam et al., 1961). Thus acidic methanol should be appropriate for extraction of hydrolyzable tannin. The applicability of acidic methanol as an extractant of plant tissues other than sorghum has not been demonstrated, but it may be useful in cases of strong interaction between tannin and other components of the tissue. Some plants contain tannin that is completely unextractable with aqueous acetone or alcohol (Bate-Smith, 1973a, 1975).

The purpose of this study was to compare several methods of leaf preser-

vation and to quantitate the extractability of tannin with various solvents. The study was conducted throughout one growing season, and the effect of leaf maturity on tannin extractability was also noted.

METHODS AND MATERIALS

The leaves used in this study were collected from trees on the Miami University campus throughout the growing season of 1986. A single tree of each type was used for all samples, and all samples were collected from low branches on the north side of the trees. Three species of trees were used: burr oak, *Quercus macrocarpa*; sugar maple, *Acer saccharum*; shagbark hickory, *Carya ovata*. Specimens have been deposited in the Miami University herbarium.

The leaves were removed from the trees and immediately put on ice. Major veins were removed with a razor blade and discarded. The leaves were cut into small pieces (~ 2 cm^2), frozen in liquid nitrogen, and ground with a mortar and pestle. The powder was either extracted immediately, or lyophilized, or oven dried at 40°C or at 90°C.

Extractions were performed either in test tubes or in miniature columns. If test tubes were used, the sample was weighed into the tube, mixed with the solvent, and centrifuged after the extraction was completed. The supernatant was removed and the final volume of extract recovered was recorded. In a faster method for extracting samples, 1-ml micropipet tips were used as columns. The end of the plastic pipet tip was sealed by touching it with a hot glass rod. A polyethylene frit cut to the proper size with a cork borer was inserted ~ 0.75 mm from the tip to support the plant tissue. The tissue was placed in the tip, on the frit, weighed, and the solvent was added. After the extraction was completed, the sealed end was cut off, the column placed in a large test tube, and centrifuged. The extract was collected in the test tube. This method provided excellent recovery of the extract for small samples (100 mg) of fresh or dried tissue.

For all the solvents, the tissue was initially extracted for 30 min at room temperature. If the solvent was to be heated, the sample was placed in an 85°C water bath for an additional 10 min of extraction.

The extracts were assayed with the radial diffusion assay (Hagerman, 1987). Wells were made in the bovine serum albumin-containing plates with a 4-mm punch, and three 8-μl aliquots of extract were applied to each well. After 94 hr, the diameter of the ring that developed was measured. The diameter squared is proportional to amount of tannin added to the well (Hagerman, 1987). The data were expressed as centimeters squared per gram dry tissue. These values could be converted to milligrams tannic acid per gram tissue or to milligrams condensed tannin per gram tissue using a standard curve (Hagerman, 1987), but those values would not be more meaningful than the unconverted raw data for the leaves that contained both types of tannin.

Tannic acid or quebracho tannin (Asquith and Butler, 1985) was added to fresh spinach or young barley leaves, and the tannin recovered from the leaves determined with the Prussian blue assay (Price and Butler, 1977). The leaves (0.34 g) were cut into small pieces, and placed in a mortar with sand, 3.0 ml of the solvent, and a 100-μl aliquot of a 100 mg/ml tannin solution. The leaves were ground with a pestle, centrifuged, and the supernatant assayed for total phenolics. Control samples without added tannin were used to determine the phenolics from the barley or spinach, and those values were subtracted from the values obtained for the spiked leaves. The Prussian blue assay was run on aliquots of tannin, and the recovery from the leaves was then calculated as percent of tannin added to the leaves.

RESULTS

Leaves of oak and maple contain both condensed and hydrolyzable tannin (Feeny and Bostock, 1968; Bate-Smith, 1977; Schultz and Baldwin, 1982); hickory contains only condensed tannin (Wilken and Cosgrove, 1964). The radial diffusion assay (Hagerman, 1987), which was used to determine the extracted tannin, does not discriminate between condensed and hydrolyzable tannin. Condensed tannin gives a lower response than hydrolyzable tannin with this assay. The response to a mixture of tannins is equal to the sum of the responses to the individual components of the mixture (Hagerman, 1987).

The extraction of tannin from lyophilized maple or oak leaves collected in midsummer was most efficient when 70% aqueous acetone was used (Table 1). Aqueous alcohol did not extract as much tannin as did the acetone. Boiling aqueous methanol apparently destroyed some tannin in the maple leaves. Heated aqueous acetone extracted less tannin than did unheated acetone (data not shown).

Similar results were obtained in experiments in which condensed or hydrolyzable tannin was added to fresh leaves just before the leaves were homoge-

TABLE 1. EXTRACTION OF TANNIN[a] FROM LYOPHILIZED LEAVES WITH VARIOUS SOLVENTS (LEAVES COLLECTED JULY 9, 1986)

Solvent	Oak	Maple
70% acetone	546 ± 87a	1381 ± 149c
50% methanol	387 ± 21b	1117 ± 133d
50% methanol, boiling	359 ± 20b	994 ± 78e
1% HCl in methanol	343 ± 35b	97 ± 177d

[a]Tannin determined by the radial diffusion assay (Hagerman, 1987) and expressed as cm^2/g dry tissue. Each value (± SD) is the mean of at least three determinations. Values followed by the same letter are the same at the 95% confidence interval (*t* test).

TABLE 2. RECOVERY OF ADDED TANNIN FROM FRESH, TANNIN-FREE TISSUE

Solvent	Recovery (%)[a]			
	Spinach		Barley	
	Condensed tannin	Hydrolyzable tannin	Condensed tannin	Hydrolyzable tannin
70% acetone	70 ± 6	79 ± 4	66 ± 1	68 ± 3
50% methanol	—[b]	—[b]	58 ± 1	53 ± 5
50% methanol, boiling	—[b]	—[b]	61 ± 3	—[b]
1% HCl in methanol	52 ± 2	79 ± 3	47 ± 8	71 ± 4

[a] Recovery of tannin added to tannin-free leaves determined with the Prussian blue assay (Price and Butler, 1977).
[b] Not determined.

nized in various solvents (Table 2). The added tannin was most efficiently recovered with aqueous acetone as the solvent. Less tannin was recovered with the alcohol-containing solvents, although acidic methanol was as effective as aqueous acetone for recovery of hydrolyzable tannin (Table 2). However, when lyophilized tannin-containing tissues were extracted, acidic methanol was much less efficient than aqueous acetone (Table 1).

The extractability of tannin was determined for leaves collected at different times during the growing season. In the early summer, much more tannin was extracted from lyophilized maple leaves by aqueous acetone than was extracted by heated methanol (Table 3). As the summer progressed, the amount of tannin extracted by acetone decreased more rapidly than the amount extracted by alcohol. In the late summer, equal amounts of tannin were extracted from the lyophilized maple leaves by aqueous acetone or by heated aqueous methanol (Table

TABLE 3. EXTRACTION OF TANNIN[a] FROM LYOPHILIZED MAPLE LEAVES COLLECTED ON VARIOUS DATES

Solvent	Date		
	May 27, 1986	July 9, 1986	August 13, 1986
70% acetone	1794 ± 36a	1381 ± 149b	692 ± 93c
50% methanol	1228 ± 54b, d	1117 ± 133d	802 ± 77c

[a] Tannin determined by the radial diffusion assay and expressed as cm^2/g dry tissue. Each value (± SD) is the mean of at least three determinations. Values followed by the same letter are the same at the 95% confidence interval (*t* test).

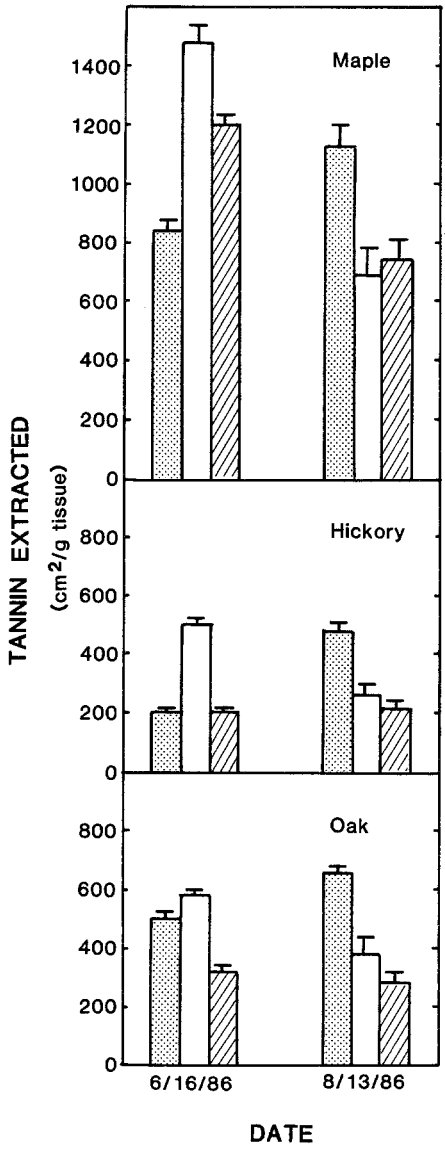

FIG. 1. Tannin extracted from fresh, lyophilized, and dried leaves. Leaves were collected on the indicated date and were either extracted immediately (▨), lyophilized and then extracted (□), or dried at 40°C and then extracted (▨). The tissue was extracted with 70% aqueous acetone and the extracts analyzed with the radial diffusion assay (Hagerman, 1987). Tannin was calculated on the basis of dry weight. Error bars show 1 SD from the mean of at least three trials.

3). Similar results were obtained with lyophilized oak or hickory leaves and with dried leaves from all three species (Figure 1). It has previously been noted that the amount of tannin in plant tissues diminishes throughout the growing season (Bate-Smith, 1973a; Butler, 1982). However, the role solvent plays in determining the apparent magnitude of the seasonal change has not been previously noted.

The extractability of tannin from leaves preserved by several methods was determined at two times during the growing season (Figure 1). Early in the season, more tannin was extracted from lyophilized leaves than from fresh leaves. Late in the season, the amount of tannin extracted from lyophilized leaves diminished, as noted above. However, the amount of tannin extracted from fresh leaves collected late in the season was greater than the amount that could be extracted from fresh leaves collected early in the season. The extraction of tannin from the dried leaves was quite efficient for some samples and less efficient for other samples. Drying at elevated temperatures was particularly harmful; no tannin could be extracted from hickory leaves that were dried at 90°C, and only a small amount of tannin could be extracted from oak or maple leaves dried at 90°C. Similar amounts of tannin were extracted from frozen samples of late-season leaves and from fresh samples of the same leaves.

DISCUSSION

It is not possible to recommend a single optimal protocol for extraction of tannin from all plant samples. Each sample has unique characteristics, including the structure of the tissue and the composition of the tannin, that determine tannin extractability (Bate-Smith, 1973a; Swain, 1979). Although a very limited number of samples were examined in the study described here, some general recommendations can be made based on these results.

Aqueous acetone appears to be the best solvent for extracting tannin from leaf tissue. Aqueous alcohol or acidic alcohol does not extract more tannin than aqueous acetone. Heated aqueous alcohol may extract less tannin than aqueous acetone. Acetone is an effective solvent because it inhibits interaction between tannin and proteins (Hagerman and Robbins, 1987) and thus prevents tannin from binding to leaf proteins during homogenization. However, acetone inhibits many of the common precipitation assays for tannin (Bate-Smith, 1973b; Hagerman and Butler, 1978; Martin and Martin, 1983), so procedures for analysis of acetone extracts must be chosen carefully. Acetone does not interfere with the radial diffusion assay because the acetone evaporates before the tannin–protein interaction takes place (Hagerman, 1987). Acetone does not interfere with most chemical assays for tannin (Price and Butler, 1977; Price et al., 1978).

To minimize artifacts associated with leaf preservation, leaves should be extracted and analyzed immediately after collection. However, if analysis of fresh tissue is impossible, tissue should be lyophilized or dried at low temperature rather than in the sun or in an oven. If tissues must be dried a higher temperature, analysis of at least a few samples of fresh tissue, for comparison with the dried tissue, would provide insight into the changes in tannin extractability associated with drying.

Tannin levels and extractability change dramatically through the growing season. Environmental conditions such as light intensity affect phenolic biosynthesis and accumulation (Balsa et al., 1979; Waterman et al., 1984). Seasonal changes in leaf morphology, moisture content, and chemistry may affect tannin extractability (Swain, 1979). Comparisons of tannin levels in plants must be made cautiously since tannin extractability is dependent on many factors. The seasonal variation in tannin extractability observed in this limited study of three temperate-climate species may not resemble the seasonal changes in other plants. Studies of the ecological significance of tannin should include investigations of seasonal variations for the plant of interest, using the plant preservation and tannin extraction techniques that will be used in the ecological study.

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDIES WITH LIVE LARVAE AND LARVAL RINSES OF THE RED IMPORTED FIRE ANT, *Solenopsis invicta* BUREN (Hymenoptera: Formicidae)

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Abstract—Behavioral and electrophysiological studies with live intact larvae and larval rinses of the red imported fire ant, *Solenopsis invicta* Buren, give undeniable evidence of a volatile material associated with the larvae of the ant that is capable of eliciting a response from brood-tending workers. In a Y-tube bioassay, worker ants were attracted equally to an airstream blown over sibling larvae or heterocolonial larvae. Workers were also attracted to a rinse of the larvae in a spot bioassay, aggregated about a piece of surrogate brood in another bioassay, and retrieved surrogate brood treated with the rinse material. A dose–response curve constructed from electroantennograms of workers revealed a receptor response of 1–100 brood equivalents.

Key Words—*Solenopsis invicta*, Hymenoptera, Formicidae, brood recognition, pheromone, fire ant, behavior, olfaction, orientation, bioassays.

INTRODUCTION

The brood of an ant colony can be considered to be not only the growing point of the colony, but also the storage tissue or energy capital (Wilson, 1971). When the colony falls upon hard times, the brood can serve as the prime source of food. It is not surprising then that the early investigators of the behavior of workers toward the brood focused mainly on the trophallaxis of either the stomodeal or proctodeal larval secretions (Stager, 1923; LeMasne, 1953; Torossian, 1961). While these studies did explain some of the feeding behavior, other

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behaviors toward the brood were left unexplained. Watkins and Cole (1966) presented the first evidence that the brood of the army ant, *Neivamyrmex apacithorax* (Emery), produced a pheromone that induced specific worker tending behavior. Later experiments by Glancey et al. (1970) showed that the brood of the red imported fire ant (RIFA), *Solenopsis invicta* Buren, also produce a pheromone that elicits particular worker behaviors (e.g., tending, grooming, moving, etc.) and is extractable and capable of being bioassayed. Robinson and Cherrett (1974) found that hexane would remove a behaviorally active material from larvae of *Atta cephalotes*, but they were unable to isolate the active fraction.

Investigations by Walsh and Tschinkel (1974) on RIFA led them to question the report of Glancey et al. (1970). Ignoring the food bioassays conducted by Glancey et al. (1970), they insisted that the latter's findings represented a food response. Walsh and Tschinkel (1974) concluded that they had found the brood pheromone, that it was a nonvolatile contact pheromone, that it was intimately tied up with the cuticle, and that the cuticle had to be contacted in order for a response to follow.

Later, Bigley and Vinson (1974) reported that the brood pheromone of RIFA was triolein, a triglyceride. This finding was questioned by VanderMeer (1983), who did not feel confident about assigning a structure based only on thin-layer chromatography.

This present paper presents the results of additional behavioral studies. The first evidence of a noncontact component of the brood pheromone is presented. Using larval rinses, we assayed workers for contact-mediated and non-contact-mediated responses to the brood pheromone. Additionally, EAG responses of brood tenders to larval rinses were measured.

METHODS AND MATERIALS

Source and Maintenance of Test Insects

Worker ants that were observed to be tending brood were collected from the vicinity of various laboratory colony brood piles. These "brood tenders" are the youngest workers and are more responsive to various pheromones (Glancey, unpublished data) than the older foraging workers. The colonies were maintained at 27 $\pm$ 1°C in Williams cells (Bishop et al., 1980) and fed honey–water and the Brooks diet. This diet, developed by T. Brooks, University of Georgia at Athens, consists of ground beef, peanut butter, eggs, sugar, salt, sorbic acid, vitamins, and water. The materials are blended with gelatin, allowed to cool and congeal, and the solidified material is cut into 1-inch squares. These squares are dipped into melted paraffin (Paraplast). When the test protocol called for the use of live larvae, the larvae were collected from colonies other than the

ones used for the collection of the brood tenders. Field larvae were collected by digging up a field colony and separating the brood from the soil.

Chemical Stimuli

Larval Rinses. The larval rinses for the behavioral bioassays were obtained by collecting field colonies and separating the larvae from the soil and workers. This separation was greatly facilitated by moving the larvae back and forth with the use of a camel's hair brush. This motion caused the larvae to cohere together due to the presence of larval hooks. These larvae could then be lifted away from the pupae. Groups of live larvae were weighed, the numbers counted, and an average weight of 0.53 mg/larva calculated. Only worker larvae were collected, mainly third and fourth instar. An estimated 250,000 larvae were collected in this manner. The larvae were rinsed in nanograde pentane for 30 sec, the extract transferred to a freezer, and the larvae discarded. The rinse was held in a freezer until used in a given bioassay.

In order to ensure that the responses obtained were not due to a food reaction, we compared the response to larval rinses with responses to pentane rinses made from American cockroaches (*Periplanata americana*). The cockroaches were obtained from the USDA's IAMARL cockroach-rearing facility located at the Gainesville Laboratory. Cockroaches, if available from the rearing section, are normally fed to our ant colonies as a source of insect protein. Previous work had shown that worker ants are attracted to and masticate a spot on filter paper to which a cockroach rinse has been applied (Glancey, unpublished data). The roaches obtained from the rearing section were weighed and rinsed with nanograde hexane. Initial tests made with the larval rinses showed that it required 500 larvae (226 mg) to elicit a response from the workers. Accordingly, a solution was made up which gave us 500 larval equivalent (LE) per 20 μl of pentane. Similarly 226 mg of roaches were rinsed in pentane and reduced to 20 μl. Both the brood rinse and the cockroach rinse were quantified by capillary GC with an external standard to give comparable amounts of extracted material (Kovats index range 900–4500) in both rinses.

Electrophysiological Experiments. Volatiles were collected from worker larvae by rinsing 1, 10, or 100 larvae in nanograde pentane for 30 sec. Excess pentane was evaporated under a stream of nitrogen until a final volume of 10 μl was reached. The 1-hexanol (>99% purity) used as a standard was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin).

Bioassays

Attraction to a Spot. A 5.5-cm piece of Whatman No. 1 filter paper was divided into four quadrants by drawing two intersecting lines through the center. A small pencil dot was placed in the middle of each quadrant. A number between

1 and 4 was assigned at random to each one of the dots. Treatments assigned randomly to the dots were 20 μl each of the larval rinse, the cockroach rinse, and the pentane solvent; a fourth treatment consisted of a cohort of live larvae (ca. 300) that were placed on the spot and removed after 15 min. After the larvae were removed, the other dots were treated. The treated filter paper was placed in the bottom half of 5.5-cm plastic Petri dish. Twenty brood tenders obtained from our laboratory colonies were placed in the disk and another dish inverted over the bottom half to keep the ants from escaping. At 1-min intervals, counts were made of the ants within 0.5 cm of the spot.

Olfactometer Bioassay. Live larvae were tested for the production of volatiles in a Y-tube olfactometer. This olfactometer was modeled after a design by VanderMeer et al. (1979) and is described completely by VanderMeer et al. (1988). The olfactometer consists of two 24/40 ground glass joints sealed to one of the arms of a 5-cm Y tube such that 1 cm of each Y-tube arm extended through half the ground glass joints. Three hundred third- and fourth-instar colony or field larvae were placed in one of the choice chambers. The other choice chamber was left blank. Compressed air was split into two streams, each stream being independently controlled by flowmeters. Airflow was regulated at 0.2 l/min into each choice chamber. Fifty brood tenders, selected at random, were chilled for 10 min (6°C) in the refrigerator and then placed in a small bronze wire cage. The open end of the cage was attached to the stem of the Y tube and the airflow turned on.

The initial choice of the first 20 ants that responded by walking into one of the choice chambers was recorded. Ants that were not trapped in the choice chamber and returned to the stem entrance were not counted. The entire olfactometer was rinsed after each test with acetone and dried. Another set of larvae from the same colony was tested with another set of workers from the same colony. However, the choice chamber in which the larvae were placed was reversed. After this test was run, the two scores were combined to give one replicate. This type of procedure eliminated any bias that was inherent in the individual choice chambers. Data were analyzed statistically by use of a chi-square test.

Surrogate Bioassay. This bioassay was developed in our research of the queen recognition pheromone (Lofgren et al., 1983). A section of a rubber needle-seal septum was treated with one of the rinses or pentane. The septum was air dried and then placed in a 9-cm Wilson cell which had all the ports sealed. The septum was placed in a 2-cm^2 area drawn upon the castone bottom of the cell, and 20 worker ants were placed in the cell. The data were quantified by counting the numbers of ants clustering in the 2-cm^2 area at 1-min intervals for 5 min. At the end of the 5-min run, the ants and septum were discarded and a new cell used for the next trial. Three different colonies were tested in this manner for three replications.

Retrieval of Brood Surrogates by Disrupted Colonies. This particular

bioassay was also designed for use in our green pheromone research. When a colony is opened and the brood and workers are scattered about the colony area, the workers immediately begin to collect the brood, lay trails back to the nest, and carry the brood along these trails. We have simulated this in the laboratory by using a laboratory colony in a large box (1.2 m^2) (Glancey et al., 1983). The rearing cell containing the colony is opened and the brood and workers scattered about the box. In the present bioassay, small pieces of colored construction paper (2 mm^2) (Union Camp Corp., Chamblee, Georgia) were soaked overnight in the rinses (larval or cockroach) or solvent. The papers were air dried, the colonies disrupted, and 10 pieces each of the various colored papers deposited in the area of disruption. Differently colored papers were used for each treatment, but no colonies received the same colored paper treated with the same rinse. One hour after the papers were deposited, observations were made of their fate. If the papers were taken inside the cell and placed with the brood, the test was scored as a positive response. Sometimes the ants had difficulty getting the paper through the small entrance hole into the cell. If the ants placed the papers beside the entrance hole because they could not get it into the cell, and if they placed some of the scattered brood next to the papers, then that test was recorded as a positive one. Five replications using five different colonies were made in this manner.

Data for the attraction and surrogate bioassays were evaluated for significance using the general linear model procedure of SAS Institute (1982) and by the Waller-Duncan *K*-ratio *t* test.

Electrophysiology Procedures. Electroantennogram (EAG) techniques utilized in these studies were modified after an earlier study (Schneider, 1957) and are described in detail elsewhere (Dickens and Payne, 1977; Dickens, 1981). In brief, Ag–AgCl capillary electrodes filled with physiological saline (Pantine, 1948; Oakley and Schafer, 1978) were used. The recording electrode was introduced into the distal end of the terminal antennal segment which was prepunctured by a sharpened tungsten needle. The indifferent electrode was inserted into the head capsule. The signal was amplified 10-fold by a Grass P-16 DC microelectrode preamplifier prior to viewing on a Tektronix 5223 digitizing oscilloscope. An *x–y* plotter recorded EAGs on graph paper for subsequent analyses and storage.

Odorous stimuli were delivered on filter paper (8 × 18 mm) inserted into glass cartridges (80 mm long; 5 mm ID) oriented toward the preparation from ca. 1 cm. Stimulus duration was 1 sec with an airflow of 1 m/sec. A range of concentrations was used to develop a dose–response curve. The stimuli were presented in order from the lowest to the highest concentrations. A 3-min interval was allowed between each stimulus. Three replications were run using three different insects. Response to the pentane control was subtracted from response to the other compounds.

Stimulation with 1-hexanol, a component of the green leaf volatile com-

plex (Visser et al., 1979), at 100 μg was used as a standard so that responses from different preparations could be compared (Dickens, 1984; Dickens and Boldt, 1985). In addition, the 1-hexanol is ideal as a standard since most insects, indeed most animals, encounter this odor in their daily environment. Six-carbon alcohols and aldehydes are omnipresent as components of the odor of green leaves (Visser et al., 1979). Thus one could predict the presence of many receptors on the antennae of the worker ants since green plants and associated green odors form a large part of their environment via the mound construction. Mean response of the brood tenders to the standard was -0.38 mV (SE = 0.04; N = 3). Each stimulus was either preceded or followed at 4 min by a stimulation with the standard. Responses to intervening test stimuli were represented as a percent of the mean of the two nearest responses to the standard (Dickens, 1978, 1981). The size of the EAG was considered to be a measure of the relative number of responding acceptors (Payne, 1975; Dickens and Payne, 1977).

RESULTS AND DISCUSSION

The results of the bioassays are given in Tables 1–4. In Y-tube olfactometer trials with intact, live larvae, it was determined that 300 immatures were capable of eliciting a statistically significant response from brood tenders (Table 1). Worker response to sibling larvae was not significantly different from worker response to heterocolonial, field-collected brood. These data strongly suggest that worker response to a brood pheromone attractant does not require contact chemoreception and that this response is not mediated by worker conditioning to colony-specific brood odors, be they heritable or environmental. Similar evidence for the presence of a volatile attractant given off by brood of the RIFA is presented in the work by Lofgren et al. (1983). In olfactometer bioassays in which ants were given the option of orienting to an airstream blown over their own colony queen or to an airstream being blown over larvae from their own colony or from an alien colony, the ants responded equally to the airstream over their own larvae and over alien larvae. The implication here is

TABLE 1. OLFACTOMETER RESPONSE OF BROOD TENDERS TO 300 LIVE, INTACT IMMATURES[a]

Response to	No. attracted ($X \pm$ SD)	No. not attracted ($X \pm$ SD)	χ^2 (1 df)
Own brood	161 (26.83 ± 2.32)	79 (13.17 ± 2.32)	10.44
Alien brood	159 (26.50 ± 2.74)	81 (13.5 ± 2.74)	9.57

[a] Laboratory-reared workers were tested against brood from their own colony and against field-collected brood; N = 6.

that regardless of the source of the brood, or the brood–worker kinship, a volatile is present that causes attraction. Observations in the field have shown that when a colony is disturbed and some of the larvae are buried beneath the soil, the worker ants are quite adept at locating each and every piece of brood. It is quite possible that the workers are locating the buried brood by the presence of the brood pheromone. It might be argued that the workers are using substrate vibrations produced by the larvae to locate them. However, it has not been shown that RIFA larvae are capable of producing sound. Lenoir (1984) has shown that, in research with the ant *Cataglyphis cursor*, colony workers recognize sibling brood immediately. If, however, workers have been adopted into the colony, these adoptees do not initially recognize the larvae as sisters. However, after six days, the adopted workers begin to tend brood indicating the inhibition has disappeared. Lenoir's work not only demonstrates the presence of a brood-tending pheromone, but also presents data that show brood kin recognition.

Further evidence for the perception by brood tenders of odorous stimuli emanating from the larvae was obtained from the electrophysiological studies. A dose–response curve constructed from EAGs to increasing larval equivalents showed receptor response to increase from a threshold of ca. 1–10 larval equivalents to a saturation level of ca. 10–100 larval equivalents (Figure 1). As seen in Figure 1, the maximum response to the brood rinse was 20% of the 1-hexanol

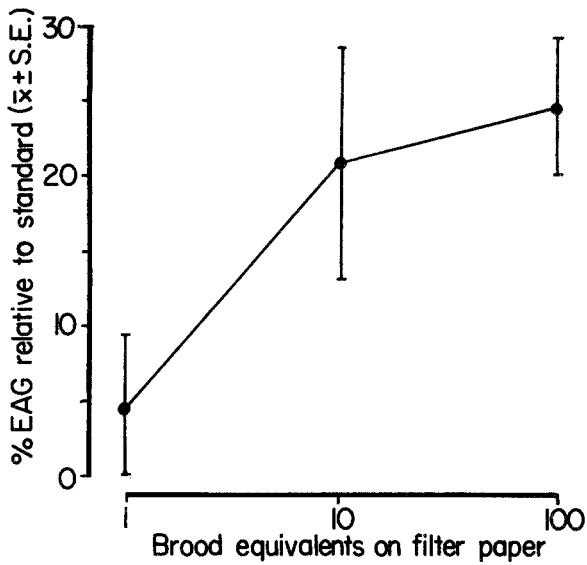

FIG. 1. Dose–response curve constructed from mean EAGs of brood tenders (N = 3) to increasing brood equivalents on filter paper. Vertical bars represent standard errors.

TABLE 2. RESPONSE OF BROOD TENDERS FED TWO DIFFERENT DIETS IN SPOT ATTRACTION BIOASSAY

Treatment	Response, % ($X \pm$ SE)	
	Normal diet	Roach diet
Brood rinse	79 ± 2.3A[a]	57.8 ± 5.7G
Larval resting spot	14 ± 2.4B	18.6 ± 4.2H
Roach rinse	4 ± 1.2C	3.0 ± 1.1I
Solvent	3 ± .75C	1.6 ± 0.6I

[a] Letters following SE in each column refer to within column comparisons (Duncan's new multiple-range test) where means followed by different letters are significantly different ($P < 0.0001$).

standard. One may wonder if this is indeed a significant response and why brood tenders should exhibit greater EAG responses to the leaf volatiles than to the brood pheromone. The green leaf volatiles may be detected by numerous receptors with broad specificity, while the brood pheromone receptors may be few in number but highly specific. A situation similar to the one described above occurs in the boll weevil (Dickens, 1984, and unpublished data). Furthermore, disregarding the peripheral receptor system, higher order neural processing may amplify and modulate plant odors and pheromones quite differently. Thus, in this case, the 20% response does indeed represent a significant value. Little or no response was obtained with the volatiles from triolein.

The results from the brood rinse experiments (Tables 2–4) show that some kind of attractive material is being extracted from the larvae. In the spot attraction test, regardless of the type of diet, the workers responded best to the rinse of the larvae (Table 1). In this test, where workers have a choice of orienting to a particular spot and settling down, the implication is that there is a contact

TABLE 3. RESPONSE OF BROOD TENDERS FED TWO DIFFERENT DIETS IN SURROGATE BIOASSAY

Treatment	Response, % ($X \pm$ SE)	
	Normal diet	Roach diet
Brood rinse	14.4 ± 3.30A[a]	20.3 ± 2.8G
Roach rinse	1.8 ± 0.56B	0.0 ± 0.0H
Solvent	1.6 ± 0.55B	2.6 ± 0.8I

[a] Letters following SE in each column refer to within column comparisons (Duncan's new multiple-range test) where means followed by different letters are significantly different ($P < 0.0001$).

TABLE 4. RESPONSE OF RIFA WORKERS FROM DISRUPTED LABORATORY COLONIES TO PIECES OF CONSTRUCTION PAPER IMPREGNATED WITH VARIOUS RINSES

Treatment	Average retrieval to colony or colony area[a]	
	Normal diet	Roach diet
Brood rinse	83	80
Roach rinse	0	0
Solvent	0	0

[a] Mean response based upon 10 pieces of paper for each rinse presented to five separate colonies.

material present that causes the workers to respond. In the natural setting of a colony, the brood tenders do not wander about, but tend to remain on the brood pile while carrying on their activities. This tendency to settle down is seen also in the response to the spot upon which the larvae had rested. These responses to the larval rinse spot or the larval resting spot agree with the findings of Walsh and Tschinkel (1974) that a pheromone is present that is intimately tied up with the cuticle. However, their conclusion was that the workers needed to contact the brood in order for a response to follow. Our data show that this latter conclusion may not necessarily be true.

Cockroach extracts were not active in tests with disrupted colonies. Ants from both tests responded to small pieces of paper treated with the larval rinse by stacking scattered brood upon the papers, by building trails from the nest to the papers, and by returning the papers to the area of the nest. No papers treated with the cockroach rinse or the solvent control were treated in this manner.

Data from both behavioral bioassays and electrophysiological studies show that volatile compounds are associated with RIFA larvae and that these compounds are capable of eliciting a response by brood tending workers. The failure of Walsh and Tschinkel (1974) to detect a volatile compound might be explained by the use of workers other than brood tenders and the extremely small amounts of the pheromone present on single larvae. We found it necessary to use at least 300 larvae to elicit an olfactometer response. Response by the workers to brood cuticle suggests that the brood recognition pheromone may have at least two components: one, a volatile component that draws the workers near the brood; and two, a contact material that causes retrieval behavior. The two components together evidently have a very shallow active space around each larva.

The question arises as to whether the pheromone is produced by the larvae or whether it is the result of some material being applied to the larvae. We know that when the RIFA queen lays an egg, she draws the ovipositor over the

egg and applies venom to it (VanderMeer, unpublished data). This material may serve not only as an antibiotic, but also as a means to alert the brood tenders to carry the egg to the brood pile. An egg-laying queen has an entourage of workers that mill about her posterior end during the egg-laying period. When an egg is deposited and the sting drawn across it, these workers enter a very highly excited state. The egg is quickly picked up and taken to the brood pile. Another possibility for the origin of the material may be the mere physical transfer of worker hydrocarbons to the brood. These hydrocarbons may facilitate the brood tender's feeding and grooming of the larvae. Such behavior is already known in the case of the myrmecophilous beetle, *Myrmecaphodius excavaticollis* (VanderMeer and Wojcik, 1982). This RIFA symbiont is capable of acquiring the RIFA hydrocarbon pattern and, being so marked, is able to move freely about the colony and obtain food directly from workers. Finally, the attention paid to the brood may simply be a result of the topical application of venom by the brood tenders. Obin and VanderMeer (1985) have shown that worker ants disperse venom through the air by raising the abdomen and vibrating the gaster (termed "gaster flagging"). Although these authors suggest that this behavior is a method of dispersing antibiotics, it may also be that it is the method of marking the brood for tending behavior. Regardless of the source of the material that causes brood tending, it is obvious that some chemical is present and that this material is capable of being extracted and tested.

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THE INFLUENCE OF DIETARY β-CARBOLINE ALKALOIDS ON GROWTH RATE, FOOD CONSUMPTION, AND FOOD UTILIZATION OF LARVAE OF *Spodoptera exigua* (HUBNER)

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Abstract—β-Carboline alkaloids are found worldwide in many plant families. Harman, harmine, and other simple β-carboline alkaloids were tested for activity against a generalist phytophagous insect, the beet armyworm [*Spodoptera exigua* (Hubner)]. Chronic dietary exposure tests (neonate to pupa) reveal potent antifeedant and possible toxic effects. Acute dietary exposure tests on fifth-instar larvae also demonstrate antifeedant activity.

Key Words—β-Carboline alkaloids, harman, harmins, *Spodoptera exigua* (Hubner), beet armyworm, antifeedant, Lepidoptera, Noctuidae.

INTRODUCTION

There are 26 angiosperm plant families and three fungi genera that contain simple β-carbolines (Allen and Holmstedt, 1980). Among these are the Leguminosae, Malpighiaceae, Symplocaceae, Passifloraceae, and Zygophyllaceae. Although isolated in small quantities from plants, these secondary metabolites have significant potency in animal systems. The simple β-carbolines have been shown to inhibit monoamine oxidase and induce tremor (Coates and Cox, 1972), to intercalate into DNA (Duportail and Lami, 1975), to interfere with UV-induced DNA damage repair systems (Chang et al., 1978), to inhibit sodium–potassium ATPase activity (Murumo et al., 1976), to be phototoxic against microorganisms (McKenna and Towers, 1981), to be antitrypanosomal (Cavin

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et al., 1987), and to cause hypothermia (Leonard and Stonier, 1971). Because of the worldwide distribution in plants, and the wide range of activity of the simple β-carboline alkaloids, their influence on the growth and development of a generalist phytophagous insect was undertaken in this study.

Polyphagous, or generalist, insects utilize a range of generalized detoxification enzymes (Krieger et al., 1971), enzyme induction systems (Brattsten et al., 1977), or simply avoid toxic plants. The activity of various β-carboline alkaloids on the growth, consumption, and food utilization of a generalist insect, the lepidopteran, *Spodoptera exigua* (Hubner), is described in this study. The simple β-carbolines under consideration are lipid soluble at pH 8–9, the pH of the insect gut. Therefore, they pass readily through the gut lining into the hemolymph. Simple β-carboline alkaloids present in an artificial diet were found excreted unchanged in the frass (Cavin, in preparation). Their low molecular weights (182–212) would allow any absorbed compounds to be at least passively transported out by means of the Malpighian tubules. However, although they are apparently not metabolized, some of the simple β-carbolines, when added to a controlled artificial diet, are perceived by the insect larvae because they reduce feeding significantly. Toxic effects were seen with long-term exposures.

METHODS AND MATERIALS

Spodoptera exigua (Hubner) larvae were obtained from a colony kept in the phytochemistry laboratory at the University of California at Irvine. The experimental groups were maintained in an incubator with a 16-hr light–8-hr dark photoperiod at 26.5°C.

The treated diet was prepared by mixing the test chemical, in methanol, with the dry portion of the insect mix (Bioserv mix No. 9219), the solvent evaporated at room temperature in a fume hood overnight, and then freeze-dried. The wet diet was then prepared according to package instructions. The control group diet was treated with methanol.

Acute Toxicity Tests. Spodoptera exigua (Hubner) were raised on insect diet to the pharate fifth stage. Maintenance of a regular dark–light schedule during rearing allowed the selection of large experimental groups which molted during the same hour of the day. Prior to each experiment, over 250 larvae were chosen at the third-instar stage and placed on fresh diet. After reaching the pharate fifth stage, just before their expected molt, a group of pharate fifth-instar larvae with an average weight of 75 mg ± 10 mg were selected for each experiment. A preliminary test on a group of such larvae determined their dry weight–wet weight ratio. The proportion of gut contents to whole body weight in larvae 24 hr after their molt into the fifth instar was also determined in another

preliminary experiment by placing larvae eating artificial diet on diet dyed a different color and collecting their frass until its color changed.

Just before molting, as described above, pharate fifth larvae were placed on artificial diet containing a simple β-carboline (prepared as above). Individual larvae were weighed, placed in plastic cups with over 500 mg prepared diet, and allowed to eat ad libitum for 24 hr. This time period was chosen as representative of a short-term exposure because the greatest amount of food is eaten during the final instar. Larvae in the control group would often enter the prepupal stage within 48 hr, so a shorter time frame was chosen. Initial larval weights and amount of diet were measured. After 24 hr, their individual wet weights, the separate dry weights of leftover food, and the dry weights of their frass were measured. The collective dry weight of each treatment group was obtained to determine the average ratio of larval dry weight to wet weight. The initial proportion of dry to wet weight of the food was determined. These data, plus the assumptions that (1) the gut contents of pharate larvae are empty and (2) the average proportion of the dry weight of gut contents in larvae 24 hr into the fifth instar remains constant, were used to determine growth and consumption indices.

Approximate digestibility (AD), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD) were determined as described by Waldbauer (1968), and the mean relative growth rate (RGR) was based on Radford (1967). Dry weight measurements were used. The initial weight was a predetermined percentage of the wet weight based on the preliminary study described above. The final weight used in determinations was the dry weight of the larva minus the predetermined percentage determined for gut contents. The mean relative consumption rate (RCR) was measured as RGR/ECI. The expected 24-hr growth rate of fifth-instar experimental larvae given their consumption rate was compared with a consumption rate versus growth rate calibration curve determined for the stock colony (Griswold, unpublished).

Chronic Toxicity Studies. A study using a control group and a group fed a 500 μg/g harman-treated diet began with pharate third-instar larvae and continued 144 hr, when most of the control group was well into the fifth instar. Their initial proportion of dry weight to wet weight was determined by a selection of larvae as described above for the pharate fifth-instar tests. RGR, RCR, AD, ECI, and ECD were determined.

Experiments were conducted on larvae raised entirely from birth on treated diets. The larvae were placed on diets with different concentrations of a compound. Harman and harmine were chosen for investigation. Two neonates were reared per cup until the second instar, at which time the smaller of the two was removed. Fresh, treated diet was administered by day six. Larval weights were measured eight days after hatching. Larval weights were compared using an analysis of variance between the groups of each experiment, then a nested anal-

ysis of variance between experimental replications. In addition, the number of days until adult emergence, and the number of abnormalities in pupal and adult formation were noted.

Data shown are mean and standard deviations. Nested ANOVA was used between experimental replicates. ANOVA was conducted to include all concentrations and, when shown to be significantly different, each group was compared with the control by means of Dunnett's (1955) multiple comparison test, except for the pharate third-instar experiment which was analyzed using the Student's t statistic because there were only two groups. Confidence limits for percentages are based on the binomial distribution (Rohlf and Sokal, 1981).

One hundred micrograms per gram equals 0.01% wet weight. Molecular weight harman = 182.22 g/mol, 100 μg = 0.54 mM/kg wet weight diet; molecular weight harmine = 212.25 g/mol, 232 μg = 1.09 mM/kg wet weight diet.

RESULTS

Acute Exposure Studies. Groups with harman-treated diet showed significant reduction of both their relative growth rate and relative consumption rate ($P \leq 0.01$) at 5 mM/kg (0.1% wet weight) (Table 1). Groups with 5 mM/kg (0.1% wet weight) harmine-treated diet also showed reduced growth and consumption rates ($P \leq 0.01$) (Table 1). The growth rate of experimental larvae, given their reduced consumption, correlates with expected values from the growth rate–consumption rate curve. The food utilization indices, AD, ECI, and ECD, revealed no significant differences among treatment groups. Tests with harmol (5 mM/kg), harmalol (10 mM/kg), 6-methoxyharman (2.7 mM/kg), and harmaline (10.9 mM/kg) showed no significant reduction of growth rate, food consumption, or food utilization.

Chronic Exposure Tests. The analysis of the effects of harman-treated diet given to pharate third-instar larvae (Table 2) reveal reduced relative consumption rates and relative growth rates over a six-day period. Although both the RGR and RCR differed significantly from the control group ($P \leq 0.001$), there were no significant differences in the other indices.

Complete larval exposure tests (Table 3) identified larval weight differences. Nested ANOVA determined no significant differences between experimental replicates, but significant differences between chemical treatment groups. An ANOVA–linear regression analysis did not allow a linear correlation to be made, but plotted points suggest that the effective dose for 50% reduction of larval weights in eight days is less than or equal to 0.8 mM/kg wet weight (0.015% wet weight) harman and 3.8 mM/kg wet weight (0.08% wet weight) harmine. The time until adult emergence of harman treatment groups increased

TABLE 1. EFFECTS OF ACUTE DIETARY EXPOSURE (24 HR) OF FIFTH-INSTAR *Spodoptera exigua* (HUBNER) LARVAE TO HARMAN AND HARMINE[a]

Concentration (μg/g)						
	N	RGR	RCR	ECI	ECD	AD
Harman						
0	20	0.83 ± 0.13	2.74 ± 0.78	0.32 ± 0.08	1.32 ± 2.81	37.9 ± 13.9
1000	20	0.46 ± 0.18 **	1.27 ± 1.36 **	0.52 ± 1.16 NS	0.22 ± 1.90 NS	−20.6 ± 276.0 NS
2000	20	0.06 ± 0.21 **	−0.36 ± 1.49 **	0.17 ± 0.28 NS	0.17 ± 0.07 NS	108.4 ± 144.1 NS

	N	RGR	*N*	RCR	ECI	ECD	AD
Harmine							
0	43	1.00 ± 0.10	25	2.42 ± 0.66	0.40 ± 0.14	0.68 ± 0.69	37.1 ± 25.7
580	38	0.89 ± 0.22 *	20	2.56 ± 0.92 NS	0.36 ± 0.08 NS	0.79 ± 0.42 NS	40.5 ± 15.6 NS
1160	38	0.70 ± 0.21 **	20	1.74 ± 0.96 **	0.21 ± 0.33 NS	0.61 ± 0.39 NS	85.0 ± 116.0 NS
2320	38	0.54 ± 0.13 **	20	0.96 ± 0.52 **	0.42 ± 0.12 NS	−0.65 ± 3.90 NS	22.3 ± 89.8 NS

[a] *$0.01 < P \leq 0.05$; **$P \leq 0.01$; NS = not significant at $\alpha = 0.05$ level; Dunnett's multiple-comparison test.

TABLE 2. EFFECTS OF LONG-TERM DIETARY EXPOSURE (144 HR) OF THIRD- TO FIFTH-INSTAR *Spodoptera exigua* (HUBNER) LARVAE TO HARMAN

Concentration (μg/g)	N	RGR	RCR	ECI	ECD	AD
0	21	0.73 ± 0.11	3.01 ± 0.73	0.25 ± 0.23	0.59 ± 0.23	41.2 ± 7.4
500	20	0.46 ± 0.13 ***[a]	1.26 ± 1.24 ***	−2.19 ± 9.95 NS	0.95 ± 2.23 NS	732.6 ± 2810.8 NS

[a] ***$P \leq 0.001$; NS = not significant at $\alpha = 0.05$; Student's t test.

TABLE 3. EFFECTS OF CHRONIC DIETARY EXPOSURE OF *Spodoptera exigua* (HUBNER) TO HARMAN AND HARMINE FROM NEONATE TO MATURITY[a]

Concentration (μg/g)	N	Larval weight day 9	% C	No Pupa No.	No Pupa %	N	Days to adult emergence
Harman							
0	55	262.6 ± 45.4	100	7/51	13.7	44	18.09
20	55	234.0 ± 46.8 **	89	9/49	18.4−	40	18.38
100	54	169.5 ± 50.0 **	64	7/50	14.0−	43	18.91
200	53	75.0 ± 16.6 **	28	2/51	3.9−	49	20.16
300	53	44.0 ± 12.2 **	17	18/50	36.0+	32	22.06
Harmine							
0	56	227.5 ± 91.3	100	2/58	3.4		
232	55	208.9 ± 92.2 NS	92	10/56	17.9+		
464	59	160.5 ± 84.4 **	70	22/59	37.3+		
696	59	131.2 ± 61.6 **	58	12/59	20.3+		

[a]NS = not significant at $\alpha = 0.05$; $^{**}P \leq 0.01$; Dunnett's multiple-comparison test. % C = percent of control.
+ Significant at the 99% confidence level; − not significant. Binomial distribution used for confidence level test of percentages.

by approximately one day per additional 0.55 mM/kg, a 22% increase for the 1.65 mM/kg treatment group. There was a significant difference (99% confidence level) between groups in the number of nonemergent adults in the harman treatment groups at 1.65 mM/kg (0.03% wet weight) and a significant difference (99% confidence level) in the number that did not pupate in the harmine treatment groups in experiments with dosages above 1 mM/kg (0.02% wet weight).

DISCUSSION

Harman and harmine both slow the growth rate of a generalist phytophagous insect. Harman is more potent than harmine (7-methoxyharman). Harmol, harmalol, 6-methoxyharman, and harmaline had no effect on growth rate in the amounts tested. The reduced growth rate in both long- and short-term experiments of *Spodoptera exigua* (Hubner) given diets treated with harman and harmine is due primarily to the antifeedant effect of those compounds. The results indicate that the experimental larvae reach their expected growth rate for their rate of food consumption. Additionally, food utilization analysis (ECD, ECI, AD) showed no significant difference between control and treatment groups. The six-day test with third- to fifth-instar larvae given harman-treated diet con-

firmed the differences between control and treatment growth and consumption rates. These partial dietary exposure tests do not indicate that there are any toxic effects of harman or harmine on *Spodoptera exigua* (Hubner) larvae.

The eventual maturation after sufficient biomass is reached and lack of a disproportionate number of abnormalities in the neonate treatment groups on less than 1.65 mM/kg (0.03%) harman (Table 3) also suggest that the reduction in growth is a result of a reduced food consumption rate and not a latent toxicity in smaller quantities of harman. However, increases in the number not able to pupate properly and adults with crumpled wings were seen when higher amounts of harman and harmine were consumed. The significant number of abnormalities in the harman treatment groups at 0.03% wet weight harman and in the harmine treatment groups indicates the toxicity of harman and harmine to sensitive pupal development at higher concentrations (>0.5 mM/kg, 0.02% wet weight). When a sufficient amount of harman or harmine is consumed, their potential toxicity becomes more evident. However, the profound antifeedant effect of harman precludes the complete study of this phenomenon at the gross level. Physiological studies of isolated insect metabolic systems would more clearly identify sensitive sites.

The antifeedant activity of harman is significant in that it prolongs the maturation of *Spodoptera exigua* (Hubner) up to 22% at quantities as low as 0.03% wet weight (Table 3). A longer period of development would increase the risk of exposure to other environmental dangers. The effective dose of harman to reduce larval weight at day 8 (when the control groups reached the middle of the fifth instar) by 50% (ED_{50}) is 0.015%. The ED_{50} for harmine is 0.08% wet weight. Even fifth-instar larvae show reduced RCRs and RGRs during short-term exposures at 0.1% wet weight ($P \leq 0.01$) (Table 1).

Harmine has been quantified at 0.546% dry weight in the leaves of *Banisteriopsis caapi* (Spruce) (Malpighiaceae) (Rivier and Lindgren, 1972) and in the seeds of *Peganum harmala* L. (Zygophyllaceae) at 1.05% dry weight (Hegnauer, 1973). Harman has been found in quantities of 0.15% dry weight in the leaves of *Prosopis nigra* (Leguminosae) (Moro et al., 1975) and 0.24% dry weight in *Symplocos racemosa* Roxb. (Symplocaceae) (Hegnauer, 1973). These quantities are much higher than the ED_{50} for *Spodoptera exigua* (Hubner). In addition, multiple β-carboline alkaloids are often present in the same plant. The additive antifeedant and toxic effect of multiple simple β-carbolines is probably significant in a plant's defense against generalist insect herbivory.

The data from this study indicate the potency and significance of alkaloids produced by plants in defense against insect herbivores. Some of the other plant alkaloids shown to have some antifeedant properties include *N*-methylflindersine (Chou et al., 1977), wilforine (Monache et al., 1984), vinblastine (Meisner et al., 1981), and the β-carboline reserpine (Janzen et al., 1977). The small amounts of alkaloids found in plants have long been predicted to be toxic to

insects as well as mammals, but only limited testing has been conducted to verify this. Research has been focused on plant secondary metabolites found in higher quantities in plants. All ecological investigations should compare the potency of secondary metabolites with actual quantities present in plants. Low ED_{50} levels of alkaloids may show them to be more significant in protection from insect herbivory than previously considered. The small amounts of alkaloids found in plants relative to other secondary plant metabolites should not deter researchers from conducting insect toxicity or antifeedant tests.

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SEX PHEROMONE OF FEMALE *Myelois cribrella* HÜBNER (LEPIDOPTERA: PYRALIDAE)[1]
Chemical Identification, Electrophysiological Evaluation, and Field Attractancy Tests

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Abstract—Females of *Myelois cribrella* contain about 20 ng/gland of the primary sex pheromone components (*Z*)-9-tetradecenyl acetate, (9*Z*,12*E*)-9,12-tetradecadienyl acetate, and (*Z*)-11-hexadecenyl acetate in proportions of 4:11:5, respectively. These physiologically active components are accompanied by a number of related compounds such as (*Z*)-9-tetradecen-1-ol, hexadecyl acetate, (9*Z*,12*E*)-9,12-tetradecadien-1-ol, (*Z*)-11-hexadecen-1-ol, octadecyl acetate, octadecan-1-ol, and eicosyl and docosyl acetates. Octadecyl acetate, the most abundant component, represents about 42 ng/female moth; however, no physiological activity could be attributed to it. In field tests, a trap baited with a 1-mg mixture of (*Z*)-9-tetradecenyl acetate, (9*Z*,12*E*)-9,12-tetradecadienyl acetate, and (*Z*)-11-hexadecenyl acetate in a ratio of 1:2:1 caught more male moths than three live female moths.

Key Words—*Myelois cribrella*, sex pheromones, Lepidoptera, Pyralidae, solid-sample injection, (*Z*)-9-tetradecenyl acetate, (9*Z*,12*E*)-9,12-tetradecadienyl acetate, (*Z*)-11-hexadecenyl acetate.

INTRODUCTION

Myelois cribrella Hübner (Lepidoptera: Pyralidae) belongs to the pyralid subfamily Phycitinae and is widely distributed in central and southern Europe

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including Great Britain and Asia. The distribution area includes Syria, Armenia, the Altai Mountains, and Japan (Leraut, 1980; Staudinger and Rebel, 1901). The adults of this nocturnal moth appear from the second half of June until the beginning of August. The larvae feed in late summer on heads of thistles and, in central Europe especially, on *Cirsium vulgare* (Savi) Ten., *Cirsium eriophorum* (L.) Scop., *Carduus nutans* L., and *Onopordum acanthium* L. In autumn, the larvae burrow into the thistle stems, make large galleries in the pith and hibernate in the stems as nearly full-grown larvae. In spring, they cut circular exit holes and pupate in the larval galleries (Beirne, 1954; Hasenfuss, 1960).

Many members of the subfamily Phycitinae are known as pests. The pheromones of a number of them, especially of those considered as stored-product pests, have been identified (Arn et al., 1986). The species *M. cribrella*, nevertheless, is not mentioned as a pest, and no information was available on its female sex pheromone. Here, we describe the characterization of its pheromone and formulation of a specific lure for the field attractancy tests.

METHODS AND MATERIALS

Insect Material. Myelois cribrella specimens were collected as pupae from dead stems of various thistles found on a hill in the vicinity of Erlangen (Bavaria, Germany). The pupae were sexed, placed in plastic boxes containing moist sawdust, and maintained under a reversed 15 : 9 hr light–dark cycle. After emergence, the females were observed carefully during the scotophase under a red lamp to observe the calling behavior and period.

Gas Chromatography. Capillary gas chromatography with flame ionization detection was performed on a Packard-United Technologies 438A instrument equipped with a splitless injector and a Shimadzu Chromatopack C-R3A data system. The "pheromone glands" of 2- to 4-day-old female moths were excised during the period of maximum calling, encapsulated in soda glass capillaries, and injected into the GC according to the solid sampling technique described by Attygalle et al. (1986). Columns and temperature programs were: (1) fused-silica capillary column (FSCC) SP-2340 (25 m × 0.22 mm), 2 min at 60°C, 60–195°C at 4°C/min; and (2) FSCC CP-19 (chemically bonded phase, 12 m × 0.2 mm), 2 min at 60°C, 60–270°C at 10°C/min.

Gas Chromatography—Mass Spectrometry. A Finnigan 9502 gas chromatograph, fitted with a Grob-type split–splitless injector and a solid sampler (Morgan and Wadhams, 1972; Attygalle et al., 1986), linked to a Finnigan 3200E quadrupole mass spectrometer with a Data System 6000 was used: Columns and temperature programs were (3) FSCC SP-2340 (25 m × 0.22 mm), 4 min at 60°C, 60–195°C at 6°C/min, splitless injection via a solid sampler, split-vent kept closed for 1 min.

Electrophysiology. Simultaneous flame ionization and electroantennographic detection (FID and EAD) was performed by splitting the gas chromatographic effluent and directing the major part over a male antenna (Struble and Arn, 1984; Arn et al., 1975). By means of implanted capillary electrodes on the antenna, the retention times of physiologically active compounds present in the effluent were determined.

Electroantennograms and recordings from single sensilla trichodea were made on excised antennae of males according to the method described by Kaissling (1974). Glass tubes containing a strip of filter paper (20 × 7 mm) on to which a known amount of a given test substance had been pipetted as a hexane solution, were used as odor sources. The receptor potentials (DC recording) and the spike pattern (AC recording) evoked by several test compounds were registered (single-cell recording, electrosensillogram, ESG) by "puffing" air over an odor source onto the antenna. Similarly, the EAG amplitudes evoked by a large number of pheromone and pheromone-like chemicals available in our laboratory were measured. Among the test compounds were all those chemicals already known as pyralid sex pheromones (Arn et al., 1986; Tamaki, 1987; Bestmann and Vostrowsky, 1980).

Chemicals. All the chemicals used in this study were from our laboratory collection of pheromones. [For the synthesis of the substances used in this study, see Bestmann and Vostrowsky, (1979).]

Field Tests. The attraction of male moths to different formulations of test substances was investigated in the hills near Ebermannstadt (Bavaria, Germany) in July 1986. The traps used were obtained from Hoechst AG, Frankfurt (Biotrap).

The bait chemicals were dispensed from short pieces of rubber tubing (3 cm × 12 mm OD, 8 mm ID) suspended 3 cm above the sticky bottom (9.5 × 25 cm). Virgin female moths (2 to 3 days old) used to bait traps were caged in a small mesh container and positioned the same way as the chemical baits. The traps were hung randomly, about 1 m above the ground in a line at a right angle to the predominant wind direction.

RESULTS AND DISCUSSION

Analysis of Gland Volatiles. Under our laboratory conditions *M. cribrella* showed maximum calling activity ca. 6–7 hr after the onset of the scotophase. During the calling period, which lasts for about 30 min, the abdomen of the insect curves upwards and the ovipositor is extruded, thus exposing the intersegmental glandular membrane between the segments VIII and IX (Figure 1). This type of calling behavior is typical for most members of Phycitinae (Krasnoff et al., 1983).

Different parts of the abdominal tip of 3-day-old female moths were excised

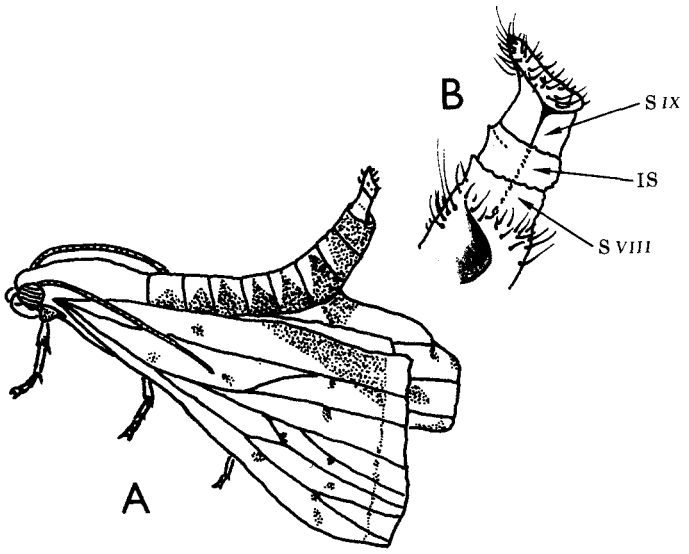

FIG. 1. (A) Female *M. cribrella* showing the characteristic calling behavior. (B) Detailed illustration of the abdominal tip. SVIII, 8th abdominal segment; SIX, 9th segment; IS, intersegmental membrane.

during the calling period and analyzed by GC via a solid-sample injection technique (Attygalle et al., 1986). Pheromone-like substances were found only in the intersegmental membrane between the segments VIII and IX (Figure 2A). For example, the membrane between the segments VII and VIII contained only hydrocarbons as volatile constituents (Figure 2B). A comparison of the two chromatograms, as shown in Figure 2, facilitated the identification of the hydrocarbon peaks in the upper profile.

The identification of the chromatographic peaks was achieved by comparison of retention times with those of authentic samples. The polar stationary phase SP-2340 used in this investigation is able to resolve most of the positional and geometrical isomers of unsaturated compounds found as lepidopteran pheromones (Heath and Tumlinson, 1984). Further confirmation was provided by combined gas chromatography–mass spectrometry. Figure 3 shows a reconstructed gas chromatogram (RGC) and several mass chromatograms obtained by subjecting female glands to GC-MS analysis. The amount of material from five glands was sufficient to obtain complete EI mass spectra of pheromone-related compounds. The mass spectra were compared by the method of Horiike and Hirano (1982) with those from a series of candidate compounds to establish the correct identity.

Acetates usually give a characteristic ion at m/z 61 due to protonated acetic

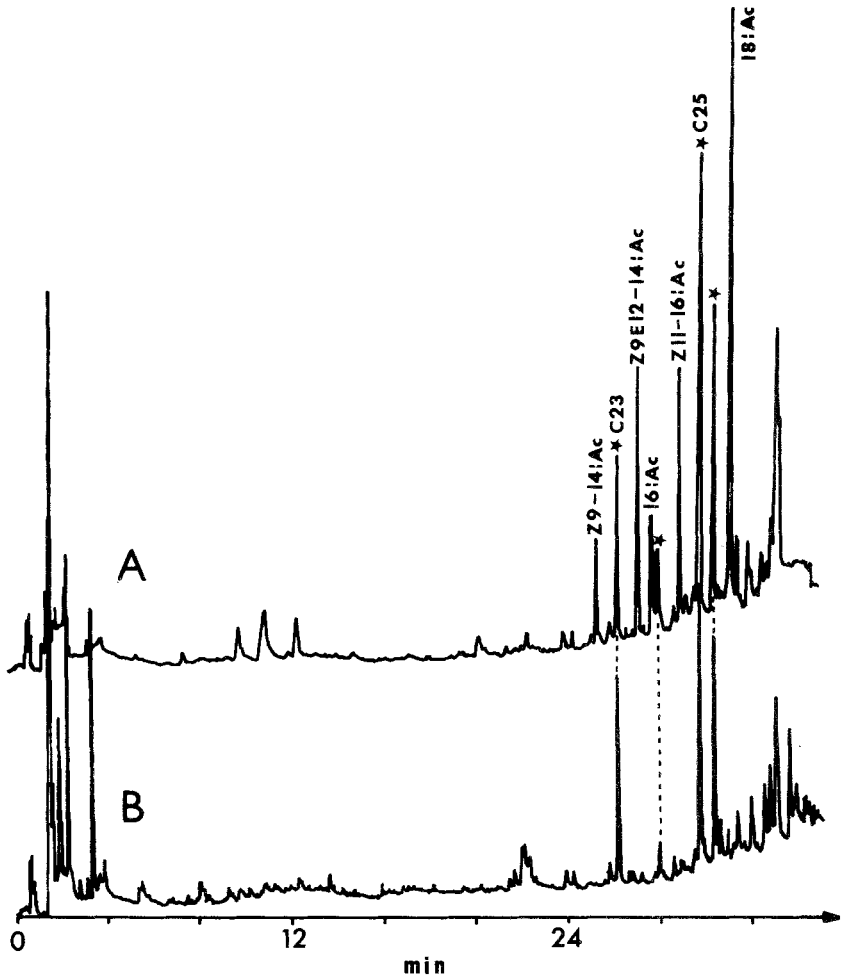

FIG. 2. Gas chromatograms obtained from the volatiles of the intersegmental membranes (A) between the abdominal segments VIII and IX, and (B) between the segments VII and VIII. The samples were introduced via a solid-sampling technique onto a 25 m × 0.22 mm FSCC coated with SP-2340. The oven temperature was held at 60°C for 2 min and programmed to 195°C at 4°C/min. The hydrocarbon peaks are marked with a star. See Table 1 for abbreviations.

acid ($CH_3COOH_2^+$). This ion was used to recognize the acetate peaks in the RGC. The mass chromatogram for *m/z* 61 has eight maxima indicating peaks 2, 5, 7, 8, 13, 17, 21, and 24 were acetates. This evidence in combination with a systematic search for the corresponding M^+-CH_3COOH ions, indicated the

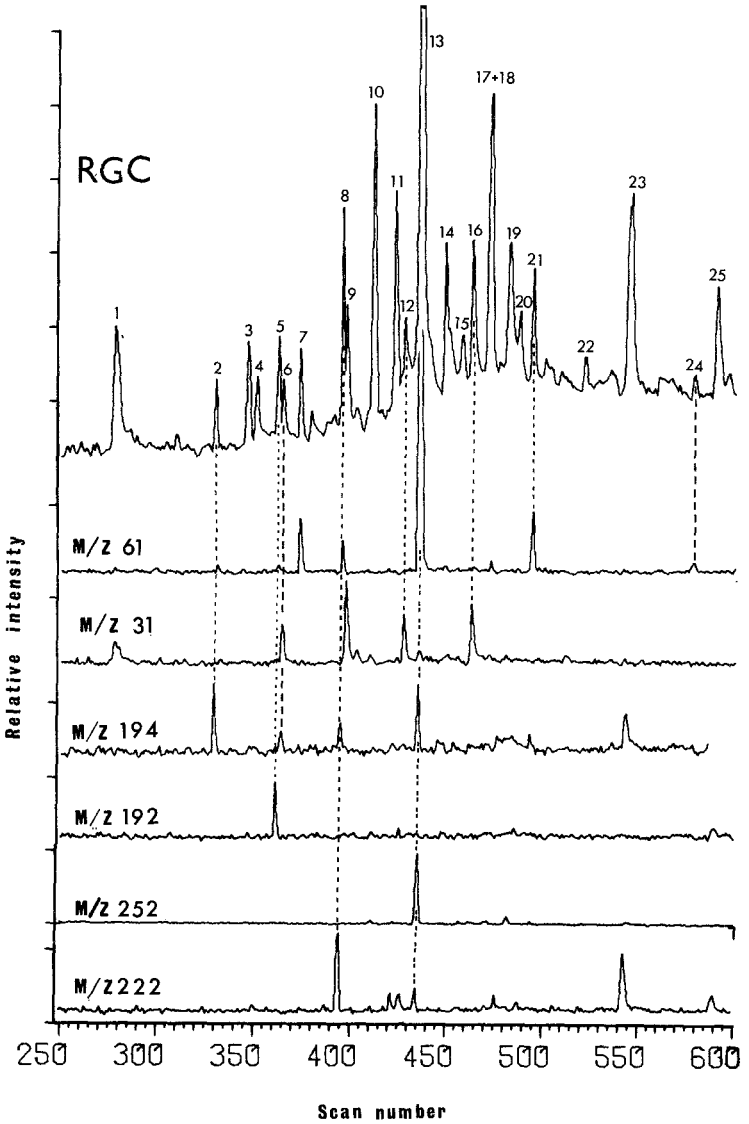

FIG. 3. A reconstructed gas chromatogram (RGC, uppermost profile) and six mass chromatograms for *m/z* 61 ($CH_3COOH_2^+$), *m/z* 31 (CH_2OH^+), *m/z* 194 ($C_{14}H_{26}^+$), *m/z* 192 ($C_{14}H_{24}^+$), *m/z* 252 ($C_{18}H_{36}^+$) and *m/z* 222 ($C_{16}H_{30}^+$), obtained from GC-MS analysis of five glands of female M. cribrella. Peak assignments refer to Table 1.

TABLE I. VOLATILE COMPOUNDS IDENTIFIED IN SEX PHEROMONE GLANDS OF *M. cribrella* FEMALES

Peak No.[a]	Compound	Abbreviation	Rel. amount (18:Ac = 100)[b]
1	Acetamide		
2	(*Z*)-9-Tetradecenyl acetate	*Z*9-14:Ac	10
3	Tricosane	C23	
4	Impurity[c]		
5	(9*Z*,12*E*)-9,12-Tetradecadienyl acetate	*Z*9,*E*12-14:Ac	26
6	(*Z*)-9-Tetradecenol	*Z*9-14:OH	2
7	Hexadecyl acetate	16:Ac	17
8	(*Z*)-11-Hexadecenyl acetate	*Z*11-16:Ac	12
9	(9*Z*,12*E*)-9,12-Tetradecadienol	*Z*9,*E*12-14:OH	2
10	Pentacosane	C25	
11	Branched hydrocarbon		
12	(*Z*)-11-Hexadecenol	*Z*11-16:OH	2
13	Octadecyl acetate	18:Ac	100
14	Unidentified		
15	Unidentified		
16	Octadecanol	18:OH	5
17	Octadecadienyl acetate		
18	Hydrocarbon		
19	Branched hydrocarbon		
20	Branched hydrocarbon		
21	Eicosyl acetate	20:Ac	5
22	Unidentified		
23	Hydrocarbon		
24	Docosyl acetate	22:Ac	2
25	Hydrocarbon		

[a] Numbers refer to peaks of Figure 3.
[b] Only pheromone-like compounds considered.
[c] System peak, appears in blank determinations.

presence of one isomer each of tetradecenyl, tetradecadienyl, hexadecyl, hexadecenyl, octadecyl, octadecadienyl, eicosyl, and docosyl acetates. Similarly the ion *m/z* 31 was used in conjunction with that due to M^+-H_2O to identify the primary alcohol peaks.

The volatile compounds identified from the gland are summarized in Table 1. Components were quantified by using a solution of *Z*11-16:Ac as an external standard. The total content of acetates and alcohols in the gland was 77 ng/female, of which octadecyl acetate, the major component, represented 42 ng.

Electrophysiological Studies. Although about 11 pheromone-like compounds are present in the gland, only three EAG signals were obtained when a

gland was subjected to GC-EAD. These three EAG signals appeared at retention times corresponding to those from Z9-14:Ac, Z9,E12-14:Ac, and Z11-16:Ac.

Of a number of mono- and diunsaturated acetates, alcohols, and aldehydes, known as phycitine sex pheromone components, Z9,E12-14:Ac evoked the highest amplitude when EAG recordings were performed. This tetradecadienyl acetate isomer is the main component of the pheromone complexes of *Cadra cautella*, *C. figulella*, *Ephestia elutella*, *E. kuehniella*, *Hypsipyla robusta*, and *Plodia interpunctuella* (all Phycitinae, see reviewing literature, Arn et al., 1986; Tamaki, 1987).

By single-cell recordings (ESG), two sensory cells specifically responding to Z9,E12-14:Ac and Z9-14:Ac were found in the sensory hairs. Cells with large spike amplitudes were activated by Z9,E12-14:Ac, and cells with small spike amplitudes by Z9-14:Ac.

Field Trials. Based on analytical and electrophysiological results, a series of baits were prepared and tested in the field for attractancy. The results are summarized in Table 2. The traps baited with a blend of Z9-14:Ac (250 μg),

TABLE 2. EFFECTS OF DIFFERENT RATIOS OF SYNTHETIC PHEROMONE AND PHEROMONE-LIKE SUBSTANCES ON FIELD CAPTURES OF MALE *M. cribrella*

	Attractant blends (μg)					Number of males captured per trap	
Trap No.	Z9-14:Ac	Z9,E12-14:Ac	Z11-16:Ac	18:Ac	Z13-18:Ac	July 2-3, 1986	July 5, 1986
1	1000						0
2		1000					0
3	300	700					0
4		700	300				0
5	500		300				0
6	250	500	250				12[a]
7	250	500	250			19[a]	
8	250	500	250			31[a]	
9	200	400	400			0	
10	250	250	500			0	
11	150	300	300	200	100	0	
12	200	200	200	200	200	0	
13	3 Virgin Females						12
14	Blank					0	
15	Blank						0

[a]The traps 6, 7, and 8 captured four, five, and nine males of *Isotrias hybridana*, respectively. The field trials were carried out in the hills near Ebermannstadt, Oberfranken, Bavaria.

*Z*9,*E*12–14 : Ac (500 μg), and *Z*11–16 : Ac (250 μg) were successful in capturing numbers of males similar to those baited with virgin females. Trap 8 captured 31 males of *M. cribrella* in two nights. Although some traps baited with this formulation were even superior to those baited with virgin females, we must not forget that the female moths were calling males only for a short period of time during the night, whereas the synthetic baits were attractive throughout the day and night. Furthermore, the microenvironment of a trap is known to play an important role; however, in practice it is impossible to provide the same microenvironment to all the traps. It was interesting to find that baits composed of one or two components only, and those with *Z*9–14 : Ac, *Z*9,*E*12–14 : Ac, and *Z*11–16 : Ac in 1 : 2 : 2 and 1 : 1 : 2 ratios did not trap any males. By examining the genitalia of the trapped moths, it was found that exclusively males of *M. cribrella* had been caught in traps 6, 7, and 8. In addition, traps 6, 7, and 8 also captured a few males of a tortricid, *Isotrias hybridana.* For this species, it is already known that a 1 : 1 mix of *Z*11–16 : Ac and *Z*9–14 : Ac make an attractive formulation (Szöcs et al., 1983). However, no *Isotrias* males were found in the trap (No. 13) with virgin females of *M. cribrella.*

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NEST PLUNDERING ALLOMONES OF THE FIRE BEE *Trigona (Oxytrigona) mellicolor*[1,2]

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Abstract—Ten volatile compounds derived from the cephalic glands of the fire bee *Trigona (Oxytrigona) mellicolor* were bioassayed for possible allomonal activities facilitating nest plundering. Two diketones, (*E*)-3-heptene-2,5-dione and (*E*)-3-nonene-2,5-dione, caused the honeybee *Apis mellifera* to display avoidance behavior and reduced defensive behavior. These diketones are produced in relatively large quantities in fire-bee cephalic glands.

Key Words—Fire bee, *Trigona (Oxtrigona) mellicolor*, *Trigona (Oxtrigona) tataira*, honeybee, *Apis mellifera*, Hymenoptera, Apidae, mandibular gland secretion, allomone, nest plundering, diketones, (*E*)-3-heptene-2,5-dione, (*E*)-3-nonene-2,5-dione.

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INTRODUCTION

The exocrine secretions of stingless bees in the genus *Trigona* possess varied functions in the chemical ecology of these apids. *T.* (*Oxytrigona*) *mellicolor* produces a remarkable mandibular gland secretion (Kerr and Costa Cruz, 1961) which causes blistering lesions of up to 2 cm in diameter, over four times the length of the bee which inflicted it, when applied to the skin of humans (Wille, 1961; T.E. Rinderer, field observation). The pain caused by such lesions, in combination with the reddish-orange color of *T. mellicolor*, explains why the bee is frequently called the fire bee.

The fire bee is also remarkable for its ability to remove honey from the nests of honeybees (*Apis mellifera*) imported from both the palearctic and Ethiopian realms (Moure, 1946; T.E. Rinderer, field observation). During nest plundering, the fire bee produces a cephalic secretion which has a strong but, to humans, pleasant floral odor (Moure, 1946; Bian et al., 1984). Yet, during a nest-plundering episode, honeybees do not defend their nest but remain motionless on the comb, hang in a cluster of bees outside the entrance of the colony, or appear to "wander" in a seemingly disorientated manner over the surface of the comb. Cephalic extracts contain a variety of compounds that we were interested in evaluating as potential allomones that might facilitate nest plundering. This report describes the results of a honeybee bioassay to determine the allomonal activities of the components of fire-bee cephalic secretions identified by Bian et al. (1984).

METHODS AND MATERIALS

Assay for Primary Effects. Thirteen observation-hive colonies were prepared from established field colonies in Baton Rouge, Louisiana. These colonies were derived from sister queens of general European commercial stock that were permitted to open-mate from the same apiary. Each observation-hive colony contained the original queen, about 0.25 kg (2500) of her adult worker bee daughters, one brood comb (16 × 45 cm) containing the queen's worker brood (about half sealed and half unsealed) and one comb (16 × 45 cm) half-filled with honey and pollen. The observation hives were then left undisturbed for four days to allow them to return to normal organization and regulation (egg laying, colony defense, foraging, etc.).

Ten chemicals [(*E*)-3-nonene-2,5-dione, or (*E*)-3-n, (*E*)-3-heptene-2,5-dione, or (*E*)-3-h, 3-hepten-2-one, dodecyl acetate, 2-decanone, tetradecyl acetate, hexadecyl acetate, pentadecane, 2-heptanone, and tetradecane] found in fire-bee cephalic gland secretions and synthesized commercially or in our laboratories (Bian et al., 1984) were prepared separately as 1:500 dilutions by

weight in paraffin oil. Additionally, a mixture containing equal proportions of each by weight was prepared as a 1:500 dilution by weight in paraffin oil. In the bioassay, the diluted compounds were presented to the observation-hive colonies as 0.5 ml of solution in 7 × 11-mm "sleeve-type" rubber septa. Two control treatments were incorporated into the design: rubber septa with no added materials and rubber septa containing 0.5 ml of paraffin oil.

The effects of the 13 treatments (exposure to 10 compounds, a mixture of the 10 chemicals, and two controls) were tested in a randomized Latin-square design with the 13 colonies. The tests were conducted on 13 consecutive days, each rain-free and with similar temperatures (ca. 30°C).

For each test, a rubber septum was held in a firm steel wire loop, filled with 0.5 ml of the appropriate solution, and inserted from the top of the hive to the center area of one side of the brood comb. Prior to the test, this side of the observation hive was fitted with a standard grid to facilitate later measurements. After 2 min, the septum was withdrawn and the number of stings implanted in it was counted. The entire procedure was recorded on video tape for later evaluation. The maximum diameter (measured through the center of the top surface of the septum) of the area surrounding the septum that had no bees at the end of the 2 min was measured using the grid image to standardize measurements. Ten seconds after the septum was in place, the reaction of the first bee to approach the vial was observed and recorded. Such reactions were observed for a total of 10 bees identified at 10-sec intervals. Reactions included, from the least defensive to the most defensive: (1) rapidly withdrawing from the area, (2) slowly withdrawing from the area, (3) remaining in the area, (4) investigating the septum, (5) "buzzing" wings in response to the septum or its contents, and (6) attacking the septum either by biting or stinging it.

Bee responses were converted to a composite defensive scale by summing the numerical values (1 through 6) of the responses of the 10 bees and then rank-transforming the 169 sums (Conover and Iman, 1981).

These scores, the numbers of stings, and the diameters of areas without bees surrounding the septa at the end of the tests, were submitted to analyses of variance according to the Latin-square design for the main factors of chemicals, days, and colonies. Videotaping errors resulted in a few missing cells for defensive behavior and diameter of area. These missing cells were estimated using the method of Steel and Torrie (1980). The error degrees of freedom were reduced appropriately to reflect the number of observed values. Mean separation evaluations were based on Duncan's multiple-range tests.

Additional information was obtained by ranking the responses to the independent variables for each dependent variable. These ranks were then analyzed with a Friedman two-way analysis of variance in order to test the null hypothesis that the independent variables had generally similar effects for the three dependent variables in the analysis (Siegel, 1956).

Assay for Synergistic Effects. Seven additional observation-hive colonies were prepared and tested in a 7 × 7 Latin-square design by the procedures described for the first experiment. Based on the results of the first experiment, seven chemicals and mixtures were used; (1) (*E*)-3-n, (2) (*E*)-3-h, (3) 3-hepten-2-one, (4) a mixture of 1 and 2, (5) a mixture of 1 and 3, (6) a mixture of 2 and 3, and (7) a mixture of 1, 2, and 3.

RESULTS

Primary Effects. Significant and consistent trends were revealed by the analysis (Table 1). Two of the dependent variables, the number of stings in the septum and the intensity of the defensive responses, differed significantly among

TABLE 1. HONEYBEE RESPONSES TO FIRE BEE CEPHALIC COMPOUNDS[a]

Treatment	*N*	Diameter (cm) of comb area cleared of bees	Number of stings	Defensive scale
(*E*)-3-Nonene-2,5-dione	13	5.83 ± 0.22a	1.03 ± 0.66a	69.3 ± 11.7ab
(*E*)-3-Heptene-2,5-dione	13	5.62 ± 0.22a	1.07 ± 0.86ab	55.5 ± 9.8a
Mix of all chemicals	13	4.41 ± 0.14b	1.20 ± 0.76abc	58.3 ± 13.0a
3-Hepten-2-one	13	4.20 ± 0.15bc	1.61 ± 0.86cd	105.2 ± 13.3bc
Dodecyl acetate	13	4.15 ± 0.15bc	1.28 ± 0.81abc	71.7 ± 11.9abc
2-Decanone	13	4.11 ± 0.15bc	1.24 ± 0.61abc	78.0 ± 13.2abc
Tetradecyl acetate	13	3.99 ± 0.15bcd	1.54 ± 1.16cd	91.9 ± 14.7abc
Hexadecyl acetate	13	3.92 ± 0.10bcd	1.49 ± 0.76bcd	89.6 ± 16.7abc
Pentadecane	13	3.89 ± 0.10cd	1.56 ± 0.76cd	102.3 ± 12.4bc
2-Heptanone	13	3.78 ± 0.08cd	1.63 ± 0.86cd	90.3 ± 13.3abc
Paraffin oil control	13	3.77 ± 0.07cd	1.87 ± 1.32d	108.1 ± 15.1c
Septum control	13	3.59 ± 0.07d	1.34 ± 0.96abc	83.2 ± 10.6abc
Tetradecane	13	3.57 ± 0.07d	1.63 ± 0.97cd	101.6 ± 14.3bc

Analyses of Variance

Source of Variation	*df*	*F*	*P*	*F*	*P*	*F*	*P*
Chemicals	12	20.63	0.0001	3.19	0.0005	2.45	0.006
Days	12	1.03	0.4230	7.36	0.0001	3.23	0.0004
Colonies	12	3.07	0.0005	4.14	0.0001	3.96	0.0001
Sampling	132 (128)[b]						

[a] Average diameter of an area without honey bees surrounding an empty septum, a septum containing paraffin oil, a septum containing paraffin oil with each of ten chemicals, or a septum containing paraffin oil and all 10 chemicals; average number of stings delivered to the septa containing the treatments; average indexes of graded defensive responses (explained in text). For each dependent variable, values are significantly different if not followed by the same letter as determined by Duncan's multiple-range tests. Each set of statistical values from the analysis of variance in the lower part of the table is for the column above.

[b] Sampling df for number of stings and (diameter and defensive scale).

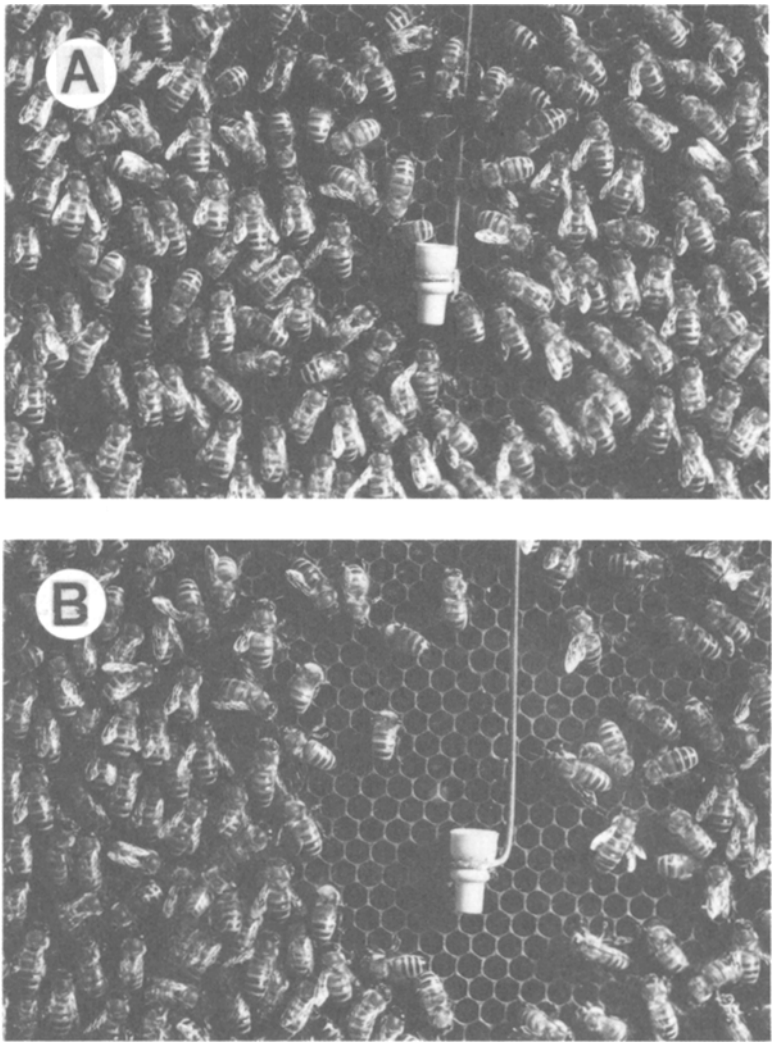

FIG. 1. (A) Area surrounding rubber septum containing paraffin oil vacated by bees. (B) Area surrounding rubber septum containing (*E*)-3-nonen-2,5-dione vacated by bees. Both A and B are responses after 2 min.

treatments, days, and colonies. The diameter of the area vacated by bees differed among treatments and colonies.

Analysis of the differences among treatments by Duncan's multiple-range tests identified strong allomonal activities by (*E*)-3-n and (*E*)-3-h. These two compounds cleared an area with a significantly larger diameter than was cleared by the other treatments (Figure 1). Also, of all the remaining treatments, the

mixture of compounds (which contained these two, but at one tenth the concentration) was most similar to them. Additionally, the septa containing these two compounds received the fewest numbers of stings, and the septa containing the mix of compounds received the next fewest. These three treatments also provoked the least intense defensive responses as measured by the defensive scale.

The Friedman two-way analysis of ranks confirmed the consistent effects of the two unsaturated diketones and the mixture containing them ($X_r^2 = 152.12$, $df = 12$, $p = 0.001$). (*E*)-3-n and (*E*)-3-h and the mixture of compounds had combined ranks of 6, 6, and 7, respectively. The next closest grouping was hexadecyl acetate and pentadecane with combined ranks of 13 and 14. The remaining treatments had combined ranks of 22–35.

Synergistic Effects. No significant or even suggestive differences were found to support the hypothesis that synergistic effects are elicited by mixtures of these compounds.

DISCUSSION

Generally, the chief honeybee response to the diketones was negative chemotaxis. Reductions in stinging and defensive behavior most probably stem from this negative chemotactic response rather than a second specific response. The reactive nature of the diketones (Michael addition) suggest that they may function as irritants, especially to sensitive antennal chemoreceptors.

The diketones also may have an important role in the defensive secretion of *T. mellicolor*. When added to the formic acid in this secretion (Roubik et al., 1987), the diketones may increase its ability to produce burning sensations and blistering either by increasing its solubility in epidermal lipids or facilitating nucleophilic additions by protonation of the carbonyl groups.

The possible roles of the diketones in both nest robbing and colony defense suggest that the existence of one behavioral potency made possible the evolution of the other. *T. mellicolor* has often been observed robbing the nests of *Apis mellifera* but not the nests of sympatric neotropical bees (Roubik et al., 1987). This simply may be a consequence of *A. mellifera* nests being more easily observed. However, *A. mellifera* lacks the elaborate entrance structure typical of neotropical stingless bee nests. Perhaps the importation of *A. mellifera* to the Americas provided a novel opportunity for the development of *T. mellicolor* nest plundering of comparatively unprotected nests. The possibility exists that nest plundering is a simple extension of other foraging-related behavior. Perhaps *T. mellicolor* has expanded the use of its nest-defense secretions to repel competitors from rich floral patches. If so, the use of the secretions in plundering the nests of recently introduced honeybees would be a further expansion of an already existing behavior.

Because the two diketones are so effective at repelling honeybees, they may have desirable commercial uses. Formulations may be developed to remove bees from hive chambers prior to honey harvesting or to reduce the incidence of persons being stung while handling bees.

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SOME COMMUNICATORY FUNCTIONS OF SCENT MARKING IN THE COTTON-TOP TAMARIN (*Saguinus oedipus oedipus*)

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Abstract—The ability of cotton-top tamarins to discriminate between scents from conspecifics and those from other tamarin species, and between scents from conspecific individuals was tested. Cotton-tops scent mark with specialized skin glands in the circumgenital area. Females possess larger glands than males and show more scent-marking behavior. In the first experiment, subjects were presented with a glass rod scented with either material collected from the surface of the scent glands of a conspecific female, with scent material from a female of a related species, or with an unscented rod. Glass rods carrying scent from conspecifics were sniffed more frequently than rods carrying scent from related tamarin species or unscented rods. A second experiment offered a choice between two glass rods, one scent marked by a *Saguinus o. oedipus* female, the second one scent marked by a *Saguinus fuscicollis* female. Shelves carrying rods that had been scent marked by conspecifics were contacted more frequently than those carrying rods marked by heterospecific females. Scent marks from conspecific females were also sniffed more frequently. A third experiment compared the level of responses to rods carrying material collected from the scent glands of female individuals to which the subjects had been habituated with their responses to rods carrying scent from females to which they had not been habituated. Contacting and sniffing responses to the scents of novel females were higher than those to the scents of females to which the subjects had been habituated.

Key Words—Primates, callitrichids, scent marking, chemical communication, chemical signals, odor discrimination, cotton-top tamarin, *Saguinus oedipus oedipus*.

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INTRODUCTION

In the South American callitrichid monkeys, chemical signals appear to play an important role in social communication. The small body size (180–800 g body weight) and the highly arboreal way of life (Hershkovitz, 1977) may have favored the use of chemical communication in these marmosets and tamarins. All species possess specialized scent glands and show a variety of scent-marking behaviors whose complexity rivals that seen in prosimians (Epple et al., 1986; Schilling, 1979). Scent-marking behaviors and their social contexts have been studied in a number of callitrichid species. These studies, recently reviewed by Epple et al. (1986), indicate that marking behavior and the signals deposited in this manner play important roles in a number of sexual and social interactions, among them pair bonding, infant–caregiver relationships, intergroup spacing, and intragroup dominance interactions.

Some of our own work has concentrated on the signal content and the chemical composition of the scent marks of the saddle-back tamarin (*Saguinus fuscicollis*).[1] We have shown that the scent marks of this species communicate the identity of species, subspecies, gender, and individual. Information on the endocrine condition and dominance status of males and on the age of the scent material also appears to be encoded in the marks (Belcher et al, 1986; Epple et al., 1979). The major volatile constituents of the scent marks, squalene and 15 esters of *n*-butyric acid, may be involved in encoding some of the messages listed above (Smith et al., 1985), although they are not the sole carriers of the communicatory information (Belcher et al., 1986).

The present paper reports on the first in a series of studies on the communicatory content and the chemical composition of the scent marks of another callitrichid monkey, the cotton-top tamarin, *S. o. oedipus*. The goal of these studies is a comparison of the behavioral and chemical aspects of scent communication in two closely related species. This may result in the detection of phylogenetic principles of encoding communicatory signals by means of chemical compounds in this primate family.

The morphologic appearance of the circumgenital scent gland of *S. o. oedipus* is similar to that of *S. fuscicollis*. The gland involves the labia majora in the female, the scrotum in the male, and extends into the suprapubic area as a pair of pubic cushions divided by a slight median sulcus (Perkins, 1969). It is deeply pigmented, slightly raised, and relatively hairless. According to our own observations, the perineal area also appears to be part of the glandular complex (Epple, unpublished). In the cotton-top tamarin, the gland is sexually dimorphic, being much larger in the female (Perkins, 1969). As in *S. fuscicollis* (Perkins,

[1]The genus *Saguinus* will be abbreviated (*S.*) in the remainder of the paper.

1966), it consists of an accumulation of holocrine and apocrine glands which are more numerous and more complex in the female (Perkins, 1969).

Cotton-top tamarins show two distinct patterns of scent marking as described by French and Snowdon (1981). Anogenital marking is performed in a sitting position ("sit rubbing," Moynihan, 1970) and results in the application of glandular secretion, urine, and perhaps genital discharge and fecal residues (French and Snowdon, 1981). Suprapubic marking ("pull rubbing," Moynihan, 1970) results in the application of skin secretions and other material adhering to the suprapubic part of the glands as the animals, assuming a sprawling position, rub this portion of the gland across the substrate. Both scent-marking patterns are much more frequenctly performed by females than by males (French and Snowdon, 1981; Wolters, 1978).

In the course of the present study, we tested the ability of the monkeys to discriminate among scents from closely related tamarin species and between scents from conspecific individuals. The pronounced sex differences in the structure of the scent glands and in the scent-marking behavior make it difficult to obtain scent material from males. Therefore, this study has used female cotton-top tamarins as scent donors.

METHODS AND MATERIALS

Housing and Maintenance. All cotton-top tamarins used as subjects lived as permanently cohabiting male–female pairs. Females of other tamarin species, which were used as donors of scent material, lived either in pairs or as breeding females in small families.

The animals were housed in stainless-steel mesh cages. The cages varied in size between 150 × 80 × 130 cm and 300 × 80 × 130 cm, depending on the number of animals in the group. The cage floors were located 85 cm above the floor of the rooms. All refuse fell out of reach of the monkeys and was collected on paper. All cages consisted of several interconnected compartments, each measuring 50 × 80 × 130 cm. They were equipped with wooden sleeping boxes, natural branches, and wooden resting shelves. All compartments were available to the animals most of the time. By inserting a sliding door made from stainless-steel mesh, it was possible to divide each cage into subunits, and temporarily separate an animal from its group mates for testing or scent collection without causing alarm or stress responses.

Each pair of cotton-top tamarins shared a small colony room with one or two groups of *S. fuscicollis*, but none of the colony rooms contained more than one pair of *S. o. oedipus*. The monkeys had no visual contact with tamarins belonging to other groups. However, even conspecific groups housed in different colony rooms exchanged calls, and the animals could probably smell other

monkeys in the colony. The animal rooms were maintained at a temperature of 26°C and 50% relative humidity. The rooms were lighted by means of fluorescent light for 12 hr a day. Infrared bulbs suspended over a shelf in the cage provided dim light during the night and an area of warmth which the animals prefer during periods of resting.

The monkeys received a mixed diet consisting of fruits, vegetables, small amounts of cooked meat, dairy products, monkey chow, and several types of cereal, supplemented with vitamins and minerals.

Subjects and Donors. Seven pairs of cotton-top tamarins were available. One of the males did not participate in the study because of his chronic bad health. With the exception of two females, all of the subjects participated in all three experiments. One female did not participate in experiment 1, the second female did not participate in experiments 2 and 3. Five of the *S. o. oedipus* female subjects, eight *S. fuscicollis* females, and three *S. l. labiatus* females served as donors of scent. None of the *S. o. oedipus* females was used as a scent donor for tests in which she or her male served as a subject.

Stimulus Materials and Scent Collection. The ability of the cotton-top tamarins to discriminate among different tamarin species and between conspecific individuals on the basis of scent was tested in three experiments. Natural scent marks and material collected from the surface of the circumgenital scent gland were offered as stimuli. Statistically significant differences in the responses of the tamarins to different scent types were interpreted as proof that the animals could discriminate among the stimuli.

Natural scent marks and material from the surface of the gland were collected on frosted glass rods. These rods were made by closing one end of a frosted glass tube (10 x 1.5 cm) to form a round smooth top. Between uses, the glass rods were cleaned in a chromic–sulfuric acid solution, rinsed with distilled water, followed by methanol and methylene chloride, and air-dried.

The glass rods could be inserted into holes drilled into heavy wooden shelves, and in this way mimicked small protruding twigs. The animals prefer to scent mark small protruberances and could be trained to mark these "glass twigs." After an extended period of training, all natural scent marks could be collected within a few minutes and used for testing immediately. To collect scent marks, clean glass rods were inserted into the holes of the wooden shelves and were removed immediately after the donor animals had scent marked them. The material collected in this way from cotton-top tamarins consisted almost exclusively of anogenital marks, since suprapubic marking was extremely rare. *S. fuscicollis* often combines anogenital and suprapubic marking, and some of the scent material collected from this species resulted from combined marking patterns (Epple et al., 1986).

At the start of the project, i.e., before the tamarins were trained to donate natural scent marks, material adhering to the surface of the gland was used as

stimulus scent. To collect this material, all donors were trained to allow their scent gland to be rubbed with the glass rod while receiving a reward. For this purpose, the clean glass rod was gently wiped over the whole surface of the gland. Each collection was completed within a few minutes. The material collected in this manner probably consisted of a mixture of glandular secretions, urinary residues, genital discharge, and environmental contaminants.

Scent Discrimination Tests. All subjects were housed and tested in cages consisting of five compartments. Three of the compartments were equipped for testing. In addition to natural branches, level wooden shelves ($7 \times 7 \times 80$ cm), which served as sample carriers, were suspended between the back and the front of the cages at a height of 65 cm above the cage floor. Each shelf was equipped with a hole into which a glass rod containing the scent sample could be inserted.

In experiments 1 and 3, only a single sample was presented to the animals. Therefore, only one sample carrier was present in the center of the testing area. In experiment 2, choice tests offering two samples were conducted. For these tests, the right and left compartments of each testing area contained one sample carrier while the center compartment did not contain a carrier. The wooden sample carriers were part of the permanent cage equipment. The cages and their equipment were washed in a cage washer at least once a month using hot water but no detergent.

The animals were allowed to use all five compartments when no tests were performed. During each test, the subject was confined to the three testing compartments, while its mate remained in the other part of the home cage. The duration of each test was 10 min for experiments 1 and 2 and 15 min for experiment 3. The total testing time was divided into intervals of 5 sec, indicated by an audible signal.

Three behavioral responses were recorded. (1) Contacting the wooden sample carrier: any physical contact with the surface of the shelf carrying the glass rod containing the sample; (2) sniffing the glass rod containing the sample: bringing the nostrils within 2 cm of the glass rod; most sniffs are performed with the muzzle actually contacting the scented tip of the rod, and usually an accelerated rate of inhalation can be observed; (3) scent marking the glass rod: anogenital marking of the glass rod in a sitting position; suprapubic marking was very rare and is not included in this category. The subjects received a score of one for each of these behaviors if they displayed the pattern during a given 5-sec interval, regardless of the frequency of display.

All animals were tested repeatedly in the course of each experiment (see below). The right–left position of the scent stimuli was counterbalanced across tests and across subjects. The order of testing of the subjects was determined at random for every experiment. The observer recorded the behavioral responses on check sheets while sitting in full view of the animals, approximately 2 m from the cage. In analyzing the results of each experiment, mean scores for

contacting, sniffing, and scent marking were computed for each subject. These data were analyzed by two-way (donor by sex) analysis of variance (experiment 1) and paired *t*-tests (experiments 1, 2, and 3). A significance level of $P \leq 0.05$ was accepted. Each animal was tested only once a day. All testing and all collection of stimulus material were done between 8 and 11 AM and 1 and 4 PM.

Experiment 1. The ability of cotton-top tamarins to discriminate between material from the surface of the circumgenital scent gland of conspecific and heterospecific females was tested. Each of the subjects was given 18 tests. During each test, a single glass rod which was either unscented (blank) or scented with glandular material from an adult female, was inserted into the sample carrier located in the center of the testing area. The following samples were presented in random order: (1) an unscented glass rod (blank), (2) a glass rod scented with material from the circumgenital gland of an adult female *S. o. oedipus*, (3) a glass rod scented with material from the corresponding gland of an adult female *S. fuscicollis*, and (4) a glass rod scented with the material from the corresponding gland of an adult female *S. l. labiatus* (it should be noted that *S. l. labiatus* lacks the pronounced suprapubic glandular ridges present in the other two species).

All subjects were familiar with the scent marks of *S. fuscicollis*. In addition to living in close proximity to this species, they had previously undergone training tests during which scent marks from conspecifics and from *S. fuscicollis* were offered on wood. *S. l. labiatus* scent was known to the subjects only as far as living in the same colony familiarized them with the odor of this species.

Each subject received three tests which presented scents from three different conspecific female donors, three tests each with scent from three different female donors of *S. fuscicollis* and *S. l. labiatus*, and nine tests with blanks. The order of stimulus presentation was determined at random. All tests lasted 10 min.

Experiment 2. The ability of the tamarins to discriminate between natural scent marks of adult *S. o. oedipus* and *S. fuscicollis* females was investigated. Each subject was given four choice tests. During each test, a glass rod that had been scent marked by a conspecific female and a glass rod that had been scent marked by a female *S. fuscicollis* were offered simultaneously. Two *S. o. oedipus* and two *S. fuscicollis* females served as donors for each subject. Scent marks from each pair of donors were presented twice. All tests lasted 10 min.

Experiment 3. The ability of the tamarins to discriminate between conspecific female individuals was tested. A habituation paradigm was used. It is based on the assumption that the animals will habituate to a scent stimulus which is presented several times in close succession, showing a decrease in investigatory responses. Investigatory responses should increase again when a novel stimulus is presented after the animals have habituated to the original one. The design

of this experiment required the presentation of up to three different fresh scent samples from each donor individual. Since donor females did not readily scent mark several glass rods in close succession, material collected from the surface of the gland was used as stimulus scent.

Each subject was given four tests. The tests consisted of three 5-min presentations of a single glass rod scented with a fresh sample of material from an adult *S. o. oedipus* female. A pause of 1 min separated the presentations. In two of the four tests, three different samples of scent material collected within a short period of time from the same individual were presented. In the two remaining tests, the first and the second glass rod carried samples from the same individual, while the third glass rod was scented with material from another female. Two adult females, A and B, served as donors for each subject. Each donor female was used for one test in which she donated all three samples (AAA, BBB), for one test in which she donated only the first and second sample, and for one test in which she donated only the third sample, (BBA, AAB). The order of the four tests on each subject was determined at random.

In analyzing the results of experiment 3, mean individual contacting, sniffing, and scent-marking responses to the third sample were compared between tests in which the third sample was from the same donor as the first and second sample and tests in which it was from a novel donor.

RESULTS

Figures 1–4 show the mean scores obtained in experiments 1–3 by males and females for contacting the sample carriers and for sniffing and scent marking the stimulus rods. These scores represent the mean number of 5-sec intervals in which the behavioral responses were displayed. The results of experiments 1 and 2 show that cotton-top tamarins distinguish the scent of conspecifics from that of other tamarin species, both on the basis of material obtained from the surface of the scent glands and on the basis of natural anogenital scent marks.

Two-way analysis of variance (donor by sex) showed that in experiment 1, in which stimuli were presented one at a time, the material obtained from the surface of the scent glands of the three species elicited significantly different levels of sniffing ($P = 0.03$). Paired comparisons by means of t test further showed that as a group, male and female subjects sniffed glass rods carrying scent material from *S. o. oedipus* females significantly more frequently than glass rods carrying *S. fuscicollis* ($P = 0.03$) or *S. l. labiatus* scent ($P = 0.01$) or blank rods ($P = 0.05$). There also were significant sex differences in contacting the sample carrier, in sniffing, and in scent marking. Male subjects contacted the sample carrier ($P = 0.01$) and sniffed the samples ($P = 0.003$) more

frequently than females, but females showed much more scent marking of the glass rod, regardless of whether it carried a sample or not ($P = 0.003$) (Figure 1).

When natural scent marks from *S. o. oedipus* and *S. fuscicollis* were presented simultaneously in experiment 2, male and female subjects contacted the shelves carrying rods marked by conspecific females significantly more frequently than those carrying rods marked by *S. fuscicollis* (male: $P < 0.01$; female: $P = 0.01$). Moreover, males ($P = 0.03$) and females ($P < 0.01$) sniffed glass rods with *S. o. oedipus* scent significantly more frequently than rods with *S. fuscicollis* scent. As in experiment 1, females showed significantly more scent marking than males ($P = 0.01$) but sex differences in the other responses were not observed (Figure 2).

The results of the habituation tests in experiment 3 are depicted in Figures 3 and 4. They show that the tamarins can discriminate between scent material from conspecific female individuals on the basis of material adhering to the surface of the scent gland. Figure 3 shows that the average number of intervals spent contacting the sample carrier and sniffing the sample by males and females as a group gradually decreased when the animals were offered three successive samples of material from the scent gland of the same individual. However, when the third scent sample was from a different individual than the first and second sample, sniffing and contacting scores obtained during the third sample presentation were higher than those obtained during the second sample presen-

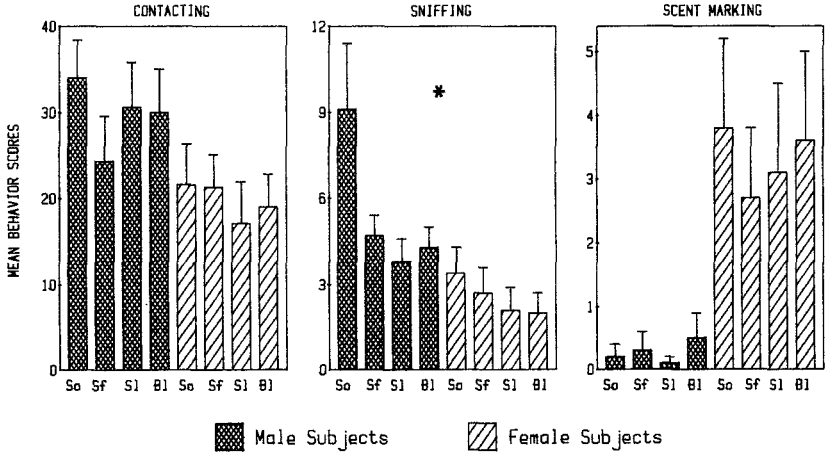

FIG. 1. Experiment 1: mean contacting, sniffing, and scent-marking scores ± SEM obtained by male and female subjects in response to single glass rods carrying gland material from *S. o. oedipus* (S.o.), *S. fuscicollis* (S.f.), or *S. l. labiatus* (S.l.) and to blanks (Bl). *Significant difference (ANOVA); P levels in text.

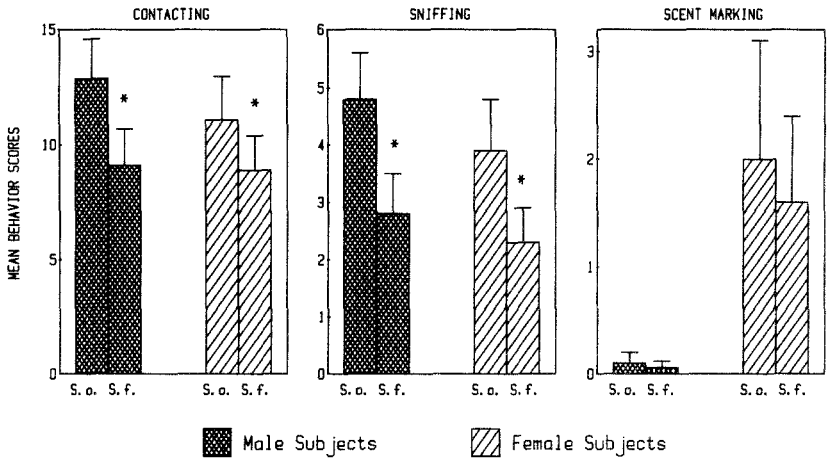

FIG. 2. Experiment 2: mean contacting, sniffing, and scent-marking scores ± SEM obtained by male and female subjects in tests offering a choice between glass rods carrying scent marks from *S. o. oedipus* (S.o.) and *S. fuscicollis* (S.f.). *Significant difference (*t* test); *P* levels in text.

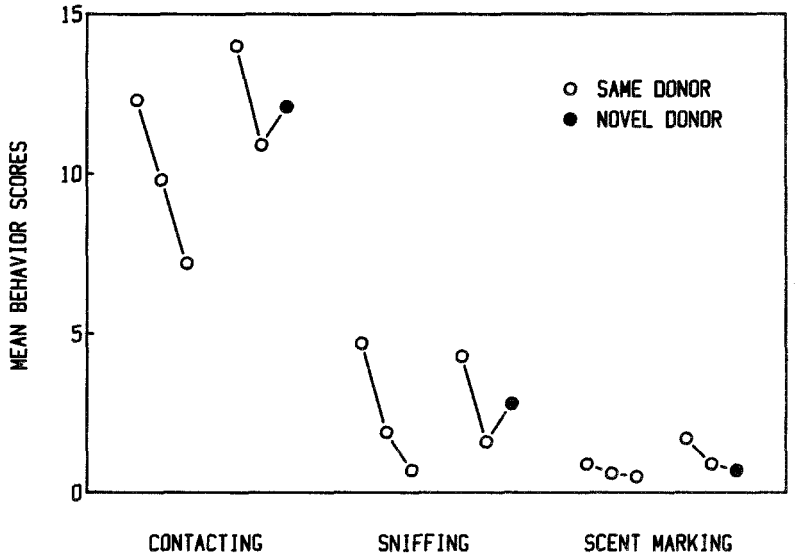

FIG. 3. Experiment 3: habituation curves showing mean contacting, sniffing, and scent-marking scores of male and female subjects as a group during three successive presentations of scent samples from female conspecifics. ○-○-○, the three glass rods carried scent from the same donor; ○-○-●, the first and second rods carried scent from the same donor; the third rod carried scent from a novel donor.

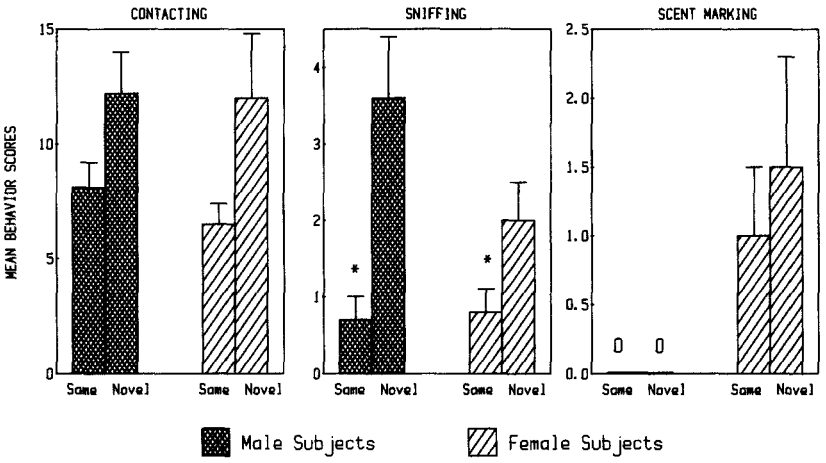

FIG. 4. Experiment 3: mean contacting, sniffing, and scent-marking scores ± SEM obtained by male and female subjects during the third of three successive presentations of scent samples from female conspecifics. Scores obtained during tests offering three successive samples from the same donor individual are compared with scores obtained during tests offering two samples from the same individual followed by a sample from a novel individual. *Significant differences (t test); P levels in text.

tation. In contrast to contacting and sniffing responses, there was little habituation and dishabituation of the scent-marking response.

Figure 4 presents the scores for contacting and sniffing obtained by males and females during the third sample presentation in experiment 3. Both sexes showed more sniffing of the sample during tests in which the scent donor was an individual to which the subjects had not been habituated during the first and second test (male: $P = 0.01$; female: $P = 0.02$). Males and females as subgroups did not discriminate between samples from the two donor individuals in terms of contacting the carriers. However, when the contacting scores of all subjects were analyzed as one group, contacting was significantly more frequent in tests offering the scent from an individual to which the subjects had not been habituated ($P = 0.01$). There were no sex differences in contacting and sniffing, but females showed significantly more scent marking than males ($P = 0.01$).

DISCUSSION

Studies on saddle-back tamarins (*S. fuscicollis*) have shown that this species can discriminate among a relatively large number of scent types. Conspecifics and other callitrichid species, subspecies of *S. fuscicollis*, males and

females, castrated and intact males, dominant and submissive males, and conspecific individuals are distinguished from each other on the basis of scent marks alone (Epple et al., 1979, 1986). The results reported in the present paper indicate that the scent of cotton-top tamarins communicates information similar to that contained in the scent marks of saddle-back tamarins. The natural scent marks and the material adhering to the scent glands of *S. o. oedipus* contain cues which facilitate species recognition. Material adhering to the surface of the glands also contains cues on which individual recognition can be based. Because of the difficulty in collecting a sufficient number of scent marks, we were not able to investigate the tamarins' ability to discriminate between individuals on the basis of their natural scent marks. However, it would not be surprising if the complex scent marks also serve to identify individuals, as was found to be the case in *S. fuscicollis* (Epple et al., 1979, 1986).

It would be interesting to establish whether natural scent marks and glandular material also contain cues for gender discrimination. In *S. fuscicollis*, gender discrimination is one of the most reliable responses to scent marks (Epple, 1974). Since male and female cotton-tops both possess scent glands, one would expect that they produce sexually dimorphic signals. However, because of the relatively small glands and the low marking activity of males, the stimulus material required for gender discrimination studies is difficult to obtain. Nevertheless, we recently have begun such studies and will report on them in due course.

Our studies show that chemical cues relating to species and individuality are present on the gland surface of an animal. These body scents may be important during interactions among individuals at close range. Chemical cues of similar signal content are also deposited in the environment, where they can influence the behavior of a recipient in the absence of the animal who deposited the scent mark. The chemical constituents of a scent mark deposited by a donor monkey may be somewhat different from those of the material adhering to the glandular surface. At present, we do not know whether both scents are equally attractive to the tamarins and whether similar communicatory signals in glandular material and in scent marks are based on similar chemical composition. It would be misleading to interpret the obvious differences in the intensities with which the subjects contacted, sniffed and scent marked the stimuli in experiments 1, 2, and 3 as an indication that glandular secretion differed from scent marks in attractiveness and/or chemical composition. Although most subjects participated in all experiments, the methods used in experiments 1–3 differed from each other in duration and in the number of scent stimuli offered simultaneously. Therefore the mean behavior scores obtained in the three experiments cannot be compared directly.

The present study confirms findings on pronounced sex differences in scent marking. French and Snowdon (1981), French and Cleveland (1984), French

et al. (1984), and Wolters (1978) found that females maintaining the breeding position in their groups showed much higher levels of anogenital and suprapubic marking than males of comparative social status. In our test situations, females also showed significantly more anogenital marking than males, while males showed more sniffing of female scents and more contacting of the sample carrier than females, at least in experiment 1. This result can be interpreted as supporting the suggestion of French and Snowdon (1981) that anogenital marking in female cotton-tops is primarily a means of communicating sexually relevant information. These authors observed an increase in anogenital marking by breeding females and in male sniffing of female genitalia and female scent marks around the time of suspected estrus (as dated from the timing of parturition). Suprapubic marking, a behavior rarely observed in the present study, has been found to be associated mainly with aggressive arousal of females, and French and Snowdon (1981) suggested that one of its functions is the promotion of intergroup spacing. From these observations, it appears possible that anogenital and suprapubic scent marks communicate somewhat different information (French and Snowdon, 1981).

Depending on the social context in which basic chemical messages relating to species, gender, and individual recognition are perceived, they can influence a wide variety of different sexual and social interactions (Epple, 1986; Epple et al., 1986). We expect that our continuing studies will provide additional information on the communicatory content and the chemical composition of the scent marks of *S. o. oedipus*. This work, together with our concurrent studies on *S. fuscicollis*, will lead to a broader understanding of chemical communication in primates.

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TETRAPONERINES, TOXIC ALKALOIDS IN THE VENOM OF THE NEO-GUINEAN PSEUDOMYRMECINE ANT *Tetraponera* sp.[1]

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Abstract—The Neo-Guinean pseudomyrmecine ant, *Tetraponera* sp. utilizes its modified sting to smear upon enemies a contact poison with strong deterring and toxic properties. The venom, which originates from the poison gland, contains a mixture of eight closely related, alkaloids (tetraponerine-1 to -8). The structure of tetraponerine-8 was established by an X-ray diffraction analysis and was reported previously. The structure of five other members of the series has now been determined by comparison of their spectral properties with those of tetraponerine-8 and of model compounds.

Key Words—*Tetraponera*, ant, alkaloids, poison gland, contact poison, tetraponerine, Hymenoptera, Formicidae, Pseudomyrmecinae.

INTRODUCTION

The Neo-Guinean pseudomyrmecine ant *Tetraponera* sp. is characterized by a defensive mechanism which is unique both in the morphology of the sting apparatus and in the structure of the venom alkaloids (Braekman et al., 1986). The modified sting is not a suitable injection device but is well adapted to deposit a liquid on a surface. GC analysis of the venom showed that it contains eight closely related derivatives named tetraponerine-1 to -8 (referred to here as T1 to T8). The structure of T8, one of the major constituents of the venom, has been determined as **1** by an X-ray diffraction analysis (Braekman et al., 1986). T8 is the first member of a new family of alkaloids based on the deca-

[1] King Leopold III Biological Station, Laing Island, Papua, New Guinea, Contribution No. 136.

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hydro-pyrido-(1,2-c) pyrrolo-(1′,2′-a) pyrimidine skeleton. In this paper, we report on the isolation and structure determination of five other alkaloids (T3 to T7) from *Tetraponera* sp. venom, which are derived from the same tricyclic ring system.

METHODS AND MATERIALS

Ants. Workers of *Tetraponera* sp. were collected near Bogia, along the north coast of Papua, New Guinea, and immediately placed in vials containing methanol. The venom of 250 individual workers was collected on bits of filter paper by "milking" the ants and was stored in hexane.

Chemical Analysis. Gas chromatographic analyses were performed on a Varian model 3700 equipped with a 25-m OV-1 capillary column, isothermally at 170°C. Gas chromatography–mass spectrometry was performed at 170°C on a Finnigan 2500 GC, coupled to a Finnigan 3000 D mass spectrometer.

Preparative gas chromatographic separations were realized on a Hewlett-Packard model 402 equipped with a 1.80-m column packed with 2% SE-30 on Chromosorb W, at 150°C. Thin-layer chromatographic (TLC) analyses were realized on Merck Al_2O_3 plates (eluent: hexane–AcOEt 8:2) or on SIL G Macherey Nagel plates (eluent: hexane–acetone–NH_4OH 25% 8:2:0.5). The plates were visualized by spraying with Dragendorff reagent. Silica gel column chromatographies were performed either by the "flash" technique (Still et al., 1978) (eluent: hexane–acetone–NH_4OH 25% 8:2:0.5) or on Merck Lobar columns (eluent: $CHCl_3$–EtOH 9:1).

Infrared spectra were obtained from neat liquid films with either a Unicam SP1000 or a Perkin-Elmer 237 infrared spectrophotometer. [^{1}H]- and [^{13}C]NMR spectra were obtained at 250 and 62.8 MHz respectively, using a Bruker WM 250 spectrometer. All spectra were recorded in C_6D_6, chemical shifts (δ) being expressed in parts per million (ppm) from internal TMS. High-resolution MS (HRMS) was performed on a Micromass 7070 F mass spectrometer.

Extraction of Ants and Purification of T3 to T7. TLC analyses of the hexane extract of the venom revealed the presence of a single orange spot on visualization (Al_2O_3) with Dragendorff reagent. Filtration of the hexane extract on alumina and evaporation of the solvent afforded 16 mg of alkaloidal mixture.

To obtain larger amounts of the venom constituents, several batches of *Tetraponera* workers were ground in CH_3OH and extracted three times with CH_2Cl_2. Typically, combined extracts of 1250 workers were evaporated under reduced pressure; the residue (161 mg) was redissolved into 15 ml of CH_2Cl_2 and extracted five times with 5 ml of 5% aqueous HCl. The acid layer was basified with NH_4OH (pH 10) and extracted with 5 × 5 ml of CH_2Cl_2. Drying and evaporation of the solvent afforded 70 mg of crude alkaloidal mixture. Capillary GC analysis of this mixture showed that it contained the same alkaloids as the venom. A series of silica gel column chromatographies (flash and Lobar),

followed by preparative GC afforded T3 (2 mg), T4 (1.1 mg), T5 (3 mg), T6 (3 mg), T7 (3 mg), and T8 (6 mg). The purity of these compounds was checked by capillary GC and TLC.

RESULTS

Capillary GC analyses of the *Tetraponera* sp. venom revealed the presence of eight components: T1–T8, numbered in order of increasing retention time. The relative proportions, given in Figure 1, were found to change slightly from

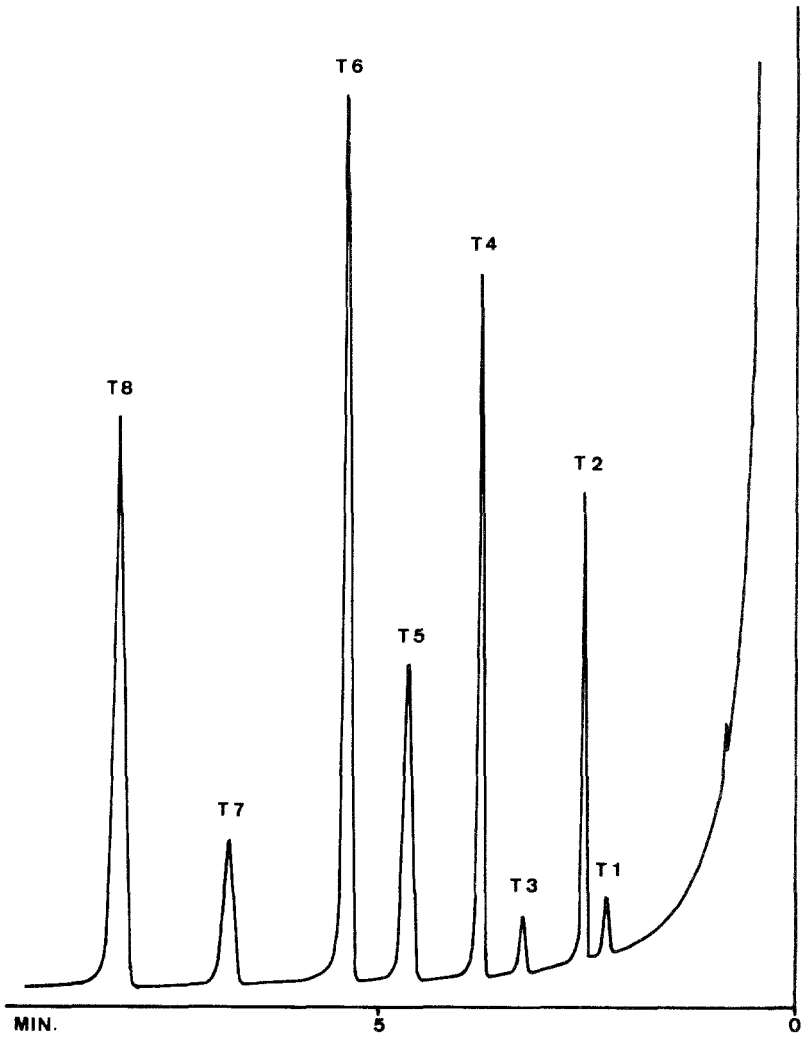
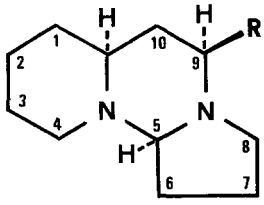
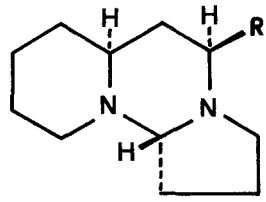


FIG. 1. Chromatogram of the crude secretion of *Tetraponera* sp. (OV-1, 170°C).

one collection to the other. Careful dissection of the Dufour and poison glands followed by TLC analyses of the hexane extract showed that these compounds are specific to the poison gland. GC-MS analysis of the mixture shows that these derivatives may be distributed into four pairs of isomers. The mass spectra of matched isomers are almost superimposable, suggesting that they are diastereomers. HRMS measurements established the empirical formula of three of the four pairs and indicated that each pair differs from the next one by a CH_2 unit. Moreover, a plot of the logarithm of the corrected retention times of the alkaloids versus the number of carbon atoms (Kovats, 1961) suggests that the eight compounds may be distributed into two series of homologs, one even (T2, T4, T6, and T8) and one odd (T1, T3, T5, and T7).

Six of these compounds could be isolated in small amounts by a combination of chromatographic methods. The structure of T8 has been established to be **1** by X-ray diffraction analysis (relative configuration 5S*, 9R*, 11S*) (Braekman et al., 1986). The structures of T3–T7 were deduced from a careful analysis of their spectral properties and will be discussed below.





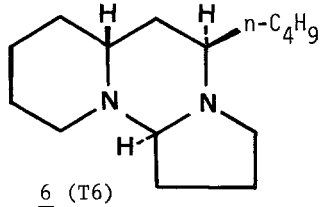
1 (T8) R= n-C_5H_{11}

2 (T4) R= n-C_3H_7

3 (T3) R= n-C_3H_7

4 (T5) R= n-C_4H_9

5 (T7) R= n-C_5H_{11}



6 (T6)

Structure of T4 (2). A comparison of the MS (Table 1), [^{1}H]NMR (Table 2), and [^{13}C]NMR spectra of T4 [oil; $KI^{OV\text{-}1}_{173°}$ = 1663; $C_{14}H_{26}N_2$ by HRMS (measured 222.208, calculated 222.210); [^{13}C]NMR: 85.5 (d, C-5), 62.7 (d, C-11*), 61.1 (d, C-9*), 51.5 (t, C-4$^+$), 48.9 (t, C-8$^+$), 37.9 (t, C-12°), 36.9 (t, C-10°), 33.0 (t, C-1), 29.7 (t, C-6), 26.2 (t, C-3), 25.1 (t, C-2), 20.3 (t, C-7), 18.7 (t, C-13), 14.8 (q, C-14)] and T8 [mp 40°C; $KI^{OV\text{-}1}_{173°}$ = 1853;

TABLE 1. COMPARISON OF MASS SPECTRA OF COMPOUNDS T3 TO T8 (in % of the base peak)

	Percent of base peak					
m/z	T8	T7	T6	T5	T4	T3
250	$M^{+\cdot}$: 51	$M^{+\cdot}$: 60				
249	97	99				
236	x[a]	x	$M^{+\cdot}$: 49	$M^{+\cdot}$: 50		
235	x	10	100	100		
222	x	x	x	x	$M^{+\cdot}$: 53	$M^{+\cdot}$: 44
221	x	10	10	x	100	62
207	22	33	10	x	11	7
194	16	21	10	10	16	16
193	100	100	29	22	91	100
180	33	40	13	16	21	16
179	12	18	70	98	16	24
166	10	13	32	25	x	x
165	11	12	13	8	6	x
152	55	69	24	18	57	54
151	17	23	16	7	17	14
138	13	20	57	33	21	34
137	9	13	18	15	11	15
123	6	9	8	15	7	17
110	13	23	20	22	15	x
97	11	20	18	12	10	16
96	47	94	67	86	44	65
84	24	37	13	10	27	36
70	15	23	62	35	17	18
68	10	16	23	13	10	14
55	18	33	31	13	18	26
43	9	34	32	10	9	34
41	19	46	63	33	27	42

[a]x, intensity less than 5% of the base peak.

$C_{16}H_{30}N_2$ by HRMS (measured 250.239, calculated 250.241); [^{13}C]NMR: 85.4 (d, C-5), 62.6 (d, C-9*), 61.6 (d, C-11*), 51.3 (t, C-4), 49.7 (t, C-8), 37.6 (t, C-10), 34.2 (t, C-1), 32.6 (t, C-12$^+$) 32.2 (t, C-14$^+$), 29.3 (t, C-6), 25.8 (t, C-3), 24.9 (t, C-13°), 24.7 (t, C-2°), 22.9 (t, C-15), 19.9 (t, C-7), 14.2 (t, C-16)] shows that both compounds possess the same tricyclic ring system with identical configuration at C-5, C-9, and C-11 and thus differ only by the presence of an *n*-propyl side chain in T4 in place of the *n*-pentyl side chain of T8. This conclusion is based on the following arguments. The relative intensities of the Bohlmann bands at 2785–2700 cm^{-1} in the IR spectra of T4 and T8 are

TABLE 2. [^{1}H]NMR SPECTRA OF COMPOUNDS T3 TO T8[a]

Attribution	T3 (3)	T4 (2)	T5 (4)	T6 (6)	T7 (5)	T8 (1)[b]
CH_3	0.94,t,7	0.89,t,7	0.93,t,7	0.91,t,7	0.92,t,7	0.90,t,7
HC-4 (eq)	2.7 − 2.9,m	2.82,ddd,11,2,2	2.7 − 2.9,m	2.94,ddd,8,8,2.5	2.7 − 2.9,m	2.82,ddd,11,2,2
HC-5	3.28,dd,4,3	2.29,dd,8,6	3.50,dd,4,3	2.88,dd,5,5	3.33,dd,4,3	2.32,dd,8,6
HC-8 (ax)	2.7 − 2.9,m	2.01,ddd,9,9,9	2.7 − 2.9,m	2.34,ddd,9,9,5	2.7 − 2.9,m	2.05,ddd,9,9,9
HC-8 (eq)	3.16,ddd,7,7,7	3.12,ddd,9,9,2	3.22,ddd,7,7,7	3.06,ddd,9,9,4	3.17,ddd,7,7,7	3.15,ddd,9,9,2
HC-11	2.7 − 2.9,m	2.09,m	2.7 − 2.9,m	2.43,m	2.7 − 2.9,m	2.12,m

[a]250 MHz, C_6D_6, δ, multiplicity, J(Hz)
[b]400 MHz.

the same, suggesting that the number of C–H bonds α to the nitrogen atoms and *trans* to the nitrogen lone pairs is the same in both compounds (Crabb et al., 1971). The mass spectra of both T4 and T8 show intense fragment ions at *m/z* 193.1714 ($C_{12}H_{21}N_2$), corresponding to side-chain cleavage between C-12 and C-13. Fragment ions arising from the tricyclic portion of the molecules (e.g., *m/z* 152, 96, 84) are found in the spectra of both compounds with practically the same relative intensities. The [^{1}H]NMR spectrum of T4 displays five 1H signals between 2.0 and 3.15 ppm, the chemical shifts and coupling constants of which are nearly identical to those of the corresponding deshielded protons of T8 (Table 2). Finally, the chemical shifts of the carbon atoms of the tricyclic ring system of T4 and of T8 are nearly the same, and those of the carbon atoms of the *n*-propyl chain of T4 are in good agreement with the δ reported for 2-propylpiperidine (Shamma and Lang, 1979). All these arguments point to structure 2 for T4.

Structure of T3 (3), T5 (4) and T7 (5). Table 1 shows that the mass spectra of T7 [oil; $KI^{OV\text{-}1}_{173^\circ} = 1809$; $C_{16}H_{30}N_2$ by HRMS (measured 250.239, calculated 250.241); [^{13}C]NMR: 75.9 (d, C-5), 57.1 (d, C-9), 53.7 (d, C-11), 51.4 (t, C-4*), 51.0 (t, C-8*), 34.6 (t), 32.9 (t), 32.7 (t), 31.3 (t), 30.9 (t), 27.7 (t), 26.9 (t), 25.6 (t), 23.6 (t), 22.6 (t), 14.7 (q, C-16)] and T8 are nearly superimposable, which suggests that these compounds are diastereomers. The IR spectrum of T7 also displays Bohlmann bands, but their relative intensities are lower than those of T8. This observation strongly suggests that the number of H atoms α to the nitrogen atoms and *trans* to the nitrogen lone pairs is smaller in T7 than in T8, implying that the two compounds differ by the configuration at one or more of the three chiral centers: C-5, C-9, or C-11.

On the basis of [^{13}C]- and [^{1}H]NMR arguments, we propose that T7 is the C-5 epimer of T8. In the [^{13}C]NMR spectrum of T7, the three most deshielded signals (δ 75.9, 57.1, and 53.7) attributable to C-5, C-9, and C-11, respectively, experience a strong upfield shift in comparison to the corresponding signals of T8 (Table 3).

The influence of the change from an equatorial to an axial alkyl group on the chemical shift of the quinolizidine carbon atoms was calculated by using 2α- and 2β-methylquinolizidine and 4α- and 4β-methylquinolizidine as model compounds (Tourwe and Van Binst, 1978; Lalonde and Donvito, 1974). The increments thus obtained were utilized to calculate the chemical shifts of the quinolizidine carbon atoms of 5-epi- and 9-epi-T8.

As can be seen from Table 3, there is a good agreement between the values calculated for the quinolizidine carbon atoms of 5-epi-T8 and those observed for the same carbon atoms of T7. These data strongly suggest that T7 and T8 are C-5 epimers and that the quinolizidine ring system is *trans*-fused in both compounds, as indicated by the chemical shifts of C-1 to C-4 (Tourwe and Van Binst, 1978). These conclusions are fully supported by the [^{1}H]NMR spectrum

TABLE 3. COMPARISON OF CALCULATED CHEMICAL SHIFTS FOR QUINOLIZIDINE CARBON ATOMS OF 5-EPI- AND 9-EPI-T8 WITH THOSE OBSERVED FOR T6–T8

	C-1	C-2	C-3	C-4	C-5	C-9	C-10	C-11
9-epi-T8	33.7	24.6	26.3	51.6	79.5	60.2	34.6	57.0
5-epi-T8	34.1	24.7	26.2	52.4	79.8	55.8	37.6	53.7
T8	34.2	24.7	25.8	51.3	85.4	61.6	37.6	62.6
T7	34.6	25.6	26.9	51.4	75.9	57.1	—	53.7
T6	—	—	—	—	83.4	64.2	—	59.7

of T7, which displays five protons in the 2.7–3.4 ppm region, whereas only two protons appear in the corresponding region of the T8 spectrum (Table 2). In T7, the HC-5 (3.33 ppm) is equatorial and *cis* to the lone pairs of both N atoms, which explains its deshielding. Furthermore, HC-4 (eq) and both HC-8, being *cis* to the nitrogen atom lone pairs, appear at about 3.0 ppm. The fifth signal at about 2.8 ppm is attributed to HC-11 which is deshielded (0.5 ppm) by a Van der Waals interaction with H_2C-6 (Bhacca and Williams, 1964).

Consequently, we propose structure **5** for T7, with the relative configuration 5R*, 9R*, 11S*.

Several attempts were made to correlate chemically T7 and T8 by cleavage of the *gem*-diamino function with reducing agents ($LiAlH_4$, $NaBH_4$, DIBAL-H, catalytic hydrogenation). These treatments led either to unchanged starting material or to complex mixtures.

Next, we turn to compound T3 [oil; $KI^{OV\text{-}1}_{173°} = 1627$; $C_{14}H_{26}N_2$ by HRMS (measured 222.208, calculated 222.210); [^{13}C]NMR: 75.5 (d, C-5), 56.7 (d, C-9), 53.0 (d, C-11), 51.0 (t, C-4*), 50.6 (t, C-8*), 34.3 (t), 33.3 (t), 32.2 (t), 30.6 (t), 26.6 (t), 25.2 (t), 22.2 (t), 20.7 (t), 14.5 (q, C-14)] and compound T5 [oil; $KI^{OV\text{-}1}_{173°} = 1713$; $C_{15}H_{28}N_2$ by HRMS (measured 236.223, calculated 236.225); [^{13}C]NMR: 76.5 (d, C-5), 58.3 (d, C-9), 54.1 (d, C-11), 51.0 (t, C-4*), 50.0 (t, C-8*), 33.2 (t), 32.5 (t), 31.6 (t), 30.2 (t), 30.1 (t), 27.4 (t), 23.2 (t), 22.0 (t), 20.4 (t), 14.4 (q, C-15)]. Their mass spectra (Table 1) and their Kovats indexes (KI) strongly suggest that they are the lower homologs of T7, possessing the same tricyclic skeleton as T7 but with an *n*-propyl or *n*-butyl side chain, respectively. The similarity observed between the [^{1}H]- and [^{13}C]NMR spectra of T3, T5, and T7 confirms this assumption. These data also demonstrate that the relative configuration at C-5, and C-11 must be the same in the three compounds.

Thus we propose structure **3** for T3 and **4** for T5.

Structure of T6. The mass spectrum (Table 1) of T6 [oil; $KI^{OV\text{-}1}_{173°} = 1751$; $C_{15}H_{28}N_2$ by HRMS (measured 236.223, calculated 236.225); [^{13}C]NMR: 83.4

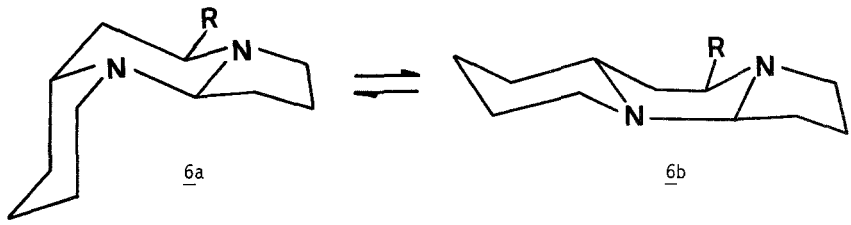


FIG. 2. The two most stable conformers of T6.

(d, C-5), 64.2 (d, C-9), 59.7 (d, C-11), 49.2 (t, C-4*), 45.9 (t, C-8*), 34.8 (t), 33.5 (t), 32.7 (t), 30.7 (t), 29.3 (t), 25.9 (t), 23.1 (t), 21.3 (t), 21.0 (t), 14.3 (q, C-15)] indicates the presence of a tricyclic system substituted at C-9 by an *n*-butyl side chain. The relative intensity of the Bohlmann bands in IR is similar to those observed in the spectra of T4 and T8. These observations, together with the KI of T6 led us to hypothesize that it could be the homolog between T4 and T8. However, its [^{1}H]- and [^{13}C]NMR spectra are clearly different from those of T4 and T8, thus precluding that it could possess the 5S*, 9R*, 11S* configuration. Moreover, since T6 is diastereomeric with T5, its configuration cannot be 5R*, 9R*, 11S*. Examination of Table 3 shows that the chemical shifts of C-5, C-9, and C-11 of T6 are very different from those calculated for 9-epi-T8 (5S*, 9S*, 11S*). It follows that T6 has structure **6**, possessing the 5S*, 9R*, 11R* configuration. Examination of molecular models shows that **6a** and **6b** are the two more stable conformers of T6 (Figure 2).

Conformation **6b**, in which the B ring adopts a boat conformation, is probably the preferred one since it has an overall shape that is very close to that of T4 and T8. This would best explain the behavior of T6 in gas chromatography and the intensities of the Bohlmann bands in IR.

Pure samples of compounds T1 and T2 could not be obtained. Nevertheless, the mass spectrum, obtained by GC-MS, suggests that they have the empirical formula $C_{13}H_{24}N_2$ (M^+ at *m/z* 208), which is compatible with a tetraponerine tricyclic skeleton bearing an ethyl side chain. The relative configuration of these compounds remains undetermined.

DISCUSSION

The presence of alkaloids in ants is well documented (Blum and Hermann, 1978; Jones and Blum, 1983). Several species belonging to the genera *Solenopsis* and *Monomorium* are well-known alkaloid producers. This is, however, the first report of saturated nitrogen heterocycles from the venom of a pseudomyrmecine ant. All other alkaloids of this type have been isolated from the myrmicine genera *Solenopsis*, *Monomorium*, and *Chelaner* (Jones et al., 1986).

Structurally, all ant alkaloids isolated till now were based on the pyrrolidine (Jones et al., 1982), piperidine (MacConnell et al., 1971; Jones et al., 1982), indolizidine (Jones et al., 1980, 1984), or pyrrolizidine (Jones et al., 1986) ring systems. Although based on piperidine and pyrrolidine rings, the tricyclic ring system of the tetraponerines has never been reported before in a natural compound.

The biosynthetic origin of ant alkaloids is still controversial. The presence of the same structural elements in the Myrmicinae alkaloids and in the tetraponerines could reflect the existence of a basic biogenetic scheme common to all these derivatives. However, it should be noted that the *Monomorium* and *Solenopsis* alkaloids, with one exception (Jones et al., 1982), all possess an odd-numbered carbon skeleton. This contrasts with the tetraponerine family where both odd- (T1, T2, T5, T6) and even-numbered (T3, T4, T7, T8) carbon skeletons are found. We plan to address the problem of the tetraponerine biosynthesis in the near future. The total synthesis of the tetraponerines is currently underway in our laboratory.

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PARTIAL INHIBITION OF PHEROMONE PRODUCTION IN *Dendroctonus ponderosae* (COLEOPTERA: SCOLYTIDAE) BY POLYSUBSTRATE MONOOXYGENASE INHIBITORS[1]

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Abstract—Female and male mountain pine beetles, *Dendroctonus ponderosae* Hopkins, were treated topically with piperonyl butoxide or sesame oil, both of which are known to inhibit polysubstrate monooxygenase activity. Beetles then exposed to vapors of the host monoterpenes α-pinene and myrcene were found to contain reduced levels of the pheromones *trans*-verbenol and ipsdienol, as well as a buildup of monoterpene precursors. Polysubstrate monooxygenase enzymes appear to be at least partially responsible for the detoxification of host monoterpenes and for the production of terpene alcohol pheromones in this species.

Key Words—*Dendroctonus ponderosae*, Coleoptera, Scolytidae, pheromones, *trans*-verbenol, ipsdienol, allelochemicals, mixed-function oxidases, monooxygenase inhibitors.

INTRODUCTION

Many plants contain allelochemicals that are toxic to herbivorous insects. In response, these insects have developed several enzyme systems that metabolize plant allelochemicals to compounds that are less toxic and/or more easily excretable (Brattsten, 1977). The polysubstrate monooxygenases (also known

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as mixed-function oxidases) are probably the most important of these enzyme systems. The products of monooxygenase-catalyzed oxidations of allelochemicals generally are not biologically active and often are excreted as glutathione conjugates.

The mountain pine beetle, *Dendroctonus ponderosae* Hopkins, is one example of an herbivorous insect that makes use of the metabolic products of allelochemical oxidation. This bark beetle breeds in the phloem of several *Pinus* species throughout western North America and thus is exposed to a variety of monoterpenes and other toxic compounds. The monoterpenes are converted to corresponding terpene alcohols and related compounds, and these are excreted by the insects. Female *D. ponderosae* are known to convert the monoterpene α-pinene to *trans*-verbenol (Hughes, 1973), an aggregation pheromone for this species (Pitman, 1971). Male *D. ponderosae* convert myrcene to ipsdienol (Hunt et al., 1986), which may act as an antiaggregation pheromone for this species (Hunt and Borden, 1988) (see Figure 1).

Although it has been hypothesized that polysubstrate monooxygenases are involved in the production of bark beetle pheromones (White et al., 1980), experimental evidence is inconclusive. Sturgeon and Robertson (1985) measured polysubstrate monooxygenase activity in midgut tissue from *D. ponderosae*, but did not evaluate the role of these enzymes in terpene metabolism and pheromone production. White et al. (1979) reported that microsomal fractions of *Dendroctonus terebrans* could produce α-pinene oxide, and other unidentified compounds, from α-pinene. White et al. (1979) hypothesized that α-pinene oxide may be the first step in the *in vivo* production of certain terpene alcohol pheromones of *Dendroctonus* species. No corresponding studies have been carried out on the oxidation of myrcene.

We have investigated the role of monooxygenase enzymes in the production of terpene alcohol pheromones in the mountain pine beetle. Our approach has been to use intact insects instead of homogenates and to assess the effects of the polysubstrate monooxygenase inhibitors piperonyl butoxide and sesame oil on the conversion of α-pinene and myrcene to their corresponding terpene alcohol pheromones. Monooxygenase inhibitors are widely used as insecticide synergists and have become a standard means of assessing the involvement of these enzymes in insecticide metabolism (Brattsten, 1979). As such, they lend themselves naturally to studies on allelochemical metabolism as well.

METHODS AND MATERIALS

Logs of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, infested with the mountain pine beetle were obtained from the southwestern interior of

British Columbia. Their cut ends were sealed with hot paraffin wax, and the logs were stored at 4°C. Adult *D. ponderosae* were obtained by placing logs in cages at 27°C, collecting the emergent beetles, and storing them on moistened paper toweling at 2–4°C until required (two weeks maximum). When needed for experiments, beetles were rewarmed to room temperature, and active individuals were selected and their sex determined using the method of Lyon (1958).

Piperonyl butoxide (analytical grade, >95%; McLaughlin, Gormley, King Co., Minneapolis, Minnesota) or sesame oil (Sigma Chemical Co., St. Louis, Missouri) were diluted in acetone and 1 μl of a 20% solution (v/v) was applied to the pronotum of each insect. Control beetles received 1 μl of acetone on the pronotum. Then female beetles were exposed to the vapors from a vial containing 25 μl of distilled α-pinene (>99%; Aldrich Chemical Co., Milwaukee, Wisconsin) in a 500-ml jar (9 cm OD) for 24 hr at 22°C. Male beetles were exposed to myrcene vapors (>99%; Phero Tech Inc., Vancouver, British Columbia) under the same conditions.

A group of control beetles was exposed to monoterpene vapors at the same time and under the same conditions as each group of inhibitor-treated beetles. This ensured that control and treatment groups had identical exposure to monoterpene vapors and further ruled out factors such as beetle age from affecting the comparisons between inhibitor and control treatments.

To assess the effect of acetone treatments, groups of female and male beetles receiving 1 μl of acetone were compared to groups receiving no treatment. All groups were exposed to monoterpene vapors as previously outlined.

In all treatment and control groups, some insects died or appeared sick following monoterpene vapor exposure. All beetles that did not appear to be healthy were excluded from pheromone analysis. Extracting and analyzing only apparently healthy beetles avoided biasing the data, as more sick beetles were present in the inhibitor treatments than in the controls.

Abdomens from individual insects were excised and extracted in double-distilled pentane; each insect represented one replicate of the piperonyl butoxide, sesame oil, or acetone control treatments. Extracts were analyzed for terpene alcohol pheromones and their precursors on a Hewlett Packard 5880A gas chromatograph equipped with a capillary inlet system, flame ionization detector, and glass capillary column (30 m × 0.50 mm ID) coated with SP-1000 (Supelco, Inc., Bellefonte, Pennsylvania). 3-Octanol was added to the pentane used to extract the abdomens and was used as an internal standard. The compounds were identified by comparison of retention times with authentic standards, and selected samples were analyzed using mass spectroscopy to ensure proper identification of compounds.

Differences in the quantities of monoterpenes and pheromones were analyzed statistically using the Mann-Whitney U test, $P > 0.05$.

TABLE 1. PRODUCTION OF *trans*-VERBENOL FROM α-PINENE IN FEMALE *Dendroctonus ponderosae* FOLLOWING TOPICAL APPLICATION WITH MONOOXYGENASE INHIBITORS

Experiment	Treatment	Mortality (24 hr)	Beetles extracted (No.)	Amount in abdomen (ng/beetle)[a]	
				α-Pinene ($\bar{X}$ ± SE)	*trans*-verbenol ($\bar{X}$ ± SE)
1	1 μl 20% sesame oil	2/15	12	1034 ± 188a	294 ± 76a
	1 μl acetone	3/15	12	231 ± 49b	1469 ± 531b
2	1 μl 20%piperonyl butoxide	5/17	11	1044 ± 233a	441 ± 138a
	1 μl acetone	0/15	11	247 ± 74b	914 ± 337a
3	1 μl acetone	1/20	10	223 ± 54a	2218 ± 383a
	Untreated	0/20	10	271 ± 60a	2034 ± 362a

[a] Values within a column for each experiment followed by the same letter are not significantly different, Mann-Whitney U Test, $P > 0.05$.

RESULTS

Female *D. ponderosae* that had been treated with sesame oil prior to α-pinene exposure contained lower levels of *trans*-verbenol than did acetone-treated controls (Table 1, experiment 1). A similar, but not statistically significant, effect occurred after beetles were treated with piperonyl butoxide (Table 1, experiment 2). Females that had been treated with sesame oil or piperonyl butoxide retained significantly higher levels of α-pinene than did acetone-treated controls (Table 1, experiments 1 and 2). Acetone-treated females contained levels of α-pinene and *trans*-verbenol that were not significantly different from those found in untreated beetles (Table 1, experiment 3).

Male *D. ponderosae* that had been treated with piperonyl butoxide, but not sesame oil, prior to myrcene exposure contained lower levels of ipsdienol than did acetone-treated controls (Table 2, experiments 1 and 2). However, males that had been treated with either inhibitor retained significantly higher levels of myrcene than did acetone-treated controls (Table 2, experiments 1 and 2). Acetone-treated males contained levels of myrcene and ipsdienol that were not significantly different from those found in untreated beetles (Table 2, experiment 3).

DISCUSSION

Unlike most bark beetles, which breed in weakened or dead host material, *D. ponderosae* attack vigorous host trees that are capable of a significant defen-

TABLE 2. PRODUCTION OF IPSDIENOL FROM MYRCENE IN MALE *Dendroctonus ponderosae* FOLLOWING TOPICAL APPLICATION WITH MONOOXYGENASE INHIBITORS

Experiment	Treatment	Mortality (24 hr)	Beetles extracted (No.)	Amount in abdomen (ng/beetle)[a]	
				Myrcene ($\bar{X}$ ± SE)	Ipsdienol ($\bar{X}$ ± SE)
1	1 μl 20% sesame oil	5/20	11	320 ± 155a	1655 ± 306a
	1 μl acetone	1/20	11	82 ± 17b	1925 ± 364a
2	1 μl 20% piperonyl butoxide	4/15	10	1463 ± 182a	107 ± 58a
	1 μl acetone	1/14	10	105 ± 23b	1820 ± 218b
3	1 μl acetone	1/20	10	173 ± 41a	1987 ± 305a
	Untreated	1/20	10	151 ± 38a	2104 ± 334a

[a]Values within a column for each experiment followed by the same letter are not significantly different, Mann-Whitney U Test, $P > 0.05$.

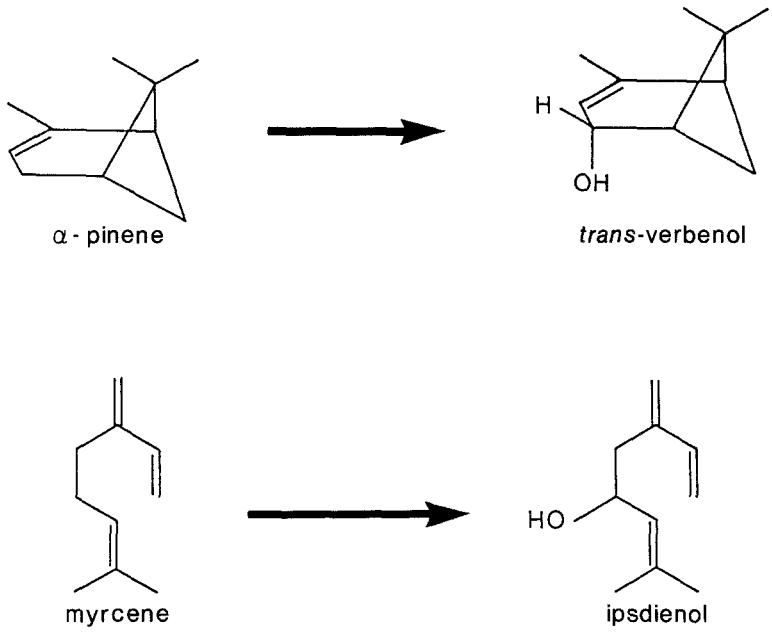

FIG. 1. Oxidation of α-pinene to *trans*-verbenol and myrcene to ipsdienol.

sive reaction. As a result, these insects are exposed to large amounts of resin containing a variety of substances known to be toxic to bark beetles (Smith, 1965; Reid and Gates, 1970; Coyne and Lott, 1976). Evidently, these insects require a rapid and efficient means of dealing with a wide range of toxic host substances.

Another result of *D. ponderosae* attacking healthy, resinous hosts is the need for rapid production of aggregation pheromones. At low attack densities host trees are capable of confining attacking adults within necrotic lesions containing high levels of monoterpenes and other toxic plant allelochemicals. Only through rapid mass attack are these insects able to achieve a density of attacking beetles sufficient to overwhelm the defensive capacity of the host (Raffa and Berryman, 1983).

Our findings that topical applications of piperonyl butoxide or sesame oil cause a decrease in the levels of certain terpene alcohol pheromones, along with a buildup in the levels of their monoterpene precursors, are supportive of the hypothesis that polysubstrate monooxygenases are involved in bark beetle pheromone production. Thus, the mountain pine beetle is able to detoxify host monoterpenes and produce the aggregation pheromone *trans*-verbenol rapidly and simultaneously using the same enzyme system. In doing so, the insects have

adapted to use the metabolic products of detoxification to produce a behavioral response, a strategy that is more energy efficient than producing pheromones *de novo* from precursors not found in the diet.

It is known that monoterpenes such as α-pinene and myrcene are potent and rapid inducers of cytochrome P-450-linked monooxygenases in certain insects (Brattsten et al., 1977). Such induction probably also occurs in *D. ponderosae* and greatly facilitates the rapid detoxification of host monoterpenes and the production of aggregation pheromones.

Polysubstrate monooxygenase inhibitors such as piperonyl butoxide possess some toxicity to insects, and it is possible that the reduction in pheromone levels and concurrent increase in precursors following inhibitor exposure could be due to sublethal toxicity. However, our dosages of piperonyl butoxide and sesame oil caused only low levels of beetle mortality (Tables 1 and 2); thus it appears unlikely that the reduction in terpene alcohol production is caused by general toxicity. In addition, a topical application of an insecticide, diazinon, at a dosage that approximated the LD_{50} for female *D. ponderosae*, did not decrease the production of *trans*-verbenol in females (authors' unpublished data). This is further evidence that the reductions in pheromone production are not due to sublethal toxicity.

There is evidence that microorganisms associated with certain bark beetle species can convert host tree monoterpenes into compounds that their insect hosts utilize as pheromones (Brand et al., 1975; Byers and Wood, 1981). However, there is also evidence that bark beetles devoid of readily culturable microorganisms can produce these terpene alcohol pheromones (Conn et al. 1984). It is possible that these reactions are being catalyzed by monooxygenase enzymes present in the insects, in their symbiotic microorganisms, or both. Further studies using polysubstrate monooxygenase enzymes *in vitro* are needed to address this question.

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MALE CONFUSION OF THE NUN MOTH WITH DISPARLURE AT HIGH AND LOW POPULATION DENSITIES

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Abstract—The feasibility of using the synthetic sex pheromone, disparlure, as a mating confusant of the nun moth (*Lymantria monacha* L.) was investigated on wild populations occurring in outbreak proportions in Poland and in low density in Czechoslovakia. The size of experimental plots ranged from 1 to 12 hectares. The amounts of pheromone applied were 10 or 20 g/hectare. Three types of slow-release formulations of a racemic mixture of disparlure were tested: (1) a spray formulation with latex as a carrier, (2) a tubing of natural rubber, and (3) sawdust of a porous material used for making tips of fiber-tip pens. All three pheromone formulations prevented significant numbers (98.6–100% in the low density situation, 90–96.2% during the outbreak) of the males from locating a discrete source of pheromone (a trap containing synthetic pheromone or a female) during the whole flight period, indicating long-lasting efficiency of the formulations.

Key Words—*Lymantria monacha*, Lepidoptera, Lymantriidae, pheromone communication, mating disruption, disparlure, slow-release formulations.

INTRODUCTION

In Central Europe, the nun moth, *Lymantria monacha* (L.) is one of the most economically important defoliators of coniferous trees, notably spruce and pine. Periodic population outbreaks have produced devastating results, as for exam-

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ple in Czechoslovakia in 1917–1927 (Pfeffer, 1954), when an area of 105,000 hectares was damaged, and more recently in Poland, where some 6.3 million hectares of forested areas had to be chemically treated during 1978 through 1984 (Śliwa and Sierpiński, 1986). Smaller outbreaks have also been reported from Germany (Altenkirch, 1986), Austria (Schmutzenhofer, 1986), and Denmark (Jensen and Nielsen, 1984). In the search for alternative and environmentally more acceptable approaches to the management of this pest, a series of field trials was carried out at the same period in Germany (Altenkirch, 1985a), Austria (Schmutzenhofer, 1986), and in Poland and Czechoslovakia (Vrkoč et al., 1981, the present study) to investigate the feasibility of using the synthetic pheromone, disparlure (Schönherr, 1972), as a mating confusant.

In this paper we report on the experiments done on low-density populations in Czechoslovakia and during an outbreak of the pest in Poland. The objectives of the studies were to obtain information concerning (1) the performance of three different slow-release formulations of disparlure and (2) the ability of the disparlure treatment to disorient and/or confuse wild males that are seeking a discrete source of the sex attractant (a pheromone cap or a calling female).

METHODS AND MATERIALS

Pheromone Formulations. A racemic mixture of *cis*-7,8-epoxy-2-methyloctadecane (disparlure, Zoecon Corp., Palo Alto, California) was formulated into one of three carriers. The first carrier was an aqueous polystyrene–butadiene latex (density 0.9662, pH 10.5, dry weight 19.3%, contents of the bound styrene 24.6%, contents of the free styrene 2.9 g/liter): the desired hectare dose of disparlure (10 or 20 g) was dissolved in a mixture of 20 ml of 75% nonpolar emulsifier and 100 ml of benzene which was then thoroughly emulsified in 5 liters of the latex. In one experiment (Bobrowice, PL, 1984), a commercially available latex paint was used as a carrier. The second was a tubing of natural rubber, mixture No. 3263, CSN 2245.14 (Gumárne SNP n.p., Dolné Vestenice, Czechoslovakia). The length of the individual pieces was 30 cm, diameter 4 mm, wall thickness 0.5 mm. The desired hectare dose (10 g) of disparlure was dissolved in 200 ml of benzene and allowed to embed into 400 g of the tubing (approximately 170 pieces) in a tightly closed vessel for 12 hr, after which time the solvent was permitted to evaporate. The third carrier was a sawdust of the material used for making the tips of fiber pens (Kohinoor-Hardtmuth n.p., 07 Dačice, Czechoslovakia). It consists of curled polyamide fibers impregnated with polymerized epoxy resin, porosity 45%, density 1.1 g/cm^3, mean effective radius of the most frequently occurring pores 2.5 and 50 μm. The desired hectare dose (10 g) of disparlure was dissolved in a mixture of 200 ml of paraffin oil and 5 liters of petrolether, which was applied to 3 kg

of the fiber-tip sawdust in a closed container and allowed to embed into it overnight. The solvent was allowed to evaporate at room temperature.

Experimental Plots and Designs of Experiments. Application of disparlure against scarce populations of the nun moth were performed in Czechoslovakia, where the last outbreaks of the pest occurred in 1965–1968. Since then, the pest has been in a latency phase. In such a situation the catches of the pheromone survey traps are most frequently in the range of 100–500 specimens per plate trap and season. The presence of the pest cannot be detected by other means.

The first experiment was done in Čížová near Milevsko, S. Bohemia (alt. 460–490 m above sea level), where the 56- to 76-year-old stands were composed of 90% spruce and 10% pine. The experiment was done in 1981. Three square plots of one hectare each were delineated for the experiment. The shortest distance between the plots was more than 300 m. Racemic disparlure formulated into the latex was applied to two of them at the doses 10 and 20 g/hectare, respectively, by means of a motor-driven sprayer. The third plot served as an untreated control. In each plot, five traps with 0.1 mg of racemic disparlure were exposed and checked regularly throughout the whole flight period of the moths.

The second Czechoslovak experiment was performed in Dobříš, Central Bohemia (alt. 450–470 m) in 1983. Composition of the stands was 80% spruce, 20% pine; the age of the trees was 60–80 years. Racemic disparlure was formulated into rubber tubing and the porous fiber-tip sawdust. Pieces of tubing were manually stapled on tree trunks (approximately one piece per five trees) in a square area of one hectare, and the fiber-tip sawdust was spread manually over another one-hectare area 700 m away. Five traps with 20 μg of (+)-disparlure were exposed on each treated plot, and the same number of traps was placed on an untreated one-hectare control plot 800 m away. Trap catches were checked regularly throughout the whole flight period.

The third experiment was performed again in Dobříš at a nearby location (alt. 550–570 m) in 1984. Composition of the stands was 90% spruce and 10% pine, age of the trees was 65–70 years. Two plots (300 $\times$ 400 m) 2 km apart were delimited for the experiment. One of the plots was treated manually with the fiber-tip pen formulation of racemic disparlure (10 g/hectare), the other was left as an untreated control. Twelve traps with 20 μg of (+)-disparlure were evenly distributed over both areas together with four traps that were each provided with a virgin female. The females originated from pupae collected in Chocianow, Poland, where the nun moth occurred in outbreak proportions that season. The caged females were first exposed on August 7 and replaced by newly emerged ones on August 10 and 14. The virgin females of the last batch were left in traps till September 5. No freshly eclosed females were available later during the season. The catches were recorded until the end of the flight season.

Two other experiments were performed in Poland, where the most devastating European outbreak of the nun moth ever recorded occurred in 1978–1984. The first experiment was carried out in Karsznica near Ustka (alt. 50–60 m above sea level) in 1982. The experiment was performed during the eruptive phase of an outbreak that culminated in 1982 and 1983. An average of 47 females per tree trunk ($N = 6$) was found that season. Four one-hectare plots (100 × 100 m), 400 m apart, were delineated from a spruce (80%) and pine (20%) stand whose trees ranged in age from 70 to 85 years. In two of the plots, the latex formulation of racemic disparlure was applied by hand sprayer in parallel strips 10 m apart. Ten grams per hectare of synthetic pheromone was disseminated in plot A, while 20 g/hectare was applied to plot B. The remaining two plots were not treated and served as controls. Five traps baited with 0.1 mg of racemic disparlure and four traps baited with a virgin female were exposed on each plot. The females were exchanged at every check, i.e., once in three or four days.

The second Polish experiment was done in Bobrowice near Zielona Gora during the first year of population decline in 1984. An average of 0.1 female and 116 caterpillars were found on sample trees. Two plots 300 × 300 m each, 1.5 km apart, were delineated from a 100% pine forest whose trees were 70–95 years old, at altitudes of 80–100 m above sea level. On one of the plots, 10 g/hectare of racemic disparlure was applied with a manual sprayer. A hectare dose of the pheromone was dissolved in 100 ml of benzene and emulsified in 1.5 liters of the latex paint, to which 3.5 liters of water and 20 ml of a detergent were added. Sixteen pheromone traps with 0.1 mg of racemic disparlure were evenly distributed on each of the plots and checked regularly during the whole flight season.

Methods of Evaluation of Male Confusion Effects. The effect of disorientation and/or confusion of the males (E_d) was calculated from the decrease in catches of pheromone traps on treated plots as compared with those on untreated ones. In some cases, live virgin females also were used as a lure. The E_d was calculated from the formula $E_d = [(K - A)/K] \times 100$, where K = the males caught at the control plot, and A = the males caught at the treated plot. Two different traps were employed. In Czechoslovakia, tin plates, 50 × 50 cm, provided with the adhesive Chemstop I (Chemika, Bratislava, Czechoslovakia) on both sides, were hung on tree trunks 1.5 m above the ground. The pheromone dispenser or a small cage with the female was placed in the middle of the plate (Skuhravý et al., 1974). In Poland, dry traps of the lantern type were used (manufactured by Producent, Warszawa). The trap consists of a plastic funnel with an upper diameter of 18 cm that is covered with a roof, under which the bait is suspended. The moths that enter the trap through the gap between the roof and funnel fall into a plastic bag suspended under the funnel. The chemicals used as a lure were either commercially available pheromone dis-

pensers (Zoecon) for the gypsy moth containing 0.1 mg of racemic disparlure, or caps of natural rubber containing 20 μg of (+)-disparlure, which was synthetized in the Institute of Organic Chemistry and Biochemistry, Praha. In each experiment, the same type of lure and trap was used both in the treated and control plots.

RESULTS

Application of Disparlure at Low Population Densities. The results of a pilot experiment (Čížová, 1981), in which two doses of disparlure were formulated in the latex, are clear-cut. Not a single male was caught on either of the pheromone-treated plots, while 282 males were found in the traps on the untreated plot between July 30 (the day of spraying) and September 24.

In the second small-scale experiment (Dobříš, 1983) two other slow-release formulations were tested. As shown in Table 1, very few moths were captured on either of the treated plots at any time after applications of the pheromone (E_d = 98.0% and 99.8% for rubber and fiber-tip sawdust preparations, respectively), whereas over 600 males were found in traps on the control plot. All differences between the catches on the untreated and on both treated plots were significant ($P < 0.05$) or highly significant ($P < 0.01$, t test).

The following year, a large-scale experiment was performed at the same location with disparlure being formulated only into the fiber-tip sawdust. Catches to the control pheromone traps as well as to the traps baited with virgin females

TABLE 1. MALES OF *L. monacha* CAUGHT BY PHEROMONE TRAPS (20 μg OPTICALLY ACTIVE DISPARLURE IN DOBŘÍŠ EXPERIMENT IN 1983 (MEAN PER TRAP AND DAY ± SD)

Date checked[a]	Untreated plot (1 hectare) (N = 5)	Plots (1 hectare) treated with 10 g of racemic disparlure formulated into	
		Rubber tubing (N = 5)	Fiber-tip sawdust (N = 5)
July 26	54.8 ± 16.3	0.4 ± 05	0
July 28	37.6 ± 10.3	0.6 ± 0.5	0
August 2	17.6 ± 4.4	0	0
August 5	1.6 ± 1.1	0	0.2 ± 0.4
August 10	1.2 ± 0.4	0.4 ± 0.9	0
August 16	4.6 ± 0.9	0	0
August 24	6.6 ± 3.2	0	0
September 21	1.0 ± 1.4	0	0

[a] The pheromone treatment was done on July 22.

were significantly higher on the untreated plot than on the pheromone-treated one, E_d being 99.1% and 98.8%, respectively (Table 2). The following season (1985), the same set of disparlure-baited traps (0.1 mg/trap) was exposed on both plots during the entire flight period. Mean catch per trap (±SD) in the control plot was 174.0 ± 105.2 males per season, whereas that in the plot treated in 1984 was 80.3 ± 50.9. The differences are not significant.

Application of Disparlure During Outbreak Population Densities. The differences in the catches of the traps exposed in Karsznica on the disparlure-treated and control plots are illustrated in Figure 1. For both doses applied, the catches on treated plots were significantly ($P < 0.05$) or highly significantly ($P < 0.01$) lower (t test) than those from control plots, except for some checks after August 23, when the flight period was already ending and very few moths were captured on either plot. Calculated from the data summarized for the whole season, the E_d was 92.0 and 95.0% in pheromone- and female-baited traps, respectively, in plots treated with 10 g of disparlure per hectare, and 94.2 and 90.5% in plots treated with 20 g of disparlure.

In the second experiment, carried out in Bobrowice, only 91 males were caught into 16 pheromone traps on the disparlure-treated plot during the whole flight period of the moths, versus 2388 males on the control plot, thus making the $E_d = 96.2\%$. The differences between the catches on the treated and control

TABLE 2. MALES OF *L. monacha* CAUGHT BY TRAPS BAITED WITH 20 μg OF OPTICALLY ACTIVE DISPARLURE OR BY VIRGIN FEMALES IN DOBŘÍŠ EXPERIMENT IN 1984 (MEAN PER TRAP AND DAY ± SD)

Date checked[a]	Untreated plot (12 hectares)		Pheromone-treated plot (12 hectares)	
	Pheromone traps ($N = 12$)	Female-baited traps ($N = 4$)	Pheromone traps ($N = 12$)	Female-baited traps ($N = 4$)
August 7 [b]	13.3 ± 8.7	—	0	—
August 10[b]	6.6 ± 5.6	3.0 ± 6.0	0	0.3 ± 0.5
August 14[b]	28.4 ± 9.9	19.3 ± 24.1	0	0
August 17	49.3 ± 15.1	9.8 ± 8.5	0	0
August 22	89.4 ± 17.8	6.0 ± 1.2	0.7 ± 1.0	0
August 29	80.7 ± 32.6	2.5 ± 1.9	0.4 ± 0.5	0.3 ± 0.5
September 5	46.5 ± 13.0	0.5 ± 0.6	0.4 ± 0.8	0
September 12	0.2 ± 0.4	—	0.1 ± 0.1	—
September 19	2.3 ± 1.9	—	0	—
October 3	0.5 ± 0.8	—	0	—

[a] The pheromone treatment was done on August 2.
[b] Dates when traps were baited with newly enclosed virgin females.
[c] The traps were not baited.

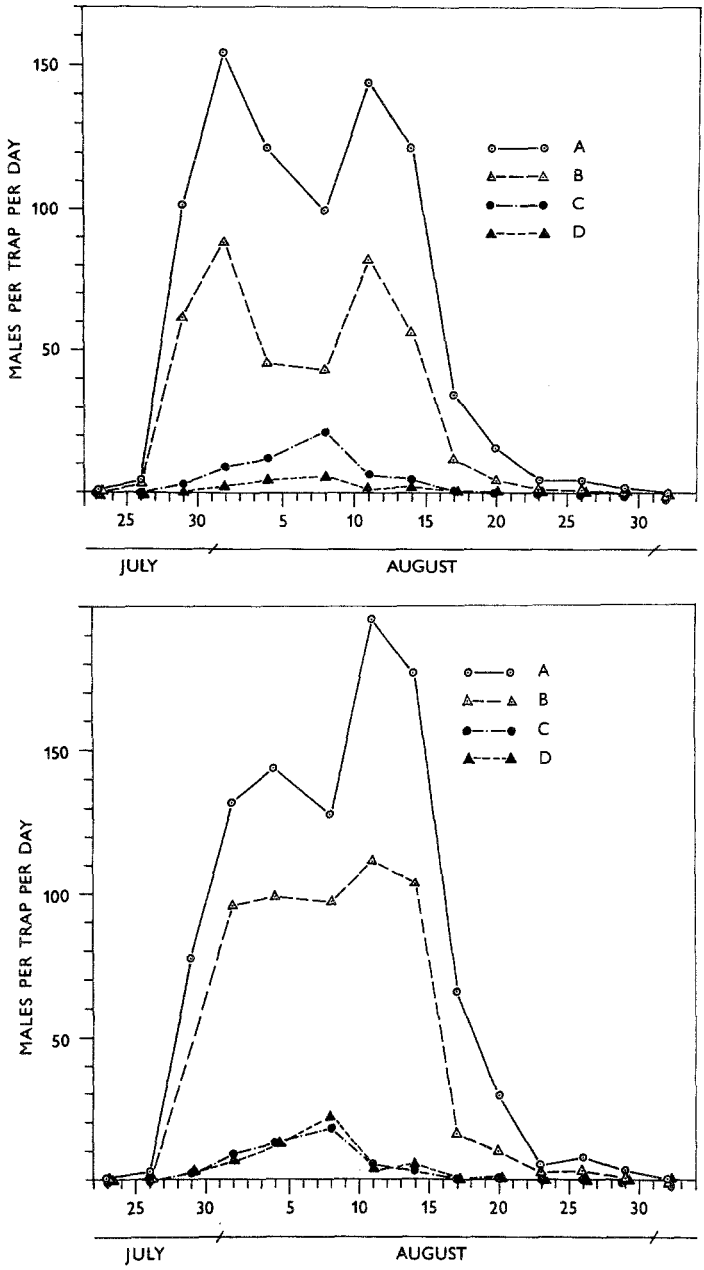

FIG. 1. The males of *Lymantria monacha* caught by the traps with 0.1 mg of racemic disparlure (circles) or by a virgin female (triangles) in the Karsznica 1982 experiment. A, B: the control plots; C, D: the plots treated with 10 (upper diagram) or 20 g/hectare (lower diagram) of racemic disparlure.

TABLE 3. MALES OF *L. monacha* CAUGHT BY PHEROMONE TRAPS (0.1 mg OF RACEMIC DISPARLURE) IN THE BOBROWICE 1984 EXPERIMENT (MEAN PER TRAP AND DAY ± SD)

Date checked	Treated plot (9 hectares)[a] (N = 16)	Control plot (9 hectares) (N = 16)
August 1	0.1 ± 0.2	0.6 ± 0.8
August 4	0.2 ± 0.5	3.3 ± 3.0
August 7	0.3 ± 0.6	9.5 ± 4.1
August 10	1.1 ± 1.0	18.9 ± 10.1
August 13	1.2 ± 1.1	13.6 ± 15.3
August 17	0.5 ± 0.7	19.1 ± 6.4
August 20	0.9 ± 1.0	7.1 ± 7.1
August 23	0.4 ± 0.6	13.7 ± 8.8
August 27	0.9 ± 1.3	14.2 ± 5.2
August 30	0.1 ± 0.3	12.3 ± 9.4
September 2	0.1 ± 0.3	6.5 ± 3.4
September 5	0	4.9 ± 2.5
September 8	0	6.3 ± 4.3
September 11	0	1.2 ± 2.0

[a] Racemic disparlure (10 g/hectare) formulated in latex was applied on July 31.

plots were highly significant ($P < 0.01$, t test) throughout the whole flight period (Table 3).

DISCUSSION

Disparlure, the sex pheromone of the gypsy moth (Bierl et al., 1970), has been recognized as a sex attractant in the nun moth also (Schönherr, 1972), and later it was found in the female of the latter species (Bierl et al., 1975). The synthetic pheromone has been used in survey traps for many years for detection and prognosis of the nun moth in Central Europe (Altenkirch, 1985a, b; Schmutzenhofer, 1986; Skuhravý et al., 1974; Maksymov, 1978; Apel et al., 1984; Jensen and Nielsen, 1984). Synthetic disparlure was also tried as an attractant for the control of the moths by trapping off the males (Boness et al., 1974) and as a mating confusant in several countries (Vrkoč et al., 1981; Altenkirch, 1985a, 1986; Schmutzenhofer, 1986). The results were mostly encouraging. Racemic disparlure also has been extensively tested in the United States as a confusant of males of the introduced gypsy moth populations with the aim of finding environmentally acceptable methods of halting the spread of the introduced pest (for review see Doane and McManus, 1981; Plimmer, 1982;

Schwalbe et al., 1983). The rationale for using the sex pheromone as a controlling agent against the nun moth in Europe comes from different premises and a different strategy should be adopted. The nun moth is a native species in the palearctic region, occurring in sparse populations all over the continent and making only occasional outbreaks, presumably as a result of ecological disturbances caused by man. Therefore, there is no reason for fighting the sparse autochthonous populations in ecologically balanced environments. Instead, the population levels of the moths should be closely watched for signs of incipient outbreaks and controlling measures applied only after a population buildup is detected.

The present paper investigates feasibility of using racemic disparlure as a mating confusant in the nun moth under the conditions of low and high population densities. Three types of slow-release formulations were tested: a spray formulation with latex as a carrier, a tubing of natural rubber, and a sawdust of porous material used for manufacturing tips of fiber-tip pens. All of them appeared suitable for the purpose. Providing that 10–20 g/hectare of disparlure were disseminated over the experimental plots (with their size ranging from 1 to 12 hectares), the disparlure concentration in the atmosphere remained high enough to interfere with the males' ability to find a discrete source of sex attractant (i.e., a dispenser loaded with racemic or optically active disparlure or a calling virgin female) for the whole flight season, which may extend for four to five weeks. In sparse density populations (approximately 10 females/hectare), the confusion effect was very high (98.6–100%) at all pheromone formulations and both doses applied. This finding is encouraging with respect to the potential use of the tested formulations in preventing the onset of a population outbreak.

A somewhat different situation appeared during the outbreak period. Although the catches of the pheromone and female baited traps of the disparlure-treated plots were significantly suppressed, the confusion effects were lower (90–96.2%) than during the non-outbreak period. These findings support the hypothesis made for the gypsy moth (Beroza and Knipling, 1972), and often also considered for other moth species, that synthetic sex attractants may have limited value as a practical measure to disrupt mating in medium or dense populations because their effectiveness will be influenced by the density of the target pest population.

With respect to the technical feasibility of dissemination of the active material, latex and the fiber-tip sawdust appear to be convenient carriers. The sawdust has particularly promising properties because it is composed of small particles that are easy to spread aerially by conventional applicators of solid materials. Moreover, it is a cheap waste by-product of the pen industry.

Questions concerning the impact of pheromone treatment on the reproduc-

tive efficiency of wild females were not addressed by the present study. Their understanding is, however, of prime importance for the confusion method to become operational. More research along this line will have to be done in the future.

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CHEMICAL DEFENSE IN BIRCH. Platyphylloside: A Phenol from *Betula pendula* Inhibiting Digestibility

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Abstract—Extracts of Scandinavian birch (*Betula pendula* Roth) buds and internodes of varying twig diameters have been investigated for their inhibitory effect on ruminant digestibility in vitro. The predominant inhibitory effect was observed in extracts containing the phenol platyphylloside, 5-hydroxy-1,7-bis-(4-hydroxy-phenyl)-3-heptan-one-3-O-β-D-glucopyranoside. Isolation of the pure compound and incubation with rumen inocula showed that digestibility was negatively related to the concentration. Significant effect was measured below the concentration naturally occurring in birch (0.8% of dry matter). Another isolated compound, the closely related rhododendrin, 4-(4-hydroxyphenyl)-2-butanol-2-O-β-D-glucopyranoside, did not depress digestibility within the concentrations found in birch. An important phenol of buds, apigenin-4′,7-dimethylether, did not have any measurable effect on digestibility, while a nonphenolic fraction of bud extract had a slight inhibitory effect on digestibility. Implications for browsers feeding on birch are briefly discussed.

Key Words—Phenol, platyphylloside, digestibility, plant defense, ruminant, birch.

INTRODUCTION

Plants produce a number of structurally different secondary metabolites of which at least some must be regarded as part of a chemical defense against foraging

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animals (Freeland and Janzen, 1974; Feeny, 1975; Swain, 1977; Rosenthal and Janzen, 1979). Studies on boreal tree–herbivore interactions demonstrate that food selection by boreal vertebrate herbivores may be explained, to some extent, by the occurrence of certain antinutritional or deterrent compounds in the plant (Pehrson, 1980, 1981; Bryant and Kuropat, 1980; Bryant, 1981a,b; Bryant et al., 1983; Palo et al., 1983; Palo, 1984). Herbivores of the boreal forest depend on birch as a main feed source during both winter and summer. However, different animals such as moose (*Alces alces*), hare (*Lepus timidus*), beaver (*Castor fiber*), and certain birds (*Tetranoidae*) utilize birch differently. All species feed selectively on different anatomical fractions and locate their feeding activity at different heights within the tree (Pullianen, 1972; Salo, 1973; Svendsen, 1980; Pehrson, 1980; Danell et al., 1987).

It seems reasonable to assume that the feeding behavior of the species studied is related to the presence of compounds that make the birch unpalatable, toxic, and/or indigestible (Bryant and Kuropat, 1980). Birch is rich in phenolic compounds, which are more or less associated with high biological activity (Palo, 1984). No doubt phenols have properties that ought to make them suitable as defense substances. Low-molecular-weight phenols are often sufficiently lipid soluble to enter animal cells, and polyphenols may interact with plant and animal macromolecules in the digestive system. Tannins and lignin are mostly found in long-lived tissues and plants growing on poor soils, and they are thought to act in a dosage-dependent fashion. Short-lived plants on richer soils tend to synthesize compounds that are active in lower concentrations, e.g., alkaloids, phenolic glycosides, and cyanogenic glycosides (Feeny, 1975; Cates and Orians, 1975; Rhoades, 1979; Coley et al., 1985).

Recent investigations have shown, that phenolic extracts of birch depress ruminant in vitro digestibility, and phenols in birch may be involved in metabolic disturbances in hares (Palo et al., 1983; 1985; Palo, 1985). However, the chemical basis of these effects was not known. The aim of the present investigation was to isolate and identify the compounds(s) responsible for the depressing effect on ruminant in vitro digestion.

METHODS AND MATERIALS

Twigs from Scandinavian birch (Betula pendula, Roth.) (Betulaceae) were collected in April, before leafing, at a site northeast of Uppsala, Sweden, and were separated into two groups with diameters of 0–1.5 mm and 1.5–5 mm, respectively. The twigs, either with or without buds, were dried at 40°C for 72 hr and then milled to pass a 1-mm sieve. Separate buds were stored at −20°C.

High-performance liquid chromatography (HPLC) was performed on a Waters chromatograph using either an analytical (Chrompac CP-TM-spher C

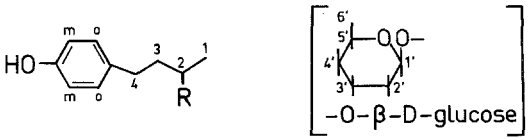
18, 4.6 × 250 mm) or semipreparative column (HiChrom Spherisorb S 10 ODS, 8 × 500 mm). Phenols were detected at 280 nm. Proton nuclear magnetic resonance spectra ([^{1}H]NMR) were recorded at 89.6 MHz on a Jeol Fx 90 Q spectrometer. Values are given relative to tetramethylsilane (TMS) as internal reference, [^{13}C]NMR spectra were recorded at 22.5 MHz. Sugars were determined with gas chromatography (GC) according to Theander and Westerlund (1985). Thin-layer chromatography (TLC) was performed on Merck HF-254 silica gel plates using the following solvents: (A) chloroform–methanol–water (80:15:1, v/v); (B) chloroform–methanol–water (40:15:2, v/v); (C) chloroform–butanone–acetic acid (10:7:2, v/v); and (D) petroleum ether (bp 40–60°C)–ethyl acetate (3:1, v/v). Phenols were detected by spraying the plates with diazotized sulfanilic acid (Fluka AG. CH. 9470) in 10% sodium carbonate (w/v), followed by 50% sulfuric acid (v/v). Catechin and leukoanthocyanins were detected with vanillin in 37% hydrochloric acid.

Some samples were hydrolyzed with pectinase (Sigma Chemical Co., No. P4625, 1.1 units/mg), which is known to have a high glycosidase activity, by incubating 5–15 mg in 2 ml water with 1 mg of enzyme added. After 20 hr in the dark at room temperature, the solution was extracted with 2 × 2 ml ethyl acetate. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. All evaporations were made under reduced pressure below 40°C.

Isolation of Major Phenols from Different Fractions

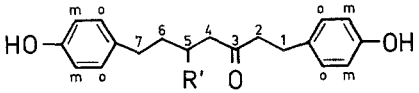
Twigs with Buds. Flour (100 g) from twigs (< 1.5 mm) was extracted with 80 ml acetone for 30 min in an ultrasonic bath in room temperature. The acetone was decanted and the flour extracted again with 80 ml acetone–water 1:1 (v/v). The two extracts were combined, the acetone was evaporated, and the aqueous phase extracted with petroleum ether (40–60°C). The remaining aqueous phase was lyophilized to a powder (8.5 g), which was dissolved in 96% ethanol and eluted on a Sephadex LH-20 column (30 × 880 mm) using (1) water, (2) 30% aqueous ethanol v/v, (3) 60% ethanol, and (4) 96% ethanol. The fractions were monitored by TLC using eluent A. Seven fractions were collected: I:1 (1.87 g); I:2 (0.45 g); I:3 (0.10 g); I:4 (0.5 g); I:5 (0.53 g); I:6 (0.49 g); and I:7 (0.64 g). Fractions I:1–4 were eluted with H_2O, fractions I:4–5 with 30% ethanol, fractions I:5–7 with 60% ethanol, and I:7 also with 96% ethanol. Part (0.3 g) of fraction I:5 was refractionated on silica (Merck Kiselgel 60, 0.040–0.063 mm, column size 20 × 300 mm) using eluent A. After examination of the fractions with TLC, one pure fraction (90 mg), later shown to be platyphylloside (Figure 1, structure 3) (cf. fraction III:4 below) was collected.

Buds. Buds (91 g, 69% dry matter) from twigs 1.5–5 mm in diameter were



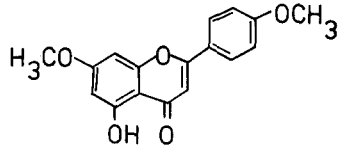
1: R = -O-β-D-glucose; Rhododendrin

2: R = H ; Rhododendrol



3: R' = -O-β-D-glucose; Platyphylloside

4: R' = H ; Platyphyllone



5: Apigenin - 4',7 - dimethylether

FIG. 1. Structural formulas of phenols isolated from birch. Numbers and letters on carbon atoms refer to [^{1}H]NMR spectra.

extracted with 3 × 200 ml acetone in a homogenizer, and the liquid was filtered and evaporated. The residue was suspended in 500 ml water and extracted with 2 × 100 ml hexane and 3 × 250 ml ethyl acetate. The two organic phases were dried over sodium sulfate and evaporated, yielding 9.0 and 11.7 g, respectively. Part (4 g) of the solid residue from acetate extract was chromatographed on a silica column (50 × 500 mm) with ethyl acetate–petroleum ether 40–60°C, 1:1 as an eluent. The fractions were monitored with TLC, eluent C. Seven fractions were collected: II:1 (0.1 g); II:2 (0.7 g); II:3 (0.4 g); II:4 (0.3 g); II:5 (0.1 g); II:6 (0.2 g); and II:7 (0.2 g). Fraction II:5 consisted almost exclusively of one compound. Part of it was recrystallized from acetone, yielding yellow needles (mp 174–175°C, not corrected) (cf. Wollenweber and Egger, 1971; Wollenweber, 1975). TLC eluent D: R_f = 0.38. Color with diazotized sulfanilic acid followed by 50% sulfuric acid: was dark yellow. The substance (Figure 1, structure 5) was identified as apigenin-4',7-dimethylether with [^{1}H]NMR (cf. Biftu and Stevenson, 1978).

Twigs without Buds (Internodes). Flour (100 g) made from internodal segments with a diameter of 1.5–5 mm was extracted with 600 ml ethanol (96%)

in an ultrasonic bath for 15 min. The solution was filtered, and the twig residue was washed with 100 ml ethanol. The combined ethanol phases were evaporated (10.9 g) and chromatographed on Sephadex LH-20, column size 4 × 50 cm using (1) water, (2) 30% aqueous ethanol (v/v), (3) 60% ethanol, (4) 96% ethanol, and (5) pure acetone as solvents. The fractions were monitored with TLC (eluent A) and seven fractions collected: III:1 (115 mg); III:2 (299 mg); III:3 (82 mg); III:4 (634 mg); III:5 (197 mg); III:6 (1273 mg); and III:7 (237 mg). Fractions III:1–2 were eluted with water, fractions III:3–5 with 30% ethanol, fractions III:5–7 with 60% ethanol, and fraction III:7 also with 96% ethanol and acetone.

Part (5 mg) of fraction III:2 was purified with semipreparative HPLC, mobile phase: linear gradient 52–100% aqueous methanol, 2.0 ml/min for 30 min; retention time was 14.3 min; yield was 4 mg. TLC, eluent A, $R_f = 0.35$, color with diazotized sulfanilic acid: yellow, darkens to red. [^{1}H]NMR (D_2O): 1.22 (1-CH_3, d, $J = 6.2$ Hz), 1.6–2.0 (3-CH_2, m), 2.65 (4-CH_2, t, $J = 7.6$ Hz), 3.0–4.0 (2-H and 2′-H to 6′-H, m), 4.50 (1′-H, d, $J = 7.6$ Hz), 6.85 (2 *m*-H, d, $J = 8.7$ Hz), 7.20 (2 *o*-H, d, $J = 8.7$ Hz). The compound was determined to be rhododendrin (Figure 1, structure 1).

Another part (15 mg) of fraction III:2 was enzymatically hydrolyzed and one product identified with GC as glucose. The aglycone (Figure 1, structure 2) was purified with semipreparative HPLC, mobile phase: linear gradient 36–100% aqueous methanol, 3.0 ml/min, 15 min; retention time 19.4 min. TLC eluent A: $R_f = 0.43$, eluent B: $R_f = 0.91$. Color with diazotized sulfanilic acid followed by 50% sulfuric acid: orange with darker contours. [^{1}H]NMR agreed with data for rhododendrol (Terasawa et al., 1973).

Fraction III:4 was almost chromatographically pure and was not further purified. TLC eluent A: $R_f = 0.17$, eluent B: $R_f = 0.52$. [^{1}H]NMR (CD_3OD): δ 1.6–1.9 (6-CH_2, m), 2.4–2.9 (1-, 2-, 4- and 7-CH_2, m), 3.0–4.0 (5-H and 2′-H to 6′-H, m), 4.27 (1′-H, d, $J = 7.3$ Hz), 6.69 (4 *m*-H, d, $J = 8.4$ Hz), 6.98 (2 *o*-H, d, *J* 8.5 Hz), 7.01 (2 *o*-H, d, $J = 8.5$ Hz). The compound was identified as platyphylloside (Figure 1, structure 3). [^{13}C]NMR agreed with data of Ohta et al. (1985). A small amount (6 mg) of the substance was enzymatically hydrolyzed and the sugar moiety identified as glucose with GC. TLC analysis of the aglycone gave $R_f = 0.65$, 0.91, and 0.78 for eluents A, B, and C, respectively. [^{1}H]NMR agreed with data for platyphyllone (Figure 1, structure 4), peviously called platyphyllonol (Terasawa et al., 1973, 1984; Nomura, 1981).

Determination of Digestibility In Vitro

The technique employed was essentially that of Palo (1985). Plant extracts or isolated substances were mixed with 500 mg of milled hay or flour from

birch twigs of diameter 1.5–5 mm in triplicate in glass filter tubes and dried at 40°C for 12 hr. The concentration of plants extracts or pure phenols was similar to that naturally occurring in birch twigs of diameter 0–1.5 mm. The tube contents were then incubated for 96 hr at 38°C with buffered rumen liquor taken from a goat fed hay (den Braaver and Eriksson, 1967). After incubation, the indigestible residues were recovered by filtration, washed with water and acetone, dried at 100°C for 24 hr, ashed at 600°C for 2 hr, weighed, and the in vitro organic matter digestibility (IVOMD) was calculated.

Twig fractions I: 1–7, bud fractions II: 1–7, crude hexane and ethyl acetate extracts of buds were tested for effects on IVOMD of hay. Substances isolated from internodes were tested at different concentrations on flour from twigs of 1.5–5 mm diameter.

Statistical analysis was performed using the Mann-Whitney U test (Siegler, 1956). Values of probability below 0.05 were considered as significant.

RESULTS

The effect of fractions I: 1–7 (twigs with buds) on IVOMD of hay is presented in Figure 2. Fraction I: 1, containing mostly low-molecular-weight car-

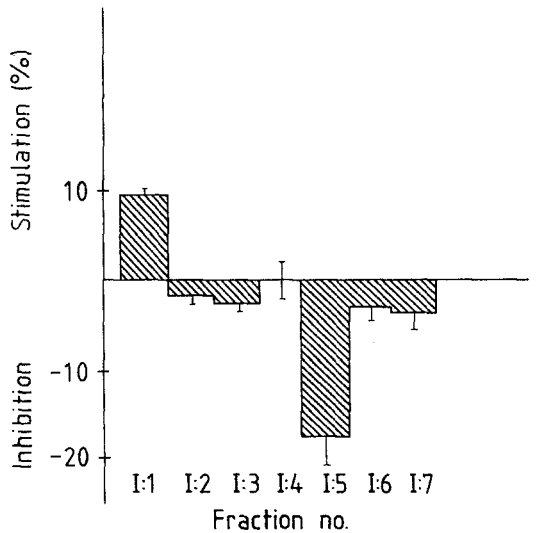


FIG. 2. Effect on in vitro organic matter digestibility of different fractions of an acetone–water extract of birch twigs including buds. Means and standard deviation ($N = 4$).

bohydrates and phenolic acids, stimulated IVOMD, while fraction I:5 has a strong inhibitory acitivity (Figure 2). This encouraged further studies on the latter fraction.

Qualitative analysis of catechins and leukoanthocyanins in fractions I:1–7 were made by spraying TLC plates with vanillin–hydrochloric acid. Fractions I:1–4 lacked catechin derivatives, while fractions I:5–7 contained vanillin-reactive substances (in increasing amounts). No correlation could be found between digestibility reduction and vanillin reactive substances.

In order to study the distribution of the compounds inhibiting IVOMD, internodes and buds were extracted separately. In fractions III:2 and III:4 one major phenolic compound was isolated from each.

Compounds Found. The [^{1}H]NMR spectrum of the substance in III:2 showed one *p*-substituted aromatic ring and four types of aliphatic protons in addition to signals from sugar protons. The NMR shift for the proton bound to the anomeric carbon showed that the sugar was aliphatically linked. The integral revealed a monoglycoside. The substance was identified as rhododendrin, which was confirmed by decoupling of the aliphatic protons. Enzymatic hydrolysis gave glucose identified with GC. [^{1}H]NMR data of the glycoside and the aglycone confirmed the compounds to be rhododendrin and rhododendrol respectively.

Fraction III:4 from internodes contained, as determined by TLC and HPLC, one major compound and minute amounts of impurities. [^{1}H]NMR data showed two almost equal, *p*-disubstituted aromatic rings in addition to 18 aliphatic protons. Decoupling of the aliphatic proton signals confirmed the structure to be platyphylloside. As for rhododendrin, the sugar moiety was aliphatically linked β-glucose. Although it has not been shown for either of the two compounds, we take for granted that it is the D-enantiomer of glucopyranose. The glycoside of fraction III:4 was identical to the major component of the active fraction I:5.

Buds contained a number of ethyl acetate soluble compounds not present in internodes. Fraction II:5 from buds crystallized spontaneously, and the substance was identified as apigenin-4′,7-dimethylether by [^{1}H]NMR.

Biological Effects Observed. The effect of the isolated compounds on IVOMD was studied at or below concentrations occurring in the plant material. The natural concentration of rhododendrin is about 0.3% in fine twigs. Neither rhododendrin nor rhododendrol had any effect on IVOMD. Platyphylloside, which has a concentration of 0.8% in fine twigs (based on dry matter), strongly affected digestibility in vitro (Figures 2 and 3). The reduction of IVOMD was directly proportional to the concentration of platyphylloside within the range studied and was significant even at concentrations below those found in fine twigs. Buds, which constitute about 7% of the twig weight, contained the

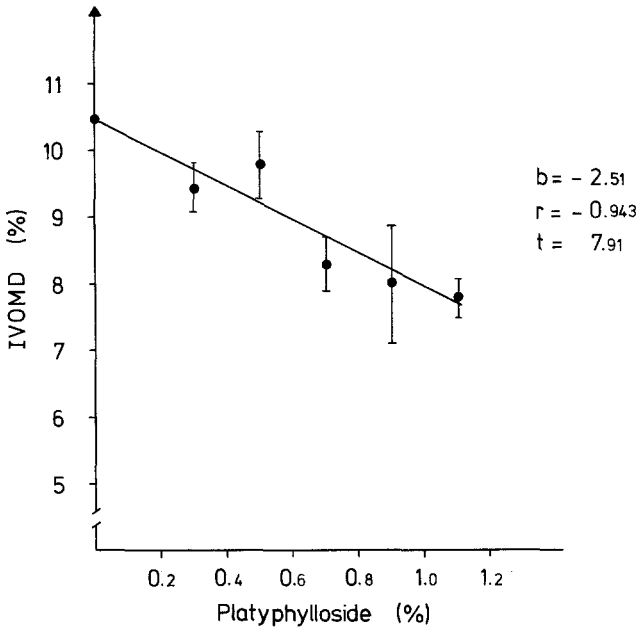


FIG. 3. The effect of addition of platyphylloside to twig flour on its in vitro organic matter digestibility. Mean and standard error ($N = 6$).

phenol, apigenin-4′,7-dimethylether, showing no effect on IVOMD. However, the ethyl acetate fraction II:1 of buds, containing mostly nonphenolic compounds had a certain depressive effect. The hexane fraction lacked effect on IVOMD.

DISCUSSION

This study confirms earlier observations that extracts of birch twigs contain substances that depress digestibility in goat rumen liquor (Palo, 1985; Palo et al., 1985). Buds contained several nonphenolic lipophilic substances not present in internodes. These were not further analyzed in this study, but a preliminary investigation of the hexane extract established that the triterpenoid, papyriferic acid, was not present in buds or internodes. This compound has been suggested as the primary defense compound in Alaskan paper birch (*B. papyrifera*) and has been shown to deter browsing by snowshoe hare (*L. americanus*) and also to depress cellulose digestion in ruminants in vitro (Reichard et al., 1984; Risenhoover et al., 1985). This does not exclude the possibility that the depression of IVOMD by the nonphenolic fractions may be partly due to some other isoprenoid substance(s). In view of the relatively modest effect of different

bud extracts on IVOMD and the fact that buds constitute only 7% of the twig weight, the overall effect of buds was considered to be small.

It is apparent from this study, that the compounds in birch that primarily depressed digestibility are found in water extracts of internodes. The platyphylloside, occurs in the fraction exhibiting the largest inhibitory effect on IVOMD.

Rhododendrin is a well-known birch phenol and was first reported by Archangelski (1901). As shown in the present study, the substance did not have any measurable effect on in vitro digestibility even at concentrations well above those found naturally. The only other study so far on possible effects of rhododendrin is by Santamore and Vettel (1978) in relation to attacks of the bronze birch borer (*Agrilus anxius*). They did not find any relationship between the concentration of rhododendrin in different birch species and the attack pattern of the insect.

Platyphylloside was originally isolated from Japanese birch (*B. platyphylla*) by Terasawa et al. (1973, 1984) and has not previously been identified in Scandinavian birch. About 80% of the observed depression of IVOMD by twig extracts in our experiments could be explained by the effect of this compound. It is very likely that the observations by Palo (1985) and Palo et al. (1985) of the inhibitory effect by water extracts from birch on IVOMD could be explained primarily by the occurrence of platyphylloside. Further support for the effect of platyphylloside is provided by the fact that its concentration shows the same seasonal decline in spring as catechin. (Palo et al., 1985; K. Sunnerheim, unpublished data). Thus the earlier reported effects on IVOMD of birch extracts and their variation with season (Palo, 1985; Palo et al., 1985) can most probably be ascribed to the presence of platyphylloside.

Results from in vitro studies may not be applicable directly to the situation in the living animal; nevertheless the mechanism of action on rumen microbes would qualitatively be the same. The rate of degradation of fibrous material in the rumen is important for the passage rate of food and hence for food intake. Although the rate of digestion has not been measured in detail in this study, we have indications that even the rate was decreased when the "total" digestibility was depressed. Substances such as platyphylloside, which depress digestibility, may thus reduce both the available energy per unit feed and the energy supply per unit time.

The optimal defense theory, as proposed by Rhoades (1979) states that the type of defense would be governed by the plant's apparency to herbivores in evolutionary time. Two major classes of defense compounds, quantitative and qualitative defenses, have been evolved. These are effective against specialist and generalist herbivores, respectively (Feeny, 1975). Polyphenols are regarded as a typical quantitative defense with a general antinutritional action (Swain, 1977, 1979).

Substances such as alkaloids and phenolic glycosides fall into the category of qualitative defense associated with a specific toxic action (Rhoades, 1979). Here we have shown that the polyphenolic fraction of birch does not exhibit the proposed action on digestibility; on the contrary, a phenolic glycoside has been shown to have the main antinutritional effect. Other observations on green alder (*Alnus crispa*), willows (*Salix* spp.), and paper birch have shown that phenolic glycosides and isoprenoids are responsible for defense properties against hares in these species (Bryant et al., 1983; Reichard et al., 1984; Tahavanainen et al., 1985).

The results of this study give further support to our hypothesis that deterrence by woody decidous plants against vertebrate herbivores is related to specific substances rather than groups of compounds.

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CHEMICAL ASPECTS OF HOST-PLANT SPECIFICITY IN THREE *Larrea*-FEEDING GRASSHOPPERS

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Abstract—The host-selection behavior of three species of grasshopper feeding on creosote bush, *Larrea tridentata*, in southern California was investigated. The species were *Bootettix argentatus*, which is monophagous; *Ligurotettix coquilletti*, oligophagous; and *Cibolacris parviceps*, polyphagous. The monophagous species is stimulated to bite by nordihydroguaiaretic acid (NDGA), a compound that is characteristic of the host plant and that may comprise up to 10% of the dry weight of the leaf. Host specificity of *B. argentatus* is enhanced by deterrent responses to compounds present in the surface waxes of all non-host-plant species. Both the oligophagous and polyphagous species are deterred by NDGA at naturally occurring concentrations. Their association with *Larrea* is probably based on tolerance of the plant chemicals rather than on dependence on specific chemicals. Factors other than the chemistry of the plant probably also contribute to the specificity of *B. argentatus* and *L. coquilletti*.

Key Words—Creosote bush, *Larrea*, nordihydroguaiaretic acid, grasshoppers, monophagy, *Bootettix*, *Ligurotettix*, *Cibolacris*, Orthoptera, Acrididae, host selection, feeding deterrence.

INTRODUCTION

Creosote bush, *Larrea tridentata*, is widespread in the Mojave and Sonoran deserts in North America. Associated with it is a guild of herbivorous insects exhibiting varying degrees of specificity for the plant (Schultz et al., 1977). Rhoades (1977a, b) demonstrated that the resin which coats the outer surface

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of *Larrea* leaves has an antidigestibility effect in vitro and obtained correlative evidence for an in vivo effect in the proscopiid grasshopper, *Astroma quadrilobatum* Mello-Leitao. While an antidigestibility effect may be the adaptive (ultimate) reason for the failure of many species to eat *Larrea* and for the specificity of some insects, it does not account for the present-day responses of insects to the plant, since some species reject it without feeding, while others are adapted to feeding on it.

In this paper we describe some of the chemical factors affecting the selectivity of three species of grasshoppers that exhibit different degrees of specificity on *Larrea*: *Bootettix argentatus* Bruner which is monophagous, *Ligurotettix coquilletti* McNeill which is oligophagous, and *Cibolacris parviceps* Walker which is polyphagous. We are concerned with establishing whether the monophagy of *B. argentatus* is determined largely by host-plant chemistry, as opposed to other ecological factors, and what sense organs are involved in selection.

METHODS AND MATERIALS

Nearly all the studies were made in 1984, 1985, and 1986 at the Boyd Deep Canyon Desert Research Center which is 10 km south of Palm Desert, Riverside County, California. The climate and flora of Deep Canyon are typical of the Colorado Desert, and the tetraploid race of *Larrea tridentata* is dominant over the alluvial plain and lower slopes of the canyon. The three species of grasshoppers feeding on *Larrea* are all relatively common.

Behavior on Plants. Observations on the feeding behavior of *Bootettix argentatus* and *Ligurotettix coquilletti* were made in open-fronted clear-plastic boxes 30 cm tall, 16 cm wide, and 8 cm deep. The open front allowed for ease of access and observation. A sprig freshly cut from the test plant was placed in a vial of water centrally on the floor of the box. Insects were transferred to the plant from vials and allowed a few seconds to settle down before others were added. The behavior of each individual was recorded continuously. In fact, most of the time the insects were stationary, and it was possible to observe and record the behavior of five insects simultaneously. Tests continued for 1 hr or until the insects left the plant, if this was earlier. Approximately equal numbers of observations were made by each observer on each plant to counteract the effects of possible observer bias. Insects were only used once in any one experiment, and they were allowed at least 24 hr in the stock cage with access to *Larrea* before being used in any other experiment. Only adults or last-instar nymphs were used.

Since *B. argentatus* feeds during the daytime (Otte and Joern, 1977), it

was tested during the day in subdued light usually at 30.0–32.5°C (range 27.5–35°C). Insects, previously collected in the field, were taken directly from stock cages containing fresh *Larrea* foliage for one series of tests simulating natural conditions (0 hr deprivation). Since selectivity of grasshoppers declines with increased time without food (Chapman and Bernays, 1977), a second series of experiments was carried out with insects deprived of food for approximately 20 h. This extreme condition provided a more rigorous test of specificity.

L. coquilletti feeds at night (Otte and Joern, 1977) and so was tested at night using red light. Under these conditions, the insects were not readily disturbed, although they could see the light and occasionally responded to it. A weak response to red light by *Locusta migratoria* L. has been recorded by Chapman (1954) and Cassier (1960). These insects were deprived of food for about 10 hr during the day to simulate normal feeding conditions; they were tested between 2000 and 2300 hr, usually at 30.0–32.5°C (range 27.5–35°C).

Cibolacris parviceps was tested behaviorally only on *Larrea*. Its readiness to feed on other plants was investigated by giving individual insects in 250-ml containers a sprig of a test plant for about 15 hr, starting at sunset. The occurrence or absence of feeding was recorded the following morning at about 0900 hours. Between tests the insects were fed on *Encelia farinosa*, a commonly occurring plant in the habitat on which they fed readily.

Preparation of Plant Extracts. Extracts of the resin on the surface of *Larrea* leaves were made using methanol, chloroform, or hexane as solvents. In each case, leaves were picked from freshly cut branches of *Larrea* plants on which *B. argentatus* was known to be present. This reduced the likelihood of selecting leaves from a bush which, for some reason, was unpalatable to this species. Bushes are known to vary in their palatability to *L. coquilletti*, and the same may be true for *B. argentatus*. Extracts were made from nine bushes. Dead leaves and reproductive parts were not included. Chloroform extracts were made by immersing the fresh leaves for 1 min at room temperature; with methanol, the leaves were immersed for 1 hr at room temperature; with hexane, they were soaked overnight at 8°C. These extracts were made from separate batches of leaves; they were not sequential. The times chosen for extraction were such that as much resin as possible was removed without the extract becoming contaminated by chlorophyll, as judged by its color. It was thus assumed that the chemicals in the extracts were largely limited to those present on the surface of the leaves. Although NDGA, when pure, is relatively insoluble in chloroform, the solubility of other resin components resulted in the bulk removal of the resin. As a result, the chloroform extract contained a high concentration of NDGA (up to 50% dry weight).

After the period of extraction, the extract was filtered, air-dried, and weighed. The residue of the leaves was oven-dried and weighed so that a mea-

sure of the dry-weight concentration of resin removed from the leaves was obtained. Subsequently the dried extracts were redissolved in the same solvents to give dry-weight concentrations on sucrose-impregnated glass-fiber disks that ranged from one to five times those on the plant.

For one series of experiments, the concentration of NDGA in a methanol extract was reduced by fractionation on an LH20 Sephadex column with methanol. Fifteen fractions were collected, and each one was examined for the presence of NDGA by comparison with the standard using thin-layer chromatography (Macherey-Nagel, Polygram SIL G/UV254, $CHCl_3$–MeOH–H_2O, 13:7:4). Fractions in which no NDGA could be demonstrated were pooled and the solvent removed. This was the NDGA-reduced fraction.

Some of these extracts were tested against all three species. Additional extracts were prepared for assay with *L. coquilletti*. This species habitually occupies some *Larrea* bushes but not others (Otte and Joern, 1975; Greenfield and Shelly, 1985). For convenience, these are called "good" and "bad" bushes, respectively. One good and one bad bush was selected by Dr. M. Greenfield on the basis of his observations. Leaf samples from each bush were oven-dried at 75°C for 3 hr and then extracted for 1 hr in methanol followed by 5 min in chloroform. In this case the extractions were sequential. Extraction in methanol reduced the dry weight of the leaves from the good bush by 14%, compared with 20% from the bad bush. Chloroform extraction resulted in a further reduction by 1.6% and 0.9%, respectively.

The concentration of NDGA in the extracts dissolved in methanol was determined on an Eyela PLC-5 liquid chromatograph system (Tokyo Rikatikai) at room temperature using a YMCA-PACK, AM-302 ODS reverse-phase column (15 cm × 6 mm) (Yamamura Chemical) equipped with Uptight precolumn (2 cm × 2 mm) (Upchurch Scientific). The mobile phase was methanol–water (7:3) at a flow rate of 1.25ml/min. The eluent was monitored by a built-in UV detector at 254 nm, and the amount of NDGA was quantified by comparison with a standard curve.

Surface extracts of nonhost plants from the habitat were made by dipping leaves into chloroform for 30 sec. Extracts were air-dried and leaves oven-dried to obtain a dry weight concentration. The extracts were tested at five times the concentration on the leaf surface because they are absorbed through the disk so that the concentration on the surface of the disk is less than the concentration applied (Woodhead, 1982).

Behavioral Observations on Responses to Plant Extracts. The responses of *B. argentatus* to contact with plant extracts or to their odor was observed in transparent plastic domes 8 cm in diameter and 3 cm high. These were made from inverted plastic champagne glasses with the stem cut off. The hole which remained served as an entry point for the insect; it was sealed with parafilm

during tests. The floor of the chamber was made of a double layer of wire gauze (1 mm mesh) on which were two semicircles of filter paper (Whatman No. 1). These were pleated so that the free edges were readily accessible to the insect for biting; *Bootettix argentatus* typically starts to feed at the edge of a leaf and had difficulty biting the edge of a disk flat on the floor of the chamber. The filter paper was untreated in control or odor experiments or treated with a chloroform surface extract of one of the test plants. In the odor experiments, a filter paper with the test extract or 1.5 g of fresh *Larrea* leaves was beneath the floor so the insect was not able to touch the extract or leaves, although the odor entered the experimental dome.

The observations were made in red light to minimize the effects of disturbance and the behavior of each insect was recorded at 30-sec intervals for 15 minutes at 28–31°C. Insects were previously deprived of food for about 21 hr (range 17–25 hr) generally at 19–23°C. They were allowed at least 15 min to acclimate at 30°C before being tested. During each experimental session two sets of insects were watched by different observers. A fresh insect was used for each treatment, and the sequence of treatments was randomized. Equal numbers of each treatment were observed by each observer in each session to minimize bias. Experiment 1 was carried out in Deep Canyon, experiment 2 at Berkeley using insects recently collected in the Mojave Desert.

Feeding Responses to Plant Extracts. The feeding responses of insects to plant extracts were determined in experiments in which the extract was added to a glass-fiber disk (Whatman GF/A, 2.1 cm diameter) already containing 5% dry weight of sucrose. Sucrose and extracts were each added to the disks in 100 μl of the appropriate solvents. This amount was just sufficient to impregnate the whole disk. Different dry weight concentrations, approximating those on leaves, were obtained by adjusting the concentrations of the solutions. The disks were air dried after addition of sucrose and after addition of the extract.

Insects were tested individually in a choice experiment in which the test disk was paired with a second sucrose-impregnated disk additionally treated with solvent only. The amounts of the two disks eaten after 24 or 48 hr were determined by weighing. The disks were presented in plastic boxes 20 × 10 × 3 cm high with gauze insets at either end. The disks were mounted on inverted thumb tacks 5 cm from each end of the box and 1.2 cm above the floor so that the insects had easy access to the edges. A pad of wet cotton was placed centrally in the box, equidistant between the disks, to provide drinking water.

In assays with extracts of good and bad bushes against *L. coquilletti*, test disks, one from each plant, were paired. Experiments were conducted in an incubator at 30°C and in darkness since some of the extracts were colored.

Commercially available NDGA (Sigma Chemical Company, St. Louis, Missouri) was tested in the same way as the extracts, being applied to the disks

in methanol. In one experiment NDGA was added to glass-fiber disks (Whatman GF/C) without sucrose; control disks in this case were treated with solvent only (no sucrose).

RESULTS

Feeding on Larrea. All three species fed readily on *Larrea* after a period of deprivation or, in the case of *B. argentatus*, even when taken directly from the host plant (Table 1). The duration of a meal was about 5 min for all species for periods of food deprivation of 10–20 hr, and in the case of *B. argentatus* and *L. coquilletti*, nearly all the insects remained on the plant in the observation box for the hour of the experiment. More than half the *C. parviceps* left the plant.

Effects of Extracts of Larrea. Leaves of *Simmondsia*, a woody shrub similar in size to *Larrea* and present in the habitat, were completely rejected by *B. argentatus* without any attempt at biting (Table 2, and see Table 6), but all the insects tested, bit, or nibbled at the leaves when these were painted with a chloroform extract of the surface resin of *Larrea* leaves. This extract did not, however, increase the palatability of *Simmondsia* leaves for *L. coquilletti*, which nibbled, but rarely fed on the plant.

Chloroform extracts added as 29–37% dry weight to sucrose-impregnated glass-fiber disks were phagostimulatory to *B. argentatus* in two of three tests (Table 3). These extracts contained 20–50% dry weight of NDGA, giving 5–16% dry weight of NDGA on the disks.

The methanol extract of *Larrea* leaf surface did not influence the selection of *B. argentatus* for sucrose impregnated glass-fiber disks in four experiments, but was a feeding deterrent for both *L. coquilletti* and *C. parviceps*. However,

TABLE 1. FEEDING ON *Larrea*: BEHAVIORAL OBSERVATIONS ON CAGED INSECTS

Species	Time deprived (hr)	Insect stage	Number of insects: Tested	Feeding	Nibbling	Biting	Palpating	Leaving	Meal length[a]: Minute ($\bar{X} \pm$ SD)	No. of insects
B. argentatus	0	adult	30	24	2	—	—	2	3.8 ± 1.7	27
	20	IV instar	12	11	—[b]	—	—	—	5.6 ± 1.6	11
L. coquilletti	10	adult	37	28	2	—	—	2	5.6 ± 3.5	14
C. parviceps	12	adult	11	7	3	1	—	7	5.6 ± 2.9	8

[a] Derived from a different data set, hence different number of insects.
[b] Minus indicates none in this category.

TABLE 2. FEEDING ON *Simmondsia*: EFFECT OF $CHCl_3$ EXTRACT OF *Larrea* OR NDGA APPLIED TO LEAVES OF *Simmondsia* ON FEEDING RESPONSES OF *B. argentatus*, 20-HR DEPRIVED, AND *L. coquilletti*, 10-HR DEPRIVED

		Number of insects						
Species	Applied to leaf	Tested	Feeding	Nibbling	Biting	Palpating	Not responding	
B. argentatus	Solvent	10	—[a]	1[b]	—	—	9	$P = 0.00006$,[c]
	Larrea extract	10	—	7	3	—	—	Fisher's exact test
	Solvent	24	—	1[b]	1	—	22	$P < 0.005$,
	NDGA 1–1.5mg/leaf	25	1	—	5	6	13	$G = 9.852$
L. coquilletti	Solvent	10	—	4	2	—	4	$P > 0.1$,
	Larrea extract	11	2	3	2	1	3	$G = 0.353$

[a] Minus indicates none in this category.
[b] Feeding on damp tissue at base of twig.
[c] Tests on 2 × 2 table, responding–not responding; solvent–extract or solvent–NDGA.

TABLE 3. RESULTS OF CHOICE EXPERIMENTS WITH SURFACE EXTRACTS OF *Larrea* ON GLASS-FIBER DISKS[a]

	dry wt (%) on disk		*B. argentatus*			*L. coquilletti*			*C. parviceps*		
Extract	Extract	NDGA	Number feeding	Test> control[b]	*P*[c]	Number feeding	Test> control[b]	*P*	Number feeding	Test> control[b]	*P*
Chloroform	29	6	20	10	>0.1	n.t.[d]			n.t.		
	33	16	23	22	<0.01	n.t.			n.t.		
	37	8	13	11	<0.05	n.t.			n.t.		
Methanol	24	8	20	14	>0.1	14	4	<0.02	12	2	<0.01
	31	7	18	10	>0.1	n.t.			n.t.		
	33	10	11	6	>0.1	n.t.			n.t.		
	33	7	12	4	>0.05	n.t.			n.t.		
Methanol, reduced NDGA	14	1.5	19	13	>0.1	15	5	>0.1	17	4	<0.01
Hexane	1	<0.005	18	12	>0.1	n.t.			n.t.		
	5	<0.025	19	14	<0.02	19	16	<0.02	17	8	>0.1

[a]Summary: + = phagostimulatory; 0 = no effect; − = deterrent.

	B. argentatus	*L. coquilletti*	*C. parviceps*
chloroform extract	+, 0	n.t.	n.t.
methanol extract	0	—	—
methanol extract, reduced NDGA	0	0	—
hexane extract, 5%	+	+	0

[b]Number eating more of test disk than of control disk.
[c]Wilcoxon signed-ranks test, two-tailed.
[d]n.t. = not tested.

when most of the NDGA and related compounds in the extract were removed by column chromatography, reducing the NDGA concentration on the disks to 1.5%, it lost its deterrence to *L. coquilletti*.

A methanol extract of a *Larrea* bush that was habitually occupied by *L. coquilletti* (good bush) was compared in a choice experiment with one from a bush that was not commonly occupied (bad bush) (see Otte and Joern, 1975; Greenfield and Shelly, 1985), both extracts being presented at about 22% dry weight on sucrose-impregnated glass-fiber disks. The extract from the good bush was eaten by *L. coquilletti* in significantly greater quantities than that from the bad bush (Table 4), although the total amount consumed (mean of 1.2 mg/insect) was much less than in any experiment that included a sucrose-impregnated disk as a control (range of means 6.5–10.5 mg/insect, five experiments). We conclude that the extract from the good bush was deterrent to *L. coquilletti*, but less so than that from the bad bush. The dry weight concentrations of NDGA on the disk were 4% and 6%, respectively. Chloroform surface extracts of the same two bushes made after the methanol extracts did not differ in palatability and were not deterrent (10.8 mg eaten per insect, Table 4). The disks contained less than 0.002% NDGA.

A hexane extract, containing less than 0.5% NDGA, of *Larrea* leaf surface was phagostimulatory for *B. argentatus* and *L. coquilletti* and had no effect on *C. parviceps* (Table 3).

Effects of NDGA. NDGA increased the acceptability of *Simmondsia* leaves to *B. argentatus* (Table 2) and was stimulating at all concentrations from 0.005%

TABLE 4. *L. coquilletti*: RESULTS OF CHOICE EXPERIMENTS WITH SURFACE EXTRACTS OF GOOD AND BAD *Larrea* BUSHES ON GLASS-FIBER DISKS

Extract	Dry weight (%) on disc	NDGA concentration (%) on disc		Number feeding	Amount eaten (mg, $\bar{X} \pm$ SD)		Good > bad[a]	P[b]
		Good	Bad		Good bush	Bad bush		
Methanol	22–23	4	6	20	0.8 ± 0.8	0.4 ± 0.7	14	<0.05
Chloroform	6–10	0.001	0.002	17	5.4 ± 5.7	5.4 ± 5.1	7	>0.1

[a] Number eating more of extract of good bush than of bad bush.
[b] Wilcoxon signed-ranks test, two-tailed.

to 20% dry weight on sucrose-impregnated glass-fiber disks (Figure 1). The degree of phagostimulation increased from 0.005% to 5% dry weight of NDGA ($t = 3.81$, $P < 0.001$), but there was no significant difference between 5% and 10% dry weight. In experiments using 6% or 13% NDGA on glass fiber disks (GF/C) without sucrose, the insects almost invariably nibbled at the test disks, but not the controls (13 of 14 and nine of 10, respectively), but the amounts of material consumed in 24 hr were negligible. The average amounts eaten were less than 0.2 mg in both experiments, and the biggest feed was 0.4 mg, compared with 3.5 ± 1.1 mg ($\bar{X} \pm$ SE, $N = 11$) in a parallel experiment with a choice between 5% sucrose and blank disks.

Both *L. coquilletti* and *C. parviceps* were deterred by 5% and 10% NDGA on glass-fiber disks (Figure 1), and in both cases significantly fewer insects fed on the test disks with the higher (10%) concentration of NDGA (*Ligurotettix*, $P = 0.009$; *Cibolacris*, $P = 0.005$; Fisher's exact test). At concentrations of 0.5% dry weight and below, *C. parviceps* did not distinguish between test and

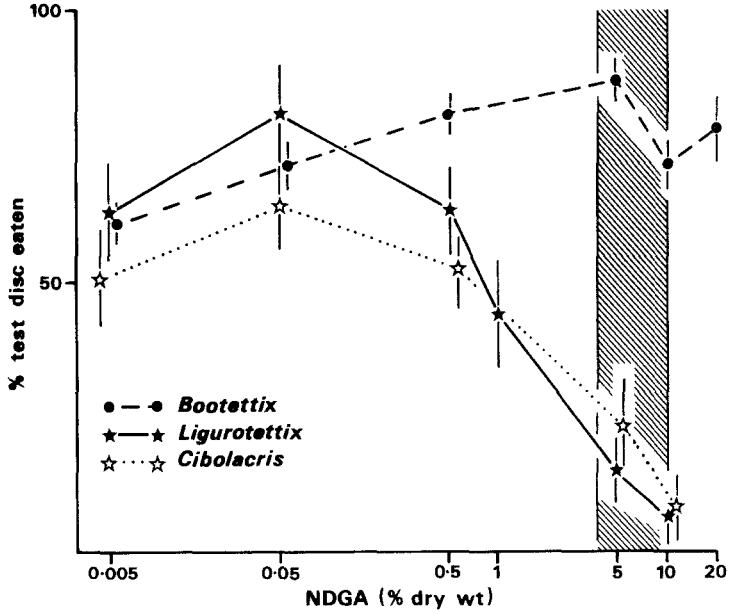

FIG. 1. Responses of each species to different concentrations of NDGA on sucrose-impregnated glass-fiber disks. Ordinate shows the amount of test disk consumed as a percentage of the total consumption by weight (test + control disks): 50% indicates no preference; >50%, phagostimulation; <50%, deterrence. Each point is based on a minimum of 16 insects. Results are of tests lasting 48 hr except for 20% which was only 24 hr. Vertical lines indicate standard errors. Hatched area shows range of normally occurring concentrations on *Larrea* leaves.

control disks. In the case of *L. coquilletti*, however, the pooled data for 0.005–0.5% dry weight indicate a significant phagostimulatory effect (Wilcoxon signed-ranks test, $P < 0.01$).

Behavioral Responses. Observations on the behavior of *B. argentatus* were carried out to determine which sensilla were involved in acceptance of the host plant. Normally, before starting to feed, a grasshopper tests the chemical quality of the potential food by touching it with the palps, "palpating," and biting. Rejection of the plant may follow either of these activities, or the insect may go on to feed. A chloroform extract of the leaf surface on filter paper induced more palpation and biting than was observed on untreated filter papers in two separate experiments (Table 5). The higher level of response with the extract may have resulted from information received via contact chemoreceptors on the tarsi or from olfactory receptors, probably on the antennae. When presented only with the odor of the extract or of *Larrea* leaves, significantly more palpation and biting occurred with the odor than without it in one experiment, but not in two others.

Responses to Other Plants. B. argentatus did not feed on any of 19 other plant species, most of which were common in the habitat at Deep Canyon. Undeprived insects rarely palpated and even after a 20-hr deprivation only a few insects palpated, bit, or attempted to nibble (Table 6). Other species of

TABLE 5. *B. argentatus*: BEHAVIORAL RESPONSES TO CONTACT WITH $CHCl_3$ SURFACE EXTRACTS OF *Larrea* AND NONHOST PLANTS (COMPARED WITH CONTROL IN SAME EXPERIMENT)

			Number of insects			
Plant	Experiment	Stimulus	Tested	Palpating and/or biting	*G* statistic[a]	*P*
Larrea	1	extract	14	12	21.007	<0.01
	2	extract	17	15	8.382	<0.05
	2	whole leaf odor	16	11	2.452	>0.05
	1	extract odor	12	6	6.831	<0.05
	2	extract odor	16	11	2.452	>0.05
Hyptis	2	extract	16	5	0.336	>0.05
	2	extract odor	16	5	0.336	>0.05
Encelia	2	extract	16	8	0.248	>0.05
	2	extract odor	16	5	0.336	>0.05
Control	1	—	16	1	—	—
	2	—	17	7	—	—

[a] *G* test, probabilities adjusted to take account of multiple comparisons.

TABLE 6. FEEDING RESPONSES OF THE THREE SPECIES TO A RANGE OF PLANTS OCCURRING IN THE HABITAT OR WITHIN THE RANGES OF THE INSECTS ELSEWHERE

Plant family and species	*Bootettix argentatus*										*L. coquilletti*					*C. parviceps*	
	Nondeprived					20-hr-deprived											
	Number tested	Feeding	Nibbling	Biting	Leaving plant	Number tested	Feeding	Nibbling	Biting	Leaving plant	Number tested	Feeding	Nibbling	Biting	Leaving plant	Number tested	Number feeding
Acanthaceae																	
Beloperone californica			not tested			8	—	—	—	3	9	—	1	2	5	not tested	
Asteraceae																	
Ambrosia dumosa	10	—[a]	—	—	8	9	—	—	—	8	10	—	7	1	1	not tested	
Bebbia juncea	10	—	—	—	9	11	—	—	—	10	10	—	—	2	3	15	14
Encelia farinosa	10	—	—	—	6	10	—	—	1	8	9	—	1	—	5	9	9
Hymenoclea salsola			not tested			10	—	—	—	6			not tested			not tested	
Perityle emoryi			not tested			9	—	—	—	6	9	—	1	3	6	not tested	
Peucephyllum schotti			not tested			10	—	1	—	9	9	—	1	2	5	not tested	
Buxaceae																	
Simmondsia chinensis	8	—	—	—	5	9	—	—	—	8	10	—	3	4	1	15	—
Chenopodiaceae																	
Atriplex canescens			not tested					not tested			10	8	1	—	—	not tested	

Fabaceae																		
Acacia greggi	10	—	—	—	6	10	—	—	—	9	10	—	8	1	—	not tested		
Cercidium floridum	8	—	—	—	5	9	—	—	—	8	11	—	5	3	1	10	8	
Krameriaceae																		
Krameria parvifolia	not tested					10	—	1	—	7	9	1	6	2	9	not tested		
Lamiaceae																		
Hyptis emoryi	9	—	—	—	6	8	—	—	—	7	10	—	4	—	8	not tested		
Nyctaginaceae																		
Boerhaavia coccinea	not tested					8	—	—	—	4	9	—	3	1	6	not tested		
Solanaceae																		
Lycium andersonii	not tested					not tested				10	10	—	—	5	not tested			
Zygophyllaceae																		
Fagonia laevis	10	—	—	—	8	10	—	—	—	8	10	7	—	—	3	not tested		
Larrea tridentata	30	26	2	—	—	12	11	—	—	—	37	28	2	—	—	10	9	
Tribulus terrestris	11	—	—	—	8	10	—	—	—	7	12	—	5	3	3	14	14	
Poaceae																		
Aristida sp.	11	—	—	—	9	11	1	1	1	6	10	—	2	1	5	15	9	
Cynodon dactylon	10	—	—	—	7	11	—	—	1	6	10	1	1	1	2	12	3	
Bouteloua aristidoides	not tested					9	—	—	—	6	8	—	—	1	5	not tested		
Triticum vulgare	not tested					9	—	—	1	6	9	1	—	1	5	not tested		

[a] Minus indicates none in this category.

Zygophyllaceae, the family to which *Larrea* belongs, were no more acceptable than plants from other families. Only on the grass, *Aristida*, was a sustained attempt at feeding made by one insect. This individual chewed at a leaf blade for 8 min, but during this time, although the leaf tissue was crushed, hardly any was removed. One other insect also nibbled and one bit on the *Aristida*. With nearly every plant offered, a majority of the insects left the plant within the duration of the experiment (1 hr) without apparently palpating on the surface. Only the tarsi had made contact with the leaves.

Surface extracts of 11 of these species were tested in choice tests on sucrose-impregnated glass-fiber disks. In all cases, the extracts were strongly deterrent and, apart from *Beloperone* and the grasses, virtually no feeding occurred on the test disks (Table 7). The chloroform extracts of *Encelia* and *Hyptis* on filter papers did not, however, influence behavior in observations lasting 15 min (Table 5).

In contrast with *B. argentatus*, one or more individuals of *L. coquilletti* bit or nibbled at all the different plants offered, and three species, *Atriplex*, *Lycium*, and *Fagonia*, were readily eaten by most insects tested. Individual insects also fed on the grasses (Table 6). *L. coquilletti* commonly left the plants on which it did not feed.

TABLE 7. *B argentatus*: RESULTS OF CHOICE EXPERIMENTS WITH SURFACE EXTRACTS OF NONHOSTS ON GLASS-FIBER DISKS

Plant family and species	Number feeding	Number eating test disk	Number eating more test than control	P^a
Acanthaceae				
Beloperone californica	16	8	3	<0.01
Asteraceae				
Ambrosia dumosa	17	0	0	<0.01
Bebbia juncea	16	1	0	<0.01
Encelia farinosa	14	0	0	<0.01
Hymenoclea salsola	11	0	0	<0.01
Perityle emoryi	13	3	1	<0.01
Peucephyllum schotti	15	0	0	<0.01
Fabaceae				
Cercidium floridum	20	1	1	<0.01
Lamiaceae				
Hyptis emoryi	17	0	0	<0.01
Poaceae				
Cynodon dactylon	15	8	2	<0.01
Triticum	16	13	3	<0.01

[a] Wilcoxon signed-ranks test, two-tailed.

The feeding responses of *C. parviceps* were only tested on eight plant species in five families. It ate all the plants (Table 6), although not always in large amounts.

DISCUSSION

Analysis of gut contents has revealed that *Bootettix argentatus* is strictly monophagous on *Larrea*; *Ligurotettix coquilletti* is oligophagous, although *Larrea* commonly constitutes a major host plant; and *Cibolacris parviceps* is polyphagous, feeding on *Larrea* only to a limited extent and not, in general, being dependent on it (Otte and Joern, 1977; Joern 1979). The present results demonstrate that the different relationships of these species with *Larrea* are at least partly governed by the chemical characteristics of the plant, extending the work of Rhoades (1977a), who clearly showed the importance of the leaf-surface resin in selection by *C. parviceps* and had circumstantial evidence relating the resin content of the leaves to acceptability by *B. argentatus* and *L. coquilletti*.

The sticky resin covering the leaves of *Larrea* may comprise 26% or more of the dry weight of the young leaves, sometimes falling to around 10% on older leaves of the same sprig and also varying between bushes. Phenolic aglycones comprise over 80% of the resin and the major component is nordihydroguaiaretic acid (NDGA), so that this single compound constitutes 4–10% of the dry weight of the leaf in samples taken from Deep Canyon (Greenfield et al., 1987). The resin also contains a range of flavonoid aglycones (Mabry et al., 1977; Rhoades, 1977a). In addition, Seigler et al. (1974) recorded the presence of a range of wax esters (even-numbered, C_{46}–C_{56}), most of which were from the external surface of the stems, but some of which were probably also on the leaves.

In the present experiments, *B. argentatus*, even after 20 hr without food, fed only on *Larrea*; none of the other plants from the habitat was eaten, and they were almost invariably rejected by the insect without biting. Clearly the insect responds to the surface chemicals or to the odors of the plants. The importance of the surface resin is confirmed by the fact that a chloroform extract of the surface induced the insects to bite and nibble at leaves of *Simmondsia* to which it was applied and to feed on sucrose-impregnated glass-fiber disks. The extract used in these experiments contained 50% dry weight of NDGA, which itself was a stimulant over a range of concentrations from 0.005–20% dry weight of the disks. The experiments in which NDGA was presented without sucrose suggest that it is a biting factor since, although virtually all the test disks were bitten, very little was ingested. These experiments were carried out with GF/C disks, not GF/A as in all other experiments, so it is possible that the failure to ingest was related to the physical differences of the disk, although we consider this unlikely.

The hexane surface extract was also phagostimulatory at 5% dry weight. This extract contained less than 0.025% NDGA, which may have produced the positive effect, although the presence of other phagostimulants cannot be excluded. Although the methanol surface extract contained NDGA at relatively high concentrations, it was not phagostimulatory. Presumably the effects of the NDGA were offset by unidentified deterrents.

The behavioral observations using a chloroform extract show that palpation and biting are induced by tarsal contact and/or olfaction. The much stronger response on contact suggests that contact chemoreception of the resin is of primary importance, but a response to odor occurred in one of three experiments, so that an olfactory response cannot be ruled out, especially since the odor presented was that of the surface extract and so will, presumably, have contained only a proportion of the many volatiles known to be present in *Larrea* (Mabry et al., 1977). An electroantennogram could be recorded from an antenna stimulated by the odor of leaves, so the odor was perceived by the insect, although the antennogram did not differ in magnitude from that produced by the leaves of nonhost plants (Wyatt, unpublished).

When presented with nonhost plants, nearly all the *B. argentatus* left the plants without attempting to feed or even palpating. This may have been due to the lack of specific phagostimulants characteristic of the host or to deterrent effects of the nonhosts. The behavioral experiments with *Encelia* and *Hyptis* provided no evidence of rejection, although the longer-term disk tests show that the surface extracts of all the nonhosts, including *Encelia* and *Hyptis*, contained feeding deterrents. This was true even of the two grass species tested, but on these more than half the insects did feed on the test disk, whereas with extracts of most other plants no feeding occurred at all. Mature grasses are generally low in biochemically active plant secondary compounds (Bernays and Barbehenn, 1987), although some strains of *Cynodon* are cyanogenic (this was not tested), and it is significant that the only nonhost on which *B. argentatus* made sustained attempts to feed was the grass *Aristida*. In this case, the combination of the fibrous food and specialized mandibles of the insect (Chapman, unpublished) apparently resulted in a mechanical barrier to feeding.

We conclude that the specificity of *B. argentatus* to *Larrea* involves a positive response to NDGA. This is the first example of an acridid adapted to respond positively to a characteristic host-plant chemical, although probably other instances are to be expected in other monophagous species (Bernays and Chapman, 1978). This parallels the suggestion by Rhoades (1977b) that the *Larrea* resin provides feeding cues to the monophagous caterpillar of the moth *Semiothisa colorata*. The specific response to NDGA in *Larrea* is complemented by the deterrent responses to surface compounds of nonhost plants, a combination similar to that exhibited by many relatively specific phytophagous insects (Chapman and Bernays, 1977). This is not to suggest that the chemical

cues are solely responsible for the specificity of *B. argentatus*. The insect is cryptic in *Larrea* foliage, and it probably responds to visual features of the plant (Otte, 1981).

Ligurotettix coquilletti exhibits a disjunct oligophagy. Otte and Joern (1975, 1977) recorded seven plants in the gut contents including *Larrea*, *Atriplex*, *Simmondsia*, and grass, and Ball et al. (1942) recorded feeding on *Franseria* (=*Ambrosia*) *dumosa*. Diet breadth, however, was more restricted within any one locality (Otte and Joern, 1977). In the current experiments, this species occasionally nibbled at *Simmondsia*, but did not eat it even when it was coated with the surface extract of *Larrea*. It did, however, eat *Atriplex*, *Lycium*, and *Fagonia* as well as *Larrea*. Populations are known to exist in areas in which *Larrea* is absent, and *Atriplex* and *Lycium* are the dominant shrubs (Greenfield, personal communication); clearly it is not dependent on any one plant species.

Methanol extracts of *Larrea* leaf surface were deterrent to *L. coquilletti*, even that from a good bush normally inhabited by this species. This extract was, however, less deterrent than that from a normally uninhabited bush. Rhoades (1977a) observed that the resin content of *Larrea* leaves from unoccupied bushes was, on average, higher than that of occupied bushes. This was also true in our case, but in the experiment the two extracts were present in similar dry weight concentrations (22–23%) on the glass-fiber disks. Our experiment suggests that the significant difference to the insects is the composition of the resin rather than, or perhaps, as well as, its quantity on the leaf. The disks from the good bush contained 4% dry weight of NDGA, compared with 6% in the disks from the bad bush. Over this range of concentrations, the deterrent effect of NDGA changes rapidly (Figure 1), and this could account for the differences in amounts eaten. Greenfield et al. (1987), in a parallel study of *L. coquilletti*, recorded longer meals on foliage with low NDGA–resin ratios.

The hexane extract of *Larrea* leaves is phagostimulatory. The compound responsible has not been identified, although low levels of NDGA, similar to those in the extract, are phagostimulatory.

L. coquilletti is cryptic on the stems of *Larrea*, and Otte and Joern (1977) point out that it is equally cryptic on the stems of *Atriplex* and *Simmondsia*. The same is true of *Lycium*. This suggests that visual cues may be important in host-plant selection by this species, although concealment on the nonhost, *Encelia*, seems equally effective (Greenfield, personal communication). If so, the chemical aspects of selection may be permissive rather than providing key sign stimuli. That the insect is not dependent on specific chemical signals, at least in the initial stages of selection, is suggested by the frequency with which biting and nibbling occur on nonhost plants. These are all ultimately unpalatable, but the deterrence in many cases depends on the internal constituents of the plant, not primarily on its surface properties.

Cibolacris parviceps is polyphagous (Otte and Joern, 1977; Rhoades,

1977a; Joern, 1979), feeding on a wide range of plants from numerous families. In the current experiments, all the extracts of *Larrea* leaf surface, as well as NDGA, were deterrent. Rhoades (1977a) and Schultz et al. (1977) showed that this species feeds preferentially on the oldest *Larrea* leaves, presumably those with the lowest resin contents. Removal of the resin with ether greatly enhanced the palatability of both young and old leaves and eliminated the differential between them (Rhoades, 1977a). The implication is that *C. parviceps* is able to tolerate *Larrea*, rather than having a specific response to it.

Bernays and Chapman (1978) suggest that polyphagous Arididae are, in general, less sensitive than oligophagous species to deterrence by plant secondary compounds, some of which may even become phagostimulatory. Host range and amounts eaten by these insects are determined by the balance between phagostimulatory and deterrent properties of a plant for that species of grasshopper. The results with *C. parviceps* are consistent with this thesis, although the meager data do not allow any more certain conclusions to be drawn.

All three species of grasshopper studied are Gomphocerinae (Jago, 1971), a subfamily comprised almost entirely of grass-feeding taxa. Otte and Joern (1977) discuss the evolutionary pressures that may have led to the current habits of these *Larrea*-feeding species. They envisage a series of steps in which insects fed on shrubs when grasses were no longer available, through the ability to feed entirely on a range of shrubs, and finally to specialization on *Larrea* associated with its high level of spatial and temporal predictability. It seems evident that the chemical relationships between the insects and the plant are largely a consequence of the pressures of desertification. *B. argentatus* have evolved a dependence on the plant, and at least one specific chemical is stimulatory over a wide range of concentrations. In contrast, it seems that both *L. coquilletti* and *C. parviceps* tolerate *Larrea*, but the requirement of *L. coquilletti* for suitable stems for concealment has perhaps become associated with a more limited tolerance of other plants than is true for *C. parviceps*. Thus the first exhibits oligophagy, the second polyphagy.

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GYPSY MOTH[1] MATING DISRUPTION: Dosage Effects

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Abstract—Small (1-hectare) plots in a dense gypsy moth (*Lymantria dispar*) infestation were treated with 5, 50, or 500 g racemic disparlure, and effects on male trap catch and mating behavior were studied. Capture of males in traps baited with 1, 10, 100, or 1000 μg (+)-disparlure declined as disruptant dosages increased. Traps with high levels of attractant caught moths when capture in those with lower baitings was dramatically reduced. While all disruptant dosages reduced trap catch, it was reducd at least 95% at all attractant levels at the 500-g disruptant application rate. Visual estimates indicated that male moth density was similar in treated and control plots; female mating success was reduced 6.5, 34.5, and 84% in plots with 5, 50, and 500 g/hectare disruptant, respectively. The duration of precopulatory and copulatory periods was similar for all females that were observed mating, regardless of disruptant treatment. It is proposed that reduced trap catch and female mating success are due to effects of atmospheric synthetic disparlure (disruptant) camouflaging natural attractant point sources.

Key Words—Gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, disparlure, mating disruption.

INTRODUCTION

The development of techniques for disrupting pheromone communication in Lepidoptera has received considerable research effort in the last decade, but field trials have often been empirical and application rates have been restricted to those that were presumed to be "economically feasible" in the real world. For the gypsy moth, determining potential effectiveness of the technique has

[1] Lepidoptera: Lymantriidae.

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been hindered further: in dense infestations where direct population measurement is accurate (through egg mass, larval and pupal counts), populations have not been significantly affected by disparlure treatment (Granett, 1976). Other studies with gypsy moth (Schwalbe, et al., 1979) and Douglas-fir tussock moth, *Orgyia pseudotsugata* (Sower and Daterman, 1977), have shown mating disruption to be inversely density-dependent. In low-density populations, direct census is difficult and imprecise and, therefore, the degree of control achieved by a disruptant treatment cannot be accurately quantified. Most field tests with gypsy moth have evaluated only disruptant dosages below 50 g/hectare; it may be theorized that the technique will be more effective against denser populations when the airborne concentration of disruptant is increased.

The purpose of the experiments in this report was to evaluate the degree of communication and mating disruption effected by vastly different atmospheric concentrations of disruptant.

METHODS AND MATERIALS

Gypsy moth mating-disruption field studies typically have been conducted on 16-hectare (or larger) plots with treatments applied by aircraft. Most controlled-release formulations that have been devised are appropriate for applying ca. 50 g disparlure (or less) per hectare. Formulation volume and cost of material render tests of greater application dosages impractical; consequently, it was necessary to miniaturize our experimental design such that a wide range of disruptant dosages could be studied.

Square, 1-hectare plots were established near Cotuit, Massachusetts. In the year of the test (1981), this site harbored a "dense" gypsy moth population that resulted in ca. 50% defoliation of oak trees. Plots were separated from one another by at least 1.5 km. Transect lines running through the plots at 10-m intervals resulted in 100 grid points in each plot. Relative airborne concentrations of racemic disparlure (disruptant) were achieved using 2.5-cm-wide Hercon tape containing 3.1 mg racemic disparlure/cm^2. A strip of tape was stapled in loops 1.5–2.5 m high on a tree at each grid point. By using strips 6.4, 63.5, and 635 cm long, plots were treated with 5, 50, or 500 g disparlure/hectare, respectively. Based upon laboratory measurements of emission rates from similar strips, daily disparlure release in the three plots was estimated at 15, 150, and 1500 mg/hectare (B.A. Leonhardt, personal communication).

Large-capacity traps fashioned out of 1.9-liter milk cartons were used to trap males in the test plots. Traps were baited by dispensing 1, 10, 100, or 1000 μg (+)-disparlure in 100 μl *n*-hexane onto 1 × 1-cm-diameter cotton wicks. These wicks were replaced every three days during the course of the studies. Laboratory emission tests indicate that, for the first three days, 10-, 100-, and

1000-μg wicks release ca. 2, 18, and 33 μg/day, respectively (Schwalbe, unpublished). Four traps of each dosage (16 traps total) were placed on a 4 $\times$ 4 grid arrangement (20 m between traps) in each plot. One trap of each dosage was randomly positioned on each line. Traps within each line were rerandomized with each observation. Pieces of dog collars 8.9 cm long and containing 8.37% (by weight) dichlorvos (Zoecon Corp., Palo Alto, California.) were suspended within the traps to kill the moths that entered. Traps were checked and rerandomized, and all captured males were counted daily. Populations were monitored in the four plots for five days (July 19–23, 1981) to give baseline trap catch prior to treatment. Treatments were applied to three plots on the evening of July 23, 1981, and traps were checked daily until July 29, 1981, to determine the effects of disruptant on capture.

Periodically throughout the day on July 31 (1130–1600 hr), observers walked slowly through the center 10 $\times$ 10-m sector of each plot and counted all male moths seen. This provided a visual census of male moth density in the plots.

Tests were also performed to determine mating activity in the plots. On July 28 and 31, and August 3, fifteen 1-day-old laboratory-reared female moths were placed on trees in each of the plots and collected three to four days later. That mating had occurred was concluded if the moth laid embryonated eggs. If an egg mass was not laid or the eggs were not embyonated, it was assumed the female had not mated. On each day that females were released, all females were observed continuously from 1130 to 1600 hr; incidence of mating, time to initiate mating, and duration of copulation were recorded.

RESULTS

Summaries of the number of male moths captured in traps baited with 1-1000 μg (+)-disparlure are presented in Figure 1A–D. Traps baited with 1–1000 μg (+)-disparlure typically yield a dose–response curve in which, as bait concentration increases, trap catch also increases to a peak at ca. 100 μg (+)-disparlure; traps baited with 1000 μg capture at about the same level as those with 10 μg pheromone. While the total number of moths captured in the various plots varied somewhat because population densities were different, the pretreatment dose–response curves were similar in all plots.

In the control plot, treatment catches were similar to pretreatment catches in traps baited with 1, 10, and 100 μg (+)-disparlure, indicating that no major change in adult population density occurred during the course of the test (Figure 1A). Traps containing 1000 μg pheromone captured 30.6% fewer moths in the presence of disruptant (61.6 moths/trap/day) than pretreatment (88.7/trap/day); the reason for this is not known.

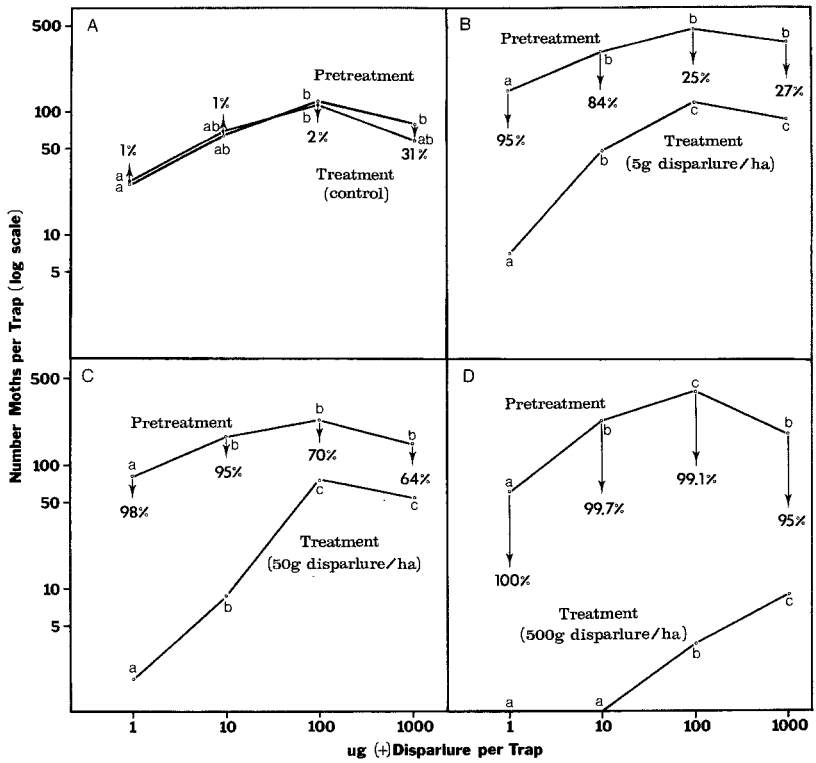

FIG. 1. (A–D) Daily number of male gypsy moths captured in four traps baited with 1, 10, 100, and 1000 μg (+)-disparlure before and after treatment of plots with 5, 50, and 500 g disparlure/hectare. Values shown below arrows on pretreatment curves are percent change in trap catch following application of disruptant. Pretreatment curves are from five daily trap readings (July 19–24, 1981); treatment results are from the six days following disruptant application (July 25–29, 1981).

In the plot treated with 5 g racemic disparlure/hectare, a general reduction in trap catch from pretreatment levels was observed in (Figure 1B). Catch in traps baited with 1 μg was suppressed to a greater degree (95%) than that in traps with 10, 100, or 1000 μg (+)-disparlure (reductions of 84, 25, and 27%, respectively). Traps containing 1000 μg (+)-disparlure caught fewer moths (89.2 males/trap/day) than traps baited with 100 μg (121 males/trap/day). While catch at both concentrations of attractant was reduced similarly, the typical "peak" of the dose–response curve was observed at the 100 μg concentration.

Increasing the application rate to 50 g racemic disparlure/hectare resulted in a further overall reduction in trap catch (Figure 1C). In this case, catch in the 1- and 10-μg traps was reduced appreciably (98 and 95%, respectively)

TABLE 1. AVERAGE NUMBER OF MALE GYPSY MOTHS OBSERVED IN CENTER 100 m^2 OF 1 ONE-HECTARE PLOTS TREATED WITH VARYING AMOUNTS OF RACEMIC DISPARLURE (COTUIT, MASSACHUSETTS, 1981)

	Treatment			
	Control	5 g/hectare	50 g/hectare	500 g/hectare
Number of observations	18	18	18	17
Number males/census	1.1 a[a]	2.3 b	2.1 b	2.9 b

[a]Means followed by the same letter are not significantly different at the 5% level according to Duncans multiple-range test.

compared with the 100- and 1000-μg traps, which were reduced only 70 and 64%, respectively. As in the 5-g disruptant/hectare plot, traps baited with 100 μg (+)-disparlure caught more moths than any other trap type.

When the disruptant treatment was 500 g/hectare, the trap catch pattern changed dramatically. No moths were capture in traps baited with 1 μg (+)-disparlure, and those baited with 10 and 100 μg had catches reduced 99.7% and 99.1%, respectively, from pretreatment levels (Figure 1D). A reduction of 95% in trap catch was recorded in traps baited with 1000 μg (+)-disparlure. The treatment dose–response curve in this plot was unique from other treatments in that the 100-μg traps caught fewer moths than those baited with 1000 μg.

Results of visual census of male moths taken on July 31 indicate that more moths were present in the treated plots than in the control plot (Table 1).

In the control plot, all recaptured female moths were mated (Table 2). Successful mating was observed in 93.5, 65.6, and 16% of the females col-

TABLE 2. MATING INCIDENCE OF FEMALE GYPSY MOTHS IN ONE-HECTARE PLOTS TREATED WITH VARYING AMOUNTS OF RACEMIC DISPARLURE (COTUIT, MASSACHUSETTS, 1981)

	Treatment			
	Control	5 g/hectare	50 g/hectare	500 g/hectare
Number mated	32 a[a]	29 a	19 b	5 c
Number not mated	0	2	10	26
Percent reduction	0	6.5	35.5	84.0

[a]Values followed by the same letter are not significantly different ($P \leq 0.01$) according to chi-square analysis with Yate's correction factor.

TABLE 3. PRECOPULATORY AND COPULATORY PERIODS OF FEMALE GYPSY MOTHS IN PLOTS TREATED WITH VARYING AMOUNTS OF RACEMIC DISPARLURE (COTUIT, MASSACHUSETTS, 1981)

	Treatment			
	Control	5 g/hectare	50 g/hectare	500 g/hectare
Number females observed mating	9	4	5	0
Precopulatory period (min)	103	74	77	–
Copulatory period (min)	64	80	60	–

lected in plots treated with 5, 50, and 500 g disparlure/hectare, respectively. Females that were mated during the observation periods were located by males as soon after release as those in the control plot (Table 3). There were simply fewer cases of mating in the treated plots and, in fact, none were observed in the 500 g/hectare plot. The duration of observed copulations was also similar, at ca. 60–80 min.

Native, unmated, and calling females could be commonly observed in the plot treated with 500 g/hectare, but searches in the environs around that plot only yielded mating or ovipositing female moths. As indicated in Table 1, male moths were present in all plots, but mating of females was noticeably rare in the plot with the 500 g/hectare treatment.

DISCUSSION

The results of these studies give insight into the mechanism of mating disruption effected by atmospheric permeation with racemic disparlure: atmospheric disparlure appears to camouflage attractant point sources. This is indicated by the finding that low disruptant levels caused substantial reductions in catch by traps baited with small amounts of attractant. Traps containing higher concentrations of attractant were consistently less affected. As disruptant treatments were increased from 5 to 50 to 500 g/hectare, catch in traps baited with increasing concentrations of attractant was consecutively reduced. If reduced trap catch was the result of males spending more time orienting to "competing" disruptant tapes (false-trail-following), treatment trap catch would have been generally reduced at all trap-bait concentrations. In effect, moth density would simply appear lower and the shape of the dose–response curves would have been similar to those before treatment. Indeed, such was not the case, as the shape of the treatment curves was very much influenced by disruptant concentration. Additionally, the racemic disparlure disruptant is a weak attractant, and

male moths would not be expected to spend much time searching such sources (Cardé, et al., 1977), nor were they observed doing so in this study. In tests with the western pine shoot borer, *Eucosma sonomana*, Daterman et al. (1982) obtained somewhat similar results, and they concluded that males were unable to locate trap baits because disruptant particles were more attractive. In such cases where the synthetic pheromone is used as the disruptant, false-trail-following (taken in the context where males were actually diverted and temporarily occupied by attractant point sources) is a tenable theory.

Adult male moth density (based on visual census) was higher in treated plots than in control plots. This rules out the possibility that high airborne levels of disruptant cause males to leave the treatment area, resulting in reduced trap catch and mating incidence. This contradicts a previous report (Granett and Doane, 1975) that moths are less numerous in areas with high airborne disparlure levels than in untreated areas. The differences observed here are thought to be due to plot location, but the possibility that moths were attracted to the treated plots cannot be ruled out. Note that pretreatment trap catch was higher in treated plots, suggesting higher local population density (Figures 1A–D).

The rarity of female mating in the plot treated with 500 g/hectare indicates that even at very high male moth densities (the daily pretreatment catch in four traps with 100 μg (+)-disparlure was 401 males in this plot), mating can be reduced. The frequent observation of unmated, calling native females in high-density sites is unusual. In high-density populations, female mating typically occurs within a few hours of eclosion and within minutes of placement (Doane, 1968). This finding suggests that the mating disruption technique could be effective in populations denser than previously hypothesized (Schwalbe et al., 1979; Cameron, 1973). Most literature on this subject alludes to the applicability of the technology to "low-density" populations.

Based upon laboratory emission measurements from disruptant dispensers similar to those used in this test, 1.5 g released/hectare/day (in the 500 g/hectare plot) was necessary to substantially reduce mating success at the high population densities in which these tests were run. Improvements in controlled-release formulations may offer new opportunities to develop the mating disruption technique for managing gypsy moth populations.

The limited observations indicating that the precopulatory period of female moths was similar in the control, 5 g, and 50 g/hectare plots is intriguing and suggests that those females that attracted mates produced enough attractant to be readily perceptible above atmospheric levels by males. There is considerable variation in pheromone production among individual females (Charleton and Cardé, 1982). Camouflaging was implicated as the mechanism of disruption in tests with pink bollworm moths (*Pectinophora gossypiella*) (Doane and Brooks, 1980) and Oriental fruit moths (*Grapholitha molesta*) (Cardé, 1980). If camouflaging is the governing principle in this mating disruption system, increasing

atmospheric concentrations should mask the presence of increasing proportions of the female population. Obviously, those that release perceptible amounts of pheromone should attract males, at least at high moth densities where long range plume-following is not necessary for locating females. Indeed, in dense populations, females may be located (via tactile cues) and mated before they commence calling (Cardé and Hagaman, 1984). The evidence that population control can be affected at low densities with lower (and economical) disruptant rates is still incomplete, however.

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FERULATE ESTERS OF HIGHER FATTY ALCOHOLS AND ALLELOPATHY IN *Kalanchöe daigremontiana*

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Abstract—Ferulate esters of normal C_{22}–C_{30} alcohols were found in the root extract of *Kalanchöe daigremontiana* and the free *n*-C_{30} alcohol, triacontanol, was found on the leaves. Ferulic acid was isolated from the vermiculite in which plants were grown. Whole plant and tissue culture experiments were done to investigate the role of ferulic acid as an allelochemical and of triacontanol as a plant growth regulator in *K. daigremontiana* and other bioassay systems. No positive growth responses to triacontanol were observed, but inhibitation of growth response of plantlets by ferulic acid was seen.

Key Words—*Kalanchöe daigremontiana*, triacontanol, ferulate esters, ferulic acid, tissue culture, allelopathy.

INTRODUCTION

Mature plants of *Kalanchöe daigremontiana* (Crassulaceae) have been shown to display an allelopathic influence on the growth and development of their own plantlets (Groner, 1974) as well as on other species of plants (Groner, 1975). Extracts of *K. daigremontiana* were shown to have an inhibitory effect on the germination and survival rates of various plant seedlings (Groner, 1975). The phenolic glucoside, bryophyllosid, was suspected to be one of the allelopathic agents present in *K. daigremontiana* (Karsten, 1965). In another context, Williams and Smith (1984) have reported that the leaves of this plant were toxic to chicks at a dosage of 8–12 mg/g body weight, but the toxic principles were not chemically characterized. Several other phenolic components and flavonoid glycosides isolated by Gaind and Gupta from the leaves of *K. daigremontiana* were not characterized fully (Homans and Fuchs, 1970; Gaind and Gupta, 1971).

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These authors did, however, analyze the sterols and alkanes present in the leaves (Gaind and Gupta, 1971).

In addition to the toxicity mentioned in these reports, *Kalachöe* species exhibit the phenomenon of asexual reproduction and are usually grown as curiosities because of the plantlets borne on leaf margins. The scientific explanation for this phenomenon has led to several investigations for plant growth regulation and for hormonal requirements during in vivo and in vitro propagations of *Kalanchöe* species (Smith and Nightingale, 1979; Kazaryan and Gevorkyan, 1985).

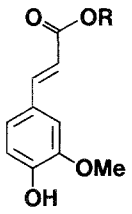
The allelopathic effect of the mature *K. daigremontiana* plant on its own plantlets is thus stimulatory as well as inhibitory. These effects, particularly the former, prompted us to investigate this phenomenon further, and to look for a possible chemical explanation (Burke, 1985; Nair and Burke, 1986). Furthermore, the availability of tissue culture methods involving *Kalanchöe* species (Thomas and Stobart, 1971; Smith and Nightingale, 1979) provided us with an opportunity to shorten the experimental time and to compare the effects of plant tissue in vitro with whole plants.

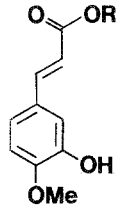
METHODS AND MATERIALS

General. Kalachöe daigremontiana were grown in the greenhouse in a bedding mix (20% peat, 15% sand, 15% red cinder, and 50% fir bark) supplied by Shelton Transfer, San Jose, California, and were harvested at eight months of age. In experiments aimed at extracting the potting medium, vermiculite was substituted for the bedding mix. Over the growth periods, the greenhouse temperature varied between a minimum of 15° C at night and a maximum of 28° C in the day. The relative humidity values ranged from 40% to 50% at night to 20% to 30% in the day. [^{1}H]- and [^{13}C]NMR spectra were done on a Varian XL-300 spectrophotometer, 300 MHz for proton and 75 MHz for carbon. High-resolution mass spectra (70 eV) were obtained on VG analytical 7070E spectrometer. GC analyses were conducted on a Perkin-Elmer Sigma 2000 gas chromatograph and OV-1 and DB-5 capillary columns, 25 m long and 0.25 mn in diameter. Unless specified, all GCs were done under gradient conditions with an initial temperature of 150°C, held for 2 min, and the temperature raised to 310°C at a rate of 10°C/min, and held for another 20 min. Both the detector and injector temperatures were 300°C. IR and UV spectra were recorded on Perkin-Elmer 1420 and Perkin-Elmer Lambda 5 spectrophotometers, respectively. To test for growth enhancement by triacontanol, three tissue culture bioassays were used measuring variations in fresh and dry weight of callus and suspension cultures. Unless otherwise indicated, the triacontanol was dissolved

in acetone and added to the media before autoclaving. The acetone concentration before autoclaving for all treatments including the control was 1%.

Extraction of Potting Medium and Isolation of Ferulic Acid. The vermiculite (200 g) in which the *K. daigremontiana* was grown was Soxhlet extracted in the dark with methanol for nine days. The methanol solution, evaporated to dryness in vacuo, gave a brown gum (610 mg) which showed seed germination and growth inhibition against lettuce and cress (Goldsby and Burke, 1987). This crude gum was triturated with chloroform, and removal of the solvent yielded a brown solid (240 mg), which, upon TLC purification (silica plates, 4:1 $CHCl_3$–MeOH) and activity-directed bioassay based on the inhibition of germination of lettuce and cress seeds, resulted in a UV-positive band, compound 1. This band was further purified by HPLC on a C18 column (5% ODS, MeCN–





1 R = H

2 R = $C_{22}H_{45}$

3 R = $C_{24}H_{49}$

4 R = $C_{26}H_{53}$

5 R = $C_{28}H_{57}$

6 R = $C_{30}H_{61}$

7 R = $C_{28}H_{57}$

H_2O, 60:40) and shown to be ferulic acid (30 mg) by spectral data and by comparison with authentic ferulic acid. In an attempt to evaluate the presence of other phenolics, the crude gum was silylated and the product subjected to GC-MS analysis. The result indicated only ferulic acid as the silyl derivative. Other minor, less polar components in the crude soil extract, which were also UV-positive on the TLC plates, did not yield silyl derivatives and were not acids or phenolics. They were not characterized. The natural and commercial samples of ferulic acid were also assayed for seed germination activity on cress and lettuce seeds.

Extraction of Roots and Isolation of Ferulate Esters. Fresh roots (wet weight 35 g) were blended with acetone (600 ml) and the resulting extract evaporated to dryness (520 mg) in vacuo. The crude extract was bioassayed using

the seed germination activity of cress and lettuce seeds as mentioned above. Since there was no activity, the UV-positive bands on TLC (silica plates, 4:1 $CHCl_3$–MeOH) were purified by TLC and the resulting product, A, (29 mg) appeared as a single spot. An attempt at further purification of this portion of HPLC was not successful.

Product A. Amorphous solid, UV (MeOH) 326 and 203 nm and with KOH in MeOH, 387 and 207 nm; IR ($CHCl_3$) 3540 (OH), 1700 (carbonyl), 1640 (olefinic), 1600 (aromatic) cm^{-1}; [^{1}H]NMR ($CDCl_3$) indicated that product A is a mixture of ferulate esters of higher fatty alcohols and sterols: The signals are δ 0.86 (3H, t, J = 6 Hz, CH_3), 0.96–1.50 (br s, CH_2), 1.69 (2H, m, CH_2), 4.16 (2H, t, J = 6 Hz, OCH_2), long chain alkyl; 5.50 (m, H-6), 3.70 (m, H-3) free sterols; 5.85 (1H, bs, exchanged with D_2O, OH), 6.26 (1H, d, J = 16 Hz), 6.91 (1H, d, J = 8 Hz, H-5), 7.07 (2H, overlapping signals, H-2, H-6), 7.58 (1H, d, J = 16 Hz), ferulate moiety. [^{13}C]NMR ($CDCl_3$) ppm, 167.38 (C=O), 144.60 (C-α), 123.03 (C-β), 146.0, 130.0 (aromatic C—O); 127.03, 115.62, 114.64, 109.18 (aromatic C), 76.57 (O—CH_2); 31.91, 29.69, 29.59, 29.54, 29.44, 29.35, 29.29, 28.75, 25.98, 22.68 (CH_2s of the fatty alcohol); 14.13 (CH_3 of fatty alcohol) 139.21, 121.23 (C-5, C-6 sterols) 72.86 (C-3 sterols); HRMS, *m/z*:6, 614.528 ($C_{40}H_{70}O_4$, M^+, req. 614.527); 5, 586.492 ($C_{38}H_{66}O_4$, M^+, req. 586.481); 4, 558.462 ($C_{36}H_{62}O_4$, M^+, req. 558.465); 3, 530.434 ($C_{34}H_{58}O_4$, M^+, req. 530.434); 2, 502.4034 ($C_{32}H_{54}O_4$,M^+, req. 502.402); 194.057 ($C_{10}H_{10}O_4$), and 177.058 ($C_{10}H_9O_3$) for the ferulate fragments. Mild alkali hydrolysis of product A afforded ferulic acid, found to be identical to an authentic sample.

Synthesis of Ferulate Ester with C_{22} Alcohol. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), 194 mg, and behenyl alcohol, *n*-$C_{22}H_{45}OH$, (326 mg) were dissolved in dry benzene (100 ml) and a drop of conc. H_2SO_4 added as catalyst. Water formed during the reaction was removed using a Dean Stark appartatus. The product on purification by TLC afforded a white solid, 135 mg, UV (MeOH) 326(ϵ = 4930), 202 (ϵ = 7630) nm, shifting with KOH in MeOH, 387 (ϵ = 7496), IR ($CHCl_3$) 3540 (OH), 1700 (C=O), 1640 (C=C), 1600 (aromatic) cm^{-1}; [^{1}H]NMR ($CDCl_3$) δ 0.86 (3H, t, J = 6 Hz, CH_3), 0.96–1.50 (br s, alkane CH_2), 1.69 (2H, m, CH_2), 4.16 (2H, t, J = 6 Hz, OCH_2), 5.85 (1H, bs, exchanged with D_2O, OH), 6.26 (1H, d, J = 16 Hz, Hα), 6.91 (1H, d, J = 8 Hz, H-5), 7.07 (2H, overlapping signals, H-2 and H-6), 7.58 (1H, d, J = 16 Hz, Hβ); [^{13}C]NMR ($CDCl_3$) ppm, 167.38 (C=O), 144.60 (C-α), 123.03 (C-β), 146.0, 130.0 (aromatic-C—O), 127.03, 115.62, 114.64, 109.18 (Ar—C), 76.57 (O—CH_2), 31.91, 29.69, 29.59, 29.54, 29.44, 29.35, 29.29, 28.75 25.98, 22.68 (CH_2 of the alkane side chain), 14.13 (CH_3); EI-MS*m/z*: 502 (100) M^+, 194 (85), 177 (65).

Extraction of Leaves of Isolation of Triacontanol. Leaves of *K. daigre-*

montiana were removed from the stem (130 g wet weight) and blended with acetone in a Waring blender. The extract (600 ml) was dried *in vacuo* under reduced pressure to yield a crude gum (6.5 g) which was dissolved in methanol and filtered. The methanol-insoluble precipitate (1.32 g) was water soluble and tested positively for oxalates. The methanol-soluble portion was evaporated to dryness and stirred with $CHCl_3$. Removal of $CHCl_3$ gave a green gum (2.7 g). All extracts were bioassayed against cress and lettuce seed germination. The extracts were further purified by TLC on silica plates and assayed for seed germination activity on cress and lettuce seeds.

Fresh leaves of *K. daigremontiana* (100 g) were washed with $CHCl_3$. The wash was evaporated to dryness in vacuo. This crude extract (250 mg) was redissolved in acetone and the solution cooled and filtered. The residue (29 mg), product B, was analyzed by GC and found to be triacontanol, [^{1}H]NMR ($CDCl_3$), δ 1.01 (3H, t, CH_3), 1.5 (52H, bs, CH_2), 2.78 (4H, m, CH_2), 3.82 (2H, t, J = 2 Hz, O—CH_2), 4.20 (1H, s, exchanged with D_2O, OH). This was identical to the [^{1}H]NMR spectrum of the standard triacontanol.

Whole Plant Experiments with Standard Ferulic Acid, Extracts from Potting Medium, and Water-Soluble Fraction from Leaf Extract. Ferulic acid, the crude extract of the potting medium in which the *K. daigremontiana* plants were grown, and the water-soluble portion containing oxalates from the leaf extracts of the same plant were dissolved in DMSO–H_2O (1:99) to obtain concentrations of 50 and 250 ppm (pH ~5.5). Ten plantlets were placed on filter paper in a Magenta box after weighing. Solutions (10 ml) of the test compounds were placed on the filter paper and kept in the growth chamber. Each concentration had three replicates with 10 plants. Fresh weights were recorded on day 0, 7, and 14. On day 14 the plantlets were sacrificed for dry weights. These results were evaluated against controls.

Whole Plant Experiments with Triacontanol. K. Daigremontiana plantlets obtained from the leaves of matured plants were used for the assay. Triacontanol was dissolved in acetone (1 mg in 100 ml). Aliquots (1 ml) of this solution were mixed with 9 ml of Hoagland's solution in a test tube. A filter paper was placed on the surface of each solution and one plantlet was placed on the filter paper. A control was set similarly. There were 10 replications for both. The plantlets were kept in a growth chamber at 25°C under a 16:8 hr light–dark photoperiod. Each plantlet was weighed at day 0, 7, 14, and 21. On day 21, they were sacrificed to obtain the dry weight.

In another experiment, triacontanol was sprayed on the plantlets. Five plantlets were placed on a filter paper in a sterilized container which already contained 10 ml of Hoagland's solution. Triacontanol (1 mg) was dissolved in $CHCl_3$ (0.5 ml) and made up in water to achieve 1 mg in 100 ml (Ries and Wert, 1982). This solution was warmed, while stirring, in order to displace the

$CHCl_3$, cooled to room temperature, and sprayed uniformly on the plantlets in the container (Ries and Wert, 1982). Ten such pots containing five plantlets each were set. A control of 10 pots minus triacontanol was also set. All the pots were kept in the growth chamber, and their weights were recorded at 0, 7, 14, and 21 days. On day 21, plantlets from each pot were sacrificed to get the dry weight.

Tissue Culture Experiments with Triacontanol. Calluses were obtained from leaves of *K. daigremontiana* grown on MS medium (Murashige and Skoog, 1962) modified with Nitsch's vitamins and supplemented with 10 mM α-naphthalene acetic acid and 1mM *N*-6-benzyladenine (Karp and Sink, 1976). The calluses were transferred six or more times before being used in the bioassay. Ten pieces of callus tissue, averaging 0.12–0.16 g each, were placed on 30 ml medium in 100 × 15-mm Petri dishes. The plates were maintained under constant low light of 39 μE/sec/m^2 at 27°C. After 14 days, the mass of callus per plate was determined collectively. Ten plates were weighed for each treatment. The initial callus weights at day 0 were substracted from the weights at day 14; consequently the data describe the growth in grams during 14 days. The callus per plate was dried, and dry mass expressed as a percentage of fresh mass. In the second experiment *N*-6-benzyladenine was replaced by triacontanol.

The hypocotyls of aseptically germinated sunflower seedlings *Helianthus annuus* L. (variety Mammoth Grey Stripe) were sliced 2 mm thick and grown on MS medium with 4.4 mM *N*-6-benzyladenine (Greco et al., 1984). The individual slices averaged 5.3 mg. The plates were cultured for two weeks under low constant light of 39 μE/sec/m^2 at 27° C. Fourteen plates with 10 hypocotyl calluses per plate were weighed per treatment. For fresh weight determinations, the calluses were weighed individually, and for dry weights, the 10 calluses per plate were combined.

Suspension cultures of alfalfa (*Medicago sativa* L.) (variety Caygo UC) were grown in SH medium (Schenk and Hildebrandt, 1972) supplemented with 25 mM α-naphthalene acetic acid and 10 mM kinetin (Stuart and Strickland, 1984). Approximately 200 mg fresh weight of cells were inoculated into 20 ml of medium in 125 ml Delong flasks. The flasks were placed on a gyratory shaker at 125 rpm at 28°C in the dark. The cells were collected after 7 or 14 days with gentle vacuum on weighed filter disks and washed with 10 ml of distilled water. For each data point, the contents of five flasks were individually weighed. The fresh and dry weights of the cells were determined.

Bioassays were developed using tissues *K. daigremontiana* because triacontanol was found on its leaves, and such tissues would provide a useful comparison between whole plants and plant tissue in cultures. Alfalfa also has been

shown to produce triacontanol. Sunflower was an additional source of tissue for the study of initiation of callus formation.

RESULTS AND DISCUSSION

The earlier chemical investigations of the biologically active chemicals associated with *K. daigremontiana* have indicated the presence of nontoxic levels of oxalates, alkaloids, and nitrates in the leaves (Williams and Smith, 1984), but these studies have not defined the allelochemicals that were produced in the vermiculite in which *K. daigremontiana* plants were grown nor those that affected the plants whose development and growth were inhibited (Groner, 1975). Our investigation on the allelopathy of *K. daigremontiana* has identified an allelochemical in the potting medium, a conjugate of this compound in the roots, and a reported plant growth regulator in leaves of this plant.

In order to identify the allelochemicals in the potting medium, *K. daigremontiana* plants were grown in the greenhouse for eight months using vermiculite. Nutrients were added over the growth period to ensure growth. The vermiculite in which the plant had grown was extracted with cold methanol. Removal of the solvent gave very little residue. Soxhlet extraction of this vermiculite in the dark with methanol for nine days gave a brown gum, the $CHCl_3$-soluble portion of which was purified. This purification was guided by our bioassay against cress and lettuce seeds. Ferulic acid, compound 1 (0.05% to the total extract) was obtained. This was distinguished from isoferulic acid by comparison of the UV spectrum and the base shift with that of the latter, by GC-MS of the silyl derivative, and by direct comparison to an authentic sample of ferulic acid. Silylation of the total extract did not indicate any other phenolics. Bioassy of ferulic acid against *K. daigremontiana* plantlets and in our sensitive evaluation against lettuce and cress seeds showed significant activities. Evaluation of activity against lettuce and cress is particularly useful as a preliminary indicator of activity because of the high percentage of germination and the speed with which these seeds germinate.

The Soxhlet extraction of a blank vermiculite on which no plant was grown, but which was processed similarly, gave no such product. The very low yield of ferulic acid from the rooting medium is to be expected since organics tend to bind very strongly and sometimes almost irreversibly to such medium (Dalton et al., 1983; Nishimura et al., 1984).

The roots of the same plant which were grown in vermiculite gave a mixture of products when extracted with acetone. Bioassay of the crude root extract and a purified fraction, product A, on seed germination of cress and lettuce, and the assay against the fungus, *Cladosporium herbarum* (Homans and Fuchs, 1970), did not show any activity. Despite the lack of activity, product A was

evaluated because, like ferulic acid, it showed strong UV absorption on TLC plates. Product A consisted mainly of *n*-alkyl-3-methoxy-4-hydroxy cinnamates, compounds 2–6. Spectral data (UV, IR, [^{1}H]NMR, and MS) of this mixture indicated the presense of traces of stigmasterol and campesterol, in addition to the ferulate esters. The HPLC and TLC methods of separation used did not achieve separation of the sterols from the ferulate esters. However, the presence of these esters and sterols coexisting in the above purified fraction of root extract, product A, was established by fractional separation under MS conditions (Figures 1 and 2) and by GC. The total ion current (TIC) showed separation into these two groups of compounds (Figure 1). The broad peak centered at scan 63 (Figure 2) represented the ferulate esters showing mass measurements of 502, 530, 558, 586, and 614 as molecular ions, M^+, for these esters and 194 for the ferulic acid fragment. Similar mass measurements of the other peaks centered at scan 45 showed molecular ions, M^+, at 412 and 400, and a spectrum which indicated stigmasterol and campesterol, respectively. High-resolution mass spectra on the mixture confirmed this distribution of compounds. A selective ion chromatogram indicated that ions at *m/z* 194 and 177 for the ferulic acid moiety coincided with the TIC of the ferulate esters and were not related to the sterols. The sterols were thus free sterols. UV and base shift comparisons supported the presence of ferulate (4-hydroxy-3-methoxy cinnamate) rather than the isomeric 3-hydroxy-4-methoxy cinnamate. The [^{1}H]- and [^{13}C]NMR spectra (see Methods and Materials) of this mixture supported the

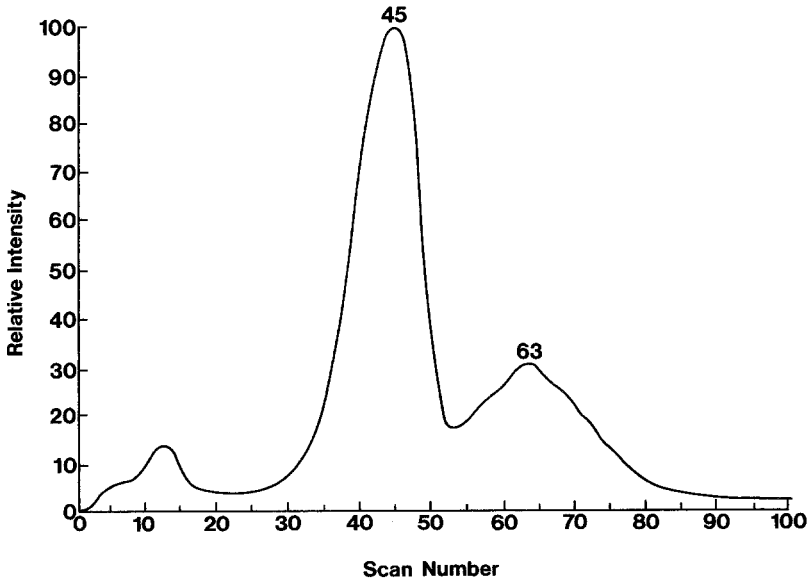


FIG. 1. Total ion current (TIC) of EI mass spectrum (70 eV) of product A.

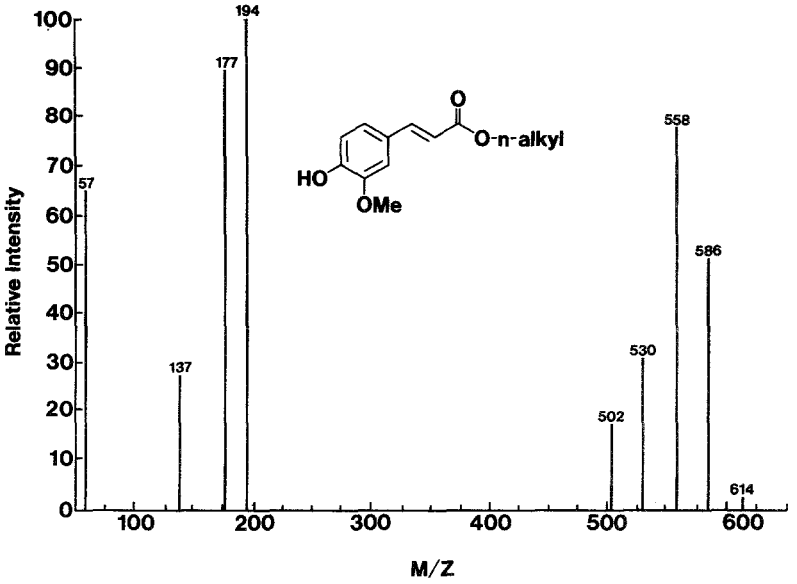


FIG. 2. EI mass spectrum (70 eV) of scan 63 of the TIC obtained from product A.

assignments from MS and UV data. In particular, the [^{1}H]NMR signal at δ 3.70 is characteristic of H-3 of a free sterol, while the triplet at δ 4.16 supports the primary ester of the fatty alcohols.

In order to verify this further, one of the ferulate esters $C_{22}H_{45}OH$ was synthesized from behenyl alcohol and ferulic acid. MS of the purified product (Figure 3) showed the expected molecular ion of 502 as the highest mass. The rest of the spectrum was similar to the spectrum of the mixture of esters. Also, other spectral data such as the IR, UV, [^{1}H]NMR, and [^{13}C]NMR were similar to that of product A. Since the molecular ion, M^+, at 502, is the base peak in this single-component spectrum, the relative ratio (8, 17, 44, 29 and 2) of the molecular ions of compounds 2–6 in the mixture of esters is a very good indication of the relative percent proportion in which these esters (C_{22}, C_{24}, C_{26}, C_{28}, and C_{30}) exist naturally in the roots of *K. daigremontiana*.

The presence of ferulic acid and ferulate esters in the rooting medium and roots, respectively, prompted us to reinvestigate (Williams and Smith, 1984) the leaves of *K. daigremontiana* for the existence of related substances. Acetone extraction of the fresh leaves revealed an extremely high concentration of water solubles (20% of the total extract by weight). Seed germination bioassay on cress and lettuce seeds of the crude extract exhibited a moderate level (40%) of inhibition of germination at 250 ppm level. When the dried acetone extract was reconstituted by trituration with methanol, the methanol-soluble portion was inactive in our bioassay against cress and lettuce seeds. However, the residue

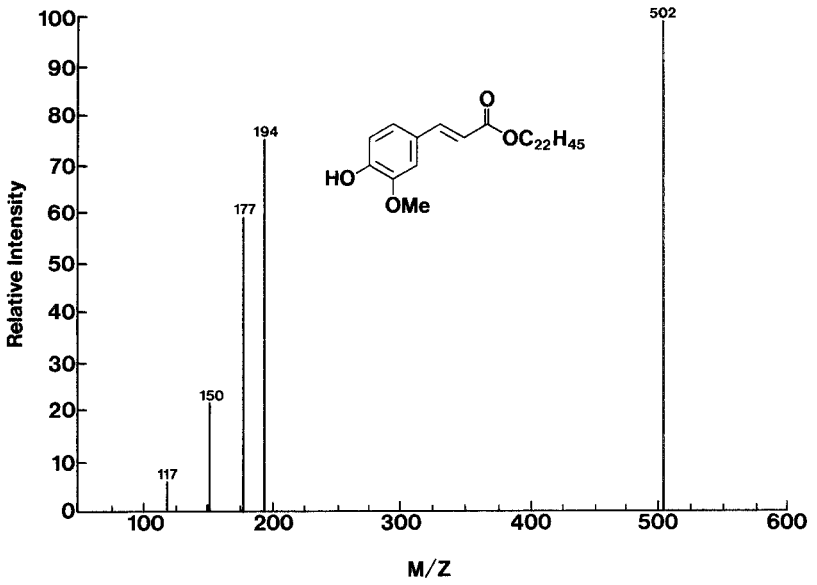


FIG. 3. EI mass spectrum (70 eV) of the synthetic ferulate ester, *n*-docosanyl-4-hydroxy-3-methoxycinnamate, compound 2.

that was water soluble and consisted largely of oxalates was toxic and inhibited the seed germination of cress and lettuce at the 250 ppm level. A fungal bioassay using the saprophyte, *C. herbarum*, was negative (Homans and Fuchs, 1970).

Alkanes of chain length varying from 22 to 35 were reported earlier in the leaves of *K. daigremontiana* (Gaind and Gupta, 1971). We therefore extracted the epicuticular leaf surfaces. Fresh leaves were washed with $CHCl_3$ and solvent then removed in vacuo. The colorless extract was redissolved in acetone and cooled. A precipitate thus formed, product B, which was filtered off and analyzed by GC. Both the crude leaf washings and the partially purified product B contained triacontanol, the C_{30} normal alcohol. Crude leaf washing contained alcohols of chain lengths C_{22}–C_{30} and the sterols. Product B contained sterols as well.

Ferulic acid is a well-known allelopathic agent (Putnam, 1983; Dalton et al., 1983; Liebl and Worsham, 1983). It has been suggested by Liebl and Worsham (1983) in their studies with prickly sida that the activity of ferulic acid as an allelochemical may be enhanced by microbial decarboxylation to its styrene derivative, a compound which is even more potent than ferulic acid. Triacontanol is a reported plant growth regulator (Ries and Wert, 1982) and is now found as a conjugate of ferulic acid in the roots and as a component of the leaves. No free ferulic acid was detected in the roots. It therefore seems that

the allelopathic activity of this plant is due to the ferulic acid exuded into the soil from the roots. This further suggests that the ferulic acid which is sequestered as esters with the long-chain fatty alcohols in the roots is nontoxic to the host plant, but the esters are cleaved to yield ferulic acid which is free to act in the soil as an allelochemical. Triacontanol, on the other hand, may act as a plant growth regulator (PGR). To examine this PGR effect of triacontanol on *K. daigremontiana*, tissue culture (Table 1) and whole plant experiments (Figures 4 and 5) were conducted.

In the tissue culture bioassays, there was no reproducible increase in the wet or dry weights during the two-week growth cycles with calluses of *K. daigremontiana* or of sunflower hypocotyls, or of suspension cultures of alfalfa (Table 1). In experiment 1, calluses of *K. daigremontiana* on media with the hormone, *N*-6-benzyladenine, increased the fresh weight by four fold. No effect was evident when treated similarly with triacontanol. The calluses of *Kalanchöe* on media without hormones showed a 30% decrease in fresh weight, but again there was no appreciable growth response to triacontanol. These results suggest that triacontanol cannot replace the hormonal requirements of this tissue. The hormone-supplemented portion of the first experiment was repeated with similar results. The dry weights did not show any response to triacontanol. The growth of the sunflower calluses was not enhanced with additions of triacontanol. When the concentration of tricaontanol was 10^{-5} M there appeared to be a slight growth inhibition with a marginal increase in percent of dry matter (Table 1).

With suspension cultures of alfalfa, no growth enhancement by triacontanol was observed. An analysis of variance for each day, as well as two-sample *t* tests, did not indicate any statistical differences between controls and triacontanol treatments. The standard deviations are large enough to mask possible minor differences in response.

Whole plant experiments with individual plantlets of *K. daigremontiana* treated with triacontanol at the roots did not show any significant difference in the fresh or dry weights (Figure 4). In addition, plantlets sprayed with triacontanol suspension (Ries and Wert, 1982) did not show any difference in the fresh or dry weights (Figure 5). Solubility of triacontanol is a major problem in conducting bioassays and may have contributed to the lack of any positive results for PGR activity of triacontanol.

Young plantlets treated with ferulic acid and crude ferulic acid extracts, isolated from the substrate in which the plantlets of *K. daigremontiana* were grown, showed considerable inhibitory effects on their growth (Figure 6). Plantlets treated with total potting medium extract and with ferulic acid had their roots decayed and the leaves chlorotic. In contrast, aqueous portions containing oxalates derived from the acetone extracts from leaf extracts showed no such activity but showed more root growth than the control, a result significantly different from that of inhibition shown in the seed germination assay.

TABLE 1. BIOASSAYS FOR GROWTH ENHANCEMENT WITH TRIACONTANOL[a]

Tissue and treatment	Triacontanol (M)				
	0	10^{-11}	10^{-9}	10^{-7}	10^{-5}
Kalanchöe callus, average growth in 14 days					
Experiment 1, with/without *N*-6 benzyladenine					
Fresh weight with *N*-6-benzyladenine (g ± SE)	4.87 ± 0.32	4.92 ± 0.42	4.38 ± 0.45	4.82 ± 0.41	4.67 ± 0.40
Fresh weight without hormone (g ± SE)	0.97 ± 0.08	0.93 ± 0.08	0.95 ± 0.09	0.95 ± 0.07	0.94 ± 0.10
Experiment 2, with *N*-6-benzyladenine					
Fresh weight (g ± SE)	4.16 ± 0.26	3.91 ± 0.28	3.74 ± 0.22	4.24 ± 0.33	3.65 ± 0.26
Dry weight as a percent of fresh weight (% ± SE)	4.5 ± 0.2	4.2 ± 0.04	4.3 ± 0.04	4.2 ± 0.1	4.2 ± 0.1
Sunflower callus, average growth in 14 days					
Fresh weight per hypocotyl slice (mg ± SE)	207.4 ± 8.2	199.9 ± 8.1	211.9 ± 9.3	203.5 ± 6.7	171.5 ± 7.3
Dry weight as a percent of fresh weight (% ± SE)	5.2 ± 0.3	5.3 ± 0.3	5.3 ± 0.3	5.4 ± 0.3	6.5 ± 0.5
Alfalfa suspension cultures, average per flask					
Day 0					
Fresh weight (mg ± SE)	190 ± 12.5				
Dry weight (mg ± SE)	25 ± 3.8				
Day 7					
Fresh weight (mg ± SE)	744 ± 125.0		702 ± 108.0	804 ± 141.0	661 ± 89.0
Dry weight (mg ± SE)	70 ± 10.0		64 ± 8.7	65 ± 8.3	54 ± 8.0
Day 14					
Fresh weight (mg ± SE)	1188 ± 427.0		2115 ± 463.0	1864 ± 629.0	1890 ± 570.0
Dry weight (mg ± SE)	89 ± 34.0		159 ± 26.9	134 ± 36.6	130 ± 37.9

[a] See text for experimental details.

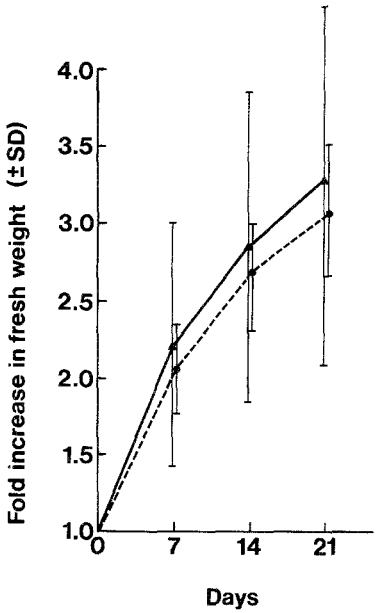


FIG. 4. Comparison of growth at days 7, 14, and 21 of plantlets of *K. daigremontiana* grown on filter paper impregnated with triacontanol (1 ppm). The fresh weight of the plantlets is expressed as -fold increase over weight at day 0 ± SD. Solid line, treated with triacontanol; dashed line, control.

In other bioassays against lettuce and cress seeds, fractions containing ferulate esters were noninhibitory. By contrast, ferulic acid showed good allelopathic activity, inhibiting 100% of seeds at 250 ppm concentrations during the seed germination assays. It is very clear that the allelopathic influence shown by *K. daigremontiana* can be attributed to free ferulic acid which, when sequestered as the nontoxic acid conjugates with the long-chain fatty alcohols, is not toxic to the host plant. These ferulate esters (compounds 2–6) are unprecedented as natural products, although the isoferulate of octacosanol, *n*-octacosanyl 3-hydroxy-4-methoxycinnamate, compound 7, isolated from the stem bark of *Erythrina senegalensis*, *E. glanca*, and *E. mildbaedii* (Fomum et al., 1986) has been reported since the completion of this work. The free ferulic acid presumably acts on young *K. daigremontiana* and other plantlets but is ineffective on the parent. Dalton et al. (1983) have indicated that ferulic acid is bound most tightly to the topsoil, where the concentration of organic matter is greatest. It is here perhaps, where ferulic acid becomes concentrated, that it is most effective on young plantlets or germinating seeds, because the young roots are in contact with this bound allelochemical.

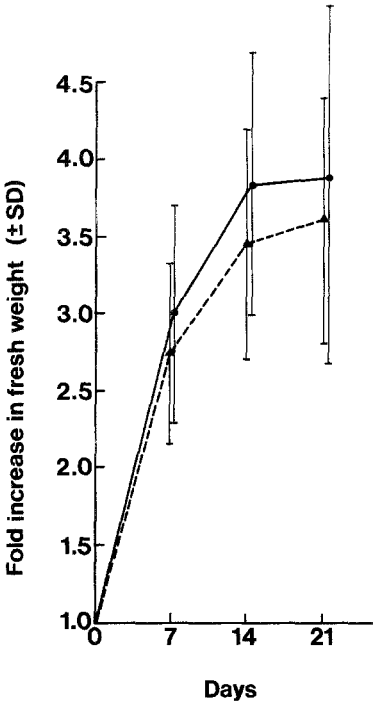


FIG. 5. Comparisons of growth at days 7, 14, and 21 of plantlets of *K. daigremontiana* after foliar spray with a solution of triacontanol (1 ppm). The fresh weight of the plantlets is expressed as -fold increase over weight at day 0 ± SD. Solid line, treated with triacontanol; dashed line, control.

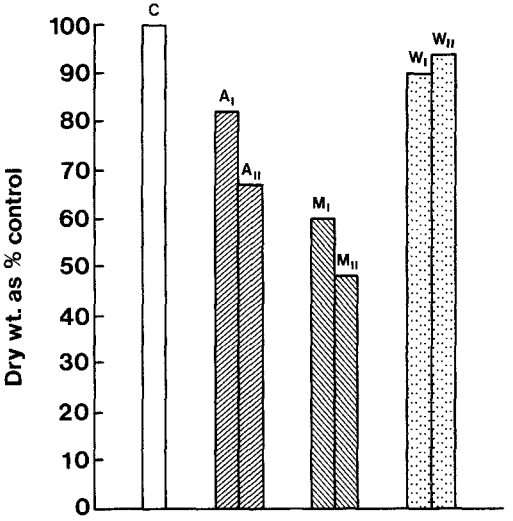


FIG. 6. Growth of plantlets of *K. daigremontiana* in solutions of ferulic acid, water-soluble portion containing oxalate and crude extract of the soil at 50 (I) and 250 (II) ppm concentrations. Data expressed as percent of control after 14 days. C = control, A = ferulic acid, M = methanol extract of the soil, W = water-soluble portion containing oxalates.

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SEX PHEROMONE COMPONENTS OF THE OBLIQUE-BANDED LEAFROLLER, *Choristoneura rosaceana*[1] IN THE OKANAGAN VALLEY OF BRITISH COLUMBIA[2]

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Abstract—(*Z*)-11-Tetradecen-1-yl acetate, (*E*)-11-tetradecen-1-yl acetate, and (*Z*)-11-tetradecen-1-ol were previously reported as the sex pheromone in New York strains of the oblique-banded leafroller, *Choristoneura rosaceana* (Harris), and (*E*)-11-tetradecen-1-ol was tentatively identified in female tip extracts. For Okanagan Valley strains of *C. rosaceana*, an additional component, (*Z*)-11-tetradecenal, was identified from female tip extracts by splitless capillary gas–liquid chromatography and mass spectroscopy and was strongly stimulatory in electroantennogram studies. In field tests, 3 mg of 96.5:2:1.5 (*Z*)-11-tetradecen-1-yl acetate, (*E*)-11-tetradecen-1-yl acetate, and (*Z*)-11-tetradecen-1-ol (containing approx. 1% *E* isomer) was not as attractive as female-baited traps, and significant numbers of European leafroller *Archips rosanus* L. were attracted. The above blend with 1% (*Z*)-11-tetradecenal added was significantly more attractive than traps baited with

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female *C. rosaceana*, but *A. rosanus* males were still attracted. Increased percentages of (*Z*)-11-tetradecenal up to 4% caused increased catches of *C. rosaceana* and decreased catches of *A. rosanus*. Low amounts of (*E*)-11-tetradecen-1-ol and (*E*)-11-tetradecenal male also contribute to increased specificity of the synthetic pheromone blend to *C. rosaceana*.

Key Words—Oblique-banded leafroller, *Choristoneura rosaceana*, Lepidoptera, Tortricidae, sex pheromone, (*Z*)-11-tetradecen-1-yl acetate, (*E*)-11-tetradecen-1-yl acetate, (*Z*)-11-tetradecen-1-ol, (*E*)-11-tetradecen-1-ol, (*Z*)-11-tetradecenal, (*E*)-11-tetradecenal.

INTRODUCTION

The oblique-banded leafroller (OBLR), *Choristoneura rosaceana* Harris, was first reported attacking apples in British Columbia in 1922 by Venables (1924). It was a serious pest of apple until 1926 when the outbreak collapsed, apparently due to the activity of parasites (Venables and Gillespie, 1926). Increased damage occurred from 1934 to 1937 (Buckell, 1937). Mayer and Beirne (1974) surveyed unsprayed orchards and native host sites in 1972 but found only very low populations of OBLR. Since 1979, however, OBLR has become an important pest of apples in the Okanagan and Similkameen Valleys (Madsen and Procter, 1982).

In 1979, preliminary attempts to monitor OBLR populations in the Okanagan Valley of British Columbia were made using a bait containing only (*Z*)-11-tetradecen-1-yl acetate (Z11-14:Ac) (Conrel Co., Needham Heights, Massachusetts) (Madsen and Madsen, 1980). Better captures of OBLR were obtained with a formulation for the European leafroller (ELR) *Archips rosanus* (L.) (Zoecon Corporation, Palo Alto, California) containing an 85:15 mixture of Z11-14:Ac and (*Z*)-11-tetradecen-l-ol (Z11-14:OH) (Roelofs et al., 1976). Traps containing another bait for OBLR in plastic capsules[9] (Zoecon Corporation, Palo Alto, California) caught few males in orchards under an integrated pest management (IPM) program in 1980, even though high levels of damage were found in 1979 (Vakenti and Peters, 1981).

Differences in response to ratios of pheromone components occur between strains of the fruit-tree leafroller (FTLR), *Archips argyrospilus* (Walker) (Roelofs et al., 1974). The response of a New York strain is dependent upon the correct *Z*:*E* ratio and the presence of a synergist, while the British Columbia strain is less specific. Specificity was linked to the presence of the sympatric species, the red-banded leafroller, *Argyrotaenia velutinana* (Walker) in New York (Roelofs et al., 1974). Similarly, ELR males in British Columbia

[9] Identity and amount of pheromone blend used in the plastic capsule formulation is not available from the company.

responded to a wide range of Z11–14:Ac and Z11–14:OH ratios, but in the Netherlands it responded better to a 90:10 acetate–alcohol mixture (Roelofs et al., 1976). However, tests in the Okanagan Valley with modifications of the ratios and amounts of the pheromone blend for eastern OBLR populations, *Z*- and *E*11–14:Ac in a 95:5 ratio and ca. 5% Z11–14:OH (Hill and Roelofs, 1979) failed to improve catches of OBLR or to eliminate cross-attraction of ELR.

We report the results of a project initiated to investigate and reevaluate the pheromone chemistry and bioactivity for OBLR in the Okanagan Valley of British Columbia.

METHODS AND MATERIALS

Insects. OBLR larvae were obtained from laboratory colonies maintained on broad beans (16:8 hr light–dark, 25°C, 60% relative humidity) or field collected and individually reared on excised apple leaves or artificial diet. Pupae were held at room temperature and natural photoperiod until adult eclosion. Emergent adults were held at 5°C until used in traps or laboratory experiments.

Analytical Procedures. Solvents were distilled and/or filtered through activity I alumina prior to use. The compounds used in this work were purchased or synthesized by standard methods. Commercial Z11–14:Ac (Albany International Co., Needham Heights, Massachusetts), was purified from a 1% *E* isomeric impurity by liquid column chromatography on 20% $AgNO_3$–silica gel. The purified acetate was used to obtain Z11–14:OH by hydrolysis, and the latter was converted into (Z)-11-tetradecenal (Z11–14:Ald) by oxidation with pyridinium chlorochromate. All compounds used were >99% pure as determined by splitless capillary gas–liquid chromatography (SC-GLC).

Abdominal tips of individual OBLR females, 1–3 days old, 2–4 hr into the scotophase, were excised and washed with freshly distilled analytical reagent grade heptane (3 μl) as described by Klun et al. (1979). Extracts were prepared by covering several excised tips with heptane and storing these for two to four days in a sealed glass ampoule at room temperature. The heptane extract was removed and washed twice with a 5% aqueous sodium bicarbonate solution (50 μl) to remove free fatty acids prior to analysis.

SC-GLC was performed on a Hewlett-Packard 5880A instrument. The fused silica columns used were (A) a 50-m, 0.2-mm-ID Carbowax 20 M (Hewlett-Packard, Avondale, Pennsylvania) and (B) a 30-m, 0.25-mm-ID DB-1, a cross-linked methyl silicone (J&W Scientific, Inc., Rancho Cordova, California). Column A was programmed at 40°C isothermal for 2 min, 30°C/min to 150°C, then 2°C/min to 210°C. Column B was operated at 80°C isothermal for 2 min, 10°C/min to 180°C, then 2°C/min to 240°C. The injector was main-

tained at 200°C with a helium carrier gas flow of 1 ml/min. A flame ionization detector was operated at a temperature of 250°C. The detection limit for (*E*)-11-tetradecen-1-yl acetate (*E*11–14 : Ac) admixed with Z11–14 : Ac under these conditions was 5% *E* in 95% *Z* for column A and 1% *E* in 99% *Z* for column B.

Double-bond position was established as described by Bierl-Leonhardt et al. (1980). The combined washes from 35 female tips were mixed with *m*-chloroperbenzoic acid (30 μg) in methylene chloride (50 μl) and sealed in a glass ampoule at 25°C. After two days, the mixture was diluted with heptane (50 μl), washed three times with a 5% aqueous sodium bicarbonate solution (50 μl), and once with water (50 μl). An aliquot was chromatographed on column B and the remainder analyzed by mass spectroscopy.

Mass spectroscopic analysis, (SC-GLC-MS), was performed on combined washes or extracts by splitless injection into a Hewlett-Packard 5985B GLC-MS, fitted with either a 30-m × 0.32-mm-ID DB-1 or a 15-m × 0.25-mm ID Carbowax C20 M column using electron impact ionization.

Electrophysiological Studies. Electroantennograms (EAGs) were performed to determine response of male OBLR antennae to functional groups of a monounsaturated (Δ11) 14-carbon chain. Standard EAG techniques (Roelofs, 1976) were used and excised antennae were suspended between AgCl-coated silver electrodes (Arn et al., 1975) in a humidified, heated stream (1 liter/min) of compressed medical air. Chemical stimuli (10 ng) were placed on filter paper in 5 ml of air, which was injected in 1 sec into the air stream 10 cm from the antenna. One antenna was used for all test compounds with a 2- to 3-min exposure to clean airflow between stimulus presentations. Solvent controls (hexane) and nonpheromonal standard stimuli (14 : Ac) were presented throughout the test series. Responses to solvent controls (hexane) prior to and after test stimuli were averaged and subtracted to correct for mechanical and solvent stimulation. Data were normalized to a standard (14 : Ac) to correct for decline in EAG amplitude as an antennal preparation aged. Data presented are based on 20 antennal preparations. Compounds tested were: Z11–14 : Ac, *E*11–14 : Ac, Z11–14 : Ald, (*E*)–11-tetradecenal, (*E*11–14 : Ald), Z11–14 : OH, and (*E*)-11-tetradecen-1-ol (*E*11–14 : OH). All compounds were >99% pure as determined by capillary gas chromatography.

Field-Trapping Procedures. In all field experiments using virgin OBLR females, a yellow, cylindrical trap (Proverbs et al., 1966) was used. This trap was effective for leafrollers (Madsen and Vakenti, 1973) and could hold females alive for up to seven days. In other experiments, Pherocon 1C traps or a similar trap from a local supplier (Ecoyoos Consulting Services, Osoyoos, British Columbia) were used. Traps were spaced at a minimum between-trap distance of 8 m within rows and were hung in apple trees at a height of 2 m. To avoid contaminated trapping surfaces that would have decreased trap efficiency (Shep-

herd, 1979; Westigard and Graves, 1976), trap bottoms were replaced if catches exceeded 10 moths/trap. Except where otherwise noted, all chemical baits were formulated on rubber cap dispensers (Arthur H. Thomas Co., Philadelphia, Pennsylvania) which were pinned inside the top portion of the trap. Details of the experimental design are reported in Tables 1–5.

1982 Field Studies. In two orchards in Cawston, British Columbia, the following formulations of OBLR pheromone were compared: three dosages (1, 3 and 5 mg) of *Z*- and *E*11–14:Ac at a 93:2 ratio and 5% *Z*11–14:OH (Hill and Roelofs, 1979), and an *A. rosanus* formulation (Zoecon AROS) containing 5 mg of an 85:15 mixture of *Z*11–14:Ac and *Z*11–14:OH (Table 1). In one orchard, another blend (Albany OBLR) formulated in a Conrel fiber packet (Hill and Roelofs, 1979) was tested. All treatments were compared against unbaited control traps and trap catches were recorded weekly.

In the Entomology Research Orchard (Agriculture Canada, Summerland, Research Station, Summerland, British Columbia), traps baited with one of seven blends of *Z*11–14:Ac, *E*11–14:Ac, and *Z*11–14:OH were compared against traps baited with OBLR females (5/trap) and unbaited controls (Table

TABLE 1. NUMBERS OF *C. rosaceana* (OBLR) MALES CAPTURED IN PHEROCON 1C TRAPS BAITED WITH 3 DOSAGES OF SYNTHETIC PHEROMONE BLEND OR 2 COMMERCIALLY AVAILABLE BLENDS, 1982, CAWSTON, BRITISH COLUMBIA[a]

Treatment	Dosage (mg)	Total number of males captured[b]	
		Zebroff orchard (June 9–July 29)	Mennell orchard (June 17–July 29)
OBLR lab[c]	1	39b	38c
OBLR lab	3	189a	57b
OBLR lab	5	173a	75ab
Zoecon AROS[d]	5	232a	96a
Albany OBLR[e]	—	—	57b
Blank control	—	0c	0d

[a] Randomized complete block layout, $N = 3$ in Zebroff orchard and 6×6 Latin square in Mennel orchard. Traps checked weekly.

[b] Treatment totals followed by the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$.

[c] *Z*11–14:Ac, *E*11–14:Ac, and *Z*11–14:OH in a 90:5:5 blend prepared in laboratory from materials obtained from Farchan Co. Willoughby, Ohio; based on OBLR pheromone determination by Hill and Roelofs (1979).

[d] *Z*11–14:Ac and *Z*11–14:OH in a 85:15 blend; based on *Archips rosanus* pheromone determination by Roelofs et al. (1976).

[e] Probably *Z*11–14:Ac, *E*11–14:Ac, and *Z*11–14:OH in a 90:5:5 blend (Hill and Roelofs, 1979) formulated in a packet of 10 microtubules by Albany International Co., Needham Heights, Massachusetts. Not tested in Zebroff Orchard.

2). [Z11–14:Ac obtained from Albany International Co., Needham Heights, Massachusetts; Z11–14:OH prepared from Z11–14:Ac containing up to 1% *E*11–14:Ac. Solvent used in preparation of mixtures unless otherwise noted, was 1,2-dichloroethane. Blends prepared at the Summerland Research Station.]

Another experiment was conducted from August 16 to September 15, 1982, in the Still Orchard, Cawston, British Columbia to compare lower amounts of *E*11–14:Ac blended with increasing amounts (1.5, 3.0, and 6.0%) of Z11–14:OH to catches in traps baited with OBLR females ($N = 5$, randomized complete block design).

1983 Field Studies. Three experiments were done in 1983, but only the results of experiment 1 are reported in Table 3. In experiment 1, eight blends of Z11–14:Ac, *E*11–14:Ac, and Z11–14:OH with the amounts of *E*11–14:Ac kept at $<3\%$ and Z11–14:OH at $\leq 2\%$ were compared against OBLR females. A 3-mg bait was used because little difference was found between a 3- or 5-mg dosage (Table 1). A second experiment tested similar blends (low *E*11–14:Ac and Z11–14:OH), but the blends were not compared against traps baited with virgin females (8 × 8 Latin square, traps checked weekly from June 6 to August 8). At the Zebroff Orchard, Cawston, British Columbia (experiment 3), eight blends prepared in the laboratory were tested to determine if the *E*11–14:Ac

TABLE 2. NUMBERS OF *C. rosaceana* (OBLR) AND *A. rosanus* (ELR) CAPTURED IN TRAPS BAITED WITH FEMALE OBLR OR DIFFERING BLENDS OF Z11–14:Ac, *E*11–14:Ac AND Z11–14:OH[a]

Treatment (percentage in 5-mg blend)			Total males captured[b]		
Z11–14:Ac	*E*11–14:Ac	Z11–14:OH	OBLR	ELR	$\bar{X}$ Ratio OBLR:ELR
94.3	5.7	—	102d	7f	14.6:1
84.7	10.2	5.1	22e	92d	0.2:1
80.6	9.7	9.7	14e	49e	0.3:1
89.2	5.4	5.4	162cd	123cd	1.3:1
84.6	5.1	10.3	122cd	183bc	0.7:1
94.2	2.8	3.0	311b	246b	1.3:1
93.3	1.0	5.7	600a	894a	0.7:1
Unbaited control			3f	2g	1.5:1
OBLR females (5/trap)			198c	10f	16.5:1

[a] 9 × 9 Latin square, Entomology Reseach Orchard, Summerland, British Columbia; traps checked weekly, June 10 to July 12, 1982. Experiment continued to July 26 but female OBLR were not replaced after July 12.

[b] Treatment totals followed by the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$.

TABLE 3. NUMBER OF *C. rosaceana* (OBLR) AND *A. rosanus* (ELR) CAPTURED IN EXPERIMENT 1) IN 1983 TO TRAPS BAITED WITH FEMALE OBLR OR DIFFERING BLENDS OF Z11-14:Ac, E11-14:Ac, AND Z11-14:OH

Experimental design, location, and dates	Treatment (percentage in 3-mg blend)			OBLR	ELR	OBLR:ELR
	Z11-14:Ac	E11-14:Ac	Z11-14:OH			
9 × 9 Latin square;	97.5	1.0	1.5	71de	276a	0.3:1
Entomology Research Orchard	98.5	1.0	0.5	148bc	191a	0.8:1
Summerland, British Columbia; traps	97.5	1.5	1.0	107cd	187a	0.6:1
checked twice weekly,	97.0	1.5	1.5	85de	174a	0.5:1
June 3-30[b]	96.5	1.5	2.0	77de	164a	0.5:1
	96.5	2.0	1.5	149b	120b	1.2:1
	96.0	3.0	1.0	67de	32c	2.0:1
	95.0	3.0	2.0	55e	40c	1.4:1
OBLR females (5/trap)				507a	29c	17.5:1

[a]Treatment totals followed by the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$.
[b]Blends prepared in laboratory using compounds obtained from Albany International, Co.; materials checked for purity by SC-GLC.

component was critical for increased attraction of OBLR males. Purified Z11–14:Ac alone, Z11–14:Ac from Albany International (up to 1% E11–14:Ac as determined by SC-GLC) with either no E11–14:Ac or 2 mg E isomer plus up to 15% E11–14:OH, purified Z11–14:Ac + 2% E11–14:Ac + E11–14:OH and Z11–14:Ac + 2% E11–14:Ac + E11–14:OH were compared (8 × 8 Latin square; traps checked weekly, June 7 to August 3).

1984 Field Tests. In three experiments in 1984 (Table 4), the presence of an additional component, Z11–14:Ald, was assessed. In experiment 1, standard OBLR blend alone (3 mg Z11–14:Ac, E11–14:Ac, Z11–14:OH) or with three dosages of Z11–14:Ald, and 0.03 mg of the standard blend alone or with three dosages of Z11–14:Ald were tested in comparison to traps baited with virgin females.

The effect of greater amounts of Z11–14:Ald on the catch of OBLR and ELR was tested in experiments 2 and 3 by adding 0.5–8.0% Z11–14:Ald to the standard 3-mg OBLR blend. In experiment 2, these treatments were compared against traps baited with 1 to 4-day-old virgin females.

The effects of adding two additional minor components possibly present in female OBLR, E11–14:OH and E11–14:Ald, on the catches of OBLR and ELR were tested by adding them in low amounts to the standard OBLR blend + 1% Z11–14:Ald and comparing it to standard OBLR + 1% Z11–14:Ald, standard ELR blend, and a blank control (Table 5).

To ensure that the Z11–14:Ald component of the OBLR bait was not being destroyed by air oxidation, 0.1 or 1.0% 2,6-di-*t*-butyl-4-hydroxytoluene (BHT) was added to blends containing aldehyde and compared to the aldehyde blend without BHT. This antioxidant has been included at various concentrations in aldehyde baits by some investigators (Nesbitt et al., 1977; Klun et al. 1980). The treatments were examined in a 3 × 3 Latin square layout in a 2-hectare cherry block at the Mennell Orchard, Cawston, British Columbia. Numbers of captured males were recorded weekly from July 27 to October 4, 1984.

Statistical Analysis. Electrophysiological data were subjected to an arcsin transformation and then analyzed by ANOVA and Duncan's multiple-range test (Duncan, 1951).

Field-test data were transformed (e^{y+1}) when necessary and subjected to analysis of variance using computer programs (ACTS AOV or LATIN SQ). The assumptions of ANOVA were tested graphically and with Bartlett's test. Means were separated with Duncan's multiple-range test (ACTS Duncan) (Duncan, 1951).

A curve-fitting program (Spain, 1982) was used to determine "best-fit" regression lines and statistics from the data to illustrate graphically differences in catch levels of OBLR in response to changing amounts of E11–14:Ac and Z11–14:OH in blends.

TABLE 4. NUMBER OF *C. rosaceana* (OBLR) AND *A. rosanus* (ELR) MALES CAPTURED IN 3 EXPERIMENTS IN 1984 TO TRAPS BAITED WITH FEMALE OBLR OR STANDARD OBLR BLEND ALONE OR WITH VARYING AMOUNTS OF ADDITIONAL COMPONENT, Z11-14 : Ald (ENTOMOLOGY RESEARCH ORCHARD, SUMMERLAND, BRITISH COLUMBIA

Exp. No.	Experimental design, location, and dates	Treatment: Amount (mg) of standard OBLR blend, Z11-14 : Ac + E11-14 : Ac + Z11-14 : OH, in 96.5 : 2 : 1.5 blend	Treatment: Additional Z11-14 : Ald as % of standard OBLR blend	Total males captured[a]: OBLR	Total males captured[a]: ELR	$\bar{X}$ Ratio OBLR : ELR
1	9 × 9 Latin square; traps checked every 2 days, July 3–15; 1- to 3-day-old virgin females used, replaced July 9; exp. continued to July 21, but no OBLR females available after July 9	3.00	0.00	91cd	53a	1.7 : 1
		3.00	0.01	86de	61a	1.4 : 1
		3.00	0.10	178bc	74a	2.4 : 1
		3.00	1.00	613a	59a	10.4 : 1
		0.03	0.00	21f	18b	1.2 : 1
		0.03	0.01	37ef	17b	2.2 : 1
		0.03	0.10	36ef	22b	1.6 : 1
		0.03	1.00	49e	20b	2.5 : 1
	OBLR females (5/trap)			270b	2c	135 : 1
2	7 × 7 Latin square; checked every 2 days, July 21–August 2; 1- to 4-day-old virgin females used, replaced July 27	3.00	0.0	23c	29a	0.8 : 1
		3.00	0.5	107abc	28a	3.8 : 1
		3.00	1.0	122ab	20ab	6.1 : 1
		3.00	2.0	185a[b]	11bc[b]	16.8 : 1
		3.00	4.0	174a	7c	24.9 : 1
		3.00	8.0	124ab	3c	41.3 : 1
	OBLR females (5/trap)			58bc	0c	
3	6 × 6 Latin square; checked weekly, August 2–September 14, 1984	3.00	0.0	3a	2	1.5
		3.00	0.5	17bc	1	17
		3.00	1.0	13b	0	—
		3.00	2.0[b]	23cd	0	—
		3.00	4.0	26cd	0	—
		3.00	8.0	40d	0	—

[a] Treatment totals followed by the same letter are not significantly different, Duncan's multiple ange test, $P < 0.05$.
[b] Captures for one replicate on one recording data recalculated because of missing data.

TABLE 5. NUMBERS OF *C. rosaceana* (OBLR) AND *A. rosanus* (ELR) MALES CAPTURED IN PHEROCON 1C TRAPS BAITED WITH 3 MG OF VARIOUS SYNTHETIC PHEROMONE BLENDS[a]

Treatment	Total males captured[b]		$\bar{X}$ ratio
	OBLR	ELR	OBLR : ELR
Standard OBLR[c] + 1.0% Z11-14 : Ald	33a	17b	1.9 : 1
Standard OBLR[c] + Z11-14 : Ald 1.0%, *E*11-14 : OH 0.03%, *E*11-14 : Ald 0.02%	29ab	4c	7.3 : 1
Standard ELR[d]	10bc	100a	0.1 : 1
Blank control	0c	2c	—

[a] 4 × 4 Latin square, Entomology Research Orchard, Summerland, British Columbia; traps checked weekly, July 25–August 24, 1984.
[b] Treatment totals followed by the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$.
[c] Z11-14 : Ac + *E*11-14 : Ac + Z11-14 : OH in a 96.5 : 2 : 1.5 blend.
[d] Z11-14 : Ac + Z11-14 : OH in an 85 : 15 blend.

RESULTS

Compounds Produced by Virgin Females. Individual female ovipositor tip washes analyzed by SC-GLC indicated the presence of two major components with retention times on columns A and B corresponding to Z11-14 : Ac and Z11-14 : OH, as reported by Hill and Roelofs (1979). Individual washes seldom contained more than 20 ng of Z11-14 : Ac. A minor component (1–1.5%) was detected fairly consistently on column B, identical to the retention characterstics of the *E*11-14 : Ac. Epoxidation of combined tip washes resulted in new peaks in the SC-GLC traces corresponding to the retention times for authentic samples of *Z*- and *E*11-14 : Ac epoxides. Analysis by SC-GLC confirmed the identity of the Z11-14 : Ac epoxide, but the mass spectrum of the minor *E* isomer was obscured by an impurity.

Another minor component (up to 1%), corresponding to Z11-14 : Ald was occasionally apparent in SC-GLC traces of individual washes. Single-ion monitoring of SC-GLC-MS traces for ions of *m/e* 70, 98, and 192 provided further evidence for a tetradecenal structure. Extracts prepared over a four-day period exhibited enhanced levels of this component, and SC-GLC retention times of this material on both columns were consistent with those for Z11-14 : Ald. A mass spectrum of this substance obtained by SC-GLC-MS analysis of these extracts was identical to that of authentic Z11-14 : Ald.

Electrophysiological Responses. Z11-14 : Ac, the major pheromone com-

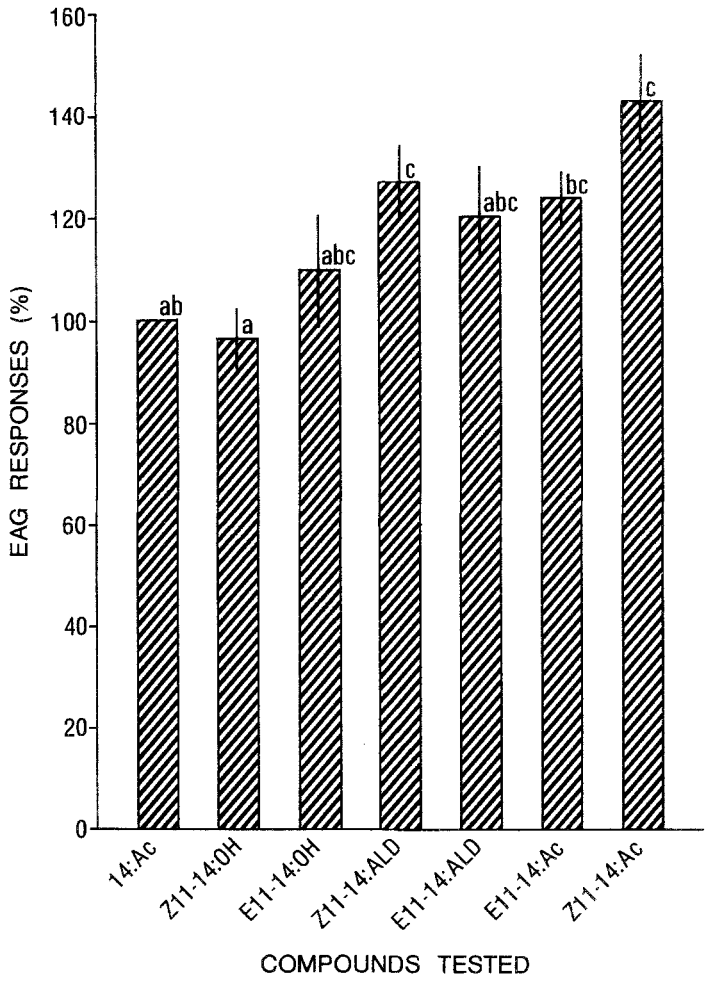

FIG. 1. EAG responses of 20 male *C. rosaceana* to a series of monounsaturated (Δ11) 14-carbon aldehydes, acetates, and alcohols. Responses were compared to a standard, 14 : Ac, and converted to percentages. Bars indicate ± 1 SE of the mean. Bars identified by the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$.

ponent, and the newly identified Z11–14 : Ald were the most stimulating of the six components tested (Figure 1). An intermediate response was obtained to *E*11–14 : Ac, but neither it nor the other standard bait component, Z11–14 : OH, were more stimulatory than the 14 : Ac standard. The mean EAG response (minus that to the solvent controls) to the nonpheromonal standard, 14 : Ac, was 537 $\pm$ 83.3 μV.

1982 Field Tests. The Zoecon AROS lure was as effective in catching OBLR males as 3 or 5 mg of the 90:5:5 blend of Z11–14:Ac, E11–14:Ac, and Z11–14:OH formulated in the laboratory (Table 1). As also found by Madsen and Madsen (1980), very few *A. rosanus* males were captured in the two orchards in Cawston; therefore, the species specificity of these blends could not be evaluated.

The 1-mg laboratory blend on rubber cap dispensers gave poor results in both orchards (Table 1). The Albany OBLR lure was effective for two weeks but catches were low after the second week.

When the percentages of Z11–14:Ac, E11–14:Ac, and Z11–14:OH in a 5-mg bait were varied, the two most effective blends (94.2:2.8:3.0 and 93.3:1.0:5.7 of the above components, respectively) were those that contained the lowest percentage of E11–14:Ac (Table 2). However, these two treatments also caught the highest numbers of ELR males. While traps baited with these two blends captured more OBLR males than traps baited with virgin females, the biological effectiveness of the females was questionable because of their age (1–7 days old prior to being placed in the field for seven days).

The blend containing 5.4% E11–14:Ac and 5.4% Z11–14:OH was comparable to the Zoecon AROS and Albany OBLR lures (Table 1), attracting fewer OBLR but almost equal numbers of ELR and OBLR (Table 2). Blends which caught the fewest ELR males also caught the fewest OBLR males.

When increasing amounts of Z11–14:OH (up to 6%) were blended with 1% or 2% E11–14:Ac and large amounts of Z11–14:Ac, the most effective treatment consisted of 2% E11–14:Ac and 1.5% Z11–14:OH, but significantly fewer males (Duncan's multiple-range test, $P < 0.05$) were captured than with traps baited with 10 OBLR females. Increasing the percentage of Z11–14:OH did not improve catch levels of OBLR.

At a percentage of 5.7% Z11–14:OH in the blend, the highest ELR catch occurred at Summerland (Table 2). Previous studies on *A. rosanus* found that catches did not differ significantly with acetate–alcohol ratios up to 50:50 (Roelofs et al., 1976). Therefore, increased amounts of Z11–14:OH in the blend would decrease specificity for OBLR. On the other hand, higher amounts of E11–14:Ac in the blend reduced catches of both species (Table 2).

1983 Field Tests. The catches of *C. rosaceana* and *A. rosanus* in only one orchard with blends containing 1–3% E11–14:Ac are presented in Table 3 (experiment 1) and Table 2. In experiment 1, a blend containing E11–14:Ac and Z11–14:OH at 2.0 and 1.5% or 1.0 and 0.5%, respectively, attracted the most OBLR and the ratio of OBLR to ELR males captured was ca. 1.0. Traps baited with OBLR females were significantly more effective than these blends. In experiment 2, the most attractive blend to OBLR, with a OBLR to ELR capture ratio >1, was also one containing 2% E11–14:Ac and 1.5% Z11–14:OH. More OBLR males were captured in response to a blend containing

1% *E*11–14:Ac and 2% *Z*11–14:OH but high numbers of ELR were also captured. In both experiments, *E*11–14:Ac at >2% appeared inhibitory to *A. rosanus* males but OBLR catches were also reduced (Table 3). Also, maintaining the percentage of *Z*11–14:OH at <2% appeared critical to obtaining increased catches of OBLR.

The relative importance of the two minor components, *E*11–14:Ac and *Z*11–14:OH was further demonstrated in experiment 3 at the Zebroff Orchard, (Table 4). While the major component, *Z*11–14:Ac alone captured many OBLR males, the addition of either 2% of the *E*11–14:Ac or 1.5% of *Z*11–14:OH substantially increased catches. The best catches were obtained when all three components were present in a 96.5:2:1.5 blend. Again, a high percentage of *Z*11–14:OH (15%) with *Z*11–14:Ac appeared to inhibit positive orientation by OBLR. Captures of OBLR in several experiments were pooled and compared against changing amounts of *Z*11–14:OH and *E*11–14:Ac in the blend. These results are graphically presented in Figure 2, which indicates the optimum percentages of *Z*11–14:OH and *E*11–14:Ac.

After two years of field tests, the optimum blend for OBLR was concluded to consist of a 96.5:2:1.5 blend of *Z*11–14:Ac (purified), *E*11–14:Ac, and *Z*11–14:OH. While large numbers of *C. rosaceana* could be attracted with this blend, it still was not competitive with five virgin OBLR females. Moreover, the specificity was poor if traps with this blend were deployed in orchards with high populations of *A. rosanus*. The blend was adequate for determining the distribution and flight activity of OBLR (Madsen et al., 1984), but results of its use for monitoring populations in orchards could be difficult to interpret, particularly if endemic female OBLR densities increased in certain orchards (Reidl and Croft, 1974). Nonetheless, it was used as a standard blend for further research in 1984.

1984 Field Tests. The numbers of OBLR males captured increased with 0.1 and 1.0% *Z*11–14:Ald added to 3 mg of the standard three-component pheromone blend (Table 4, experiment 1). The addition of 1.0% *Z*11–14:Ald to the standard blend resulted for the first time in more OBLR males captured than in traps baited with virgin females. The numbers of *A. rosanus* did not decrease with the addition of up to 1.0% of the aldehyde, but the higher catches of OBLR caused the OBLR–ELR ratio to increase.

The species specificity increased as more *Z*11–14:Ald was added to the standard OBLR blend (Table 4, experiment 2). These data suggest that a blend containing between 2 and 8% *Z*11–14:Ald would combine the advantages of superior attractiveness to OBLR and minimal attractiveness to ELR. In experiment 3 (Table 4), the blend with 8% *Z*11–14:Ald continued to perform well, both with respect to OBLR catches and species selectivity.

The results obtained late in the OBLR and ELR flight periods with the addition of 0.03% *E*11–14:OH and 0.02% *E*11–14:Ald to the four-component

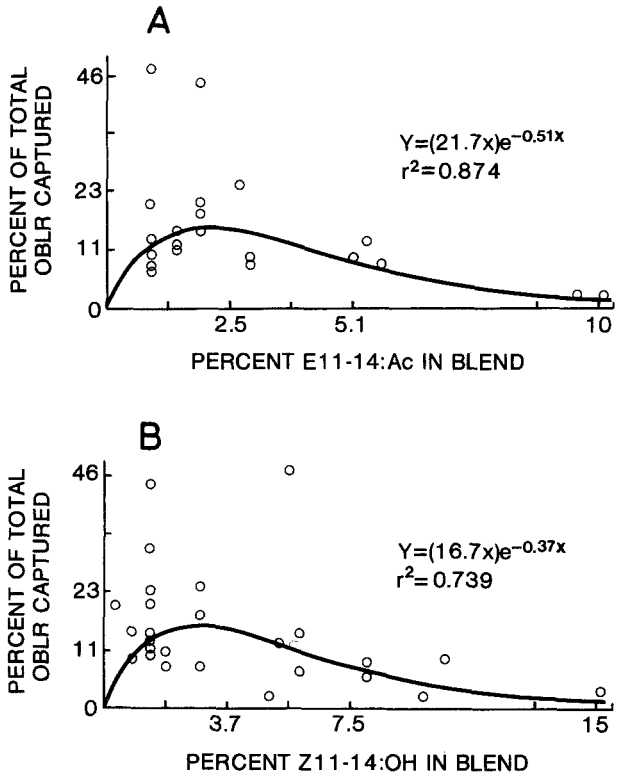

FIG. 2. Maxima function curves (with equations) describing the influence of changing percentages of *E*11–14 : Ac (A) and *Z*11–14 : OH (B) on catches of OBLR males in traps baited with different synthetic pheromone blends. Captures with each blend are expressed as a percentage of the total OBLR catch in an experiment to allow comparison of data from several experiments.

blend above suggest that these two minor components might impart even higher specificity for OBLR without lowering the attractiveness to OBLR males (Table 5). These percentages were chosen in an attempt to match the *Z*:*E* ratio found with the more abundant Δ11–14 Acs. Further studies are necessary to validate a species selective, six-component blend during peak flight periods of OBLR and ELR.

No apparent degradation of *Z*11–14 : Ald at the 1% level on the rubber caps with the standard OBLR blend were observed as there were no significant differences in the numbers of males captured from July 27 to October 4, 1984, between lures treated with 0.0, 0.1, and 1.0% BHT.

DISCUSSION

The addition of Z11–14 : Ald to the three-component standard OBLR blend (confirmed by EAG responses as well as field tests) supports the hypothesis that a major pheromone component for *C. rosaceana* was yet to be found. Similar seasonal and diurnal activity periods for OBLR (unpublished results) and ELR (AliNiazee, 1976) would demand pheromonal species specificity as demonstrated by female OBLR and blends containing Z11–14 : Ald, and possibly E11–14 : Ald and E11–14 : OH. [Peak hourly catches for OBLR in synthetic pheromone traps occurred from 2200–2300 hr, similar to ELR catches in female-baited traps (AliNiazee, 1976). Mixed ELR and OBLR populations were found on 16 native hosts in a recent survey.]

In the eastern United States, Hill and Roelofs (1979) found that the three-component blend of Z11–14 : Ac, E11–14 : Ac, and Z11–14 : OH was the most attractive bait for OBLR. The optimal ratio of the three components was 90 : 5 : 5 (Roelofs and Novak, 1981), considerably different from the 95.5 : 2 : 1.5 blend that we found to be optimal. In contrast to our results, Hill and Roelofs (1979) did not find Z11–14 : Ald in female tip extracts, and increased trap catches were not achieved with the addition of the aldehyde. Thus, geographic variation in pheromone content, as found for other moths such as *Archips argyrospilus* (Walker) (Roelofs et al., 1974), *Agrotis segetum* (Schiff.) (Arn et al., 1982), and *Ostrinia nubilalis* (Hübner) (Klun et al., 1973), appears to occur in *C. rosaceana*. In British Columbia, *C. rosaceana* feeds on a wide range of deciduous hosts that overlaps in distribution with the coniferous hosts of other *Choristoneura* species such as *C. occidentalis* Freeman, *C. biennis* Freeman, and *C. orae* Freeman. Possibly, additional pheromone components are required to impart specificity between a greater number of sympatric species in British Columbia than in New York State. In eastern Canada, Z11–14 : Ald was reported to be an effective attractant for *Choristoneura conflictana* and the addition of 1.0% Z11–14 : Ald to Z11–14 : Ac increased captures of *C. rosaceana* (Weatherston et al., 1976). Our findings support the above authors' hypothesis that reinvestigation of extracts from female OBLR would reveal the presence of Z11–14 : Ald.

In 1983 and 1984, experiments on monitoring populations of *C. rosaceana* were conducted in the Okanagan Valley using the standard three-component OBLR blend. High numbers of males can be trapped in orchards that sustain little or no fruit damage (Madsen et al., 1984). For practical monitoring considerations, replacing the three-component blend with a more attractive and species-selective six-component blend might further complicate the interpretation of numbers of moths caught. However, the problem of contamination of traps with ELR would be solved.

In mating disruption studies with *C. rosaceana* in New York State, Roelofs and Novak (1981) found that the n ural blend (90:5:5 *Z*11–14:Ac, *E*11–14:Ac, *Z*11–14:OH) gave the best disruption of male orientation to traps in small test plots. Our better understanding of the sex pheromone complement of *C. rosaceana* in British Columbia will make it possible to optimize the disruption technique for this locale.

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GLUCOCAPPARIN VARIABILITY AMONG FOUR POPULATIONS OF *Isomeris arborea* NUTT.

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Abstract—Glucocapparin (methylglucosinolate), a putative defense compound, was found to vary between desert and nondesert populations of *Isomeris arborea* (Capparaceae): Plants from desert populations contained greater concentrations than nondesert plants in four of the five organs analyzed. Immature leaves at desert sites had average glucocapparin concentrations of 9.2 mg/g and 8.4 mg/g, while nondesert sites averaged 6.0 mg/g and 4.6 mg/g. Mature leaves from desert sites had average concentrations of 12.8 mg/g and 7.9 mg/g; leaves from plants at nondesert sites contained approximately one third to one half of those concentrations. A similar pattern was observed in capsule walls and seeds but not in flower buds; for these, nondesert plants contained a slightly higher concentration of glucocapparin. Our studies show that nitrogen and glucocapparin concentrations fluctuate throughout the year and contribute to the observed variability among populations during any particular season. Glucocapparin may fluctuate seasonally as much as 37% in immature leaves and 78% in mature leaves. In a controlled experiment, glucocapparin concentration varied inversely with nitrogen fertilizer treatment. The plants treated with fertilizer lacking nitrogen ranged from 10.1 mg/g to 10.9 mg/g glucapparin, which was roughly twice the concentration of those supplied with 20 mM nitrogen in the fertilizer.

Key Words—Capparaceae, chemical defense, glucocapparin, glucosinolate, *Isomeris arborea*.

INTRODUCTION

According to some current theories of defensive secondary compounds, glucosinolate–myrosinase systems can be expensive to maintain (cf. Rhoades, 1979). One would reasonably expect, therefore, that the same species growing

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in areas of different resource availability would be found to differ in concentration of defensive compounds and that these differences would be variable within individuals in response to changing resource availability (Coley et al., 1985). Although these predictions are straightforward, relatively few studies have focused on determining whether or not they are met or what environmental resources might be involved.

Ecological research on the concentration of plant defensive compounds has focused on differences due to elevation (De Araujo, 1976; Jones, 1977), moisture gradients (Louda and Rodman, 1983a,b; Louda et al., 1987), and disjunct distribution (Wisdom and Rodriguez, 1982); few studies describe the variation in defensive compounds among plant populations. We report here our studies of the concentration of glucocapparin found in the organs of plants from four distinct populations of *Isomeris arborea* Nutt.

I. arborea is well suited to studying variation in defensive compounds both among populations and within individuals. Although restricted to southern California and northern Baja, it occurs in a diversity of plant communities, including creosote bush scrub, valley grassland, and coastal sage scrub. Stands of *I. arborea* within these communities may contain hundreds of bushes, but they are often small in area and widely separated with little opportunity for gene flow among them. The plant communities themselves are even more disparate (Munz, 1968; Barbour and Major, 1977).

Within California, *I. arborea* is the only woody member of the Capparaceae. Plants of this family produce glucosinolates, with some known to contain as many as four or five (Kjaer and Thomson, 1963). All of the organs of *I. arborea* that have been examined (immature and mature leaves, flower buds, capsule walls, seeds) (Blua and Hanscom, 1986), however, contain only glucocapparin (= methylglucosinolate), which eliminates the complication of having to assay for several compounds and to consider possible interactions among them.

Concentrations of phytotoxins (such as glucocapparin, in species which have been studied, generally increase in plants exposed to a variety of environmental stresses (McKey, 1974; Rhoades, 1979). On the other hand, concentrations of digestibility-reducing compounds in some perennials generally decrease with environmental stresses (Rhoades, 1979). *I. arborea* is unusual because it is a perennial containing a defensive compound commonly found in annual species (Blua and Hanscom, 1986). Our studies (unpublished) show that *I. arborea* is subjected to water and nitrogen stress of different magnitudes, depending on the population in which it is found. We predict the glucocapparin in stressed *I. arborea* will behave in a manner similar to that in other phytotoxin-containing plants growing under various degrees of environmental stress.

Although the geographic populations of *I. arborea* are uniform with respect to the presence of the same glucosinolate, they show significant quantitative

variation in several other physiologic and morphologic traits (Tobiessen, 1976; Iltis, 1957), and four subspecies are described in the taxonomic literature (Munz, 1968). This suggests that we could also expect to find differences in glucocapparin concentration among populations. We further hypothesized that glucocapparin concentration would be plastic within individuals from the same population, varying in response to availability of important resources. To test these ideas, we sampled several widely separated natural stands and also grew plants from a single population under different, controlled greenhouse conditions.

METHODS AND MATERIALS

Collection of Plant Material. Plant material was collected from four sites within southern California, representing the four plant communities where *Isomeris arborea* is most frequently found: Yaqui Well in the low desert and Short Canyon in the high desert, both of which are creosote bush scrub plant communities; Bakersfield, a valley grassland plant community; and Sorrento Valley, a coastal sage scrub plant community. The precise locations were: (1) Yaqui Well: junction of State Highway 78 and county road S3, 0.1 miles southwest of Tamarisk Grove Campground, Anza Borrego State Park, San Diego County; (2) Short Canyon: 1 mile west of U.S. Highway 395 at Brady's convenience station, 0.3 miles north of the junction of State Highway 14 and U.S. highway 395, Kern County; (3) Bakersfield: 6 miles east of Oildale at the edge of the Poso oil field, Kern County; and (4) Sorrento Valley: Flint Kote Road, Torrey Pines State Preserve, San Diego County.

Immature (not fully expanded) and mature leaves, flower buds, capsule walls, and seeds were collected from five plants in each of the four populations and from an additional five plants in the Sorrento Valley population. All vegetative organs and flower buds were collected in the late spring (May). Capsule walls and seeds were collected as they became available in spring and summer. In addition, leaves from ten Sorrento Valley plants were collected in winter (November), early spring (March), late spring (May), and summer (August). Each time plant material was collected, it was immediately placed on Dry Ice and transfered to a freezer for storage prior to analysis.

Greenhouse Experiment. To test the hypothesis that leaf glucosinolate concentrations increase with water and nitrogen stress, a completely randomized 3 $\times$ 2 factorial experiment was used. One factor was the application of three nitrogen levels in the fertilizer: 0, 2, and 20 mM nitrogen in a modified Van de Elst solution. Our preliminary studies showed that the application of these concentrations resulted in low, intermediate, and high leaf Kjeldahl nitrogen in our test plants. Total Kjeldahl nitrogen (TKN) was measured with an automated spectrophotometer (Technicon Auto Analyzer II, Terrytown, N.Y.).

The second factor was the difference in amount of water supplied. Individual plants considered unstressed were irrigated with deionized water daily, while those comparatively water stressed were irrigated twice weekly. A difference in the degree of stress was monitored by measuring dawn water potential with a vapor pressure osmometer (Wescor No. 5100B, Logan, Vermont).

Four *I. arborea* clones of similar size and development were used as replicates within each combination of nitrogen concentration and irrigation treatment. Plants were grown in full sunlight in five-gallon pots containing U.C.II(C) soil mixture. All plants were allowed in equilibrate at their assigned nitrogen concentration for two weeks before withholding water from water-stressed plants. Treatments lasted eight weeks, at which time immature and mature leaves from each plant were collected for glucosinolate and TKN analysis and dry weight determination.

Glucocapparin Analyses. Glucocapparin was identified and quantified by methods described by Blua and Hanscom (1986).

Statistical Procedures. Although a conventional two-way ANOVA, with the main effects being location and plant organs, would appear to be an appropriate method of data analysis, we took a sample of each of the five plant organs that were studied from each of five plants at each site. Thus, the 25 samples taken at each site are not independent. Therefore, we chose to analyze our data in two parts: (1) a one-way ANOVA for differences among sites was carried out on the means taken across organs for each plant, with the five resulting plant means at each site serving as replicates, and (2) at each site, a randomized block ANOVA was carried out to test for differences among organs, with the plants serving as blocks. Thus, in this second analysis, the data from each site were treated separately. This statistical analysis precludes the possibility of testing the interaction term for significance, and we will present evidence along different lines in our results that suggest that the pattern of differences among organs is not the same at different sites.

The greenhouse experiment was designed to be analyzed with ANOVA to detect significant differences in the glucocapparin content of immature and mature leaves from plants treated with the three nitrogen and the two irrigation levels. The following statistical model (Montgomery, 1984) is consistent with the experimental design:

$$y_{ijkm} = \mu + \alpha_j + \beta_k + \gamma_m + P_{i(jkm)} + (\alpha\beta)_{jk} + (\alpha\gamma)_{jm} + (\beta\gamma)_{km} + (\alpha\beta\gamma)_{jkm} + E_{ijkm}$$

where α_j, β_k, and γ_m are the effects associated with nitrogen fertilization, irrigation treatment, and leaf age class respectively; and $P_{i(jkm)}$ is the unique contribution associated with the ith plant nested in nitrogen fertilization j, irrigation treatment k, and leaf age class m. Interaction effects are shown in parenthesis.

E_{ijkm} is the error of measurement associated with determining Y_{ijkm}, the glucocapparin concentration of leaves of age class m from plant i grown under nitrogen fertilization j with irrigation treatment k.

RESULTS

Differences Among Field Sites and Among Organs. Of the organs examined, mature seeds contained the highest concentrations of glucocapparin at all four sites (Table 1). Because these concentrations were so consistently high relative to those of the other four organs, a separate one-way ANOVA for differences among sites just for mature seeds was carried out. This analysis indicated that mean concentrations of glucocapparin in mature seeds did not differ significantly among sites. In contrast, a one-way ANOVA among site means, calculated across all organs exclusive of mature seeds, indicated highly significant differences among sites ($F = 32.1$; $df = 3, 16$; $P < 0.001$; Table 1).

The randomized block ANOVA to test for differences among organs (excluding seeds) at each site indicated that the organ means were highly significantly different at all four sites. At each site the capsule walls were consistently lowest in glucocapparin concentration, with the mean across populations of 2.5 mg/g fresh weight. But there was little similarity in the pattern of glucocapparin concentrations of immature leaves, mature leaves, and flower buds among the different sites. The differences among these three organs were slight and probably not significant for Sorrento Valley (the coastal site) and Short Canyon (high desert). The mean across these three organs at Short Canyon, however, was nearly 70% higher than the mean across organs at Sorrento Valley. The means for Yaqui Well (low desert), in contrast, ranged from 3.4 mg/g for flower buds, which was the lowest value for any of these three organs at

TABLE 1. GLUCOCAPPARIN CONCENTRATIONS IN SAMPLES OF *Isomeris arborea* COLLECTED FROM 4 POPULATIONS IN LATE SPRING, EXCEPT FOR SEEDS WHICH WERE COLLECTED IN SUMMER[a]

Site	Immature leaves	Mature leaves	Flower buds	Capsule walls	Seeds
Yaqui Well	9.2 ± 1.0	12.8 ± 1.7	3.4 ± 0.9	2.5 ± 0.9	18.9 ± 4.4
Short Canyon	8.4 ± 0.7	7.9 ± 1.9	7.5 ± 1.2	4.0 ± 1.0	14.9 ± 1.3
Bakersfield	6.0 ± 0.7	3.8 ± 0.7	6.5 ± 0.8	1.9 ± 1.0	19.3 ± 4.3
Sorrento Valley	4.6 ± 1.0	4.0 ± 0.8	5.6 ± 0.6	1.8 ± 1.4	14.3 ± 4.3

[a] Seeds were not included in the ANOVA. Values are milligrams per gram fresh weight ± standard deviation. One-way ANOVA for differences among sites: $F = 32.1$; $df = 3, 16$; $P < 0.001$.

any site, to a high of 12.8 mg/g for mature leaves, which was the highest value. The situation for the Bakersfield site was different from any of the others, with mature leaves having a concentration of only 3.8 mg/g, while flower buds and immature leaves at Bakersfield differed little from one another and had intermediate concentrations of glucocapparin. From the differences in these patterns at each site, taken together with the difference among sites of means across organs, we infer that there is an interaction between site and organ.

Seasonal Patterns. Data obtained at Sorrento Valley several times during the year indicated that glucocapparin concentration in leaves changed significantly as the growing season progressed into the drier summer months (Table 2). Again, the pattern of differences was not straightforward: the interaction term between leaf age and season was significant, and season, as a main effect, was highly significant. But leaf age as a main effect was not significant. For both immature and mature leaves, the period of greatest concentration of glucocapparin in the samples occurred during the summer, although Tukey's procedure for unplanned multiple comparisons (Steel and Torrey, 1980) indicated that collection date was significant only for mature leaves ($P = 0.01$). The increase in mature leaves was quite pronounced, with glucocapparin concentration in summer being 78% higher than in late spring.

Greenhouse Experiment. Total Kjeldahl nitrogen and leaf dry weight increased as higher concentrations of nitrogen were supplied in the fertilizer treatment (Table 3), which was anticipated. Glucocapparin concentration declined with increasing concentrations of nitrogen in the fertilizer (Table 3). In addition, immature leaves contained a greater glucocapparin concentration than mature leaves, which agrees with our earlier observations of plants from this population grown under greenhouse conditions (Blua and Hanscom, 1986). No trend in glucocapparin concentration with respect to irrigation treatment was observed in this experiment, even though the water potential of well-watered plants was consistently higher than that of the water-stressed plants. All com-

TABLE 2. CONCENTRATIONS OF GLUCOCAPPARIN IN IMMATURE AND MATURE LEAVES OF *Isomeris arborea* COLLECTED 4 TIMES DURING THE SEASON AT SORRENTO VALLEY[a]

Time of collection	Immature leaves	Mature leaves
Winter	6.0 ± 2.3	[b]
Early Spring	6.1 ± 1.1	4.9 ± 1.1
Late Spring	5.1 ± 1.3	4.6 ± 1.1
Summer	7.0 ± 1.9	8.2 ± 2.3

[a] Values are mean milligrams per gram fresh weight ± standard deviation.)
[b] Mature leaves not available.

TABLE 3. KJELDAHL NITROGEN CONTENT OF IMMATURE AND MATURE *Isomeris arborea* LEAVES DRY WEIGHT, AND GLUCOCAPPARIN CONCENTRATION (GCN) FOR EACH OF 3 NITROGEN TREATMENTS COMBINED WITH IRRIGATION (+ WATER) AND WITHOUT IRRIGATION (− WATER)

	Immature leaves			Mature leaves		
Treatment	Nitrogen (mg/g dry wt ± SD)	Dry weight (g)	GCN (mg/g fresh wt ± SD)	Nitrogen (mg/g dry wt ± SD)	Dry weight (g)	GCN (mg/g fresh wt ± SD)
0 N + Water	21.7 ± 1.9	0.43 ± 0.14	10.1 ± 1.4	18.2 ± 0.8	0.18 ± 0.10	8.6 ± 0.0
0 N − Water	19.9 ± 0.5	0.69 ± 0.38	10.9 ± 1.9	17.9 ± 2.7	0.39 ± 0.05	8.3 ± 0.5
Lo N + Water	23.7 ± 0.8	0.73 ± 0.34	9.6 ± 1.4	19.9 ± 1.3	1.07 ± 0.20	6.2 ± 0.5
Lo N − Water	21.8 ± 0.9	1.4 ± 0.38	8.3 ± 1.5	20.6 ± 1.1	1.07 ± 0.20	6.3 ± 0.3
Hi N + Water	32.2 ± 5.9	7.0 ± 2.19	6.6 ± 0.3	33.7 ± 4.3	6.6 ± 0.61	4.8 ± 0.9
Hi N − Water	39.7 ± 1.6	4.53 ± 1.17	7.6 ± 1.3	39.6 ± 1.2	4.94 ± 1.52	5.6 ± 0.3

binations of interactions between nitrogen fertilization, irrigation treatment, and leaf age class was found to be insignificant.

DISCUSSION

The mean glucocapparin concentrations were found to vary significantly among four *Isomeris arborea* populations. The differences in values between plants in the desert and in the nondesert populations were especially obvious: the glucocapparin concentration in the coastal sage scrub (Sorrento Valley) and valley grasslands (Bakersfield) populations were similar but quite different from their counterparts in the desert (Yaqui Well and Short Canyon). Our data suggest that plants growing in different populations respond to environmental driving variables that are unique to those environments, particularly the availability of key resources such as soil nitrogen, perched ground water, and perhaps other resources not covered in this study. In controlled experiments conducted in other laboratories, glucosinolate concentration responded to environmental factors such as water stress (Bible et al., 1980), nitrogen (Freeman and Mossadeghi, 1972), soil sulfate (Josefesson, 1970), and soil type (Ju et al., 1980). We speculate that two key variables, soil nitrogen content and the water resources available to the plants during the growing season, differed from population to population and thereby contributed to the glucocapparin concentration observed among the four *I. arborea* populations studied.

The presence of herbivores may constitute another factor that leads to variability in concentration of plant toxins among populations of a plant species. Rhoades (1979) lists several studies in which the concentration of a toxin increased in as little as 12 h after the plants producing them were attacked by herbivores. The direct effect of herbivores on the concentration of glucocapparin in *I. arborea* was not tested in this study. However, herbivore load is not independent of other stresses and may have been a contributing factor to the results we obtained. It is unclear to us at this point how to separate the direct effects of herbivory from indirect effects, such as the change in plant water potential, and other driving variables that come about as a result of the physical injury to the plant by the herbivores.

Because of seasonal extremes, neither herbivores nor plants remain active throughout the year in the desert populations. But the effect of herbivory may be just as great during the growing season, even though the herbivore load may be much lighter than in populations, such as the coastal sage scrub, where herbivores and growth of plants occur throughout the year. The combined effects of herbivory and relatively more extreme environmental stresses may have led to a higher mean glucocapparin concentration in desert plants.

In controlled experiments, glucocapparin concentration in *I. arborea* var-

ied inversely with nitrogen fertilizer treatment (Table 3). This trend was observed in annual species (Eaton, 1942; Trzebny, 1964; Josefsson, 1970) and has now been confirmed in *I. arborea*, which differs from many other perennials in that it contains a toxin as a chemical defense. The adaptive significance of this phenomenon is subject to speculation, and it may be that toxins are relatively common in fast-growing tropical trees. We observed a decline in the production of glucocapparin when nitrogen was not a limiting factor during rapid growth. This is partially explained by Eaton's (1942) observation that the production of many metabolites increased at a greater rate than glucosinolate when nitrogen was not a limiting resource.

Water stress and its interaction with nitrogen fertilizer treatment were not found to influence glucocapparin concentration in *I. arborea* (Table 3). In contrast to our observations, Louda et al. (1987) found an increase in the glucosinolate concentration of an annual caper growing in a dry environment. Their data were in agreement with those of Wolfson (1980, 1982), and Louda and Rodman (1983a,b). We were unable to recreate the degree of water stress in our greenhouse plants that we observed in field specimens (Wilford-Beanan, 1985), and the evidence, by inference from the field studies cited, is that water stress may likewise trigger an increase in glucocapparin concentration.

Glucocapparin concentration may vary inversely with leaf age or may be static upon leaf maturation (full leaf expansion). The temporal variation in glucocapparin in mature leaves observed in the Sorrento Valley population may represent variation in mean leaf age rather than a seasonal fluctuation of glucocapparin. In a study of Brussels sprouts (Cruciferae), mature and "old" leaves contained statistically similar concentrations of allylisothiocyanate, whereas immature leaves had much higher concentrations (VanEmden and Brashfor, 1969). In our study, the glucocapparin concentration observed in mature leaves increased significantly when the plants were most stressed (Table 2). Again, the evidence suggests that environmental stresses, particularly availability of key resources, is the driving factor for fluctuations of toxins in *I. arborea*.

It is noteworthy that glucocapparin concentration in young leaves did not vary significantly through the growing season. We speculate that *I. arborea* has evolved a capacity to buffer the physiologic environment of immature leaves and other tissues which are undergoing development because developing tissues would experience more stress from environmental extremes than mature tissues. Immature leaves may also have a higher glucocapparin concentration because they are not fully expanded, resulting in the toxin being more concentrated. As the leaves mature, glucocapparin concentration declines and then rises as environmental stress increases.

Coley et al. (1985) argue that resource availability is the major determinant of the amount and type of a plant's chemical defense. Their evidence indicates that plant species which grow slowly because of resource limitations evolve

"immobile defenses" such as polyphenols and fiber, and plants that are capable of fast growth when resources are abundant evolve "mobile defenses." The defensive chemistry of *I. arborea* is consistent with their views. First, *I. arborea* grows rapidly, with a net assimilation rate of over 400 mg dry matter/dm^{-2}/day (unpublished data), when the plants are treated with fertilizer with a high nitrogen content and in the absence of herbivory. Secondly, *I. arborea* contains a "mobile defense," glucocapparin (Blua and Hanscom, 1986), the concentration of which fluctuates depending upon the environment in which it is found. Finally, among the four populations we studied, glucocapparin concentrations were highest in plants growing in populations in which the environmental stresses were most evident. More specifically, our controlled studies point to nitrogen as the variable that is most related to glucocapparin concentration. We speculate that selection pressure will favor plants which increasingly invest in defensive chemicals during times of resource limitation.

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ATTRACTION OF ADULT *Diabrotica* (COLEOPTERA: CHRYSOMELIDAE) TO CORN SILKS AND ANALYSIS OF THE HOST-FINDING RESPONSE

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Abstract—Volatile chemicals from corn silks attracted *Diabrotica virgifera virgifera* LeConte and *D. barberi* (Smith and Lawrence). The behavioral response of both species of beetles to the host plant was typified by four distinct phases: perception, random movement, orientation to the source, and search with reorientation. The perception phase was composed of stationary behaviors, while the random, orientation, and search phases were composed of directed and nondirected movements. Each of the movement phases had a characteristic response pattern composed of the ratio of upwind, lateral, and downwind walking and flight movements, which affected net displacement of the beetle in the flight tunnel. The perception phase occurred within and between the other phases and was responsible for initiating changes from one movement phase to another (based on the presence or absence of volatiles from corn silks). Host finding was flexible, and the response pattern fit a flow-chart type of response, rather than a single stereotyped sequence of behaviors.

Key Words—Rootworm adults, *Diabrotica* spp., Coleoptera, Chrysomelidae, attraction, corn, flight tunnel, ethograms, host finding, bioassay.

INTRODUCTION

Although the northern corn rootworm, *Diabrotica barberi* (Smith and Lawrence), and the western corn rootworm, *D. virgifera virgifera* LeConte, have long been pests of corn, little is known about their host-finding behavior and

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host-mediated chemical ecology. The two species are closely associated with corn due, in part, to their restricted larval host range (Branson and Ortman, 1971) and adult feeding behavior (Branson and Krysan, 1981). Larvae feed on the roots of corn, but they have no long-range ability to distinguish these roots from those of nonhost plants and are restricted in the distance they are able to travel in search of hosts. Selection of a suitable host is dependent upon the oviposition habits of the adult female (Branson and Krysan, 1981).

The feeding behavior of adults of the two species differs significantly but reflects their close association with corn (Branson and Krysan, 1981). Newly emerged *D. v. virgifera* adults initially feed on corn leaves, but then move to the pollen-bearing tassels, silks, and young kernels, when these food sources become available (Ball, 1957; Hintz and George, 1966). When pollen becomes unavailable and the silks dry, *D. v. virgifera* remains in the cornfield and resumes leaf-feeding (Ludwig and Hill, 1975). A short time after the silks dry, *D. v. virgifera* disperses between cornfields (Hill and Mayo, 1980), although a small number of beetles feed on cucurbits and other plants (Metcalf et al., 1982; Ferguson et al., 1983). Conversely, *D. barberi* adults do not feed on the leaves, but feed on the pollen, silks, and young kernels of corn (Ludwig and Hill, 1975). When pollen becomes unavailable and the silks begin to dry, *D. barberi* females leave the cornfield to feed on the pollen of other plants nearby, but many return to corn to oviposit (Cinereski and Chiang, 1968). Several days after the silks have dried completely, *D. barberi* disperses to other plants more distant from the cornfield, including weedy oats and soybeans (Hill and Mayo, 1980; Matin and Yule, 1984).

The preference of both species for corn tissues, the behavioral change from leaf-feeding to pollen- and silk-feeding by *D. v. virgifera*, and the ability of weed-feeding *D. barberi* females to relocate corn for oviposition suggest the existence of feeding (Bruss, 1981) and oviposition (Cinereski and Chiang, 1968; Branson and Krysan, 1981) attractants in corn. This field evidence is further supported by laboratory reports that their activity is affected by corn silks (Bruss, 1981).

The confirmation of chemical attractants in corn to adult rootworms and an understanding of host-finding behavior are vital to any study of the chemical ecology of corn rootworms. Further, such information should prove useful for monitoring and trapping field populations. The objectives of this paper are to determine that corn silks produce volatile attractants, and to describe the host-finding behavior of adult *D. barberi* and *D. v. virgifera.*

METHODS AND MATERIALS

Adult Collection and Holding. Beetles were collected from cornfields near the University of Guelph, Guelph, Ontario. Beetles were tested over a two-

week period beginning when the first few gravid females were found and continuing over the time when beetles were feeding on the pollen of nearby weeds and presumably returning to the cornfields to oviposit. Adults were collected 24 hr prior to testing and housed in 30 × 30 × 30-cm Plexiglas cages. All cages were kept in a room that was well lit with natural light to maintain the light–dark cycle experienced by beetles in the field. Beetles were not isolated by species or sex, but they were held as collected to minimize handling. Beetles were starved for 24 hr prior to testing to predispose them to host finding. A moist paper towel was provided as a source of water, and cages were held in a room with high humidity. Tests were conducted during the morning and early evening to coincide with the 6–10 AM and 5–10 PM periods of maximum locomotive activity found by VanWoerkom et al. (1980). Test individuals were selected on the basis of good color, anatomic completeness, and normal walking and flight activity.

Source of Volatiles. Three whole ears of corn with mature silks were used as the source of volatiles for most tests. Mature silks were >7 cm long, yellow in color, and many individual silks often had a faint brown streak down their length indicating recent pollination. These silks had the characteristic odor of corn. Ears were collected from fields ca. 20 min prior to their use in assays. Ears were replaced with fresh ones every hour during assays.

Silks were also removed from the corn ears and tested separately to identify the source of volatiles and to eliminate visual effects of corn ears. The silks from three ears were strapped lengthwise to three 4 × 8-cm strips of 20-mesh aluminum screen. The strips with silks were suspended at the upwind end of the flight tunnel 8 cm from the top and 10 cm apart. Fresh silks were used for every group of 10 bioassays.

A 500-ml flask that contained silks from three ears was also used as a source of volatiles. The flask was placed outside the tunnel and concealed from view with aluminum foil. Two glass funnels 5 cm in diameter at the mouth were positioned at the upwind end of the flight tunnel and connected to the flask by Teflon tubing.

In all tests, the volatiles were pulled through the tunnel at 0.33 m/sec. using a centripetal exhaust fan. This system pulled air through the flasks containing corn silks at a rate of 1 liter/min. The dimensions of the odor plumes were estimated using smoke emitted from heated paraffin wax in the flasks or directly behind the corn ears or silks. The odor plumes were similar for the three treatments.

Bioassays. Two assay systems were used to study host-finding behavior. The first, which was used to determine the existence of volatile attractants from corn, used a 1.2 × 0.4 × 0.4-m Plexiglas flight tunnel. The upwind two thirds of the tunnel was divided into two 0.2 × 0.7-m chambers to provide a two-choice test. Airstreams from the time chambers mixed downwind in the release area. At least 2 min before each bioassay, groups of 10 beetles of mixed sex of

either *D. barberi* or *D. v. virgifera* were placed in a release cage, 5 × 5 × 12 cm, positioned ca. 15 cm from the downwind end of the tunnel. Three ears of corn with silks were used as a source of volatiles in one of the upwind chambers; the second chamber was left unbaited. Fresh beetles were used for each replication, and the baited and control treatments were randomly assigned to either side of the chamber. Bioassays were performed in a greenhouse to provide natural light. The types of behaviors displayed and the number of beetles that located the odor source following each release were recorded on videotape for a 10-min period.

A second assay system used the same 1.2 × 0.4 × 0.4-m Plexiglas flight tunnel, but it was not divided into two chambers and treatment and control beetles were tested separately. This system was used to record the frequency and sequencing of particular behaviors of individual beetles so that comprehensive ethograms could be constructed. Individual beetles were released in the downwind end of the tunnel as in the previous test, and their behaviors were recorded on videotape for a 5-min period. All aluminum screens, Plexiglas, and release cages were thoroughly rinsed with methanol after each series of 10 bioassays. This same assay system was used with funnels connected to a flask of silks as a source of volatiles.

Videotapes of beetle responses from both bioassays were transcribed from a television monitor using both stop-action and standard-speed modes. Species, sex, date of assay, and the occurrence and sequence of all behaviors were tabulated.

Statistical Analysis. Treatment differences for the flight tunnel bioassays were tabulated using chi-square analysis with Yates' correction for continuity. For analysis, behaviors in the flight tunnel were classified as random phase when no volatiles were present, orientation phase when beetles were in an odor plume, and search phase when beetles had left an odor plume and until it was reentered. Frequencies of walking (WW) and flight behaviors (HPF) were compared for the three phases using an analysis of variance.

An ethogram was constructed by tabulating the frequencies of all observed behaviors in first-order, preceding–following, transition matrices. Each cell in the matrix was tested using chi-square analysis, and standard normal deviates were calculated and applied to a binomial test for individual transitions (Teal et al., 1981). Transitions with deviates having probabilities of $P < 0.05$ were used to construct an ethogram of the most probable sequence involved in host finding.

RESULTS AND DISCUSSION

Role of Volatiles in Host Finding. During initial tests using groups of 10 beetles and whole ears of corn as a source of attractants, all the beetles flew ($N = 300$), with 91.7% flying upwind (Table 1). The 12 beetles (4%) that initially

TABLE 1. RESPONSE OF *Diabrotica barberi* AND *D. v. virgifera* TO VOLATILES FROM CORN EARS WITH SILKS IN TWO-CHOICE TESTS[a]

Species	*N*	Flying (%)	Direction of initial flight (% of those flying)				Final position after 10 min (% of all beetles)		
			Vertical	Downwind	Upwind	Crosswind	Release area	Unbaited chamber	Corn-baited chamber
D. barberi	150	100	5.3	89.3	94.6	3.3	0	30.7a[a]	69.3b
D. v. virgifera	150	100	2.7	94.0	96.7	1.3	0	22.7a	77.3b
Total	300	100	4	91.7	96.3	2.3	0	26.7a	73.3b

[a]Numbers analyzed by chi-square 2 × 2 test of independence with Yates' correction. Percentages in the same row followed by different letters are significantly different ($P < 0.05$).

flew downwind subsequently flew at least part way up the tunnel. Eventually, 73.3% of the beetles found the corn during the 10-min bioassay period, while 26.7% of the beetles remained in the unbaited chamber ($P < 0.05$). In an assay system similar to ours, but without attractants, VanWoerkom et al. (1983) found that upwind flights of WCR were always uncommon. We assume that the higher percentage of upwind flights in our study was due to the attractancy of corn.

In subsequent 5-min assays with individual beetles, in which whole ears of corn were used as bait ($N = 75$), 96% of the beetles flew; 55.6% of them initially flew upwind, while 74.6% were at least half way up the flight tunnel at the end of the assay (Table 2). Eventually, 61.3% of all beetles found the corn ears (Table 2). *Diabrotica v. virgifera* were significantly better at finding the corn (28/36 = 77.8%) than were *D. barberi* (18/39 = 46.2%) (Table 3). Males of both species were equally adept as females in finding the corn. In the untreated controls, 92.9% of the beetles flew ($N = 85$), but only 7.6% of these beetles flew upwind, and only 32.9% reached the upwind end of the flight tunnel (Table 2).

Two additional treatments were used to eliminate all volatiles except those from silks and to eliminate any visual attraction due to color, shape, or orientation of the ears. In the first, where corn silks were strapped to aluminum screens, all the beetles flew at least half way up the flight tunnel ($N = 20$), and 75% of all beetles found and fed on the silks (Table 2). In the second treatment in which silks were in a hidden flask, upwind flight occurred 55.6% of the time ($N = 30$), and 70% of the beetles landed on the glass funnel that was the source of silk volatiles (Table 2). These results clearly demonstrated that volatiles from corn silks were attractive to *D. barberi* and *D. v. virgifera*.

The beetles first walked upward to the edge of the release container where they could encounter a directional airflow that carried volatile substances. After reaching the upper edge, the beetles paused temporarily (P), waved their antennae (PW), or walked about the edge of the cage while waving their antennae (WW). The latter two behaviors were often repeated several times and were interrupted by a third behavior, head held up without antennal movement (HU). Flight usually followed the head up position.

The preflight behaviors were similar to those reported by VanWoerkom et al. (1983) for *D. v. virgifera* in the absence of any attractant. The "hind legs bent as if crouching" behavior reported by VanWoerkom et al. (1983) is equivalent to the head up (HU) behavior that we report. They found that without attractants, flight was instantaneous at wind speeds up to 0.5 m/sec, whereas flight was seldom instantaneous in our tests at wind speed of 0.33 m/sec with corn as an attractant. Instead, one or more PW and HU behavioral sequences occurred with a minor amount of WW behavior. The PW and HU behaviors appeared to be perceptory behaviors and were followed by upwind flight by 97% of beetles tested in groups of 10 and by 75% of individual beetles. How-

TABLE 2. RESPONSE OF *Diabrotica* TO WHOLE EARS OF CORN WITH SILKS, CORN SILKS, AND UNTREATED CONTROLS[a,b]

			Direction of initial flight (% of those flying)				Final position after 5 min (% of all beetles)			
									Upwind end	
Treatment	*N*	Flying (%)	Vertical	Downwind	Upwind	Crosswind	Release area	Mid-tunnel	Not on corn	On corn[a]
Whole ears of corn	75	96a[b]	19.4a	12.5a	55.6a	12.5a	25.3a	1.3a	12.0a	61.3a
Silks strapped to screens	20	100a	30a	5a	50a	15a	0a	15b	10a	75a
Silks in flask connected to funnels	30	90a	25.9a	11.1a	55.6a	18.5a	13.3a	10b	6.7a	70a
Untreated control	85	92.9a	34.2a	38.0b	7.6b	20.2a	56.5b	10.6b	32.9b	—

[a] Includes beetles on screens and release funnels.
[b] Numbers analyzed by chi-square with Yates' correction. Percentages within a column followed by different letters are significantly different ($P < 0.05$).

TABLE 3. RESPONSE OF MALE AND FEMALE *D. barberi* AND *D. virgifera virgifera* TO WHOLE EARS OF CORN WITH SILKS AND UNTREATED CONTROL[a]

Species and treatment	Sex	*N*	Flying (%)	Direction of initial flight (% of those flying)				Final position after 5 min (% of all beetles)			
				Vertical	Downwind	Upwind	Crosswind	Release area	Mid-tunnel	Upwind end: Not on corn	Upwind end: On corn
D. barberi											
Whole ears of corn	M	22	100a	22.7a	9.1a	50.0a	18.2a	36.4a	0a	13.6a	50.0a
	F	17	100a	23.5a	11.8a	47.1ac	17.6a	23.5a	5.9a	29.4a	41.2a
Untreated control	M	14	92.9a	38.4a	15.4a	23.1bc	23.1a	50.0ab	14.3a	35.7a	—
	F	14	85.7a	41.7a	25.0a	0b	33.3a	71.4b	7.1a	21.4a	—
D. v. virgifera											
Whole ears of corn	M	25	96a	16.7a	12.5a	66.6a	4.2a	16.0a	0a	0a	84.0a
	F	11	81.8a	11.1a	22.2a	55.6a	11.1ac	27.3ac	0a	9.1a	63.6a
Untreated control	M	24	100a	45.8b	16.7a	4.2b	33.3bc	50.0bc	12.5a	37.5b	—
	F	33	90.9a	20.0a	23.3a	6.7b	50.0b	57.6b	9.1a	33.3b	—

[a]Beetles were tested individually in an undivided flight tunnel. Numbers analyzed by chi-squared with Yates' correction. Percentages for a species and in the same column that are followed by different letters are significantly different ($P < 0.05$).

ever, only 1% of beetles flew upwind in the absence of attractants (Van-Woerkom et al., 1983). More importantly, their beetles did not orient themselves facing into the wind until wind speeds were greater than 2.0 m/sec. At a wind speed of 0.33 m/sec, most beetles in our tests oriented themselves upwind when exposed to corn volatiles.

After taking flight upwind, most beetles flew only a short distance. These 10- to 20-cm flights were called hop flights (HPF). This type of behavior was more common than flight up the entire length of the tunnel, called full flight (FF). Beetles typically proceeded upwind by a series of HPF and WW movements. Upwind HPF and WW were interspersed with several pauses (PW), during which time the beetles lifted their heads (HU), lifted their elytra (E), passed their foretarsi through their mouthparts (FT), rubbed their hind legs against each other (HL), and extended their hind wings (HW). These stationary behaviors also appeared to be perceptory behaviors, since they were usually followed by corrections (changes) in the direction of movement. Perception phases, consisting of one or more stationary behaviors, occurred infrequently, usually three to four times, in beetles that entered the chamber baited with the corn ears. These beetles were typically less diverse in their behavior than beetles that entered the unbaited chamber. Behavior included three to four HPF upwind and three to four WW upwind sequences, before ending up on the corn silks. Beetles that entered the unbaited chamber typically displayed the perceptory phase more than 10 times. Beetles in the unbaited chamber (random phase) displayed a higher proportion of lateral and downwind directed HPF and WW behaviors (Table 4). Apparently, after losing the attractant, the corrective movements are lateral and downwind relative to the airflow. In most cases, this action led the beetles out of the unbaited chamber and back into the release area where the two airstreams mixed and where they could reorientate to the corn. Thus, although initial upwind flights left 59% of the beetles in the unbaited chamber, ultimately 73.3% of the beetles ($N = 300$) found the corn ears (Table 1). The same host-finding responses were shown by beetles tested individually in the undivided flight tunnel. Beetles that flew upwind often flew past the source of volatiles, but subsequently paused and then made downwind and lateral WW and HPF movements that eventually placed them back in the plume of volatiles. A large part of successful host finding appeared to be due to this ability to relocate the volatile plume in the airstream and then reorientate to the source.

Most beetles that failed to respond to the volatiles from corn, and most of those in the controls, walked about the release area in all directions. Many beetles also flew downwind or laterally, so that net displacement in the flight tunnel was downwind.

Sequential Analysis of Host-Finding Behavior. The observations above were confirmed by analysis of 190 successful host-finding sequences using individual beetles. Host-finding behavior was complex. *Diabrotica barberi* and *D.*

TABLE 4. MEAN NUMBERS OF WALKING WITH ANTENNAE WAVING (WW) AND HOP FLIGHT (HPF) BEHAVIORS PER *Diabrotica* ADULT CLASSIFIED ACCORDING TO PHASE OF HOST-FINDING BEHAVIOR AND DIRECTION OF MOVEMENT

Behavior[a]	Phase of behavior[b]	*N*	Direction of movement		
			Upwind	Lateral	Downwind
Walking with antennae waving (WW)	Random	50	2.56	2.88	1.28
	Orientation	51	4.71	1.41	0.27
	Search	36	2.25	3.86	2.50
Hop flights (HPF)	Random	50	0.08	0.68	1.00
	Orientation	52	1.33	0.58	0.02
	Search	34	0.68	0.15	0.27

[a] The interaction between both WW and HPF behaviors and direction of movement was significant ($P < 0.001$).

[b] Phase response: random, in the absence of host volatiles; orientation, in the presence of host volatiles; search, host volatiles present but behaviors recorded after beetles had left the odor plume and until they reentered it.

v. virgifera located the volatile source using three movement phases: random activity, orientation to the source, and search with reorientation. The existence of these three distinct movement phases was confirmed in two ways. First, each phase had a characteristic response pattern composed of the ratio of lateral, upwind, and downwind WW and HPF movements (ANOVA: $P < 0.001$) (Table 4). Also, first-order, preceding–following, transitions were calculated and found to be distinct for behaviors in each of the three movement phases.

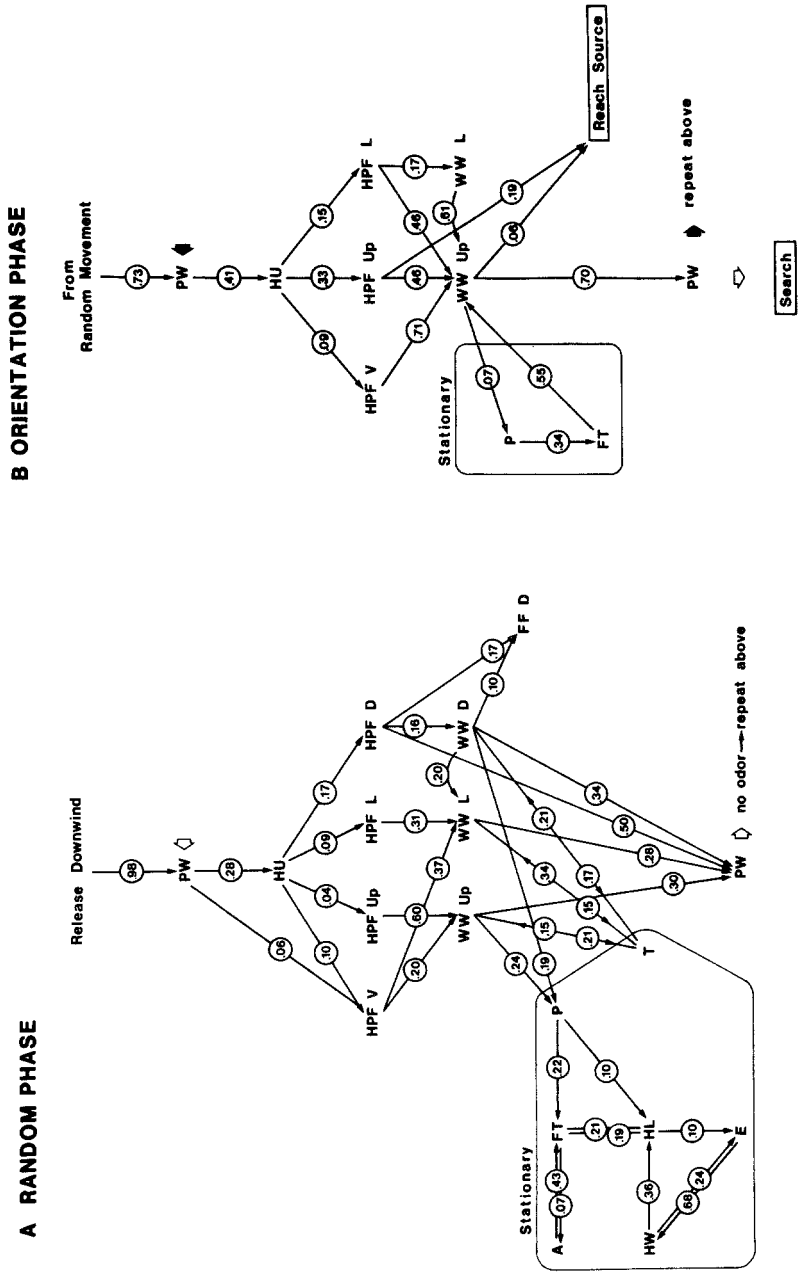
Sequential analysis of host-finding behavior was accomplished by first tabulating the frequencies of transitions from one behavior to the next, then consolidating the frequencies into a transition probability matrix using only first-order transitions. Self-transitions (i.e., when, after a predetermined time interval, the beetle remains in the same behavioral state) were not included in these matrices (the behavior was recorded as having occurred once). The chi-square value for the expected frequency of transition, based on the chance association of the two behaviors, was then calculated. The expected and observed frequency transitions were then tested by chi-square for any significant deviation from random. Standard normal deviates (Z) were calculated by taking the square root of the final chi-square value and applying it to a binomial test by selecting only those deviates with values of $Z = 1.96$–2.56 (for $P < 0.05$) or higher (more significant). Transitions with deviates yielding probabilities of $P < 0.05$ were used in construction of an ethogram to indicate the most probable behavioral sequence. A separate ethogram was constructed for each of the random,

orientation, and search phases (Figure 1). Each phase was characterized by the types of behavior shown by the beetles and by the conditional probability of occurrence of each preceding–following transition. As evidenced by the occurrence of several significant "following" behaviors and the low conditional probability of each multiple transition, host-finding behavior was not "stereotyped," but was instead plastic (Baker and Cardé, 1979).

In the random phase, the WW behavior occurred upwind and laterally more frequently than downwind, while HPF movements were more frequently directed downwind and laterally (Table 4). First-order transitions indicated that the conditional probability of flight was significant in all directions, as was WW (Figure 1A). This combination of HPF and WW usually resulted in serpentine movement with no net displacement of the beetles from the release area (79% of beetles), although downwind flight was also common (11%). Perception behavior was complex in the random phase. Inactivity was common, and beetles often went through a series of diverse stationary behaviors over a lengthy period of time. The perceptory behaviors of beetles occurred after WW in any direction and were followed by a change in the direction of movement.

Orientation was characterized by lateral and downwind HPF and WW occurring at much lower frequencies than upwind HPF and WW (Table 4). Also, first-order transitions indicated that the conditional probability of the HU to HPF upwind transition was high, as was the transition to WW upwind from HPF in any direction (Figure 1B). However, the response of beetles remained plastic, with both vertical and lateral HPF behaviors being significant occurrences. This combination of WW and HPF movements resulted in a net displacement of the beetles upwind. Host finding during the orientation phase involved only a few behaviors relative to the random phase. Transitions from one behavior to the next occurred quickly, with little interruption by lateral or downwind movements, and with few perceptory pauses. Also the entire sequence of stationary behaviors occurred less often and the perception phase had a lower diversity of stationary behaviors (boxes Figure 1A and B). The fewer pauses during orientation suggest that WW involves some perception and that PW was probably initiated by a lack of odors. Also, the perception phase occurred only after WW in both the random and orientation phases, further suggesting that beetles perceive odors while WW.

The search phase occurred only after an orientation phase. Search behavior was characterized by more frequent lateral WW than upwind and downwind WW, and downwind and upwind HPF occurring more frequently than lateral HPF (Table 4). This combination of WW and HPF resulted in displacement of the beetles downwind, lateral, or both, and thereby helped the beetles to relocate the odor plume. While searching, most beetles found the volatile source by reorienting with only one or two upwind HPF or WW movements. First-order transitions confirmed behavioral observations, whereby beetles leaving


A RANDOM PHASE
Release Downwind
PW
HU
HPF V
HPF Up
HPF L
HPF D
WW Up
WW L
WW D
FF D
T
P
Stationary
A
FT
HL
HW
E
PW no odor—repeat above
B ORIENTATION PHASE
From Random Movement
PW
HU
HPF V
HPF Up
HPF L
WW Up
WW L
Stationary
P
FT
Reach Source
PW repeat above
Search

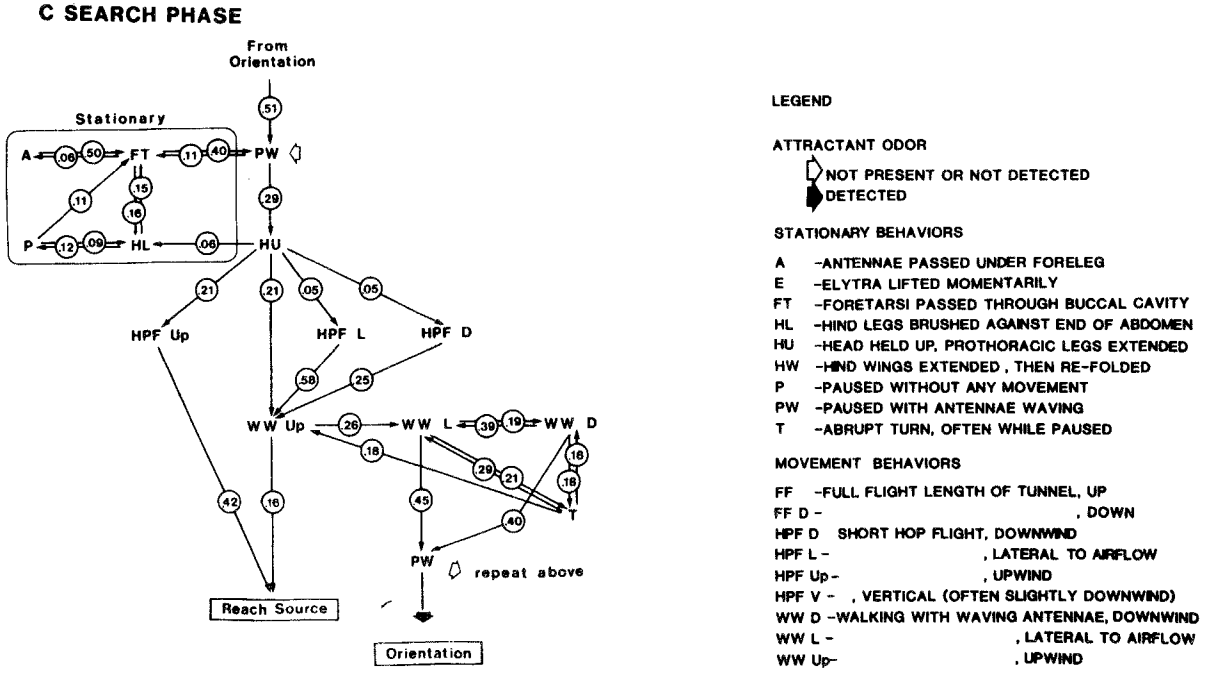


FIG. 1. Ethogram of the response of *Diabrotica* to the presence or absence of silk volatiles in a flight tunnel: (A) random—volatiles absent; (B) orientation—volatiles present; (C) search—volatiles present but beetles not in the odor plume. Circled values are the conditional probabilities of occurrence.

the odor plume paused, then flew upwind directly to the source of volatiles ($P = 0.12$), but most beetles flew downwind or laterally, then proceeded upwind by WW ($P = 0.71$) (Figure 1C). Beetles that did not encounter the odor plume when WW upwind subsequently moved laterally or downwind. Beetles WW in all directions frequently turned (T), presumably in an attempt to relocate the odor plume, again suggesting that the WW behavior was perceptory and initiated changes in the direction of movement.

A perception phase is used during the orientation behavior of other insects. Linn and Roelofs (1983) reported that at certain points in the flight sequence, male *Grapholitha molesta* (Busck) were very sensitive to changes in the chemical signal. They interpreted these "key" behaviors in the sequence as points at which males made decisions with respect to this type of mechanism when locating their host (Hawkes et al., 1978). Flies that overshoot the odor source land, then change their flight behavior to relocate the odor plume. The PW–HU sequence and the other concurrently occurring stationary behaviors of *Diabrotica barberi* and *D. v. virgifera* appear to be the key behaviors during which these beetles made decisions about their subsequent movement pattern. The ratio of directional movements following the PW–HU sequence was the same as that prior to this sequence whenever the chemical signal remained unchanged. When the chemical signal was changed, as when the beetles encountered corn volatiles in the airstream or moved out of the volatile plume, the ratio of directional movements following the PW–HU sequence and the conditional probabilities of movement in different directions (Figure 1) also changed. Therefore, changing the chemical signal had the effect of changing the net displacement of the beetles through the flight tunnel at the next perceptory phase. Moreover, the frequency of the perception phase itself was found to be significantly higher (ANOVA: $P < 0.05$) during the random phase and search phases than after the orientation phase, perhaps as a result of the same change in chemical signal.

Orientation to a host plant is commonly thought to involve zigzag flight, much like that observed when moths proceed up a plume of sex pheromone (Shorey, 1973). However, Hawkes et al. (1978) reported that *Delia radicum* orientates to its host without using zigzag flight. Instead, *D. radicum* uses a series of short upwind flights and landings in the odor plume, interspersed with a small amount of walking. The principal effect of the host odor was to increase the take-off rate and to direct flight upwind. Similarly, Dindonis and Miller (1980) reported that *Delia antiqua* (Meig.) uses a series of short flights when relocating the volatile plume from a nontransient emitter. They suggested that these short flights, punctuated by frequent landings, prevented the fly from straying far from the area where it was last stimulated by the odor. This behavior would maintain a high probability of restimulation, provided wind direction was not exceedingly variable (Dindonis and Miller, 1980). Both species of corn

rootworms frequently used short flights (HPF) and walking (WW), punctuated by the PW–HU sequence, to locate the source of volatiles during bioassays in a flight tunnel. Long flights (FF) up the entire length of the flight tunnel occurred infrequently ($P = 0.17$). Thus it appears that the host-finding behavior of these beetles has some similarities with that used by *D. radicum* and *D. antiqua*.

The random and search phases of host finding differed in the pattern of directional movements and net displacement of the beetle through the flight tunnel, even though both phases were initiated by the absence of silk volatiles. This implies that the beetles can differentiate between the absence of attractants, and the absence of attractants given their previous detection. In this study, beetles that overshot the odor plume switched their behavior from orientation to plume relocation, as indicated by the change in the ratio of directional movements (Table 4), and the change in the conditional probabilities of these movements (Figure 1). Hawkes et al. (1978) reported that female *Delia radicum* flying in a plume of host volatiles land as soon as they overshoot the odor source. Females then turn downwind, circle, and reenter the plume. This behavior is similar to the search phase of host finding used by *Diabrotica barberi* and *D. v. virgifera*. The search phase was responsible for 37.3% of successful host finding over that realized by beetles using orientation alone (24.0%), based on bioassay where 61.5% of beetles found the corn ears (Table 2).

Modeling Host-Finding Behavior. Given the perceptory role of stationary behaviors, the characteristic pattern of directional movement behaviors in each of the random, orientation, and search phases that followed perception (Table 4), and ethograms of the most probable transitions (Figure 1), we produced a simplified model of the host-finding response. Starting from any movement or stationary phase, the behavioral response and net displacement of the beetle can be predicted (Figure 2). At the next perceptory phase, which occurred frequently in the absence of an attractant odor, there was a high probability that the beetle would WW downwind, laterally, or upwind in about equal proportion if no attractant was detected (Table 4) (Figure 1A). If an attractant odor was detected, then there was a high probability that the beetle would make HPF and WW movements in an upwind direction (Table 4) (Figure 1B). This behavior usually placed the beetle on the corn silks. If the beetle overshot or walked out of the odor plume (loss of attractant odor), subsequent movement was characteristic of the search phase (Figure 1C). Searching beetles paused and performed stationary behaviors, then made HPF and WW movements in a lateral and downwind direction (Table 4) (Figure 1C), often with several repetitions. This behavior usually placed the beetle back in the odor plume. One or two upwind reorientation movements, HPF or WW, completed the search phase, placing the beetle on the corn silks (Figure 1).

The establishment that corn silks contain volatile chemical attractants for

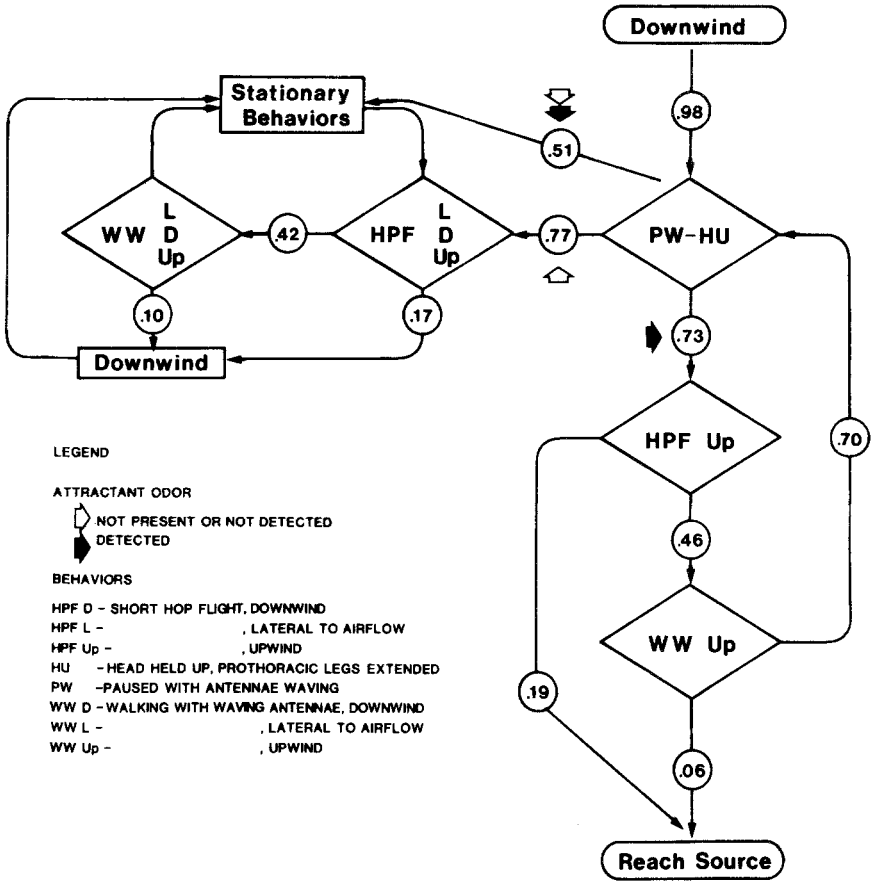


FIG. 2. Computer flow-chart equivalent of the ethograms (Figure 1) of host finding by *Diabrotica*.

Diabrotica beetles and the full description of host-finding behavior should facilitate further studies. Identification of biologically active compounds from corn silks may have an important application in trapping or monitoring programs for these important agricultural pests.

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TRAIL-FOLLOWING BEHAVIOR OF *Reticulitermes hesperus* BANKS (Isoptera: Rhinotermitidae)

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Abstract—The behavior of *Reticulitermes hesperus* Banks pseudergates (workers) was assessed on artificial trails containing different concentrations of sternal gland extract. On nongradient trails, more pseudergates were recruited to trails of greater pheromone concentration, they traveled a greater distance without pausing, and their rate of locomotion increased over that observed on trails of lesser concentration (positive orthokinesis). Of the individuals pausing before completing trails of high concentration, fewer left the trails or reversed direction (negative klinokinesis) than on trails of lower concentration. Termites walking down concentration gradients failed to complete these trails to the low-concentration termini. At a point representing an average decrease of slightly more than 10-fold in the original concentration of pheromone, individuals reversed their direction of travel and returned to the high-concentration terminus. Termites walking up pheromone gradients proceeded to the high-concentration termini without reversing direction. *R. hesperus* pseudergates are thus able to orient along a gradient of trail pheromone by longitudinal klinotaxis.

Key Words—Termite, *Reticulitermes hesperus*, Isoptera, Rhinotermitidae, pheromones, trail following, orientation.

INTRODUCTION

Reticulitermes spp. (Isoptera: Rhinotermitidae) establish chemical trails with pheromones secreted by the sternal gland, an epidermal gland located in the anterior portion of the fifth abdominal segment beneath the fourth abdominal sternite (Mosconi-Bernardini and Vecchi, 1964; Smythe and Coppel, 1966;

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Quennedey, 1971; Liang et al., 1979). The trail pheromone of *Reticulitermes virginicus* Banks was identified by Matsumura et al. (1968) as *cis*-3,*cis*-6,*trans*-8-dodecatrien-1-ol. This compound is attractive to other *Reticulitermes* spp. (Matsumura et al., 1972) and has been implicated as the pheromone of *Reticulitermes lucifigus* var. *santonensis* (Feytaud) (Ritter and Coenen-Saraber, 1969) and *Reticulitermes speratus* Kolbe (Honda et al., 1975). It may also be closely related to that of other species (Howard et al., 1976) or represent one component in a multicomponent trail pheromone (Kaib et al., 1982; Prestwich et al., 1984).

Traniello (1982) and Hall and Traniello (1985) consider termite trail-following behavior to consist of both recruitment and orientation components. Additionally, there are two aspects to orientation: orientation to the lateral boundaries of the trail space (cf., Bossert and Wilson, 1963) and orientation along the longitudinal axis of the trail. With respect to longitudinal orientation, we describe here the concentration-dependent responses elicited by *Reticulitermes hesperus* Banks pseudergates by different concentrations of sternal gland extract on artificial trails. Orientation responses were evaluated both on trails drawn with single concentrations of sternal gland extract and on trails containing discrete (incremental) concentration gradients of extract.

The significance of evaluating trail orientation with respect to pheromone gradients lies in the intriguing possibility that differences in pheromone concentration could indicate directionality on trails. Although behavioral assays with *Hospitalitermes sharpi* (Holmgren) (Jander and Daumer, 1974), *R. flavipes* (Runcie, 1983), and *Trinervitermes trinervoides* (Sjöstedt) (Tschinkel and Close, 1973) indicated that these species could not distinguish the direction of their nest from the direction of foraging areas, these assays employed short segments of naturally laid trails. Any differences in trail pheromone concentration over a short distance might not be sufficient to elicit changes in termite orientation. Leuthold (1975) suggested, based on observations of grass-feeding termites, *Trinervitermes* spp., that orientation could be facilitated by the presence of a pheromone gradient over an extensive network of trails.

These studies emphasize mechanisms of individual termite orientation. Rather than attempting to draw conclusions about behavior to unknown stimuli on natural trails, we chose to define the behavioral responses to a series of known stimuli on artificial trails. Thus, with the individual behavioral parameters established, investigation of the unknown natural stimuli, in the appropriate social context, can follow.

METHODS AND MATERIALS

Source of Insects and Gland Extracts. In behavioral assays we used undifferentiated *R. hesperus* pseudergates (workers) older than the third instar (as

determined by size) from two colonies. One of these was removed from severely infested Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) wood substructure framing in a home in Alameda County, California (Oakland). The other colony was from a Douglas-fir soil-retaining board on the grounds of a University of California family housing complex in the same county (Smyth). After collection, termites were removed from the wood and placed in plastic trays containing a small block of the Douglas-fir, damp cotton, and several pieces of Whatman No. 1 filter paper as feeding substrates. These trays were maintained in a humidity chamber at 94 ± 5% relative humidity, 21–25°C (Grace, 1986). Individuals from the two colonies were kept separate from each other and assayed only with extracts of pseudergates from the same colony.

Stock sternal gland extracts containing trail pheromone (1× dilution) were prepared by removing the fourth and fifth sternites from 10 *R. hesperus* workers (immobilized by exposure to Dry Ice) and extracting these sternites in 1 ml dichloromethane (Baker analyzed) for 24 hr. Tenfold (0.1×), 100-fold, and several lesser dilutions (0.0625× and 0.03125×) were prepared from the stock solution. Artificial trails containing higher concentrations of trail pheromone than the stock solution were prepared by repeatedly overlaying trails with the stock solution.

Assay Conditions. Each artificial trail consisted of a straight 100, 150, or 200-mm line, 1–2 mm in width, drawn on Monroe No. 41 parchment tracing paper (5 × 25 cm) with a microliter syringe containing 1 μl of extract per 50 mm of trail. A thin, uniform line of solvent could be more readily applied to this particular paper than to others that were tested. Preliminary tests indicated that trails drawn on this paper with pure solvent and with extracts of the paper itself did not elicit any noticeable behavioral response (e.g., trail following, arrestment, repellence). A light pencil line on the underside of the paper served as a guide for extract application and as a reference during the assay. Our extract application rate of the stock glandular extract (1×) at 1 μl/50 mm of trail was equivalent to a concentration of 2×10^{-4} glandular equivalents (GE) per millimeter of trail.

The papers were placed on a glass surface and uniformly illuminated by overhead fluorescent lighting (13.5–19.5 foot-candles). All assays were performed at ambient conditions (21–25°C, 35–45% relative humidity), which were standardized throughout each experiment by regulating the laboratory temperature. In each assay, an artificial trail was drawn with extract and the solvent allowed to evaporate for ca. 15 sec. A single *R. hesperus* pseudergate was gently deposited from a small glass vial onto one end of the trail. Timing of the assay and measurement of distance traveled on the trail was begun when the insect moved a distance greater than one body length in any direction. In experiments where distance traveled on the trail was the critical parameter, only the distance traveled on new (previously untraveled) portions of the trail was considered. The points of initiation and termination of locomotion along the trail

axis were marked lightly in pencil at the base of the paper for later measurement. In all of our experiments, a new trail was drawn for each individual assay, and each insect and each trail were used only once to prevent any effects from behavioral conditioning or trail reinforcement.

Orientation on Nongradient Trails. Two experiments were performed on nongradient artificial trails drawn with different dilutions of sternal gland extract. The first experiment was designed to determine whether a concentration-dependent relationship existed in the response to artificial trails and longitudinal orientation upon them. We recorded the distance traveled in 0.5, 1.0, 2.0, or 3.0 min by pseudergates from the Oakland colony on 200-mm artificial trails of either stock (1×), 0.1× or 0.01× dilute extract and control trails of dichloromethane. Any movement along the trail axis was scored as a positive response, with the short movements recorded along the control trails representing the background level of behavioral "noise" in the assay. Twenty-five individual assays were performed for each time interval with each of the three concentrations of glandular extract and the dichloromethane control, for a total of 16 independent treatments.

Our second experiment was conducted to examine concentration-mediated differences in orientation behavior following initiation of trail following. Pseudergates from the Oakland and Smyth colonies were deposited at one end of 150-mm trails containing glandular extract (from their respective colony-mates) in either 10×, 1×, or 0.1× concentration. Trails of the 0.01× concentration and solvent controls were not included in this experiment since these elicited very little or no trail following in the first experiment. We recorded the distance traveled on each trail without pausing or leaving the trail, the time taken to travel that distance (rate of locomotion), and the behavior of those that did not immediately complete the 150-mm trail (i.e., continuing forward after pausing on the trail, leaving the trail, or reversing direction). Thirty individuals from each colony were tested with each of the three concentrations of glandular extract.

Orientation on Gradient Trails. Three separate experiments were performed on artificial trails consisting of a concentration gradient of sternal gland extract. In the first experiment, a pseudergate from the Oakland colony was deposited at either the high or the low concentration end of a 100-mm artificial trail containing a 20-fold arithmetic gradient of either the stock 1× sternal gland extract or its 10-fold dilution. These 0.1×–2× and 1×–20× arithmetic gradients were created by repeatedly overlaying successive 5-mm increments of the trail with either the stock 1× extract or the 0.1× dilution. Thus, individuals deposited at one end of the trail were initially either walking up or down a steep gradient of trail pheromone. Under these conditions we recorded the number of termites completing trails in a 3-min period, the time taken to complete trails, and the number reversing their direction of travel before reaching the end of the trail. Behavior of pseudergates walking up or down a pheromone gradient was

compared to behavior on nongradient trails containing only the 1× extract or the 0.1× dilution. Twenty-five individual assays each were performed walking up the gradient, down the gradient, and on nongradient trails of the 1× stock extract, while 20 individual assays in each of these three treatments were performed with the 0.1× dilution.

A second experiment was performed to supplement observations made during the first experiment with respect to the distance traveled by individuals walking down a pheromone gradient before reversing their direction of travel. Twenty-five pseudergates from the Oakland colony were individually tested walking down 20-fold gradients (100 mm in length) and on nongradient artificial trails drawn with 0.0625× and 0.03125× dilutions of the stock extract. These dilutions were based upon the earlier observation that a 0.01× dilution of the stock extract elicited very little trail following. The proportions of individuals completing trails and reversing direction without completing trails were compared on gradient and nongradient trails at each dilution.

Our third experiment measured the behavior of 30 pseudergates each from the Oakland and Smyth colonies walking either up or down a 10×–1×–0.1× incremental logarithmic pheromone gradient in individual assays on 150-mm artificial trails. We compared the distances traveled without reversing direction and the proportion of pseudergates in each treatment completing trails without reversing direction.

Analyses. Statistical analyses employed two-tailed t tests and analysis of variance (ANOVA) with the rank transformation (SAS Institute, 1982). This ANOVA is equivalent to the Kruskal-Wallis test with the F approximation (Conover and Iman, 1981; Quade, 1966). Comparison of means was performed with the Tukey-Kramer method (Kramer, 1956) or the Ryan-Einot-Gabriel-Welsch (REGW) multiple F test ($\alpha \leqslant 0.05$). Proportions were compared with multiple Z tests using Bonferroni's inequality to maintain $\alpha \leqslant 0.05$ (Dixon and Massey, 1983).

RESULTS

Orientation on Nongradient Trails. In the first experiment on nongradient trails, the proportion of pseudergates initiating trail following increased significantly as the concentration of glandular extracts increased (Table 1). With the 23% overall response recorded to the dichloromethane control trails representing random movements, almost twice as many individuals (41%) responded to the 100-fold (0.01×) dilution of glandular extract. The number of respondents increased significantly (90% and 99%) at the 0.1× and 1× concentrations. Conversely, there was no apparent relationship between the length of the exposure time and the number of individuals responding to trails (Table 1).

The mean distance traveled on the 200-mm trails by responding pseuder-

TABLE 1. NUMBER RECRUITED AND MEAN DISTANCE TRAVELED IN DIFFERENT TIME INTERVALS BY *Reticulitermes hesperus* PSEUDERGATES ON 200-mm ARTIFICIAL TRAILS OF STERNAL GLAND EXTRACT

Trail concentration[a]	Total percent recruited[d]	Number recruited (N) and mean distance ± SEM(mm)[c]			
		0.5 min	1.0 min	2.0 min	3.0 min
1×	99a	(25) 181.72 ± 7.92a	(25) 186.60 ± 8.96a	(25) 191.92 ± 6.03a	(24) 196.33 ± 3.67a
0.1×	90b	(23) 94.22 ± 13.20b	(22) 126.73 ± 14.99b	(23) 124.70 ± 14.70b	(22) 149.00 ± 13.81b
0.01×	41c	(7) 26.86 ± 9.09c	(12) 23.08 ± 3.92c	(12) 25.08 ± 4.53c	(10) 37.30 ± 13.09c
Control	23d	(6) 10.33 ± 2.22c	(6) 13.00 ± 1.91c	(6) 13.50 ± 2.87c	(5) 17.20 ± 3.51c

[a] 1× = 10 sternal glands per milliliter dichloromethane, applied at the rate of 1 μl per 50 mm of trail. Control = dichloromethane.
[b] Total percent recruited in all four time periods (N = 100). Different letters indicate that each proportion is significantly greater than the one appearing immediately below it in the column (one-tail Z test of proportions, $\alpha \leq 0.05$).
[c] N = number of pseudergates (Oakland colony) recruited to trails in 25 individual assays with each concentration of extract in each time interval. SEM = standard error of the mean. Means in the same column followed by different letters are significantly different (ANOVA of ranks, Tukey-Kramer test, $\alpha \leq 0.05$).

gates also increased with increasing concentration of glandular extract (Table 1). Of the 100 total termites tested with each dilution of glandular extract, 88 completed trails drawn from the stock 1× concentration, 32 completed trails at the 0.1× concentration, and none completed trails of 0.01× concentration or the dichloromethane alone. There was no statistically significant relationship (ANOVA, $\alpha \leqslant 0.05$) between the distance traveled and the length of the exposure period. This can be attributed to the relatively short length of the trail (200 mm) and the rapidity with which trail following was initiated under our assay conditions.

Results from our second experiment (Table 2) indicate that differential movement along trails of different pheromone concentration is due, at least in part, to concentration-dependent differences in rates of locomotion. Individuals from both the Oakland and Smyth colonies walked faster on trails of 1× and 10× concentrations of glandular extract than on trails of 0.1× concentration. Oakland pseudergates also walked faster on 10× than on 1× trails.

The distance traveled without pausing, reversing direction, or deviating from the 150-mm trail was also greater at higher concentrations of glandular extract (Table 2). The mean distances traveled by pseudergates from both colonies at the 1× and 10× concentrations differed significantly from the distances traveled on trails of 0.1× concentration.

Individuals that did not immediately complete the 150-mm trails evidenced three behaviors: (1) continuing forward on the trail after pausing, (2) leaving the trail, or (3) turning around and reversing their direction of travel on the trail. Combining the results from both colonies (Figure 1), 28% of the pseu-

TABLE 2. MEAN RATE OF LOCOMOTION AND MEAN DISTANCES TRAVELED WITHOUT VARIATION IN BEHAVIOR BY *Reticulitermes hesperus* PSEUDERGATES ON 150-mm ARTIFICIAL TRAILS DRAWN WITH STERNAL GLAND EXTRACT[a]

Colony	Concentration[b]	Rate ± SEM (mm/sec)[c]	Distance ± SEM (mm)[c]
Oakland	0.1×	5.30 ± 0.52c	122.97 ± 7.73b
	1×	7.33 ± 0.54b	143.00 ± 3.93a
	10×	10.11 ± 0.74a	146.90 ± 3.10a
Smyth	0.1×	6.22 ± 0.48b	87.33 ± 9.69b
	1×	8.32 ± 0.35a	131.37 ± 6.37a
	10×	8.64 ± 0.65a	120.70 ± 7.88a

[a] Treatment $N = 30$.
[b] 1× = 10 sternal glands per milliliter dichloromethane, applied at the rate of 1 μl per 50 mm of trail.
[c] Means in each column within each colony followed by different letters are significantly different (ANOVA of ranks, REGW multiple F test, $\alpha \leqslant 0.05$). SEM = standard error of the mean.

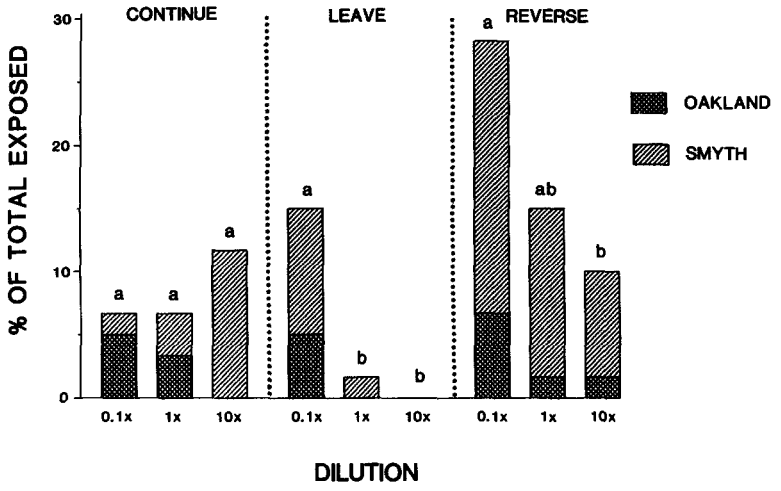

FIG. 1. Behavior of *Reticulitermes hesperus* pseudergates after pausing on 150-mm artificial trails drawn with three different dilutions of sternal gland extract. Thirty individuals from two colonies (Oakland and Smyth) were tested with each dilution ($N = 60$). Different letters over bars in each category indicate that proportions are significantly different (two-tail Z test of proportions, $\alpha \leqslant 0.05$).

dergates on trails of low 0.1× concentration of glandular extract reversed their direction of travel, in contrast to 15% of those on trails of 1× concentration and 10% of those on trails of 10× concentration. Significant differences occurred between the 0.1× and 10× concentrations. Although fewer termites left the trail in each treatment than reversed direction, the relationship to pheromone concentration was similar to that observed for directional reversals. However, the proportion leaving the trail at both the 1× and 10× concentrations was significantly different from the 0.1× concentration. More individuals continued forward after pausing on trails of the 10× concentration than was the case with either of the lower two concentrations, although these differences were not statistically significant.

Orientation on Gradient Trails. R. hesperus pseudergates on artificial trails containing a 20-fold gradient of pheromone exhibited significant differences in behavior when compared to pseudergates on trails of constant pheromone concentration (Table 3). In the 3-min assay period, fewer individuals walking down a pheromone gradient completed the 100-mm trails than was the case with those walking up a gradient or on nongradient trails of 1×, 0.1× or 0.0625× pheromone concentration. With the more dilute sternal gland extract (0.1×), more individuals walking up a 20-fold gradient completed the trails than did those walking on nongradient pheromone trails. This significant difference did not

TABLE 3. RESPONSES OF *Reticulitermes hesperus* PSEUDERGATES ON 100-mm ARITHMETIC GRADIENT AND NONGRADIENT TRAILS[a]

Concentration at low end of gradient[b]	Trail gradient	Percent completing trail[c]	Time to complete trail (sec ± SEM)[d]	Percent reversing direction[c]
1×[e]	Down	76a	36 ± 6a	52a
	No gradient	96b	27 ± 4a	16b
	Up	96b	20 ± 2a	8b
0.1×[e]	Down	30a	48 ± 7a	85a
	No gradient	70b	31 ± 5a	40b
	Up	100c	41 ± 8a	25b
0.0625×[f]	Down	56a	24 ± 2a	44a
	No gradient	76b	20 ± 1a	16b
0.03125×[f]	Down	56a	21 ± 2a	44a
	No gradient	60a	18 ± 1a	20b

[a] Termites (Oakland colony) were tested walking up or down 20-fold (20–1) arithmetic gradients and on nongradient trails corresponding to the pheromone concentration found at the low end of the gradient trails.

[b] 1× = 10 sternal glands per milliliter dichloromethane, applied at the rate of 1 μl per 50 mm of trail.

[c] Proportions within each concentration followed by different letters are significantly different (Z test of proportions, $\alpha \leqslant 0.05$).

[d] Means within each concentration followed by the same letter are not significantly different (ANOVA of ranks with Tukey-Kramer test, or t test of ranks, $\alpha \leqslant 0.05$). SEM = standard error of the mean.

[e] $N = 25$ (1×), or $N = 20$ (0.1×). Termites were observed for 3 min. Those reversing direction could return to complete the trail.

[f] $N = 25$. Termites were observed until they either completed the trail, reversed direction, or left the trail.

appear in tests with the 1× extract, suggesting the presence of an upper limit for pheromone perception and response under our assay conditions. With those termites completing trails, the time taken to do so did not differ significantly with respect to the trail gradient.

More termites walking down a 20-fold pheromone gradient reversed their direction of travel before (or without) completing the trail than did those walking up a gradient or on nongradient trails (Table 3). This difference was significant at every dilution of gland extract assayed. Thus, the response to a negative gradient was not to wander off the trail as the pheromone concentration decreased, but to reverse direction and walk in the direction of increasing concentration. The proportion of pseudergates completing nongradient trails declined to 60% with the 0.03125× dilution (Table 1). However, half of these

individuals (five) did not respond to the low concentration of pheromone at all, while the 11 termites not completing the gradient trails all reversed direction after initially responding to the trails and walked back to the high concentration termini.

The similarity in the distances traveled by termites down gradient trails of different pheromone concentration before reversing direction (Table 4) indicates that this is not a static klinokinetic response to an absolute threshold level of trail pheromone. Rather, the turning response appears to be initiated by a particular proportional decrease in the original concentration of pheromone detected. However, the relatively small sample size and high degree of variability in individual responses indicate that further trials are necessary to substantiate this hypothesis.

Termites exposed to logarithmic gradients on the artificial trails behaved similarly to those on arithmetic gradients (Table 5). Very few of the pseudergates walking down the 10×–1×–0.1× gradient completed the trails to the low-concentration termini. Rather, they reversed direction and walked back up the pheromone gradient. Although the mean distances traveled down the gradient trails at which these reversals occurred differed significantly between the

TABLE 4. DISTANCES TRAVELED BY *Reticulitermes hesperus* PSEUDERGATES BEFORE REVERSING DIRECTION ON 100-mm ARITHMETIC GRADIENT AND NONGRADIENT TRAILS[a]

Concentration at low end of gradient[b]	Mean distance ± SEM (mm)[c]		
	No Gradient[d]	Down gradient[d]	Up gradient[e]
1×	45 ± 10a (*N* = 4)	72 ± 6a (*N* = 13)	60 ± 20a (*N* = 2)
0.1×	34 ± 10a (*N* = 8)	60 ± 7a (*N* = 17)	55 ± 16a (*N* = 5)
0.0625×	23 ± 4a (*N* = 4)	65 ± 8a (*N* = 11)	
0.03125×	40 ± 11a (*N* = 5)	62 ± 9a (*N* = 11)	

[a] Termites (Oakland colony) were tested walking up or down 20-fold (20–1) arithmetic gradients and on nongradient trails corresponding to the pheromone concentration found at the low end of the gradient trails.

[b] 1× = 10 sternal glands per milliliter dichloromethane, applied at the rate of 1 μl per 50 mm of trail.

[c] SEM = standard error of the mean.

[d] Means in the same column followed by the same letter are not significantly different (ANOVA of ranks, Tukey-Kramer test, $\alpha \leqslant 0.05$).

[e] Means are not significantly different (*t* test of ranks, $\alpha \leqslant 0.05$).

TABLE 5. RESPONSES OF *Reticulitermes hesperus* PSEUDERGATES ON 150-mm LOGARITHMIC GRADIENT TRAILS[a]

Colony	Trail gradient	Percent completing trail[b]	Time to complete trail (sec ± SEM)[c]	Percent reversing direction[b]
Oakland	Down	17a	29 ± 2a	83a
	Up	90b	24 ± 2a	10b
Smyth	Down	0a	—	100a
	Up	73b	32 ± 3	27b

[a] 30 pseudergates from each colony were tested walking either up or down 150-mm trails containing three 10-fold dilutions of sternal gland extract in successive 50 mm increments (0.1×-1×-10×). Termites were observed until they either completed the trail, reversed direction, or left the trail.
[b] Proportions from the same colony in each column followed by different letters are significantly different (Z test of proportions, $\alpha \leqslant 0.05$).
[c] Means for the Oakland colony are not significantly different (*t* test of ranks, $\alpha \leqslant 0.05$). SEM = standard error of the mean.

Oakland and Smyth colonies (Table 6), identical concentrations of pheromone (e.g., 1×) were associated with both of these mean distance values. With respect to the gradient, this 1× concentration represents a 10-fold decrease in the amount of pheromone to which the pseudergates were initially exposed. Summing the results from the Oakland and Smyth colonies, slightly more termites starting at the 10× terminus reversed direction when they encountered the 1× portion (29 workers) of the trail than the 0.1× portion (22 workers). This variation in individual response agrees with our observations on arithmetic gradients

TABLE 6. DISTANCES TRAVELED BY *Reticulitermes hesperus* PSEUDERGATES BEFORE REVERSING DIRECTION ON 150-mm LOGARITHMIC GRADIENT TRAILS[a]

Colony	Mean distance ± SEM (mm)[b]	
	Down gradient	Up gradient
Oakland	86 ± 7a (N = 25)	25 ± 6b (N = 3)
Smyth	65 ± 5b (N = 30)	48 ± 11b (N = 8)

[a] Termites were tested walking up or down 150-mm trails containing three 10-fold dilutions of sternal gland extract in successive 50-mm increments (.1×-1×-10×).
[b] Means in the same column followed by different letters are significantly different (*t* test of ranks, $\alpha \leqslant 0.05$). SEM = standard error of the mean.

(Table 4) and suggests that the mean proportional decrease in trail pheromone needed to trigger a turning response is slightly in excess of a 10-fold change.

DISCUSSION

Although termite trail pheromones may consist of multiple chemical components, as suggested by Kaib et al. (1982), Traniello (1982), Traniello and Busher (1985), Runcie (1983) and Prestwich et al. (1984), certain aspects of trail-following behavior appear to be modulated quantitatively. The behavior of *R. hesperus* on nongradient trails of sternal gland extract indicates that initiation of trail following, rate of movement (orthokinesis), and distance traveled without any variation in behavior are directly related to the concentration of the compound(s) eliciting trail following. Reversing direction on the trail (klinokinetic behavior), and propensity to leave the trail, on the other hand, are negatively related to pheromone concentration. The positive orthokinesis we observed is in agreement with the suggestion by Prestwich et al. (1984) that the speed of termite trail-following may be related to discrimination of pheromone. Van Vorhis Key et al. (1981) also suggested from their work with *Iridomyrmex humilis* that speed of locomotion might be a relevant factor in trail-following assays; they subsequently (Van Vorhis Key and Baker, 1982) incorporated locomotory rate into their index of anemotaxis induced in *I. humilis* by trail pheromone.

Our laboratory assays were designed to illuminate individual orientation in response to specific chemical stimuli. Initiation of trail following in response to sternal gland extracts is one component of, but not necessarily analogous to, termite recruitment to trails under field conditions. Other social and environmental stimuli are likely of great importance in affecting their behavior. However, concentration-dependent responses in initiating trail following, rate of locomotion, distance traveled without leaving the trail, and a reduced klinokinetic response would all be advantageous to *R. hesperus* in locating the nest after a disturbance or in enhancing foraging efficiency. More individuals could be recruited to well-traveled trails, presumably containing a higher concentration of pheromone, and would be able to reach the nest or forage more rapidly. Individuals encountering trails of low pheromone concentration would tend to leave these trails or reverse direction on them, increasing their probability of encountering a well-traveled trail and minimizing the time spent following old and possibly unprofitable trails.

Detection of a change in pheromone concentration by *R. hesperus* pseudergates is dependent upon successive longitudinal sampling of trail increments and temporal processing of this information. This orientation mechanism has been referred to as longitudinal klinotaxis (Ewer and Burrell, 1950; Kennedy,

1978) to distinguish it from lateral klinotaxis, or side-to-side sampling. Orientation to the lateral boundaries (edges) of the trail-space may involve either lateral klinotaxis or tropotaxis, independent of orientation along the longitudinal axis of the trail. Kennedy (1986) recently proposed the alternative label schemakinesis to emphasize the internally programmed, self-steered basis of this response to longitudinal gradients.

The ability to detect and respond to changes in trail pheromone concentration suggests the possibility that differential deposition of pheromone could indicate directionality on natural trails. Such a mechanism does not necessarily require that individuals have the ability to vary the amount of pheromone deposited. As Leuthold (1975) has suggested for grass-harvesting termites, concentration of termite activity in particular areas of a gallery network could passively polarize the system in an additive fashion. Although behavioral studies to date have not documented the existence of pheromone gradients on termite trails, chemical analyses of trail increments may prove useful in this regard. Identification of the trail pheromone(s) is crucial to such investigations.

Concentration-dependent responses to crude glandular extract do not imply that a single component is responsible for eliciting the multiple behaviors comprising the trail-following response. Traniello (1982) and Traniello and Busher (1985) suggested that both an ephemeral recruitment component and a very persistent orientation component were present in glandular extracts from the termite *Nasutitermes costalis* (Holmgren). Similarly, additional components in the pheromone blend may excite locomotion or suppress klinokinesis. Researchers must develop assays designed to measure these single behaviors rather than a general trail-following response if such semiochemicals are to be isolated and identified.

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THE ROLE OF LEAF LIPIDS IN FOOD SELECTION BY LARVAE OF THE TOBACCO HORNWORM, *Manduca sexta*

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Abstract—The role of leaf lipids in food plant selection by larvae of *Manduca sexta* was investigated by measuring preference responses in a two-choice preference test using glass fiber filter paper disks laced with extract (test) or water (control). The larvae respond to the petroleum ether extract of whole leaves of the host-plant *Lycopersicon esculentum* (tomato) extract in a concentration-dependent manner. At "natural concentration" it is the most strongly stimulating extract or compound yet tested using the disk test. This response is affected by food plant experience of the larvae, suggesting stimulation by plant-specific compounds in the extract. The extract contains volatile compounds that attract the larvae. In contrast, it does not promote continued feeding on an agar–cellulose diet that incorporates the extract. Also stimulating are the extracts of leaf surfaces of two hosts, *L. esculentum* and *Solanum pseudocapsicum*, and two acceptable nonhosts, *Brassicae napus* and *Vigna sinensis*, indicating the presence of nonpolar feeding stimulants at the leaf surface. However, similar leaf-surface extracts of the unacceptable plant *Canna generalis* were inactive, although the surface extraction process renders this plant acceptable. Leaf-surface extracts of *L. esculentum*, *S. pseudocapsicum*, and *B. napus* evoke feeding responses that are qualitatively comparable to those of their corresponding leaves. However, no such parallel is found for surface extracts of *V. sinensis* and *C. generalis*. Thus, nonpolar compounds at the leaf surface of host and some acceptable non-host-plant species strongly stimulate feeding and hence must play an important role in food selection by the tobacco hornworm.

Key Words—*Manduca sexta*, Lepidoptera, Sphingidae, feeding preference, leaf lipid extracts, plant surface, hosts, nonhosts.

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INTRODUCTION

Larvae of the tobacco hornworm, *Manduca sexta* (Johan.), feed only on some members of the family Solanaceae (Madden and Chamberlin, 1945; Yamamoto and Fraenkel, 1960a). Many studies have tried to elucidate the chemical basis of this specificity by testing various host-plant extracts and fractions thereof, as well as some solanaceous allelochemicals (Yamamoto and Fraenkel, 1960b; Fraenkel et al., 1960; Howard, 1977). These studies showed that only polar constituents of host plants are important in stimulating feeding. Bordner et al. (1983) presented a tentative structure of one polar host-recognition factor.

In contrast to the above studies on polar compounds, Morgan and Crumb (1928) and Städler and Hanson (1978) demonstrated that nonpolar material extracted from host leaves also stimulated feeding; moreover, the feeding responses to the extracts resemble very closely those of their corresponding leaves (Städler and Hanson, 1978). de Boer and Hanson (1982) showed that nonpolar feeding stimulants of a host plant are neutral lipids. Many of these lipids can be found on the outer surface of the leaves and, upon contact, may signal the palatability of the plant to the insect before it initiates feeding (Chapman, 1977; Woodhead and Chapman, 1986). Following these leads, the present investigation attempts to characterize the feeding responses of *Manduca sexta* to nonpolar extracts of both host and nonhost plants.

METHODS AND MATERIALS

Larvae of the tobacco hornworm, *Manduca sexta* (Johan.) (Lepidoptera, Sphingidae) were reared from eggs obtained from a culture maintained on an artificial diet (Yamamoto, 1969) at the U.S.D.A. at Beltsville, Maryland. Larvae were reared on cut foliage of tomato, *Lycopersicon esculentum* Mill. var. Better Boy and Jerusalem cherry, *Solanum pseudocapsicum* L. var. Jubilee. The rearing conditions were 16:8 hr light–dark and 27 ± 3°C. Tests were done in the light at the same temperature.

Bioassays. The preference test of Städler and Hanson (1978) was used to assay the feeding preferences of the larvae. Four test and four control disks of glass fiber filter paper (Whatman GF/A, 14 mm in diameter) were placed in ABAB . . . order around the circumference of transparent containers (10 cm in diameter). The floor of the containers was a layer of paraffin wax into which were inserted pins holding the disks ca. 5 mm above the substrate. Small retainers (3 mm in diameter) punched from acetate sheets (or Teflon when chloroform was used as solvent) kept the disks from sliding down the pin. A wire screen covered a moist piece of filter paper on the floor of the container. A freshly moulted fifth-instar larva, starved for 12–24 hr after the moult, was placed in the center of the container. The containers were covered with transparent lids

to maintain high humidity. When the larva had eaten approximately 50% of the total area of either test or control disks, the experiment was stopped and the area consumed of each disk was estimated visually. Larvae were tested only once, then discarded.

The test disks were laced with 0.1 ml of the test solution which is enough to wet each disk completely. The control disks received 0.1 ml of solvent only. After evaporation of the organic solvent from test and control disks, each disk was wetted with 0.1 ml distilled water because *Manduca* does not eat dry disks. The evaporating solvents apparently left no active residue; when larvae were given a choice between filter paper disks pretreated with hexane or chloroform and untreated disks, no difference in feeding preferences could be demonstrated.

The difference in food choices between test and control disks is expressed by a choice index (range -100 to $+100$) which measures the mean percent of total consumption of test disks minus that of control disks. Since the consumption scores were not normally distributed, a two-tailed Mann-Whitney test was used for the analysis of the raw scores to determine the significance of difference in food choices (*P*-choice). Most tests were carried out with 20 larvae or more.

To examine whether feeding responses to plant extracts are plant-specific, two groups of larvae were used in preference tests. Each group was reared on a different food plant species which induces feeding preferences specific for the rearing plant (Jermy et al., 1968; de Boer and Hanson, 1984) and their extracts (Städler and Hanson, 1978).

In some experiments, differences in disk texture could interfere with chemosensory-based feeding responses, such as choices between leaves and certain dewaxed leaves (chloroform treatment makes the leaves less turgid). In these cases, the leaf disks were presented in a "sandwich" (analogous to the test designed by Jermy, 1966), in which the leaf disk was placed between two glass fiber disks. These disks were then wetted with 0.2 ml of distilled water which "bonded" the multilayered disks into one integral substrate. Two layers of glass fiber filter paper disks spotted with 0.2 ml distilled water were used as control disks in these experiments.

The feeding continuation test of de Boer et al. (1977) was used to screen for compounds that promote continued feeding. The leaf extract was incorporated in a 4% agar–4% cellulose diet at concentrations equivalent to $0.5\times$ and $0.1\times$ on filter paper disks, whereas the control diet only received the solvent. The extract was added to cellulose, and the solvent was allowed to evaporate before mixing with warm agar. The same was done with the solvent for control diet. Larvae were placed individually on either the test or control diet in a no-choice situation and their production of fecal material (dry weight) over a 24-hr period was measured as an indication of the amount of food consumed. The significance of differences between feces production of the larvae on the test diet and those on the control diet was determined using Student's *t* test.

The orientation test (modified from Saxena and Schoonhoven, 1978) examined the presence of attractants or repellents in the extract by observing turning behavior of individual larvae towards a filter paper disk treated with extract or distilled water (control). A freshly moulted fifth-instar larva, starved for 24–48 hr, was allowed to climb on the bars of an aluminum grid, tilted upwards at about a 45-degree angle. At a right angle intersection, a control and a test disk were positioned approximately 0.5 cm from the center bar on which the larva was walking. After reaching the test intersection, the larva could turn to the control or the test disk or continue its movement along the center bar. The larva was not allowed to contact the disks. The choice of each larva was recorded and the larva discarded. The position of the test and control disks was randomized, and the experiment was run under red light conditions to avoid interference of visual stimuli. Choice scores from at least 30 larvae were averaged, and the means were analyzed for differences between test and control turns using the binomial test.

Extractions. Leaves of *L. esculentum* were freeze-dried before extraction with petroleum ether at room temperature. The extract was filtered and the solvent was removed. Leaf-surface extracts were made by dipping whole leaves in hexane or chloroform at room temperature for periods of either 30 sec or 2 min as indicated for each experiment. The solvent was agitated gently by a magnetic stirring bar to facilitate the removal of the surface lipids. This method removes lipids from the leaf surface with very little contamination by internal leaf lipids (Hamilton and Hamilton, 1972). The "natural concentration" of the extract was determined by two methods. For the "area method," the surface area of the leaves extracted, compared to that of a filter paper disk, determined the proportion of the extract to be spotted on each filter paper disk. For the "weight/volume method," the water content of the leaf was determined by drying a sample of leaves. The weight of all leaves extracted, corrected for percentage water content, yielded the concentration in milligrams of extract per milliliter of water. Both methods yielded similar results. All dried extracts were stored below 0°C in the dark.

The following plant species were used for extraction of phytochemicals: tomato, Jerusalem cherry, cowpea (*Vigna sinensis* Savi. var. Black-eyed Pea), rape (*Brassica napus* L. var. Dwarf Essex), and canna (*Canna generalis* Bailey var. Rosamond Cole). All plants were grown in the campus greenhouse; in addition, canna was grown outdoors.

RESULTS

Whole-Leaf Extract of Host Plant. The extract of *L. esculentum* at the natural concentration is 0.61 times as stimulating as fresh leaves (Figure 1). Thus the leaf chemicals that stimulate larvae to feed are partially extracted by

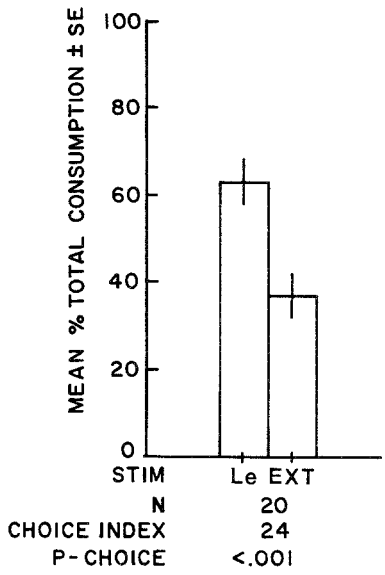

FIG. 1. Food choice between leaf disks of tomato, *L. esculentum* (Le), and filter paper disks laced with a petroleum ether extract of freeze-dried tomato leaves (EXT). The leaf disks were sandwiched between two moistened paper disks. The tomato extract is only slightly less preferred than whole leaves. Columns represent mean percentage of total consumption scores, with bars representing ± SEM. Choice index is the difference between consumption scores (%) on the two choices, and P-choice is the probability that this could occur by chance (Mann-Whitney test). Abbreviations: STIM, stimuli in choice tests; N, number of test repetitions (animals).

petroleum ether. Larvae reared on *L. esculentum* show a maximal preference for the natural concentration of the extract (Figure 2A). A threshold response is seen at 100-fold dilution (0.01×). The supranormal concentration (10×) is slightly less stimulatory than the natural concentration. Larvae that were reared on another host plant (*S. pseudocapsicum*) and therefore were not "induced" on *L. esculentum* show a completely different concentration–response curve for the extract of *L. esculentum* (Figure 2B); the stimulation of feeding in general is much weaker and the concentration–response curve is flatter. Moreover, the extract inhibits feeding slightly but significantly at 0.1× concentration.

In the orientation test, a significant number of larvae turned more often towards the disk with 3.3× extract than to the control disk ($P < 0.001$, $N = 93$). This signifies attractive volatiles in the extract. At lower extract concentrations (0.33× and 0.033×), no significant attraction could be demonstrated.

In the continuous feeding test, the larvae consumed equal amounts of the control diet and the diet with 0.5× and 0.1× extract ($P > 0.05$, $N = 40$; $P > 0.10$, $N = 38$, respectively). Thus, the petroleum ether extract of leaves of

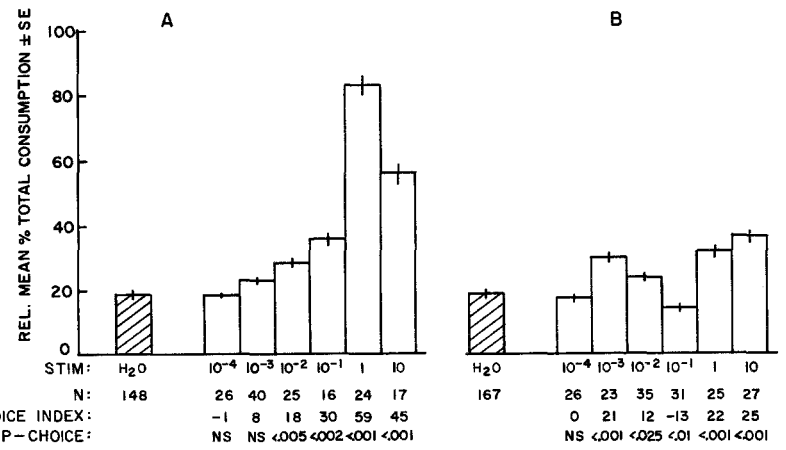

FIG. 2. Feeding responses to a petroleum ether extract of leaves of *L. esculentum* (Le) by larvae reared on: (A) *L. esculentum* or (B) *Solanum pseudocapsicum* (Sp). Each extract concentration (1 = natural concentration) was tested vs. water in two-choice bioassays. Response to each concentration was normalized to the grand mean of water consumption. A strong, dose-dependent stimulatory effect was seen at the natural concentration for Le-reared larvae; this was much weaker for Sp-reared larvae. Abbreviations: see Figure 1.

L. esculentum contains compounds that attract the larvae, but it does not seem to stimulate continued feeding on an agar–cellulose diet at the concentrations tested. This failure was examined by testing the validity of this bioassay using a known stimulant (sucrose, 0.1 M) incorporated into the diet. Larvae consumed twice as much of this diet compared with control diet, thereby confirming an earlier report (de Boer et al., 1977). Furthermore, because of the possibility that the active principle may be thermolabile and thus inactivated during preparation of the diet, we examined heat-pretreated (60°C for 3 min) extract (0.5×) in the standard filter paper disk feeding preference test. The results showed that larvae still strongly prefer this preheated extract over water ($P < 0.001$, choice index = 67, $N = 24$). Thus, it is unlikely that the active principle was destroyed by heat.

Leaf-Surface Extracts of Host and Nonhost Plants. Leaf-surface extracts using two different solvents (hexane and chloroform) were tested in the two-choice preference bioassay with water as control. The results in Figure 3A show that larvae strongly prefer host-plant extracts to water. Thus a feeding stimulant comparable to that seen for the whole-leaf extract (Figure 2A) can be obtained from just the leaf surface.

Leaf-surface lipids were similarly extracted from two weakly acceptable nonhosts (*Brassica napus* and *Vigna sinensis*) and one unacceptable nonhost

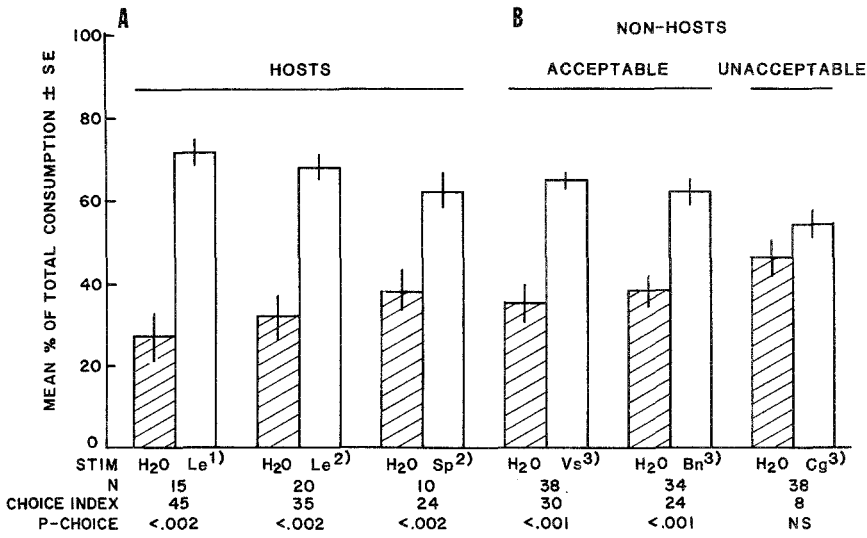

FIG. 3. Feeding responses to leaf-surface extracts of different plant species. Each extract was tested vs. water in a two-choice test. All larvae were reared on leaves of Le, except for those tested on Sp which were reared on Sp. The leaf-surface extracts of acceptable plants (Le, Sp, Vs, Bn) stimulate feeding, whereas that of the unacceptable plant (Cg) is neutral. Abbreviations: Le, *L. esculentum*; Sp, *S. pseudocapsicum*; Vs, *Vigna sinensis*; Bn, *Brassicae napus*; Cg, *Canna generalis*. Extraction procedures: 1) 120-sec dip in hexane; 2) 30-sec dip in chloroform; 3) 30-sec dip in hexane. Details as in Figure 1.

(*Canna generalis*). Extracts from the acceptable nonhost plants are stimulatory, whereas the extract of the unacceptable plant is neutral (Figure 3B).

The stimulating effectiveness of the leaf-surface extracts of *L. esculentum* was examined in a concentration–response series. Larvae show an increased preference for the chloroform and hexane leaf surface extracts at increasing concentrations (Figure 4). At higher than natural concentrations (3.3×) a slightly reduced stimulation is seen. Thus, the leaf-surface extract of *L. esculentum* stimulates feeding in a concentration-dependent manner just as does the extract of whole leaves (Figure 2A).

After surface extraction of acceptable leaves, the remaining leaf ("dewaxed leaf") still elicits feeding, but usually less strongly than the untreated leaf (Figure 5), suggesting that some stimulatory compounds have been removed. The only exception found is for *L. esculentum* dewaxed with hexane instead of chloroform (Figure 5, first pair of columns); dewaxed and untreated leaves are equally acceptable, irrespective of whether the dewaxing period lasts 30 sec (standard) or 120 sec.

In contrast, dewaxed leaves of the unacceptable *C. generalis* elicit much

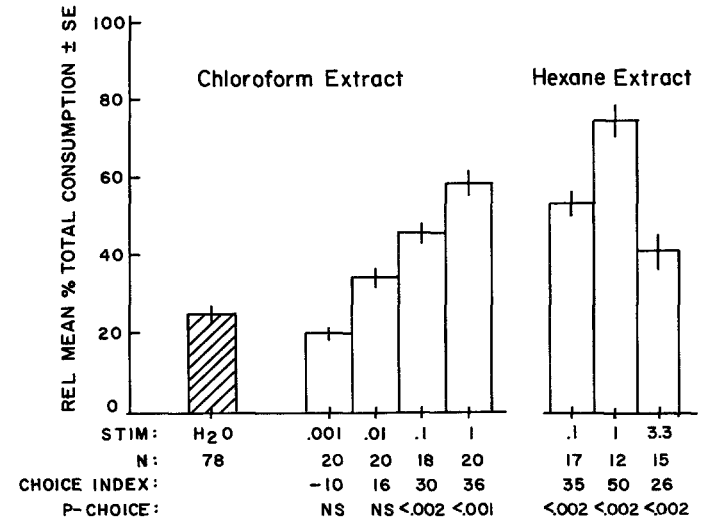

FIG. 4. Feeding responses to leaf-surface extracts of tomato (*L. esculentum*). The extract was obtained by dipping leaves in chloroform (30 sec) or hexane (120 sec) and tested at different concentrations vs. water in two-choice tests. Both leaf-surface extracts stimulated feeding in a concentration-dependent manner. Details as in Figures 1 and 2.

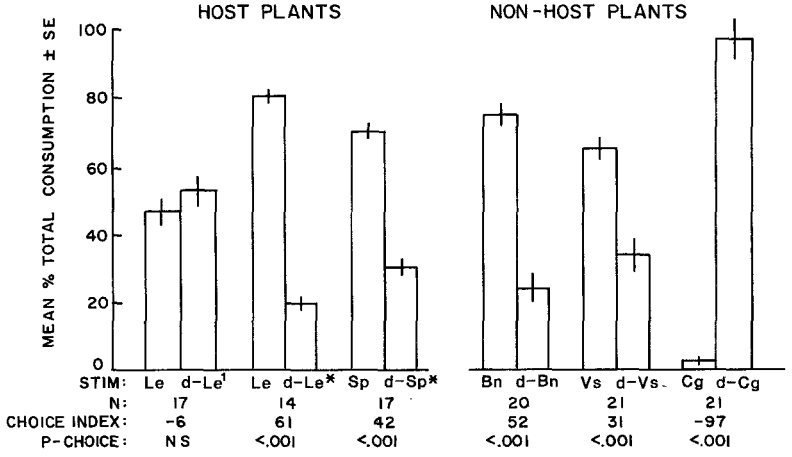

FIG. 5. Change in leaf acceptability following surface extraction. Larvae were given a choice between normal and dewaxed leaf disks. Leaves were dewaxed by dipping in hexane or chloroform (*) for 30 or 120 sec (¹). In experiments using chloroform for dewaxing, the leaf disks were presented between two moistened filter paper disks (sandwich method). Removal of leaf surface material generally decreased feeding on acceptable species (Le, Sp, Bn, and Vs) and increased feeding on the unacceptable species (Cg). Details as in Figures 1 and 3.

more feeding than untreated leaves (Figure 5, last pair of columns). This suggests that a deterrent principle in the leaf waxes of this plant is being extracted. However, no such feeding-deterrent activity could be demonstrated (Figure 3, last pair of columns). To control for possible textural changes brought about by the dewaxing process, an experiment was performed using leaf disks sandwiched between two filter paper disks; however, the larvae still prefer dewaxed leaves over untreated leaves (choice index = -96, $P < 0.001$, $N = 7$). Thus unacceptability of *C. generalis* must be due to chemical rather than physical factors.

Reconstitution experiments in which the leaf-surface extract of *C. generalis* was added back to its dewaxed leaves did not restore the unacceptability of *C. generalis*. Other experiments showed that merely spotting fresh leaf disks with solvent (and permitting it to evaporate) also rendered them acceptable. Thus, the feeding deterrent activity must be destroyed by the solvent used, and the strongly deterrent character of *C. generalis* is lost in the surface-extraction process.

The question arises whether the leaf-surface chemicals are qualitatively representative of the entire leaf in eliciting feeding behavior of *M. sexta*. To test this, normal leaf disks were paired with filter paper disks wetted with water in two-choice tests; the results are compared to those of the corresponding surface extracts in Figure 3. Table 1 shows that the feeding scores of the surface extracts of *L. esculentum*, *S. pseudocapsicum*, and *B. napus* are comparable with those of their corresponding leaves (all are stimulatory), whereas those of *V. sinensis* and *C. generalis* are totally different. Thus the leaf-surface extracts do not always elicit feeding responses qualitatively similar to those of the corresponding leaves.

Specific activities (discrimination per milligram extract) of all extracts stimulating feeding were compared at natural concentrations (1×), using water

TABLE 1. COMPARISON OF FEEDING RESPONSES OF *M. Sexta* LARVAE TO FRESH LEAVES VS. SURFACE EXTRACTS[a]

Plant	Choice index			
	Whole leaves	*N*	Surface extract	*N*
L. esculentum	67	24	45	15
S. pseudocapsicum	34	24	24	10
B. napus	44	20	24	34
V. sinensis	−32	27	30	38
C. generalis	−100	21	8	38

[a]Leaves and extracts were each tested vs. water in two-choice tests. See Figure 3 for details.

TABLE 2. COMPARISON OF SPECIFIC ACTIVITIES OF VARIOUS EXTRACTS OF DIFFERENT PLANT SPECIES FOR FEEDING STIMULATION BY LARVAE OF *M. sexta*.[a]

Plant	Extract		Natural concentration (mg/ml)	Choice index[b]	Specific activity[c]	*N*
	Leaf part	Solvent				
L. esculentum	whole	petether	2.8	59	21	24
	surface	hexane	0.3	45	149	15
	surface	chloroform	1.3	35	27	20
S. pseudocapsicum	surface	chloroform	0.3	24	80	10
B. napus	surface	hexane	4.8	24	5	34
V. sinensis	surface	hexane	0.4	30	75	38

[a] See Figures 2 and 3 for details.
[b] Measured at natural concentration (1×).
[c] Specific activity = choice index/mg extract.

as the common control. Table 2 shows that the hexane leaf-surface extract of *L. esculentum* has the highest specific activity of the extracts tested, indicating relatively few contaminants (neutral and/or inhibitory compounds) and/or high concentration of stimulant(s). Hence, this extract seems most promising for further isolation and identification of the nonpolar feeding stimulatory principle(s).

DISCUSSION

The petroleum ether extract of whole leaves of *L. esculentum* clearly stimulates feeding in the tobacco hornworm. This finding confirms Städler and Hanson's (1978) results showing that nonpolar extracts elicit feeding. Using the disk test, the extract accounts for about 61% of the stimulatory activity of whole tomato leaves, indicating that the extraction was incomplete and/or that compounds other than nonpolar chemicals also contribute to the attractiveness of this food plant. Polar compounds and compounds of intermediate polarity also show feeding stimulatory activity (Yamamoto and Fraenkel, 1960b; Städler and Hanson, 1978).

Feeding stimulation by this simple extract is remarkably strong. The optimal feeding responses to the extract is four times that of the water control (Figure 2A) and twice that of the common stimulant sucrose at optimal concentration (Städler and Hanson, 1978). Thus, the petroleum ether extract of *L. esculentum* leaves is the most strongly stimulating extract or compound so far tested using this preference test. A supranormal concentration shows lower feeding activity

than a normal concentration, indicating a saturation in the stimulatory response of the larvae, or perhaps the presence of deterrent compounds that become effective at higher concentrations. Indeed, feeding deterrent activity was shown in one of the fractions of this extract (de Boer and Hanson, 1982).

In contrast to the unimodal concentration–response curve obtained with *L. esculentum*-reared larvae, a bimodal response to the same extract is found with larvae reared on *S. pseudocapsicum* (Figure 1B). These larvae are stimulated to feed less strongly than larvae reared on *L. esculentum* and are slightly deterred from feeding by the 0.1× extract. The latter demonstrates the presence of a deterrent in the extract to which *S. pseudocapsicum*-reared larvae respond but to which the larvae reared on *L. esculentum* do not respond. This difference in feeding preference of these two groups of larvae illustrates clearly an induction of feeding preference for some component(s) of the *L. esculentum* extract. These findings agree with those of Städler and Hanson (1978), who also found an induction of feeding preference for a nonpolar extract of *L. esculentum* using tobacco hornworm larvae reared on the same two plant species. Thus, the extract of *L. esculentum* contains compounds determining the specific "chemosensory profile" of this plant recognized by larvae that have been induced to prefer this plant.

The disk preference test measures larval feeding responses that are the end result of activation of one or more distinct behavioral acts, such as orientation, biting, continued feeding, and cessation of feeding. This bioassay cannot determine which component of feeding behavior is elicited by the extract. For this reason, two other bioassays were used to examine the effect of the extract on orientation behavior and continued feeding of the larvae.

The orientation test showed that the petroleum ether extract of *L. esculentum* leaves contains volatile compounds which attract the larvae. Apparently the active chemical(s) must have a low volatility because the extract had been dried twice and still elicited an attraction from 0.5 cm away. These results support and complement those of a study on the chemosensory basis of food selection in *M. sexta*, which shows that olfaction plays a role in the discrimination between *L. esculentum* and certain other plants (Hanson and Dethier, 1973; de Boer and Hanson, 1987a).

In contrast, no stimulation of continued feeding could be demonstrated by this extract using larvae in the 24-hr feeding test. Experiments using sucrose as the stimulant showed that the bioassay is functional in detecting feeding stimulatory activity. Heat treatment, such as that during diet preparation, does not inactivate the extract. Several possibilities for the lack of activity can be suggested: Perhaps the active principle is inactivated by the ion-exchange properties of the agaropectine, a constituent of agar; the active principle may bind to the agar or cellulose and thus not be available to stimulate the larval chemore-

ceptors; the extract contains no compounds that elicit long-term feeding, only attraction, short-term biting, and swallowing; and the animals may adapt to the presence of the stimulus over 24 hr.

The above results demonstrate the importance of using more than one bioassay to screen for behaviorally active compounds. But because the filter paper disk preference test appeared to be the most sensitive, further studies to monitor feeding stimulatory activity were done using this bioassay.

Since the first contact of the larva with its food occurs at the leaf surface, which contains mainly lipids, further study was focused on the role of leaf-surface lipids in food-plant selection. These compounds have been reported to affect feeding in several phytophagous insect species (Chapman, 1977; Woodhead and Chapman, 1986). The leaf alcohols *n*-hexacosanol and *n*-octacosanol promote feeding in silkworm larvae, *Bombyx mori* (Mori, 1982). Lipid-soluble material from the surface of *Poa annua* leaves stimulates biting in nymphs of *Locusta migratoria* (Bernays et al., 1976). In contrast, this insect is strongly deterred from feeding by surface material of sorghum leaves, which was shown to be mainly due to the compound *p*-hydroxybenzaldehyde (Woodhead, 1982). Klingauf et al. (1971) demonstrated that the insect *Acyrthosiphon pisum* is stimulated to probe longer by certain alkanes of the leaf surface of *Vicia fabae*. The above studies suggest a relationship between the leaf cuticular compounds and feeding stimulations, as we found for the tobacco hornworm.

One of the interesting findings is that leaf-surface extracts of hosts as well as acceptable nonhosts contain feeding stimulatory compounds on the leaf surface, whereas that of the unacceptable plant *C. generalis* did not affect feeding at all. The failure of the reconstitution experiment confirms that no deterrent is extracted. Nevertheless, the dewaxing process or just adding solvent to the leaf makes this plant acceptable (although still less acceptable than wet filter paper or tomato leaves; de Boer and Hanson, unpublished). This effect is the more remarkable because untreated leaves of this plant deter feeding so strongly that the larvae starved to death rather than consume any leaf material. A possible explanation for the reduced deterrency following dewaxing is that repellent odors were removed. The presence of such odors was suggested by the results of an orientation experiment in which larvae preferred turning towards a "neutral" plastic disk instead of a leaf disk of *C. generalis* ($P < 0.05$, $N = 77$). It is also possible that the solvent used for dewaxing destroys a strong deterrent.

One remarkable result is that, despite large texture differences between leaves and filter paper, some leaf-surface extracts on filter paper mimic the whole leaf in feeding responses as well as they do (Table 1). This indicates that nonpolar compounds in the leaf surface are major contributors to the feeding stimulation. In contrast, larvae apparently do not recognize the leaf-surface extract of *V. sinensis* as faithfully representing whole leaves of this plant. In fact, the extract stimulates feeding, whereas the whole leaf is slightly deterrent.

Apparently, interior chemicals or other unidentified factors play a major role in feeding on *V. sinensis*.

Jermy (1966) suggested that the oligophagous character of phytophagous insects is mediated mainly by plant compounds having feeding inhibitory activity in nonhost plants. Indeed, feeding in *M. sexta* is inhibited by various deterrents (de Boer et al., 1977; Städler and Hanson, 1978; de Boer and Hanson, 1987b). However, the strong stimulation of feeding by lipid extracts signifies the importance of feeding stimulants for food selection as well. Botanical specificity, on the other hand, does not seem to be conferred by these surface lipids.

In conclusion, lipid extracts of leaves contain attractants and feeding stimulants for larvae of *M. sexta*. The primary repository of these stimulants can be found at the leaf surface of two hosts and two acceptable non-host-plant species. In contrast, no activity was found in leaf-surface chemicals of an unacceptable nonhost plant. The results of this study underscore the importance of leaf-surface lipids in food plant selection by the tobacco hornworm.

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IDENTIFICATION OF SEX PHEROMONE COMPONENTS OF *Spodoptera sunia* Guenée (LEPIDOPTERA: NOCTUIDAE)[1]

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Abstract—Moths belonging to the species *Spodoptera sunia* have been recognized as a new pest of cotton in Central America. By means of electrophysiologic investigations, solid sample injection gas chromatography, and combined gas chromatography–mass spectrometry, (*Z*)-9-tetradecenyl acetate, (9*Z*,12*E*)-9,12-tetradecadienyl acetate, (*Z*)-9-tetradecen-1-ol, and (*Z*)-11-hexadecenyl acetate, in a ratio of 100:5:31:20, were identified in the pheromone gland of female insects. These substances should serve as a base for the development of a pheromone-monitoring system for this lepidopteran pest.

Key Words—Sex pheromone analysis, *Spodoptera sunia*, solid sample injection, Lepidoptera, Noctuidae.

INTRODUCTION

Members of the genus *Spodoptera* (Lepidoptera: Noctuidae, Amphypyrinae) appear worldwide. A number of species of this genus are known as pests that cause damage to cereals, field crops, grasses, and flowers. Over the last few years, especially in Central America, *Spodoptera sunia* Guenée has been recognized as a new pest of cotton, showing resistance or at least insensitivity

[1] Pheromones, 60. Pheromones 59: Bestmann, H.J., Attygalle, A.B., Brosche, T., Erler, J., Platz, H., Schwarz, J., Vostrowsky, O., Wu Cai-Hong, Kaissling, K.E. and Chen Te-Ming. 1987. *Z. Naturforsch.* 42c:631–636.

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towards a wide range of standard insecticides. It is likely that *S. sunia* may have experienced a favorable selection pressure, due to the extensive use of certain pesticides, resulting in a steady increase of its population.

The correct identification of a pest species is one of the most important requirements in deciding the appropriate method of control. It is rather tedious, or even impossible, to differentiate young larvae of *S. sunia* from those of other sympatrically occurring species of *Spodoptera*, because of the overlapping color patterns of their larval skin. The development of a species-specific lure for capturing males of *S. sunia* can be useful to monitor selectively the presence of this species among other pests.

In the literature, no information on the pheromone of *S. sunia* was found. Therefore, a determination of the composition of its female sex pheromone complex appeared useful. By means of electrophysiologic studies, solid sample injection gas chromatography of isolated pheromone glands, and combined gas chromatography–mass spectrometry, the chemical structures of the pheromone components were determined. The analytic results will be useful in formulating a synthetic lure for monitoring the abundance and distribution of this lepidopteran pest.

METHODS AND MATERIALS

Insect Material. Spodoptera sunia moths were reared on a semisynthetic diet at Hoechst AG, Frankfurt. The strain, originating from Colombia, has been maintained as a laboratory colony for several years.

The larvae were allowed to pupate and were sexed prior to hatching. The segregated sexes were placed in plastic boxes containing moist sawdust and maintained on a reversed 15 : 9 hr light–dark cycle. After emergence, the female insects were observed carefully during the scotophase under a red darkroom lamp to observe the calling behavior and period.

Electroantennography. Using the electroantennogram (EAG) technique (Schneider, 1957) with male moth antennae, the stimulus potentials evoked by a large number of pheromone and pheromone-like chemicals (filter paper strips loaded with the test chemicals) were measured, and the response amplitudes were compared with each other. The test compounds selected were those known as noctuid sex pheromones, especially those identified from females of other species of *Spodoptera* (for recent reviews, see Tamaki, 1987; Arn et al., 1986; Bestmann and Vostrowsky, 1981).

Encapsulation of Pheromone Glands. The samples were prepared according to the method described by Attygalle et al. (1987). The pheromone glands of 2- to 4-day-old female moths were excised during the period of maximum calling activity. One to five glands prepared in this way were sealed in a soda

glass capillary (2 cm × 2 mm) and used for immediate analysis or stored at −20°C.

Gas Chromatography. Capillary gas chromatography with flame ionization detection was performed on a Packard-United Technologies 438A instrument equipped with a splitless injector and Shimadzu Chromatopac C-R3A data system. Columns and temperature programs were: (A) fused silica capillary column coated with SP-2340 (100% cyanopropyl silicone, Chrompack, 25 m × 0.22 mm), 2 min at 60°C, 60–195°C at 4°/min; and (B) fused silica capillary column coated with CP-19 (chemically bonded 50:50 phenyl–methyl silicone, Chrompack, 12 m × 0.2 mm), 2 min at 60°C, 60–270°C at 10°/min.

Samples sealed in glass tubes were chromatographed by a solid sample injector (Attygalle et al., 1987).

Gas Chromatography–Mass Spectrometry. A Finnigan 9502 gas chromatograph, fitted with a Grob-type split–splitless injector and a solid sampler, linked to a Finnigan 3200E quadrupole mass spectrometer with a Data System 6000 was used: (C) fused silica capillary column coated with SP-2340 (50 m × 0.22 mm), 4 min at 60°C, 60–195°C at 6°/min, splitless injection by a solid sampler, split-vent was kept closed for 1 min.

RESULTS AND DISCUSSION

Electrophysiological Studies. In order to obtain some information about the chemical nature of the sex pheromone of *S. sunia*, electrophysiologic studies were carried out. A series of mono- and diunsaturated aldehydes, alcohols, and the corresponding acetates was employed as the stimulus sources, and the EAG amplitude evoked by each compound was measured. Of all the compounds tested, (9*Z*,12*E*)-9,12-tetradecadienyl acetate was the most active. It could evoke response amplitudes of 1.6 mV and 2.9 mV, for doses of 0.2 μg and 2 μg at the stimulus source, respectively. This tetradecadienyl acetate isomer is known as a pheromone component of *Spodoptera eridania* (Jacobson et al., 1970; Teal et al., 1985), *S. exempta* (Beevor et al., 1975), *S. exigua* (Persoons et al., 1981; Steck et al., 1982; Tumlinson et al., 1982), *S. litura* (Nesbitt et al., 1973; Tamaki et al., 1973), and *S. littoralis* (Tamaki, 1987).

In a few species of Spodoptera, (9*Z*,11*E*)-9,11-tetradecadienyl acetate is known as a pheromone component (Nesbitt et al., 1973). However, this tetradecadienyl acetate isomer was about 100 times less active than (9*Z*,12*E*)-9,12-tetradecadienyl acetate in releasing EAG activity. This was determined by comparing the loadings required, at the stimulus source, by the two compounds to evoke the same EAG response.

The second most effective test chemical was (*Z*)-9-tetradecenyl acetate (0.6 mV and 2.0 mV at 0.2 μg and 2.0 μg, respectively). This compound is often

found in the pheromone complexes of other species of Spodoptera (Steck et al., 1982, and references cited above).

Analysis of Gland Volatiles. Under our laboratory conditions, *S. sunia* females showed maximum calling activity ca. 7 hr after the beginning of the dark period. During the calling period, which lasts for about 20–30 min, the females raise their wings slightly and expose the ovipositors. Various parts of the abdominal tip of 3-day-old female moths were excised during the calling period and analyzed by GC for their pheromone content. Almost all the components of the sex pheromone complex were found only in the ventral side of the intersegmental membrane, between the VIIIth and IXth abdominal segments (densely stippled area in Figure 1). This area becomes enlarged and bulbous during the calling period.

The segments VIII and IX, and the intersegmental membrane between segment VII and VIII contained no detectable amounts of pheromone-like substances. The dorsal region of the intersegmental membrane contained only traces of pheromone components, which may be due to contamination. Steinbrecht (1982) has obtained similar results by EAG evaluations of hexane extracts made from different parts of the abdominal tip of *S. exempta.*

The section of the intersegmental membrane, containing the highest concentration of volatile pheromone components, was dissected carefully under a binocular microscope and sealed in a glass capillary to be used for GC-MS analysis by a solid sample injector (Attygalle et al., 1987).

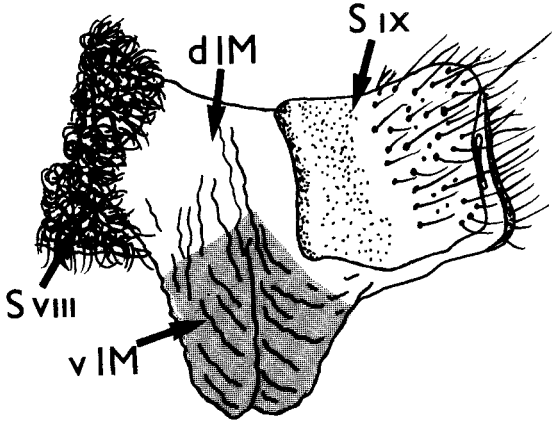

FIG. 1. An illustration of the abdominal tip of a female *Spodoptera sunia* (S VIII = 8th abdominal segment; S IX = 9th abdominal segment; dIM = intersegmental membrane, dorsal side; vIM = intersegmental membrane, ventral side). The pheromones are located during the calling period only in the ventral side (densely stippled area) of the intersegmental membrane.

Figure 2 shows a reconstructed gas chromatogram (RGC, top chromatogram in the figure) from a GC-MS analysis of three "pheromone glands" on column C. All the signals were well resolved for accurate integration of peak areas. Complete EI-mass spectra of the four pheromone-like components could be obtained using only three female glands. The identification of the chromato-

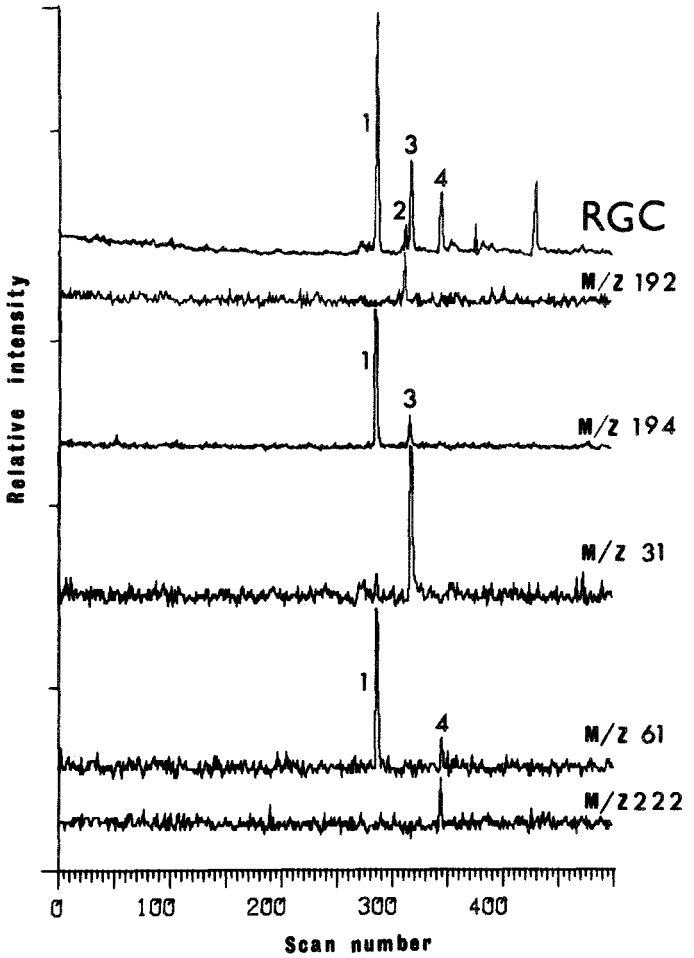

FIG. 2. A reconstructed gas chromatogram (RGC) and four mass chromatograms for m/z = 192 ($C_{14}H_{24}^+$), m/z = 194 ($C_{14}H_{26}^+$), m/z = 31 ($CH_2{-}OH^+$), and m/z = 61 ($CH_3COOH_2^+$). 1 = (*Z*)-9-tetradecenyl acetate, 2 = (9*Z*,12*E*)-9,12-tetradecadienyl acetate, 3 = (*Z*)-9-tetradecen-1-ol, and 4 = (*Z*)-11-hexadecenyl acetate. Three glands from 3-day-old females were chromatographed on a 50 m × 0.22 mm FSCC coated with SP-2340 via a solid sample injection technique.

graphic peaks was achieved by mass chromatographic analysis of characteristic fragment ions, and elucidation of mass spectra by the method of Horiike and Hirano (1982). Under identical mass spectroscopic conditions, the spectra of a series of *Z* isomers of tetradecenyl acetate and hexadecenyl acetate were measured, and the intensities of various peaks in each ion cluster of these spectra were compared to those obtained from peaks 1 and 4 in Figure 2. This way peaks 1 and 4 could be identified tentatively as 9-tetradecenyl acetate and 11-hexadecenyl acetate. The stationary phase SP-2340 (columns A and C) can resolve essentially all the positional and geometric isomers of monounsaturated compounds found in pheromones (Heath and Tumlinson, 1984). This was verified by using a standard mixture containing all positional isomers of tetradecenyl acetate. Furthermore, column A could afford baseline separations of four geometric isomers of 9,12-tetradecadienyl acetate.

A mass chromatographic search for characteristic ions known for previously identified pheromone components from other species of *Spodoptera* was made. The results are shown in Figure 2. The main component of the *S. sunia* pheromone complex was found to be (*Z*)-9-tetradecenyl acetate (1). This was corroborated by the coincidence of the maximum of abundance of fragment ions $m/z = 194$ ($C_{14}H_{26}^+$, generated by the elimination of acetic acid) with that of $m/z = 61$ due to protonated acetic acid $CH_3COOH_2^+$. A small amount of the diunsaturated (9*Z*,12*E*)-9,12-tetradecadienyl acetate (2) was also found (mass chromatogram of $m/z = 192$, $C_{14}H_{24}^+$, in Figure 2).

The second major component, 3, is (*Z*)-9-tetradecen-1-ol, as shown by the contemporal appearance of ion $m/z = 194$ ($C_{14}H_{26}^+$, generated by the elimination of H_2O) and $m/z = 31$ ($CH_2{-}OH^+$) in the corresponding mass chromatograms in Figure 2. The third major component, signal 4 in the figure, was found to be (*Z*)-11-hexadecenyl acetate (mass chromatogram $m/z = 61$, $CH_3COOH_2^+$, and $m/z = 222$, $C_{16}H_{30}^+$).

The identification of the four peaks was confirmed by a comparison of retention times obtained from columns A and B with those of authentic samples. The relative amounts of (*Z*)-9-tetradecenyl acetate, (9*Z*,12*E*)-9,12-tetradecadienyl acetate, (*Z*)-9-tetradecenol, and (*Z*)-11-hexadecenyl acetate were 100, 5, 31, and 20, respectively. In 3-day-old females, the major component, (*Z*)-9-tetradecenyl acetate, represents about 30 ng/insect. This amount is several times more than that reported for *S. exempta* by EAG estimations (Steinbrecht, 1982; Beevor et al., 1975).

The two acetates, (*Z*)-9-tetradecenyl acetate, 1, and (9*Z*,12*E*)-9,12-tetradecadienyl acetate, 2, are common to almost all other known pheromone complexes from this genus. However, a recent analysis by Tumlinson et al. (1986) shows that the latter component plays no role in the pheromone blend of *S. frugiperda*. (*Z*)-11-Hexadecenyl acetate, 4, has been found only in *S. eridania* (Teal et al., 1985) and *S. frugiperda* (Tumlinson et al., 1986), and this may be

an essential sex attractant component necessary for speciation and isolation. (*Z*)-9-tetradecenol, 3, gave at least 100 times lower EAG responses than those from 2 with male *S. sunia* antennae and could be considered rather as a biosynthetic precursor. Alcohols corresponding to pheromone acetates are often found in more comprehensive pheromone analyses, especially in those carried out by a solid sample injection technique. In at least a few examples, the biosynthetic role of the alcohols as precursors of pheromone acetates (Bestmann et al., 1987) and aldehydes (Teal and Tumlinson, 1986) has been established. Nevertheless, since (*Z*)-9-tetradecenol has also been identified in other *Spodoptera* species (Steck et al., 1982; Tumlinson et al., 1982; Mitchell et al., 1983; Teal et al., 1985), the possible role of the alcohol in the pheromonal communication of *S. sunia* can be evaluated only by field trails.

Based on the compounds identified as the major components of the pheromone gland of *Spodoptera sunia*, extensive field experiments, to be conducted in Central and South America, are planned for the development of a monitoring method that can be incorporated into integrated pest control.

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LECTIN STUDIES OF SURFACE CARBOHYDRATES AND INDUCTION OF GLAND SECRETION IN THE FREE-LIVING STAGES OF *Schistosoma mansoni*

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Abstract—Secretion from glands was observed when cercariae of *Schistosoma mansoni* were exposed to certain lectins. Lectins from *Maclura pomifera*, *Pisum sativum*, and *Triticum vulgaris*, were effective at 7.5 μg/ml. The effects of cercarial gland secretion caused by *T. vulgaris* agglutinin and *Arachis hypogaea* agglutinin were blocked by pretreatment with the inhibiting glycan. Discharge of glands was not visualized after exposure of miracidia to lectins. The distribution of five labeled lectins was determined on live miracidia and cercariae. Only *T. vulgaris* agglutinin generally labeled the cercarial and miracidial bodies. Specific labeling occurred with the other lectins on the anterior, in glands or on their secretions, in flame cells in both stages, and on an unidentified ring of cells in miracidia. The possible mechanisms involved in changes caused by the lectins are discussed.

Key Words—*Schistosoma mansoni*, lectins, cercariae, miracidia, secretions, surface carbohydrates.

INTRODUCTION

Sugars can occur generally as receptors in biologic systems (Monsigny et al., 1983), and recent studies with nematodes have shown that the carbohydrate moieties of glycoproteins function in the reception and recognition of chemotactic signals (Zuckerman and Jansson, 1984; Bone and Bottjer, 1985). Exposure of worms to mannosidase and sialidase completely inhibits normal

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chemotaxis (Jansson et al., 1984), while partial inhibition results from treatment with the mannose/glucose-specific lectin concanavalin A (Con A) or the sialic-specific lectin *Limulus polyphemus* agglutinin (limulin) (Jeyaprakash et al., 1985). Electrophysiologic measurements of receptors are possible in large invertebrates (Dusenbery, 1980), but are impractical in small invertebrates. However, the demonstration that mannosyl (McClure and Zuckerman, 1982) and sialyl (Spiegel et al., 1982; Jansson and Nordbring-Hertz, 1984) residues are proximate to the cephalic sensillae of four nematode species, when combined with the behavioral changes caused by molecules reacting with sugars, suggests that sugars are almost certainly involved in chemotactic responses in nematodes.

When miracidia of *Schistosoma mansoni* approach snails, their behavior changes (MacInnis, 1965). Certain fatty acids, particularly 2-tetradecanoic acid, stimulate cercarial penetration (Haas, 1984). These compounds may act on glycoprotein receptors. Therefore, the effects of lectin exposure on miracidia and cercariae were investigated, and labeled lectins were used to determine if the sites of receptors could be identified.

METHODS AND MATERIALS

Organisms. A Puerto Rican strain of *S. mansoni*, originally obtained from the Center for Tropical Diseases, University of Lowell, Massachusetts, was maintained in albino *Biomphalaria glabrata* and Charles River CD1 mice. Miracidia were hatched from eggs released by homogenization of infected murine livers, and cercariae were collected by placing patent snails in a small quantity of dechlorinated tap water under a bright light for 2 hr. Both miracidia and cercariae were used within 1 hr of collection.

Lectin Studies. Twenty-five to 50 miracidia or 50–100 cercariae were incubated in 0.1 ml of 5 mM Tris (pH 7.2) buffer or buffer plus lectin (see Table 1 for lectins tested) in multiwell tissue culture plates at room temperature for 0.5 hr. Two drops of Lugol's iodine were added to each well for observation of gland discharge in both lectin-treated and untreated miracidia and cercariae.

The unlabeled lectins tested for their physiologic effects, their abbreviated names, and their sugar specificities are as follows: *Arachis hypogaea*, PNA, *d*-galactose and beta-(1-3)*N*-acetyl galactosamine; *Concanavalia ensiformis*, Con A, alpha-*d*-glucose and alpha-*d*-mannose; *Griffonia simplicifolia*, GS1, *d-n*-acetyl glucosamine; *Glycine max*, SBA, alpha-*d-n*-acetyl galactosamine and *d*-galactose; *Limax flavus*, LFA, sialic acid; *Limulus polyphemus*, LPA, sialic acid; *Maclura pomifera*, MPA, alpha-*d*-galactose; *Pisum sativum*, PSA, alpha-*d-n*-acetyl glucosamine and *d*-mannose; *Solanum tuberosum*, STA, beta(1-4)*d-n*-acetyl glucosamine; *Triticum vulgaris*, WGA, beta-*d-n*-acetyl glucosamine;

Ulex europaeus, UEA-1, alpha-1-fucose and *d-n*-acetyl glucosamine. Lectins were tested from 500 μg/ml down to 7.5 μg/ml in 5 mM (pH 7.2) Tris buffer.

For the labeling trials, both fluorescein (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) conjugates of Con A, LFA, LPA, PNA, and WGA were tested. Concentrations were 25 μg protein/ml in 5 mM Tris buffer, pH 7.2. The specificity of the lectin was established by preincubation with the appropriate inhibiting saccharide. Miracidia or cercariae were placed on microscope slides and the label visualized on a Nikon fluorescence microscope equipped with FITC and TRITC filters. For photography, labeled miracidia and cercariae were immobilized by warming the slides immediately before photography.

To test further for sialic acid, ^{125}I-labeled LFA was prepared by the chloramine-T reaction (Talmage and Claman, 1967) according to Kahane and Tully (1976). This procedure is more sensitive than the fluorescent technique and should indicate even small numbers of sialic acid molecules. The test was performed on heat-inactivated cercariae.

Labeling was attempted also with WGA, UEA, PNA, and LFA conjugated with ferritin. Live miracidia and cercariae were treated with lectin conjugate at 25 μg/ml in 5 mM Tris buffer, pH 7.2, as described by Zuckerman et al. (1979). Then the specimens were fixed, embedded in araldite, and processed for transmission electron microscopy (TEM) by standard procedures. Micrographs of random sections through specimens were taken on a JEOL 100S electron microscope operated at 80 kV.

TABLE 1. PERCENTAGE OF CERCARIAE OF *Schistosoma mansoni* SHOWING GLAND DISCHARGE IN LECTIN SOLUTIONS[a]

	Concentration (μg/ml)						
Lectin	500	250	125	62.5	30	15	7.5
MPA	–	–	–	–	100	100	85 ± 6
PSA	–	–	–	–	–	–	81 ± 6
WGA	–	–	–	100	89 ± 2	87 ± 4	72 ± 4
PNA	–	–	–	94 ± 3	93 ± 3	87 ± 7	10 ± 5
Con A	–	–	–	100	68 ± 10	18 ± 9	–
SBA	93 ± 7	95 ± 5	83 ± 5	78 ± 11	62 ± 16	40 ± 16	9 + 8
UEA	90 ± 8	28 ± 9	22 ± 10	–	–	–	–
GS1	100	18 ± 3	–	–	–	–	–
STA	3 ± 1	–	–	–	–	–	–
LFA	0	–	–	–	–	–	–
LPA	0	–	–	–	–	–	–

[a] Values are mean of four determinations ±SD. –, not tested.

Tegumental damage to *S. mansoni* adults after binding of Con A or *Ricinus communis* agglutinin can occur (Simpson and McLaren, 1982). Therefore the cercariae and miracidia were examined for similar damage after treatment with lectins. Miracidia or cercariae were exposed first to 250 μg/ml PNA or WGA, which was greater than the doses causing gland discharge in cercariae. Then specimens were prepared for TEM as described previously. The untreated cercariae and miracidia were used for controls.

Lectins and *N,N*-diacetyl chitobiose penta-*o*-acetate were purchased from EY Laboratories (San Mateo, California), with the exception of unlabeled LPA, *d*-(+)-galactose and alpha-methyl-D-mannoside which were from the Sigma Chemical Co. (St. Louis, Missouri).

RESULTS AND DISCUSSION

A range of lectins caused discharge of glands in cercariae (Figure 1 and Table 1). WGA at 15 μg/ml resulted in discharge of glands of 91% of cercariae, but prior incubation of cercariae in 5 μg/ml of the specific blocking agent *N,N*-diacetyl chitobiose penta-*o*-acetate reduced this to 5% compared with 1% in untreated controls. In similar tests, 300 μg/ml galactose blocked 30 μg/ml PNA (Figure 1B) and 125 μg/ml alpha-methyl-D-mannoside blocked 30 μg/ml Con A, demonstrating the specificity of the binding of these lectins to chemoreceptors. However, 300 μg/ml *d*-galactose did not block 30 μg/ml MPA and 250 μg/ml *N,N*-diacetyl chitobiose penta-*o*-acetate did not block 30 μg/ml PSA, indicating that the binding of these two lectins may be nonspecific. The same lectins used on cercariae were tested on miracidia (except STA) at 500 μg/ml. No discharge of glands was visible before or following addition of Lugol's iodine, and more sensitive techniques may be required to visualize secretions as they occur. Some miracidia died or rounded up and swam more slowly in the two highest doses of GS1, but attempts to block this effect with a similar concentration of *N,N*-diacetyl chitobiose penta-*o*-acetate were not successful, suggesting that the effect of GS1 on miracidia is nonspecific.

To determine if the position of possible receptors could be identified, live active miracidia and cercariae were incubated in FITC and TRITC conjugated lectins (Figure 2, Table 2). Both miracidia and cercariae (Figure 2E and F) were labeled by LPA and LFA, which suggested the presence of sialic acid. In competitive displacement trials fluorescent Con A, WGA, and PNA were displaced, indicating that *n*-acetyl glucose and galactose were on the surface. Ferritin-labeled WGA also labeled densely the surface glycocalyx or miracidia (Figure 3) and cercariae. The position of the miracidial plates was visualized following the WGA (Figure 2B) and Con A fluorescent treatments. No labeling of miracidia or cercariae was observed with the PNA, LFA, or UEA ferritin

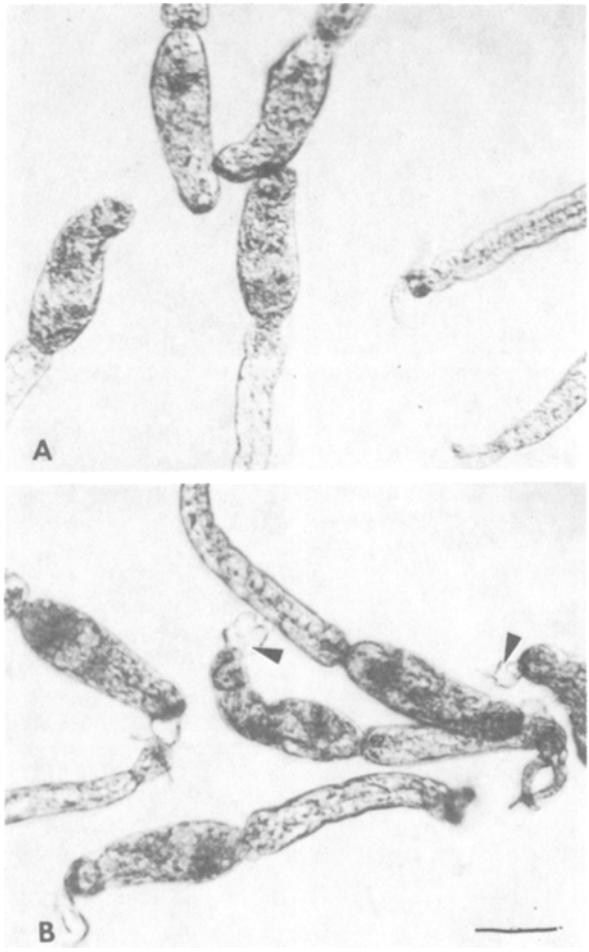

FIG. 1. Effects of lectins on gland discharge in cercariae of *Schistosoma mansoni*. (A) normal cercariae; (B) cercariae treated with PNA (30 μg/ml) showing secretion of preacetabular glands (arrowheads) Bar = 100 μm.

conjugates, but it is probable that if fucose residues occur on only localized surface areas of these schistosome stages, the residues would not be detected using this technique. Transmission electron microscopy of miracidia and cercariae exposed to 250 μg/ml WGA or PNA did not reveal damage to the tegument.

With three exceptions, all of the tested lectins induced discharge of cer-

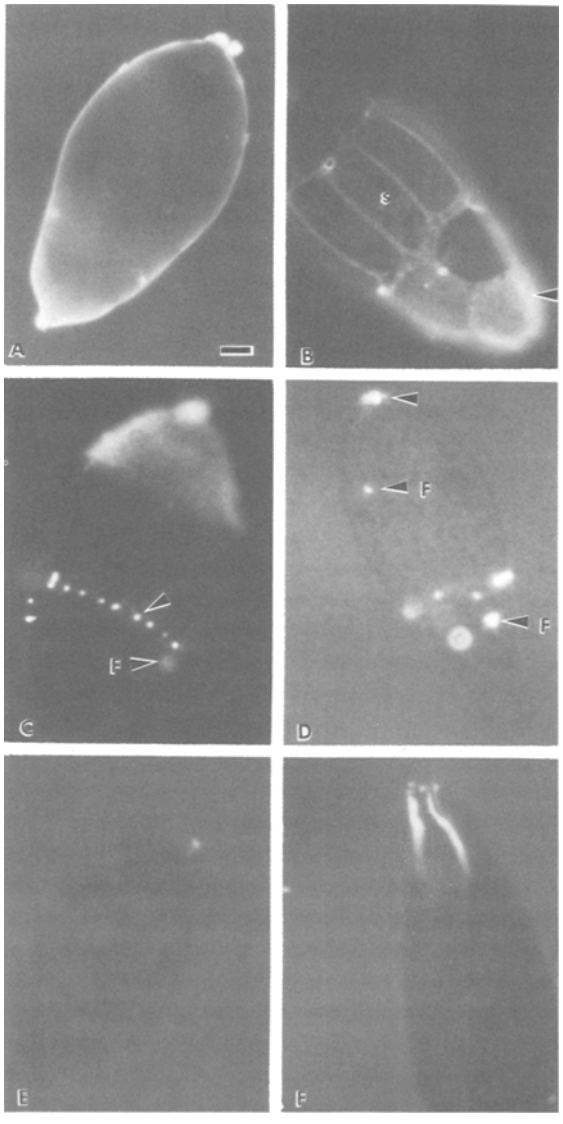
A
B
S
C
F
D
F
F
E
F

TABLE 2. LECTIN BINDING TO *Schistosoma mansoni* CERCARIAE AND MIRACIDIA[a]

Stage, location	Lectin				
	LPA	LFA	PNA	WGA	Con A
Miracidia					
Anterior end	+	+	++	+	++
Secretions		+	+	+	++
Penetration glands	+	++			
Flame cells			++		++
Uniform surface label				++	
Corona of unidentified cells (2/3 posteriad)			++		++
Plates				++	++
Cercariae					
Anterior end	+	+	++	+	+
Secretions	+	+	++	++	++
Penetration glands	++	++	++	+	
Flame cells				+	
Tail			+	+	
Uniform surface label	+			++	

[a] The results of FITC, TRITC, and ferritin-conjugated tests are combined. + = weak intensity; ++ = strong intensity.

carial glands (Figure 1B). Three of the active lectins (WGA, MPA, and PSA) caused significant discharge at the lowest dose tested, 7.5 μg/ml, indicating a very high sensitivity of the cercariae to lectin exposure. Linder (1985, 1986) used fluorescent lectins to visualize secretions as cercariae moved over skin lipids on a slide. However, our results suggest that the lectins affected the cercariae and, therefore, the events observed did not represent the normal situation.

The uniform distribution of *n*-acetyl glucosamine over the whole miracidial surface (Figure 2A), but not under the plates which are shed (Figure 2B), suggests that the lectin is not reaching most of what becomes the surface of the sporocyst. The labeling patterns found in our study agree with those of Samu-

FIG. 2. Binding of fluorescent conjugated lectins to miracidia and cercariae of *Schistosoma mansoni*. (A) General surface labeling of a miracidium by TRITC-WGA. (B) Outline of ciliary plates (arrowheads) on miracidium treated with TRITC-WGA. Some plates have been shed by the miracidium. (C) Miracidium treated with TRITC-PNA. Flame cells (arrowheads) and corona of discrete unidentified structures (arrowheads) shown: (D) Miracidium treated with FITC-PNA showing label to the flame cells (arrowheads F) and the anterior area. (E) Labeling of preacetabular glands of a cercariae by FITC-LFA. (F) Labeling of preacetabular glands of a cercariae by FITC-LFA. Bar on A = 8 μm and applies to entire plate.

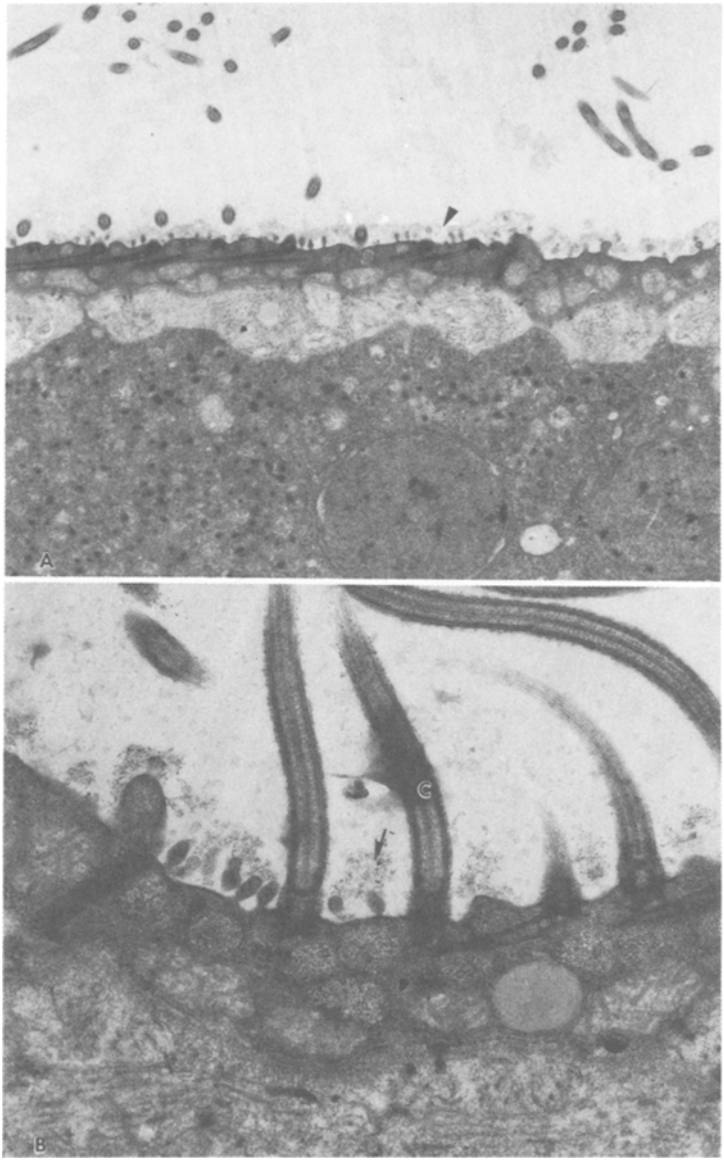

FIG. 3. WGA–ferritin conjugate label on the miracidial surface of *Schistosoma mansoni*. (A) Generalized view of ferritin label (arrowhead) on the surface glycocalyx, ×8000; (B) glycocalyx at the base of the cilia; (C) heavily labeled with ferritin (arrowhead), ×25,000.

elson and Caulfield (1985), who used Con A, but disagree with those of Murrell et al. (1978), who reported that Con A but not WGA bound generally to cercariae. Lectins can, however, reach other structures. PNA labeled a ring of cell- or pore-like structures of unknown origin which must be rich in galactose or galactose-like molecules (Figure 2C and D). LFA, which is specific for sialic acid or one of its analogs, labeled a number of structures and presented an enigma (Figure 2E and F). One of the proofs of the presence of sialic acid is the ability to displace the lectin with sialic acid, but this did not occur in tests with both the fluorescent and iodinated conjugates. Sialic acid has been reported in a number of helminths using indirect methods, but conclusive chemical determinations have not been undertaken (Schauer, 1982). We conclude that the labeling observed with LFA does not prove the presence of sialic acid in the schistosome stages examined.

The relationship of glycans localized on the cuticular surface proximate to nematode chemosensilla was discussed by Zuckerman and Jansson (1984) and has been shown to be important in chemoreception by the animal parasite *Trichostrongylus colubriformis* (Bone and Bottjer, 1985). Based on the current work, several receptors with different recognition sugars are involved in the triggering of gland discharge. This could explain why skin lipids cause almost complete discharge of the preacetabular glands (Austin et al., 1972), but relatively little occurs with 2-tetradecanoic acid (Haas, 1984). In *Caenorhabditis elegans*, enzymatic digestion of surface mannosyl residues interrupted the nematode's response behavior to a food source, supporting the view that this surface sugar derives from sensilla exudates and that sugars are the recognition molecules of receptors positioned within the chemosensilla. Based on the present results, we propose that galactosyl residues associated specifically with the chemosensilla of *S. mansoni* cercariae are critical to the recognition of the mammalian host. The role of galactose and related residues has precedent as being vital in molecular recognition between organisms, one example being the report of *n*-acetylgalactosamine on the nematode cuticle surfaces being critical in the recognition of the nematode by the nematophagous fungus *Arthrobotrys oligospora* (Nordbring-Hertz and Mattiasson, 1979). A better understanding of the molecular basis of chemical recognition by schistosomes is needed, but the abundance of surface sugars complicates the use of lectins to identify the structures or to try to purify the receptor molecules.

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COYOTE ESTROUS URINE VOLATILES

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Abstract—Samples of female coyote urine were taken once or twice each week during the winter and spring for two years. Headspace analysis was employed with Tenax GC trapping and GC-MS. Tenax trapping was started in less than 1 hr after sampling, and mild conditions were used to minimize losses of highly volatile and labile compounds. Thirty-four compounds were identified. They include sulfur compounds, aldehydes and ketones, hydrocarbons, and one alcohol. The principal constituent is methyl 3-methylbut-3-enyl sulfide, which usually comprised 50% or more of the total volatiles observed. The concentration of many constituents varied widely. This appeared to be quasiperiodic for five of the constituents, with a period of a few weeks, and with pronounced maxima at the peak of estrus. Apparently these compounds are 3-methyltetrahydrothiophene, methyl 3-methylbutyl sulfide, octanal, dodecanal, and bis(3-methylbut-3-enyl) disulfide. One or more of these compounds may have pheromonal activity in coyote relationships.

Key Words—Canidae, coyote urine, volatiles, estrous urine, urine volatiles.

INTRODUCTION

Deposition of urine is one of the means of communication used by coyotes (Bekoff and Wells, 1986). Among other functions, it appears to be important in mating behavior; scent marking is a common act performed during courtship

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rituals. It has long been known by trappers that the odor of urine collected during estrus is more attractive to coyotes than is urine taken at other times.

A number of studies have been made to identify the volatile chemicals present in the urine and in other secretions of canids. The tenative identification of several carboxylic acids, sulfides, and other compounds in coyote estrous urine has been reported by Murphy et al. (1978), and by Teranishi et al. (1981). The analytic approach was to use liquid–liquid extraction first, to separate the constituents into acid, base, and neutral fractions. No more recent analytic work with this approach has been done by these authors. However, one of the original findings was that the acid fraction was more attractive than the others. This finding was one of the factors that led to a comprehensive program of testing the attractancy of both a series of aliphatic carboxylic acids and their trimethylammonium salts (Fagre et al., 1983).

Urine of the red fox, collected during the winter season (breeding season), has been reported to contain several unusual volatile compounds, including two sulfides and two terpene-derived ketones (Jorgenson et al., 1978). Urine from captive red foxes has been studied by Bailey et al. (1980). They found some of the same compounds and also methyl 3-methylbutyl sulfide, and related their concentrations to the reproductive state of the animal. Wolf urine has been investigated by Raymer et al. (1986). They reported over 70 compounds, including eight sulfides and disulfides found also in other canids, and showed the dependence of some of them on reproductive hormones. Studies of the volatile constituents of female dog urine (*Canis familiaris*) have been made by Schultz et al. (1985). Major constituents found were two aliphatic sulfides. The list of minor constituents includes disulfides, trimethylamine, and other compounds.

Studies of the volatile constituents of coyote and dog anal sac secretions during estrus have been made by Preti et al. (1976). The major constituents reported were low-molecular-weight (C_2–C_6) carboxylic acids and trimethylamine. These same compounds, along with putrescine and cadaverine, have been reported as principal volatile constituents of red fox anal sac secretions by Albone et al. (1974).

Goodwin et al. (1979) studied vaginal secretions of the dog during the state of estrus and reported the presence of methyl *p*-hydroxybenzoate in samples taken on days of full estrus only. They described pheromonal activity for this compound. However, when similar testing was done by other investigators (Kruse and Howard, 1983), no pheromonal activity was observed.

The present paper reports on the identification and relative amounts of some of the volatile constituents of female coyote urine obtained from early winter well into spring of two years. These periods included the normal mid-winter estrous season. The analyses were run on vapors from the headspace of

fresh urine samples at room temperature. Gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC-MS) were employed.

METHODS AND MATERIALS

Collection of Urine Samples. Three female coyotes were kept in separate but adjoining 9-m^2 outdoor pens, each with a small shelter at one end. These animals were maintained on a standard diet of commercial dog food, dry kibble, and water. Coyote 1 was 3 years old and had pups in April of the previous year. Coyote 2 was 3 years old and never had pups. Coyote 3 was 2 years old and never had pups. Coyote 3 failed to donate urine after March 2 of the first season. Only coyote 1 was used in the second season.

Samples were collected early in the morning, usually twice each week from early January through March, then irregularly until early June in the first season (1981). Samples were taken from December 21 to April 26 in the second season (1982). For each collection, a clean pan, 20 cm wide, 25 cm long, 5 cm deep, was placed on the ground near the sheltered area of the pen and left there. The coyote usually sniffed and scent marked the pan, donating a urine sample, within a few minutes. Often, three or four scent-marking episodes were needed to obtain enough volume for analysis, but all samples were collected within a half hour of the initial urine donation. The urine was then poured into a glass bottle, stoppered, and kept in an ice bath until the start of the analysis, which was usually about 30–60 min later. The volume of sample varied from 3 to 30 ml during the first season. Generally, larger donations of urine were made during the second season. A 10-ml sample was taken each time for a GC analysis, and in 12 instances, the remainder, 9–42 ml, was used for GC-MS analysis.

Headspace Analysis. Purified nitrogen was passed over the surface of the urine sample. Entrained volatiles were carried by the gas into a 0.6-cm-diam., 7.5-cm-long stainless-steel tube packed with Tenax GC where they were adsorbed by the Tenax. The adsorbed volatiles were subsequently back-flushed into a liquid-nitrogen-cooled cold trap. The cold trap was flash-heated with a beaker of glycerol at 140°C or a heat-gun to move the volatiles into the GC column. Once the volatiles were loaded on the column, the GC run was done as usual. A stainless-steel open-tubular column, 0.75 mm ID and 150 m long, coated with methyl silicone oil containing 5% Igepal, was used. Details of the apparatus and procedure have been previously described (Schultz et al., 1971, 1985). An integrator–plotter, Hewlett-Packard model 3385A, was employed to trace the chromatogram and determine peak areas.

GC-MS. The procedure for trapping and back-flushing the volatiles for GC-MS was essentially the same as that described above. The GC column and

operating conditions of flow rate and temperature program were similar. The mass spectrometer was a Finnigan model 4500 equipped with an INCOS data system.

Separate runs on mixtures of pure paraffinic hydrocarbons from C_5 to C_{16} were made with the same GC and GC-MS systems and operating conditions as used with the urine volatiles in order to minimize the possibilities of mismatching any of the peaks and to permit calculation of retention indices.

RESULTS AND DISCUSSION

A list of identified constituents of the headspace vapor from female coyote urine is shown in Table 1. Peak numbers in column 1 refer to peaks in the chromatograms in Figures 1 and 2. Except where noted otherwise, all of these compounds were identified by matching their mass spectra and Kovats indices with those of authentic compounds. By far the most prominent constituent was methyl 3-methylbut-3-enyl sulfide (peak 46). This compound was synthesized by one of the authors (D.J.S.) using published methods (Wilson et al., 1978). The mass spectrum of the synthetic compound closely matched that of peak 46. In nearly half the urine samples, this compound amounted to 50% or more of the total volatiles observed. This compound has been reported to be a constituent of the urine of the red fox (*Vulpes vulpes* L.) during the winter season (Jorgenson et al., 1978) and of the wolf (*Canis lupus*) (Raymer et al., 1986). This compound, or perhaps a closely related isomer, was found also in the urine of the domestic dog (Schultz et al., 1985) but only as a minor constituent. Conversely, methyl propyl sulfide and methyl butyl sulfide were found to be major constituents of female beagle urine but only minor constituents of coyote urine. The other sulfur compounds in Table 1 are related to the three discussed above.

It was not surprising to find carbonyl compounds since they have earlier been found to be urine constituents (Matsumoto et al., 1973). Those in Table 1 comprise incomplete homologous series of aldehydes and methyl ketones from C_3 to C_{12}, but constituents were found in both of these classes only at the C_4 and C_5 levels. Hydrocarbons were represented by several terpenes, paraffins, 1-undecene, and toluene. Only one alcohol was found in substantial quantity, 3-methylbut-3-en-1-ol, which was always present, with relatively little variation in concentration. It might be one of the precursors of some of the sulfur compounds.

The most striking feature of the data is the great variation in concentration and ratios of concentrations of one constituent to another, with numerous ups and downs separated by only a few days. For the largest constituent, methyl 3-methylbut-3-enyl sulfide (peak 46), the concentration varied widely with time,

TABLE 1. COYOTE URINE VOLATILE CONSTITUENTS

Peak number[a]	Compound name	Kovats index	
		Found	Determined or literature
5	Acetone	484	474
6	2-Propanol	503	481
7	Isobutyraldehyde	537	539
9	2-Butanone	575	577
10	Hexane	600	600
12	Chloroform[b]	609	611
19	2-Methylbutanal	640	643
22	2-Pentanone	664	666
25	Pentanal	675	677
26	Trichloroethylene[b]	689	686
27	Methyl propyl sulfide	701	702
29	Dimethyl disulfide	726	732
30	3-Methylbut-3-en-1-ol	745	710
32	Toluene	751	755
36	Hexanal	777	772
37	Methyl butyl sulfide	801	803
39	3-Methyltetrahydrothiophene	847	846
41	*p* (or *m*)-Xylene[c]	855	853 (*p*) 852 (*m*)
43	Methyl 3-methylbutyl sulfide	864	862
44	2-Heptanone	870	872
46	Methyl 3-methylbut-3-enyl sulfide	883	881
47	Methyl pentyl sulfide	902	902
48	α-Pinene	927	929
49	Dimethyl trisulfide	949	940
50	β-Pinene	967	968
52	Myrcene	982	984
	Octanal	982	979
54	Decane	1000	1000
56	*p*-Cymene	1012	1013
58	Limonene	1019	1024
63	Acetophenone	1061	1056
67	Nonanal	1085	1087
68	1-Undecene	1089	1087
69	Methyl isopentenyl disulfide[c]	1093	
76	Decanal	1187	1188
78	Dodecane	1200	1200
87	Dodecanal	1391	1391
90	Bis(3-methylbut-3-enyl) disulfide	1441	1437

[a] Peak numbers refer to the chromatograms in Figures 1 and 2.
[b] Probably an artifact.
[c] Tentative.

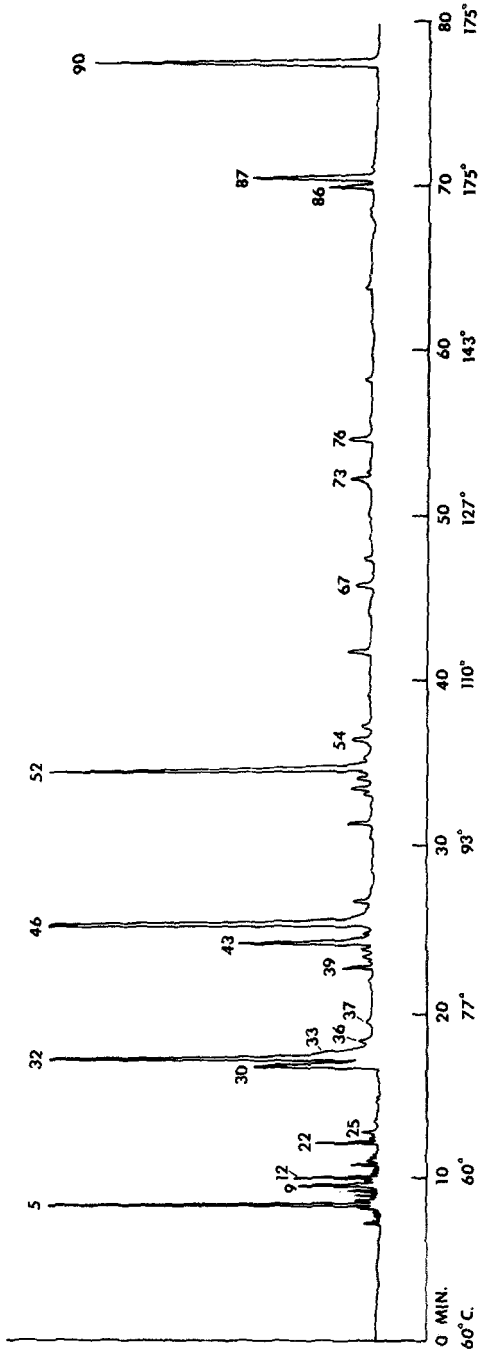

FIG. 1. Chromatogram of headspace volatiles from female coyote urine, coyote 1, on February 25, first year. Column: methyl silicone.

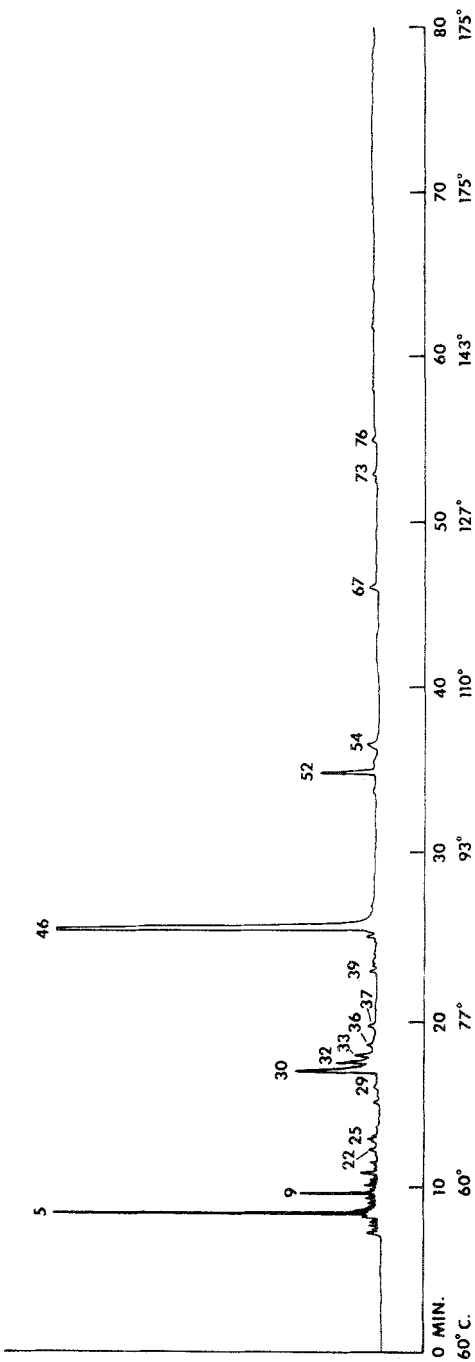

FIG. 2. Chromatogram of headspace volatiles from female coyote urine, coyote 1, on February 13, first year. Column: methyl silicone.

but there was no apparent relationship between its concentration and the state of estrus. Several of the other constituents varied from zero to moderate or fairly high concentrations. For example, the chromatogram of Figure 1 shows a fair number of substantial peaks. (Peak areas, measured in counts by the automatic integrator, are roughly proportional to concentrations of the constituents in the urine headspace; response factors were not determined.) This chromatogram is for coyote 1 on February 25, first season. The chromatogram for the same coyote on February 13 is shown in Figure 2. Peaks 43, 86, 87, and 90 are at, or near, zero, and peaks 32, 39, and 52 are much smaller than they are in Figure 1. However, in this particular comparison, peak 46 was nearly the same size, 20,300 vs. 21,000 counts.

Five of the constituents, with one or more of the coyotes, show patterns that suggest the variation is quasiperiodic. Plots of the first-year data for 3-methyltetrahydrothiophene (peak 39), methyl 3-methylbutyl sulfide (peak 43), and dodecanal (peak 87) are shown in Figures 3, 4, and 5, respectively. 3-Methyltetrahydrothiophene and dodecanal show maxima at intervals of a few weeks. Methyl 3-methylbutyl sulfide shows only two maxima, both in February. [Bailey et al. (1980) reported that the concentration of this compound in urine of the male red fox was significantly increased in February.] The other constituents that suggest a quasiperiodic variation are octanal (part of peak 52) and bis(3-methylbut-3-enyl) disulfide (peak 90). These five constituents showed pronounced maxima within, or near, the last week of February, which appeared

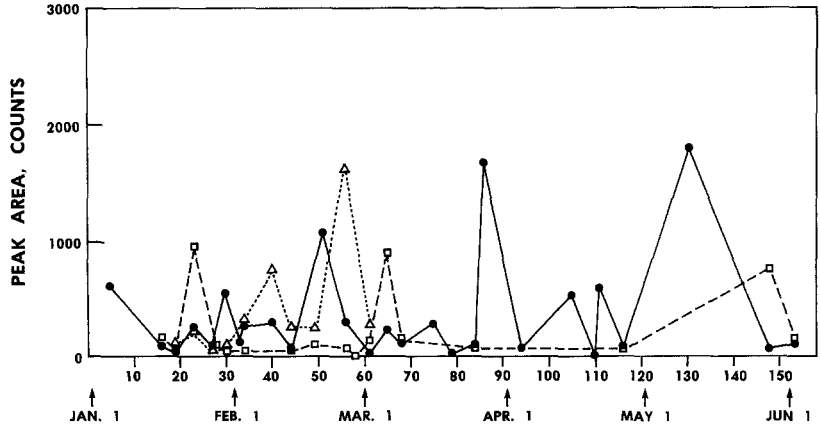

FIG. 3. Variation in concentration of 3-methyltetrahydrothiophene (peak 39) in the headspace with date, first year. Solid circles, coyote 1; squares, coyote 2; triangles, coyote 3.

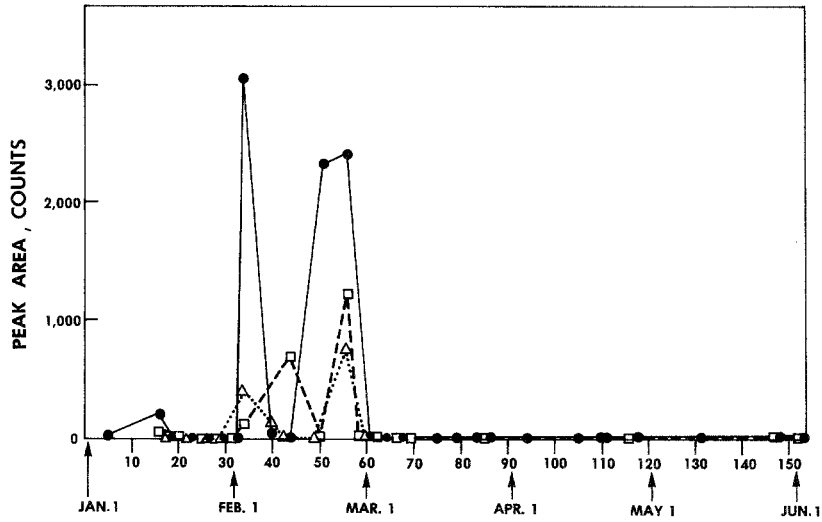

FIG. 4. Variation in concentration of methyl 3-methylbutyl sulfide (peak 43) in the headspace with date, first year. Legend is as in Figure 3.

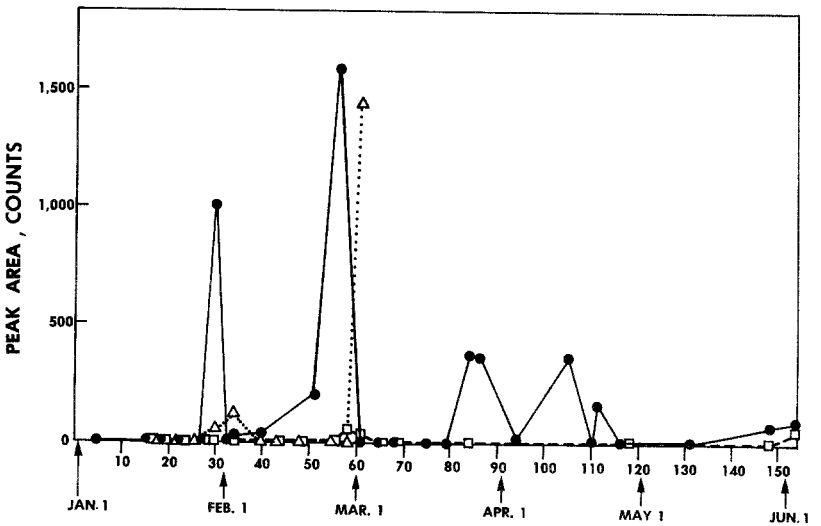

FIG. 5. Variation in concentration of dodecanal (peak 87) in the headspace with date, first year. Legend is as in Figure 3.

to be the height of the estrous season, as judged by sexual behavior of male–female pairs in nearby pens.

In the second year, when samples were available from only one of the coyotes, 1, similar variations were observed. The data for 3-methyltetrahydrothiophene (peak 39) are plotted in Figure 6. There is good agreement of the pattern compared with that for this compound with the same coyote in the first year (see Figure 3). However, from the samples collected, methyl 3-methylbutyl sulfide gave no substantial peak in February. High concentrations might have occurred within the five- or nine-day gaps when no samples were taken. Dodecanal gave only one substantial peak all season. This was in one of the GC-MS runs, and the identification as dodecanal was definite. The peak area, calculated with a conversion factor, was 1450 counts. In agreement with data of the first year, this peak was observed in a sample taken on February 26.

In the data presented above, there is a suggestion that the variations in concentration of these five constituents reflect hormonal cycles. However, this hypothesis is very tentative. Before it could be established, many more analyses would be required, with many more animals and more frequent sampling.

One of the other aliphatic aldehydes, nonanal (peak 67), is noteworthy. In the first year, its peak area varied between zero and 1920 counts, but on January 16, the sample from coyote 1 showed a very high peak of 23,000 counts.

In the first year, consistency of the numerical data was only fair, due mainly to the use of urine samples of different volumes. However, we believe that discrepancies from this cause are not serious enough to change any of the con-

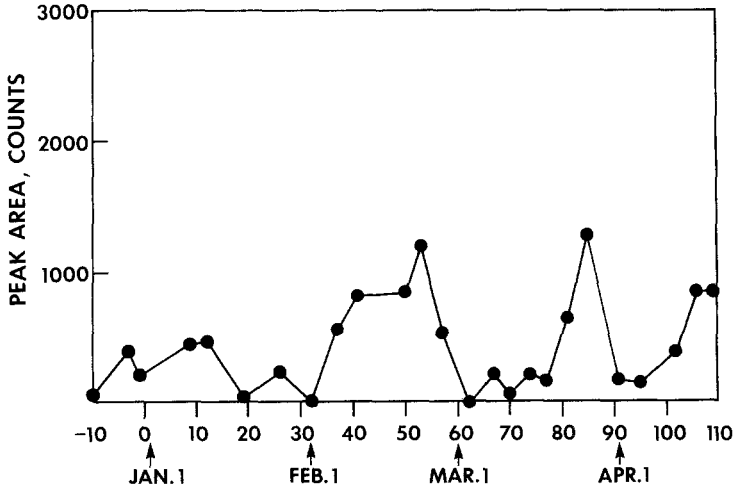

FIG. 6. Variation in concentration of 3-methyltetrahydrothiophene (peak 39) in the headspace with date, second year, for coyote 1.

clusions of this paper. In the second year, the urine sample volume was consistent at 10 ml.

No information was obtained about the possible presence of carboxylic acids, phenolic compounds, or mercaptans in the urine headspace because the GC column used does not permit the passage of small amounts of these compounds as definite peaks.

Some aldehydes, including octanal, nonanal, and dodecanal, have been shown to be coyote attractants (Scrivner et al., 1984), but further behavior studies are necessary to establish whether any of the compounds identified in coyote estrous urine have pheromonal activity.

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DEFENSIVE ODOR EMISSION FROM LARVAE OF TWO SAWFLY SPECIES, *Pristiphora erichsonii* AND *P. wesmaeli*[1]

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Abstract—The emission of species-specific odors by *Pristiphora erichsonii* and *P. wesmaeli* is accompanied by a characteristic defensive behavior called "snap bending." When the larvae are disturbed, blends of volatile compounds are emitted from ventral glands. The odor of *P. erichsonii*, a colonial species, is composed of bornyl acetate, borneol, *trans*-pinocarveol, myrtenol, benzaldehyde, and tetradecyl, hexadecyl, and octadecyl acetates, whereas that of the solitary *P. wesmaeli* is composed of 3-carene-10-al, linalool, myrtenal, and benzaldehyde. The role of these compounds in the defensive behavior of the larvae is discussed.

Key Words—Larch sawfly, *Pristiphora erichsonii*, Hymenoptera, Tenthredinidae, 3-carene-10-al, benzaldehyde, bornyl acetate, larval snap-bending.

INTRODUCTION

Larvae of the larch sawflies, *Pristiphora erichsonii (Hartig)* and *Pristiphora wesmaeli* (Tischbein) (Hymenoptera, Tenthredinidae), produce characteristic scents, easily distinguished by human olfaction. Upon being disturbed, the larvae emit the scents and quickly flex their abdomens to attain a characteristic stiff S-shaped posture, called "snap-bending" ("Schnickender haltung," Nägeli, 1935) (Figure 1).

[1] Hymenoptera: Tenthredinidae.

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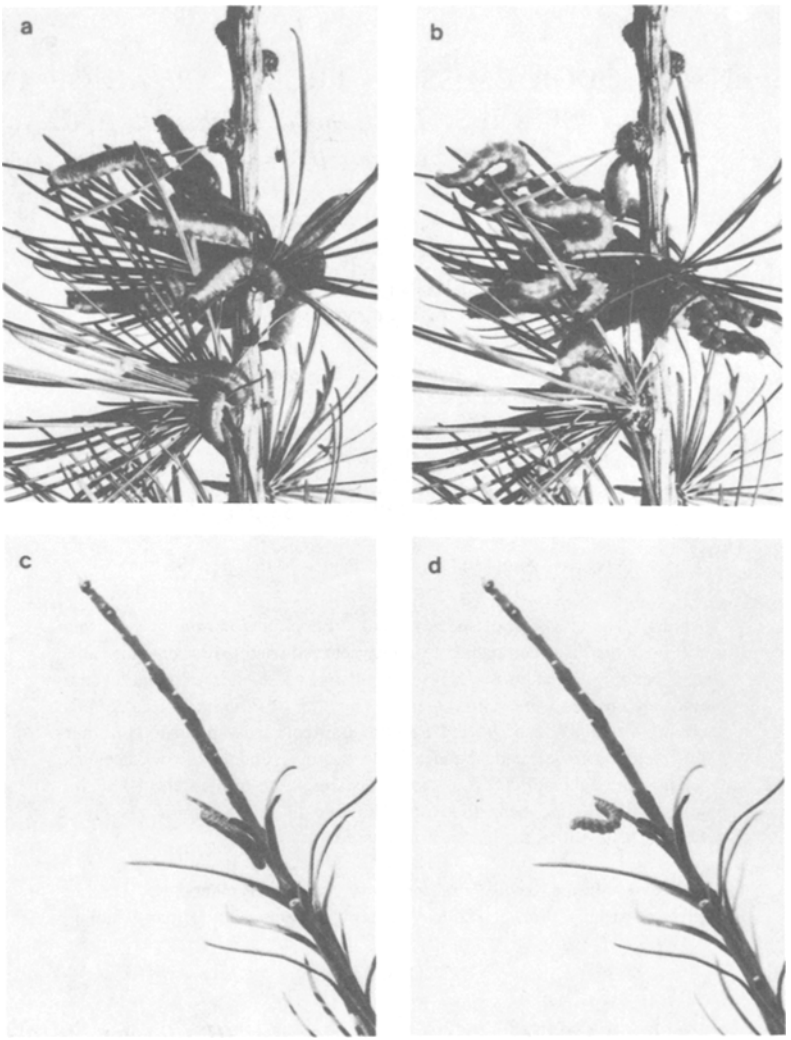

FIG. 1. A colony of *Pristiphora erichsonii* larvae (a, b) and a single *P. wesmaeli* larva (c, d) on needles of *Larix decidua* in the field. In a and c the larvae are feeding undisturbed, while in b and d the same larvae are shown in the snap-bending posture.

Glands producing volatile secretions are situated ventrally on the first seven abdominal segments in several Tenthredinidae (Yuasa, 1923; Maxwell, 1955; Boevé and Pasteels, 1985). The glands on the second to seventh segments are protrusile, those on the first segment are not. It has been suggested that the latter produce the odor emitted by snap-bending larvae (Benson, 1950, p. 76).

The sympatric *P. erichsonii* and *P. wesmaeli* differ in several respects, including feeding and oviposition behavior. *P. erichsonii* attack needles growing from tufts produced the preceding summer, whereas *P. wesmaeli* feed only on the long shoot needles initiated the same summer. Larvae of *P. erichsonii* live colonially with the most pronounced aggregation during the early instars, while *P. wesmaeli* larvae are solitary (Pschorn-Walcher and Zinnert, 1971). *P. wesmaeli* larvae are light green with two somewhat lighter bands along the body, and they exhibit homochromy in that they resemble larch needles. *P. erichsonii* larvae are greyish-green with a black head and are easier to discover at distance.

We have isolated compounds from the secretions which emanate from gland cells of the first abdominal segment of each species of sawfly larvae. The volatile components have been identified in an attempt to understand the role of these volatiles in the behavior related to the different life histories of these two species.

METHODS AND MATERIALS

Feeding larvae of *P. erichsonii* and *P. wesmaeli* were collected from *Larix* spp. near Uppsala in eastern Sweden or near Vårgårda in southwestern Sweden during the years 1975, 1979–1981, and 1985. In the laboratory, the larvae were fed with fresh foliage of *Larix decidua* (Miller).

To collect the volatiles emitted from the first abdominal segment, the larvae were induced to snap-bend and the glandular openings were wiped with 3 × 12-mm pieces of filter paper held with forceps. This procedure was repeated four to six times for each larva, with at least 12 h of undisturbed feeding between collections. The filter papers were immediately extracted in either redistilled pentane or pentane:ethyl acetate (95 : 5 v/v). The extracts were stored at −20°C and concentrated by evaporation at 42°C. Analyses were made on a coupled gas chromatograph–mass spectrometer (GC-MS), Finnigan 4021, San Jose, California. Glass capillary columns coated with either FFAP/OV-17 (6 : 5) (0.3 mm × 33 m) or Superox FA (0.25 mm × 56 m) or a fused silica column coated with OV-351 (0.2 mm × 25 m) were used. All compounds were identified by comparing their retention characteristics and mass spectra with those of synthetic reference compounds. The mass spectra were scanned from *m/e* 31 to

300, and, for the quantitative determinations, the area under each peak in the reconstructed ion chromatogram was measured. A response factor of 1.0 was used for all compounds.

The gas chromatographic phases were purchased from Alltec Assoc. Bellefonte, Pennsylvania and Supelco Inc. Deerfield, Illinois. The 3-carene-10-al was derived was derived by oxidation of 3-carene-10-ol synthesized as described in Lanne et al. (1987).

In a series of experiments at the laboratory, groups of the bug *Anthocoris nemorum L.* (Heteroptera, Anthocoridae) were placed one by one in Petri dishes (diameter 70 mm) containing a piece of filter paper with synthetic compounds (0.5–1 μg of each compound). As control, insects were studied in the same situation but without odors added to the filter paper. Three replicates were made of each test.

RESULTS

The gland on the ventral side of the first abdominal segment of *P. erichsonii* larvae was studied by light microscopy and was found to consist of a thin layer of glandular cells in a pocket. The gland is not protrusible, but the glandular pocket was observed to open and close regularly while the larvae were snap-bending. The secretions from these glands of *P. erichsonii* and *P. wesmaeli* were analyzed.

Gas chromatograms of the volatiles from the two species (Fig. 2) show that they are made up of several different compounds (Table 1). Benzaldehyde was the only compound common to both species, although present in different relative amounts. In *P. erichsonii*, the four oxygenated monoterpenes found in appreciable amounts were bornyl acetate, borneol, myrtenol, and *trans*-pinocarveol. Of these, bornyl acetate was the major component. The *P. wesmaeli* secretion contained three other oxygenated monoterpenes: linalool, myrtenal, and 3-carene-10-al. The mass spectrum of 3-carene-10-al, the major compound in *P. wesmaeli*, was compared with that of the synthetic reference (Figure 3). The *P. erichsonii* secretion also contained straight-chain tetradecyl, hexadecyl, and octadecyl acetates. Individual larvae of *P. erichsonii* and *P. wesmaeli* contained on the average 50 ng bornyl acetate and 1 ng 3-carene-10-al, respectively. Different instars of *P. erichsonii* and *P. wesmaeli* were analyzed. Third and fifth instars were compared for *P. erichsonii*. These analyses, based on 150 larvae of each instar, showed that none of the components in the secretion differed more than a few percent relative to the main component, bornyl acetate. Three groups of *P. wesmaeli* were analyzed, the first (120 larvae) consisted of a mixture of second- and third-instar larvae, the second group (75 larvae) consisted of third-instar larvae, and the third group (41 larvae) a mixture of fourth- and fifth-instar larvae. For *P. wesmaeli*, the amount of linalool and myrtenal did not differ more than 3% relative to 3-carene-10-al.

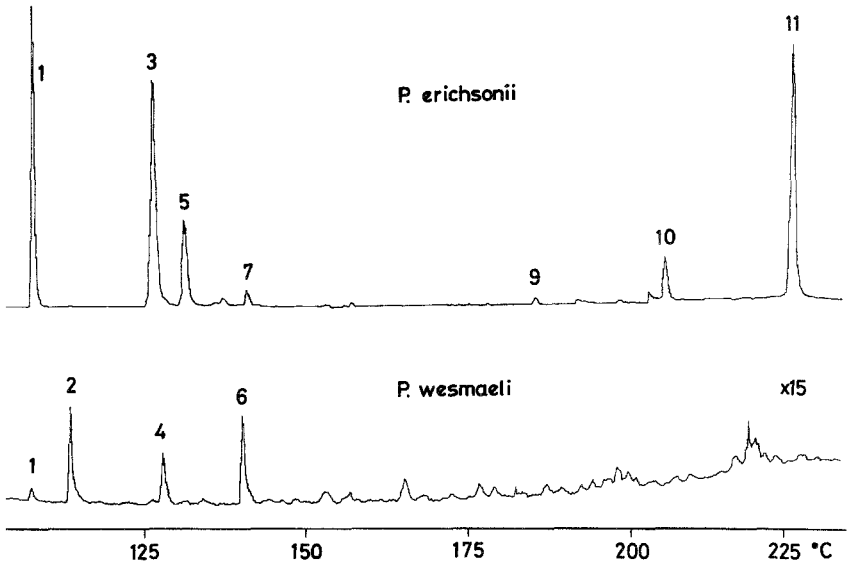

FIG. 2. Examples of capillary gas chromatograms of volatile secretions from *P. erichsonii* and *P. wesmaeli*. The GC column was coated with FFAP/OV-17 (see Methods and Materials). Compound 8 was not evident in this analysis as it coeluted with bornyl acetate on this particular column.

TABLE 1. VOLATILE COMPOUNDS IDENTIFIED IN GLANDULAR SECRETION OF *Pristiphora erichsonii* AND *P. wesmaeli* LARVAE

No.	Compounds	Relative amount[a]	
		P. erichsonii[b]	*P. wesmaeli*[c]
1.	Benzaldehyde	60	0.5
2.	Linalool		30
3.	Bornyl acetate	100	
4.	Myrtenal		27
5.	Borneol	14	
6.	3-Carene-10-al		100
7.	Myrtenol	2	
8.	*trans*-Pinocarveol	7	
9.	Tetradecyl acetate	1	
10.	Hexadecyl acetate	3	
11.	Octadecyl acetate	36	

[a] Expressed as percentage of the major compound in the respective secretion.
[b] Mean value of five extracts of instars 3, 4, and 5. More than 400 larvae were analyzed.
[c] Mean value of three extracts of instars 1–5. Seventy-six larvae were analyzed.

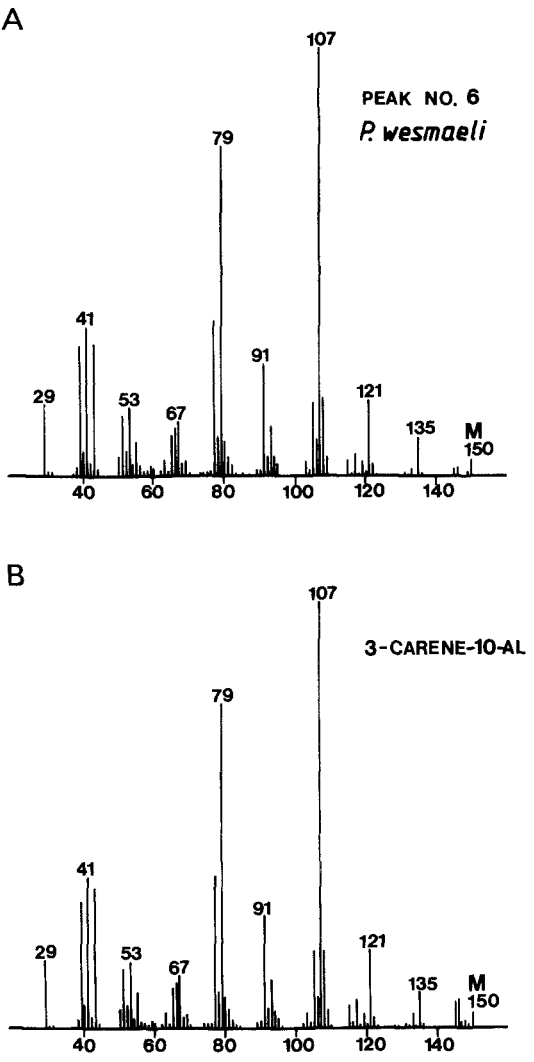

FIG. 3. Mass spectra of (A) compound 6 in *P. wesmaeli*, and (B) synthetic 3-carene-10-al.

Needles of *Larix sibirica* (Ledebour) and *L. decidua* were extracted and analyzed. Bornyl acetate, the only detectable volatile compound common to both *P. erichsonii* larvae and needles, was present in trace amounts in *L. sibirica*.

Mechanical or visual stimuli were used to evoke snap-bending and odor

emission under experimental conditions in the field and in the laboratory. Under natural conditions in the field, attacks by heteropteran predators such as *Orius* sp., *Anthocoris nemorum*, and *Ligyrocoris silvestris* L., were observed to release snap-bending behavior and odor emission. The attacking insects normally reacted to the behavior and excretions of the larch sawfly larvae by retreating and then grooming their antennae. When the predator *A. nemorum* was exposed to compounds found in the secretion of *P. erichsonii* in the laboratory, the insects were alerted and increased the intensity of their activities. They groomed their antennae, twitched, ran around, and attempted to fly. A mixture of borneol, bornyl acetate, myrtenol, and tetradecyl and hexadecyl acetate evoked a strong response, and when the compounds were tested one by one; hexadecyl acetate gave the strongest response. Benzaldehyde also stimulated the insects but only for a short while.

DISCUSSION

The spectrum of defense mechanisms known for larvae of Tenthredinidae include: (1) rapid changes in position (Figure 1b and d); (2) hiding (Figure 1a and c); (3) camoflage (homochromy) (Figure 1a and c); (4) display, which may be aposematic (Figure 1b and d); and (5) deterrence. Coupling between these modes of defensive behavior is common and augmentative (Benson, 1950). Glandular ejections of ill-smelling or protective liquids by sawfly are effective against spiders, which are chemotactically sensitive (Bristowe, 1941). Recently, Boevé and Pasteels (1985) have shown that the secretions from Nematinae sawfly larvae with well-developed ventral glands in the abdominal region protect the larvae from attacks by ants. Our field observations of *A. nemorum* and other heteropteran predators also show that the sawfly larval secretions protect the larvae from predators. Furthermore, our laboratory experiments indicate that the predators are affected by the glandular compounds of *P. erichsonii*.

The patterns of oxygenated monoterpenes found in the larch sawfly larvae show great similarity to the patterns of such compounds found in the hindguts of many bark beetles. In bark beetles, many of the oxygenated monoterpenes were originally suggested to result from detoxification of host terpenes (White et al., 1980), but the compounds are known now, in some cases, to be used as aggregation pheromones (Wood, 1982; Borden, 1982). The ability to oxygenate host monoterpene hydrocarbons is widespread in several insect orders. Two plausible precursors of *trans*-pinocarveol, myrtenol, and myrtenal are α-pinene and β-pinene, while 3-carene is probably the precursor of 3-carene-10-al. These three monoterpene hydrocarbons are present in many conifers (Karrer, 1976). It is also possible that the insects selectively sequestered some of the trace oxygenated monoterpenes that can be present in a conifer host (Heemann and

Francke, 1977). If this is the case for the larch sawflies, the different amounts of monoterpenes in *P. erichsonii* and *P. wesmaeli* should reflect their different choices of food.

Most studies of larval odors have dealt with defensive secretions (for a review see Blum, 1981). The Tenthredinidae *Neodiprion sertifer* (Geoffrey) larvae, upon being disturbed, perform a type of snap-bending similar to that of larch sawfly larvae, but their heads are lifted rather than their abdomens. In this disturbed situation, they discharge the content of diventricular pouches in their foreguts. These pouches contain sequestered host-plant terpenoic resins (Eisner et al., 1974) that deter predators.

Boevé et al. (1984) and Boevé and Pasteels (1985) have investigated eight species of Tenthredinidae, subfamily Nematinae, all of which have glands that produce secretions repellent to ants. These glands are situated ventrally on the second to seventh abdominal segments and are protrusible. The authors have, as have we, isolated the secretions by wiping the glands with pieces of filter paper. Seven compounds were identified by analysis of these secretions: *trans*-2-hexenol, *trans*-2-hexenal, *trans*-4-oxo-2-hexenal, geranial, neral, *cis*- and *trans*-dolichodial, and benzaldehyde. Since the openings of the gland we have studied and the protrusible glands that Boevé and coworkers investigated are closely situated on the larvae, it cannot be ruled out that the benzaldehyde we found could have been produced in the eversible glands on the midabdominal region rather than in the glands of the first abdominal segment.

Several authors have reported varying compositions of the secretions of papilionids from the different larval instars (Seligman and Doy, 1972; Burger et al., 1978; Honda, 1980). We did not observe this phenomenon. However, we did not try to determine whether there are differences in the amounts of volatiles in the different instars.

In contrast to *P. wesmaeli*, the aposematic *P. erichsonii* larvae are colonial, and simultaneous snap-bending can occur. It may be possible, therefore, that the volatiles not only have a deterring effect on predators but also may have an effect in alarming the whole colony. If all the larvae in a colony snap-bend simultaneously, very large amounts of deterring compounds would be released, and the visual scaring-off effect would be complemented.

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Book Review

Chemical Signals in Vertebrates 4; Ecology, Evolution, and Comparative Biology.
D. Duvall, D. Muller-Schwarze, and R. M. Silverstein (eds.).
New York and London: Plenum Press, 1986. $95.00, 742 pp.

This volume, the proceedings of a conference held in July of 1985, is a surprisingly thorough and well-balanced survey of the current status of the field of chemical communication in vertebrates. There are a total of 54 papers included, distributed into sections as follows: General Considerations, four; Chemistry, four; Fishes, five; Amphibians, five; Reptiles, seven; Birds, two; Mammals, 27. Although half the chapters concern mammals, this volume includes much more work on nonmammalian vertebrates than any other general reference of which I am aware.

A large proportion of the chapters in this book include substantial amounts of review material, further enhancing its value as a reference. As one engaged in research on mammalian chemical communication, I found many such chapters on nonmammalian vertebrates to be particularly interesting and informative. On fishes there are valuable reviews on alarm signals and their evolution by Smith; reproductive pheromones by Stacey, Kyle, and Lily; and chemosensory orientation and homing (including an update on salmon homing) by Johnsen. On amphibians there are interesting reviews on salamander courtship pheromones by Houck, kin recognition in toads and frogs by Waldman, and a combined experimental and review paper on territoriality in salamanders by Jaeger. For reptiles there is a review of snake pheromone trails by Ford; lizard pheromones by Cooper and Vitt; homing and orientation in aquatic amphibians and reptiles, especially sea turtles, by Owens, Comuzzie, and Grassman; and, from Garstka and Crews, a summary of their extensive examination of pheromones and reproduction in garter snakes. Wenzel reviews information on the olfactory system and some of its functions in procellariiform birds (albatrosses, fulmars, petrels, shearwaters), a fascinating group that contains 10 of the top 12 avian species in terms of the size of the olfactory bulb.

A number of the 27 papers on mammals also have a broad scope. Three areas in which chemical signals influence endocrine function are reviewed: Vandenbergh on the suppression of ovarian function, Keverne and Rosser on

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pregnancy blocking, and Drickamer on puberty-influencing signals in house mice. Other relatively broad summaries not mentioned elsewhere in this review include that by Wysocki, Bean, and Beauchamp on the mammalian vomeronasal system; Fadem on chemical communication in marsupials and monotremes, and Doty on cross-cultural studies of taste and smell among humans.

It is difficult to generalize about a book that covers such a broad spectrum of species and issues, but two themes seemed to emerge from this collection. The first, probably intended by the organizers of the conference, was a widespread concern with evolutionary issues. This concern was the explicit focus of a number of articles and was clearly an issue of importance in a host of others. In addition to the chapters mentioned elsewhere in this review, papers with a strong evolutionary bias include those by Maderson on the characteristics and evolution of the tetrapod skin as a source of chemical signals, Graves et al. on den location as an adaptive feature in rattlesnakes, Gosling on the economics of territorial scent marking, and Jannet on scent glands of *Microtus*. Adams and Johnsen reported on the similarities and differences in the chemical control of feeding in herbivorous and carnivorous fish, and Dawley considered chemical isolating mechansims in salamanders.

As a second theme, it seems clear that virtually all researchers in the field now accept, at least implicity, that most chemical signals in vertebrates are likely to be complex mixtures of components and not single substances and that the perceptual processes underlying communication are likely to be similarly complex. Jemiolo, Andreolini, and Novotny, for example, identified several components from urine of house mice that may be important in the delay of puberty in that species; the effects can be obtained when these substances are presented in normally ineffective urine but, apparently, not when presented alone. Muller-Schwarze et al. reported that responses of beavers to artificial scent marks from other beavers was maximal when the stimulus contained both castoreum and anal gland secretions, less to either secretion alone, and lower still to fractions of these components. The most through work on the complexity of the chemical signals comes from studies of scent communication in marmosets and tamarins, reported by Epple, Belcher, and Smith. They show, for example, that the same chemical compounds are found in the scent secretions of males and females of two different subspecies but that the ratios of these compounds differed. Animals of a particular species and sex maintained consistent ratios over long periods of time, thus suggesting that such quantitative differences could be used for species and gender recognition. For gender recognition, the entire range of volatiles may be necessary; splitting the volatiles into two fractions resulted in loss of gender-specific responses from marmosets. All three of these studies suggest the importance of a complex chemical mixture that is necessary for communication. Galef's experiments further show that the olfactory signal itself must appear in the proper context—in this case in order

for an observer rat to be influenced by the odors of food that another has eaten they must come from the head region of a live (but not necessarily mobile) rat. Surprisingly, the odors do not necessarily come from food particles on the surface of the body—rats that have been tube-fed provide sufficient stimuli for other rats so that they alter their food preference.

The rich panorama of observation and experimental results ranges from fish to elephants (Rasmussen et al.) and is bound to be educational for almost any reader. The highlights will vary for different readers; for me they included relatively concrete examples of behavior as well as the latest analytical approaches to chemical communication. What could be a more striking (although not exactly a subtle) example of chemical signaling behavior than the male salamanders that slap their scent gland directly over the nares of the female they are courting? In other species the males first abrade the skin of females, and then rub the gland on this abraded area (reported by Houck). Equally amazing, and perhaps not unique, are the demonstrations that the sexual contact pheromone of female garter snakes is a lipid produced in the liver (!) and transported in the blood to specialized vessels (but not to glands) in the skin (Garstka and Crews). Ford reports experiments on the classic issue of the directionality of scent trails and comes up with a new mechanism by which this can occur: he shows that snakes can determine the directionality of a trail by discrimination of the higher concentration of scent on the side of objects that the snake has pushed off from, and that are therefore in the direction that the trail-laying snake was going. In the course of their review on homing and orientation, Owens, Comuzzie, and Grassman report earlier studies by Manton in which green sea turtles remembered an arbitrary discrimination for at least one year, results that should be more generally known. Except for homing studies, aspects of olfactory memory have been largely ignored, yet a variety of observations suggest that many animals have phenomenal abilities in this domain.

Other striking discoveries are reported. Schreibman et al present results that document the amazing growth and differention of olfactory structures in platyfish that are correlated with reproductive development. Mason and Clark demonstrate that the plants selected by starlings for their nest material on the basis of chemical cues are effective in inhibiting the growth of ectoparasites and bacteria. Porter shows that spiny mice recognize their siblings and that they do so by learning their chemical signatures. He has demonstrated that experience with one sibling is sufficient to permit young to recognize other siblings but that experience of an individual with its own odor is not. Cues from the mother are also sufficient: siblings reared apart treat one another as siblings if they are nursed by the same mother but not if they are nursed by different mothers. Perhaps the most biologically hi-tech research included is that on discrimination of closely related strains of house mice: Beauchamp, Gilbert, Yamazaki, and Boyse report that inbred, congenic mice with a mutation in one gene in the

major histocompatibility compex, which results in a substitution of 3 amino acids on one protein, can still be discriminated from the original strain on the basis of chemical cues! Furthermore, experiments on the source of the strain-distinctive odors have shown that they originate from cells in the hematopoietic system. When mice have this entire system destroyed by irradiation and then receive bone-marrow transplants from donors of either the same inbred strain or a heterozygote strain, the recipient smells like animals of the donor type to other mice. Thus, marrow cells that are involved in cell recognition within the body are also involved in production of substances that allow mice to discriminate between closely related individuals on the basis of odor cues.

I strongly recommend this book as a reference to anyone interested in this field. The book is large (684 pages of text plus 57 additional pages of indexes) and, although not actually encyclopedic, it does cover an impressive range of topics. Most chapters are clear and offer insights into the current state of the art and science of chemical communication in vertebrates.

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Letter to the Editors

WHAT IS CHEMICAL ECOLOGY?[1]

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What is chemical ecology; what direction should it take in the future; and what can chemical ecologists do to promote, facilitate, and advance the science? I will present a personal viewpoint and do not seek consensus. A debate on these issues may be valuable and would indicate what diversity of opinions exists about our science. Pluralism in opinion and approach usually promotes vigorous science when the issues are aired openly. My comments should not be construed as criticism of past work or approaches. Here I will suggest that the body of studies in the *Journal of Chemical Ecology* reflects the current definition of chemical ecology stated in the journal and in the statement of purpose of the International Society of Chemical Ecology. I will argue for a broadening of our approach and for the explicit inclusion of more ecology.

Let me first state my personal perspective. I do not consider chemical ecology to be an autonomous discipline, but see it rather as an approach to ecology—viewing ecological interactions from a chemical perspective. Chemistry is a valuable tool or probe for increasing ecological understanding because it frequently plays a crucial role, is discrete and quantifiable, and is often manipulable. This approach is not necessarily restricted to any one level of ecological organization, and it strives to understand the role of the chemistry together with the diversity of physical, biotic, and historical factors that shape ecological interactions. Although such an approach is integrated within ecology, the science remains clearly identifiable because of its unique perspective—much in the same way that physiological ecology and ecological genetics retain their identities.

In order to examine some current emphases in chemical ecology, I sur-

[1] Work done while on sabbatical at the Department of Biology, University of York, England.

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veyed a total of 305 papers published in the *Journal of Chemical Ecology* in 1985 and 1986. I categorized papers into approaches or areas of study that constituted their main focus (Table 1). The bulk of papers (84%) dealt with methods, natural-products chemistry, biochemistry, pharmacology, toxicology, behavior, and organismal biology. These papers contained very little reference to ecology. The remainder dealt with some ecological aspects; the majority (12%) concerned studies of autecology or species pair interactions. There were very few papers dealing with higher levels of ecological organization, such as population or community ecology, and none addressing ecosystem ecology. The few papers on community ecology all dealt with allelopathy. Evolutionary ecology papers (primarily plant–insect coevolution) were limited in number (1%); there was only one review paper, and there were no papers that were primarily conceptual or theoretical. Studies that attempted to examine the relative importance of chemical factors versus other ecological factors were very rare (1%). Almost all studies dealt with identified or partially characterized and/or isolated organic compounds (92%). A few were nonchemical (7%), where no specific compounds were directly involved or no isolation and/or fractionation was attempted); and only 1% addressed inorganic compounds.

Clearly, studies of natural-products chemistry, studies within organisms, behavior, and organismal-level studies dominate over ecological studies in the journal. The current balance of papers appears to reflect the statement of purpose of the *Journal of Chemical Ecology*, the official organ of the International Society of Chemical Ecology (ISCE). "The *Journal of Chemical Ecology* (and

TABLE 1. DISTRIBUTION OF PAPERS (N = 305) PUBLISHED IN *Journal of Chemical Ecology* IN 1985 AND 1986, CLASSIFIED BY APPROACH OR AREA OF STUDY

Subject area	Papers (%)	
Methods	6	
Natural-products chemistry (isolation, characterization, synthesis)	29	
Biochemistry/pharmacology/toxicology (including biosynthesis, growth inhibition effects, physiology, chemoreception)	19	84
Behavior (primarily assays and behavioral roles of chemicals)	23	
Organismal biology	7	
Autecology (ecology of species, including social ecology)	4	
Species interactions	8	
Population ecology	1	
Community ecology	2	16
Ecosystem ecology	0	
Evolutionary ecology	1	
General chemical ecology (reviews, theory, concepts)	<1	

ISCE) is devoted to promoting an understanding of the origin, function and significance of natural chemicals that mediate interactions within and between organisms.'' The statement of purpose recognizes that the study of chemical interactions is central to our science. Isolation and identification of compounds, and determining their mode of action, are also explicitly stated. The word ''significance'' implies that we should also examine nonchemical phenomena in order to understand the importance of the chemical phenomena. ''Interactions within... organisms'' validates suborganismal and behavioral approaches; and ''interactions... between organisms'' may imply some aspects of autecology or species interactions. There is no reference to ecology in the statement.

It is ironic that a journal and a society that use the word ecology in their titles should have statements of purpose that lack any specific reference to ecology. Perhaps the relative dearth of ecological papers in the journal is a reflection of the statement. Authors of manuscripts involving chemistry that have a more ecological emphasis may not consider submitting to the journal as a consequence. A brief examination of such journals as *Ecology* and *Oecologia* over the last three years shows that an increasing number of more ecologically oriented papers involving chemistry are appearing there, rather than in the *Journal of Chemical Ecology*. Although studies of natural-products chemistry or biochemistry are essential to chemical ecology, they are not necessarily, in themselves, chemical ecology, just as an ecological study alone is not chemical ecology. While the *Journal of Chemical Ecology* cannot, nor should, publish all papers that cover the entire range of studies from natural products chemistry to ecology, our science may be better served if there was a broader and more balanced representation of approaches in the journal, and if the journal contained the very best examples of chemical ecology.

Chemical ecology is a new and relatively small area of science that can ill afford fragmentation into different camps. Much more is to be gained by broadening our science and encouraging a multiplicity of approaches and perspectives. A broad approach might increase membership of the society and readership of the journal. We would all gain additional insight and perspective that would improve our own studies. Ultimately we might increase the impact of our field of science to both basic and applied research. I argue that this can be done without diluting our discipline or excluding scientists whose individual contributions to chemical ecology may lie at opposite ends of a spectrum.

How could these lofty goals be accomplished? A few suggestions come to mind, and no doubt the creative readership of the *Journal of Chemical Ecology* and members of ISCE can come up with many better ideas. We could alter the statement of purpose of the journal and the society to broaden scope and more explicitly include ecology (e.g., ''...devoted to promoting ecological understanding of the origin, function, and significance of naturally occurring organic and inorganic chemicals and the interactions that they mediate.'') We could

increase the editorial board of the journal to reflect a wider range of approaches. We could change our statement of editorial policy to explicitly welcome submission of papers in areas that we feel are currently underrepresented and actively solicit submission of papers in these areas. Outside of the journal, we could find ways to promote broad, multidisciplinary interest and collaboration, such as occasional joint meetings of ISCE with organizations of similar size and scope that have mutually beneficial aims. We could provide funds to students through ISCE to attend meetings, do research in underrepresented areas, or build bridges between different approaches within our science. We could carefully balance the structure of ISCE meetings to reflect our diversity and actively solicit the attendance of scientists who do not currently attend.

Chemistry is important in most ecological interactions. Roles include: exploitation of resources by producers, consumers and decomposers; communication and defense within and between species; dynamics of populations; organization of communities; and structure and function of ecosystems. Exploring, understanding, and integrating the diverse roles of chemistry in the operation of ecological systems is an exciting, important challenge and a new frontier in chemical ecology. The approach to these complex problems is necessarily diverse, multidisciplinary, and collaborative. It includes a mixture of approaches with techniques ranging from the elucidation of the structure of natural products, biochemical and behavioral assays, to quantitative analysis and experimental manipulation of such parameters as organismal, population, community, or ecosystem characteristics. Most importantly, it requires the interaction of scientists with diverse talents and backgrounds. Chemical ecology grew out of the mutualistic desires of the chemist and ecologist to work together, and we have been very successful at developing these collaborations. As our discipline matures, we should not lose sight of the reasons for and benefits from these interactions and continue to keep a broad perspective that is the essence of our future productivity and success.

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Announcement

INTERNATIONAL CONFERENCE ON CHEMICAL SIGNALS

The 5th International Conference on Chemical Signals in Vertebrates will take place in Oxford, England, August 8–10, 1988. Sessions will include semiochemistry, reproduction and primers, neurophysiology and anatomy, social and spatial organization, predators and prey, and applications. Inquiries should be addressed to the conference chairman, Dr. David Macdonald, Department of Zoology, South Parks Road, Oxford, England, U.K. 0X1 3PS.

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BIOLOGICAL SUPPRESSION OF NITRIFICATION BY SELECTED CULTIVARS OF *Helianthus annuus* L.

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Abstract—The allelopathic potential of different cultivars of *Helianthus annuus* against nitrification was studied using the soil incubation method. The results indicated that aqueous extracts and residues of roots and shoots of all test cultivars significantly reduced the nitrification rate in soil. However, cultivars Local and Citosol were found to be more inhibitory to nitrification than others.

Key Words—Allelopathy, *Helianthus annuus*, nitrification inhibition, biological control.

INTRODUCTION

The nitrification process is considered important to the regulation of nitrogen leaching in soils. Such leaching may severely reduce the productivity of plants and cause great losses in the energy requirement for growing crops (Alexander, 1977; Rice, 1984). Moreover, it is reported to increase water pollution and disease incidence in plants (Huber et al., 1977; Alexander, 1977; Rice, 1984). In attempting to enhance utilization of nitrogen fertilizers, nitrification inhibitors have been extensively used.

Inhibition of nitrification by allelopathic plant species has been reported in natural and manipulated ecosystems. Greenland (1958) found that low levels of nitrate under grassland were due to suppression of nitrification by toxic materials exuded from grass roots. Neal (1969) reported that aqueous root extracts of 12 grasses and forbs were inhibitory to the nitrifying organisms *Nitrosomonas* and *Nitrobacter*. Rice and Pancholy (1972, 1973, 1974) revealed that nitrification is significantly inhibited by climax species. The phenolic com-

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pounds identified from soil under climax vegetation were found to be inhibitory to *Nitrosomonas* and *Nitrobacter*. Lodhi (1981) indicated that the aboveground parts of corn in a corn field significantly reduced ammonium oxidation. Moore and Waid (1971) reported that root exudates of rye grass, wheat, lettuce, salad rape (*Brassica napus*), and onion were inhibitory to nitrification. Root exudates of rye grass, wheat, and onion had the most pronounced and persistent inhibitory effects. Alcohol extracts of neem seeds were successfully used as nitrification inhibitors in agricultural land in India (Sahrawat and Parmar, 1975). Subsequent work on the neem plant showed that leaf residues and defatted neem seed cake can also be used as inhibitors of nitrification (Santhi et al., 1986). AlSaadawi et al. (1986) evaluated several cultivars of *Sorghum bicolor* for their ability to inhibit nitrification and found that aqueous extracts and decaying residues significantly inhibited nitrification. Some cultivars appeared more inhibitory than others.

Helianthus annuus is one of the important crops in the world. It is reported to be an allelopathic species (Wilson and Rice, 1968; Hall et al., 1982) and has considerable variations for this trait (Leather, 1982, 1983). However, most of the research activity concerning the allelopathic potential of this species or its cultivars against weeds and crops, and information concerning the allelopathic activity against nitrification, is not available. Therefore, the present work was conducted to determine the allelopathic activity of *H. annuus* against nitrification and to find out whether differences in allelopathic activity among selected cultivars exist.

METHODS AND MATERIALS

Plant and Extract Preparations. Five seeds each of *Helianthus annuus* cultivars Local, Pemir, Peredovik, Record, Citosol, and Cheremn were planted in plastic pots, each containing 7 kg of loamy soil. The pots were placed under field conditions and watered with tap water whenever necessary. At the flowering stage, plants of all test cultivars were harvested, separated into shoots and roots, and air-dried for several days under shade. The air-dried shoots and roots were ground to pass a 2-mm sieve.

Five grams of roots and tops of each cultivar were Soxhlet extracted with 500 ml methanol for 10 hr. The methanol extract of each treatment was evaporated to dryness in a flash evaporator at 50°C. The residues of each treatment were dissolved in 250 ml of distilled water to make a final concentration of 2% of the original weight of roots or shoots. The controls were prepared in the same manner except that plant materials were ommitted.

Biological Activities of Sunflower Cultivars Against Nitrification. For extract bioassays, soil with characteristics listed in Table 1 was collected to a

TABLE 1. SOME PHYSICAL AND CHEMICAL PROPERTIES OF SOIL USED FOR NITRIFICATION EXPERIMENTS

Soil property	Value[a]
pH (saturation extract)	8.5
EC (saturation extract, mS/cm at 25°C)	3.8
NH_4-N (ppm)	12.4
NO_3-N (ppm)	9.3
Organic matter (%)	0.1
$CaCO_3$ (%)	27.9
Sand (%)	39.7
Silt (%)	38.5
Clay (%)	21.8

[a] Each value is an average of three replications.

depth of 10 cm in April 1986. The soil was mixed thoroughly, air-dried under shade, and passed through a 2-mm sieve. Samples of 100 g of the air-dried soil were placed in 100-ml plastic beakers, and the beakers were preincubated at 28°C under darkness for seven days before adding the substrate $(NH_4)_2SO_4$.

After incubation, each beaker of the control treatment received 12 ml of an appropriate concentration of $(NH_4)_2SO_4$ necessary to make the nitrogen concentration added 300 ppm. Treatments were run in the same manner, except that aqueous solution of $(NH_4)_2SO_4$ was replaced by an equal amount of extract solution of roots or shoots of the test cultivars containing the same amount of $(NH_4)_2SO_4$ as was added to each control pot. All beakers were covered with perforated polyethylene sheets to allow aeration and incubated at 28°C under darkness. The experiment was arranged in a randomized complete block design with four replications.

The amounts of NH_4 nitrogen and NO_3 nitrogen were measured at 0, 4, 8, and 12 days after incubation using the MgO-Devarda alloy method (Bremner, 1965).

For residue bioassays, 1 g of powder of roots or shoots of each test cultivar was mixed with 100 g of soil and placed in a 100-ml plastic beaker. Control treatments were made by mixing 1 g of peat moss with 100 g of soil to keep the organic matter the same (AlSaadawi et al., 1985). Each beaker received 12 ml of aqueous solution containing enough $(NH_4)_2SO_4$ to make the NH_4 nitrogen added 300 ppm. The incubation conditions, experimental design, and measurements of NH_4 and NO_3 nitrogen were the same as mentioned in the previous experiment.

RESULTS

In all bioassay experiments, the amounts of NH_4 nitrogen converted in the incubated soil was significantly correlated with the amount of NO_3 nitrogen produced over the periods of incubation (Table 2). All bioassay experiments revealed significant correlation between NH_4 nitrogen converted and NO_3 nitrogen produced.

Shoot extracts of all test cultivars significantly reduced nitrification rates at all incubation periods (Table 3). However, the reduction was significantly different among the extracts of the test cultivars at the first and second periods of incubation. Shoot extracts of Cheremn cultivar had the greatest ability to reduce nitrification when compared to the shoot extracts of other cultivars. At the third period of incubation, shoot extracts of all test cultivars revealed the same potential of nitrification inhibition.

Root extracts of all test cultivars significantly reduced the nitrification rate at all incubation periods (Table 4). Root extracts of Citosol and Cehremn cultivars caused greater inhibition than those of others at all incubation periods except at four days.

Shoot residues of all cultivars significantly inhibited the nitrification rate at all incubation periods (Table 5). Shoot residues of all cultivars except Citosol revealed the same ability of nitrification inhibition at the first period of incubation. However, at the second and third incubation periods, shoot residues of some cultivars exhibited greater inhibitory action than others.

Root residues of all cultivars caused significant inhibition of nitrification at all periods of incubation. The inhibitory potential of test cultivars was the

TABLE 2. LINEAR REGRESSION EQUATIONS BETWEEN AMMONIUM CONVERTED AND NITRATE PRODUCED IN NITRIFICATION BIOASSAYS (12 OBSERVATIONS)[a]

Bioassay	Intercept (a)	Regression coefficient (b)[b]	Correlation coefficient of determination (r^2)[c]
Root extract	5.59	1.01	0.983
Shoot extract	−2.04	1.00	0.991
Root residues	6.49	0.93	0.926
Shoot residues	30.98	0.61	0.670

[a]Nitrate nitrogen produced and ammonium nitrogen converted are considered as dependent and independent variables respectively.
[b]Significant at $P \leq 0.01$ level according to t test.
[c]Significant at $P \leq 0.01$ level.

TABLE 3. EFFECT OF SHOOT EXTRACTS OF DIFFERENT SUNFLOWER CULTIVARS ON NITRIFICATION RATE[a]

Test cultivar	Nitrification rate (%) after[b]		
	4 days	8 days	12 days
Control	21.23 a	51.60 a	71.95 a
Cheremn	14.48 f	44.77 d	66.55 b
Peredovik	15.80 e	46.20 c	65.28 b
Record	18.33 b	47.25 bc	63.53 b
Citosol	16.33 cd	47.85 b	62.25 b
Local	17.63 bc	46.90 bc	65.25 b

[a] Percent nitrification $= \left(\frac{NO_3\text{-}N}{NH_4\text{-}N + NO_3\text{-}N}\right) \times 100.$

[b] Means within each incubation period followed by the same letter are not significantly different at 0.05 level according to Duncan's new multiple-range test.

same at the first and third periods of incubation (Table 6). However, at the second incubation period, Record cultivar showed the lowest inhibitory action.

When the allelopathic potential of all bioassay experiments was pooled and presented against time to reflect the allelopathic activity of each cultivar, the results were as shown in Table 7. Citosol and Local cultivars showed more

TABLE 4. EFFECT OF ROOT EXTRACTS OF DIFFERENT SUNFLOWER CULTIVARS ON NITRIFICATION RATE[a]

Test cultivar	Nitrification rate (%) after[b]		
	4 days	8 days	12 days
Control	19.87 a	57.35 a	72.05 a
Cheremn	14.90 c	46.83 c	62.75 d
Peredovik	17.08 b	51.20 b	63.87 c
Record	14.63 c	50.50 b	63.82 c
Citosol	12.73 d	45.30 d	59.00 e
Local	14.40 c	50.90 b	64.95 b

[a] Percent nitrification $= \left(\frac{NO_3\text{-}N}{NH_4\text{-}N + NO_3\text{-}N}\right) \times 100.$

[b] Means within each incubation period followed by the same letter are not significantly different at 0.05 level according to Duncan's new multiple-range test.

TABLE 5. EFFECT OF SHOOT RESIDUES OF DIFFERENT SUNFLOWER CULTIVARS ON NITRIFICATION RATE[a]

Test cultivar	Nitrification rate (%) after[a]		
	4 days	8 days	12 days
Control	26.45 a	52.60 a	64.05 a
Cheremn	19.95 c	40.97 b	53.68 bc
Peredovik	19.33 c	40.68 b	54.40 b
Record	20.63 bc	39.97 bc	52.18 cd
Citosol	21.55 b	40.80 b	51.25 d
Local	19.68 c	38.23 c	51.88 cd

[a] Percent nitrification $= \left(\frac{NO_3\text{-}N}{NH_4\text{-}N + NO_3\text{-}N}\right) \times 100.$

[b] Means within each incubation period followed by the same letter are not significantly different at 0.05 level according to Duncan's new multiple-range test.

inhibition to nitrification than the other test cultivars [b values (ppm of NO_3-N produced per day) of Citosol and Local cultivars equal 5.07 and 5.03, respectively].

The Cheremn cultivar exhibited the lowest ability to inhibit nitrification (b = 5.33). There was no appreciable difference in allelopathic activity between Peredovik and Record cultivars.

TABLE 6. EFFECT OF ROOT RESIDUES OF DIFFERENT SUNFLOWER CULTIVARS ON NITRIFICATION RATE[a]

Test cultivar	Nitrification rate (%) after[b]		
	4 days	8 days	12 days
Control	32.30 a	53.80 a	69.83 a
Cheremn	21.40 b	45.45 c	63.70 b
Peredovik	21.35 b	45.27 c	60.23 b
Record	21.45 b	48.05 b	62.53 b
Citosol	21.42 b	45.47 c	62.43 b
Local	21.87 b	46.27 bc	60.75 b

[a] Percent nitrification $= \left(\frac{NO_3\text{-}N}{NH_4\text{-}N + NO_3\text{-}N}\right) \times 100.$

[b] Means within each incubation period followed by the same letter are not significantly different at 0.05 level according to Duncan's new multiple-range test.

TABLE 7. ALLELOPATHIC POTENTIAL OF DIFFERENT SUNFLOWER CULTIVARS AGAINST NITRIFICATION (96 OBSERVATIONS)[a]

Cultivar	Intercept (a)	Regression coefficient (b)[b]	Correlation coefficient of determination (r^2)[c]
Control	1.45	5.93	0.973
Cheremn	−0.68	5.33	0.970
Peredovik	−0.25	5.26	0.973
Record	−0.05	5.23	0.966
Citosol	−0.05	5.07	0.968
Local	−0.23	5.03	0.963

[a] Nitrification and incubation periods are considered as dependent and independent variables, respectively.
[b] Significant at $P \leq 0.01$ level according to t test.
[c] Significant at $P \leq 0.01$ level.

DISCUSSION

The high correlation between ammonium converted and nitrate produced in all bioassay experiments suggests that the soil incubation method is very useful in studying nitrification under laboratory conditions. The high correlation also indicates that almost all the ammonium converted is biologically oxidized to NO_3 via bacterial nitrification. A similar observation was reported by AlSaadawi et al. (1985, 1986), who studied the allelopathic potential of sorghum cultivars against nitrification by the soil incubation method. In all bioassay experiments, 12 days were found to be enough to convert about 95% of ammonium added.

Aqueous extracts of roots and shoots of all test cultivars inhibited nitrification, indicating that the extracts have water-soluble nitrification inhibitors. This explanation is further substantiated by the decaying residues, in which the decaying roots and shoots of the cultivars revealed significant inhibition to nitrification. No attempts were made to isolate, identify, and quantify the inhibitors. However, several reports on the allelopathy of *Helianthus annuus* showed that numerous phenolic compounds occur in different parts of sunflower plants (Wilson and Rice, 1968; Hall et al., 1982). Most of these compounds were found to inhibit nitrifying bacteria and thus the nitrification process (Rice and Pancholy, 1974). It is possible that variations in the inhibitory potential among the test cultivars resulted from different amounts of inhibitor(s) in the test species. Fay and Duke (1977) evaluated the allelopathic potential of 3000 accessions of

Avena sp. germ plasm against weeds, and they found that the most allelopathic accessions contained three times the concentration of the allelopathic agent scopoletin as was present in a commercial oat cultivar.

These results suggest that *Helianthus annuus* is inhibitory to nitrification in soil, and this may help augment the nitrogen efficiency of the added fertilizer. Barnes and Putnam (1983) reported that *H. annuus* has potential as a smother crop or a cover crop in no-tillage systems. The residues of smother crops are used to reduce soil erosion, improve water retention, and control weeds when the smother crop used is highly allelopathic. This study suggests another possible advantage for no-tillage cropping in that the residues of the smother crop can also inhibit nitrification. No definite conclusion can be drawn until field evaluations are made. However, such findings should be taken into consideration since a reduction in the requirement for commercial nitrification inhibitors could be possible by using allelopathic cover crops.

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GERANIUM DEFENSIVE AGENTS
III. Structural Determination and Biosynthetic Considerations of Anacardic Acids of Geranium

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Abstract—Ozonolysis, dithioether derivatization, and EI and CI mass spectrometry were used to establish the location of the double bond in the side chain of the two major anacardic acids in geranium (*Pelargonium hortorum*) trichome exudate. The point of unsaturation was shown to be between C-5 and C-6 counting from the methyl end of the side chain, contradicting the earlier hypothesis that the olefinic bond was probably at the 9–10 position based upon expected biosynthetic considerations. The two major components are thus 6-[(*Z*)−10′-pentadecenyl]salicylic acid ($C_{22}H_{34}O_3$) and 6-[(*Z*)-12′-heptadecenyl]salicylic acid ($C_{24}H_{38}O_3$). This may indicate that the precursor of these anacardic acids is a saturated fatty acid since the location of a double bond at the 5–6 position is unusual among the unsaturated fatty acids. Capillary GLC and HPLC of the trichome exudate indicated the presence of small amounts of other anacardic acid analogs possessing such features as odd numbers of carbon atoms and saturated side chains.

Key Words—Trichome, geraniums, *Pelargonium*, defensive agents, anacardic acids, double-bond location, insect resistance.

INTRODUCTION

Previous work (Gerhold et al., 1984; Craig et al., 1986) on the mite resistance chemicals produced by geraniums indicated that the trichome exudate was a mixture of two components, namely, *o*-pentadecenyl salicylic acid (A) and *o*-heptadecenyl salicyclic acid (B) (Scheme 1). The position of the *cis* double bond

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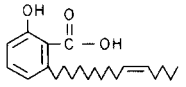
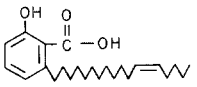
Compound A (C_{22}) 40 %



Compound B (C_{24}) 60 %



SCHEME 1.

in the side chain was not determined but was postulated to be nine carbons from the methyl end of the chain, corresponding to that of the commonly occurring unsaturated fatty acid. This study is concerned with determination of the double-bond location in both compounds and analysis of the exudate using the greater resolving power of capillary gas chromatography.

METHODS AND MATERIALS

Plant Source. Geraniums were maintained in a greenhouse environment using standard cultural practices. The geranium variety used (71-17-7) was previously determined to be mite resistant by Stark (1975).

Exudate Collection. Exudate was collected by two different methods. The first method (pressing a microscope slide against the leaf surface) was performed as described by Gerhold et al. (1984). The slide was rinsed with methylene chloride, and this extract was concentrated to a small volume and used directly for analysis. The second method used was a simple dip of the flower pedicel and sepals in methylene chloride. The sample was then treated in a manner similar to the microscope slide collection.

HPLC Separation. Preparative high-pressure liquid chromatography (HPLC) was used to isolate the individual anacardic acids. HPLC was performed using a Waters model ALC/GPC 244 with 6000A pumps, a model 480 variable wavelength UV detector, a WISP 710A automatic injector, and a data module. For preparative HPLC, a 60 cm × 10 mm C_{18} Bondapak Porasil B column (25–75 μm particle size) was used to achieve separation of up to 250 μg per injection of exudate. The solvent used was 5% 0.1 N acetic acid–95% acetonitrile mobile phase at 3 ml/min. The central two thirds of each of the peaks representing the two compounds was collected. Solvent was removed using a flash evaporator at 40°C. The sample was redissolved in acetonitrile and evaporated again several times until no odor of acetic acid could be detected. The sample was then weighed, redissolved in methylene chloride, and stored at −20°C. Chromatographic analysis of the isolated compounds on GLC and HPLC (25 cm × 4 mm C_{18} Spherisorb ODS Column, 5 μm particle size, 20% 0.1 N acetic acid–80% acetonitrile) revealed that the individual compounds isolated were >98% pure.

Capillary Chromatography. A Varian 2700A gas chromatograph equipped with a flame ionization detector was used for all analyses. For the GLC analysis and subsequent GC-MS analysis of the aliphatic aldehyde portion of the ozonolysis product, a 30 m × 0.53 mm RTX-1 (Restek Corp., Port Matilda, Pennsylvania, cross-bonded 100% dimethyl polysiloxane) column operated at 110°C with a gas flow of 10 ml/min was used. For the analysis of the underivatized compounds, the dimethylated derivatives, the dithioether derivatives, and the aromatic aldehyde portion of the ozonolysis products, a 20 m × 0.53 mm RTX-5 (Restek Corp., cross-bonded 95% dimethyl–5% diphenyl polysiloxane) column operated at a temperature program of 260–300°C with a gas flow of 10 ml/min was employed.

GLC-MS Analysis. Mass spectral analysis of the aliphatic aldehyde products of ozonolysis was performed using chemical ionization (CI) with methane as the ionizing gas. Electron impact mass spectral (EI) analysis was performed on the underivatized anacardic acids, the dimethylated derivatives, the ozonolysis-produced aldehyde containing the aromatic ring, and the dithioether derivative. All GLC-MS analyses, both EI and CI, were performed using a Finnigan model 3200 GLC-MS mass spectrometer. Probe samples were analyzed on a Kratos model MS–950.

Methylation. Methylation of both compounds was achieved by treating a small amount of the pure compound (0.1–1 mg) dissolved in a few drops of methanol with an ethereal solution of diazomethane. The reaction required overnight treatment at room temperature to methylate the phenolic moiety in agreement with Gellerman and Schlenk (1968).

Ozonolysis. Ozonolysis was performed using a microozonizer kit (Supelco, Inc., Bellefonte, Pennsylvania) for ozone generation. The reaction was carried out in an acetone–Dry Ice bath and was continued until a yellow color appeared in a KI indicator solution. Approximately 0.1–0.3 mg of methylated anacardic acid dissolved in 0.5 ml of methylene chloride was ozonized in each trial. Reduction of the ozonide to the aldehyde was achieved using a threefold excess of triphenyl phosphine (Stein and Nicolaides, 1962). Methyl oleate and palmitate were ozonized as controls to check the procedure.

Dimethyl Disulfide (DMDS) Derivatization. DMDS derivatization was performed on the methylated compounds according to the procedure of Buser et al. (1983). An aliquot of the anacardic acids (0.05–0.1 mg) was dissolved in 1.75 ml of hexane. DMDS (2.5 ml) and iodine solution (0.25 ml of a 60 mg I_2/ml diethyl ether) were added to the reaction vial, and the reaction was allowed to run overnight at ca. 40°C. Iodine was included as an initiator in the free radical addition reaction. After completion, 2.5–5 ml of sodium thiosulfate (5% solution in water) was added to the reaction mixture for removal of the iodine. The organic phase was removed and concentrated for subsequent GLC and GC-MS analysis.

RESULTS AND DISCUSSION

Chromatographic Analyses. HPLC analysis of the exudate revealed primarily compounds A and B, identified by Gerhold et al. (1984). A small broad peak (<1%) of the total material (absorbing at 208 nm) which eluted after compound B was collected and analyzed by mass spectroscopy, revealing a spectrum similar to those of compounds A and B, but apparently produced by C_{22} and C_{23} compounds with saturated side chains. Peaks for molecular ions and fragments representing losses of water and CO_2 and a base peak of 108 *m/z* are present to substantiate this conclusion. Trace amounts of other compounds also produce small "shoulder" peaks on the peaks produced by compounds A and B in both HPLC and GLC chromatograms. The presence of small amounts of compounds with minor structural differences from compounds A and B is not surprising and has been shown to occur with anacardic acids in the *Anacardiaceae* as well (Yalpani and Tyman, 1983; Tyman et al., 1984; Gellerman and Schlenk, 1968). The length of the side chain as well as the number and position of double bonds are all somewhat variable in extracts from the *Anacardiaceae*, usually showing a predominance of one or two of the variations.

GLC mass spectral results from a crude trichome collection (microscope slide method) indicated the presence of C_{22}, C_{23}, C_{24}, and C_{25} homologs with saturated side chains, as well as C_{18}, C_{23}, and C_{26} homologs with unsaturated side chains, all representing less than 1% of compounds A and B. In all probability, the other minor peaks associated with the trichome exudate represent similar modifications.

Mass Spectral Analysis. The mass spectrum of isolated compound A (EI, probe) is presented in Figure 1A. Methylation stabilizes the aromatic portion of the molecule preventing the loss of the carboxylate group during GLC (Figure 1B). Molecular ions were observed at *m/z* 374 and *m/z* 402 for A and B, and significant peaks occurred at M-31 and M-32, indicating losses of CH_3O- and CH_3OH, respectively. The mass region from 100 to 200 *m/z* is nearly identical for the two compounds A (C_{22}) and B (C_{24}). The base peak occurs at *m/z* 161 produced by loss of water from the ion produced by α-cleavage of the side chain from the aromatic ring (*m/z* 179). Substantial peaks occur at *m/z* 148 and *m/z* 121, indicating losses of $-OCH_3$ and $-\overset{\overset{O}{\|}}{C}-OCH_3$, respectively, from the *m/z* 179 fragment. These data are consistent with those reported earlier (Gerhold et al., 1984).

Mass spectral analysis of the lower-molecular-weight straight-chain aldehyde products of ozonolysis was somewhat ambiguous. Although a large amount of pentanal was present in the reaction product, smaller amounts of hexanal and the corresponding alcohols of both compounds were present. In addition, isomers of both compounds, as well as dimers representing the condensation products of both pentanal and hexanal, were present. Reducing the time between

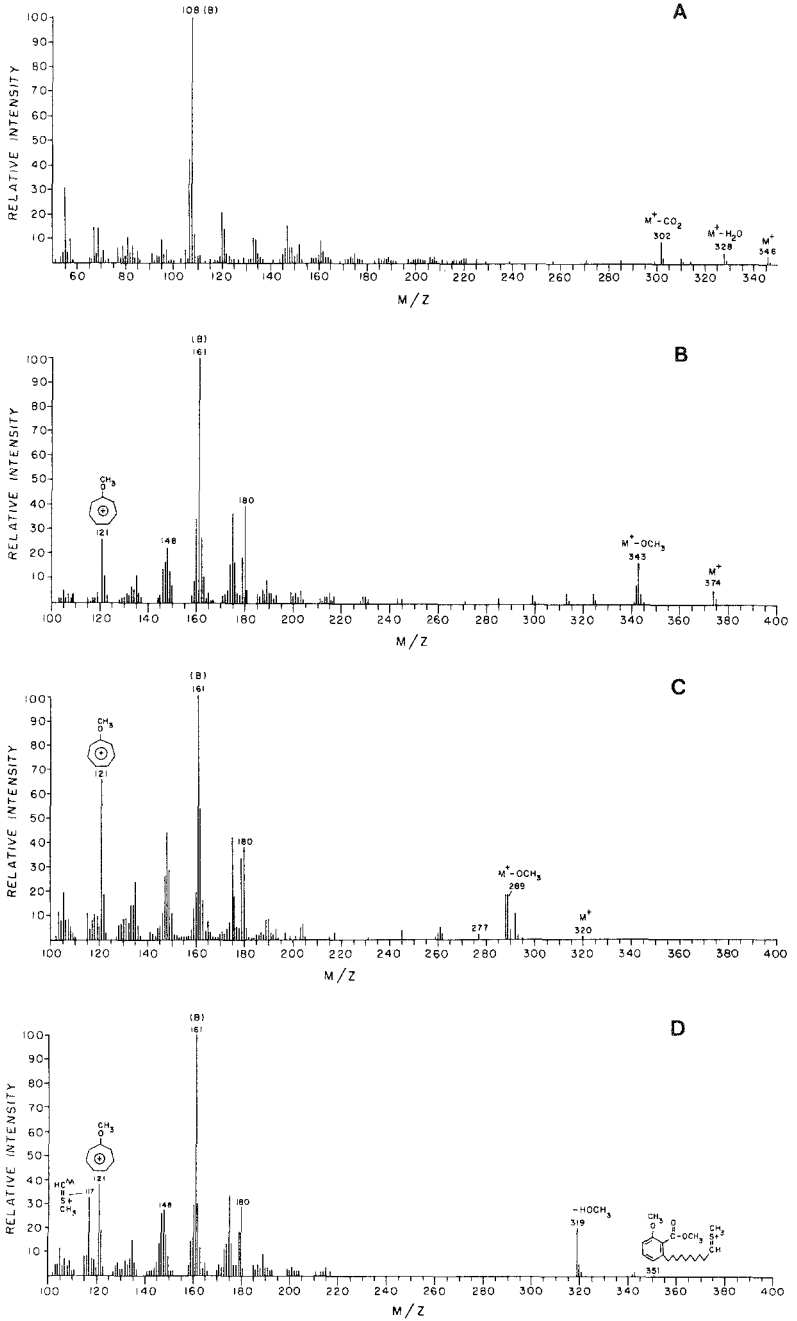


FIG. 1. EI Mass spectra of isolated and derivatized C_{22} anacardic acid from geranium (compound A); (A) isolated compound (probe); (B) methylated derivative; (C) aldehyde product possessing the chain and aromatic ring after ozonolysis of compound A; (D) dithioether derivative of compound A.

ozonolysis and mass spectral analysis resulted in a less complex product. It should be noted that ozonolysis of chromatographically pure oleic acid as a control sample also produced small amounts of C_8 and C_{10} aldehydes in addition to the expected C_9 aldehyde.

In order to resolve this ambiguity, the larger-molecular-weight aldehydes containing the aromatic portions of the molecules were analyzed by GLC-MS. GLC capillary analysis produced a single high-molecular-weight peak for each anacardic acid ozonized. GC-MS analysis then showed the aldehyde to represent a molecule with a side chain five carbon units shorter than the parent compound in both cases, implying no other positional isomers present for the double bond (Figure 1C). The mass region from 100 to 200 *m/z* remained, for practical purposes, identical to that of the methylated but unozonized compound. For compound A, a molecular ion of *m/z* 320 and significant peaks of *m/z* 288, 289, and 261 (loss of CH_3O-, CH_3OH, and $-CO-CH_3$, respectively) are all consistent with a compound having a 10-carbon straight-chain aldehyde moiety, indicating that the double bond of the parent compound occurred between carbons 10 and 11 of the side chain (counting from the ring) (Figure 1C). Results from ozonolysis of compound B are entirely analogous with corresponding peaks occurring 28 mass units higher. Thus, in B, the double bond lies between carbons 12 and 13 of the side chain.

DMDS derivatization of the methylated anacardic acids produced compounds with a methyl thioether group attached to the carbons joined by the double bond. GC analysis of the dithioether derivative of both A and B indicated a single pure product and no evidence for positional isomers of the double bond. The presence of the methyl thioether groups on adjacent carbons renders the bond between them susceptible to cleavage during mass spectral fragmentation (Buser et al., 1983), thus enabling the determination of the original double-bond location. As with the ozonolysis products, the region of the spectrum between *m/z* 100 and *m/z* 200 is essentially identical with that of the methylated compound with one notable exception (Figure 1D). That exception is the large increase of a previously very minor peak at *m/z* 117. This is the fragment expected from the cleavage of the bond between the adjacent methyl thioether groups for the instance when the bond occurs five carbons from the methyl end of the side chain. Although the molecular ion was not detected, a small peak at $M-117$ and a large peak at $M-117-CH_3OH$ were detected, providing further support for the location of the bond five carbons from the methyl end. The excellent correspondence of the ions from the lower mass end of the spectrum with that of the dimethyl ether ester before DMDS derivatization is good evidence that only the desired derivatization occurred and the aromatic portion of the molecule remains intact. Figure 2 depicts the mass spectral fragmentation of the DMDS and ozonide derivatives relative to compound A.

The results of this investigation indicate that the double-bond location pro-

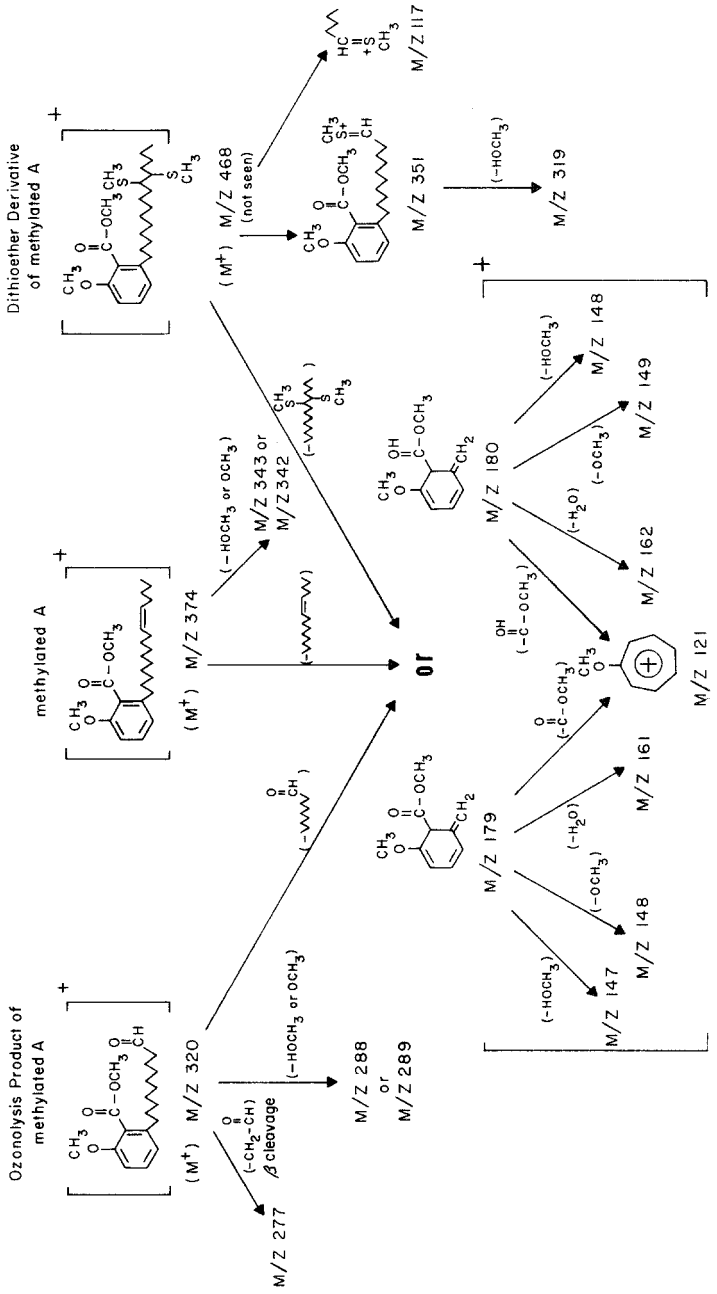


FIG. 2. Mass spectral fragmentation scheme of methylated compound A and of ozonized and dithioether-derivatized products.

posed by Gerhold et al. (1984) for the anacardic acids of geranium is incorrect. This location was based upon the premise that oleic acid and palmitoleic acid might be the precursors from which compounds A and B were synthesized. Establishment of the double-bond location occurring five carbons from the end of the side chain may be an indication that a saturated fatty acid is the precursor for these two compounds. This somewhat unusual double-bond location may provide insight into the proposed biosynthetic scheme which has been suggested to occur by way of a polyketide intermediate that undergoes cyclization via an intramolecular aldol type condensation (Geissman, 1963; Gerhold, 1984). It seems unlikely that the additional two carbon units in the side chain of compound B over that of A occur as the result of condensation and subsequent reduction of an extra acetate unit. A more likely prospect is that the anacardic acid synthetase system will accept a range of fatty acid precursors and that compounds A and B are the result of the respective processing of C_{16} and C_{18} fatty acids by the enzyme system. Desaturation of the alkenyl side chain may occur external to the anacardic acid synthetase system (the enzyme system may be specific for this double bond position). Alternatively, desaturation may be performed as part of the anacardic acid synthetase system either as the initial step in the pathway or as the final step in synthesis on the completed anacardic acid carbon skeleton. In any case, the enzyme(s) desaturates the molecules a fixed number of carbon units from the methyl end of the chain, a fairly rare occurrence since most double bonds are inserted relative to the carboxyl end of the molecule in fatty acids.

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ENNIATINS FROM *Fusarium avenaceum* ISOLATED FROM BALSAM FIR FOLIAGE AND THEIR TOXICITY TO SPRUCE BUDWORM LARVAE, *Choristoneura fumiferana* (CLEM.) (LEPIDOPTERA: TORTRICIDAE)

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Abstract—Material extracted from hyphae of *Fusarium avenaceum*, isolated from foliage of balsam fir, *Abies balsamea*, was toxic to spruce budworm larvae when incorporated into insect diet. The major insecticidal component of the toxic fraction was identified by chemical and spectroscopic methods as enniatin complex, rich in enniatin A/A_1. Possible ecological implications of these observations are considered.

Key Words—Enniatins, cyclodepsipeptides, *Fusarium avenaceum, Abies balsamea*, balsam fir, *Choristoneura fumiferana*, Lepidoptera, Tortricidae, spruce budworm, insecticide, toxin.

INTRODUCTION

The spruce budworm, *Choristoneura fumiferana* (Clem.), is one of the most destructive defoliators of the spruce–fir forests in eastern North America. Population levels of the insect in New Brunswick have been observed to oscillate, with major outbreaks followed by dramatic declines occurring in 30 to 40-year cycles (Greenbank, 1963; Royama, 1984). Royama (1984) suggested that, besides parasitoids and probably diseases, a complex of unknown causes con-

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tributed to mortality during collapse of a budworm population. Discussions among N. Whitney (Biology Department, University of New Brunswick), T. Royama, E. Eveleigh (Canadian Forestry Service—Maritimes), and one of us (D.B.S.) resulted in the suggestion that phylloplane fungi may have a role in the unknown causes of budworm mortality.

Recently a body of information has accumulated suggesting that plant–fungal associations can provide some protection to the plant against phytophagous insects (Carroll, 1986; Hinton and Bacon, 1985; Siegal et al., 1985). In New Brunswick, Miller et al. (1985) reported that balsam fir trees infested with spruce budworm had a different phylloplane fungal community than budworm-free trees, and some fungi from infested trees were toxic to larvae when ingested. It is possible that this plant–fungal association may result in adverse effects on the spruce budworm, comparable to reported cases where plants are protected from phytophagous insects by endophytic fungi (Carroll, 1986; Clay et al., 1985).

One phylloplane fungus toxic to budworm was *Fusarium avenaceum* (Miller et al., 1985). This fungus is known to be a pathogen of a wide range of plants, including conifers (Booth, 1971). It occurs mainly in the soil as a saprophyte, but it can infect the roots of susceptible plants, and it has been isolated from foliage (Miller et al., 1985). It was also reported to colonize an insect pest of grain (Miczulski and Machowicz-Stefaniak, 1977) and can grow saprophytically on budworm larvae (D.B.S., unpublished data). Other *Fusarium* species (e.g., *F. lateritium*) associated with insects produce biochemicals toxic to insects (Claydon and Grove, 1984; Grove and Pople, 1980). The purpose of this study was to investigate the biochemical basis of the toxicity of *F. avenaceum* to spruce budworm larvae.

METHODS AND MATERIALS

Instrumentation. Melting points were determined on a hot-stage apparatus and are uncorrected. Infrared spectra were recorded on a Pye Unicam PU 9516 infrared spectrophotometer, and ultraviolet spectra on a Philips PU 8800 UV/Vis spectrophotometer. Proton and carbon magnetic resonance spectra were run on a Varian XL-200 instrument. Optical rotations were measured using a Perkin Elmer 241 polarimeter. High-resolution mass spectra were provided through the courtesy of Dr. A. Hogg, University of Alberta.

Stock Cultures. Fusarium avenaceum (Corda ex Fr.) Sacc. (isolate No. 120, FA120) isolated from balsam fir foliage (Miller et al., 1985) was kept on 2% malt extract agar (MEA) slants. In an attempt to preserve the physiological traits, several slants were made from a culture subcultured only twice from the original isolate. These slants were used for subsequent culturings of the fungus.

A culture of FA120 is deposited in the herbarium at the Biosystematics Research Centre, Ottawa, Ont. (DAOM No. 196490).

Culture Conditions. FA120 was grown as described by Miller et al. (1985). A MEA stock culture was macerated in 50 ml of sterile, distilled water and 2.5 ml of the suspension was added to a 250-ml Erlenmeyer flask containing 50 ml of the following medium: NH_4Cl (3 g), $FeSO_4 \cdot 7H_2O$ (0.2 g), KH_2PO_4 (2 g), peptone (2 g), yeast extract (2 g), malt extract (2 g), and glucose (20 g) in 1-liter distilled water. Flasks were placed on a rotary shaker (220 rpm) at 28°C for two days. The resulting inoculum was homogenized and added (5%, v/v) to 1-liter Roux bottles containing 400 ml of a medium consisting of glucose (20 g), yeast extract (5 g), and peptone (5 g) per liter distilled water. Cultures were incubated at 28°C in the dark for 14 days.

Extraction of Metabolites and Isolation of Enniatins. Mycelia were harvested by vacuum filtration (Whatman No. 1 paper) and freeze-dried in preparation for extraction (2 liters culture medium yielded 16 g of hyphae, dry weight). The culture filtrate was extracted immediately. Freeze-dried hyphae (10 g) were ground in a mortar, then extracted with methylene chloride (CH_2Cl_2) (45 ml) at ambient temperature using a Polytron homogenizer. The resulting slurry was filtered at the pump on Whatman No. 1 filter paper, and the residual hyphal powder reextracted twice with 45-ml portions of CH_2Cl_2. The combined filtrates were filtered through celite, then evaporated to dryness on a rotary vacuum evaporator affording 1.659 g of extract. In an initial attempt to separate lipids from more polar material, the CH_2Cl_2 extract was partitioned between hexane (75 ml) and methanol–water (1 : 1, v/v) (100 ml). The upper layer was separated, dried over $MgSO_4$, and evaporated to yield 1.208 g of hexane extract. The lower phase was evaporated in vacuo giving 0.183 g of aqueous methanol extract.

The hexane extract, dissolved in carbon tetrachloride (CCl_4), was applied to a column of silica gel (Baker, average particle diameter 40 μm) packed in CCl_4. Material eluted with CCl_4–ether (3 : 2) was monitored by thin-layer chromatography (TLC) [silica gel plates, eluent CCl_4–ether (1 : 1), spots rendered visible with iodine vapor], and three major fractions were thus separated.

Fraction 1, $R_F \sim 0.85$, was further purified by preparative TLC on silica gel. Characterized as an unsaturated fatty acid fraction on the basis of its spectra, this material was not investigated further.

The principal component of fraction 2, $R_F \sim 0.51$, also further purified by preparative TLC on silica gel, was identified as ergosterol by comparison of melting point and spectra with those of an authentic sample.

The third fraction (502 mg), $R_F \sim 0.23$, showed ions in the mass spectrum at m/z inter alia 681.4560 (3.90%) (M^+ enniatin A), 667.4393 (4.35%) (M^+ enniatin A_1), 653.4235 (2.04%) (M^+ enniatin B_1), 228.1598 (8.41%) ($C_{12}H_{22}NO_3$), 214.1443 (2.63%) ($C_{11}H_{20}NO_3$), 100.1125 (100%) ($C_6H_{14}N$),

86.0973 (40.91%) ($C_5H_{12}N$). This fraction was crystallized from ethanol–water at −50°C. (In cases where material from the column did not appear homogenous on TLC, it was further purified by preparative TLC before recrystallization.) Colorless needles (312 mg) consisting principally of enniatin A (major component) and enniatin A_1 plus *N*-methylleucine containing enniatin(s) (minor components) were obtained. This material was toxic to spruce budworm larvae in the bioassays (Table 4, see below). The crystallized product had mp 120–121°C, $[\alpha]_D^{20}$ − 88.9° (c 1.04, $CHCl_3$); IR (KBr) 2970, 2935, 2880, 1735, 1665, 1465, 1415, 1385, 1380, 1370, 1305, 1260, 1185, 1150, 1130, 1115, 1095, and 1015 cm^{-1}; [^{1}H]NMR (200 MHz, $CDCl_3$, major peaks) δ 0.84–1.09 (36H, m), 1.26–1.44 (6H, m), 1.87 (occluded H_2O), 2.08 (3H, m), 2.28 (3H, m), 3.12 (9H, s), 4.67 (3H, d, J = 9.5 Hz), 5.13 (3H, d, J = 8.3 Hz). [^{13}C]NMR data (major peaks) and tentative assignments are presented in Table 1. Mass spectrum inter alia *m/z* 681.4568 (3.85%) ($C_{36}H_{63}N_3O_9$, M^+ enniatin A), 667.4406 (1.44%) ($C_{35}H_{61}N_3O_9$, M^+ enniatin A_1), (enniatin B <1%), 455.3116 (3.17%) ($C_{24}H_{43}N_2O_6$), 228.1596 (7.76%) ($C_{12}H_{22}NO_3$), 100.1126 (100%) ($C_6H_{14}N$), 86.0973 (10.76%) ($C_5H_{12}N$).

The enniatin complex was the major component of the aqueous methanol extract and could similarly be isolated by column chromatography and crystallization. This product, however, consistently contained a contaminant, absent in the hexane extract, which was visible as a superimposed dark spot when the TLC plate was sprayed with anisaldehyde reagent and heated (Krebs et al.,

TABLE 1. [^{13}C]NUCLEAR MAGNETIC RESONANCE DATA (MAJOR PEAKS) FOR "PURIFIED" ENNIATIN A

Carbon	Chemical shifts[a]	Multiplicity
C-1	170.31 [a]	s
C-2	61.90	d
C-3	34.00	d
C-4	25.30	t
C-5	10.97	q
C-6	16.28 [b]	q
C-7	33.15	q
C-8	169.15 [a]	s
C-9	75.61	d
C-10	29.89	d
C-11	18.68 [b]	q
C-12	18.48 [b]	q

[a]Chemical shifts in ppm relative to TMS as internal standard. Solvent $CDCl_3$. Superscript letters denote interchangeable assignments. For carbon numbering system, see structure of enniatin A in Figure 1.

1969). The contaminant, identified as ergosterol peroxide by its spectra and by comparison with an authentic sample (cf. Windaus and Brunken, 1928), could be separated from the enniatin fraction by elution with methanol through a column of Sephadex LH-20.

Hydrolysis of Recrystallized Enniatins (Mainly Enniatin A). A portion (104 mg) of the recrystallized fraction was heated under reflux with 6.1 M HCl (4.0 ml) in a nitrogen atmosphere for 20 hr. The resulting solution was evaporated to dryness in vacuo, and the residue was redissolved in water (15 ml). The solution was extracted four times with ether (10 ml each), and the combined extracts were washed with brine, dried over Na_2SO_4, and evaporated to dryness under reduced pressure. The product, essentially homogenous on TLC, was crystallized from benzene–petroleum ether (bp 35–52°C), yielding colorless crystalline material, mp 63–64°C, identified as D-2-hydroxyisovaleric acid by its optical rotation and by comparison of its solution spectra with those of an authentic sample of racemic 2-hydroxyisovaleric acid.

The aqueous solution, containing the ether-insoluble hydrochloride salts of *N*-methylamino acids, was evaporated to dryness. The residue was redissolved in the minimum volume of water, and the solution adjusted to pH 7 by addition of triethylamine. After addition of an excess of absolute ethanol, the solution was cooled to −50°C. The white precipitate that separated was collected at the pump and washed with cold ether–ethanol mixture. The product was analyzed by chromatography on cellulose plates (Brinkman Polygram cel 300 UV_{254} 0.1 mm), *tert*-amyl alcohol–acetic acid–water (20:1:20), with detection by buffered (pH 7) ninhydrin (Audhya and Russell, 1973). By comparison with standards, the amino acid products of the hydrolysis were identified, in order of increasing R_F, as *N*-methylvaline, *N*-methylisoleucine, and *N*-methylleucine. Visual inspection of the spots indicated that *N*-methylisoleucine was the predominant amino acid, and that *N*-methylvaline and *N*-methylleucine were minor products. A variety of solvent systems was tested in an attempt to distinguish the threo and erythro forms of *N*-methylisoleucine. Although marginal differences in R_F were observed using the above solvent system, the presence of both diastereoisomers in the hydrolysis mixture could not be rigorously established but may perhaps be inferred from the research of others (Audhya and Russell, 1974).

Bioassay. Hyphae, extracts from hyphae and culture filtrate, and crystalline product from FA120 were tested for toxicity to spruce budworm in an assay where larvae were reared on a meridic diet (McMorran, 1965) containing these materials. On a wet weight basis, the concentrations used were hyphae (1%), extracts (0.04%), and crystalline product (0.04% and 0.004%). The hyphae were ground to a powder and mixed well with freeze dried diet, while extracts and crystalline material were dissolved in approximately 30 ml of CH_2Cl_2, mixed thoroughly with diet, then the solvent evaporated under vacuum. In each

case 0.1 g of treated, dry diet was loaded into conical, plastic vials (8 × 38 mm inside diameter, 4.0 ml capacity) and rehydrated to 80% with sterile distilled water (0.4 ml) (A. W. Thomas, Canadian Forestry Service—Maritimes, Fredericton, New Brunswick, personal communication). Controls had diet either untreated, or treated with CH_2Cl_2 and the solvent evaporated. Laboratory stock spruce budworm larvae (second instar) from the Forest Pest Management Institute, Sault St. Marie, Ontario, were put singly into vials. The vials were capped, inverted, and incubated at 25 ± 2°C under intermittent light (16 hr light–8 hr dark) for 14 days.

After incubation, larval survival was recorded, the survivors were killed by freezing, then the instar determined by measuring head capsule width, and dry weight was recorded after freeze drying.

Fir Foliage Inoculations. Balsam fir foliage up to 3 years old was harvested from seedlings. Approximately 10 g of needles was put into a 250-ml Erlenmeyer flask with 5 ml distilled water and autoclaved. A culture of FA120 was macerated as described above and 2 ml of the inoculum suspension was added to each flask. These cultures were incubated at 25°C in the dark for eight weeks. After incubation, the cultures were extracted and checked for the presence of enniatins.

RESULTS AND DISCUSSION

Hyphae and hyphal extracts of FA120 were toxic to spruce budworm larvae when incorporated into a meridic insect diet at 1 and 0.04%, respectively (Tables 2 and 3, see below). Some toxic material was also present in the extract of the fungal culture filtrate (Table 2), but was not investigated because of the paucity of material. The biological activity was associated with a major fraction

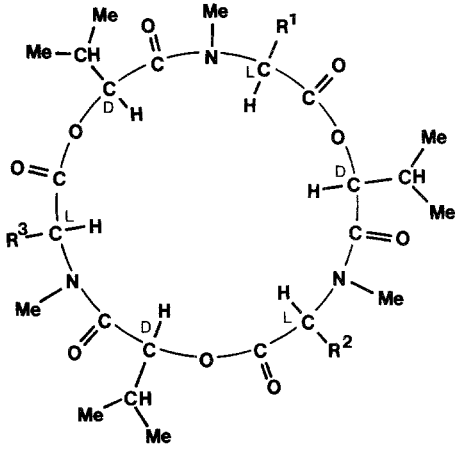
TABLE 2. PERCENTAGE SURVIVAL OF SPRUCE BUDWORM LARVAE FED HYPHAE OR CULTURE FILTRATE EXTRACT OF *Fusarium avenaceum* (ISOLATE 120)

Treatment	*N*	Survival (%)	No. of survivors to each instar			
			III	IV	V	VI
Control diet[a]	74	93	1	55	6	7
Hyphae	25	44[b]	11	0	0	0
Control CH_2Cl_2[c]	48	96	1	30	6	9
Culture filtrate extract	25	76	9	4	4	2

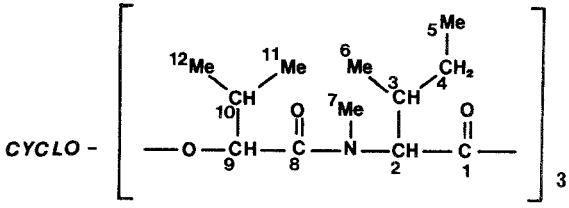
[a] Untreated diet (McMorran, 1965).
[b] Weak movements only, no development.
[c] Diet treated with methylene chloride. Hyphae fed at 1%, extracts at 0.04%.

with physical properties and spectra characteristic of the cyclodepsipeptide enniatins (Russell, 1966). Other components of the hyphal extract identified were an unsaturated fatty acid fraction, ergosterol, and ergosterol peroxide. The latter was toxic to spruce budworm cells at 10 ppm (C. Clark, Biology Department, University of New Brunswick, personal communication). Ergosterol peroxide fed to budworm larvae at concentrations up to 400 ppm had no toxic effects.

The enniatins are a group of cyclohexadepsipeptide antibiotics produced by various *Fusarium* species including *F. lateritium* and *F. sambucinum* (Cook et al., 1948; Russell, 1966). These compounds are ionophores possessing the general structure 1 (Figure 1), in which the R groups may be Pr^i, Bu^s, or Bu^i.



1



Enniatin A

FIG. 1. Structures of enniatins.

They are normally isolated as complexes which may contain enniatin A (R^1 = R^2 = R_3 = Bu^s), A_1 (R^1 = R^2 = Bu^s, R^3 = Pr^i), B_1 (R^1 = Bu^s, R^2 = R^3 = Pr^i), and B (R^1 = R^2 = R^3 = Pr^i) (Audhya and Russell, 1974). The presence of *N*-methylleucine in hydrolysates of natural enniatin mixtures suggests the existence of a series of enniatins containing this amino acid in place of one or more *N*-methylisoleucine residues (enniatin C has R^1 = R^2 = R^3 = Bu^i). While the enniatin complex has often defied attempts at separation, enniatin B has been obtained pure from a *Fusarium* species (Plattner and Nager, 1948), and enniatin A has been purified by chromatography of mycelial extracts of *F. sambucinum* (Audhya and Russell, 1974). The latter authors demonstrated that their "pure" enniatin A contained both threo and erythro diastereoisomers of *N*-methylisoleucine.

Chromatography of hyphal extracts of FA120 yielded a fraction whose mass spectrum showed molecular ions corresponding to enniatins A, A_1, and B_1, with A_1 slightly more abundant than A. After recrystallization, B_1 was no longer detected in the mass spectrum, and the relative abundance of the molecular ion of A was significantly enhanced relative to that of A_1. [Ions $(C_6H_{14}N)^+$ and $(C_5H_{12}N)^+$ derived from *N*-methylisoleucine and *N*-methylvaline residues, respectively (cf. Wulfson et al., 1964) are also diagnostic in this regard, with the caveat that *N*-methylleucine residues may also give rise to a $(C_6H_{14}N)^+$ ion. Indeed, minor peaks at 37.97(t), 23.31(q), and 21.56(q) ppm in the [^{13}C]NMR spectrum suggested the presence, in low concentration, of compound(s) containing the *N*-methylleucine residue.] Acid hydrolysis of the recrystallized enniatin mixture gave, as expected, D-2-hydroxyisovaleric acid, and a mixture of the amino acids *N*-methylisoleucine, *N*-methylvaline, and *N*-methylleucine, in which the former predominated, and the other two were minor products. It can be concluded that enniatin A (Figure 1) is the principal component of the recrystallized fraction from FA120.

To our knowledge, enniatin B (Tirunarayanan and Sirsi, 1957; Minasyan et al., 1978) and "avenacein" (mp 139°C) (Cook et al., 1948) are the only cyclohexadepsipeptide metabolites previously reported from *F. avenaceum*.

The results of bioassays showing the effects on spruce budworm larvae of FA120 hyphae, a culture filtrate extract, crude hyphal extracts, and crystalline enniatins (see above) are presented in Tables 2–4. Table 3 gives results for hexane and aqueous methanol fractions which had their origin in an initial attempt to separate the crude hyphal extracts into lipids and more polar material by solvent partition. Enniatins were present in both phases and were, in fact, the major components of the (smaller) aqueous methanol fraction. The toxicity data in Table 3 reflect differences in relative concentrations of enniatins in the lipid and polar fractions as well as a dose–response relationship that is not expected to be linear.

TABLE 3. PERCENTAGE SURVIVAL OF SPRUCE BUDWORM LARVAE FED FRACTIONS OF METHYLENE CHLORIDE EXTRACT FROM HYPHAE OF *Fusarium avenaceum* (ISOLATE 120)

Treatment	*N*	Survival (%)	No. of survivors to each instar				
			II	III	IV	V	VI
Control CH_2Cl_2[a]	75	97	4	2	36	18	13
Hexane	22	86	3	7	4	4	1
Methanol–water	25	32[b]	0	8	0	0	0
Hyphal residue	25	100	2	4	16	1	2

[a]Diet (McMorran, 1965) treated with methylene chloride.
[b]Weak movements only, no development. Extracts added at 0.04%, residue at 1%.

The recrystallized enniatin A-rich fraction showed pronounced toxicity to spruce budworm larvae at a concentration of 0.04% (400 ppm) in the diet. The toxicity was manifested as mortality or retarded development (Table 4). At the conclusion of this assay, the larvae were found on the lid or side of the rearing vial. Those that were not dead showed weak movements only, and little evi-

TABLE 4. PERCENTAGE SURVIVAL AND DRY WEIGHT OF SPRUCE BUDWORM LARVAE FED ENNIATINS AT 0.004% AND 0.04% FROM HYPHAE OF *Fusarium avenaceum* (ISOLATE 120)

Treatment	*N*	Survival (%)	No. of survivors to each instar				
			II	III	IV	V	VI
Control CH_2Cl_2[a]	50	96	1	12	16	18	1
Enniatin A/A_1 0.004%	25	100	0	6	13	6	0
Enniatin A/A_1 0.04%	26	42[b]	6	4	1	0	0

	Average dry weight (mg)/larva by instar		
	IV	V	VI
Control CH_2Cl_2	0.50 ± 0.09 (14)[c]	1.12 ± 0.50 (18)	4.92(1)
Enniatin A/A_1 0.004%	0.56 ± 0.12 (13)	1.02 ± 0.37 (6)	—[d]
Enniatin A/A_1 0.04%	ND	—	—

[a]Diet (McMorran, 1965) treated with methylene chloride.
[b]Weak movements only, no development.
[c]Number of larvae is given in parentheses.
[d]No insect.

dence of feeding was observed (food in the gut or frass in the vial), suggesting that the toxicity may be accompanied by feeding deterrent effects. Little evidence of toxicity was observed at 0.004% (40 ppm) and larval dry weight was not significantly different from that of the controls (Table 4); however, the enniatin A-rich fraction caused damage to spruce budworm cell cultures at concentrations as low as 5 ppm (C. Clark, Biology Department, University of New Brunswick, personal communication).

The enniatins, like other cyclodepsipeptides, are ionophores capable of forming complexes with alkali metal cations. Their antibiotic and other biological properties appear to be associated with their ability to affect ion transport across membranes (Shemyakin et al., 1965), and this may account for the observed toxicity to spruce budworm cells at low concentration.

While the phytotoxicity and antibiotic properties of the enniatin complex are well known, and other cyclodepsipeptides such as beauvericin (Hamill et al., 1969) and bassianolide (Kanaoka et al., 1978) from the entomopathogenic fungus *Beauveria bassiana*, are known to have insecticidal activity, only one previous report was found in which the effects of the enniatin complex on insects were investigated. Grove and Pople (1980) reported that enniatin complex and enniatin A from *F. lateritium* had moderate insecticidal activity—an order of magnitude lower than carbaryl—when injected into adult *Calliophora erythrocephala* (blowfly) and *Aedes aegypti* (mosquito) larvae. Comparison of these data with the toxicity of enniatins to spruce budworm larvae (Table 4) is complicated by differences in the test insects and in the methods of dosing and by the deterrent effects of the material on budworm feeding.

It is apparent that enniatins can affect spruce budworm larvae adversely at concentrations that might be produced by *F. avenaceum* on budworm-infested foliage, although preliminary attempts to isolate enniatins from (detached and autoclaved) foliage inoculated with the fungus were inconclusive.

Jensen (1977) suggested that production, by microorganisms like fungi, of biochemicals toxic to insects was advantageous to the fungus in habitats where it was subjected to predation by the insect. Likewise, Carroll (1986) suggested that trees harbor fungi that produce insecticidal toxins in the foliage, thereby reducing predation by insect herbivores. The results reported here may lend support to these ideas. If this is the case in the balsam fir–spruce budworm system, then the presence of *F. avenaceum*, and perhaps other phylloplane fungi, might cause budworm mortality contributing to the "unknown" death syndrome described by Royama (1984).

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ALLELOCHEMICAL RESISTANCE OF BALD CYPRESS, *Taxodium distichum*, HEARTWOOD TO THE SUBTERRANEAN TERMITE, *Coptotermes formosanus*

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Abstract—The heartwood of bald cypress, *Taxodium distichum* (L.) Rich., resisted feeding attack by the Formosan subterranean termite, *Coptotermes formosanus* Shiraki. Hexane-extracted heartwood, however, was consumed at >12 times the amount of sound heartwood eaten. A bioassay using *T. distichum* sapwood as a feeding substrate was employed to assess the antitermitic activity of successive hexane, acetone, and methanol extracts of heartwood shavings and isolates derived from the active hexane extract. Two fractions, eluted from the crude hexane extract by liquid chromatography, significantly reduced termite feeding compared to the parent extract, while a third fraction was less active than the original hexane extract. Each fraction contained one major component. All three components were structurally related diterpenes. The two most active heartwood constituents were identified by GC-MS and NMR as ferruginol and manool, while the third and least active, but most prevalent, compound in heartwood was identified as nezukol. Results of bioassays suggest that these allelochemicals act principally as feeding deterrents with accompanying termite mortality due to starvation.

Key Words—Taxodiaceae, *Taxodium distichum*, bald cypress, Isoptera, *Coptotermes formosanus*, Rhinotermitidae, Formosan subterranean termite, antitermitics, feeding deterrents, diterpenes, nezukol, ferruginol, manool.

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INTRODUCTION

Oshima (1919) first demonstrated that natural products occurring in termite-resistant woods were more essential in conferring resistance than the woods' physical qualities. Unlike cellulose and lignin, these allelochemicals do not contribute to the structural integrity of wood, are solvent extractable, and occur in relatively small amounts, usually in heartwood (Wolcott, 1953; Sandermann and Dietrichs, 1957; Bultman and Southwell, 1976). A number of diverse compounds from resistant wood species have been reported to be toxic to termites, including the acetylenic sesquiterpenoid, chamaecynone, from *Chamaecyparis pisifera* D. Don (Saeki et al., 1973); 7-methyljuglone, a naphthoquinone from *Diospyros virginiana* L. (Carter et al., 1978); and the iridoid glycoside, loganin, from *Guettarda speciosa* L. (Yaga and Kinjo, 1985). Termite repellents in wood include an unknown saponification-deactivated compound from *Eucalyptus microcorys* F. Muell. (Rudman and Gay, 1961) and 2-furfuraldehyde from *Pinus sylvestris* L. (Becker et al., 1971). Scheffrahn and Rust (1983) isolated free carboxylic acids from *Pinus lambertiana* L. wood which deter termite feeding, and Carter et al. (1981) presented evidence that some Nearctic woods contain unknown compounds that are toxic to gut symbionts of termites. Most of these and other antitermitic agents in resistant woods probably encompass several categories of activity (Becker et al., 1972; Carter and Dell, 1981).

Bald cypress, *Taxodium distichum* (L.) Rich., is a widespread North American conifer ranging from Delaware to the Florida Keys and west to Texas (Harlow and Harrar, 1969). Bald cypress heartwood has been reported to resist attack by the drywood termite, *Cryptotermes brevis* (Walker) (Wolcott, 1957), and three subterranean rhinotermitid species, including *Coptotermes formosanus* Shiraki (Smythe and Carter, 1970a). In one case, bald cypress timbers used to construct buildings in Florida remained free of damage for over three centuries, although adjoining native woods were heavily damaged by termites (Ellis, 1936). Organic extracts of bald cypress heartwood deposited onto filter paper reduced feeding and increased mortality of *Reticulitermes flavipes* (Kollar) (Carter and Smythe, 1972, 1974). The compounds mediating this antitermitic activity were not identified.

We now report the chemical nature of major antitermitic compounds isolated from *T. distichum* heartwood and their activity against the Formosan subterranean termite, *C. formosanus*.

METHODS AND MATERIALS

Wood and Termites. T. distichum heartwood used in this study was seasoned "old-growth" lumber (7–21 annual rings/cm) collected in central Florida prior to 1944 and stored under indoor conditions until this study (1986). A

portion of wood was processed into shavings, for later extraction by use of a Wiley Mill. Construction grade *T. distichum* sapwood (2–4 rings/cm), also grown in Florida, was obtained from a commercial dealer. Foraging groups from three colonies of *C. formosanus* were harvested from field traps located in Hallandale, Florida, and separated from wood and debris by published methods (Tamashiro et al., 1973; Su and Scheffrahn, 1986) over the course of this study. Freshly collected (< 1 week) workers (larvae of at least third instar, 2.9–3.8 mg/individual) and soldiers (in foraging group proportions) were used in bioassays.

Bioassays. Four feeding and mortality tests were conducted to assess the antitermitic activity of (1) sound and hexane-extracted heartwood, (2) successive solvent extracts of heartwood shavings, (3) fractions of heartwood hexane extract eluted from a silica-gel column, and (4) purified major components isolated from deterrent fractions in (3). Bald cypress sapwood was used for the feeding bioassay substrate as it was found to be one of the most preferred woods in a related study (Scheffrahn and Su, unpublished). For tests 1–3, wood slices (35 × 19 × 1.2 mm thick, ca. 500 mg) were cut so that the plane of the large surface was perpendicular to the grain. Slices were oven dried at 100°C for 2 hr, transferred to airtight vials, and weighed. In accordance to the exact weight of each slice, gravimetrically determined concentrations of test solutions were deposited by pipet to yield a 2% (wt solute/wt wood) slice retention after drying. Solution concentrations were adjusted to permit saturation of slices without runoff. Wood slices were placed on the bottom of 74-ml cylindrical ointment jars and covered with 25 cm^3 of cleaned sand and 6 ml of water. Eighty-eight workers and six, three, or five soldiers (tests 1, 2, and 3, respectively) were placed on the moist sand. Foragers from the same colony were used in bioassays 2 and 4, while termites from two different colonies were used in tests 1 and 3. Jar lids were loosely fitted and experimental units were stored in the dark at 29°C for two weeks.

Surviving workers were counted, and wood slices were brushed free of sand, redried, and reweighed to determine dry weight loss resulting from worker feeding. Survivors were transferred to fresh jars provisioned with untreated sapwood slices for an additional two weeks to observe for latent effects from exposure to test materials.

The fourth bioassay was conducted to determine activity of the major component isolated from each of three fractions of the hexane heartwood extract. Because quantities of these pure isolates were limited, a small-scale version of the original bioassay procedure was adopted. Test isolates were deposited at 1% (w/w) on 12-mm-diam × 1.2-mm-thick sapwood disks weighing ca. 55 mg. Disks were placed in the bottom of 40-ml jars to which were added 10 cm^3 sand, 2.5 ml water, and 40 *C. formosanus* workers. Wood consumption and worker mortality were assessed after three days.

Treatments in all four bioassays were replicated four times. Feeding and mortality control units contained untreated sapwood, while starvation controls containing no wood were included to determine mortality due solely to starvation. For each bioassay, wood consumption (mg) and worker mortality (%) at each time period were subjected to analysis of variance. Significant differences among means were detected by Student-Newman-Kuels test at $\alpha = 0.05$ (Steel and Torrie, 1980).

Extraction, Isolation, and Identification. Bald cypress heartwood shavings (240 g) were extracted successively with hexane, acetone, and methanol (ca. 2.7 l each) for 8 hr each using a side chamber extractor (2200 ml capacity to siphon) to yield extractives at 4.0, 1.0, and 2.7% w/w, respectively. A 780-mg portion of crude hexane extract, concentrated in vacuo, was eluted with $CHCl_3$–hexane (1 : 1) from a 2- × 40-cm glass column which was slurry packed with 50 g of silica gel 60 (70–230 mesh). Thin-layer chromatography (TLC) of fractions was performed on Kodak chromatogram sheets coated with silica gel. Sheets were developed with 20% $CHCl_3$ in hexane and visualized with iodine vapor. Similar fractions were combined to produce three major fractions. Fraction yields were measured gravimetrically by solvent evaporation of 1-ml volumes.

Gas chromatography (GC) was performed with a Tracor 540 chromatograph equipped with a flame ionization detector and a DB-5 fused silica capillary column (J & W Scientific, 30 m × 0.325 mm ID; 0.25 μm film thickness) with helium as carrier at a flow of 29 cm/sec. Oven temperature was programmed from 150°C to 260°C at 5°C/min with injection port at 200°C and detector at 300°C. Further purification of components B and C for [^{1}H]- and [^{13}C]NMR analyses was accomplished by GC with a Hewlett Packard 5890A instrument fitted with a thermal conductivity detector using helium at 40 ml/min as carrier. Glass columns, 1.8 m × 6.4 mm OD × 4 mm ID, packed with 10% Silicone GE SE-54 (100–120 mesh) and 10% Silar-10C (100–120 mesh) on Gas Chrom QII (Alltech Associates, Inc.) were temperature programmed from 170°C to 260°C at 4°C/min and from 100°C to 230°C at 5°C/min with 15 min final hold to elute components B and C, respectively. Effluents corresponding to the major peaks were collected by a 15-cm × 1.5-mm glass tube fitted to the detector effluent port with a sleeve of Teflon tubing.

[^{1}H]- and [^{13}C]NMR spectra were obtained with a JOEL FX-200 instrument at 200 and 25 MHz, respectively, in $CDCl_3$ and TMS added as internal standard. GC-mass spectrometry of the crude hexane extract was performed on a DB-5 fused-silica bonded-phase capillary column (J & W, 15 m × 0.32 mm ID) with a temperature program from 100°C to 230°C at 5°C/min interfaced with a Finnigan 4021 MS system. EI spectra were obtained with a source temperature of 250°C and an electron energy of 70 eV. CI spectra were obtained with a reagent gas (methane or isobutane) at a pressure of 0.2 torr, a source

temperature of 150°C, and an electron energy of 70 eV. MS of ferruginol was obtained with a VG ZAB-1FHF mass spectrometer by direct probe at 70 eV.

RESULTS

C. formosanus workers consumed over 12-fold more of the hexane extracted *T. distichum* heartwood slices than sound heartwood (Table 1). Sapwood slices were eaten only slightly more (ca. 15%) than extracted heartwood. Nevertheless, this difference was significant. Mortality was significantly higher after the two-week exposure and two-week postexposure period for workers exposed to intact heartwood. When termites were exposed to sapwood slices treated at 2% with successive solvent extracts of heartwood shavings, only the hexane extract significantly reduced feeding below control levels, while acetone and methanol extractives had no effect on wood consumption (Table 2). Mortality on the hexane treatments was intermediate between the starved termites and the other treatments at two weeks. Both feeding and survival on hexane extract treatments were lower than levels on other extracts or control treatments even after returning the termites to untreated sapwood substrate for two weeks. The combined results in Tables 1 and 2 indicated that the antitermitic compounds in bald cypress heartwood could be extracted with, and retain activity in, hexane.

Three major fractions in the elution order A, B, and C were obtained by silica-gel chromatography of the hexane extract in yields of 40, 4.7, and 4.8%, respectively, of the total crude solids. Bioassay results of these fractions, an ethyl acetate–methanol column strip, and the original hexane extract are given in Table 3. Fractions B and C reduced feeding at levels significantly below their parent extract. Fraction A and the strip elution were significantly less active

TABLE 1. WOOD CONSUMPTION BY AND MORTALITY OF *C. formosanus* WORKERS EXPOSED 2 WEEKS TO *T. distichum* WOOD[a]

Wood condition	Consumption (mg) 2 weeks	Mortality (%) 2 weeks	Mortality (%) 4 weeks[b]
Heartwood	16.3 a	13.9 a	33.5 a
Hexane Extracted Heartwood[c]	206.5 b	8.8 b	17.9 b
Sapwood	234.8 c	8.8 b	16.4 b

[a] Means ($N = 4$) followed by same letter in each column not significantly different ($\alpha = 0.05$, SNK test).
[b] Termites transferred to untreated sapwood at two weeks.
[c] Slices refluxed 6 hr in 500 ml hexane.

TABLE 2. WOOD CONSUMPTION BY AND MORTALITY OF *C. formosanus* WORKERS EXPOSED 2 WEEKS TO SUCCESSIVE EXTRACTS OF *T. distichum* HEARTWOOD[a]

Extract 2% w/w	Consumption (mg)		Mortality (%)	
	2 weeks	4 weeks[b]	2 weeks	4 weeks[b]
Hexane	56.5 a	56.2 a	10.2 ab	26.4 ab
Acetone	153.5 b	92.0 b	5.7 b	17.9 bc
Methanol	181.5 b	89.7 b	4.8 b	15.6 c
Control	153.7 b	84.7 b	4.0 b	17.6 bc
Starvation	—	—	15.1 a	34.1 a

[a]Means ($N = 4$) followed by same letter in each column not significantly different ($\alpha = 0.05$, SNK test).
[b]Termites transferred to untreated sapwood at two weeks.

than the original extract. None of the deposited fractions induced mortality levels beyond those of the unfed termites either immediately after the two-week exposure or two weeks thereafter when termites were transferred to untreated sapwood slices.

GC and TLC analyses of fractions A–C indicated that each contained a single major component also designated A, B, and C. Respective TLC R_f values were 0.68, 0.28, and 0.36, and GC retention times were 15.5, 19.3, and 14.2 min with purities (based on percent area) of 99, 89, and 79%, respectively.

TABLE 3. WOOD CONSUMPTION BY AND MORTALITY OF *C. formosanus* WORKERS EXPOSED 2 WEEKS TO COLUMN FRACTIONS OF HEXANE EXTRACT OF *T. distichum* HEARTWOOD[a]

Fraction 2% w/w	Consumption (mg) 2 weeks	Mortality (%)	
		2 weeks	4 weeks[b]
A	84.5 c	6.5 bc	21.6 b
B	18.3 a	8.2 c	31.8 bc
C	22.9 a	5.7 abc	29.0 bc
EtOAc/MeOH strip	87.1 c	3.4 ab	9.6 a
Crude Hexane Extract	60.9 b	3.7 ab	26.4 bc
Control	125.4 d	2.0 a	9.1 a
Starvation	—	8.5 c	36.6 c

[a]Means ($N = 4$) followed by same letter in each column not significantly different ($\alpha = 0.05$, SNK test).
[b]Termites transferred to untreated sapwood at two weeks.

GC-MS (EI and CI) spectra revealed the molecular weight of component A to be 290. Identification was established by [^{1}H]- and [^{13}C]NMR, as well as by its melting point (42–43 °C). The spectral data (within ± 0.08 ppm) and melting point of A agreed with literature values (Corbett and Smith, 1967; Rao et al., 1982) for nezukol (8β-hydroxysandaracopimar-15-ene) (Figure 1A). Spectrometric data for component A (nezukol) were as follows: EI-MS, *m/z* 290 (M^+, $C_{20}H_{34}O$, 12%), 275 (M − CH_3, 75), 272 (M − H_2O, 9), 257 (M − CH_3 − H_2O, 20), 247 (2), 221 (5), 205 (10), 193 (9), 179 (26), 165 (10), 149 (14), 137 (20), 136 (23), 123 (27), 121 (23), 109 (48), 95 (51), 81 (65), 69 (65), 55 (88), 43 (58), 41 (100); [^{1}H]NMR (200 MHz, $CDCl_3$) δ 0.84 (s, 3H, methyl), 0.86 (s, 3H, methyl), 0.99 (s, 3H, methyl), 1.21 (s, 3H, methyl), 4.78 (d, 1H, J_{cis} = 9 Hz, vinyl, 4.85 (d, 1H, J_{trans} = 18 Hz, vinyl), 5.73 ppm (dd, 1H, J_{trans} = 18 Hz, J_{cis} = 9 Hz, vinyl); [^{13}C]NMR (25 MHz; $CDCl_3$) δ 15.6, 17.0, 17.8, 18.4, 21.6, 24.3, 33.3, 33.5, 36.4, 37.2, 38.1, 39.4, 42.1, 43.5, 51.5, 56.6, 57.0, 72.5, 108.5, 151.6 ppm. Multiplicities were determined by the INEPT experiment (Morris and Freeman, 1978) and agree with assignments made by Rao et al. (1982).

Component B was identified by EI-MS and [^{1}H]NMR as ferruginol (Figure 1B) based on published spectral values (Wenkert et al., 1976; Dev. et al., 1985) and proton coupling experiments. The [^{13}C]NMR data were also consistent with the ferruginol structure. Spectrometric data for component B (ferruginol) were as follows: EIMS, *m/z* 287 (M + 1, 23), 286 (M^+, $C_{20}H_{30}O$, 100), 272 (14), 271 (67), 229 (12), 215 (11), 201 (32), 189 (35), 187 (13), 175 (40), 163 (12), 159 (10), 149 (19), 147 (14), 137 (11), 109 (11), 95 (10), 83 (13), 81 (10), 69 (53), 57 (14), 55 (13), 43 (18), 41 (10); [^{1}H]NMR (200 MHz, $CDCl_3$) δ 0.91 (s, 3H, methyl), 0.94 (s, 3H, methyl), 1.2 (d, 6H, methyls), 2.84 (m, 2H, $-CH_2$-7), 3.11 (m, 1H, isopropyl−CH−Ar), 4.62 (s, 1H, −OH), 6.63 [s, 1H, H−Ar (C-14)], 6.83 ppm [s, 1H, H−Ar (C-12)]; [^{13}C]NMR (25 MHz; $CDCl_3$) δ 19.3, 21.6, 22.6, 22.7, 24.8, 26.8, 29.7, 29.7, 33.3, 33.4, 37.5, 38.9, 41.7, 50.4, 111.0, 126.6, 127.3, 131.4, 148.7, 150.7 ppm.

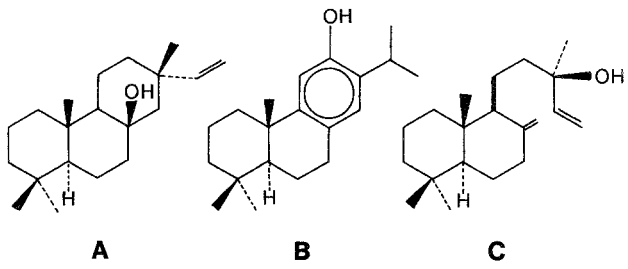

FIG. 1. Major components in the hexane extract of *T. distichum* heartwood: nezukol (A), ferruginol (B), and manool (C).

Component C was identified by GC-MS (EI and CI) as manool (Figure 1C), which was further supported by comparison of [^{13}C]NMR data of the purified material with literature values (Almqvist et al., 1975). Spectrometric data for component C (manool, $C_{20}H_{34}O$) were as follows: CI-MS (CH_4), *m/z* 331, 319, 291 (M + 1); EIMS, *m/z* 272 (M - H_2O, 3), 257 (M - H_2O − CH_3, 27), 244 (3), 229 (2), 204 (7), 203 (7), 189 (14), 177 (16), 175 (12), 161 (12), 148 (10), 147 (10), 137 (93), 123 (39), 121 (34), 109 (40), 107 (43), 95 (89), 93 (58), 81 (96), 79 (48), 71 (52), 69 (66), 55 (77), 43 (93), 41 (100); [^{1}H]NMR (25 MHz; $CDCl_3$) δ 0.68 (s, 3H, methyl), 0.80 (s, 3H, methyl), 0.87 (s, 3H, methyl), 1.28 (s, 3H, methyl), 4.47 (bs, 1H, vinyl), 4.80 (bs, 1H, vinyl), 5.06 (d, 1H, vinyl), 5.21 (d, 1H, vinyl), 5.92 ppm (dd, 1H, vinyl); [^{13}C]NMR (25 MHz; $CDCl_3$) δ 14.5, 17.7, 19.4, 21.7, 24.5, 28.0, 33.6, 33.6, 38.4, 39.1, 39.9, 41.4, 42.2, 55.6, 57.3, 73.7, 106.3, 111.6, 145.2, 148.8 ppm.

Results of bioassays with the pure isolates (Table 4) were in general agreement with those of the fractions from which they were derived (Table 3). Ferruginol and manool reduced feeding at an equal level of significance with only minute termite grazing evident on the wood disks. At 1% w/w, nezukol did not significantly reduce feeding below control levels. At 8% w/w, feeding on nezukol-treated disks was similar to crude extract deposits of 1%. Again, as in the initial bioassay (Table 1), termites consumed 12-times as much extracted heartwood as untreated heartwood. Because of the abbreviated bioassay period, no significant differences in mortalities for any of the treatments were observed.

TABLE 4. WOOD CONSUMPTION BY AND MORTALITY OF *C. formosanus* WORKERS EXPOSED 3 DAYS TO PURE ISOLATES OF *T. distichum* HEARTWOOD HEXANE EXTRACT AND HEARTWOOD DISKS[a]

Isolate (ww) or wood	Extracted from wood (%)	Consumption (mg)	Mortality (%)
Ferruginol 1%	0.2	2.2 a	1.7 a
Manool 1%	0.2	2.3 a	1.3 a
Nezukol		23.7 cd	0.0 a
1%	1.6		
8%	—	12.7 b	1.9 a
Crude hexane extract 1%	4.0	13.0 b	0.0 a
Heartwood	—	1.6 a	1.9 a
Hexane extracted heartwood[b]	—	19.9 c	0.6 a
Control	—	27.2 d	0.6 a
Starvation	—	—	1.3 a

[a] Means (N = 4) followed by same letter in each column not significantly different (α = 0.05, SNK test).
[b] Disks refluxed 6 hr in 70 ml hexane.

DISCUSSION

Known as "the wood eternal" (Wolcott, 1957), bald cypress heartwood has long been recognized for its durability in service. Wolcott (1950) was first to investigate the resistance of this wood to termite attack. Results from his feeding studies using *C. brevis* implied an allelochemic resistance mechanism. He observed that increased "gum" content in heartwood was related to increased resistance. Wolcott (1950) classified *T. distichum* sapwood as "very susceptible" to attack by *C. brevis*. Smythe and Carter (1969) exposed the eastern subterranean termite, *R. flavipes*, to bald cypress heartwood blocks and observed poor survival (0–22%) after eight weeks, but when provisioned with pine sawdust as an alternative food source, survivorship was high (81%). We interpret their results to indicate that termite mortality in treatments with heartwood alone was a result of starvation, and not toxicity, as was speculated by Smythe and Carter (1969). Results of further tests by Smythe and Carter (1970a,b), using *C. formosanus*, *Reticulitermes virginicus* (Banks), and *R. flavipes* varied with wood source, but overall poor survival of termites exposed to bald cypress heartwood blocks or its sawdust was evident. *C. formosanus* workers, however, did not avoid contact with heartwood sawdust in a repellency bioassay (Smythe and Carter, 1970b).

Carter and Smythe (1972, 1974) verified that allelochemicals played an important role in bald cypress resistance by using filter paper as a bioassay substrate to test heartwood extracts against *R. flavipes*. As reported in our study, they found antitermitic activity to be greatest in the nonpolar (pentane) extract. Carter and Smythe (1972, 1974) did not quantify feeding but assessed extract activity based on mortality observed after eight weeks of continuous exposure to treated filter papers. Although 70 and 100% mortality were reported by Carter and Smythe (1974) at four and eight weeks, respectively, on pentane extract deposits; 94 and 100% mortality occurred in their starvation controls. This suggests again that mortality resulted from starvation in the bald cypress extract treatments. The role of bald cypress heartwood constituents as toxicants to gut symbionts was suspected after defaunation of intestinal protozoa was observed in *R. flavipes* workers exposed to pentane extract deposits (Carter, 1976) and reduction of symbiont populations in guts of *C. formosanus* workers exposed to *T. distichum* heartwood blocks (Carter et al., 1981). Cause and effect, however, are uncertain, as defaunation of essential protozoa in *R. flavipes* can occur after one week of induced starvation (Carter, 1976).

The antitermitic activity of manool, ferruginol, and to a lesser extent, nezukol against *C. formosanus* and occurrence of these compounds in bald cypress heartwood explain the findings reported in previous studies of bald cypress heartwood–termite interactions. Our study focused on a single source of heartwood and, although we identified and tested the three major components

in the hexane extract, over 20 compounds were detected by GC in the crude extract. Heartwood extractives are known to vary quantitatively and qualitatively along a radial sector of a single bole (Rudman, 1964), among trees in one area, or among geographically isolated tree populations (Rudman and Gay, 1964). In addition to allelochemical content, the physical characteristics of wood influence, to a lesser extent, feeding behavior. Behr et al. (1972) demonstrated an inverse relationship between amount consumed by *R. flavipes* and the specific gravity and hardness of wood. Likewise, Bultman et al. (1979) observed a general trend for decreased feeding by *C. formosanus* with increased density among 42 species of wood. The density of sapwood and extracted heartwood used in our study was 0.49 and 0.59 g/cm^3, respectively. This may explain why control feeding on untreated sapwood slightly surpassed the quantities of extracted heartwood consumed (Tables 1 and 4).

It is interesting to note that fresh core samples of heartwood removed from living bald cypress trees release 2-furfuraldehyde in concentrations sufficient to retard development of silkworm, *Bombyx mori* L., larvae (Jones et al., 1981a). This compound has an inhibitory effect on enteric microorganisms in *B. mori* and thus may decrease their nutritional contribution (Jones et al., 1981b). 2-Furfuraldehyde, isolated from *P. sylvestris* wood, was found to repel the subterranean termite, *Heterotermes indicola* (Wasmann), from treated inert substrates (Becker et al., 1971). 2-Furfuraldehyde deposits evaporate from wood within several days, losing their potency. Although 2-furfuraldehyde was not detected in our well-seasoned heartwood sample, the presence of this or unknown volatile compounds in heartwood used in other studies may have additionally influenced bioassay results.

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HOST-PLANT ACCEPTANCE BY GEOGRAPHIC POPULATIONS OF THE COLORADO POTATO BEETLE, *Leptinotarsa decemlineata*
Role of Solanaceous Alkaloids as Sensory Deterrents

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Abstract—We used a detailed analysis of feeding behavior to investigate the role of solanaceous alkaloids as sensory-based feeding deterrents for the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Experiments were conducted on three geographic, host-adapted populations of beetles to determine whether evolutionary changes in host use have been accompanied by behavioral adaptations to alkaloids. Solanine and tomatine, steroidal glycoalkaloids found in two regional host plants, did not reduce leaf consumption or significantly alter behavior patterns of newly emerged beetles, including those from populations that normally will not feed on plants containing the compounds. Atropine, a tropane alkaloid found in several taxonomically related nonhost species, caused a significant increase in sampling behavior (indicating direct action on the sensory system) and reduced acceptance of treated potato leaves. We propose that variable acceptance of host plants among regional populations of *L. decemlineata* has evolved independently of adaptations to alkaloids at the sensory level. To establish that secondary compounds such as atropine influence host choice in nature, field observations are needed to confirm that beetles routinely encounter, and sample, nonhost species.

Key Words—*Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, feeding deterrents, host recognition, host-adapted populations, host races, solanine, tomatine, atropine, alkaloids, chemoreception.

INTRODUCTION

This paper describes experiments designed to clarify the role of solanaceous alkaloids as feeding deterrents for the Colorado potato beetle *Leptinotarsa*

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decemlineata (Say). An oligophagous species that feeds on the alkaloid-bearing Solanaceae, *L. decemlineata* has served as a case study in developing theories regarding the defensive function of plant secondary compounds (e.g., Fraenkel, 1959; Harborne, 1982). Although it has long been recognized that food-plant selection in *L. decemlineata* is based on a complex of stimuli (Jermy, 1961), several studies have stressed an important role for alkaloids as feeding deterrents and toxins. The alkaloids are thought to restrict the host range of the beetles and reduce feeding on "resistant" plants (Stürckow and Löw, 1961; Hsiao and Fraenkel, 1968; Hsiao, 1974; Sinden et al., 1978, 1980; Dimock and Tingey, 1985). In several papers, authors imply, or explicitly state, that alkaloids act directly on the chemosensory system to inhibit feeding. As a result, it has become generally accepted that alkaloids exert a strong influence on the host choice behavior of *L. decemlineata*. However, this conclusion is based largely on long-term feeding experiments using beetles confined on alkaloid-treated diets or leaves. Because alkaloids and other toxic compounds may reduce feeding after consumption, such methods do not demonstrate involvement by the sensory system, nor do they indicate how beetles would behave if they were free to leave an unacceptable host.

In this paper, we focused on the influence alkaloids have on initial host-plant acceptance and first-meal feeding. Newly emerged, unrestrained beetles were videotaped as they approached and contacted leaves infiltrated with alkaloids to levels equaling or exceeding those reported in the literature. When new adults first contact a plant, they perform a series of stereotyped sampling behaviors, the durations of which vary according to food-plant preference (Harrison, 1987). Our approach here was to correlate any reduction in feeding on alkaloid-treated plants with changes in sampling behavior, thereby demonstrating effects of the compounds at the sensory level.

Experiments were conducted on three geographic populations of *L. decemlineata* from North America that use different local hosts. Within these populations varying degrees of specialization in larval digestive efficiency (Hsiao, 1978) and adult food-plant preference (Harrison, 1987) have evolved. One of our objectives in this study was to determine whether these changes in adult behavior have been associated with adaptations to specific alkaloids at the sensory level. Our intent was not to provide an extensive list of foreign substances that might deter feeding, but to concentrate on a small number of ecologically meaningful compounds and ask whether they influence host acceptance among regional populations of beetles.

METHODS AND MATERIALS

Collection sites for beetles (and their dominant regional host plants) were: Benson, Arizona (*Solanum elaeagnifolium* Cav.); Edmonton, Alberta (*Solanum*

tuberosum L.); and Beltsville, Maryland (*Lycopersicon esculentum* Mill). Beetles were reared on their respective regional hosts under conditions described previously (Mitchell and Harrison, 1984). Plants were grown in a greenhouse equipped with supplemental lighting at 16 : 8 light–dark.

Experiments were conducted using newly emerged, unfed adults of both sexes. Behavior was recorded using an apparatus designed to allow videotape monitoring of unrestrained beetles (Harrison, 1987). Each beetle was allowed free access to a leaf until 3 min without feeding elapsed. This time interval is a good indicator of the end of the first meal (Harrison, unpublished data). Leaf area consumed was measured using a Licor Area Meter, model 3100 (accuracy = 1 mm^2).

Leaves were treated chemically by passive transpiration. Leaf petioles were cut with a sharp razor blade, weighed, immersed in a 5-ml test tube containing the alkaloid solution, and sealed with Parafilm. Because the alkaloids used were nonvolatile, they accumulated in leaves as transpiration occurred. From a knowledge of the percent water content of each plant species (Table 1) and the concentrations of test solutions, we determined the approximate level of alkaloid accumulating in a leaf by weight loss of the alkaloid solution. As a rule, we replaced the amount of water in each leaf with an equal amount of alkaloid solution. Therefore, we estimate that the concentration of alkaloid added to each leaf approximately equaled that of the test solution.

To determine whether alkaloids were being distributed throughout the treated leaves, several leaf petioles were placed in a solution of fluorescein, a water-soluble fluorescent dye, and periodically viewed under UV light. After 30 min, dye was visible in all parts of the leaves. Because treating leaves with alkaloids took 2–4 hr, we concluded that the test solutions were distributed evenly within the leaves.

Control leaves were placed in deionized water for the same length of time as treated leaves before being used. Control and treated leaves were interspersed randomly in time during the experiments. All alkaloids were obtained from Sigma Chemical Co. (St. Louis, Missouri).

TABLE 1. TOTAL GLYCOALKALOID (TGA) LEVELS AND PERCENT WATER CONTENT OF LEAVES FROM THREE SOLANACEOUS HOST PLANTS OF *Leptinotarsa decemlineata*

Plant	Water (%)[a]	TGA (mg/g dry wt)
Solanum tuberosum (potato)	91	5.2 (approx 0.5 mM)
Solanum elaeagnifolium	86	0.85 (approx 0.08 mM)
Lycopersicon esculentum (tomato)	88	7.8 (approx 0.8 mM)[b]

[a] Calculated by subtracting dry weight from wet weight. Dry weight determined by oven-drying ten leaves at 100°C for 24 hr.

[b] From Sinden et al. (1978); flowering plants grown at 16:8 light–dark.

Total Glycoalkaloid Determinations. In order to treat leaves to a desired total glycoalkaloid (TGA) level, natural levels of glycoalkaloid in two of the *Solanum* species used in this study were measured using techniques described by Fitzpatrick and Osman (1974), and Fitzpatrick et al. (1978) (Table 1). TGA levels for *L. esculentum* were approximated using values reported by Sinden et al. (1978).

To verify that alkaloids were accumulating in treated leaves at the desired concentrations, we also measured the TGA content of a group of alkaloid-treated leaves. With the high levels of alkaloid present in treated leaves, a precipitate formed in a separation phase resulting in a TGA value of only 70% of that predicted. However, in accuracy tests of these techniques, recovery rates of standards were highly variable and ranged from 46% to 78% (Coxon, 1984). Given a minimum 22% loss, analysis of treated leaves therefore accounted for at least 92% (= 70 + 22) of the predicted alkaloid content.

Behavioral Analysis. Definitions of the behavioral measures used are as follows (for complete descriptions, including illustrations, see Harrison, 1987):

Examine is defined as time spent by beetles on the leaf surface beginning with first leaf contact and ending with transition to *macerate*. Beetles typically palpate and move their antennae over the leaf surface during this period. This measure was included to determine whether alkaloid treatments induced changes in leaf surface properties that may have affected subsequent feeding.

Macerate is a period following *examine* during which the leaf edge was "squeezed" by repetitive movements of the mandibles. Because plant saps containing alkaloids are first encountered at this time, variations in macerating behavior can be used to investigate action of alkaloids on the gustatory system. Duration of the first (preingestive) macerating bout and frequency of macerating bouts during the test period were recorded. On preferred plants, this behavior was usually followed by feeding.

Feeding behavior was quantified by first comparing the number of beetles to reject leaves as food. For beetles that did feed, the amount consumed, feeding rate, and number of feeding sites on the leaf were measured.

Data collected as frequencies were analyzed using G tests. To compare the number of sampling and feeding sites among treatment groups using this statistic, data were separated into groups of one (the usual pattern on primary hosts) and greater than one. The remaining data were analyzed using one-way analysis of variance (ANOVA). All probabilities reported are based on directional (one-tailed) tests. Statistics were calculated with the aid of BMDP statistical software (Dixon, 1983).

Experiment 1. This experiment was designed to compare effects of three alkaloids within a single beetle population. Beetles from Alberta were individually videotaped on *S. tuberosum* (potato) leaves treated with deionized water (control), or with a 2 mM solution of solanine, tomatine, or atropine. The steroidal glycoalkaloid, solanine, is a principal alkaloid in *S. tuberosum* and many

other tuber-bearing *Solanum* species (Schreiber, 1979; Gregory et al., 1981). Tomatine, also a steroidal glycoalkaloid, occurs in relatively high concentrations in many *Lycopersicon* species (including tomato) where it is often the only alkaloid present (Roddick, 1974). Atropine, a tropane alkaloid, is found in several genera of Solanaceae (e.g., *Atropa* and *Datura*; Evans, 1979) that are not regularly used as hosts by *L. decemlineata*.

Experiment 2. Irrespective of their geographical origin, feeding by adult beetles on tomato is significantly lower than on primary *Solanum* hosts (Bongers, 1970; Harrison, 1987). In this experiment, we tested the hypothesis that reduced feeding on tomato is caused by its resident alkaloid—tomatine—acting as a sensory deterrent. Tomato-reared beetles from Maryland were randomly assigned to leaves treated with either 2 mM tomatine or water. This concentration of tomatine has been correlated with a reduction in feeding by beetles from Maryland on certain *Lycopersicon* species (Sinden et al., 1978).

Experiment 3. When reared as larvae on their regional host, *S. elaeagnifolium*, most adult beetles from Arizona will not feed on potato leaves on first contact (Harrison, 1987). In this experiment, we asked whether solanine was a factor in this initial rejection of potato. Beetles were randomly assigned to *S. elaeagnifolium* leaves treated with either 0.5 mM solanine or water. This concentration of solanine was double that normally found in greenhouse-grown potato leaves (Table 1).

RESULTS

Experiment 1. Of the three alkaloids tested, atropine had the most pronounced effect on beetles, with 40% refusing to feed despite repeated sampling (Table 2). Interestingly, beetles that fed consumed as much as those offered control leaves, although their feeding rate was somewhat (but not significantly) lower. There was also an increase over controls in the duration of the first macerating bout on atropine-treated leaves ($F = 7.94$, $df = 1$, $P < 0.01$) and a trend toward increased numbers of sampling and feeding sites. These changes in behavior indicate that atropine did act via the sensory system to inhibit feeding.

Increasing the solanine concentration in potato leaves fivefold did not influence the beetles' behavior. Leaf consumption was not reduced, and there was no increase in plant sampling.

Although first-meal feeding on tomato by beetles from Alberta is negligible (Harrison, 1987), tomatine had no significant effect on these beetles when infiltrated into potato leaves. Duration of the first macerating bout ($F = 0.18$, $df = 1$, NS, using square root transformation) and its variance ($F = 2.54$; $df = 9,9$, NS) did not differ from controls. There was no overall reduction in first-meal feeding or in feeding rate.

Experiment 2. Increasing the existing tomatine content in tomato leaves by

TABLE 2. SUMMARY OF BEHAVIORS OF BEETLES FROM ALBERTA ON ALKALOID-TREATED *S. tuberosum* LEAVES (conc = 2 mM)[a]

	Sampling			Feeding			
Treatment (N = 10)	Examine (sec)[b]	Macerate (sec)[c]	Maceration sites (N)	Reject (N)	Amount (mm^2)	Rate (mm^2/min)	Sites (N)
Control	3.3 ± 0.62	3.2 ± 0.41	1.0, 1–5	0	31 ± 2.2	6.2 ± 0.71	1.0, 1–4
Solanine	3.6 ± 0.50	2.8 ± 0.65	1.0, 1–2	0	37 ± 5.2	6.0 ± 0.75	2.0, 1–3
Tomatine	3.3 ± 0.59	4.1 ± 1.04	2.0, 1–5	0	31 ± 4.8	5.8 ± 0.49	1.5, 1–2
Atropine	3.5 ± 0.47	8.4 ± 1.95	4.0, 1–8	4	31 ± 6.1	3.9 ± 0.61	3.5, 1–4
	F = 0.06	F = 4.29[d]	G = 11.6	G = 12.5	F = 0.45	F = 1.83	G = 5.76
	df = 3,34	df = 3,36	df = 3	df = 3	df = 3,32	df = 3,32	df = 3
	P = 0.98 NS	P = 0.01	P = 0.009	P = 0.006	P = 0.72 NS	P = 0.70 NS	P = 0.12 NS

[a] Sampling data include nonfeeding beetles if present in treatment group. Values given are means ± standard errors or medians and range.
[b] Duration of examining the leaf surface prior to *macerate*.
[c] Duration of the first macerating bout.
[d] After square root transformation.

TABLE 3. SUMMARY OF BEHAVIORS OF BEETLES FROM MARYLAND ON *L. esculentum* LEAVES TREATED WITH 2 mM TOMATINE OR WATER[a]

	Sampling[b]			Feeding			
Treatment ($N = 10$)	Examine (sec)	Macerate (sec)	Maceration sites (N)	Reject (N)	Amount (mm^2)	Rate (mm^2/min)	Sites (N)
Control	1.8 ± 0.26	7.5 ± 1.6	1.0, 1–3	0	18 ± 6.2	4.6 ± 0.73	1.0, 1–2
Tomatine	3.7 ± 0.85	5.8 ± 1.1	1.0, 1–2	1	13 ± 3.5	6.2 ± 0.81	1.0, 0–3
	$F = 4.01$	$F = 0.75$	$G = 0.00$	$G = 1.44$	$F = 0.41$	$F = 2.09$	$G = 0.09$
	$df = 1,17$	$df = 1,17$	$df = 1$	$df = 1$	$df = 1,17$	$df = 1,16$	$df = 1$
	$P = 0.06$ NS	$P = 0.40$ NS	$P = 1.00$ NS	$P = 0.23$ NS	$P = 0.53$ NS	$P = 0.17$ NS	$P = 0.76$ NS

[a] Values given are means ± standard errors or medians and range.
[b] Definitions as in Table 2.

TABLE 4. SUMMARY OF BEHAVIORS OF BEETLES FROM ARIZONA ON *S. elaeagnifolium* LEAVES TREATED WITH 1 mM SOLANINE OR WATER[a]

	Sampling[b]			Feeding			
Treatment ($N = 7$)	Examine (sec)	Macerate (sec)	Maceration sites (N)	Reject (N)	Amount (mm^2)	Rate (mm^2/min)	Sites (N)
Control	2.3 ± 0.63	3.3 ± 0.42	1.0, 1–2	0	21 ± 3.8	2.4 ± 0.40	1.0, 1–2
Solanine	2.7 ± 0.27	2.6 ± 0.52	1.0, 1–1	0	24 ± 2.4	2.8 ± 0.29	1.0, 1–1
	$F = 0.17$	$F = 1.06$	$G = 1.46$	—	$F = 0.54$	$F = 0.62$	$G = 1.46$
	$df = 1,12$	$df = 1,12$	$df = 1$		$df = 1,12$	$df = 1,12$	$df = 1$
	$P = 0.68$ NS	$P = 0.32$ NS	$P = 0.23$ NS		$P = 0.48$ NS	$P = 0.44$ NS	$P = 0.23$ NS

[a] Values given are means ± standard errors or medians, range.
[b] Definitions as in Table 2.

2 mM to yield an estimated TGA level of 2.8 mM did not change the behavior of beetles from Maryland (Table 3). Having one beetle reject a tomatine-treated leaf was not unusual because two of 12 beetles tested on untreated tomato leaves in a previous experiment did the same (Harrison, 1987). Consumption of tomatine-treated leaves did not differ from controls, and there were no consistent trends in other variables measured.

Experiment 3. Sampling and feeding behavior by beetles from Arizona did not change with the addition of 0.5 mM solanine to their regional host plant *S. elaeagnifolium* (Table 4). This TGA level represents twice that normally found in potato (which the beetles largely reject) and several times the amount estimated in their regional host *S. elaeagnifolium* (Table 1).

DISCUSSION

In this study we used a direct behavioral approach to investigate the role of solanaceous alkaloids as sensory-based feeding deterrents for *L. decemlineata* adults. This function has long been implied for a number of alkaloids, including tomatine, but our findings indicate that neither solanine nor tomatine act on the sensory system to influence food-plant acceptance, even when these compounds are tested at relatively high concentrations.

Using the same experimental protocol as employed here, Harrison (1987) demonstrated that most beetles from Arizona will not feed on potato despite repeated sampling. In the present study, however, solanine did not inhibit feeding by beetles from Arizona on their regional host, *S. elaeagnifolium*. It is therefore unlikely that solanine is a cause of the initial rejection of potato by these beetles. Increasing the solanine level in potato leaves approximately fivefold also did not alter the behavior of beetles from Alberta which feed locally on potato.

Perhaps more surprising are the results obtained with tomatine. Tested at levels characteristic of "resistant" tomato varieties (Sinden et al., 1978), tomatine did not inhibit first-meal feeding by beetles from two populations. Also, there were no significant changes in sampling behavior that would indicate tomatine acted on the sensory system. In a recent electrophysiological study (Mitchell and Harrison, 1985), we found that tomatine and solanine have similar effects on chemosensory cells in adult beetles. After long applications, both compounds elicit bursting activity (first reported by Stürckow, 1959) associated with cell injury. There is no evidence of alkaloid-sensitive cells or "deterrent receptors" (Dethier, 1980; Schoonhoven, 1982) in *L. decemlineata*. Although cell injury is potentially one of the mechanisms by which alkaloids could disrupt feeding (Schoohoven, 1982; Mitchell and Sutcliffe, 1984), there is no indication that tomatine or solanine had this effect in the present study.

Our results do not necessarily contradict studies reporting inhibition of

adult feeding by tomatine. The reduced consumption reported by Stürckow and Löw (1961) and the reduction in feeding rate reported by Sinden et al. (1978) were both based on long-term feeding bioassays. A postingestive accumulation of tomatine affecting general activity levels (including feeding) may be responsible for these results. Similarly, slower growth and development by *L. decemlineata* larvae on tomatine-treated diets (Hsiao and Fraenkel, 1968; Hsiao, 1974) probably results from the compound's toxicity. This conclusion is supported by the observation that surviving larvae actually eat more tomato than potato to complete development (Latheef and Harcourt, 1972), suggesting that tomatine at nominal levels may not inhibit larval feeding via the sensory system.

It would be interesting to know if the same general conclusion applies to acetylated *Solanum* glycoalkaloids, the leptines. Sinden et al. (1986) recently reported dramatic reductions in feeding in both field and laboratory experiments when high-leptine-content individuals of *S. chacoense* were compared with low-leptine-content individuals from the same clone. Their experiments were not designed to differentiate sensory-mediated and longer-term deterrent effects of the alkaloids. In their laboratory choice experiments, adult beetles always consumed some of the high-leptine leaf disks, but within 2 hr a significant reduction in feeding could be measured. The leptines could have been acting via the sensory system, as atropine did in this study, or they could have been acting internally, following initial consumption. Unfortunately purified leptine samples were not available when the studies reported here were carried out.

Kogan (1976) has proposed that *L. decemlineata* may be physiologically adapted to steroidal alkaloids but not to tropane alkaloids, thereby allowing expansion of the beetles' host range to include tomato but not, for example, *Datura* or *Atropa* species. This hypothesis is supported by results of the present study, in that atropine (a tropane alkaloid) apparently caused a sensory-based inhibition of feeding. However, these results must be interpreted with caution. For oligophagous insects, conclusions regarding the role of deterrent compounds cannot be made from laboratory studies alone because visual and olfactory cues may be sufficient to preclude routine contact with many nonhosts in the field (Prokopy and Owens, 1983; Visser, 1986). Moreover, nonhost plants that are encountered may be rejected on the basis of leaf structure properties before plant fluids that normally contain secondary compounds are first sampled (Harrison, 1987). To establish that nonhost compounds such as atropine naturally deter feeding in *L. decemlineata*, we need to know if these compounds are routinely contacted by beetles in nature. This could be accomplished through a detailed study of the foraging behavior of adult beetles.

Recent studies have shown that the perception of host plants by *L. decemlineata* is based on an integration of complex stimulus patterns. Selective, upwind orientation to *Solanum* species is dependent on precise ratios of green-leaf volatiles which form an appropriate blend or "Gestalt" for the insects (Ma

and Visser, 1978; Visser and Avé, 1978). Food-plant acceptance involves a complex of stimuli as well, and beetles may be capable of discriminating among closely related *Solanum* species by examining the surface of their leaves (Harrison, 1987). These findings, and the results of the present study, indicated that selective host-choice behavior by beetles may occur independently of alkaloids. While acknowledging that many plant secondary compounds probably restrict host choice, either by direct action on the sensory system or by postingestive means, we hypothesize that specificity for *Solanum* hosts (Tower, 1906; Hsiao, 1981; May and Ahmad, 1983) is maintained primarily by the beetles' sensory system, which is selectively tuned to plants containing the appropriate combinations of "positive" stimuli.

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STRAWBERRY FOLIAGE HEADSPACE VAPOR COMPONENTS AT PERIODS OF SUSCEPTIBILITY AND RESISTANCE TO *Tetranychus urticae* KOCH

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Abstract—Headspace components from strawberry foliage have been isolated by nitrogen entrainment and Tenax trapping. Traps were eluted with hexane, and components were analyzed by gas chromatography–mass spectrometry. Fifteen compounds were identified by comparison with authentic standards, *trans*-2-hexenal, 1-hexanol, *trans*-2-hexen-1-ol, *cis*-3-hexen-1-ol, hexyl acetate, *cis*-3-hexenyl acetate, 6-methyl-5-hepten-2-ol, 1-octanol, 1-octen-3-ol, linalool, α-terpineol, methyl salicylate, ethyl salicylate, benzyl alcohol, and 2-phenylethanol. The relative amounts of these components were compared at flowering and after fruit harvest when plants were more resistant to the two-spotted spider mite, *Tetranychus urticae* Koch. The predominant components, *cis*-3-hexen-1-ol and its acetate, did not change markedly between the sampling periods, but methyl salicylate increased approximately 10-fold after fruit harvest. Methyl salicylate at low concentrations under bioassay conditions did not affect mite behavior. The biosynthetic relationship of this compound to other phenols which have been implicated in plant resistance is discussed.

Key Words—*Fragaria ananassa*, two-spotted spider mite, *Tetranychus urticae*, Acari, Tetranychidae, headspace compounds, methyl salicylate, seasonal resistance.

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INTRODUCTION

The twospotted spider mite (TSSM), *Tetranychus urticae* Koch, can be a serious pest for a large number of crop plants including strawberry. In early work with strawberry–TSSM interaction we observed that strawberry plants are susceptible to attack in the preflowering and flowering period (April–May) but become resistant after fruit harvest (mid- to late June) (Dabrowski et al., 1971). Volatile compounds have been implicated in plant–parasite interactions (Seigler, 1983), and mites respond to certain volatile compounds in bioassays (Rodriguez et al., 1976). One of the most recently developed methods for assaying volatiles from plants is headspace trapping. In one approach to this procedure volatiles emitted by plant material are entrained in a stream of gas flowing over foliage and are adsorbed on a porous polymer such as Tenax. After a trapping period of several hours, the volatiles are eluted from the Tenax with a solvent and separated by gas chromatography.

In the present experiments, strawberry leaves from several cultivars were bioassayed for susceptibility to mites at flowering and after fruit harvest. At the same time, fresh foliage was analyzed for the compounds present in headspace samples trapped on Tenax to determine the types of volatiles emitted. Comparisons were made between the headspace volatiles trapped during the two periods.

METHODS AND MATERIALS

Plant Material. Fully expanded leaves were harvested at flowering (late April to early May) and after fruit harvest (mid- to late June) from field-grown strawberry *Fragaria* × *ananassa* Duch. plants and used the same day for bioassay or headspace analysis.

Scanning electron micrographs were made of the top and bottom surfaces of freshly harvested leaves.

Detached Leaf Disk Bioassay. Disks (44 mm diam.) were cut from fresh strawberry leaves and placed on moist paper in Petri dishes. Tanglefoot was applied around the cut edges of each disk, and five teneral female TSSM were placed on the leaf disk surface. The dishes were placed in continuous photoperiod at 23°C for one week, after which the number of TSSM progeny were counted. There were five replications for each test conducted.

Headspace Analysis. One hundred grams of freshly harvested foliage was used for each headspace analysis. Each leaf was cut into two to three pieces and placed in a 5-liter round bottom flask which was immersed in a water bath maintained at 30°C. A stream of nitrogen (high purity, compressed) was filtered through a molecular sieve and passed over the foliage via Teflon tubing at a flow rate of 500 ml/min for 20 hr. The stream of gas containing the entrained volatiles passed from the flask into 1.5 g of Tenax (Alltech, Inc.) packed in a

glass tube attached to the flask. Prior to use, each Tenax trap was washed with 50 ml of hexane and then conditioned for 1 hr at 250°C using filtered nitrogen at 500 ml/min. After headspace vapor adsorption, the Tenax was removed from the tube and eluted with 15 ml of hexane (Burdick and Jackson Co.). The hexane eluate was concentrated to approximately 1 ml using a microstill with a Vigreux column. Aliquots of the hexane concentrate were submitted to gas chromatographic analysis as described below.

GC-MS Analysis and Quantitation. Headspace samples were separated using a 60-m × 0.32-mm Supelcowax 10 fused silica capillary column held isothermal at 60°C for 1 min and then programmed at 3°C/min to 220°C. GC-MS was carried out using a Hewlett Packard model 5985A instrument operated at 70eV. Identification of components was based on comparisons of mass spectra and cochromatography with authentic standards. Headspace component peaks were integrated electronically, and octadecane was used as an external standard for quantitation. Two headspace analyses were performed for each of the three cultivars at both sampling periods, and the average values for the peak areas were calculated.

RESULTS AND DISCUSSION

Observations in previous years of strawberry plants grown in the field have shown that mite populations build up during the preflowering and flowering period and then decline significantly after fruit harvest. Results from leaf disk bioassays utilizing field-grown plants in 1985 (Table 1) also showed that the plants were susceptible at flowering and relatively resistant to TSSM approxi-

TABLE 1. BIOASSAY DATA SHOWING NUMBER OF PROGENY SEVEN DAYS AFTER PLACING FIVE TENERAL FEMALE *T. urticae* ON STRAWBERRY LEAF DISKS

Date and plant growth stage	Cultivar		
	Sunrise	Red Chief	Scott
1985			
Flowering (4-22)	118*[a]	119*	178*
Mid-Harvest (5-16)	137*	210*	217*
Post-Harvest (6-13)	11**	19**	44**
1986			
Flowering (5-9)	221*	206*	251*
Mid-Harvest (5-27)	224*	130*	211*
Late Post-Harvest (7-1)	69**	3**	2**

[a] Means in each column followed by the same number of asterisks are not significantly different at 5% level, Duncan's multiple-range test.

mately two to three weeks after fruit harvest. This confirmed our observations with intact plants in the field. It was determined that the plants were still susceptible to mites during fruit harvest but became resistant in about two weeks postharvest (end of harvest was about June 1). When bioassays were performed in 1986 (Table 1), similar susceptibility at flowering and resistance following fruit harvest were observed. Sampling in late May during the mid-period of fruit harvest showed that the plants were still susceptible. This confirmed the 1985 data and indicated that a period of about two to four weeks following fruit harvest is critical in the development of resistance. Harvesting the fruit removes a metabolic sink for the leaf photosynthate, and this process may alter the metabolism of the plant and concurrently increase resistance. In addition, environmental factors such as temperatures, water availability, and photoperiod may also function in the development of resistance.

In some plants, leaf trichomes have been associated with the production of volatile compounds (Loomis and Croteau, 1980). The volatile 2-tridecanone is believed to be secreted by trichomes of the wild tomato (*Lycopersicon hirsutum*) and is toxic to certain insects (Williams et al., 1980). It was considered of interest to determine the trichome characteristics of the strawberry leaves used in these studies, and scanning electron micrographs indicated the presence of two types of trichomes on the cultivars (Figure 1). The most prominent was a long slender structure that narrowed to a pointed tip and did not appear to be secretory. The second was quite small in comparison but possessed a globular-

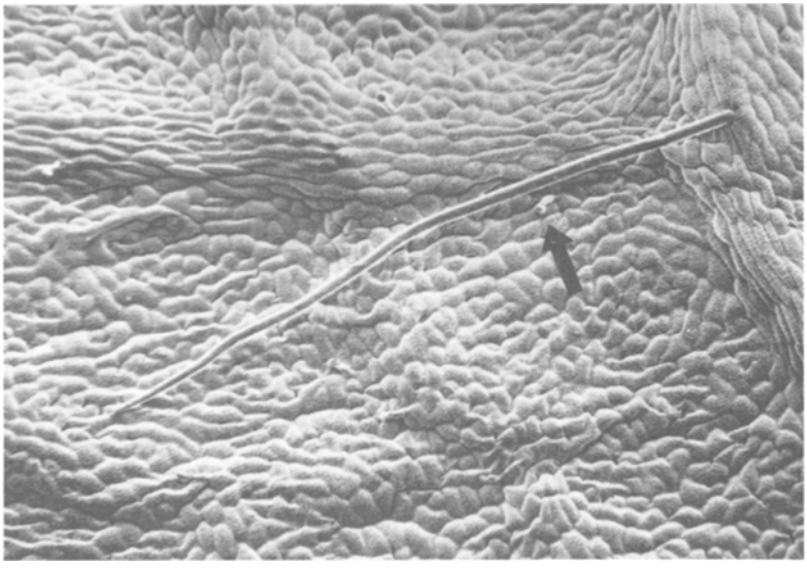

FIG. 1. Scanning electron micrograph (×150) of the lower surface of a leaf removed from a flowering plant (Scott) showing large and small (arrow) trichomes.

shaped tip that may indicate the structure was secretory. General observations of the leaves did not indicate dense populations of trichomes on the cultivars although exact counts were not done.

Table 2 lists the compounds that were identified in a headspace sample from strawberry leaves. Among these compounds were *cis*-3-hexen-1-ol and its corresponding acetate ester, which were the most abundant compounds trapped by this procedure. This result was similar to those reported for intact foliage headspace from other plants (Buttery et al., 1984, 1985). Among the headspace components with pleasant odors were the terpenoid linalool (floral odor) and methyl salicylate (wintergreen). In addition to monoterpenes, the headspace sample also contained a component believed to be a sesquiterpene (mol wt 204) with major ions in decreasing intensity at *m/e* 41, 93, 69, 55, 79, 91, 77, 107, 119, and 123. The biosynthesis and secretion of volatile and nonvolatile terpenes has been associated with trichomes in some plant species (Loomis and Croteau, 1980).

Alcohols and esters accounted for the majority of compounds identified under the trapping conditions used. The yield of strawberry headspace components was estimated at around 5–10 ppm (fresh weight basis), which was

TABLE 2. IDENTIFICATION AND RELATIVE AMOUNTS OF COMPOUNDS ENTRAINED BY NITROGEN FROM HEADSPACE OF STRAWBERRY FOLIAGE

Compound	Kovats' index[a]	Mass spectral data[b]	Peak area units[c]	
			Flowering[d]	After harvest[d]
trans-2-Hexenal	1241	41, 55, 69, 42	—	19
Hexyl acetate	1290	43, 56, 61, 84	16	20
cis-3-Hexenyl acetate	1333	43, 67, 82, 41	1960	886
1-Hexanol	1366	56, 43, 55, 42	152	140
cis-3-Hexen-1-ol	1396	41, 67, 55, 82	4820	3160
trans-2-Hexen-1-ol	1427	57, 41, 43, 82	66	22
1-Octen-3-ol	1458	57, 43, 41, 72	23	95
6-Methyl-5-hepten-2-ol	1471	41, 95, 45, 69	7	31
Linalool	1556	71, 41, 43, 93	679	394
1-Octanol	1568	41, 56, 55, 43	14	6
α-Terpineol	1722	59, 93, 43, 121	10	10
Methyl salicylate	1820	120, 92, 152, 121	26	332
Ethyl salicylate	1854	120, 92, 166, 121	3	43
Benzyl alcohol	1903	79, 108, 77, 107	125	259
2-Phenylethanol	1942	91, 92, 65, 122	139	100

[a] Supelcowax 10 column.
[b] Four most intense peaks in decreasing order of intensity above *m/e* 40.
[c] Average of two determinations.
[d] Measured at susceptible (flowering) and resistance (postharvest) periods for Scott as shown by bioassays.

greater than the yields obtained from other plants such as wheat and tobacco that were examined by this method in preliminary experiments. The compounds identified accounted for greater than 80% of the total quantity of the headspace samples trapped from the cultivar Redchief in these experiments.

In the present experiments nitrogen was used in the headspace analysis procedure to prevent oxidation of the trace quantities of compounds entrained and trapped on Tenax. Recent experiments (T.W. Kimmerer, University of Kentucky, personal communication) showed that the volatiles acetaldehyde and ethanol increased in leaves, and alcohol dehydrogenase, which reduces acetaldehyde, increased in tree roots under a nitrogen atmosphere. In future experiments, it would be interesting to compare the types of strawberry headspace components entrained in air to those in an inert gas such as nitrogen.

Interestingly, several of the compounds found in the headspace analysis, including 3-hexen-1-ol, methyl salicylate, and linalool, were also found in an earlier study when foliage was subjected to steam distillation (Kemp et al., 1968). One notable difference was that the major component found in the steam distillate, nonanal, was not identified among the headspace volatiles, although it may have been present in relatively small amounts. A similar result was observed with tobacco tissue earlier (Andersen et al., 1986). Perhaps this compound is compartmentalized and not readily released into the headspace. Also, 1,2-dihydro-1,1,6-trimethylnaphthalene, found in distillates (Stoltz et al., 1970), was not detected in the headspace samples; the low volatility of the compound compared to the other components isolated may account for this result. This rather unusual type of hydrocarbon, which is probably formed from a carotenoid, was recently reported as a component of volatiles from black tea (Mick and Schrier, 1984). In addition to studies on foliage, numerous studies have been carried out on the isolation and identification of the aroma components from strawberry fruit (Winter and Willhelm, 1964; McFadden et al., 1965; Tressl et al. 1969; Pyysalo et al., 1979). All of the compounds found in the foliage headspace have also been found in strawberry fruit.

A comparison of the quantities of the headspace components of the cultivar Scott at flowering and after fruit harvest is presented in Table 2. From these data it can be seen that some headspace components such as the C_6 components apparently decreased, while others such as 1-octen-3-ol and benzyl alcohol increased. However, one of the most marked changes occurred for methyl salicylate, which increased approximately 10-fold from flowering to postharvest and became a major component in the postharvest headspace components. These compositional changes from flowering to postharvest were found in the three cultivars tested as shown for the representative compounds in Table 3. Similar changes occurred for ethyl salicylate with increased amounts in Redchief; however, generally this compound was a minor constituent of the samples. The accumulations of the salicylate esters may reflect their increased synthesis or greater release from bound forms in plants later in the season.

TABLE 3. RATIO OF AMOUNTS OF SOME HEADSPACE VOLATILES AFTER FRUIT HARVEST TO THOSE AT FLOWERING FOR THREE CULTIVARS IN 1985

Compound	Cultivar		
	Scott	Redchief	Sunrise
3-Hexen-1-ol	0.7	1.0	1.0
1-Octen-3-ol	4.1	3.1	5.3
Linalool	0.6	0.5	0.8
Methyl salicylate	13.0	12.6	8.1
Ethyl salicylate	13.7	37.7	8.5
Benzyl alcohol	2.1	1.3	2.3
2-Phenylethanol	0.7	0.6	0.9

In a bioassay used previously to test attractancy or repellency of strawberry essential oils (Rodriguez et al., 1976), a "standard essential oil mixture" (SEOM) was utilized that contained the compounds in relative proportions of strawberry foliage essential oil. The SEOM formed the base for other test mixtures containing varied levels of other compounds such as methyl salicylate. In those bioassay tests, the SEOM containing varied levels of methyl salicylate generally attracted TSSM females at concentrations of 0.001% and repelled them at 1.0%. Currently, in preliminary tests using this bioassay but testing only methyl salicylate per se, the compound failed to elicit attractancy at the dilutions tested. It is interesting that salicylates are phenolic compounds believed to be formed through the shikimic acid pathway via benzoic acid (Klambt, 1962). Many phenolics are synthesized in plants, and frequently members of this class are repellents, feeding deterrents, and toxins and have been generally associated with plant resistance to herbivores (Levin, 1976; Feeny, 1976; Stipanovic, 1983). Nonvolatile phenols can account for a significant portion of plant tissue weight (Andersen and Todd, 1968; Feeny, 1970) and function as defense factors against herbivores. The build-up of the volatile salicylates may be accompanied by similar accumulations of nonvolatile phenols, and the possible role of this class of compounds as determinants of mite resistance of strawberries merits further study.

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RESPONSES OF MALE CODLING MOTHS (*Laspeyresia pomonella*) TO CODLEMONE AND OTHER ALCOHOLS IN A WIND TUNNEL

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Abstract—The primary pheromone (*E,E*)-8,10-dodecadien-1-ol (I, codlemone), 11 further alcohols, and binary blends of both were evaluated for attractiveness as defined by the percentage of *L. pomonella* males showing oriented upwind flight terminated by landing at the source within 10 min after release. Sources of I were attractive from 10^{-5} μg to 10^{1} μg, with approx. 70% males responding at 10^{-3}–10^{-1} μg. Most other alcohols also showed attractiveness, but the dose–response curves differed greatly with respect to the range of effective lure doses as well as the maximum response level reached at any dose. (*E,E*)-7,9-Undecadien-1-ol (III) revealed a dose–response curve similar in shape to I but shifted towards higher concentrations. (*E,E*)-8,10-Tridecadien-1-ol (II) and (*E*)-8,10-undecadien-1-ol (IV) exhibited threshold values of 10^{-5} μg (same as for I) and were effective over, respectively, seven and nine decades of source load, but they did not reach a response level of 40% at any test amount. (*E*)-9,11-Dodecadien-1-ol (VI) and the monoenes (*E*)-8-, (*E*)-10-, and (*Z*)-10-dodecen-1-ol (VIII–X) showed weak attractivity restricted to one to three test doses. Upwind approaches that broke off a few centimeters from the source were rarely seen in tests with I but frequently occurred with some of the analogs. When combined with 10^{-3} μg of I, all alcohol analogs showed "inhibitory" properties, although the amounts required to obtain a significant lowering of response differed by up to 10^{5}-fold. This amount was the lowest (10^{-4} μg) for the positional isomer VI, and the highest (10^{1} μg) for undecan-1-ol (XI) and dodecan-1-ol (XII). With some mixtures, the presence of the inhibitor appeared to cause an alteration in landing behavior. No synergistic effects were seen in these tests. The results are briefly considered with respect to the sensory perception of the test stimuli and the involvement of minor components in the female pheromone blend.

Key Words—Codling moth, *Laspeyresia pomonella*, Lepidoptera, Tortricidae, codlemone, sex pheromone, parapheromones, inhibitors, olefinic alcohols, wind tunnel, orientation flight.

INTRODUCTION

Virgin female codling moths, *Laspeyresia pomonella* (L.) (Tortricidae: Olethreutinae), release a sex pheromone whose major component has been partially identified as (*E*,*E*)-8,10-dodecadien-1-ol by Roelofs et al. (1971) and Beroza et al. (1974). The synthetic compound (codlemone) is highly attractive to conspecific males; potential minor pheromone constituents have recently been isolated from the effluvia of calling females, as will be considered further below, but synergistic effects over and above codlemone alone have not yet been demonstrated in the field. However, several investigators have reported a decrease of captures of codlemone-baited traps due to the addition of certain other chemicals. A lowering effect of codlemone stereoisomers was recognized early (Roelofs et al., 1971, 1972), although 8,10-dodecadienol samples containing less than 50% of *E*,*E* isomer still retained attractivity. Arn et al. (1974) reported a reduction of codlemone trap captures due to admixture of certain monounsaturated alcohols and acetates in a 1 : 1 ratio. Hathaway et al. (1974) and George et al. (1975) demonstrated strong inhibitory effects for several (*E*,*E*)-8,10-dodecadienyl esters and ethers; the most potent codlemone inhibitors found by these authors were the acetate and ethyl ether analogs, which in amounts of 5% of the pheromone almost abolished trap captures. The mode of behavioral action as well as sensory perception of these various attraction-modifiers remain unexplored.

We have studied behavioral and sensory responses of male codling moths to the primary attractant (codlemone), selected parapheromones (nonpheromonal compounds showing attractivity), and attraction modifiers (increasing or decreasing the response to codlemone or a parapheromone). A wind-tunnel assay was used in conjunction with field trapping tests and electrophysiological recordings from single receptor cells. The wind-tunnel setup was designed to quantify attractive as well as inhibitory or synergistic properties of test compounds, as measured by the percentage of orientation flight responses elicited from a male test population by single compounds and compound mixtures, respectively. This study describes the laboratory behavioral setup and presents results for codlemone and 11 further alcohols.

METHODS AND MATERIALS

Insects. Pupae of *L. pomonella* were obtained from Eidgenössische Forschungsanstalt, Wädenswil, Switzerland, and Bundesanstalt für Pflanzenschutz, Vienna, Austria. The two cultures originated from local wild populations and were reared on semisynthetic diets. The pupae as well as the eclosed male moths were kept under continuous light (ca. 1500 $\pm$ 10 lx) at room temperature (ca.

23°C) until the experiments, which were carried out using 43- to 73-hr-old moths. Care was taken to avoid contact of the males with female odor or synthetic pheromone prior to testing.

Chemicals. The following 12 alcohols (abbreviation and code number are in parentheses) will be treated in this study: (*E*,*E*)-8,10-dodecadien-1-ol (*E*8,*E*10–12:OH, I, codlemone); (*E*,*E*)-8,10-tridecadien-1-ol (*E*8,*E*10–13:OH, II); (*E*,*E*)-7,9-undecadien-1-ol (*E*7,*E*9–11:OH, III); (*E*)-8,10-undecadien-1-ol (*E*8,10–11:OH, IV); (*E*,*E*)-6,8-decadien-1-ol (*E*6,*E*8–10:OH, V); (*E*)-9,11-dodecadien-1-ol (*E*9,11–12:OH, VI); (*Z*)-8-dodecen-1-ol (Z8–12:OH, VII); (*E*)-8-dodecen-1-ol (*E*8–12:OH, VIII); (*Z*)-10-dodecen-1-ol (Z10–12:OH, IX); (*E*)-10-dodecen-1-ol (*E*10–12:OH, X); undecan-1-ol (11:OH, XI); and dodecan-1-ol (12:OH, XII).

Compounds I–V were generously supplied by the Laboratoire des Médiateurs Chimiques INRA/CNRS at Brouessy; VI, by the Tropical Products Institute, London; and VII–X by the Institute for Pesticide Research, Wageningen. Steric purity was 99% or better at receipt; possible isomerization was not checked continuously. The saturated analogs XI and XII were obtained commercially in purissimum quality (Fluka Chemicals, Neu-Ulm, BRD). The chemicals were serially diluted in *n*-heptane and applied onto the dispenser in amounts ranging from 10^{-6} to 10^{3} μg. All samples were stored at −18°C.

Flight Tunnel. The flight tunnel (115 × 50 × 50 cm) was of clear PVC and had a flight compartment of 80 cm. Its upwind and downwind ends were closed with a perlon screen (mesh width 2 mm); the side walls were covered with black cardboard up to a height of 35 cm, with white cardboard beyond. The floor consisted of a stationary black-and-white chessboard pattern (each black and white square 2 × 2 cm). Air was sucked through the tunnel by means of a ventilator (air speed: 0.3 m/sec, measured at the center of the flight compartment), its outlet leading outside the building. The flight tunnel was diffusely illuminated from above by means of eight 60-W bulbs with a light intensity of about 1500 lx (measured at the center of the flight compartment), dimmable down to 15 lx by means of a variable resistor.

Stimulus Application. The chemicals were applied on a flat cotton pad (weight: 3 ± 0.5 mg; diameter 1 cm^2) in 10-μl amounts from the serial dilutions. The pad was positioned in the center of a glass tube (diameter 2.5 cm) whose tapered outlet (diameter 1 cm) touched the upwind screen of the flight compartment from outside, 30 cm above the tunnel floor. The chemical(s) was actively blown into the flight compartment by means of a pump (60 liter/hr) connected with the glass tube. The difference between stimulus and wind-tunnel air velocity equalized after a few centimeters, as indicated by substituting the odor source with a smoke source (NH_4Cl) as well as by measurement with a hot-wire anemometer.

In tests for inhibitors, two identical glass tubes were connected one behind the other; the first was loaded with codlemone, the second with a test analog.

Testing Procedure. An experiment was started by placing a cotton-screen cage (10 × 8 × 8 cm) containing 10–12 males into the flight compartment at 65 cm from the upwind end of the tunnel, at the same height as the pheromone applicator. The moths were then "activated" by "artificial dusk," during which light intensity was halved, by 5-min intervals, down to about 15 lx. During these 30 min of "activation" clean air was continuously applied to the moths from the glass tube applicator. The cotton pad loaded with the test substance was then positioned within the glass tube, and after a further 30 sec the cage with the males was opened. This procedure ensured that before opening the cage, the males had already perceived the test substance(s). During the following 10 min, males landing at the source were counted; in addition, the time interval between opening the cage and landing was recorded for each moth. Landed males were sucked out from the flight compartment to avoid double counts. Males that did not "activate" (as indicated by the lack of walking movements or wing fluttering) or that were sitting on the floor of the tunnel at the end of the 10-min registration period were not taken into account. Each male was tested only once.

A test formulation that elicited no landing at the source in three replicated runs (each including 10–12 males) was not tested further. All other formulations were investigated in five runs, and mean values and standard deviations of responses were calculated for these five replications.

Between experimental series, the level of male activity and the optimal functioning of the experimental setup was verified by control tests of 10^{-3} μg of I (about 70% of responding males). After each test, glass tubes and pheromone applicators were washed with *n*-hexane and oven-dried at 100°C; clean air was sucked through the tunnel for at least 30 min before starting the next test.

RESULTS

Male Behavioral Actions. Little motor activity of the males in the release cage was noted as long as full lights were on (1500 lx). When subjected to the "artificial dusk," most males were observed walking while fanning wings and/or attempting to fly. Once synthetic pheromone (I) or parapheromone was added to the air stream, these activities were further enhanced and additional males—in most cases all—became "active."

On opening the cage, in the presence of stronger stimuli (e.g., 10^{-3}–10^{-1} μg of I), most males immediately oriented towards the odor source, whereas with weaker stimuli (e.g., 10^{-5} or 10^{-4} μg of I) most males first distributed

themselves throughout the flight compartment, outside the odor plume. However, because of frequent spontaneous flights, they accidentally restored contact with the plume and orientation flights towards the source were then induced. These differences in behavior are reflected by a different time distribution of landings at the source, as considered further below. Landings usually occurred directly on the glass tube outlet (touching the upwind screen of the flight compartment) or within 2 cm from it, both with strong and weak stimuli. In control experiments (pure air applied only), this landing place was not preferred to any other parts of the tunnel walls. However, with many pheromone–inhibitor blends, males frequently stopped their approach flight before reaching the source; the distance from the source at which the flight broke off usually increased with the dose of inhibitor. These parameters were not systematically evaluated, since very short upwind flight approaches were not surely distinguished from spontaneous flights.

Flight tracks in response to stronger stimuli usually were rather straight upwind, with cross-wind excursions not exceeding approx. 2 cm. With weaker stimuli, convoluted flight tracks with enlarged cross-wind excursions in both the horizontal and vertical plane, partly resulting in a "spiral flight," dominated. It is noteworthy that with most attractant–inhibitor blends, flights that were terminated by landing at the source were not obviously different from those with the respective amount of codlemone alone: the "inhibitor," therefore, primarily affected the number of approach flights, rather than the flight pattern itself.

Attraction Response to Codlemone. "Successful flights" (i.e., approach flights terminated by landing at the source) were induced over the range of 10^{-5}–10^{1} μg of I (Figure 1); the lowest test amount of 10^{-6} μg did not elicit any such response. On increasing the source load from 10^{-5} to 10^{-3} μg, the proportion of responding males increased to 70%. The dose–response curve then formed a plateau extending over three decades of source load; with further increase, from 10^{-1} μg up to 10^{1} μg, the percentage of successful flights strongly decreased (and may be expected to become zero at 10^{2} μg, not tested).

Except for the lowest attractive amount of 10^{-5} μg, most responding males reached the source within the first 1 min of registration (Figure 2). The high value of responses at 10^{-3}–10^{-1} μg is evidently due to upwind starts within this first 1-min interval, i.e., starts directly from the release cage shortly after opening. However, even within this plateau of the dose–response curve, the increase of lure dose from 10^{-3} to 10^{-1} μg further shifted responses towards the very beginning of the registration period; with 10^{-2} and 10^{-1} μg of I some males reached the source only 5 sec after release. (These very short response times are not apparent from the histograms because of their class interval). With lure doses of 10^{0} and 10^{1} μg, too, the few males that responded all arrived at

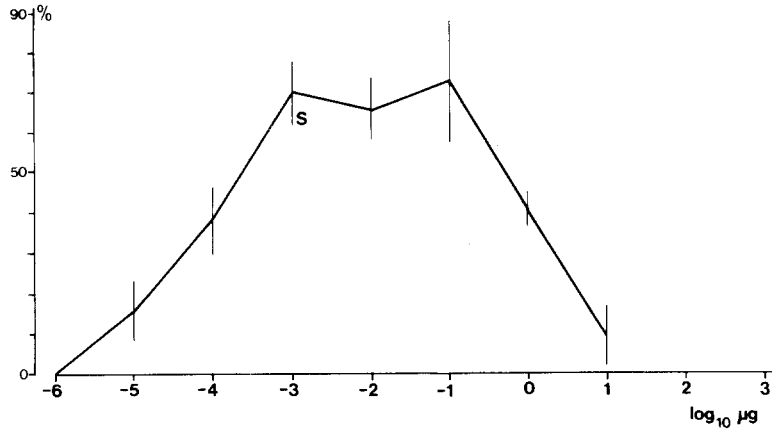

FIG. 1. Response of male *L. pomonella* towards eight different amounts of codlemone (I) in a wind tunnel. Values represent percentage (mean ± SD) of males displaying orientation flight completed by landing on the source, within 10 min of registration (S denotes standard response to 10^{-3} µg, used as the reference in tests on compound mixtures, presented in Figure 5).

the source within 1 min; the strongly reduced efficacy of these very high doses is, thus, obviously a result of the lack of responses after this interval.

Attraction Response to Analogous Alcohols. The mono- and diunsaturated alcohol analogs were tested for attractancy in the same manner as used for I. All of these, with the exception of the *Z*8 monoene (VII), elicited approach flights terminated by landing at the source. Compound VII was tested at 10^{-4} and 10^{-3} µg in eight runs; only one male (of 96 tested) displayed an approach flight, but this flight stopped 2 cm from the source. Among the other eight alcohols, the attractant power, as measured by both the threshold dose and the peak value of responding males, was highly different (Figure 3). Although some analogs (e.g., *E*8,10–11:OH, IV) showed very low threshold values of 10^{-5}–10^{-4} µg, none of these reached a peak value of 70% as measured for I.

With respect to this peak value, *E*7,*E*9–11:OH (III) was the most potent analog. This compound yielded a dose–response curve similar to that of I but shifted towards a dose about 100-fold higher and not exceeding response values of 50% at the plateau, which again extended over three decades of lure dose (Figure 3). The C_{13} homolog (II) showed a lower threshold value than found for III, differing by less than 10-fold from the codlemone (I) threshold. Attractive properties of II, as measured by the number of responding males, extended from 10^{-5} to 10^{3} µg, thus covering a broader range of effective lure dose than found with any other test compound during the present study. However, the

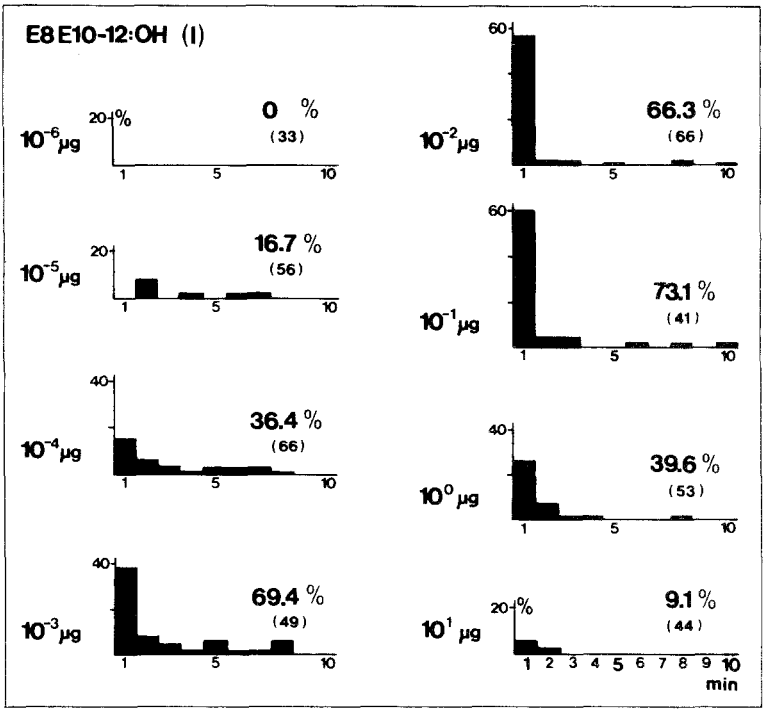

FIG. 2. Time distribution of orientation flight responses of male *L. pomonella* to codlemone (I) sources (same data as in Figure 1, specified for 1-min intervals; number of "activated" test males given in parentheses).

slope of the dose–response curve for II is much flatter compared to the dose–response curve for I or III, not forming a plateau and not reaching a response value of 40% at any test dose (Figure 3).

An even flatter dose–response curve applies to *E*8,10–11 : OH (IV); despite the very low threshold, responses to this compound did not reach a level of 20% at any test amount (Figure 3). The two other dienes, V and VI, showed response curves restricted to two or three decadic steps of stimulus amount and not exceeding a peak value of 10%. The threshold doses for these two compounds differed about 10^4-fold, thus totally separating the range of effective lure dose (Figure 3). Response curves restricted to a narrow range of lure dose were also obtained for the *Z*10 and *E*10 monoenes (IX and X). In the experiments with X, however, the number of approach flights that stopped shortly (1–5 cm) before reaching the source was strongly increased within the range of 10^{-3}–10^0 µg, exceeding by approx. sixfold the number of flights completed by

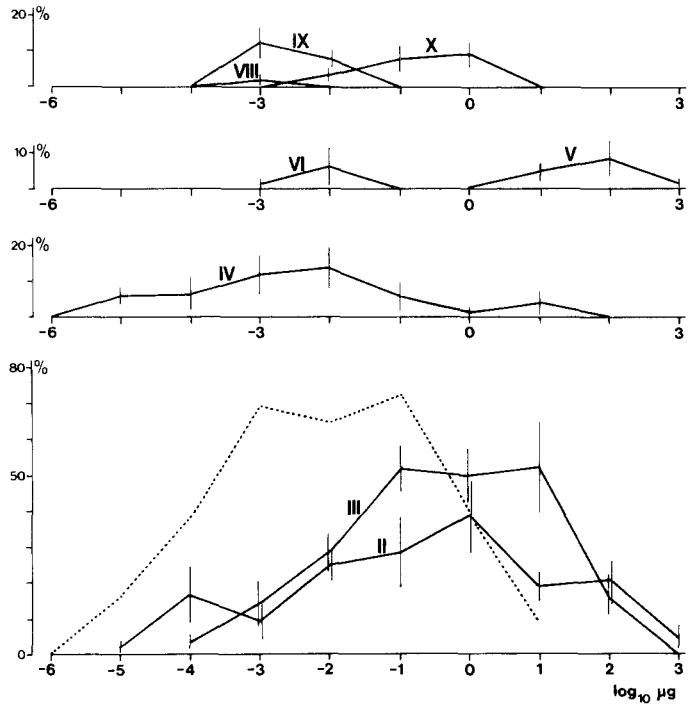

FIG. 3. Response (as in Figure 1) of male *L. pomonella* to nonpheromonal alcohols II–IX (dotted line shows response to I, taken from Figure 1).

landing at the source. These incomplete flights, however, were not counted because of the predetermined parameters for evaluation of responses. The dose–response curve for X, therefore, might not sufficiently reflect the compound's real attractivity. Finally, with the *E*8 monoene (VIII), tested in the range of 10^{-4}–10^{-2} µg, only one male arrived at the source (Figure 3). This compound is, therefore, almost as ineffective as the *Z*8 isomer (VII), mentioned above.

Time distributions of landings are exemplified in Figure 4 for two analogous compounds, *E*7,*E*9–11:OH (III) and *E*8,*E*10–13:OH (II). The dose–response curve for III is similar to that of I, as pointed out above. The data presented in Figures 2 and 4 show that the time distributions are also similar between I and III when corresponding parts of the two dose–response curves are compared. Thus, at low lure doses of III (10^{-4} and 10^{-3} µg), landings were more or less equally distributed over the 10-min period, whereas with increasing lure dose, the time interval from release until landing continuously decreased (Figure 4). In the range of the plateau of the dose–response curve, 10^{-1}–10^{1}

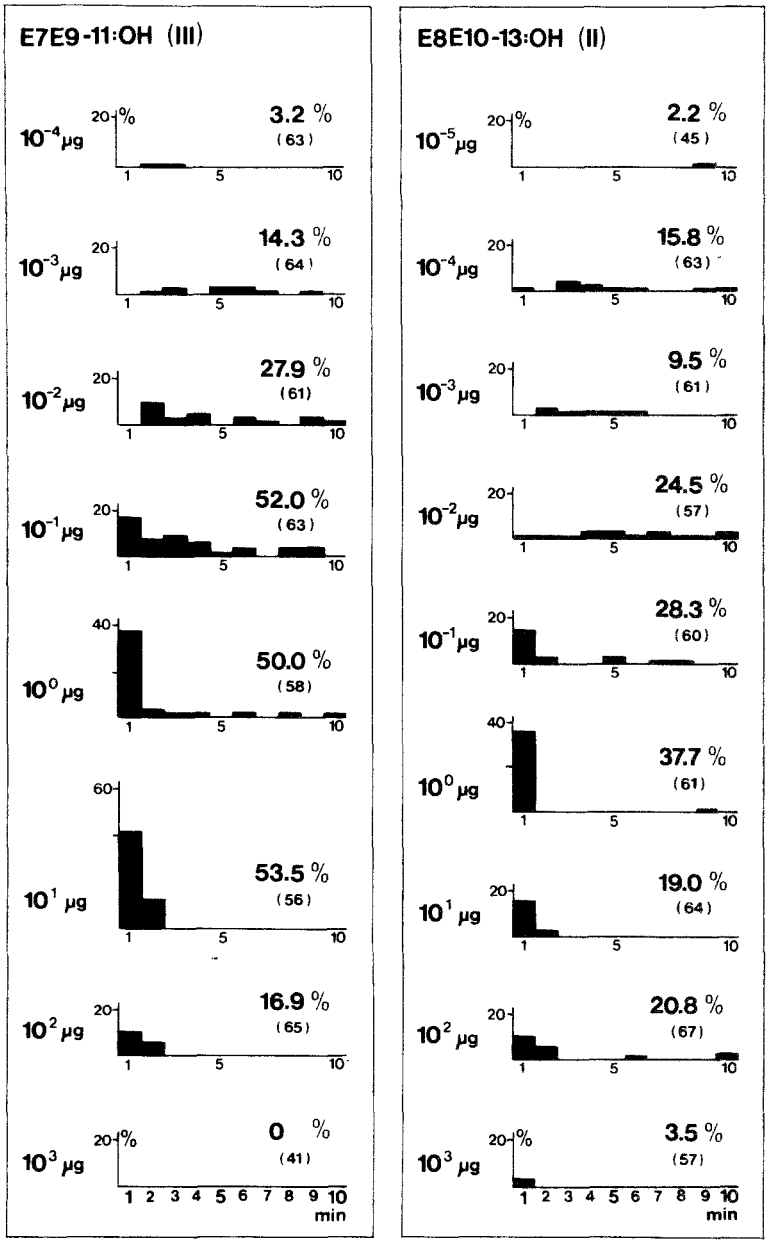

FIG. 4. Time distribution (as in Figure 2) of orientation flight responses of male *L. pomonella* to nonpheromonal alcohols III and II.

μg, responses during the first 1-min interval became dominant. This closely compares to respective parts of the codlemone (I) dose–response curve.

For compound II, which has revealed a flattened dose–response curve lacking a plateau (Figure 3), the decrease in response times with increasing lure dose is evident also (Figure 4). Thus, males preferentially arrived at the source during the first 1 min of registration at 10^{-1}–10^{3} μg of II but not at 10^{-5}–10^{-2} μg (Figure 4). Closer comparison between I and II is precluded by the different shapes of the dose–response curves, but it is noteworthy that at the peak value of II, 10^{0} μg, almost all (22 of 23 responding) males arrived within the first 1 min, although the response rate was only 37.7%. By comparison, 10^{-4} μg of I revealed a response rate of 36.4%, but more than half the arrivals occurred after the first 1-min interval (Figure 2).

In the case of compounds with only a weak attractivity, restricted to a narrow range of lure doses (compounds V, VI, IX, X), the landing responses were irregularly distributed over the 10-min period, with only a weak preference for the first 1-min interval.

Inhibitory Effects of Analogous Alcohols. The alcohols II–XII were also tested for their ability to reduce the number of oriented flights towards a source of codlemone (I) when simultaneously delivered with it into the same airstream. All binary mixtures considered here were made with 10^{-3} μg of I, the onset of the plateau of its dose–response curve (Figure 1).

All alcohols were effective in reducing responses to 10^{-3} μg of I. Among the 11 compounds, however, the threshold doses required to obtain a significant lowering in the number of responding males differed up to 10^{5}-fold (Figure 5). Furthermore, the slope of the "inhibition curves," as measured from the first significant lowering up to the total abolishment of responses, varied greatly; total inhibition required an increase of dose of less than 10^{2}-fold for some analogs (e.g., XI and XII) but more than 10^{5}-fold for others (e.g., II). With three compounds, II, III, and V, total inhibition was not obtained even on addition of 10^{3} μg (the highest stimulus amount used in this study).

The *E*9,11–12:OH (VI) was the most inhibitory alcohol. This positional isomer of the pheromone significantly reduced responses as a 10% admixture to 10^{-3} μg of I, although a further 10^{3}-fold increase of stimulus amount was required to abolish responses totally (Figure 5A). A similar flat "inhibition curve," but shifted towards 10^{4}-fold higher doses as compared to VI, was obtained for *E*8,*E*10–13:OH (II) (Figure 5A). However, as was apparent from a comparison with the "attraction curve" of this compound (Figure 3), within the range of 10^{-1}–10^{3} μg of II, the number of responding males did not significantly differ whether or not 10^{-3} μg of I was added. Similarly, with admixture of 10^{1}–10^{3} μg of *E*7,*E*9–11:OH (III) to 10^{-3} μg of I, the number of responding males was the same as for the respective amount of III alone.

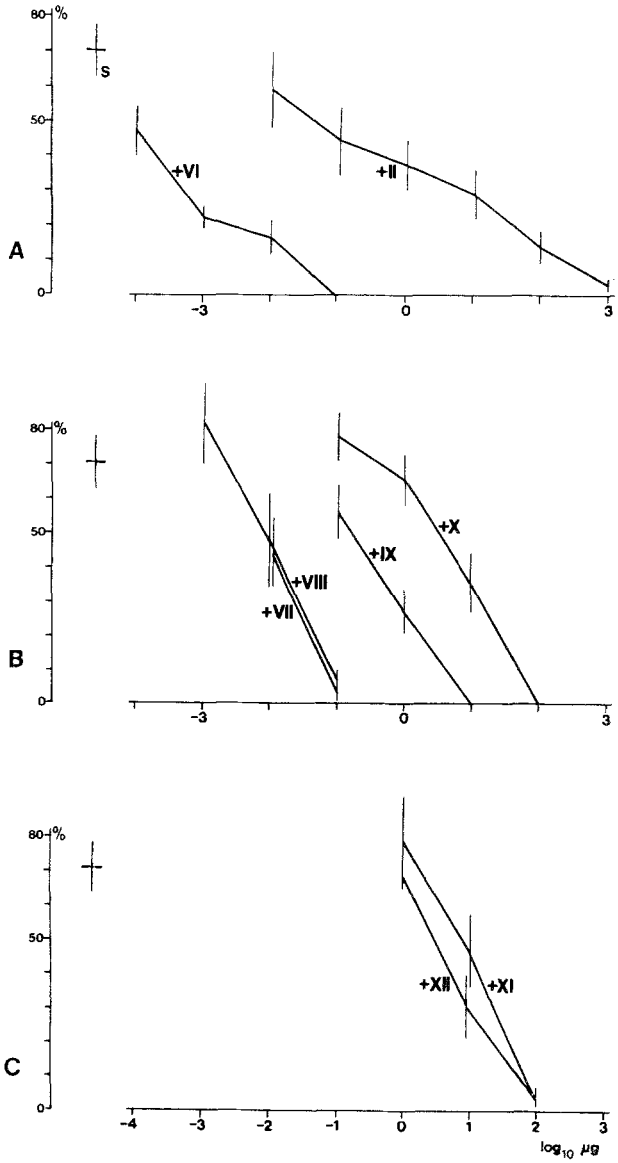

FIG. 5. Response of male *L. pomonella* to binary mixtures of 10^{-3} µg of I with varying amounts (abscissa) of alcohols II–XII (bar at upper left indicates response to 10^{-3} µg of I alone, taken from Figure 1).

The "inhibitory curves" obtained for the monounsaturated alcohols VII–X were, by comparison, much steeper, reaching total inhibition of responses to I within two decadic steps of stimulus amount (Figure 5B), although the threshold doses were up to 10^5-fold above that of *E*9,11–12 : OH (VI). The same holds for the saturated analogs XI and XII (Figure 5C).

The C_{10} homolog (V) showed the least inhibitory properties of all alcohol analogs listed. With 10^1 μg of V added to 10^{-3} μg of I, the number of responding males was significantly reduced; however, even in the presence of 10^3 μg, about 30% of the test males successfully oriented towards the 10^{-6}-fold lower amount of I present in the mixture (results not specified by figures).

Approach flights not terminated by landing were generally found to be more frequent in experiments with binary mixtures than with 10^{-3} μg of I alone. The inverse, however, was found with the *E*10 monoene (X): In the presence of 10^{-3} μg of I, the number of flights that stopped before reaching the source was far lower as compared to tests with X alone, where uncompleted flights predominated over flights terminated by landing.

DISCUSSION

Mating flights of male codling moths in the field usually occur within 1 hr before and after sunset (Wong et al., 1971; Batiste et al., 1973a,b; Mani et al., 1974). This periodicity is reflected by a pronounced circadian rhythm of male responsiveness to female sex pheromone in laboratory tests. Castrovillo and Cardé (1979) and Arn et al. (1985) studied pheromonal behavioral responses in *L. pomonella* males kept under a 16 : 8 hr light–dark photoperiod, using a tube olfactometer and a wind tunnel, respectively. In their studies, responses towards synthetic female pheromone were highest within the first 3 hr of scotophase. However, even with males at a circadian stage 3 hr prior to scotophase, the full level of 70–80% of upwind orientation responses could be induced by lowering light intensity from 1500 to 15 lx (Castrovillo and Cardé, 1979).

In the present work, "artificial dusking" was used throughout to induce high pheromonal responsiveness in the *L. pomonella* males. Differing from the procedure applied by the above authors, the males were kept in continuous light (1500 lx) from the late pupal stage until test, including 43–73 hr of adult life. Various tests (R. Preiss, unpublished results) showed that *L. pomonella* males, submitted to this pretreatment followed by the 30 min "dusking," revealed the same level of approx. 70% orientation flight responses towards 10^{-3} μg of codlemone (I), irrespective of the actual time of day. This permitted us to conduct our test program around the clock.

In our experiments and those of Arn et al. (1985), males had to orient by flight within an odor plume, whereas in the experiments of Castrovillo and Cardé (1979), moths had to walk upwind within an homogeneous odor stream.

The dose–response curves for I as measured by the two different methods both run through a maximum, although only in our experiments did the dose–response curve form a pronounced plateau, extending over three decadic steps of lure dose. Within this plateau the only observed effect of increased lure dose was a reduced response time as measured from male release until landing at the source.

In all three studies, the dose–response curves strongly declined at very high lure doses. The cause of this "overdose effect" is unknown. In our wind-tunnel experiments, the overdose stimuli primarily affected the number of approach flights rather than the flight pattern itself; e.g., flights stopping before reaching the source were not found to occur more frequently at these high doses.

Further comparison of the dose–response curve for I (Figure 1 and 2) with those obtained by other researchers with tube olfactometers (Castrovillo and Cardé, 1979) or wind tunnels (Arn et al., 1985; Einhorn et al., 1986) is limited by a number of factors, including the spatial arrangement of the experimental setup, the type of odor dispenser used, the kind of behavioral action(s) evaluated, and the predetermined time of observation. Unpublished wind-tunnel results showed that rubber caps (such as used by Arn et al., 1985) release olefinic test compounds at an approx. 10^3-fold lower rate compared to filter papers or cotton pads; this could explain in part the marked difference in threshold dose for I between the study of Arn et al. (1985) and ours. Arn et al. also evaluated the percentage of males completing orientation flight by landing at the source. However, in their tests males that did not take off within 1 min after release or that touched parts of the tunnel wall other than the odor source were taken as nonresponding (and discarded). This differs from our arrangement in which males were allowed to start or restore contact with the plume during flight over the entire 10-min exposure period.

The olfactometer study of Castrovillo and Cardé (1979) evaluated male upwind orientation within a period restricted to 30 sec, which resulted in a high threshold value (10^{-2} μg) for I. We are fully aware that, in our setup too, the number of males reaching the source (and thus, the attractivity of the compound as given in the Results section) strongly depended on the predetermined duration of the exposure period following male release. Restricting exposure to, e.g., 1 min would have resulted in a higher threshold dose and a lower percentage of responding males, as can easily be seen from the time distribution of responses to I (Figure 2). With analog II, the flat dose–response curve would, in the case of a 1-min exposure period, start at a higher lure dose and the maximum at 10^{-2} μg would become more pronounced. Conversely, in the case of unlimited exposure time, the percentage of responding males would theoretically approach 100% for any effective lure, if other limiting factors would not interfere. In our wind-tunnel experiments, some males were indeed found to approach the source even after the 10-min exposure period.

It also should be kept in mind that, in our wind-tunnel assay, orientation flight is measured over only a short distance (< 1 m). It is conceivable that a field bioassay, where orientation towards the odor source over greater distances is evaluated, should be even more sensitive in discerning the more effective attractant. In fact, whereas some of the alcohol analogs mentioned in this paper induced a considerable rate of orientation flight towards the source (mostly terminated by landing at the source), in field experiments these same compounds in lure doses of up to 1000 μg revealed only poor, if any, captures of *L. pomonella* males (E. Priesner and E. Mani, unpublished results). Likewise, threshold values for inhibitory effects of compounds in admixture to codlemone were lower in field experiments than in the wind-tunnel tests. Thus, Hathaway et al. (1974) found that the addition of undecanol (XI) to codlemone (I) in a ratio of 20 : 1 reduced trap catches of *L. pomonella* males by approx. 50%. In our wind-tunnel tests a more than 1000-fold overdose of XI over I was required to reduce orientation flight responses (Figure 5B). A lower threshold dose for field as compared to wind-tunnel responses was also found for some other codlemone inhibitors, including some highly potent ester and ether analogs of codlemone (E. Priesner and R. Preiss, in preparation).

It was interesting to find that most test alcohols elicited attraction responses from *L. pomonella* males when offered as single compounds, but also reduced responses to I when simultaneously delivered with it into the same airstream. This shows that the same chemical in the same test species may be classified as both an "attractant" and an "inhibitor," depending on the kind of behavioral test.

In this context the reduction of the percentage of responding males at overdose stimulus amounts must again be considered. This phenomenon was not only found with I where responses declined from 73% at 10^{-1} μg to 10% at 10^{1} μg (Figure 1); it similarly occurred with other more attractive analogs, such as *E*7,*E*9–11 : OH (III) and *E*8,*E*10–13 : OH (II) (Figure 3). Accordingly, "inhibition curves" such as obtained for binary mixtures between higher amounts of III or II with 10^{-3} μg of I (codlemone) cannot be considered as reflecting responses evoked by the very low amount of I present in these mixtures: These "inhibition curves" are not different from the respective parts of the "attraction curve" of the analogous compound alone, and thus evidently represent the decreasing response to overdose stimuli of these compounds themselves, apparently unaffected by the admixture of the very small amount of I (see Figures 3 and 5A for compound II).

Contrary to this, the dose–response curves obtained for the binary mixtures of the other eight alcohol analogs with 10^{-3} μg of I can be considered as "true inhibition curves": these curves (Figure 5) are fully separated from the respective "attraction curves" of the test compounds alone (Figure 3) and should thus

merely reflect the response to the portion of 10^{-3} μg of I present in the test mixtures, continuously reduced due to the dose of the other mixture component.

We have attempted to interpret the various attractive and inhibitory effects shown by the 11 test alcohols by considering their mode of sensory perception by types of receptor cells present on the male antenna. Electrophysiological recordings (E. Priesner, in preparation) have shown that the olfactory hair sensilla (s. trichodea) on male antennae of *L. pomonella* contain three different types of alcohol-sensitive receptor cells that are specific, respectively, to *E*8,*E*10–12:OH (I), *Z*8,*E*10–12:OH (a codlemone stereoisomer, not considered in this study), and *E*9,11–12:OH (compound VI of this report). No further types of alcohol receptors were found. Each test alcohol produced its own particular pattern of nerve impulse activity across the three cell types. Thus, codlemone acted upon its own type of specialist cell but also elicited weak responses from the two other cells. Similarly, other more attractive alcohols, such as II, III, and IV, had their main stimulatory effect on the codlemone cells.

We were, therefore, surprised to find that even a compound such as analog VI, which is primarily perceived via its own type of specialist cell, did induce orientation flight responses. Yet, in the electrophysiological measurements, compound VI at higher doses evoked nerve impulse discharges from the codlemone receptor cells as well, at a rate approximately 100-fold below the key stimulus (I). In the wind-tunnel experiments too, compound VI revealed a response threshold value shifted approximately 100-fold from the codlemone (I) threshold (Figures 2 and 4). These and further results are leading us to propose that the various attractive effects of test chemicals observed in the wind-tunnel tests should be triggered via excitation of the codlemone receptor cells, eventually counteracted according to simultaneous activation of further cell types. Results of the electrophysiological analysis, and a detailed interpretation of wind-tunnel and field trapping results in terms of receptor responses, will be presented elsewhere (E. Priesner, in preparation).

The codling moth sex pheromone, long considered to be a single-component attractant system, appears to include additional (secondary) components as evidenced from male behavioral responses to live females or female extracts in comparison to synthetic codlemone (Bartell and Bellas, 1981; Einhorn et al., 1984; R. Preiss, unpublished results) and the results of chemical analyses. Einhorn et al. (1984), Arn et al. (1985), and Guerin et al. (1985) isolated from extracts of pheromone glands of unmated *L. pomonella* females a number of further compounds, including the following eight alcohols (in parentheses, percent amount relative to I): *E*8,*Z*10–12:OH (3–5%), *Z*8,*E*10–12:OH (0.5%), *E*9–12:OH (4–9%), *E*8–12:OH (0.8%), 10:OH (0.2%), 12:OH (30–50%), 14:OH (10–17%), 16:OH (2–13%), and 18:OH (4%). These eight alcohols were also present, at similar proportions, in effluents emanating from calling

females (Arn et al., 1985). In wind-tunnel tests conducted by the latter authors, 12:OH (XII) when added to I appeared to enhance flight responses, whereas the other alcohols in relative amounts such as found in the extracts had no apparent synergistic effects (Arn et al., 1985; Einhorn et al., 1986). The data presented by Arn et al. suggest that the addition of XII "widens" the dose range over which males are optimally attracted to I, thus "restoring" male responses to both too low or too high an amount of the primary attractant. However, as also stated by Arn et al., the addition of XII to the nonoptimal dose of I mainly caused an improvement of the orientation flight itself (leading more males up to the source), rather than an increase in the number of males starting a flight response.

We have not studied, in our wind-tunnel experiments, additions of XII to amounts of I lower than 10^{-3} μg or greater than 10^{-1} μg (the plateau of its dose–response curve); however, a "restoring" effect such as found by Arn et al. is unlikely to occur in our setup, where orientation flights towards different test amounts of I (including those below and above the dose–response plateau) almost all led up to landing at the source (the main dose effect of I in our setup being the number of males starting a flight response, not the flight pattern itself). Electrophysiologically, we did not find a cell type specialized for compound XII; rather, this saturated analog at higher test amounts excited all three types of alcohol receptors mentioned above. It is also noteworthy that compound XII did not show synergistic effects in field trapping tests (Einhorn et al., 1984; Arn et al., 1985).

The compounds specifically acting on the two other types of alcohol receptors mentioned above were *Z*8,*E*10–12:OH and *E*9,11–12:OH. Behaviorally, the *Z*8,*E*10–12:OH showed strong modifying effects on male responses to I, not considered in this report, but the extremely low amount of this stereoisomer found in the female gland effluents makes it an unlikely candidate for a secondary pheromone constituent. The occurrence of the *E*9,11 positional isomer (compound VI of this study) in the *L. pomonella* female pheromone, and its modifying effects in field trapping tests, have not yet been reported.

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CHEMICAL TRAIL MARKING AND FOLLOWING BY CATERPILLARS OF *Malacosoma neustria*

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Abstract—Chemical trail marking and following by gregarious caterpillars, *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae), was studied in the laboratory. As in other species of *Malacosoma*, larvae deposit a trail pheromone from a sternal secretory site when searching the host for food. Larvae in the vanguard of foraging columns establish chemical trails as they explore new territory. Marking behavior diminishes as successive unfed foragers utilize the trail. These exploratory trails are subsequently overmarked by fed larvae returning to the tent. Other foragers follow the trails of fed larvae in preference to trails of unfed larvae. Thus, like the eastern tent caterpillar, *M. americanum*, successful foragers of *M. neustria* recruit colony-mates to feeding sites. The chemical activity of both recruitment and exploratory trails degrades slowly, suggesting that the trail pheromone of *M. neustria* is a nonvolatile substance. Caterpillars of *M. neustria* readily follow the nonvolatile trail pheromone which has been identified from *M. americanum*, 5β-cholestane-3,24-dione.

Key Words—Trail pheromones, trail marking, 5β-cholestane-3,24-dione, tent caterpillars, *Malacosoma nuestria* L., Lepidoptera, Lasiocampidae, recruitment, leader-follower behavior.

INTRODUCTION

Despite recent advances in research on trail pheromones of caterpillars, the chemical basis of silk trail following has been established for only a few species (reviewed in Fitzgerald and Peterson, 1988). The most thoroughly studied of these is the eastern tent caterpillar, *Malacosoma americanum* (F.), which pro-

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duces a steroidal trail pheromone, 5β-cholestane-3,24-dione, from a sternal secretory site (Fitzgerald and Edgerly, 1982; Crump et al., 1987). Another North American species of tent caterpillar, *M. disstria* Hübner, also deposits a chemical marker (Fitzgerald and Costa, 1986), which suggests that chemical trail marking is common in *Malacosoma*. This paper investigates chemical trail marking and following in a third species of tent caterpillar, *M. neustria* L.

Caterpillar trail pheromones may serve to maintain aggregations or to facilitate exploration (Fitzgerald and Costa, 1986), but they can also function as recruitment signals to indicate pathways leading to food (Fitzgerald, 1976; Fitzgerald and Peterson, 1983). *M. americanum* is the only caterpillar reported so far to use its chemical marker to recruit colony-mates to food finds (Fitzgerald, 1976). Eastern tent caterpillars forage from a stable, centrally located tent. Larvae mark trails during exploration of new territory, and the trails are reinforced (overmarked) by fed larvae as they return to the tent. These recruitment trails lead directly to food and are followed preferentially by hungry larvae (Fitzgerald, 1976).

Here the hypothesis that *M. neustria* caterpillars produce a chemical marker which recruits others to food sources was tested in the laboratory. Experiments were done to (1) localize the site of secretion of the trail marker, (2) observe the marking behavior of fed and unfed larvae, (3) test the preference of larvae for trails of fed caterpillars, (4) investigate aging of the trail, and (5) test the responses of caterpillars to the trail pheromone identified from *M. americanum*.

METHODS AND MATERIALS

Insects. Malacosoma neustria is a polyphagous species widespread in Europe and Asia (Stehr and Cook, 1968). Unhatched egg masses were collected from oak (*Quercus robur* L.) near Almere, The Netherlands. Eggs were stored at 6°C and 80% relative humidity. Egg masses were removed from refrigeration, hatched, and larvae were kept in plastic rearing containers. Young leaves of apple (*Malus* sp.) or oak were provided daily. All experiments were conducted at a controlled temperature of 21°C.

Y-Maze Bioassay. Comparisons of trails were made using a Y-maze procedure modified after Fitzgerald and Edgerly (1979). Groups of larvae (N = 8–10) established trails across long strips of 3-mm-wide white filter paper, suspended horizontally between two rods. The strips were then cut into 3-cm-long sections and assembled into Y mazes, with a 60° angle between the arms. The maze was attached with masking tape to the bottom of a glass Petri dish, which was cleaned periodically with 90% ethanol to prevent contamination by pheromone and silk. The choices of caterpillars were tested by placing third-instar larvae one at a time on the stem of the maze and allowing them to crawl to the choice point. A choice for either side was granted if the caterpillar completely

passed onto one of the branches. The arms of the maze were replaced after each replicate, but the stem was reused. To exclude artifacts due to side preferences, the position of experimental and control branches was alternated. To prevent direct overhead light from influencing the behavior of the larvae, experiments were conducted in the closed end of a cardboard box which was open at one end to allow observation. The box was laminated with sheets of white filter paper to further eliminate visual cues.

Secretory Site. Caterpillars of *M. americanum* and *M. disstria* produce their trail pheromone from a sternal cuticular site between the anal prolegs on the tip of the last abdominal segment. To lay a trail, the abdomen is pressed against the substrate as the caterpillar walks (Fitzgerald and Edgerly, 1982; Fitzgerald and Costa, 1986). Third- and fourth-instar caterpillars of *M. neustria* were observed with a hand lens to determine whether they laid trails in this manner. Larvae were allowed to crawl onto the filter-paper strips spontaneously without handling by connecting the strips to the rearing container. Larvae were fed ad libitum up to 6 hr before the experiments or were kept chilled overnight without food. Experiments were initiated when most of the larvae in the group were observed to be active.

In each of the 18 replicates of this experiment, the degree of trail marking was estimated for the first, fifth, and tenth larva to cross the trail as each traversed the middle 10 cm of the 30-cm-long strip. Since larvae do not always maintain continuous marking, their behavior was classified according to the approximate amount of time they were observed with their tip in contact with the trail: marking (>50% of the time with tip in contact with the trail), discontinuous marking (>5% but <50% tip contact), and not marking (<5% tip contact).

Tests to determine the site of pheromone secretion were conducted using the "tip-wiping" method (Fitzgerald and Edgerly, 1982; Fitzgerald and Costa, 1986). A folded piece of filter paper was wiped across the last abdominal segment of a single large (fourth-instar) caterpillar to make a residue trail of 3 cm length. As a control, surface residue was wiped from the same caterpillars' anus onto a different piece of filter paper. A smaller caterpillar (second-instar) was placed at one end of the test trail and another was placed at one end of the control trail. Each was observed to determine whether it followed the crease to the trail terminus within 3 min. The test was repeated 16 times.

A second experiment was carried out by obstructing the abdominal tip of eight fourth-instar caterpillars with liquid plastic (New Skin, Beecham Properties, St. Helen's, England). Control caterpillars ($N = 8$) were treated with New Skin on the abdomen between the first pair of abdominal prolegs. The trails made by treated insects were compared to the trails made by control caterpillars using the Y-maze test protocol described earlier.

Recruitment. Recruitment behavior was tested by comparing the overmarking of trails by fed and unfed caterpillars and observing the response of

other caterpillars to these trails in choice tests. One paper strip was connected at the far end to a sprig of foliage, and a control strip was connected to a bare wooden rod. Test and control trails were made simultaneously by letting eight starved larvae per trail traverse each strip. Larvae were allowed to crawl onto the strips without handling. Three of these larvae were allowed to visit the feeding site (or the site without food) and return to the starting point. The overmarking of trails by each group of three larvae was observed on their return. The strips were then cut, and the trails of fed and unfed caterpillars were compared using the Y-maze bioassay. A section of the trail of fed larvae was used as the stem of the Y-maze in all tests. The experiment was repeated six times using apple foliage and four times using oak foliage.

Trail Aging. The trails of other species of *Malacosoma* degrade slowly, but differences between trails of fed larvae and trails of unfed larvae of *M. americanum* persist over a period of 6 hr (Fitzgerald, 1976; Fitzgerald and Costa, 1986). *M. neustria* trails were aged at room temperature and were compared with fresh trails using the Y-maze bioassay as previously described. Eight larvae traversed each strip to establish a test trail. After 2 hr of aging, the test trail was compared to a fresh trail prepared in the same manner. The 2-hr-old test trail was then aged for an additional 4 hr (for a total of 6 hr of aging) and compared to a second fresh trail. The same sections of the aged trail were used in the comparison with fresh trails at 2 and at 6 hr. To avoid contamination, larvae were prevented from crawling more than midway onto a trail arm. The used arms of the Y maze were reversed, and the unused half was abutted to the stem of the Y for the subsequent test. Fresh and aged exploratory trails were compared in two separate tests, as were fresh and aged recruitment trails in which three of the eight larvae were allowed to feed and return. A section of the fresh trail was used as the stem of the Y maze in each of these tests.

In addition, aged trails of fed larvae were compared with aged trails of unfed larvae at 2 and at 6 hr using the protocol already described. A section of the fed caterpillars' trail was used as the stem of the Y maze in each of the tests.

Response to Chemicals. The response of *M. neustria* caterpillars to the trail pheromone of *M. americanum*, 5β-cholestane-3,24-dione, was tested. A stock solution of 0.1 mg pheromone/ml solvent was made by dissolving the synthetic substance in methanol (Merck). In addition, a 0.1 mg/ml solution of 5β-cholestan-3-one was also tested. This compound, which differs from the identified trail pheromone by lacking an oxygen at C-24, elicited trail following in caterpillars of *M. americanum* but was not a component of their secretion (Crump et al., 1987).

Test compounds were applied to white paper using microcapillary pipetes calibrated to deliver 4 μl in a 2-mm-wide streak onto a 10-cm length of paper. A positive response was recorded when the caterpillar walked from one end of the trail to the other. Responses of caterpillars to 5β-cholestane-3,24-dione were

tested at five trail dosages ranging from 0.001 to 0.1 mg/ml (N = 20 at each dosage). As a control, 20 larvae were tested with pure methanol. In addition, the responses of 20 larvae to trails prepared from 0.1 mg/ml 5β-cholestan-3-one were also tested.

Statistical Analysis. Results were analyzed using a G test (Sokal and Rohlf, 1981), either testing for significant differences between treatments or for a difference from the expected 1 : 1 ratio in the Y-maze tests.

RESULTS

Exploratory Marking and Secretory Site. Malacosoma neustria larvae were observed dragging their abdominal tips against the substrate. When 10 larvae were let onto a 30-cm-long paper strip and the marking of the first, fifth, and tenth larva in the column was observed, the frequency of marking declined with caterpillar order (Figure 1). The caterpillars often crossed the paper strip without reversing direction, so that columns of foragers were evidently led by larvae in the vanguard. Although larvae were sometimes reluctant to initiate a trail independently of other caterpillars, only 11 of 180 larvae (6.1%) reversed direction after passing 15 cm onto the strip.

The abdominal tip was determined to be the site of marker secretion by the wiping test. All larvae tested followed the 3-cm residue trail to its terminus

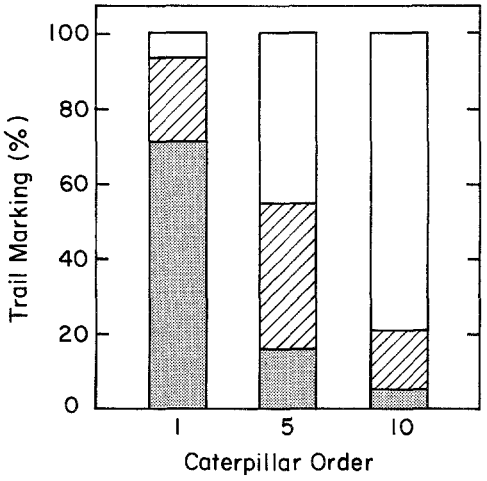

FIG. 1. Percentage of *M. neustria* trail marking as a function of order on the trail. Shading = marking; cross-hatch = discontinuous marking; open = not marking (see text). The frequency of marking vs. not marking is significantly greater for caterpillars at position 1 than for caterpillars at position 10 ($P < 0.005$, two-way comparison G test), N = 18 tests.

($N = 16$; time to follow trail = 25.5 ± 11.0 sec, mean ± SD). Only one of 16 larvae followed the control trail in the 3-min test period.

Furthermore, in two separate Y-maze tests, larvae were able to discriminate trails made by control caterpillars from trails made by caterpillars with their abdominal tip covered with New Skin. In the first test, 17 of 18 larvae chose the control trail and in the second test all 18 larvae chose the control trail ($P < 0.005$, G test).

Recruitment. The results of the recruitment experiments are given in Table 1. Caterpillars returning after feeding overmarked their trails, but unfed larvae did not overmark. Overmarking occurred to apple as well as to oak foliage. In Y-maze tests, significantly more larvae chose the trails of fed larvae over the trails of unfed larvae.

Trail Aging. The results of the trail aging experiments are given in Table 2. Both exploratory and recruitment trails were only slightly less attractive than fresh trails of the same type after 2 hr of aging, but after 6 hr the fresh trails were clearly favored. Recruitment trails were strongly preferred over exploratory trails at 2 hr and at 6 hr of aging.

Chemical Trail Following. Larvae of *M. neustria* readily followed the pheromone of *M. americanum*. The response of caterpillars diminished with

TABLE 1. RECRUITMENT BEHAVIOR OF *M. neustria* TO FOLIAGE OF APPLE AND OAK

	Number overmarking		Number following trail of	
Test	Fed larvae	Unfed larvae	Fed larvae	Unfed larvae
I. Apple				
1	3	0	15	0
2	3	0	14	1
3	3	1	11	4
4	3	0	11	4
5	3	0	13	2
6	3	0	13	2
Totals	18	1	77	13
	$P < 0.005$[a]		$P < 0.005$[b]	
II. Oak				
1	3	0	11	4
2	3	0	12	3
3	3	0	14	1
4	3	0	12	3
Totals	12	0	49	11
	$P < 0.005$[a]		$P < 0.005$[b]	

[a] Two-way comparison G test, marking vs nonmarking of fed and unfed larvae.
[b] G test of goodness of fit to 1 : 1 ratio.

TABLE 2. AGING OF *M. neustria* EXPLORATORY AND RECRUITMENT TRAILS[a]

Trail choice (A/B)	Hours of aging (A/B)	Number following (A/B)		P^b
E/E	0/2		12/6	
E/E	0/2		10/8	
		Totals	22/14	NS
E/E	0/6		16/2	
E/E	0/6		15/3	
		Totals	31/5	<0.005
R/R	0/2		11/7	
R/R	0/2		12/6	
		Totals	23/13	NS
R/R	0/6		14/4	
R/R	0/6		17/1	
		Totals	31/5	<0.005
R/E	2/2		15/3	
R/E	2/2		16/2	
		Totals	31/5	<0.005
R/E	6/6		15/3	
R/E	6/6		16/2	
		Totals	31/5	<0.005

[a] E = exploratory trails, R = recruitment trails.
[b] *G* test of goodness of fit to 1:1 ratio. NS = not significant.

decreasing concentration of 5β-cholestane-3,24-dione, from 100% positive response at 0.1 mg/ml until no response was observed at 0.001 mg/ml (Figure 2). No response was observed to the methanol control, out of 20 larvae tested. In addition, all 20 larvae tested followed a trail of 0.1 mg/ml 5β-cholestan-3-one.

DISCUSSION

The results demonstrate a chemical trail marking system in *Malacosoma neustria* remarkably like that of its congener, *M. americanum*. The similarities include: (1) marker secretion from the abdominal tip, (2) conspicuous dragging of the abdomen when marking a trail, (3) overmarking of exploratory trails by fed larvae returning to the group, (4) preference by foragers for overmarked trails, (5) gradual deterioration of pheromone activity, together with (6) persistence of differences between exploratory and recruitment trails.

The similarity of the two species is further indicated by their positive responses to 5β-cholestane-3,24-dione, the pheromone identified from *M.*

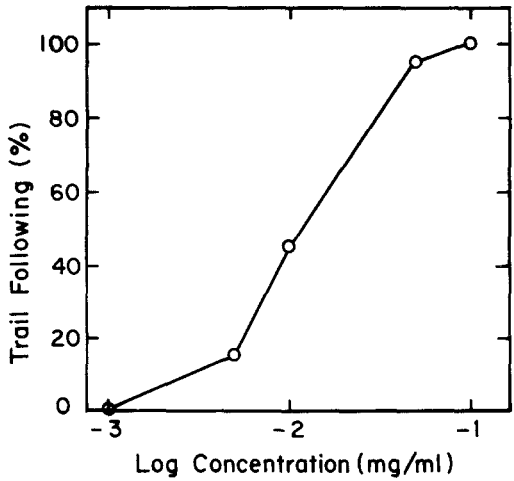

FIG. 2. Percentage of *M. neustria* following a 10-cm-long trail of 5β-cholestane-3,24-dione (N = 20 at each concentration).

americanum, and to 5β-cholestan-3-one. The gradual fade-out of the *M. neustria* trail pheromone is an indication that it is a high-molecular-weight, nonvolatile substance, like the pheromone of *M. americanum*.

The only outstanding difference between *M. americanum* and *M. neustria* trail-marking systems noted here is the apparent leader–follower behavior of *M. neustria* caterpillars. Both *M. americanum* and *M. disstria* explore unmarked substrates in groups, advancing only short distances together before turning back (Edgerly and Fitzgerald, 1982; Fitzgerald and Costa, 1986). Here, *M. neustria* larvae explored more independently. In addition, since the lead caterpillar consistently marked and those that followed marked less (Figure 1), larvae in the vanguard of foraging columns appeared to play a more active role in the establishment of exploratory trails than those in the rear. However, it is not known whether certain caterpillars are more likely to lead than others. Wellington (1957) reported leader–follower behavior in *M. californicum pluviale* Dyar and attributed this to individual differences in activity level. Laux (1962) also described activity differences in larvae of *M. neustria*, as did Greenblatt and Witter (1976) for *M. disstria*. However, these studies have been criticized on the basis of inappropriate experimental methods and statistical analysis (Edgerly and Fitzgerald, 1982; Papaj and Rausher, 1983). Moreover, in an extensive study of *M. americanum*, activity levels of caterpillars varied, but there was no indication that caterpillars assumed discrete behavioral roles (Edgerly and Fitzgerald, 1982). Larvae of *M. disstria* were also reported to diminish their marking behavior as successive waves of foragers utilized the trail (Fitzgerald and Costa, 1986).

M. neustria is only the second species of lepidopterous larvae reported to recruit to food. The fact that *M. americanum* has a well-developed recruitment system but *M. disstria* does not was attributed to differences in foraging systems by Fitzgerald and Costa (1986) and Fitzgerald and Peterson (1988). *M. americanum* larvae forage from a centrally located resting place to which they return after feeding. In contrast, *M. disstria* moves repeatedly from one resting–feeding site to the next, a syndrome described as "nomadic" by Fitzgerald and Costa (1986). Preliminary field observations of *M. neustria* suggest that it too has a central-place foraging system like that of *M. americanum* and uses its web as a resting site between periodic forays to food. This supports the conjecture of Fitzgerald and Peterson (1988) that gregarious caterpillars with central-place foraging systems may commonly exhibit recruitment behavior.

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ISOLATION OF THE TRAIL RECRUITMENT PHEROMONE OF *Solenopsis invicta*

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Abstract—The *Solenopsis invicta* trail pheromone is synthesized by the Dufour's gland and is released through the sting apparatus. The recruitment subcategory of the *S. invicta* trail pheromone was shown to be composed of a mixture of the orientation pheromone, (*Z*, *E*)-α-farnesene and an unidentified homosesquiterpene consisting of three rings and one double bond (C−1). C−1 is present in worker Dufour's glands at only 75 pg per worker equivalent. This is the first report that demonstrates that different exocrine products from the same gland control different subcategories of behavior related to mass recruitment.

Key Words—*Solenopsis invicta*, Hymenoptera, Formicidae, imported fire ant, Dufour's gland, trail pheromone, pheromone, (*Z*, *E*)-α-farnesene, sesquiterpene.

INTRODUCTION

Ant trail pheromones represent one of the most elaborate known forms of chemical communication in social insects (Wilson, 1971) and were discussed in recent reviews of the behavior and chemistry of mass recruitment (see Hölldobler, 1978; Attygalle and Morgan, 1985). Trail pheromones are essential components of efficient foraging and mass recruitment by *Solenopsis invicta* Buren (Wilson 1962b) and are a good example of trail pheromone complexity. Wilson (1959) reported that the Dufour's gland was the source of fire ant trail phero-

mones and that the contents of the gland were emitted through the sting onto the substrate on which the trail-laying worker walks. The pheromone attracts workers who then orient (move) along the trail (Wilson, 1962a). Depending on the context, the Dufour's gland contents also released colony emigration, alarm recruitment, or settling behavior (Wilson, 1962c).

The investigation of the chemical nature of fire ant trail pheromones began with Wilson (1959), who recovered bioassay active material from a steam distillate of whole ants. However, the crude material was not stable over long periods of time (Walsh et al., 1965). Barlin et al. (1976) characterized the trail pheromone of *S. invicta* as a sesquiterpene hydrocarbon, based on analogy with gas chromatograph (GC) and GC-mass spectrometric (GC-MS) data for *Solenopsis richteri* Forel. This was confirmed by Vander Meer et al. (1981), who isolated (*Z,E*)- and (*E,E*)-3,7,11-trimethyl-1,3,6,10-dodecatetraene (α-farnesenes) and two homofarnesenes later identified as (*Z,E*)- and (*E,E*)-3,4,7,11-tetramethyl-1,3,6,10-dodecatetraene (Alvarez et al., 1987). An allofarnesene was also reported (Williams et al., 1981); however, we synthesized and tested this compound in our laboratory and showed that it did not induce trailing behavior (Williams, 1985).

The isolation of the farnesenes and homofarnesenes in the previous research was directed by a bioassay that specifically measured the orientation response of ants (movement along a trail). However, these compounds were ineffective in a bioassay designed to measure worker recruitment or attraction associated with the trail pheromone. We report here the isolation and partial identification of the Dufour's gland components from *S. invicta* that recruit workers to a trail.

METHODS AND MATERIALS

Point Source Recruitment Bioassay. Ten positions were marked symmetrically on the bottom of a plastic tray (7 × 44 × 56 cm) along a circle radiating 20 cm from the tray center. Queenright colonies consisting of one to three Williams nests (Banks et al., 1981) were reared from queens collected around Gainesville, Florida, and used in the bioassays. The cells were placed on top of each other in the center of the plastic tray. In addition, workers in the foraging arena of the mother colony tray were transferred to the bioassay tray. Each test colony contained approximately 25,000–75,000 workers. The ants were given at least 1 h to equilibrate in their new surroundings. Blotter paper squares (2 × 2 cm) with 1.5-cm-diam. circles marked in the center were placed on slightly larger aluminum squares. Up to eight test samples, a Dufour's gland extract standard, and a solvent blank (volume applied = 5 μl; one worker equivalent, WE) were applied to the paper squares and placed randomly on the numbered positions. The ants responded to active test samples by aggregating on the treated spots, often chewing the paper (see Wilson 1962c). The number of

ants aggregated within a 1.5-cm circle on the blotter paper squares were counted at 5-min intervals for 30 min. The sum of the six counts was regarded as the result for each position. Each test was replicated using six different *S. invicta* colonies. Experiments were evaluated by ranking sample responses based on Dufour's gland standard = 100 and solvent blank = 0. This method of scoring was used to minimize the day-to-day variability of the responsiveness of the ants. Using this method, if a sample response is greater than the Dufour's gland control, its ranking is greater than 100. Likewise if a sample's score is less than the solvent control, then its ranking will be less than 0.

Olfactometer Bioassay. The olfactometer (modeled after a design by Vander Meer et al., 1979) consisted of two 24/40 ground glass joints (Figure 1b); each ring was sealed to one of the arms of a 5-cm Y tube such that 1 cm of each Y-tube arm extended through the male half of one of the ground glass joints (Figure 1 d). A 5-cm piece of 0.6-cm-ID tubing was ring sealed 1 cm into the female half of the ground glass joints (Figure 1, a and e). A baffle (Figure 1c) at the center of the Y tube controlled air streams and prevented premature mixing of the sample, and gave the ants a clearer choice. The baffle also narrowed the openings to the choice chambers to the minimum size required

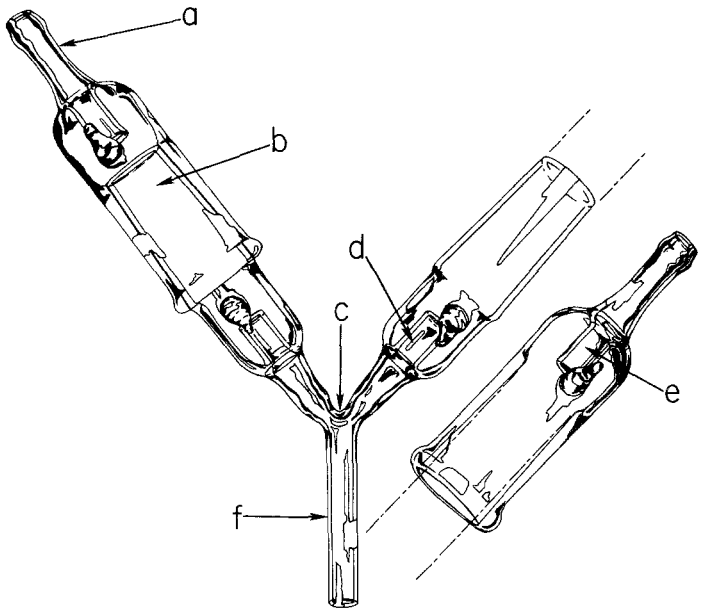

FIG. 1. Drawing of Y tube used in recruitment bioassays. Unit consists of two 24/40 ground glass joints ring-sealed to a Y stem. Legend: a, air inlet tube; b, sample chamber; c, baffle; d, ring seal tube (front); e, ring seal tube (rear); f, entrance stem.

for passage of a major worker. A test sample (10 μl/WE) and a solvent blank (10 μl) were applied to two filter paper strips (0.3 × 2.5 cm, Whatman No. 1), air dried, and each placed in one of the choice chambers (Figure 1, b). Compressed air (breathing air quality) was split into two streams and passed into the two choice chambers. Each stream was regulated to 0.2 liters/min. for a total effluent flow rate of 0.4 liters/min. Approximately 50–70 ants from laboratory colonies were confined in a 2.5-cm piece of 0.9-cm-ID Tygon tubing sealed at one end with wire gauze. The open end of the tubing was attached to the entrance stem (Figure 1, f). The initial choice of the first 20 ants that walked down the entrance tube and into one of the arms of the Y tube was recorded. Ants that were not trapped in a choice chamber and came back to the entrance stem were not counted if they made another choice. After each test, the olfactometer was rinsed with acetone and dried. Each test sample was retested with worker ants from the same colony, but the choice chamber in which the sample and control were placed was reversed. This procedure eliminated any bias inherent in the individual olfactometers. A complete replicate was the sum of the results from the two tests. Data were analyzed statistically by a chi-squared test, and comparisons between means were made with the Newman-Keuls test.

Gas Chromatography. Gas chromatography (GC) was performed on a Varian 3700 gas chromatograph equipped with a flame ionization detector (FID). Small-scale preparative GC work was carried out using the FID and a 10:1 glass splitter. The effluent was collected in glass capillary tubes and eluted into vials with hexane. The GC columns used are designated as follows: Column A—DB-1 (J and W Scientific, Inc.), fused silica, 0.322 mm ID × 15 m, 0.25 μm film thickness; Column B—Superox (SGE, Inc.), glass, 0.5 mm ID × 27 m; Column C—OV-101 on 120/140 Gas Chrom Q (Applied Science), 4 mm ID × 1.8 m; Column D—3% OV-17 (Applied Science, Inc.) on Gas Chrom Q (120/140 mesh). Quantitative data were obtained with a Varian CDS 401 data processor and paraffin internal standards.

Dufour's Gland Extract. Dufour's glands were extirpated in water from randomly chosen worker ants and macerated in hexane. Pooled Dufour's gland extracts were used to determine parameters for recruitment pheromone isolation, GC quantification, and as standards in all bioassays.

Dufour's Gland Extract Column Chromatography. Dufour's gland extract (100 WE in 150 μl hexane) was applied to a 1.5-cm × 0.4-cm bed of silica gel (37–53 μm, Whatman, Inc.) and eluted sequentially with 6 ml each of hexane, methylene chloride, and methanol to separate the solute into hydrocarbon, nonpolar lipids, and polar lipids, respectively (Christie, 1973). The solvent for each fraction was evaporated under a stream of nitrogen to a volume of 0.5 ml and used in the point source recruitment bioassay.

Preparative GC of Dufour's Gland Extract. A Dufour's gland extract (100 WE) was separated into fractions by preparative gas chomatography (FID, 10:1

splitter, column C), 145°C for 22 min then 145–250°C at 10°C/min. Two procedures were used: (1) The mixture was separated into a volatile region (solvent front through the retention time of heptadecane) and a nonvolatile region (everything beyond heptadecane). (2) Individual fractions were collected at 1-min intervals for the first 21 min. Fractions 1–10 were from the solvent front to just after the elution of heptadecane. Fraction 22 contained all material eluted beyond 22 min. The fractions were collected in capillary tubes cooled with Dry Ice, and the sample was eluted into a 2-ml vial with 500 μl of hexane. These fractions were used for subsequent bioassays.

Isolation of Non-Dufour's Gland Saturated Hydrocarbons. The postpharyngeal gland (located in the head) is a rich source of species-specific hydrocarbons (Vander Meer et al., 1982). Five postpharyngeal glands were extirpated and crushed in 150 μl of hexane. This solution was applied to a Pasteur pipet silica column and eluted with hexane to isolate only the hydrocarbons (Christie, 1973). The hexane eluent was evaporated to a small volume and quantitatively analyzed by GC.

Extraction of Worker Ants. Whole worker ants (904 g, an estimated 904,000 workers at 1 mg per individual) were rinsed two times in hexane (1.5 liters) for 15 min and filtered. The ants were then homogenized in hexane (1.5 liters) for 2 min using a Virtis blender at a medium setting. The homogenate was filtered over a bed of Celite and the residue washed and filtered several times with fresh hexane. The filtrate was concentrated under vacuum to 200 ml. The insoluble residue was further homogenized with methanol (1.5 liters), filtered, washed several times with fresh methanol, and the combined filtrate was concentrated under vacuum to 200 ml. The hexane fraction was analyzed by thin-layer chromatography (TLC) using Polygram sil G/UV_{254} precoated plastic sheets (Macherey-Nagel, 40 × 80 mm) and an eluting solution for lipid separation (Christie, 1973).

Fractionation of Hexane Extract. The hexane extract (200 ml) was fractionated by gravity column chromatography on silica gel (800 g, Analtech; 35–75 μm). The sample was sequentially eluted with hexane (2 liters), methylene chloride (1.5 liter), and methanol (1 liter). These fractions were designated G-1, G-2, and G-3, respectively. The three fractions were concentrated under vacuum to 200 ml. The hexane eluent (G-1) was further concentrated under vacuum to yield 3.2 ml of a colorless liquid.

Separation of Saturated and Unsaturated Hydrocarbons. An aliquot (30 μl, 8500 WE) of the total hydrocarbon fraction (G-1) above was diluted in hexane (1 ml) and chromatographed using silver nitrate-impregnated silica gel (10% $AgNO_3$, Adsorbosil-LC-Ag, Applied Science, Inc., State College, Pennsylvania; 1.3 × 23 cm bed size). The sample was eluted stepwise with 0–50% ethyl ether in hexane (1 ml/min; 8.5 ml/fraction). Twenty fractions (Ag) were collected, and each was filtered through sodium chloride to remove potentially

interfering AgCl. The percentage of ethyl ether was increased based on GC analysis (column A) of fractions: fractions 1–6, hexane; fractions 7–11, 5% ethyl ether; fractions 12–16, 10% ethyl ether; fractions 17 and 18, 20% ethyl ether; and fractions 19 and 20, 50% ethyl ether. The fractions were designated Ag-1 to Ag-20.

Isolation of Active Components. Preparative GC of active fractions was carried out on columns C or D using an FID and 10:1 split. Kovats indices (KI) were calculated and coinjections made on columns A and B. Chemical ionization (C1) GC-MS were obtained on a Finnigan model 1015 SL upgraded to a 3200 GC-MS system. Hydrogenations (10% Pd/C in hexane) were carried out using a Supelco, Inc. microhydrogenator.

RESULTS

Preliminary studies using Dufour's gland extracts indicated that the active components of the recruitment pheromone were hexane soluble and could be eluted from a silica gel gravity column with hexane (Table 1). These physical properties defined the active components as hydrocarbons (Christie, 1973). Preparative GC of the Dufour's gland hexane extract yielded full bioassay activity from only the volatile region (solvent front through *n*-C-17 paraffin) of the chromatogram (Table 1). However, all pheromone activity was lost on preparative GC of the volatile region into 10 one-minute fractions. Recruitment pheromone activity was regenerated on recombination of all 10 fractions, which suggested a multicomponent pheromone system.

It was not practical to continue the elucidation of the recruitment trail pheromone complex using Dufour's gland extracts, since extirpation of adequate numbers of glands would be too labor intensive. Therefore, we extended the separation procedures developed for Dufour's gland extracts to the isolation of

TABLE 1. POINT SOURCE BIOASSAY OF COLUMN CHROMATOGRAPHY AND PREPARATIVE GAS CHROMATOGRAPHY FRACTIONS OF DUFOUR'S GLAND EXTRACTS

Sample[a]	Recruitment ranking[b]
A. Hexane	96.1 ± 15.5
B. Methylene chloride	29.5 ± 10.7
C. Methanol	9.9 ± 10.7
D. GC volatile region	164.0 ± 31.7
E. GC nonvolatile region	54.0 ± 15.3

[a] All samples were tested at 1 WE.
[b] Mean response (±SE) based on Dufour's gland response = 100 and solvent response = 0 (N = 6).

bioassay-active materials from whole ant extracts (Figure 2). Whole ants were first rinsed with hexane to remove cuticular hydrocarbons, which would add to the contamination of the hydrocarbon trail pheromone components. Then the ants were extracted sequentially with hexane and methanol. Analytical thin-layer chromatography of the hexane extract showed a complex mixture of substances with a range of polarities. Following the procedure used for Dufour's gland extracts, the whole ant hexane extract was separated into hydrocarbons, nonpolar lipids, and polar lipids (G-1 to 3) by sequential elution with hexane, methylene chloride, and methanol, respectively, from a silica gel column (Christie, 1973). Point source bioassays of the three fractions clearly showed that pheromone activity was only in the hexane fraction (G-1, Figure 2), which corresponded to hydrocarbons.

GC analysis of hydrocarbon fraction G-1 showed that the vast majority of hydrocarbons were the known saturated normal, methyl, and dimethyl branched

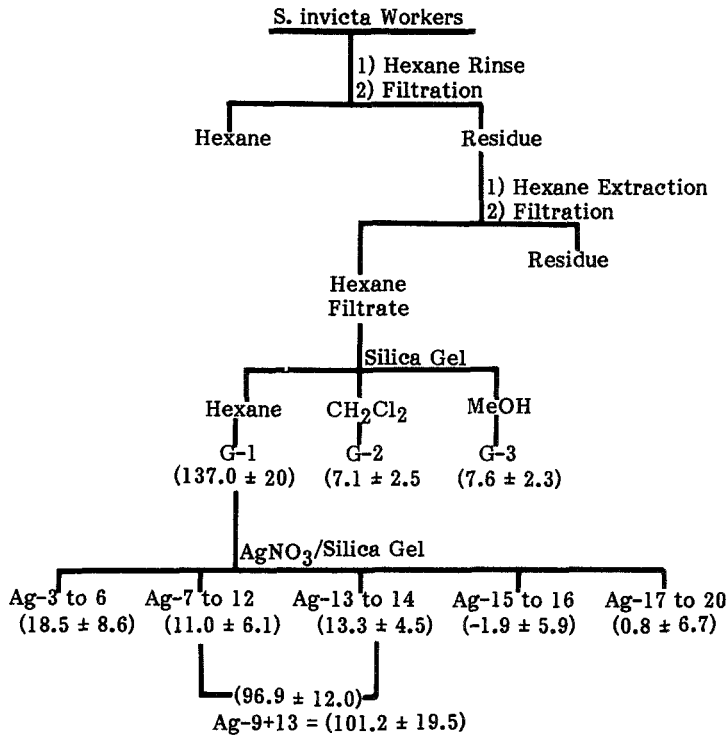

FIG. 2. Separation flow chart for the isolation of the *S. invicta* trail recruitment pheromone from worker extracts. Point source bioassay results are shown in parentheses (mean ± SE, $N = 6$) for the indicated fraction. Ranking is based on response to Dufour's gland = 100 and the response to the solvent blank = 0.

compounds specific to *S. invicta* (Lok et al., 1975; Nelson et al., 1980; Thompson et al., 1981). Our previous work on the trail orientation pheromone indicated that at least a part of the recruitment pheromone might be an unsaturated hydrocarbon. Consequently, to separate hydrocarbons with varying degrees of unsaturation, the total hydrocarbon fraction G-1 was chromatographed on a silver nitrate-impregnated silica gel column and eluted with hexane containing increasing amounts of diethyl ether. A total of 20 fractions were collected. The fractions were grouped into the following five categories based on changes observed in the GC chromatograms of each fraction: Ag-(3–6), Ag-(7–12), Ag-(13–14), Ag-(15–16), and Ag-(17–20). Point source bioassays of the five groups showed that none of the individually tested groups approached the activity induced by the Dufour's gland standard (Figure 2). However, bioassay results for various combinations of the five groups indicated that Ag-(7–12) in the presence of Ag-(13–14) gave full Dufour's gland activity (Figure 2). In addition, when these two groups were combined with the saturated hydrocarbon group (isolated in Ag-3, by GC analysis), activity greater than Dufour's gland was generated. These results led to detailed bioassay investigation of fractions within the Ag-(7–12) and Ag-(13–14) groupings. The combination of Ag-9 and -13 gave activity equivalent to that of Dufour's gland extracts, and these fractions were selected for preparative GC.

Ag-9 was initially separated into four major GC sections, Ag-(9A–D). Bioassays, always with an equivalent amount of Ag-13, indicated that only Ag-9C was active. Ag-9 was GC prepped again (Table 2), subdividing the active zone, Ag-9C, into four fractions, Ag-9(C-1 to C-4). The active area contained a single peak that was a minor component in Ag-9. This component was designated C-1. It was homogeneous on two capillary GC columns (KI 1640 on

TABLE 2. POINT SOURCE RECRUITMENT BIOASSAY RESULTS FOR PREPARATIVE GAS CHROMATOGRAPHY FRACTION Ag-9

Preparative GC fraction[a]	Recruitment ranking[b]
Ag-9A	26.3 ± 12.8
Ag-9B-1	9.7 ± 2.8
Ag-9B-2	11.4 ± 5.5
Ag-9C-1	130.1 ± 22.8
Ag-9C-2	12.8 ± 8.8
Ag-9C-3	21.0 ± 13.2
Ag-9C-4	30.6 ± 17.2

[a] All samples and Dufour's gland extracts were tested at 1 WE. All fractions were bioassayed with an equivalent amount of Ag-13.

[b] Mean response (± SE) based on Dufour's gland response = 100 and solvent response = 0 (N = 6).

column A and 1796 on column B). Knowing where to look on the GC chromatogram facilitated the discovery of C-1 in Dufour's gland extracts. Quantitative analysis of *S. invicta's* Dufour's glands (from a pooled sample of 100) showed that component C-1 was present at about 75 pg per worker. GC-MS of C-1 showed it to have a molecular weight of 218, indicative of a C_{16} hydrocarbon with four degrees of unsaturation (rings and/or double bonds). Hydrogenation followed by GC-MS showed the uptake of two hydrogens, which means that the compound has three rings and a single double bond.

Preparative GC of Ag-13 monitored by bioassays in combination with active monoene fraction, Ag-9, led to the isolation of a component (Table 3) that was shown to be identical to (*Z,E*)-α-farnesene, the major trail orientation pheromone, by direct comparison with a synthetic sample (Vander Meer et al., 1981). (*Z,E*)-α-Farnesene is present in the Dufour's gland of *S. invicta* at about 6 ng per worker (Vander Meer et al., 1981).

Results of the Y-tube olfactometer tests (Table 4), confirmed those of the point source bioassay in that a combination of (*Z,E*)-α-farnesene and component C-1 was required for attraction. Neither component had significant activity when tested alone. In the specific behavior of attraction, the mixture was indistinguishable from an equivalent amount of Dufour's gland extract. Therefore, the combination of (*Z,E*)-α-farnesene and C-1 (at a ratio of 6 ng:75 pg/WE, respectively) is responsible for the attraction or recruitment of worker ants. However, in the point source bioassay, the two-component mixture elicited only 85% of the activity found with an equivalent amount of Dufour's gland extract. The two natural homofarnesenes did not enhance the activity of C-1 in the olfactometer bioassay (Table 4).

Other components known to be present in Dufour's gland extracts, hep-

TABLE 3. POINT SOURCE RECRUITMENT BIOASSAY RESULTS FOR PREPARATIVE GAS CHROMATOGRAPHY FRACTION Ag-13

Preparative GC fraction[a]	Recruitment ranking[b]
Ag-13A	3.7 ± 1.4
Ag-13B	11.0 ± 3.8
Ag-13C	53.7 ± 5.3
Ag-13D	23.7 ± 11.1
Ag-13E	14.0 ± 6.4
Ag-13F	11.8 ± 3.4
Ag-(9 + 13)	55.3 ± 20.2

[a] All samples and Dufour's gland extracts were tested at 1 WE. All fractions were bioassayed with an equivalent amount of Ag-9.

[b] Mean response (± SE) based on Dufour's gland response = 100 and solvent response = 0 (N = 6).

TABLE 4. OLFACTOMETER RESULTS FOR ORIENTATION AND RECRUITMENT TRAIL PHEROMONE COMPONENTS

Sample tested[a]	Response (%)[b]
Dufour's gland extract	79.5 ± 2.9 A
C-1	60.0 ± 3.0 B
(*Z*,*E*)-α-farnesene	63.0 ± 2.2 B
C-1 + (*Z*,*E*)-α-farnesene	76.5 ± 1.4 A
C-1 + (*Z*,*E*) + (*E*,*E*)-α-homofarnesenes	59.4 ± 1.5B

[a] All samples were tested at 1 WE.
[b] Mean percent response (±SE) to the sample side of the olfactometer ($N = 5$). Results with different letters are significantly different ($P < 0.05$).

tadecane, nonadecane, homofarnesenes, and five heptacosanes, (Vander Meer et al., 1981; Barlin et al., 1976), were inactive in the point source bioassay by themselves and at physiological levels did not enhance the activity of the (*Z*,*E*)-α-farnesene and C-1 mixture.

DISCUSSION

Ant recruitment systems use a wide variety of mechanisms ranging from the tandem running first reported for *Leptothorax acervorum* (Möglich et al., 1974) to the multiple recruitment systems of the African weaver ant *Oecophylla longinoda* (Hölldobler and Wilson, 1978). Tandem running is initiated when the recruiter or calling ant releases an attractive secretion from its poison sac through the sting. When the responding worker touches the calling ant, tandem running occurs. Only a single nestmate is recruited, and it is suggested that tandem running is the evolutionary precursor to odor trail communication. Tandem running has since been observed in ponerine (Hölldobler and Traniello, 1980) and formicine (Hölldobler, 1974) ant species. At the other end of the spectrum is the highly evolved and complex weaver ant system, which involves multiple glandular secretions combined with a variety of tactile signals to cope with a large number of context related recruitment situations (Hölldobler and Wilson, 1978). Examples of recruitment mechanisms that fit in between the two extremes mentioned above have been reviewed by Hölldobler (1978). In examples where more than one gland is involved in recruitment, it is likely that more than one chemical or blend of chemicals is required; i.e. *Myrmica rubra* lays an odor trail with its poison sac contents and deposits attractive spots with the contents of its Dufour's gland (Cammaerts-Tricot, 1974).

S. invicta has a highly evolved and efficient mass recruitment system with

the Dufour's gland as the sole source of the chemicals responsible for releasing this behavior (Wilson, 1959, 1962a,b). *S. invicta* is, to our knowledge, the first example reported in which different chemicals produced in the same exocrine gland control subcategories of trailing behavior. The mass recruitment behaviors elicited by the Dufour's gland contents include attraction, orientation (Wilson, 1962c), and orientation priming (Vander Meer et al., submitted). In addition, depending on the context, Dufour's gland secretions can elicit colony emigration, alarm recruitment, and settling (Wilson, 1962c).

While it was clear that Dufour's gland secretions had multiple functions, it was not evident until this report that different chemical combinations originating from the same gland released different behaviors. This was because previous research on the trail pheromone of *S. invicta* did not differentiate between subcategories. The bioassay usually consisted of streaking test material on a piece of paper and then substituting it for a portion of an already established trail to a food source (Barlin et al., 1976; Jouvenaz et al., 1978; Vander Meer et al., 1981; Williams et al., 1981). This was a good bioassay that gave unambiguous results; however, only the orientation element of the total trail pheromone response was measured. This became clear when the activity of the orientation components was compared to Dufour's gland extracts in two bioassays designed to quantify the recruitment subcategory of the trail pheromone. These initial observations demonstrated that the chemicals isolated and identified as having orientation activity did not, by themselves, elicit recruitment.

In addition to attraction, the point source bioassay measured the settling or aggregation (Wilson, 1962c) behavior of the workers to Dufour's gland extracts or test samples. This bioassay could effectively test up to 10 samples at one time and was used to guide the chemical separation and isolation of the bioactive components. Initially, a combination of three classes of hydrocarbons (saturated, monoenes, and di- to tetraenes) was found to give the best bioassay results; however, quantitative analysis of the saturated hydrocarbon fraction showed that there were exceptionally large amounts of the species-specific saturated hydrocarbons (53 μg/WE) ubiquitous to *S. invicta* (Vander Meer et al., 1982). Previously Thompson et al. (1981) showed that three to four times less material (10–20 μg) released aggregation and biting behaviors in *S. invicta* workers. However, quantitative analysis of these same hydrocarbons from Dufour's gland extracts showed that the hydrocarbons were present at only 16–20 ng per worker. Bioassays of the active unsaturated classes of hydrocarbons, along with the saturated hydrocarbons at the low concentrations found in the Dufour's gland, did not enhance the activity of the unsaturated fractions. Consequently, we felt justified in removing the saturated hydrocarbons from consideration as components of the recruitment part of the trail pheromone.

Further point source bioassays and chemical separations led to: (1) the isolation of a single homosesquiterpene, C-1, with three rings and a single dou-

ble bond, that was present in minute amounts (ca. 75 pg/Dufour's gland WE) and (2) the previously isolated orientation pheromone, (*Z*,*E*)-α-farnesene (ca. 6 ng/Dufour's gland WE). The combination of the two isolated chemicals at physiological concentrations gave 85% of the recruitment activity of an equivalent amount of Dufour's gland extract. These results confirmed that different behaviors associated with the trail pheromone are elicited by different combinations of chemicals derived from the Dufour's gland.

The recruitment point source bioassay was very useful during the isolation and purification of the recruitment pheromone complex, due to its simplicity and reproducibility. However, this bioassay detected multiple behaviors (e.g., attraction, aggregation, and mastication). This problem was avoided by using a Y-tube olfactometer bioassay (Figure 1), which measured only worker attraction. The response of the ants to a mixture of C-1 and (*Z*,*E*)-α-farnesene was indistinguishable from the response to an equivalent amount of Dufour's gland extract (based on the amount of (*Z*,*E*)-α-farnesene). Therefore, this mixture is responsible for the attractive properties of the Dufour's gland contents. Interestingly, the two homofarnesenes did not enhance the attractive qualities of C-1, which indicates an as yet unknown function for these compounds.

The chemistry of ant trail pheromone systems has been the subject of research for many years (Attygalle and Morgan, 1985). Several leaf-cutting ants use methyl 4-methylpyrrole-2-carboxylate produced in their poison gland (Riley et al., 1974). In another *Atta* and several *Myrmica* species, 3-ethyl-2,5-dimethylpyrazine (Cross et al., 1979; Evershed et al., 1982), is the trail following substance. Another pyrazine, 2,5-dimethylpyrazine is a trail following pheromone of *Tetramorium caespitum* (Attygalle and Morgan, 1983). The true trail pheromone of the pharoah's ant was identified as (+)-(3*S*,4*R*)-3,4,7,11-tetramethyltrideca-(6*E*,10*Z*)-dienal (faranal; Ritter et al., 1977). Another aldehyde, (*Z*)-9-hexadecenal is part of the trail pheromone complex of *Iridomyrmex humilus* (Van Vorhis Key and Baker, 1982). Several fatty acids found in the rectal fluid of *Lasius fuliginosus* (Huwyler et al., 1975) and *Pristomyrmex pungens* (Hayashi and Komae, 1977) have been demonstrated to be trail pheromones.

In all of the known examples cited above, a trail-following (i.e., orientation) bioassay was used to measure the ants' response to isolated chemicals. The results presented in this paper demonstrate the pitfalls inherent when a single bioassay is used to measure a complex behavioral event (i.e., trailing or mass recruitment) and that care must be taken to devise bioassays that clearly define the behavior of interest. We have shown for the first time that different exocrine products release different behaviors associated with mass recruitment and that subcategories of trailing behavior can be controlled by different blends of chemicals produced in the same exocrine gland.

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EFFECTS OF COTTON LEAF SURFACE ALKALINITY ON FEEDING OF *Spodoptera littoralis* LARVAE[1]

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Abstract—The high leaf surface pH in cotton (*Gossypium hirsutum*) var. Acala SJ2 was bioassayed against *Spodoptera littoralis* larvae. Weight gain and leaf consumption of the larvae feeding on leaves devoid of alkalinity, due to daily washing, were recorded. Untreated cotton, with a leaf surface pH of 9.5–10.0 was used as control. The gland exudates contained potassium and magnesium cations, and the gland surface and intergland leaf areas were rich in calcium and phosphorus and low in K or Mg. The role of this plant antibiosis in the insect–host-plant relationship is discussed.

Key Words—Leaf surface pH, *Gossypium hirsutum*, cotton, *Spodoptera littoralis*, Lepidoptera, Noctuidae, larval weight, leaf consumption, alkaline glands exudation, ion microanalysis.

INTRODUCTION

An alkaline leaf surface is an uncommon character in the plant kingdom. However, in cotton species and varieties (Smith, 1923; Oertli et al., 1977; Harr et al., 1980) and in a few other malvaceous species (Harr et al., 1984), a high pH, ranging from 9.0 to 11.0, has been recorded. This alkalinity originates from multicellular epidermal glands, exuding magnesium and potassium cations (Ellman and Entwistle, 1982). This alkalinity has shown antimicrobial activity by inactivating insect nuclear (Andrews and Sikorowski, 1973; Young et al., 1977) and cytoplasmatic (Ali and Sikorowski, 1986) polyhedrosis viruses

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applied to the plant, but the effect of this plant antibiosis against insects is unknown. Therefore, the feeding capacity of *Spodoptera littoralis* larvae on *Gossypium hirsutum* var. Acala SJ2 was recorded in comparison with the feeding on the same cotton devoid of alkalinity by leaf washing. In addition, the ion profile of the leaf surface was analyzed to characterize the phylloplane activity against the larva.

METHODS AND MATERIALS

The commercial cotton (*Gossypium hirsutum*) var. Acala SJ2 was planted in pots in a greenhouse at 25–35°C and 60–80% relative humidity with 16:8 hr light–dark photophase. A sand–peat mixture, 1:1, fortified weekly with an aqueous nutrient mixture was supplied to the plants. In the field the cotton was raised in an ARO experimental field plot in Bet Dagan.

Leaf washing to eliminate the leaf surface alkalinity was carried out as follows: potted cotton plants were bent aside to be dipped in 0.06% aqueous polyoxyethylene sorbitan monooleate (Tween 80) solution for 15 min. The solution was stirred to increase the washing action on the leaves. Then the plants were washed with glass-distilled water and dried with an air blower. Unwashed leaves were used as the control.

The pH of the washed and the unwashed leaves was measured on both leaf surfaces by means of Radiometer electrodes G242C and K401 connected to a portable pH meter. The intact leaf was fixed flat, 300 ml bidistilled water was pipetted on the leaf surface, and the electrodes were immersed in the water, touching the leaf. The pH was recorded after 5 min. Since 10 min of recording did not increase the pH by more than 20%, the recording time throughout the work was exactly 5 min. The same pH reading technique on intact leaves was used in the laboratory and in the field. Four leaves sampled in three different plants in a single pot were used for pH measurements in a bioassay.

Larvae were obtained from an insect colony bred on an alginate diet (Navon, 1985). Neonates were caged singly on the washed and on the control plants, placed in the greenhouse (30–35°C; 16:8hr light–dark photophase. The larvae were transferred daily onto freshly washed and onto untreated plants. The pH measurements 24 hr after washing showed that the surface alkalinity was not restored. The cage was made of two glass cylinders (15 mm diam. × 5 mm). The outer side of each of the cylinders was closed with a 300-mesh metal screen used for ventilation. The two cylinders were held together on both sides of the leaf by a hair clip. Similarly, third-instar larvae weighing 8 mg were caged by two plastic cylinders (32 mm diam. × 18 mm), closed with a 150-mesh metal screen (Navon et al., 1987). The two units were held together by an uncoiled wire clip. The feeding period was four, five, and 7 days, after

which the unconsumed leaf area of the cage diameter was cut and measured with a Li-Cor area meter, model 3100 (Li-Cor, Inc., Lincoln, Nebraska. The leaf area from cages without larvae was used as the control. The leaf area consumed by the larvae was determined by subtracting the leaf area which remained after feeding from that of the control leaves. Between 10 and 15 larvae were used per experiment, with four or five replicates.

The leaf surface-ion profile was analyzed as follows: Young cotton leaves of potted plants with an average size of 22 ± 1 cm^2 were fixed in aqueous 3% glutaraldehyde solution, dehydrated in alcohol series, washed in acetone, and dried with CO_2 in a critical-point dryer. Then the leaf preparation was coated with gold. The alkaline glands were observed and counted in a Stereoscan 180 scanning electron microscope (SEM). Ion microanalyses of the glands' content and surface were conducted by an energy dispersing spectra (EDS) technique using a Proxan cation analyzer (Elscint, Haifa, Israel).

RESULTS AND DISCUSSION

Table 1 shows that the high pH of the leaf surface of cotton in the field was similar to that of the greenhouse plants: both were close to pH 10 on the leaf underside. Washing the leaf caused a drop in the surface pH by 2.7 units. For comparison, in another malvaceous species, *Hibiscus rosa-sinensis*, the leaf surface pH was below neutral.

Table 2 shows that the weight of the neonates was doubled when they fed for seven days on washed leaves in comparison to feeding on untreated cotton.

TABLE 1. LEAF SURFACE pH OF COTTON DEVOID OF ALKALINITY FOLLOWING WASHING[a]

Plant species	Leaf treatment	pH units (means ± SE) of leaf surface	
		Upper side	Under side
G. hirsutum var. Acala SJ2			
Greenhouse plants	Washing	7.1 ± 0.4b*	7.2 ± 0.4b
	Control	9.8 ± 0.2a	9.9 ± 0.2a
Field plants	Washing	7.2 ± 0.6b	7.4 ± 0.4b
	Control	9.4 ± 0.2a	9.9 ± 0.2a
Hibiscus rosa-sinensis	Washing	6.7 ± 0.1c	6.3 ± 0.1c
	Control	6.8 ± 0.04c	6.5 ± 0.1c

[a] Within each column, means followed by a common letter do not differ significantly ($P < 0.05$), according to ANOVA and Duncan's new multiple-range test.

TABLE 2. EFFECT OF DAILY WASHING OF COTTON LEAVES ON *Spodoptera littoralis* LARVAL WEIGHT GAIN AND CONSUMPTION[a]

Leaf treatment	Feeding period (days)	Larval weight gain (mg ± SE)	Leaf area consumed (cm^2 ± SE)
Neonate larvae			
Washing	7	5.1 ± 0.7a	6.5 ± 0.4a
Control	7	2.5 ± 0.3b	3.5 ± 0.4b
Third-instar larva			
Washing	3	36.0 ± 3.3c	5.2 ± 0.4c
Control	3	27.0 ± 3.0c	4.1 ± 0.6c
Washing	4	50.5 ± 3.6b	7.0 ± 0.4b
Control	4	40.8 ± 4.5b	5.2 ± 0.7c
Washing	7	72.0 ± 6.8a	11.8 ± 0.9a
Control	7	57.7 ± 4.9b	7.7 ± 0.7b

[a] Within each column, means followed by a common letter do not differ significantly ($P < 0.05$), according to ANOVA and Duncan's new multiple-range test.

Feeding capacity was increased by 1.8 times on the washed cotton. Similarly, third-instar larvae grew significantly faster on the cotton with the neutral pH within at least seven days. At shorter feeding periods, this pH effect was not significant. This experiment shows that, for both larval stages, the cotton leaf from which the alkalinity has been washed off was more acceptable than the control cotton pH close to 10.0.

Figure 1 shows that the ion content in the exudates released from the gland punctured by the SEM electronic beam, was rich in potassium and magnesium and poor in calcium, with twice as much K as Mg. On the gland surface, phosphorus content was markedly higher than that of Ca, K or Mg. On the intergland leaf surface, Ca was most abundant, whereas potassium and magnesium were very low. Sulfur was equally represented in all the ion analyses. In cv. Deltapine 16, raised in an aqueous medium (Ellman and Entwistle, 1982) phosphorus was missing, but the ion profile of the glands edudates was as in Acala SJ2.

Potassium and magnesium are abundant elements in plant tissues. These cations, more than others, are required in artificial diets by phytophagous insects (Dadd, 1970). In a suitable *S. littoralis* diet (Levinson and Navon, 1969), K and Mg were major elements in the mineral mixture (Beck et al., 1968). Therefore, it is expected that the larvae will accept high amounts of the two cations offered at neutral pH, but would tolerate less the cations in an alkaline milieu, such as the cotton leaf surface. SEM observations revealed that the glands 20–30 μm in size were evenly distributed on the leaf surface and veins, and the mean distribution of these glands was 0.118 ± 0.02 (SE) per mm^2 leaf surface.

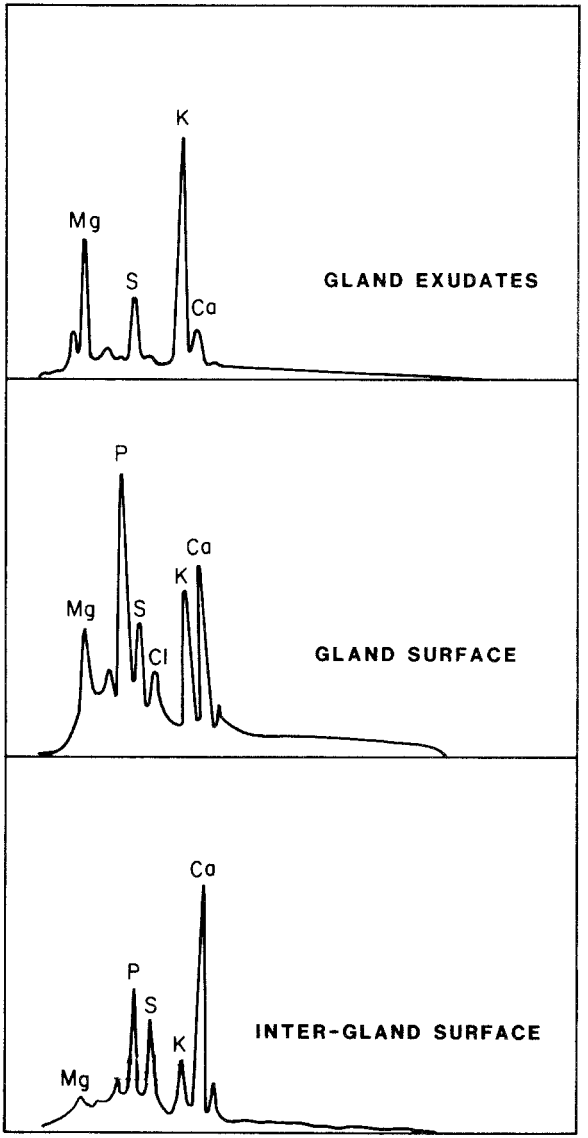

FIG. 1. Ion microanalysis of gland exudates, gland surface, and intergland surface in cotton (*Gossypium hirsutum*) var. Acala SJ2.

The leaf area consumed by the neonate at a single meal was 0.2 ± 0.03 mm^2, an area larger than the distance between glands. This explains the observation that the neonates could not avoid ingesting the glands. In spite of the high leaf-surface pH, *S. littoralis* is regarded as a serious cotton defoliator. Nevertheless, it seems that the alkaline phylloplane, in addition to other plant antibioses, could prolong the larval period on this crop. This leaf surface effect may be increased, even beyond that recorded in commercial cotton; a leaf surface pH of 11.0 was determined in the malvaceous species *Kitaibelia vitiifolia* (Harr et al., 1984). Possibly, cotton cultivars with similar phylloplane alkalinity could be selected to increase cotton resistance to larval pests.

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EFFECTS OF THREE PHENOLIC COMPOUNDS ON *Lemna gibba* G3[1]

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Abstract—*Lemna gibba* L. G3, (duckweed) was used as a bioassay organism to test the allelochemical effects of salicylic acid (SA), ferulic acid (FA), and umbelliferone (UM). Growth rate (K), dry weight (DW) and total chlorophyll (CHL) production were measured after seven days of growth. The bioassay procedure used 50 ml of E medium with and without sucrose in 125-ml Erlenmeyer flasks plus the selected concentration of allelochemical. At concentrations of 50 μM and greater, SA caused inhibition of K and DW production in *L. gibba* G3, while the threshold for CHL reduction was 20 μM. FA inhibited the DW and CHL production at 100 μM when the compound was autoclaved in E medium containing sucrose. Treatments of UM were least toxic with an inhibition threshold of 500 μM for K and DW production in medium without sucrose. UM did not reduce CHL production until 750 μM. In some cases, different thresholds of inhibition were observed depending on the presence or absence of sucrose and tartaric acid in the medium, and whether or not the chemicals were autoclaved with the medium.

Key Words—Allelopathy, allelochemical, duckweed, *Lemna gibba*, salicylic acid, ferulic acid, umbelliferone, growth rate, chlorophyll.

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INTRODUCTION

Allelopathy, or the biochemical interaction between all types of plants including microorganisms (Rice, 1984), produces marked impacts in a variety of terrestrial and aquatic ecosystems (Mandava and Rao, 1984; Putnam, 1983). The specific chemicals involved in many allelopathic situations remain obscure, but allelochemical activity has often been attributed to several secondary plant products such as simple phenolic acids, coumarins, terpenoids, flavonoids, alkaloids, cyanogenic glycosides, and glucosinolates (Putnam and Duke, 1978). Acid phenolics, such as salicylic acid, have long been recognized as plant growth inhibitors (Leopold, 1964). The series of cinnamic acids are also common inhibitors, both as the free acids and the closely related lactones. Abundant among these are ferulic acid, which belongs to the series, and the naturally occurring coumarin, umbelliferone, which is one of the lactone group (Robinson, 1980). Some of the natural inhibitors may also increase growth through a synergistic effect promoting auxin-induced growth at low concentrations (Leopold, 1964). However, the mode of action of the numerous potential allelochemicals may not be the same (Einhellig and Rasmussen, 1979).

Currently, there is considerable interest in developing strategies for capitalizing on allelopathy in agricultural production either by avoiding negative impacts or by exploiting allelopathic mechanisms for additional pest control and in approaches to growth regulation (Putnam, 1983). These challenges enhance the necessity for finding organisms that are sensitive and can be used for identifying allelochemical activity and mechanisms of action if they are not difficult to propagate in bioassays.

Previous work using *Lemna minor* 5 and *Lemna obscura* 7133 demonstrated that these two species provided sensitive bioassays of allelochemicals (Einhellig et al., 1985; Leather and Einhellig, 1985). Inhibition of growth and reproduction was detected in the range of 50–1000 μM for the allelochemicals tested. It was suggested that other *Lemna* species be evaluated for use as bioassays and that certain members of this genus have unique features that may be particularly appropriate for considering some mechanisms of allelochemical action.

Lemna gibba L. is among the largest of the duckweeds, and its fronds are characterized by being swollen or gibbous on the abaxial side. It has rapid growth, relative structural simplicity, and can be grown in aseptic culture which simplifies work with organic compounds (Hillman, 1961; Steinberg, 1943; Walsh and Palmer, 1979). As with the other Lemnaceae, it is easily cloned and can be maintained in controlled temperature, light, and nutritional conditions more easily than most other angiosperms. The plant consists of a frond with two reproductive pockets at the pointed or proximal end from which new fronds and a root are produced (Blodgett, 1904). The frond is formed by chlorenchy-

matous cells separated by large intercellular spaces that are filled with air and other gases responsible for the gibbosity (Blodgett, 1904; Hillman, 1961). The predictability of morphological and physiological characteristics suggests *L. gibba* would be suitable to be used as a bioassay organism.

The objective of this research was to study *L. gibba* G3 as a bioassay for allelochemicals and to consider any impact on chlorophyll production as a potential indicator of interference.

The known allelochemicals salicylic acid, ferulic acid, and umbelliferone were tested for effects on this species. Salicylic acid was included because others (Cleland and Ajami, 1974; Cleland and Tanaka, 1979, 1982; Tanaka et al., 1979) have reported its alteration of growth and flowering in *L. gibba* G3.

METHODS AND MATERIALS

Culture Conditions. The aquatic duckweed, *Lemna gibba* L. strain G3 maintained aseptically, was used exclusively in this research. All cultures were grown in cotton-stoppered 125-ml Erlenmeyer flasks with 50 ml of E medium (Cleland, 1979), prepared at a pH of 4.6, and sterilized by autoclaving (15–20 min) Clones containing a total of 12 fronds were transferred to each flask using a culture loop. The cultures were allowed to grow for seven days in an environmental chamber at 28°C under constant (236 μE/sec/m^2) fluorescent and incandescent light.

The allelochemicals, salicylic acid (SA; 2-hydroxybenzoic acid), ferulic acid (FA; 4 hydroxy-3-methoxycinnamic acid), and umbelliferone (UM; 7-hydroxycoumarin), were added as the dry chemical to the medium in the first experiments prior to autoclaving and in later experiments after autoclaving. Four replications in a completely randomized design were prepared for each treatment.

To determine the effects of organic amendments on the response of *L. gibba* G3 to allelochemicals, E medium with and without sucrose and tartaric acid was prepared with known concentrations of the allelochemicals and autoclaved. Cultures were prepared using plants grown in complete E medium. In another experiment, E medium with and without sucrose was prepared and autoclaved, then the allelochemicals were added to the medium after autoclaving. In order to test the effects of prior conditioning of the plants, half the cultures were prepared using *L. gibba* G3 that had been grown for a minimum of two weeks in complete E medium; the other half of the cultures utilized plants previously grown without sucrose and tartaric acid. Controls were cultured according to each experimental protocol but without the allelochemical.

Data Collection. Cultures were inspected daily and, on day 7, fronds were

removed from the flasks, counted, each placed in a test tube, and the CHL extracted. The fronds were immersed in 5 ml of 95% ETOH for 24 hr, then the ETOH–CHL solution was decanted into a second container. The fronds were soaked again twice in fresh ETOH for 2-hr periods. Solutions were combined from each extraction, most of which occurred in the first 24 hr, and adjusted to a final volume of 20 ml. The ETOH–CHL solutions were kept in darkness at room temperature and, immediately following the extraction sequence, absorbances (A) of each CHL extract were read using spectrophotometry at 665 and 649 nm and values for CHL-a, CHL-b, and total CHL were determined (Wintermans and DeMots, 1965; Einhellig and Rasmussen, 1979).

The fronds were allowed to air-dry in a hood at room temperature for one week, then weighed (DW). The growth rate (K) was calculated according to Hillman (1961):

$$K = \frac{\log_{10}(F_d) - \log_{10}(F_0)}{d}$$

where F_0 is the initial frond number and F_d the final frond number obtained from the count on day 7, and d the number of days.

All experiments were repeated at least once. The results were analyzed by the Student's t test.

RESULTS AND DISCUSSION

Lemma gibba G3 showed high sensitivity to SA. In preliminary range-finding tests in which concentrations between 100 and 1000 μM were applied, an almost complete inhibition of frond production, indicated by the growth rate (K), and biomass production (DW) was observed in E medium, with and without sucrose (data not presented). Daily inspection of the cultures showed that 24 hr after treatment, all the fronds exhibited bleaching or the loss of CHL. Four days after treatment, there was a small degree of recovery detected at 100 and 500 μM SA. A few daughter fronds began to grow, and several morphological changes were apparent. Plants were small in size, different in shape, had brown-yellow edges, and very small (<0.5 cm) or no roots. This growth might have occurred because the uptake of SA is rapid and linear for at least the first 24 hr (Ben-Tal and Cleland, 1982). These investigations determined that there was virtually no transfer of SA from the mother to daughter fronds, indicating that the SA was apparently sequestered within the cells. In this way the oldest generation absorbs some of the compounds, thus lowering its concentration in the medium and allowing the daughter fronds to resume some growth.

Results of experiments to test the effects of allelochemicals on *L. gibba*

G3 in E medium with and without organic constituents are shown in Table 1. The threshold for inhibition of all measured attributes was reached at a lower concentration with SA than with FA or UM. In cultures containing sucrose and tartaric acid, inhibition was typically greater. The inhibition threshold was lower for total chlorophyll production, where a 50% reduction occurred with 20 μM SA compared to a 70 μM threshold for those plants tested in medium without organic amendments. The threshold for FA-induced chlorophyll reduction was also lower in cultures with organic amendments; 100 μM vs 750 μM without amendments. These differences between the two culture media are contrary to

TABLE 1. ALLELOPATHIC EFFECTS OF SALICYLIC ACID, FERULIC ACID, AND UMBELLIFERONE ON GROWTH RATE (K), DRY WEIGHT (DW), AND TOTAL CHLOROPHYLL (CHL) PRODUCTION OF *Lemna gibba* G3[a]

Treatment (μM)	Percent					
	K		DW		CHL	
	W/OS[b]	W/S[c]	W/OS	W/S	W/OS	W/S
Salicylic acid						
0	100.0	100.0	100.0	100.0	100.0	100.0
20	103.0	93.4	98.3	71.8	96.0	50.2*
50	105.9	86.8*	85.8*	54.6*	81.5	43.6*
70	101.4	84.2*	68.9*	49.7*	63.2	29.4*
100	80.3*	72.5*	60.8*	38.9*	36.2*	20.5*
150	7.5*	12.9*	12.3*	5.7*	3.8*	1.6*
Ferulic acid						
0	100.0	100.0	100.0	100.0	100.0	100.0
100	113.0*	109.7	104.3	79.3*	114.3	58.9*
500	107.3*	82.7*	96.4	58.4*	118.7	48.7*
750	74.5*	105.2	41.5*	73.9*	40.4*	55.0*
880	44.8*	32.8*	17.7*	12.4*	14.8*	7.1*
1000	14.7*	15.7*	13.6*	7.3*	4.0*	2.7*
Umbelliferone						
0	100.0	100.0	100.0	100.0	100.0	100.0
100	111.5	105.3	93.6	104.2	94.9	118.2*
500	114.9	95.8*	76.7*	103.5	89.8	116.5*
750	106.6	112.2	66.6*	97.5*	61.5*	103.1
880	93.7*	119.2	62.7*	104.0	42.0*	70.5*
1000	86.7*	80.2*	57.7*	47.2*	29.7*	28.1*

[a] Chemicals autoclaved with the growth medium. Means (as % of control) in a column for each chemical marked with an asterisk are different from control at 0.05 level according to Student's *t* test.

[b] Treatments prepared in E medium without sucrose and tartaric acid.

[c] Treatments prepared in E medium with sucrose and tartaric acid.

those found by Einhellig et al. (1985) using *L. minor* 5 in small volume bioassays for allelochemicals. Their results showed that culturing duckweed without organic amendments resulted in lower thresholds of inhibition. Perhaps the larger size of *L. gibba* G3 and the greater volumes of medium utilized in the tests presented here are factors that influenced these results. Unlike SA and FA, treatments of UM were most inhibitory (500 μM, DW) in cultures without sucrose.

At the lowest concentration of FA and UM tested, stimulation of K, DW, and CHL was apparent (Table 1). CHL production was significantly stimulated in sucrose cultures with 100 and 500 μM UM. It has frequently been reported that inhibitory allelochemicals are often stimulatory at very low concentrations (Rice, 1984). Leather and Einhellig (1985) reported stimulation of *L. obscura* 7133 by several allelochemicals.

In a few instances where tests were conducted in medium without sucrose, preconditioning of the *L. gibba* G3 for two weeks prior to the test affected the threshold of inhibition (Table 2). Using cultures taken from stock grown without organic amendments, the SA threshold for affecting DW and CHL was slightly lower than when the stock culture had been grown with sucrose and tartaric acid. Conditions of growth for *L. gibba* G3 prior to transferring to flasks with FA and UM did not appear to make a substantial difference in the inhibitory effects of these treatments. Likewise, stimulation of K was obtained with 500 μM UM under both culture preconditions.

Although the experimental design and conduct of tests at different times does not allow direct statistical comparisons, the apparent effect of autoclaving SA is noteworthy. The thresholds of inhibition were lower for K, DW, and CHL when tested without organic amendments in the first experiments (Table 1) than those obtained under similar conditions in the second experiment shown in Table 2 (from stock W/S). It is possible that decomposition by heat enhanced the effects of SA. Similar results were reported for gibberellic acid (GA_3) on *Lemna* (Pieterse, 1976). Further study to determine the action of heat on SA is necessary. Comparisons between Tables 1 and 2 show autoclaving caused no apparent effect on the action of FA. The threshold for inhibition of DW by UM was slightly lower when this allelochemical was in the medium during autoclaving. We attribute this lower threshold to greater solubility of UM resulting from the heat during autoclaving.

Morphological changes occurring at and above the threshold of inhibition were similar to those observed and previously described for the preliminary tests with SA. At 500 μM FA and UM, bleaching was apparent, but was not severe. The fronds were smaller than in controls, not well developed, and clumped (daughter fronds did not separate from mother fronds). Fronds were less gibbous and had shorter (0.5 cm or less) or no roots. At 1000 μM FA and UM, the pattern of injury was similar but much more severe than at 500 μM. The

TABLE 2. ALLELOPATHIC EFFECTS OF SALICYLIC ACID, FERULIC ACID, AND UMBELLIFERONE ON GROWTH RATE (K), DRY WEIGHT (DW), AND TOTAL CHLOROPHYLL (CHL) PRODUCTION OF *Lemna gibba* G3 AS INFLUENCED BY PREVIOUS STOCK CULTURE CONDITIONS.[a]

Treatment (μM)	Percent					
	From stock W/S[b]			From stock WO/S[c]		
	K	DW	CHL	K	DW	CHL
Salicylic acid						
0	100.0	100.0	100.0	100.0	100.0	100.0
20	107.6	89.1	71.3	93.0	95.3	101.3
50	103.7	89.0	85.8	97.4	89.7	91.9
70	95.1	88.3	70.1	102.4	83.2	95.2
100	105.6	81.6	109.0	102.8	63.2*	42.5*
150	74.8*	28.9*	20.8*	70.9*	34.6*	22.3*
Ferulic acid						
0	100.0	100.0	100.0	100.0	100.0	100.0
100	96.9	100.4	91.6	105.6	97.1	113.3
500	95.8	66.7	57.5	103.6	72.5*	80.1
750	74.4*	54.7*	42.8*	85.5*	53.0*	35.6*
880	58.9*	31.9*	18.4*	64.1*	39.6*	17.1*
1000	22.5*	11.3*	2.8*	53.3*	36.6*	10.7*
Umbelliferone						
0	100.0	100.0	100.0	100.0	100.0	100.0
100	107.5	103.0	114.1	109.0	101.8	106.7
500	113.2*	88.3	88.7	116.6*	89.4	88.2
750	118.8*	81.6	72.5*	114.5	81.6*	74.8
880	108.1	92.2	59.2*	105.9	63.5*	42.7*
1000	102.3*	67.0*	40.2*	101.5	52.3*	26.9*

[a] Treatments were made in nutrient culture without sucrose, and allelochemicals were added after autoclaving. Means (as % of control) in a column for each chemical marked with an asterisk are different from control at 0.05 level according to Student's t test.
[b] Treatments were prepared using stock plant material from cultures maintained with sucrose.
[c] Treatments were prepared using stock plant material from cultures maintained without sucrose.

colonies were more clumped than with 500 μM treatments. Nevertheless, morphological changes were less than the same concentrations of SA.

L. gibba G3 responses to the three allelochemicals (SA, FA, and UM) tested showed good sensitivity. The threshold levels for inhibition were at slightly higher concentrations than for other species of duckweed, but generally they are lower than for other bioassays commonly used in the study of allelopathy (Einhellig et al., 1985; Leather and Einhellig, 1985, 1986). SA was the most potent inhibitor of the three allelochemicals tested, causing growth alter-

ations at micromolar concentrations well below those observed for FA and UM. The action of SA diminishing CHL production was the most sensitive indicator. The three compounds generally produced similar responses in *L. gibba* G3 at the minimal concentrations at which they began affecting the plant for all the parameters tested. Responses were found to be more easily identified and consistent in all tests performed with E medium not enriched with sucrose and tartaric acid. The enrichment, however, sometimes resulted in more severe inhibition of *L. gibba* G3 by the allelochemicals. Although values for CHL-a and CHL-b were determined, there were no differences in threshold levels of inhibition from total CHL caused by allelochemicals tested, nor were there differences in the ratio of CHL-a to CHL-b (data not shown).

Because of its size and the volume of medium required for growth, *L. gibba* G3 was not suitable as a bioassay organism under the conditions reported earlier (Einhellig et al., 1985; Leather and Einhellig, 1985) but could be used in the further study of allelochemical interactions and mechanisms. Its physical characteristics of predictable size, shape, gibbosity, pigmentation, and root length exhibit adequate sensitivity to the effects of substances on the plant even before the analysis of data. Effects on CHL production are readily apparent by inspection, and quantification of CHL was easily obtained. Effects on CHL were the most sensitive indicator of the allelochemical effects in the case of SA.

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COMPARISON OF EARTHWORM- AND FISH-DERIVED CHEMICALS ELICITING PREY ATTACK BY GARTER SNAKES (*Thamnophis*)

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Abstract—Materials eliciting increased tongue flicking and prey attack in garter snakes were isolated from both earthworm and fish prey. New extraction methods based on chloroform–methanol mixtures are valuable adjuncts to the more typical aqueous preparations. Both high- and low-molecular weight components from earthworms and fish were active. The similarity between the active chemicals in these two classes of prey was established using several methods of analysis. These included chromatography, carbohydrate and amino acid analyses, and nuclear magnetic resonance.

Key Words—Chemoreception, garter snakes, *Thamnophis sirtalis*, chemical cues, vomeronasal system, *Lumbricus terrestris, Pimephales promelas.*

INTRODUCTION

The prey of garter snakes (*Thamnophis*) is quite diverse and includes earthworms, fish, slugs, leeches, salamanders, frogs, and rodents. However, some garter snake species specialize more than others on certain kinds of prey (Carpenter, 1952; Drummond, 1983), even when several prey classes are available in the habitat. The occurrence of such prey preferences is not limited to adult snakes but is also shown by ingestively naive newborn snakes.

Snakes of the genus *Thamnophis*, as well as many others, use chemical cues to discriminate among various prey items (Burghardt, 1970, 1980; Arnold,

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1981). Surface chemicals can be easily removed from various prey and used to elicit feeding responses. The most common method involves presenting aqueous prey extracts to snakes with cotton swabs and recording both the number of tongue flicks and the latency of any prey attack. Extensive comparative studies show that newborn snakes may also respond to a wide range of prey chemicals highly correlated with the normal prey preferences of the species in the laboratory and field. Several studies have addressed the sensory mechanisms by which snakes detect prey chemical cues and the tongue–vomeronasal system appears largely responsible (Burghardt, 1970; Halpern and Kubie, 1984).

The wide range of inter- and intraspecific variability in the chemical responsivity of snakes to various prey, coupled with the strong genetic component underlying neonatal preferences (Burghardt, 1969, 1970; Arnold, 1981), makes this system a prime candidate for the study of evolutionary mechanisms of prey preference and chemosensory releasing mechanisms, as well as the partitioning of feeding behavior among diverse prey by sympatric guilds of related snakes. To fully carry out this task, however, more precise information is needed on the specific chemicals in various prey that snakes use in selecting prey.

To date, the chemistry of prey recognition has been limited to analysis of aqueous extracts (warm water washes) of earthworms (*Lumbricus terrestris*). Sheffield et al. (1968) showed that the nonvolatile components were critical in eliciting prey attack. Fractionation using Sephadex chromatography provided active components in at least two different molecular-weight ranges: substances more than 5000 daltons and fractions (in the vitamin B_{12} marker range) of about 1350 daltons, although the larger component was more effective. More recently, Halpern et al. (1984) have also investigated earthworm extracts in an attempt to identify active materials. They unequivocally report only a single nonvolatile component with a molecular weight in excess of 65,000 daltons. Recently, they have shown the similarity of the chemical composition of this fraction to the collagen found in earthworm cuticle (Halpern et al., 1986; Kirschenbaum et al., 1986). These workers used, it should be noted, a bioassay based only on relative tongue-flick rate and duration measures, not the more definitive attack measure (snakes tongue flick in response to many kinds of stimuli, not just food-related ones).

The ability of garter snakes to discriminate among extracts from different prey implies that different cues are recognized. However, even within the same species, initial and experience-altered preferences show that individual snakes discriminate the chemical cues from different prey. In the present study, we compare the effective chemicals from two diverse prey: a fish, the fathead minnow, and the well-studied nightcrawler earthworm. We tested Eastern garter snakes, a species that eats fish and worms both in the field and in captivity. The main question we seek to answer here is how similar or different chemically are the effective chemical cues from such diverse prey taxa.

METHODS AND MATERIALS

Animals

Freshly obtained earthworms (*Lumbricus terrestris*) and fish (fathead minnows, *Pimephales promelas*) were used to obtain the extracts. The predator species were primarily wild-caught or laboratory-reared Eastern garter snakes (*Thamnophis sirtalis*) from Michigan (Wayne County) stock with a few tests also including local (Knox county, Tennessee) animals. All animals were tested in their home cages, which varied depending on their size from plastic boxes to 60-liter aquaria. Snakes were tested at a temperature of 25 $\pm$ 2°C.

Prey Extraction Procedures

Aqueous Prey Extractions. Preparation of aqueous prey extracts followed Burghardt (1969). We extracted prey animals for 2 min with a ratio of 3 g of prey to 10 ml of distilled H_2O at 60°C. The solution was decanted and centrifuged for 10 min at 2500 rpm. The aqueous extract was then frozen or lyophilized to preserve activity. The amount of material obtained from prey ranged from 2 to 3 g/liter. References to this aqueous extract obtained from the earthworm and fathead minnow will be referred to as AQ-EW and AQ-FM, respectively. In the codes for test extracts, the extraction procedure is indicated first with the type of prey extracted (EW or FM) listed after the dash.

Methanol–Chloroform Extractions of Aqueous Extract. The lyophilized aqueous extract (0.71 g) was extracted for 15 min with 100 ml of 1 : 4 methanol–chloroform. The solution was filtered and extracted with 100 ml of 1 : 1 methanol–chloroform. The two solutions were combined and evaporated to dryness (Wold and Selset, 1977). Approximately 6% and 23% by mass of the materials obtained from this AQmc extraction from earthworm and fathead minnow, respectively, were soluble in the methanol and chloroform extracts. From this extraction procedure, there were materials that were soluble and insoluble in the methanol–chloroform solutions. The soluble compounds were labeled $AQmc_{sol}$-EW and $AQmc_{sol}$-FM and the insoluble compounds $AQmc_{insol}$-EW and $AQmc_{insol}$-FM.

Methanol Extractions of Aqueous Extract. The lyophilized aqueous extract (1.75 g) was extracted for 15 min with 75 ml of methanol. The insoluble materials were filtered with a fine glass frit and extracted again in the same manner. The insoluble compounds were extracted four additional times for 10 min with 25 ml of methanol and filtered between each extraction. All the methanol extracts were combined and evaporated to dryness. Approximately 24% and 52% by mass of the material extracted was soluble in the methanol extractions from the earthworm and fathead minnow aqueous extracts, which are labeled AQm_{sol}-EW and AQm_{sol}-FM, respectively.

Chloroform–Methanol Prey Extractions. In this procedure, 1000 g of prey was extracted for 4 min at 23°C with a solution containing 2000 ml of methanol and 1000 ml of chloroform. The solution was decanted and the prey extracted again with 1000 ml of chloroform. After 4 min, the second solution was decanted from the prey, combined with the first, and filtered with a coarse glass frit to remove particulate matter. The filtered solution was poured into a 5-liter separatory funnel and extracted with 1000 ml of distilled H_2O. The resulting emulsion was allowed to stand at 4°C, during which time it separated into two layers. After two days, the biphasic solution was allowed to warm to room temperature. The water layer was removed and filtered with a medium glass frit. Both the water and organic layers were evaporated to dryness. About 3–5 g of material were obtained per kilogram of prey, and 31% and 40% by mass of these materials were obtained from the earthworm and fathead minnow water layers, respectively. This extraction procedure is referred to as the CM extraction. The materials from the organic layer were coded CMo-EW and CMo-FM. Those from the water were labeled as CMw-EW and CMw-FM.

Chromatographic Separations

All chromatographic separations, unless otherwise stated, were conducted on a low-pressure liquid chromatography system. The system included an FMI lab pump (model RP-SV), Laboratory Data Control refractive index detector (model 1107H), and a Scientific Manufacturing Industries, fraction collector (model 12050).

Bio-Gel P-10 and P-2, 50–100 mesh and Sephadex G-200 filtration gel, 40- to 120-μm particle size were utilized. For all gel filtrations, the column-packing material was swollen and eluted with 10% ethanol in distilled water. The column size used for the analytical separations was 1.1 × 30 cm, and for the preparative separation was 3 × 23 cm. The flow rates for the analytical and preparative columns were approximately 0.4 ml/min and 1.4 ml/min, respectively. The pressure in the system was maintained below 4 psi in all cases. The fraction collector was adjusted to collect fractions at time intervals of 5 min for the analytical separations and 15 min for the preparative separations. The sample injections ranged from 30 to 40 mg for the analytical separations, while 0.50 g was used for the preparative separations.

Sephadex A-25 (diethylaminoethane anion exchange resin), 40- to 120-μm particle size, was packed in an analytical column. The exchange resin was utilized in the chloride form. The column was eluted with water and a step gradient of NaCl solution from 0.25 M to 1.0 M in increments of 0.25 M. The flow rate was 1.0 ml/min.

An analytical silica gel column was packed with Silica Gel 60 (particle

size 0.04–0.063 nm) from EM Laboratories Inc. The column was filled with 17 g of silica and vibrated until the packing material finished settling. The column was packed and eluted with 3:2 methanol–chloroform by volume. The flow rate of the system was 0.5 ml/min.

Silica gel separations were also conducted with gravity eluted (3 × 75 cm) columns. Silica gel, 60–200 mesh (60 g) (J.T. Baker Chemical Co.) was packed with 3:2 methanol–chloroform. The sample (0.30 g) dissolved in methanol was then evaporated until dry with 5 g of silica applied to the top of the silica column and the column eluted with 3:2 methanol–chloroform. In the separation of the fathead minnow material, the column was subsequently eluted with 3:2 methanol–chloroform that was saturated with H_2O.

Silica Thin-Layer Chromatography (TLC). Silica gel G (type 60, EM Laboratories Inc.) was used. The TLC plates were eluted with 3:2 methanol–chloroform or 3:2 methanol–chloroform saturated with water. Separations were visualized using iodine vapor or by treating the plate with an alpha-naphthol–sulfuric acid solution (1.0 g of alpha-naphthol dissolved in 5 ml of concentrated sulfuric acid diluted after 24 hr with 95 ml of ethanol) to detect carbohydrates. After spraying with the alpha-naphthol–sulfuric acid solution, plates were heated in a drying oven at 140°C for 2 min. Carbohydrates were visualized as purple spots on the TLC plate.

Dialysis Procedure

Sixty-four centimeters of 6000–8000 molecular weight cutoff dialysis tubing was boiled for 10 min in 950 ml of 2% sodium bicarbonate with 1 mM of disodium ethylenediamine tetraacetate in water. The dialysis tubing was cooled to room temperature in the solution, and two knots were tied in the bottom of the tubing. Aqueous prey extract (18 ml) was poured into the dialysis tubing and then was enclosed by tying two additional knots at the upper end. The filled dialysis membrane was inserted into a beaker containing 100 ml of distilled water and allowed to stand at 4°C for 24 hr. The dialysis solution was changed every 24 hr for three days.

Analysis of Active Components

Molecular Weight Estimates. Materials that were not retained by the Bio-Gel P-10 gel filtration column were estimated for molecular weight on a Sephadex G-200 column (1.1 × 30 cm). G-200 resin, 40- to 120-μm particle size, was reconstituted and packed with water. A mixture of molecular weight standards was used for calibration (Bio-Rad). The molecular weight standards and samples were eluted with water at a flow rate of 3.2 ml/hr. The fractioning range of Sephadex G-200 for globular proteins is rated from 5000 to 600,000

daltons (Grodon and Richard, 1972). In this study the upper limit for fractionation was assumed to be 600,000 daltons; however, the lower limit was estimated to be 17,000 daltons since the 1350- and 17,000-molecular-weight markers had the same retention volume. A graph was plotted of $\log_{10}$ of the molecular weight marker versus its K_{av} value ($K_{av} = (V_e - V_0)/(V_t - V_0)$ where V_e is the elution of the molecular weight marker, V_0 is the void volume of the column, and V_t is the total volume of the column). A least-squares approximation was made to obtain the best linear correlation, and this was used as a calibration plot to estimate the molecular weights of the unknown samples.

Components that were retained by the Bio-Gel P-10 gel filtration column were calibrated for molecular weight on a Bio-Gel, 50–100 mesh, P-2 column (1.1 × 30 cm). The column was packed with water, and the flow rate for the separation was 1.1 ml/min. The low-molecular-weight samples and the gel filtration standards that were used to calibrate the G-200 column were injected and eluted with water. Bio-Gel P-2 has the fractioning range of globular proteins of 100 to 1800 daltons. No calibration plot was obtained from the Bio-Gel P-2 column since sufficient standards were not available.

Sugar Analysis. Samples analyzed for carbohydrates were derivitized by the following method: 2.0 ml of aqueous 2 N trifluoroacetic acid was added to 30 mg of sample and the solution heated for 2 hr at 120°C. The hydrolyzed solution was evaporated to dryness and treated with 3 ml of water. The water was evaporated to dryness, and this procedure was repeated twice. The sample was reconstituted with 3 ml of water containing 25 mg of $NaBH_4$ and stirred for 16 hr. The $NaBH_4$ solution was acidified with Dowex 50W-X2 to pH 2 and filtered with a fine glass frit. The water from the filtrate was removed by codistillation with four 5-ml portions of methanol. Next, 3 ml of a 1 : 1 mixture of pyridine and acetic anhydride was added and the sample stirred for 16 hr. The pyridine, excess acetic anhydride, and other by-products of the reaction were removed from the sample by codistilling with four 5 ml portions of toluene. With each codistillation, the mixture's concentration was increased, finally evaporating the sample to dryness. The mixture was reconstituted with 300 μl of methylene chloride for gas chromatography (GC) and gas chromatography–mass spectrometric (GC-MS) analysis (Jansson et al., 1976).

Standards of sugars were derivitized in the same way as the samples analyzed for carbohydrates except that the hydrolysis with 2 N trifluoroacetic acid was omitted. The following sugars were used as standards in the amounts indicated: glucose (4.8 mg), galactose (2.0 mg), mannose (1.8 mg), xylose (4.1 mg), arabinose (1.9 mg), and myoinositol (2.2 mg). The standard was reconstituted with 900 μl of methylene chloride for GC and GC-MS analysis.

Gas chromatography was conducted with 0.4-μl sample injections using a 1.8-m × 2-mm-ID glass column containing 3% SP-2330 on 100–120 Supel-

coport. The samples were injected at an initial temperature of 180°C with an initial temperature hold of 6 min. The temperature ramp rate was 2°C/min to a final temperature of 240°C. The nitrogen carrier gas was at a flow of 20 ml/min, and a flame ionization detector was employed. The identity and concentrations of the unknown sugars were determined by comparing retention times and peak areas to known sugar standards of known concentrations.

GC-MS analysis was employed to confirm the identity of the acetylated sugars using Hewlett Packard models 5710H GC and 5980H MS. The same type of column and separation parameters were utilized for the GC-MS as for the GC separations with a helium flow rate of 25 ml/min and sample size of 10 μl. Electron impact was used at an ionization potential of 25 eV.

Amino Acid Analysis. Samples for amino acid analysis were reconstituted with water at a concentration of 1.0 mg/ml. An estimated 10 nm of compound from the 1.0 mg/ml sample was inserted into a small vacuum vial along with constant boiling HCl (0.4–1.0 ml). The resulting solution was frozen with liquid nitrogen and vacuum sealed. The vial was heated to 105°C and after 23 hr the sample was frozen and the vial opened and dried under vacuum over KOH. The sample was analyzed by the Analytical Services Facility at the University of Tennessee, Knoxville, using a reverse-phase column with a high-pressure liquid chromatograph. A precolumn derivitization method was used with phenol isothiocyanate for detection at 254 nm. A 0–60% acetonitrile gradient program was run at 37°C.

Nuclear Magnetic Resonance. Proton and carbon-13 nuclear magnetic resonance (NMR) of mixtures were obtained using a NT 200-WB Nicolet NMR. All samples were submitted for analysis in D_2O with acetonitrile as the reference. Presaturation experiments were conducted with all samples to reduce the HOD peak found in all proton magnetic resonance spectra.

Bioassay Procedures

The number of snakes tested in each bioassay varied between five and 12, depending on the number of extracts and the number of responsive snakes available for testing. Typically each snake was tested only once on each stimulus in a given bioassay session, in a counterbalanced fashion. Occasionally, when fewer snakes were available due to heavy recent feeding, illness, or skin shedding, suitable snakes were tested twice on each stimulus but with reversed stimulus orders. Snakes were never run on a bioassay more than once a week, usually the day before their weekly scheduled feeding.

After several bioassays, the snakes were screened a few hours before the bioassay by testing their responsiveness to aqueous extracts of the prey chemical stimuli being tested in that bioassay. Such screening resulted in a more discrim-

inating bioassay, since nonresponsive snakes were eliminated and were thus excluded from the statistical evaluation. It was also found that the snakes were more responsive if they were fed on half food rations one to two weeks before the bioassay. The food rationing technique usually resulted in a three-week feeding schedule where the snakes were given all the food they could eat every third week, and their food supply was reduced by half on the first and second weeks.

All extracts were reconstituted in distilled H_2O to a concentration representing, as nearly as possible, that found in the original extracts, less estimated losses on columns, etc. The number of stimuli in each bioassay ranged from three to seven, always including a distilled-water control and an AQ or CM extract of the prey being tested. All tests were conducted blind.

In the bioassay, a 15-cm wooden cotton-tipped swab was dipped in the extract and introduced into the snake's home cage about 2 cm from its head. It remained in that position as closely as possible (by following snake movements) for 30 sec or until the snake gave an open-mouth attack. The number of tongue flicks directed towards and away from the swab and the latency of any attacks were recorded. If the snake did not tongue flick in 10-15 sec after being presented with a stimulus, the end of the swab was brushed lightly on the snake's back to induce the snake to emit at least one tongue flick. For any bioassay series, the time between tests was kept constant, usually about 10-15 min and never less than 7 min. This basic methodology has been used in many studies (e.g., Sheffield et al., 1968; Burghardt, 1969, Arnold, 1981).

The working definition of an active component was a compound or mixture of compounds that both (1) elicited a minimum of two prey attacks by two different garter snakes of the same species and (2) exhibited a tongue flick–attack score statistically greater than water. Due to an initial shortage of garter snakes, the first criterion of this definition was relaxed if a replicate test series demonstrated statistical significance using the tongue flick–attack score and a subsequent bioassay based on the results led to satisfaction of the first criterion.

Statistical analysis to distinguish active from inactive stimuli was accomplished by comparing the extract data to distilled water controls and to the AQ or CM extract. The water control represented no activity, whereas the extract represented strong activity. The basic data for the statistical analyses were the number of attacks, attack latency time, and the number of tongue flicks each snake exhibited toward each stimulus. These were combined into a tongue flick–attack score (Burghardt, 1969) in which any attack received a higher score than any number of tongue flicks.

A two-way analysis of variance (repeated measures—stimulus $\times$ subjects) was run on the data for all the extracts tested in the bioassay excluding data from snakes that neither attacked any stimulus nor gave at least five tongue flicks toward at least one stimulus. If the variances were not homogeneous

(Hartley's F-Max test), a square root transformation was performed before all parametric tests. A nonparametric Friedman's two-way analysis of variance (ANOVA) was also performed. If the overall ANOVAs were significant, planned comparisons of prey extracts with water were made. A nonparametric Wilcoxon signed rank-matched pairs test (or a dependent *t* test if the number of subjects was six or fewer) was calculated. One-tailed *P* values are presented since, in all cases, only an increase in responsivity over water was being evaluated. All statistical calculations were performed using the Statgraphics package (Version 1.1) on an IBM-PC.

RESULTS

Only bioassay results for the major separations on which final conclusions are based will be detailed.

Separations of Earthworm Extracts

The lyophilized aqueous earthworm extract was separated on an analytical Bio-Gel P-10 gel filtration column which fractionates globular proteins from 1800 to 20,000 daltons (Gordon and Richard, 1972). However, this extract contained materials that did not pass through the column and prevented its reproducible and repeated use. In an attempt to remove the contaminants before the separation, the lyophilized material (AQ-EW) was extracted with methanol and chloroform to obtain $AQmc_{sol}$-EW. This extract was moderately active.

The material that was insoluble in the methanol–chloroform extract ($AQmc_{insol}$-EW) was separated on the P-10 gel filtration column and two major fractions of compounds were obtained. The first fraction was a multicomponent mixture ($AQmc_{insol}$-EW-HMW_{P10}) eluting close to the void volume of the column, indicating a molecular weight of at least 20,000 daltons or greater. The second fraction contained a mixture of compounds ($AQmc_{insol}$-EW-LMW_{P10}) that eluted close to the column volume, indicating a molecular weight of approximately 1800 daltons or less. Both fractions were active, although the low-molecular-weight fraction was shown to be active only for the second test.

Since it was found that the methanol–chloroform extracts from the lyophilized aqueous extracts ($AQmc_{sol}$-EW) were active, another extraction method was developed using methanol as the extraction solvent. Methanol was chosen since it was assumed that the active components were polar because of their solubility in water. The materials extracted by methanol from the lyophilized aqueous extracts were coded AQm_{sol}-EW and were active, as seen in Table 1.

The materials obtained from AQm-EW were separated on the Sephadex A-25 column. The column was injected with 0.47 g of material that had been reconstituted with water. All activity occurred in the material eluting at the void

TABLE 1. BIOASSAY RESULTS OF METHANOL EXTRACTS OF AQ-EW[a]

	Stimuli[b]		
	Water	AQ-EW	AQm_{sol}-EW
Mean tongue flick–attack score	21.00	51.84	53.40
Statistical comparison to water[c]		$t = 2.46$	$t = 2.90$
		$P < 0.05$	$P < 0.05$
Number of attacks	1[d]	4	3

[a] Six *Thamnophis sirtalis* were tested once each on six different stimuli. (One snake omitted.)
[b] Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 10.89$, $df = 20$, $P = 0.05$; $F = 4.00$, $df = 5, 20$, $P < 0.05$.
[c] Dependent t test, $df = 4$.
[d] Subject previously attacked the five other stimuli in the bioassay.

volume of the column, which was clearly active. Of eight snakes tested, five attacked the swab (as compared to eight attacks to the AQ-EW). The first fraction, which included all of the compounds eluting at the void volume of the column, contained 71% by mass of the materials injected on the column.

The active material obtained from AQm_{sol}-EW was applied to a silica low-pressure liquid chromatography column. No active fractions could be obtained when eluting with 3:2 methanol–chloroform. In view of later results it was assumed that the active components from the extract were destroyed by the acidic packing material or adhered to the column.

Since it was observed that some active components from the lyophilized aqueous extract of the earthworm could be extracted with methanol, a procedure was sought that would directly extract the prey with organic solvents. A procedure of Blight and Dyer (1959) fulfilled these requirements, and our adaptation of this procedure is referred to as the CM extraction.

Extraction of the prey with organic solvents followed by extraction of the organic layer with water provided active material easier to chromatograph. The organic layer from the chloroform–methanol prey extract (CMo-EW) contained no activity (Table 2), whereas the water layer was very active. The compounds obtained from the water layer were separated on the preparative Bio-Gel P-10 column into two fractions: a multicomponent high-molecular-weight fraction ($CMw\text{-}EW\text{-}HMW_{P10}$) eluting at the void volume of the column and a low-molecular-weight fraction ($CMw\text{-}EW\text{-}LMW_{P10}$) eluting approximately at the volume of the column. Both the low- and high-molecular-weight fractions were active, as seen in Tables 2 and 3.

TABLE 2. BIOASSAY RESULTS OF CM EXTRACTIONS OF EARTHWORMS: PART I[a]

	Stimuli[b]			
	Water	AQ-EW	CMw-EW-HMW_{P10}	CMo-EW
Mean tongue flick–attack score	6.43	29.37	24.70	6.43
Statistical comparison to water[c]		$Z = 2.28$	$Z = 2.11$	$Z = 0.08$
		$P < 0.02$	$P < 0.02$	not significant
Number of attacks	0	6	5	0

[a] Seven *Thamnophis sirtalis* were tested once each on seven different stimuli.
[b] Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 20.22$, $d = 36$, $P = 0.005$; $F = 7.39$, $df = 6, 36$, $P < 0.0001$.
[c] Wilcoxon signed rank-matched pairs test, $N = 7$.

Separations of Fathead Minnow Extracts

Fathead minnows were extracted as described in Sheffield et al. (1968). As in the case of the earthworms, chromatographic separation of this material was complicated by column clogging, and it was submitted to many of the same procedures to provide workable materials. Application of trhe AQmc extraction to the fathead minnow lyophilized aqueous extract provided soluble ($AQmc_{sol}$-FM) compounds that were active (Table 4). Chromatography was conducted on the insoluble material in the chloroform–methanol extractions ($AQmc_{insol}$-EW) using a Bio-Gel P-10 analytical gel filtration column and two fractions were obtained. A high-molecular-weight fraction ($AQmc_{insol}$-FM-HMW_{P10}) eluted at the void volume of the column, and the low-molecular-

TABLE 3. BIOASSAY RESULTS OF CM EXTRACTIONS OF EARTHWORMS: PART II[a]

	Stimuli[b]		
	Water	AQw-EW	CMw-EW-LMW_{P10}
Mean tongue flick–attack score	6.40	37.01	25.54
Statistical comparison to water[c]		$Z = 2.65$	$Z = 2.29$
		$P < 0.005$	$P < 0.02$
Number of attacks	0	6	4

[a] Ten *Thamnophis sirtalis* were tested once each on five different stimuli.
[b] Square-root transformation on all data for parameter statistics. Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 15.06$, $df = 36$, $P = 0.005$; $F = 10.13$, $df = 4, 36$, $P < 0.0001$.
[c] Wilcoxon signed rank-matched pair test, $N = 10$.

TABLE 4. BIOASSAY RESULTS OF HIGH- AND LOW-MOLECULAR-WEIGHT COMPONENTS FROM AQmc-FM EXTRACT[a]

	Stimuli[b]				
	Water	AQ-FM	$AQmc_{sol}$-FM	$AQmc_{insol}$-FM-HMW_{P10}	$AQmc_{insol}$-FM-LMW_{P10}
Mean tongue flick–attack score	8.88	53.00	49.35	34.98	18.63
Statistical comparison to water[c]		$t = 4.56$ $P < 0.01$	$t = 8.27$ $P < 0.002$	$t = 2.57$ $P < 0.05$	$t = 2.28$ $P < 0.05$
Number of attacks	0	6	7	3	0

[a] Five *Thamnophis sirtalis* were each tested twice on five different stimuli; one subject omitted. Scores on both tests were combined for each snake.
[b] Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 12.60$, $df = 12$, $P = 0.01$; $F = 9.05$, $df = 4, 12$, $P < 0.001$.
[c] Dependent t test, $df = 3$.

weight fraction ($AQmc_{insol}$-FM-LMW_{P10}) eluted at the column volume. Both the high- and low-molecular-weight fractions were active (Table 4), although the low-molecular-weight fraction was less stimulating than the high-molecular-weight compounds.

The methanol extract made from the fathead minnow lyophilized aqueous extract (AQm-FM) was also active (Table 5) and was separated on a Sephadex A-25 column in the same manner as the earthworm AQm-EW material. Not all fractions were tested for activity—only the ones that corresponded to active

TABLE 5. BIOASSAY RESULTS OF A-25 CHROMATOGRAPHY OF AQm-FM[a]

	Stimuli[b]			
	Water	AQm-FM	AQm_{sol}-FM	AQm-FM void vol_{A25}
Mean tongue flick–attack score	10.25	58.40	43.20	41.10
Statistical comparison to water[c]		$t = 4.84$ $P < 0.01$	$t = 2.81$ $P < 0.05$	$t = 2.28$ $P = 0.05$
Number of attacks	0	3	2	2

[a] Five *Thamnophis sirtalis* were each tested once each on five different stimuli; one subject omitted.
[b] Square-root transformation on all data for parameteric statistics. Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 10.38$, $df = 12$, $P < 0.05$; $F = 5.26$, $df = 4, 12$, $P = 0.01$.
[c] Dependent t test, $df = 3$.

regions from the earthworm A-25 separation. The active compounds (AQm-FM-void vol_{A25}) from the fathead minnow were eluted at the void volume of the column and amounted to 64% by mass of the compounds that were injected.

The soluble material obtained from the extraction (AQm_{sol}-FM) was separated on a low-pressure liquid chromatography silica gel column. The extract (150 mg) was reconstituted with 4 ml of 3:2 methanol–chloroform, injected on an analytical silica column and eluted with 3:2 methanol–chloroform. One active fraction (Table 6) (AQm_{sol}-FM-$Silica_1$) was obtained that had an R_f by silica gel TLC of 0.48 when eluting with 3:2 methanol–chloroform. This fraction had a carbohydrate component that was visualized by the alpha-naphthol–sulfuric acid solution.

The active material (AQm_{sol}-FM-$Silica_1$) could not be reisolated using gravity-eluted silica columns. When such a column was eluted with 3:2 methanol–chloroform, no active fraction was obtained from AQm_{sol}-FM. Next, the same column that was eluted with 3:2 methanol–chloroform was eluted with 3:2 methanol–chloroform that was saturated with water. This later elutent provided an active component labeled AQm_{sol}-FM-$Silica_2$. The activity of the fraction is shown in Table 7. This active component had an R_f by silica gel TLC of 0.08 when eluted with 3:2 methanol–chloroform. This material was visualized using the alpha-naphthol sulfuric acid solution, thus indicating a carbohydrate component.

The CM extraction was implemented on the fathead minnow in the same manner as with the earthworm. The organic layer (CMo-FM) from the extraction, in contrast to EW, was slightly more active than water (Table 8). The water layer contained most of the activity. A dependent t test was performed comparing responses to the water and organic layers. The t test showed that the

TABLE 6. BIOASSAY RESULTS OF SILICA GEL CHROMATOGRAPHY OF FATHEAD MINNOW: PART I[a]

	Stimuli[b]		
	Water	AQ-FM	AQm_{sol}-FM-$Silica_1$
Mean tongue flick–attack score	12.80	51.76	30.06
Statistical comparison to water[c]		$Z = 1.89$	$Z = 1.62$
		$P < 0.05$	$P < 0.05$
Number of attacks	0	4	2

[a] Five *Thamnophis sirtalis* were tested once each on five different stimuli.
[b] Square-root transformation on all parameteric statistics. Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 17.96$, $df = 24$, $P < 0.01$; $F = 5.92$, $df = 6, 24$, $P < 0.001$.
[c] Wilcoxon signed rank-matched pairs tests, $N = 5$.

TABLE 7. BIOASSAY RESULTS OF SILICA GEL CHROMATOGRAPHY OF FATHEAD MINNOW: PART II[a]

	Stimuli[b]		
	Water	AQ-FM	AQm_{sol}-FM-$Silica_2$
Mean tongue flick–attack score	3.20	29.96	23.54
Statistical comparison to water[c]		$t = 3.88$	$t = 2.65$
		$P < 0.01$	$P < 0.05$
Number of attacks	0	3	2

[a] Five *Thamnophis sirtalis* were tested once each on three different stimuli.
[b] Square-root transformation on all parameteric tests. Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 6.00$, $df = 8$, $P < 0.05$; $F = 12.28$, $df = 2, 8$, $P < 0.005$.
[c] Dependent t-test, $df = 4$.

water layer was significantly more active than the organic layer ($t = 4.30$, $P < 0.01$, $df = 5$).

A Bio-Gel P-10 separation was conducted on the active water layer (CMw-FM). Two fractions were obtained from the preparative P-10 column. A high-molecular-weight fraction (CMw-FM-HMW_{P10}) eluted at the void volume of the column, and a low-molecular-weight fraction (CMw-FM-LMW_{P10}) eluted approximately at the volume of the column. Both fractions appear as unsymmetrical peaks in the chromatogram and thus must be multicomponent mixtures. Both fractions are active as indicated in Table 9.

Silica gel chromatography was conducted on the low-molecular-weight components from the CM fathead minnow extraction (CMw-FM-LMW_{P10}). No

TABLE 8. BIOASSAY RESULTS OF CM EXTRACTION OF FATHEAD MINNOW: PART I[a]

	Stimuli[b]		
	Water	AQ-FM	CMo-FM
Mean tongue flick–attack score	9.18	43.80	20.23
Statistical comparison to water[c]		$Z = 2.10$	$Z = 2.10$
		$P < 0.02$	$P < 0.02$
Number of attacks	1	5	1

[a] Six *Thamnophis sirtalis* were tested once each on six different stimuli.
[b] Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 13.50$, $df = 25$, $P < 0.02$; $F = 5.53$, $df = 5, 25$, $P < 0.002$.
[c] Wilcoxon signed rank-matched pairs test, $N = 6$.

TABLE 9. BIOASSAY RESULTS OF CM EXTRACTION OF FATHEAD MINNOW: PART II[a]

	Stimuli[b]			
	Water	CMw-FM	CMw-FM HMW_{P10}	CMw-FM LMW_{P10}
Mean tongue flick–attack score	10.71	52.71	50.09	40.51
Statistical comparison to water[c]		$Z = 2.28$	$Z = 2.28$	$Z = 2.28$
		$P = 0.01$	$P = 0.01$	$P = 0.01$
Number of attacks	0	6	5	3

[a] Seven *Thamnophis sirtalis* were tested once each on four different stimuli.
[b] Square-root transformations on all data for parametric test. Overall two-way ANOVA results for stimuli: Friedman's, $\chi^2 = 13.29$, $df = 18$, $P < 0.005$; $F = 17.76$, $df = 3, 18$, $P < 0.001$.
[c] Wilcoxon signed rank-matched pairs test, $N = 7$.

active fractions were found when a gravity-eluted silica column was eluted with 3 : 2 methanol–chloroform and 3 : 2 methanol–chloroform that was saturated with water.

Analysis of Active Components

The bioassay results clearly reveal the biological similarities in the extracts of the two prey. A number of chemical similarities have also been found, although the materials from the two species are clearly different by a number of criteria.

Aqueous Prey Extracts (AQ). Aqueous extracts of both the earthworm and fathead minnow were active. Other similarities in the AQ prey extracts are that the methanol–chloroform extracts from the lyophilized extracts are also active. The high- and low-molecular-weight components of the AQmc extracts from these prey also showed definite activity documented by an increase in the tongue flick–attack score. These similarities are noteworthy since the earthworm and fish are from different phyla and live in different environments.

The mass composition of the AQmc extract obtained from both prey is shown in Figure 1. There are similarities in the solubility properties of these prey extracts, that is, the amount of material obtained per liter of extract is similar for both, and the majority of the material from the AQmc extracts was insoluble in the methanol–chloroform solutions.

Chloroform–Methanol Extracts (CM). As observed with the AQ extractions, the CM extractions gave similar results for both fathead minnow and earthworm. The active components from both were primarily in the water layer of the CM extraction. The high- and low-molecular-weight materials from the water layer elicited prey attacks from both the earthworm and fathead minnow

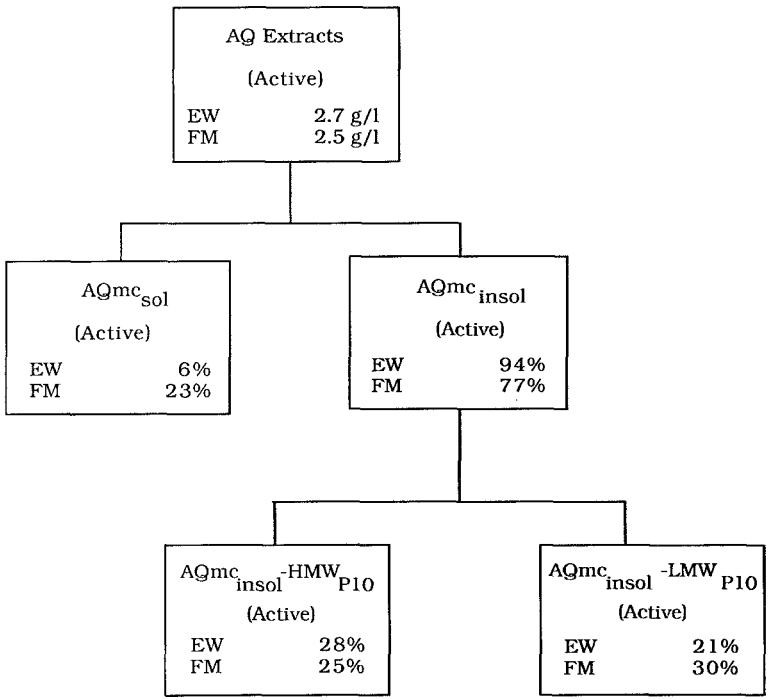

FIG. 1. Percent composition of aqueous prey extract. AQ, aqueous; EW, earthworm; FM, fathead minnow; mc, subsequent methanol–chloroform extraction; sol, soluble; insol, insoluble; HMW, high-molecular-weight fraction; LMW, low-molecular-weight fraction; P10, gel filtration column used.

(Tables 2, 3, and 8). The low-molecular-weight materials from each extract consisted of six times more material by mass than the high-molecular-weight materials. The percent composition by mass of the CM extraction for both prey is shown in Figure 2.

Molecular Weight Estimates. Molecular weight estimates of the high- and low-molecular-weight materials from the CM extraction for both the earthworm and fathead minnow were made. The high-molecular-weight materials were estimated on the basis of a molecular weight calibration plot obtained from Bio-Rad gel filtration standards on a Sephadex G-200 column. In the estimation of the high-molecular-weight materials from the CM extraction of both prey, the Sephadex G-200 column separated the material into two distinct fractions. In the case of the earthworm, the major constituent had an estimated molecular weight of 432,000 daltons. The minor constituent partially adhered to the col-

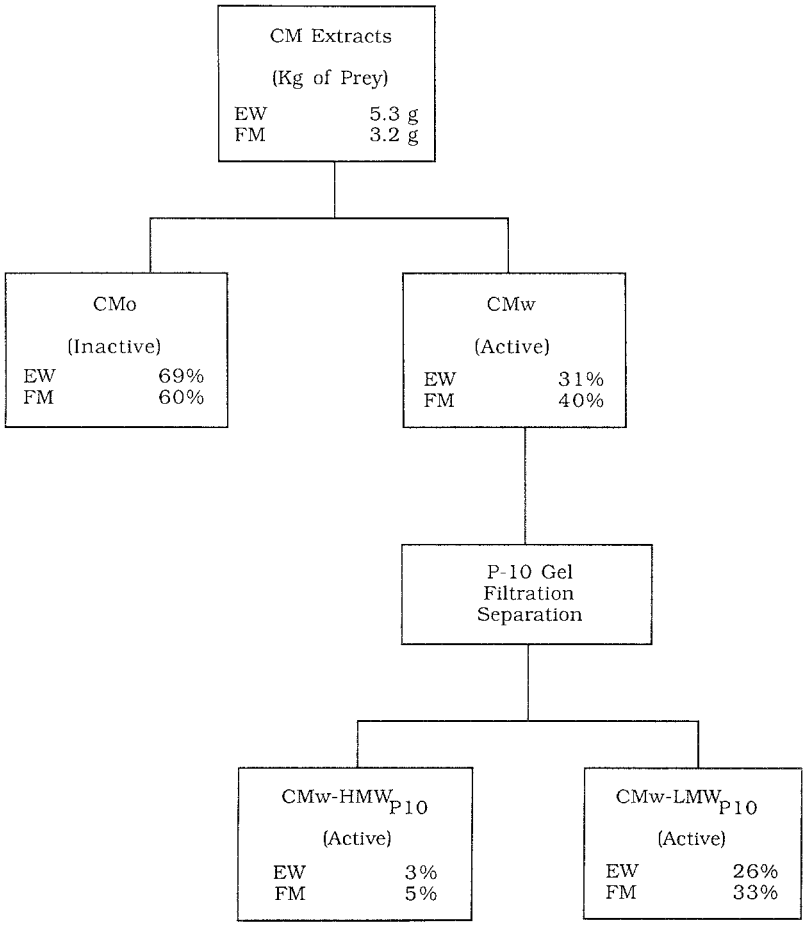

FIG. 2. Percent composition by weight of the direct chloroform–methanol (CM) extraction. o, organic layer; w, water layer; other abbreviations as in Figure 1.

umn and eluted after the void volume; thus its molecular weight could not be estimated. With the fathead minnow, the major constituent was estimated to have a molecular weight of approximately 519,000 daltons and the minor constituent 27,000 daltons. The estimates of the earthworm and fathead minnow high-molecular-weight materials along with their K_{av} values are shown in Table 10.

The low-molecular-weight materials obtained from the CM extraction from both prey were not estimated for molecular weight due to the lack of an accurate

TABLE 10. HIGH-MOLECULAR-WEIGHT CALIBRATION DATA

Sample	Total amount injection on column (mg)	Peaks obtained from chromatogram (K_{av})			
Standard	4.5				
Molecular weight $\times 10^{-3}$		670	158	44	17
					1.35
K_{av} value		(0.00)	(0.31)	(0.86)	(0.97)
CMw-EW-HMW$_{P10}$	1.7				
Molecular weight estimation $\times 10^{-3}$		432			[a]
K_{av} value		(0.07)			(1.05)
CMw-FM-HMW$_{P10}$	1.7				
Molecular weight estimation $\times 10^{-3}$		519		27	
K_{av} value		(0.02)		(0.90)	

[a] Material partially adhered to the column which resulted in the inability to estimate molecular weight.

calibration plot. The data obtained from the P-2 calibration column are presented in Table 11.

The low-molecular-weight materials from the earthworm and the fathead minnow are very similar (Table 11). All the materials from each extract eluted between the 1350-molecular-weight marker and the void volume of the column.

TABLE 11. LOW-MOLECULAR-WEIGHT CALIBRATION DATA

Sample	Total amount injection on column (mg)	Peaks obtained from chromatogram (K_{av})				
Standard	4.5					
Molecular weight $\times 10^{-3}$		670			1.35	
		158				
		44				
		17				
K_{av} value		(0.0)			(0.72)	
Sodium Azide	1.7					
Molecular weight $\times 10^{-3}$						0.65
K_{av} value						(0.83)
CMw-EW-LMW$_{P10}$	3.0					
K_{av} value			(0.06)	(0.28)	(0.56)	
CMw-FM-LMW$_{P10}$	3.0					
K_{av} value			(0.06)	(0.22)	(0.61)	

Since Bio-Gel P-2 resin fractionates globular proteins of molecular weight 100–1800 daltons (Gordon and Richard, 1972), it is assumed that the upper limit of fractionation for the resin under these separation conditions is 1800 daltons and the estimated molecular weight range for the low-molecular-weight materials from both prey is between 1350 and 1800 daltons.

Amino Acid Analysis. Amino acid analysis was conducted on the high- and low-molecular-weight materials from the water layer of the chloroform–methanol extractions of the earthworm and fathead minnow. The results of the analyses are shown in Table 12 along with the amino acid composition of glycopeptides and collagen obtained from the earthworm cuticle determined by Muir and Lee (1970).

The methods of determining the amino acid composition in our study did not provide a measure of the hydroxyproline content. Hence, the concentration of other amino acids will be artificially high since it is undoubtedly present in at least the earthworm samples, given the previous analyses of earthworm surface-derived substances. We have concentrated on establishing the presence of both high- and low-molecular-weight materials and defining methods for the high-yield isolation of the interesting low-molecular-weight substance. The composition reported here refers to fractions that are still mixtures and may only be useful as indicators of similarity rather than indicators of identity to previously isolated fractions. Nevertheless, qualitative comparisons of the amino acid composition of the high- and low-molecular-weight earthworm fractions isolated in this study to similar data available for earthworm cuticle, earthworm collagen, and the earthworm F2 fraction (e.g., Halpern et al., 1986) are in order.

The most significant observation is the similarity of the major amino acids in all five materials. Thus, alanine, glycine, serine, glutamate, and threonine are major components in all. Presumably hydroxyproline is also an important component in our extracts but that remains to be determined. The principal differences are in the relative amounts of the amino acids. In the high-molecular-weight F2 and in the glycopeptide and collagen analyzed by Muir and Lee (1969, 1970), glycine is the major amino acid by far. The differences in glycine content are too great to be explained by our lack of hydroxyproline analysis and must reflect either a serious difference in the materials being studied or the presence of an unindicated impurity in the previously studied materials.

There are other apparently significant differences in composition. For example, EW-HMW has more than twice the amount of leucine found in any other fraction and EW-LMW has similarly high amounts of serine. Since we know that we are dealing with fractions that have more than one component, it is not possible to state definitively that these elevated amounts are due specifically to active material. On the other hand, the high serine content is quite provocative in view of the probable glycopeptide nature of these substances.

Several of the amino acids most common in both earthworm fractions also

TABLE 12. PERCENT TOTAL OF AMINO ACID RESIDUES

Amino acid	Earthworm cuticle glycopeptide[a]	Earthworm cuticle collagen[a]	CMw-EW-HMW$_{P10}$	CMw-EW-LMW$_{P10}$	CMw-FM-HMW$_{P10}$	CMw-FM-LMW$_{P10}$
Lysine	trace	2.1	6.8	2.1	10.0	3.2
Histidine	0	0.1	1.1	2.3	0.8	2.7
Arginine	0	2.3	3.9	1.3	2.1	59.3
Aspartic acid	8.8	6.1	4.3	4.1	3.2	0.4
Hydroxyproline	9.7	15.9	[b]	[b]	[b]	[b]
Threonine	9.8	5.2	7.7	4.5	8.1	1.6
Serine	11.4	8.9	11.0	25.0	6.7	2.1
Glutamic acid	6.0	7.5	8.2	9.7	8.5	6.7
Proline	0.9	1.0	6.5	2.6	3.0	1.4
Glycine	38.2	32.0	11.3	16.9	12.8	11.2
Alanine	12.0	9.8	11.6	17.6	12.5	5.0
Valine	1.0	2.5	5.5	3.4	4.5	1.4
Methionine	0.0	trace	0.2	0.7	0.6	0.2
Isoleucine	1.5	2.1	4.2	2.1	5.8	0.9
Lucine	2.3	3.0	8.8	3.3	12.9	2.1
Tyrosine	0.0	0.4	2.7	2.1	1.5	0.5
Phenylalanine	trace	0.9	5.8	1.9	6.8	0.9
Cysteine	[b]	[b]	0.5	0.2	0.2	0.2

[a] From Muir and Lee (1970).
[b] Not determined.

appear to be important components of the high-molecular-weight fathead minnow fraction (e.g., glycine, alanine, leucine, glutamate, threonine, and serine). In addition, this fraction and the high-molecular-weight earthworm fraction share a high lysine concentration, significantly higher than in the other materials analyzed.

The low-molecular-weight fathead minnow fraction was found to have an extremely high concentation of arginine or a substance that mimics its behavior in the automated amino acid analysis. The significance of this obervation is not clear, but it could account for the difficulty experienced in attempted chromatography on silica gel-based absorbents. (Although it appears likely from our NMR analysis that this value is an artifact, the chromatographic similarity demonstrated in the amino acid analysis would also be reflected in other chromatographic systems.) Due to this apparently high arginine content, all other amino acids appear to be at much lower concentration than in the other fractions in Table 12. However, if the relative concentrations of the other amino acids are compared without considering the high arginine content, many of those occurring in high concentrations in other extracts are again found to be important. As in the high-molecular-weight fraction from the fish, lysine is relatively more important than in most other materials.

Carbohydrate Analysis. Silica thin-layer chromatography conducted with 3:2 methanol–chloroform on the high- and low-molecular-weight materials from the CM extractions indicated the presence of sugars when the plates were developed using alpha-naphthol–sulfuric acid spray. Hydrolysis of the individual materials followed by acetylation provided mixtures that could be analyzed by gas chromatography. Comparison of retention times of these hydrolysis mixtures with known acetylated sugars allowed identification. The identities of the sugars allowed identification. The identities of the sugars were also confirmed by mass spectrometry (Jansson et al., 1976).

The percent composition of sugars was estimated by comparing peak areas in the gas chromatograms to those of available standards of known concentrations separated under the same conditions. Since known concentrations of derivatized fucose and arabinose were not obtained for concentration data, an integration constant was calculated from the average of the peak area responses per mg of each known acetylated sugar. This averaging was implemented since it was assumed that all acetylated sugars should result in a similar relative response by the GC detector. Two inositol isomers were found in the low-molecular-weight materials of the earthworm. Both isomers resulted in the same mass spectrometric data but different GC retention times (Sherman et al. 1970). One isomer was determined to be myoinositol from GC retention time data. The integration constant was assumed to be the same for both isomers.

The percent composition of sugars (Table 13) for the high- and low-molec-

TABLE 13. PERCENT COMPOSITION OF SUGARS IN HIGH- AND LOW-MOLECULAR-WEIGHT FRACTIONS FROM CMw

Sample	Arabinose	Fucose	Galactose	Glucose	Myoinositol	Unknown inositol	Mannose	Ribose	Xylose
Earthworm cuticle[a]		trace	13.8				trace		1.3
CMw-EW-HMW$_{P10}$	2.0	19.6	39.2	5.9	1.0	0.5	19.6	[b]	9.8
CMw-EW-LMW$_{P10}$	0.9	1.6	6.9	13.8	46.2	23.1	4.6	2.3	1.4
CMw-FM-LMW$_{P10}$	1.8	16.4	61.3	12.3	4.1	0.8	2.0	2.0	0.8
CMw-FM-LMW$_{P10}$	2.6	5.9	5.3	39.5	19.7	2.6	3.3	26.3	2.6

[a] From Muir and Lee (1970).
[b] The standard mass spectra for the acetalated ribose does not match this compound's mass specta but results in a peak at the same GC retention time as other ribose derivatives.

ular-weight materials from both prey demonstrated relatively high glucose, fucose, galactose, and mannose contents in all four samples. Myoinositol was found in each sample. The low-molecular-weight materials of the earthworm had two isomers of inositol, which comprised a large portion of the sugar components.

The specific carbohydrate composition associated with the earthworm surface extracts has not been as extensively documented as the amino acid composition. Muir and Lee (1970) demonstrated a high galactose content and documented the presence of galactosyl di- and trisaccharides in purified cuticle collagen. In unpurified extracts they also found significant quantities (2–10% of neutral sugars) of fucose and xylose along with mannose and amino sugars in lower concentrations.

Both the high- and low-molecular-weight earthworm fractions identified in this study as attack stimuli differ significantly in the neutral carbohydrate analysis after hydrolysis of lyophilized materials compared to the previously studied materials. Galactose still appears to be the major monosaccharide unit in the high-molecular-weight sample but is a minor constituent of the low-molecular-weight fraction.

While the diminished galactose content of the high-molecular-weight fraction is in conflict with the Muir and Lee (1970) findings with crude cuticle collagen extracts, finding fucose and xylose in an approximately 2 : 1 ratio is in accord with their study. However, we find significantly greater quantities of both and a surprisingly large amount of mannose along with previously unreported glucose. Purification of the collagen by Muir and Lee resulted in a substance almost free of aldohexoses other than galactose. Thus, it appears that we have isolated a fraction rich in one or more components that Muir and Lee (1970) ultimately removed during the purification process.

The low-molecular-weight earthworm attack-eliciting material provides a completely different carbohydrate profile. Instead of a substantial amount of galactose, we find it as a minor component. Moreover, the major component appears to be myoinositol, a substance unreported in earlier studies of earthworm surface substances. This is accompanied by an unidentified inositol and relatively low amounts of simple aldohexoses.

Comparison of the materials isolated from the earthworm and those from fathead minnows shows some surprising similarities. Galactose and fucose are the predominant hexoses in both of the high-molecular-weight fractions. The principal difference is the increased glucose content and the reduced xylose content of the fish extract. The two low-molecular-weight fractions are less similar. While both show diminished galactose and fucose content relative to the respective high-molecular-weight fractions, the glucose, myoinositol, and ribose concentrations differ significantly.

Since many other sugars are present in the CMw extraction of the earth-

worm than are found in the earthworm cuticle, it might be that the materials obtained from the CM extraction are different than those obtained solely from earthworm cuticle.

Nuclear Magnetic Resonance. Carbon and proton NMR spectra were obtained for the low-molecular-weight fractions from the CM extraction of both earthworms and fathead minnows. Although the materials examined were crude mixtures and serious spectral interpretation awaits purer samples and more careful attention to pH effects, the spectra reflect the general carbohydrate and peptide nature of the materials. Thus, even with the low-signal-to-noise ratio imposed by the sample size, the carbon spectra of both samples displayed multiple resonances in the 50–80 ppm range, as expected for the saccharide oxygenated carbons. Likewise, signals typical of peptide linkages were apparent in the 170–185 ppm range. Furthermore, the low concentration of aromatic amino acids is reflected in both proton and carbon spectra in which there are essentially no aromatic carbon or hydrogen signals.

Perhaps the most significant feature of the spectra was the absence of a strong guanidino carbon resonance in the fathead minnow carbon spectrum. Although it is a nonprotonated carbon that can be difficult to observe, if the high percentage of arginine indicated by the amino acid analysis is real, one would have expected a clearly assignable resonance of significant intensity. Such is not the case, and we must conclude that the value measured for arginine is largely (or totally) an artifact.

DISCUSSION

Biologically active materials from the earthworm (*Lumbricus terrestris*) and fathead minnow (*Pimephales promelas*) have been isolated with two extraction procedures. These materials generally exhibit similar chemical properties as demonstrated by gel filtration data, amino acid analysis, sugar analysis, and comparison of NMR spectra.

Both high- and low-molecular-weight fractions were obtained. There are similarities in the solubility properties of these prey extracts, that is, the amount of material obtained per liter of extract is similar for both, and the majority of the material from the AQmc extracts was extractable from the methanol–chloroform solutions. These similarities are noteworthy since the earthworm and fish were of similar size and had comparable surface area-to-weight ratios.

As observed with the aqueous extracts, the CM extraction resulted in similar observations between the fathead minnow and the earthworm. The active components from both were found primarily in the water layer of the CM extraction. Much weaker activity was found in the organic layer. The high- and low-molecular-weight materials from the water layer from both the earthworm

and fathead minnow elicited prey attacks. The low-molecular-weight materials from each extract consisted of six times more material by mass than the high-molecular-weight materials for both prey.

The compounds isolated from the earthworm using the CM extraction method exhibited similar chemical and physical properties to active earthworm material isolated in the Sheffield et al. (1968) study and in Halpern's laboratory (e.g., Reformato et al., 1983). Although the molecular weights estimated in the present work differ from previous estimates, the overall properties indicate that our high-molecular-weight earthworm component is similar but not identical to previously isolated materials.

An important difference between this work and previous studies on *Thamnophis* is the identification of low-molecular-weight materials with prey identification activity. The activity of a low-molecular-weight fraction was noted by Sheffield et al. (1968) but has not been reported in recent publications (e.g., Kirschenbaum et al., 1985, 1986). These workers may not have found this active low-molecular-weight fraction since the bioassay method they utilized differed from our own (see p. 856). Perhaps the bioassay procedure used may lead to different conclusions as to what materials are active, since different chemicals may be involved in different phases of feeding or the methods differ in sensitivity. Our low molecular weight material may, ultimately, derive from the high molecular weight compounds. It should be noted that by using methylene chloride extracts of snake skins, Weldon and Schell (1984) were able to elicit prey attacks from ophiophagous snakes. These chemicals may have also been of relatively low molecular weight.

Reformato et al. (1983) reported that activity of the earthworm material seemed to diminish with the decrease of the carbohydrate content of the material. Interestingly, both active fractions of CMw-EW-HMW_{P10} and of CMw-EW-LMW_{P10} had very high carbohydrate contents. This supports these authors' view that carbohydrates may be related to the activity of the material. In the same paper they reported that the active material resulted in a positive assay for proteins. The studies reported here showed that both active fractions obtained from the CM extraction of the earthworm indicate the presence of proteins or peptides by the amino acid analysis.

The complete purification and identification of the active material from the earthworm (*Lumbricus terrestris*) and fathead minnow (*Pimephales promelas*) had not yet been achieved. However, new methods have been discovered that extract the active material from the prey through direct chloroform–methanol extraction and the methanol extraction of the lyophilized water extract. We have also found, in preliminary studies, that the mosquito fish, *Gumbusia affinis*, contains components chemically similar to the fathead minnow. Likewise, *Thamnophis butleri* seems to react to the same active fractions of both fish and earthworm as does *Thamnophis sirtalis*. Thus, these procedures may lead to the

determination of whether different species of snakes that will eat the same prey species are utilizing similar or different chemical cues in identifying it.

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RESPONSES OF BRACONID PARASITOIDS *Microplitis croceipes* (CRESSON) AND *M. demolitor* WILKINSON TO STEREOISOMERS OF KAIROMONE 13-METHYLHENTRIACONTANE

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Abstract—The responses of *Microplitis croceipes* (Cresson) and *Microplitis demolitor* Wilkinson to the *R* and *S* stereoisomers of the kairomone 13-methylhentriacontane were evaluated. Singly and in combination at 5, 50-, and 500-ng dosage levels each were tested. There was a linear dosage response by both species to the three dosages. Furthermore, there was no difference between responses to the two stereoisomers or their combination by either species at any dosage level. The effects of the two stereoisomers were fully interchangeable and additive. This is the first report of parasitoid responses to stereoisomers of a kairomone.

Key Words—*Microplitis croceipes*, *Microplitis demolitor*, Hymenoptera, Braconidae, parasitoids, semiochemicals, kairomones, 13-methylhentriacontane, stereoisomers, enantiomers, *Heliothis zea*, Lepidoptera, Noctuidae.

INTRODUCTION

The relationship of biological activity to stereostructure of insect pheromones has been a subject of considerable research. Insects generally respond to one stereoisomer of a pair (or a set, if more than one asymmetric center is present

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in the pheromone's chemical structure) and to a lesser extent, if at all, to the other or others of the set (Silverstein, 1979; Brand et al., 1979). In some cases the optimal response is to a specific ratio of stereoisomers (Lanier et al., 1980). In other situations, however, the biological response is to one of the enantiomers, and the presence of the other enantiomer is inhibitory (Tumlinson et al., 1977; Birch et al., 1977).

Although a number of hydrocarbon pheromones and other semiochemicals have been identified from insects, only a few have been synthesized in high configurational purity. Consequently, the relationship of stereostructure to the biological activity of these compounds has not been studied. No known study of stereostructure of any class of kairomones or the resulting influence on biological activity of entomophagous insects has been reported.

We report here the biological testing of the stereoisomers of 13-methylhentriacontane, a kairomone from the frass of corn earworm larvae, *Heliothis zea* (Boddie), that influences the host location behavior of the larval parasitoid, *Microplitis croceipes* (Cresson) (Lewis and Jones, 1971; Jones et al., 1971). This hydrocarbon also elicited the host-seeking response in *M. demolitor* Wilkinson, a closely related braconid recently imported into the United States from Australia (Nordlund and Lewis, 1985). Comparative evaluations of the level of responses elicited by the *R* and *S* stereoisomers singly or in combination (1 : 1) for each parasitoid species was the objective of this study.

METHODS AND MATERIALS

The synthesis of (*R*)- and (*S*)-13-methylhentriacontane in >99.6% configurational purity has been reported (Sonnet, 1984). Samples for this study were >95% pure chemically, containing 5% hydrocarbons such as docosane that were not expected to influence behavioral response.

The host insects were reared according to Burton (1970), and the parasitoids of both species were reared according to Lewis and Burton (1970) at ca. 26°C and 70% relative humidity. The age of the parasitoids used ranged from four to eight days, with equal numbers of replications (27 for *M. croceipes* and 10 for *M. demolitor*) for each treatment category and each group of parasitoids.

The bioassay procedure of Jones et al. (1971) was used. Samples of the materials were placed on filter paper in the bottom of a 150 × 15-mm Petri dish. A female parasitoid was placed in the dish and her response observed as she walked across the sample. These parasitoids are negatively geotactic and positively phototactic, so the direction of movement could be guided by rotating the dish in a vertical plane with the sample side (bottom) toward the light. Individual parasitoids were each allowed three passes over the sample and scored according to antennal examination of the material. A score of 3, 2, 1, or 0 was

assigned based on whether the individual responded on the first, second, third, or none of the passes.

Three dosages, 500, 50, and 5 ng, of each treatment were evaluated by applying 5-μl samples of the materials to filter paper at concentrations of 0.1, 0.01, and 0.001 mg/ml of the respective dosages.

The responses of 81 *M. croceipes* individuals (from three separate rearing dates) were tested for each treatment category. The responses of 30 *M. demolitor* individuals were tested, again involving individuals from three different rearing dates.

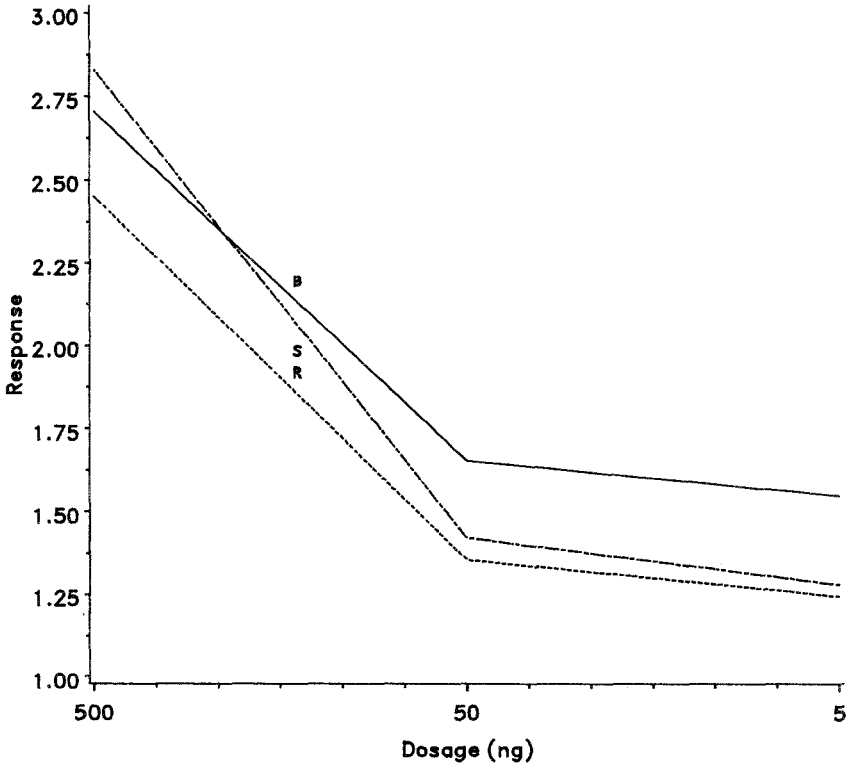

FIG. 1. Responses of *M. croceipes* to varying dosages (500, 50, and 5 ng) of stereoisomers of 13-methylhentriacontane, R, response to the *R* isomer; S, response to the *S* isomer; B, response to a mixture of equal amounts of the two isomers with the indicated dosage being the total amount of both. All treatments behaved linearly ($P < 0.0001$), and there were no treatment differences ($P < 0.05$) or treatment × dosage interactions ($P < 0.05$). Equations generated for responses were B: $Y = 1.5358 + 11.6902X$; R: $Y = 1.2325 + 12.1788X$; S: $Y = 1.2641 + 15.6638X$.

Linear regression techniques were used to test for treatment differences and interactions between treatments and dosage levels.

RESULTS AND DISCUSSION

The linear regression tests for *M. croceipes* and *M. demolitor* (Figures 1 and 2) showed: (1) no significant differences in the responses to the *R*, *S*, or combination of stereoisomers ($P < 0.05$); (2) dosage levels were all related linearly ($P < 0.0001$); and (3) there were no interactions between dosage levels and

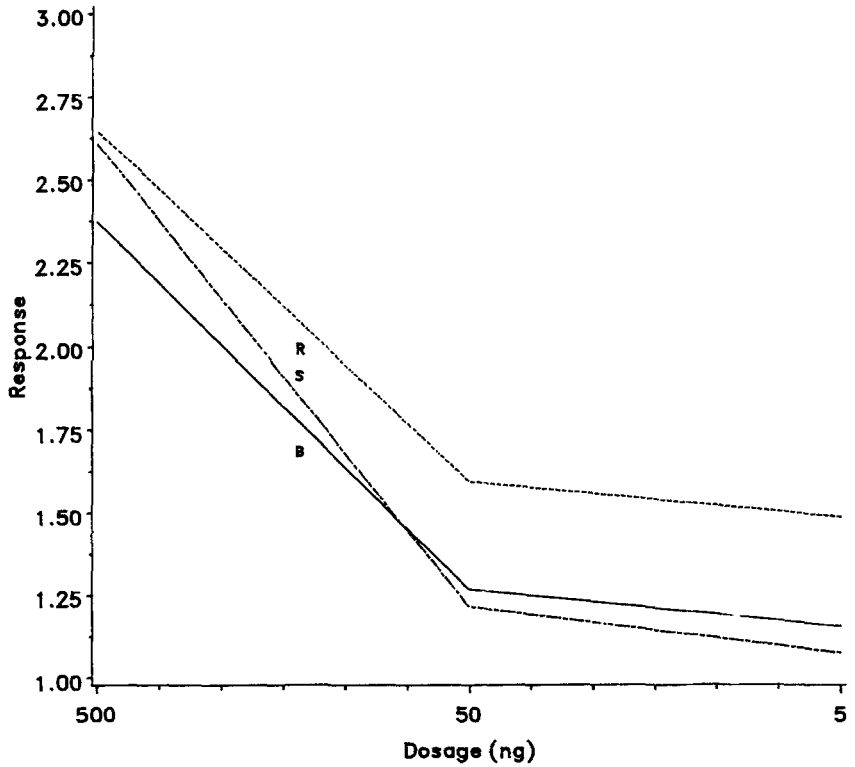

FIG. 2. Responses of *M. demolitor* to varying dosages (500, 50, and 5 ng) of stereoisomers of 13-methylhentriacontane, R, response to the *R* isomer; S, response to the *S* isomer; B, response to a mixture of equal amounts of the two isomers with the indicated dosage being the total amount of both. All treatments behaved linearly ($P < 0.0001$), and there were no treatment differences ($P < 0.05$) or treatment × dosage interactions ($P < 0.05$). Equations generated for responses were B: $Y = 1.1463 + 12.2623X$; R: $Y = 1.4796 + 11.6617X$; S: $Y = 1.0611 + 15.4655X$.

the *R*, *S*, and combination treatments. (The equations describing the responses are presented in the figure legends.)

Microplitis croceipes and *M. demolitor* both respond to 13-methylhentriacontane even though they occur in areas widely different geographically, and *M. demolitor* has a wider host range than *M. croceipes* (Nordlund and Lewis, 1985). Our results show that they have similar dose responses to each stereoisomer and the combination. The possibility of the comparisons being confounded due to impurities is unlikely, since the responses to each stereoisomer and the mixture were all similar.

This is the first report of evaluations of a parasitoid response to stereoisomers of a kairomone. The fact that there was no measurable difference in the response to the two stereoisomers by either species is interesting. Furthermore, the effect of the stereoisomers appears fully interchangeable and additive, as shown in the responses to their combinations as compared to each of the stereoisomers alone. It is important to note that the results of Jones et al. (1971) showed that the position of the methyl group up or down the carbon chain influenced the response strongly, in that dramatically lower responses were found for 11- and 15-methylhentriacontane as compared to 13-methylhentriacontane. Moreover, most studies of stereoisomers of materials mediating intraspecific responses demonstrate a bias for one stereoisomer over the other.

It is not known at this time whether the host and its frass possess both isomers. Such information would assist in determining the biological significance of the results.

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SYNERGISTIC INTERACTION BETWEEN POTATO GLYCOALKALOIDS α-SOLANINE AND α-CHACONINE IN RELATION TO DESTABILIZATION OF CELL MEMBRANES: ECOLOGICAL IMPLICATIONS

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Abstract—In studies of the lysis of rabbit erythrocytes, red beet cells, and *Penicillium notatum* protoplasts by the potato glycoalkaloids α-solanine and α-chaconine, the latter was consistently the more membrane-disruptive compound and erythrocytes the more susceptible cell type. A 1:1 mixture of solanine and chaconine produced pronounced synergistic effects in all three test systems. In beet cells, such effects were apparent from an early stage of treatment and persisted over a period of several hours. With erythrocytes and fungal protoplasts, the synergism was maximal with mixtures containing approximately 70% chaconine, whereas with beet cells it peaked at approximately 40% chaconine. Synergistic interactions between solanine and chaconine also occurred with regard to cholesterol binding in vitro, with a maximum response corresponding to the 50% mixture. The implications of these findings for the nature and efficacy of chemical defense systems in plants are discussed.

Key Words—Potato, *Solanum tuberosum*, glycoalkaloids, α-solanine, α-chaconine, cell membrane disruption, in vitro sterol binding, synergistic interactions.

INTRODUCTION

It is now generally accepted that plants are able to withstand herbivores and microbial pathogens largely because they have evolved effective chemical

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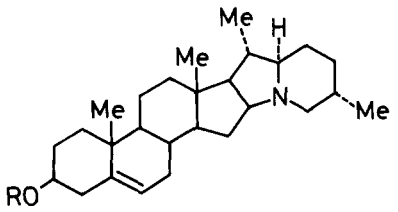
defense systems (Wallace and Mansell, 1976; Harborne, 1978; Rosenthal and Janzen, 1979; Cooper-Driver et al., 1985). However, relatively few detailed biochemical investigations of such defenses have been carried out compared with the vast range of secondary compounds elaborated by plants (Bell and Charlwood, 1980). Of the studies that have been reported, the majority have been concerned with single compounds or single classes of compounds. This approach has yielded interesting and valuable information but suffers from two major limitations: (1) it fails to acknowledge that the behavior and physiology of feeding herbivores or invading microorganisms are often based on contact with a wide diversity of plant chemicals rather than a single compound, and (2) within such a "multichemical" defense system, certain compounds may interact to produce effects qualitatively or quantitatively different from those predictable from the effects of individual compounds.

Much work on chemical defense in the potato plant (*Solanum tuberosum* L.) has centered on the two major steroidal glycoalkaloids, α-solanine and α-chaconine (Figure 1). These compounds, which share a common aglycone (solanidine) and differ only in their carbohydrate moiety, have powerful physiological and pharmacological activity (Roddick, 1986) that has resulted in numerous human and livestock poisonings and fatalities (Morris and Lee, 1984). In addition, there is evidence that these glycoalkaloids, due to their toxicity and/or bitterness, play some role as resistance factors against insects (Tingey, 1984) and, possibly to a lesser extent, as antifungal compounds (Roddick, 1987). In such organisms, deterrence by glycoalkaloids is attributable in large measure to the membrane destabilizing properties of these compounds (Mitchell and



Solanidine, R = H

α-Solanine, R = glu-gal-
rham

α-Chaconine, R = rham-glu-
rham

FIG. 1. Structures of potato glycoalkaloids.

Harrison, 1985; Roddick, 1987). A number of studies in which single glycoalkaloids were tested on a wide variety of organisms from viruses to mammals (e.g., Thorne et al., 1985; Sharma et al., 1979) consistently showed chaconine to be the more potent of the two compounds. We observed similar differential activity with regard to lysis of phosphatidylcholine–sterol liposomes (Roddick and Rijnenberg, 1986) and, more recently, have demonstrated that, in combination, solanine and chaconine produce a pronounced and highly reproducible synergistic effect (Roddick and Rijnenberg, 1987). The synergism was quite specific and did not occur if one of the glycoalkaloids was substituted by related compounds, e.g., tomatine, digitonin, or β_2-chaconine. However, because this study employed synthetic lipid membranes, the biological (and particularly ecological) implications of the synergism cannot be properly assessed. Work in this field has therefore been extended to include the responses of membranes of living cells, and in this communication we report the effects of solanine and chaconine, both singly and in combination, on the integrity of representative cells from animals, plants, and fungi.

METHODS AND MATERIALS

Source of Glycoalkaloids. Solanine and chaconine were extracted from *S. tuberosum* cv. Wauseon and purified by the method of Swain et al. (1978). Authentic samples were also purchased from Sigma Chemical Company, Poole, U.K.

Preparation and Treatment of Erythrocytes. Rabbit blood was collected into heparinized tubes and used either fresh or after storage at 4°C for up to two days. The cells were washed twice with 9× the blood volume of phosphate-buffered saline, pH 7.4, with centrifugation at 400*g* for 10 min after each wash. The final erythrocyte pellet was resuspended in its original volume of buffered saline, and a portion of this suspension removed and diluted to 2% with buffered saline. A 3-ml aliquot of the 2% suspension was incubated with 1 ml of glycoalkaloid solution in buffered saline at 37°C for 2 hr. Glycoalkaloid solutions were prepared by dissolving in the minimum volume of 0.1 M HCl and making up to volume with buffered saline. Erythrocytes were counted using a hemacytometer and the percentage of lysed cells calculated by comparing with counts in the control containing buffered saline only. In solanine–chaconine interaction experiments, the two glycoalkaloids were prepared as 50 μM solutions and mixed in the ratios indicated. The total alkaloid concentration in all test mixtures was 12.5 μM.

Preparation and Treatment of Beet Disks. Cylinders (1 cm diam.) of the swollen tap root of red beet (*Beta vulgaris* L.) were removed with a cork borer and cut into disks 2 mm thick. These were washed in running tap water for 16

hr and lightly blotted to remove excess water. Ten disks were placed in a 50-ml conical flask along with 10 ml of test solution. Glycoalkaloid solutions were prepared by dissolving in acid as above and making up to volume with phosphate–citrate buffer, pH 7.2. Flasks were closed with cotton wool plugs and placed on a reciprocating shaker at 100 rpm. Mixtures were incubated at 25°C, and at various times the test solutions were decanted and their absorbance at 535 nm measured to quantify betanin loss and, where appropriate, returned to the flask. In interaction experiments, 50 μM solutions of solanine and chaconine were used and mixed in the ratios indicated. The total alkaloid concentration in flasks was 50 μM.

Preparation and Treatment of Fungal Protoplasts. The fungal culture used was *Penicillium notatum* Westling, IMI 17968, maintained on a 2% agar-solidified medium containing 2% malt agar at 20°C. Protoplasts were prepared by a method based on that of Hamlyn et al. (1981). Liquid cultures were initiated by inoculating a medium consisting of 2% sucrose, 0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.5% trace element solution (42 mg/liter $CuCl_2 \cdot 2H_2O$, 317 mg/liter $ZnSO_4 \cdot 7H_2O$, 15 mg/liter $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 32 mg/liter $MnSO_4 \cdot 4H_2O$, 398 mg/liter $FeSO_4 \cdot 7H_2O$, and 400 mg/liter EDTA) with a spore suspension in sterile distilled water to give a final spore load of 10^6/ml of medium. The culture was incubated at 25°C for 63 hr, after which the fermenter (21) contents were vacuum-filtered through fine nylon (mesh size approx. 100 μm) which retained mycelium but not spores or small mycelial fragments. The mycelial mat was washed with 20 ml 100 mM MES–NaOH buffer, pH 5.8, containing 0.8 M KCl, after which 5 g of mycelium was removed and resuspended in 50 ml of the same buffer. To 7.5 ml of this suspension in a beaker was added 2.5 ml of a 2.4% (w/v) solution of Novozym 234 (Novo Bio Labs, Bagsvaerd, Denmark) in 100 mM MES–NaOH buffer. The yield of protoplasts was adjusted by altering the amounts of mycelium and Novozym used, but the ratio of the two components was maintained as given above. The mixture was stirred gently at 25°C, and the extent of protoplast formation was monitored by hourly microscopic examinations. After 3 hr of incubation, the digest was gravity-filtered through single muslin and the protoplasts concentrated by centrifugation of the filtrate at 3000*g* for 5 min. The pellet was resuspended in 20 ml of 20 mM MES–NaOH buffer containing 0.8 M KCl and then filtered through double muslin. The filtrate was centrifuged at 1200*g* for 5 min and the supernatant discarded. The protoplast pellet was resuspended in 7.5 ml of 50 mM potassium hydrogen phthalate containing 1.2 M sorbitol with the pH adjusted to 7.2 with tetramethylammonium hydroxide. This suspension was subjected to a final filtration through single muslin before use. Protoplasts were treated with glycoalkaloids by adding 462.5 μl of suspension to 37.5 μl of alkaloid solution in 0.002 M HCl in a 5-ml specimen tube. Reaction mixtures were left at 25°C for 1 hr, after which an aliquot was removed and protoplast

number determined using a hemacytometer. The percentage of protoplasts lysed was calculated by comparing numbers in test samples and controls containing acid only. In interaction experiments, stock solutions of 0.67 mM alkaloid were used to prepare solanine–chaconine mixtures. The total alkaloid concentration in all treatments was 50 μM.

Glycoalkaloid–Sterol Complex Formation In Vitro. The extent of complex formation was determined by measuring the amount of unbound sterol remaining in solution. Glycoalkaloids and cholesterol were dissolved in 96% (v/v) ethanol at strengths of 1 mM and 20 mM, respectively. Solutions were mixed in the ratio of 2.0 ml : 0.1 ml in 15 ml polypropylene tubes, heated at 90°C for 5 min and cooled at room temperature for 1 hr and at 4°C overnight (16 hr). Tubes were centrifuged at 27,000*g* for 30 min at 4°C and the supernatant decanted. A 0.5-ml aliquot was transferred to a 5-ml vial and the solvent evaporated in a nitrogen stream at room temperature. The residue was dissolved in 0.25 ml of ethyl acetate containing 0.04% (w/v) 5α-cholestane. The cholesterol concentration of this solution was determined by GLC for which 5α-cholestane acted as internal standard. A 1-μl sample was injected into a 2-m × 6-mm glass column packed with 3% OV-101 on Gas Chrom Q. The nitrogen flow rate was 50 ml/min and the column and detector (flame ionization) temperatures 275 and 325°C, respectively. Cholesterol was quantified using a precalibrated peak integrator.

Experimental Procedure. All treatments were replicated as indicated in the text, and each experiment was carried out at least twice. Data were analysed using a *t* test and by calculation of least significant differences (LSD) at $P = 0.05$.

RESULTS

As a prerequisite to investigating the interaction between solanine and chaconine, the lytic action of the individual glycoalkaloids against mammalian erythrocytes, red beet cells, and *P. notatum* protoplasts was examined. Dose–response curves are shown in Figure 2.

The greater membrane-lytic effect of chaconine was confirmed in all three systems. Rabbit erythrocytes proved the most susceptible to chaconine, showing lysis at around 1 μM (Figure 2a), whereas beet and protoplasts were unaffected by concentrations less than 10 μM (Figures 2b and c). Similarly, a maximal effect on blood was achieved with 25 μM chaconine (Figure 2a), whereas beet and protoplasts required around 60 μM and 100 μM, respectively (Figures 2b and c). Erythrocytes and beet showed a similar reduced sensitivity to solanine, lysis being first observed at 15–20 μM (Figures 2a and b). However, whereas 100 μM solanine caused almost 100% hemolysis, it elicited only

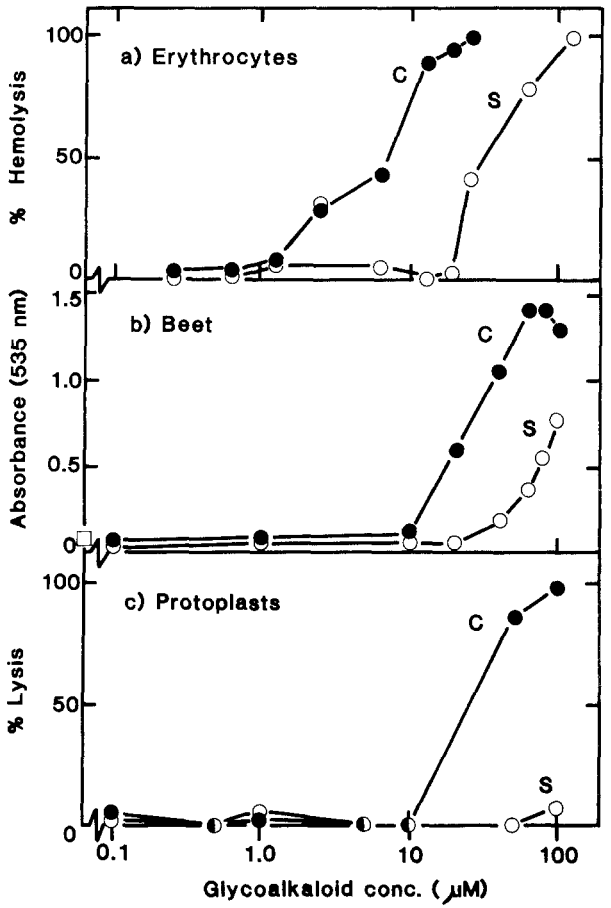


FIG. 2. Semilog plot showing effects of solanine and chaconine on disruption of (a) erythrocytes, (b) beet root cells, and (c) *P. notatum* protoplasts. Cells were treated in buffered solutions, pH 7.2–7.4, for 2, 4, and 1 hr, respectively. Lysis was assessed in erythrocytes and protoplasts by hemacytometer counts relative to controls and in beet cells by measuring the absorbance of the bathing solution at 535 nm. Each point is the mean of three replicates (five counts per replicate) for erythrocytes and five replicates for beet and protoplasts. S = solanine; C = chaconine.

40% loss of betanin from beet cells (Figures 2a and b). In contrast, fungal protoplasts were virtually unaffected by solanine concentrations up to 100 μM (Figure 2c).

From these data, we decided to explore the combined effects of solanine and chaconine in a 1 : 1 ratio using each compound at 6.25 μM against eryth-

rocytes and 25 μM against beet and protoplasts. At these levels, solanine did not lyse any of the cells and chaconine effects (where present) were less than maximal, thus facilitating the detection of any additive or synergistic responses. In all three test materials, the solanine–chaconine combination gave rise to pronounced synergisms (Table 1). In fact, with beet, the glycoalkaloid mixture was more disruptive than full-strength chaconine. With erythrocytes and *Penicillium* protoplasts, however, the mixture, while synergistic, was less so and approximately the same as full-strength chaconine. A time-course study on beet indicated that the synergism occurred from the outset of the experiment and persisted over a period of several hours (Figure 3). The actual magnitude of the synergism increased for the first 3 hr, after which it remained relatively constant.

The fact that potato tissues do not always accumulate solanine and chaconine in a 1:1 ratio (chaconine is usually more abundant than solanine) prompted us to examine the effects of other glycoalkaloid combinations ranging from 100% solanine to 100% chaconine in steps of 10% (Figure 4). The most hemolytic glycoalkaloid mixture comprised approximately 70% chaconine and 30% solanine, with some indication of a slight supraoptimal effect at higher chaconine levels (Figure 4a). The curve joining the values corresponding to zero, half-strength (6.25 μM), and full-strength (12.5 μM) chaconine only, confirms that the data in Figure 4a represent a series of real synergistic effects and not a dose–response curve for chaconine. Consistent with the results in Table 1, the 50% alkaloid mixture was less hemolytic than 100% chaconine (Figure 4a). In beet tissue, stronger synergisms were observed than in erythrocytes,

TABLE 1. EFFECT OF SOLANINE AND CHACONINE AND THEIR INTERACTION ON DISRUPTION OF ERYTHROCYTES, BEET ROOT CELLS, AND *P. notatum* PROTOPLASTS

Treatment[a]	Hemolysis (%)	A_{535}	Protoplast lysis (%)
Control	—	0.03 ± 0.007	—
Solanine (FS)	11.4 ± 5.59	0.11 ± 0.009	17.9 ± 7.31
Chaconine (FS)	89.0 ± 0.32	0.38 ± 0.080	70.9 ± 4.75
Solanine (HS)	3.2 ± 1.64	0.03 ± 0.005	2.3 ± 1.41
Chaconine (HS)	51.6 ± 3.56	0.15 ± 0.019	4.5 ± 1.03
Solanine (HS) + chaconine (HS)	70.6 ± 2.60*	0.88 ± 0.061***	73.8 ± 1.53***

[a]FS = full strength = 12.5 μM for erythrocytes, 50 μM for beet and protoplasts; HS = half strength. Cells were treated in buffered solutions, pH 7.2–7.4, for 2, 4, and 1 hr, respectively. Lysis was assessed in erythrocytes and protoplasts by hemacytometer counts relative to controls and in beet cells by measuring the absorbance of the bathing solution at 535 nm. Each value represents the mean of five replicate determinations ± standard error. *, *** = Significantly different from Σ solanine (HS) + chaconine (HS) (all combinations) at 5%, 0.1% level.

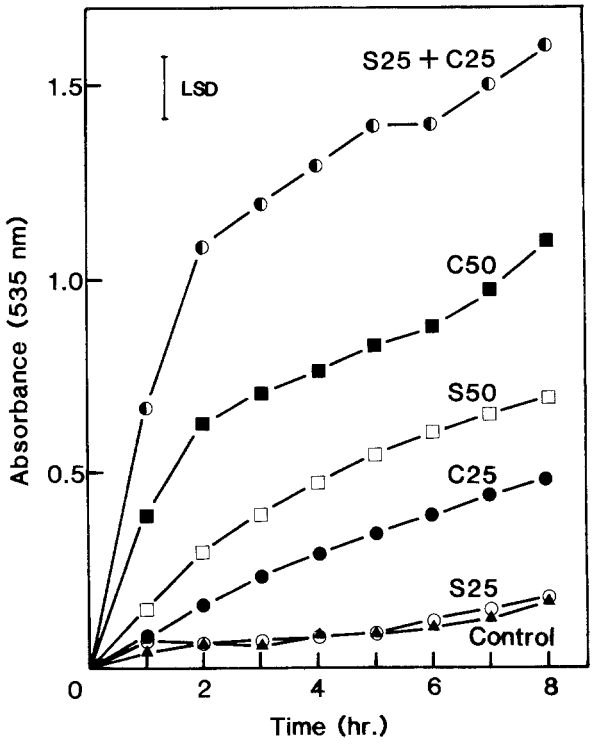


FIG. 3. Effects of solanine and chaconine and their interaction on leakage of betanin from root cells of red beet over a period of time. At various intervals, incubation solutions were decanted, their absorbance at 535 nm measured and returned to flasks. Each point is the mean of five replicates. S = solanine; C = chaconine. Values following abbreviations represent concentration in μM.

with a marked peak response corresponding to approximately 40% chaconine (Figure 4b). The earlier observation that a 50% glycoalkaloid mixture was significantly more disruptive than 100% chaconine (Table 1) was again borne out in this experiment (Figure 4b). The synergistic effects of the solanine–chaconine mixtures on *Penicillium* protoplasts (Figure 4c) bore more resemblance to those on erythrocytes than on beet, with a maximal response again at around 70% chaconine. However, in this case, the 50% mixture and 100% chaconine were of similar efficacy, an observation in keeping with the data in Table 1.

Since membrane destabilization by glycoalkaloids is thought to result from their complexing with membrane sterols (Arneson and Durbin, 1968; Roddick and Drysdale, 1984), experiments were conducted to determine whether solan-

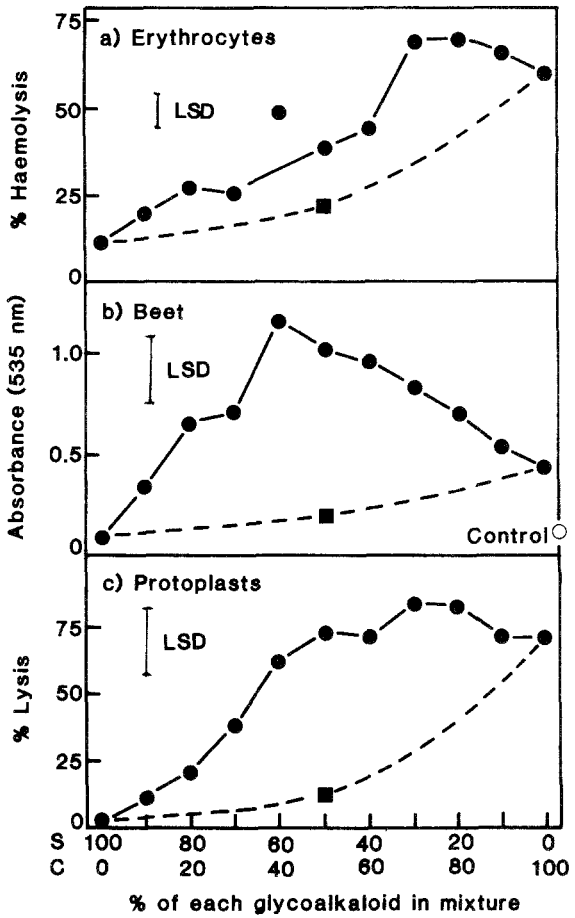


FIG. 4. Effect of relative amounts of solanine and chaconine on disruption of (a) erythrocytes, (b) beet root cells, and (c) *P. notatum* protoplasts. The total glycoalkaloid concentration was 12.5 μM for erythrocytes and 50 μM for beet and protoplasts distributed between solanine and chaconine as shown. Treatments, assessment of cell disruption, and replication were as in Figure 2. ■ = 6.25 μM chaconine only in (a), 25 μM chaconine only in (b) and (c); S = solanine; C = chaconine.

ine and chaconine also interacted synergistically in relation to cholesterol binding in vitro. Using similar glycoalkaloid mixtures, synergistic responses were observed which peaked with the 50% solanine–chaconine mixture (Figure 5). As previously (Figure 4), synergisms are particularly apparent when data are compared with those for single alkaloids (Figure 5, broken lines). In contrast

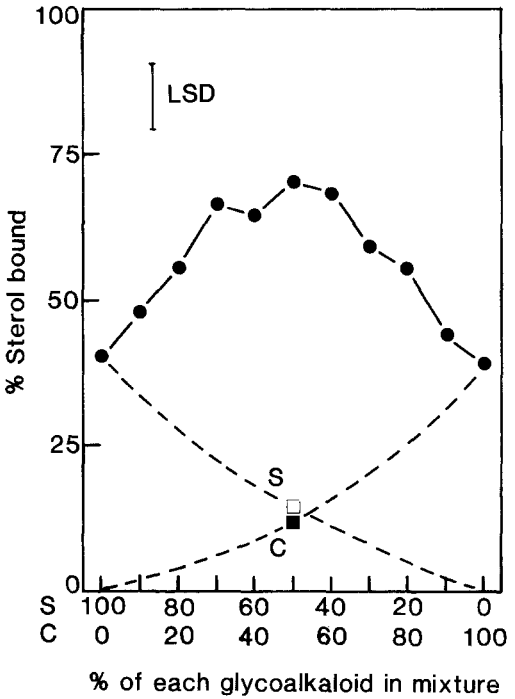


FIG. 5. Effect of relative amounts of solanine and chaconine on glycoalkaloid binding to cholesterol in vitro. The total glycoalkaloid concentration was 1 mM distributed between solanine and chaconine as shown. Reactants were prepared in 96% ethanol and incubated for 1 hr. Cholesterol binding was determined by measuring unbound sterol by GLC relative to the control. Each point is the mean of five replicates. ■ = 500 μM chaconine only; □ = 500 μM solanine only; S = solanine; C = chaconine.

to their differential lytic effects, both solanine and chaconine showed a similar affinity for cholesterol in vitro, an observation which is consistent with earlier reports (Roddick and Rijnenberg, 1986).

DISCUSSION

The greater lytic effect of chaconine than solanine on erythrocytes, beet cells, and *Penicillium* protoplasts is in keeping with numerous reports of similar differential activity against *Herpes simplex* (Thorne et al., 1985), *Alternaria solani* (Sinden et al., 1973), *Fusarium caeruleum* (McKee, 1959), erythrocytes (Tschesche and Wulff, 1964), nematodes (Allen and Feldmesser, 1971), Colorado potato beetle (Mitchell and Harrison, 1985), mice (Sharma et al., 1979) and liposomes (Roddick and Rijnenberg, 1986) and also in taste tests (Zitnak

and Filadelfi, 1985). Total lack of activity of solanine, as found here against *Penicillium* protoplasts, has also been observed with *Herpes* virus (Thorne et al., 1985) and liposomes (Roddick and Rijnenberg, 1986). The carbohydrate moiety is thus an important determining factor in the biological activity of these compounds, although no biochemical explanation of this phenomenon yet exists. The possibility that this effect might be explained by solanine and chaconine differing in their response to degradative enzymes and/or in their ability to reach binding sites in membranes cannot be ruled out.

The synergism between solanine and chaconine previously observed in relation to destabilization of synthetic phosphatidylcholine–sterol liposomes (Roddick and Rijnenberg, 1987) is now also shown to hold for membranes of living cells from taxonomically diverse groups. Further, the synergisms caused by different solanine–chaconine mixtures are not a simple reflection of the differential lytic ability of the two compounds but show an optimum response at particular ratios. The reasons for this remain obscure, but it is probably significant that the mixtures of solanine and chaconine usually found in potato tissues (60–70% chaconine in tubers; Fitzpatrick et al., 1977; Bushway et al., 1980; 70–80% chaconine in shoots; Ahmed and Müller, 1979; Gregory et al., 1981) are those apparently most effective against animal and fungal membranes. However, whether solanine and chaconine interact synergistically against animals and fungi is not yet known.

Data for in vitro cholesterol binding by different solanine–chaconine mixtures correlate well with those for lysis of beet cells (correlation coefficient $r = 0.77$, $P < 0.01$). On the other hand, with erythrocytes and *Penicillium* protoplasts, cell disruption remained high at higher levels of chaconine, while sterol binding fell away, and no significant correlations were observed between sterol binding and cell disruption ($r = 0.008$ and 0.35, respectively). Although cholesterol is a minor sterol in plants and fungi, the major sterols of these organisms, i.e., sitosterol and ergosterol, complex with potato glycoalkaloids in vitro to a similar extent (Roddick and Rijnenberg, 1986). Consequently, it remains open to question whether the solanine–chaconine synergism is due to sterol binding, whether it is achieved through some other mechanism (e.g., penetration into the membrane), or whether different mechanisms operate in different types of membranes. We have previously expressed reservations concerning the relevance of in vitro sterol binding data to in vivo cell toxicity (Roddick and Drysdale, 1984; Roddick and Rijnenberg, 1986), and similar caution probably needs to be exercised in this case.

Although under natural conditions potato glycoalkaloids encounter erythrocytes only rarely (Nishie et al., 1971) and *Penicillium* protoplasts and beet cells not at all, the responsiveness of these cells suggests that the synergism between solanine and chaconine in relation to membrane disruption is a general biological phenomenon rather than a rare event restricted to particular types of

cells. In addition to their lytic properties, solanine and chaconine may protect plants through other effects, e.g., taste (Zitnak and Filadelfi, 1985) and anticholinesterase activity (Orgell, 1963; Alozie et al., 1978), but whether these parameters are affected synergistically by the two compounds remains to be established.

To date, most studies on the biological activity of potato glycoalkaloids have employed single, pure compounds, but our demonstration of a synergistic interaction between solanine and chaconine questions the validity of extrapolating from single compound effects to in situ interactions between potato tissues and other living systems. In addition, the possibility that solanine and chaconine might further interact with other potato compounds cannot be ruled out. Our findings also raise the question of the extent to which synergisms might occur between other glycoalkaloids, e.g., between solasonine and solamargine, which share a common aglycone (solasodine) and possess the same carbohydrate moieties as solanine (solatriose) and chaconine (chacotriose), respectively, as well as between other quite different secondary plant compounds.

The idea that plant allelochemicals may function in an interactive capacity is a relatively recent one. Commercial insecticide synergists have been known for some time (Metcalf, 1967) but few biochemical/ecological studies of naturally occurring compounds have been conducted in this area. Berenbaum (1985) demonstrated a synergistic interaction between myristicin and xanthotoxin with regard to their deleterious effect on larvae of *Heliothis zea*, while Miyakado et al. (1983) found that two amides of *Piper nigrum*, pellitorine and piperine, although individually nontoxic to *Callosobruchus chinensis*, were highly so in a 1 : 1 combination. Piperine and myristicin (also nontoxic) apparently enhance toxicity by interfering with the activity of detoxifying multifunction oxidase enzymes and are therefore referred to as "quasi" synergists (Berenbaum, 1985). Saponins may also function in a quasisynergistic capacity by affecting the penetration, transport, etc., of biologically active molecules (Freeland et al., 1985). In contrast, the synergism between solanine and chaconine is more an "analog" type (Berenbaum, 1985) where two chemically related compounds of differing biological activity interact such that the efficacy of one or both is increased. However, it is not known if chaconine increases the activity of solanine, or vice versa, or both. Structural analogs are widespread within many groups of secondary plant compounds and may have been selected for partly because chemical defense systems incorporating synergistically interacting components could be energetically less expensive, and partly because resistance to such a "multichemical" defense system would probably not arise so readily (Dolinger et al., 1973). Certainly, such arguments help rationalize the apparent dilemma of considerable material and energy resources being channeled into the synthesis of solanine even though this compound is biologically less effective than chaconine.

There is currently a growing realization that the outcome of plant–herbivore and plant–pathogen conflicts may be determined by the total biochemical profile of plant tissues rather than by single allelochemicals. The additional, and related, possibility that the biological/ecological impact of a particular biochemical environment may derive to a significant extent from interactions between component chemicals finds further support in the data presented here.

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SEX PHEROMONE BIOSYNTHESIS OF (*E,E*)-8,10-DODECADIENOL IN CODLING MOTH *Cydia pomonella* INVOLVES *E*9 DESATURATION

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Abstract—Sex pheromone biosynthesis in the codling moth *Cydia pomonella* (Lepidoptera; Tortricidae) was studied by topical application of deuterated fatty acids in DMSO to pheromone glands. The incorporation of deuterium label into fatty acids and alcohols in the pheromone gland was monitored by gas chromatography with flame ionization detection and mass spectrometry in the selected ion monitoring mode. Dodecanol, (*E*)-9-dodecenol, (*E,E*)-8,10-dodecadienol, tetradecanol, and hexadecanol were found in gland extracts. The application of [12,12,12-$^2H_3$]dodecanoic acid resulted in labeled dodecanol, (*E*)-9-dodecenol, and (*E,E*)-8,10-dodecadienol, as well as the corresponding labeled acids. No label was incorporated into tetradecanol or hexadecanol or any acid with more than 12 carbon atoms. The application of labeled tetradecanoic or hexadecanoic acid introduced label not only into the 12-carbon alcohols, but also into tetradecanol, or tetradecanol and hexadecanol, respectively. The application of (*E*)-[11,11,12,12,12,-$^2H_5$]9-dodecenoic acid, whose facile synthesis is described, resulted in labeled (*E*)-9-dodecenol and (*E,E*)-8,10-dodecadienol. The (*E,E*)-8,10-dodecadienol so produced was characterized by an ion at *m/z* 186, equivalent to $[M]^+$ of a dienol labeled with four deuterons. Thus, one deuterium label is lost when the labeled (*E*)-9-monoene is converted to the (*E,E*)-8,10-diene. We conclude that (*E,E*)-8,10-dodecadienol is synthesized by chain shortening (β-oxidation) of palmitic acid to dodecanoic acid, followed by an unusual *E*9 desaturation and subsequent conversion of this intermediate into the conjugated precursor, which is finally reduced to the pheromone alcohol. The evolutionary significance of *E*9 desaturation being responsible for pheromone production in an Olethreutinae species is discussed.

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Key Words—pheromone, Lepidoptera, Tortricidae, *Cydia pomonella*, biosynthesis, palmitic acid, (*E*)-9-dodecenoic acid, (*E,E*)-8,10-dodecadienol, *E*9 desaturation, capillary gas chromatography, deuterium, labeled precursors.

INTRODUCTION

Available information concerning pheromones in many lepidopteran families indicates that most of the pheromone components can be biosynthetically derived from hexadecanoic (palmitic) acid, by chain shortening through β-oxidation and desaturation, involving a Δ 11-desaturase (Roelofs and Bjostad, 1981, 1984). Z11 desaturation of hexadecanoic acid followed by chain shortening and subsequent reduction and acetylation can produce the whole homologous series (Z)-11-hexadecenyl, (Z)-9-tetradecenyl, (Z)-7-dodecenyl, and (Z)-5-decenyl acetate, as was shown in the turnip moth (Löfstedt et al., 1986a). If chain shortening precedes desaturation, Δ11-unsaturated 14-carbon compounds and their homologous chain shortened Δ 9-unsaturated 12-carbon compounds can be produced. Such compounds are the predominant pheromone components within the Tortricidae (Roelofs and Brown, 1982).

Interestingly, many tortricids within the Olethreutinae subfamily have Δ 8- and Δ 10-unsaturated, or Δ 8,10 doubly unsaturated pheromone components. Analysis of pheromone precursors in two primitive New Zealand tortricids supported the idea of Δ 10 desaturation being responsible for the production of Δ 8- and Δ 10-unsaturated compounds (Löfstedt and Roelofs, 1985). Roelofs and Brown (1982) suggested that the Δ 8,10 doubly unsaturated pheromone components could be produced by Δ 10 desaturation, followed by chain shortening and a new Δ 10 desaturation. Alternatively, they suggested that the production of these pheromone components could be accounted for by a Δ 11-desaturase producing 11-tetradecenoate moieties. The Δ 11-tetradecenoate could then be biosynthetically converted into a Δ 10,12 conjugated system and subsequently chain shortened to the Δ 8,10-12 carbon compounds. Such a pathway was confirmed in the silkworm moth *Bombyx mori*, which stores large amounts of (Z)-11-hexadecenoate (Z11–16:acyl) (Bjostad and Roelofs, 1984) that are converted to (*E*, Z)-10,12-hexadecenoate (*E*,Z10,12–16:acyl), the immediate fatty acid precursor of bombykol (*E*,Z)-10,12-hexadecadienol (Yamaoka et al., 1984).

The major pheromone component of the codling moth was identified as (*E*,*E*)-8,10-dodecadienol (*E*,*E*8,10–12:OH) (Roelofs et al., 1971). In addition to other geometric isomers and the corresponding aldehyde and acetate, the codling moth pheromone gland was also found to contain dodecanol (12:OH), tetradecanol (14:OH), hexadecanol (16:OH), and (*E*)-9-dodecanol (*E*9–12:OH) (Arn et al., 1985; Einhorn et al., 1984). The occurrence of *E*9–12:OH

led Arn et al. (1985) to suggest that (*E*)-9-dodecenoate (*E*9–12 : acyl) could be a monounsaturated intermediate in biosynthesis of the pheromone. In fact *E*9–12 : acyl was the only unusual monounsaturated fatty acyl moiety found by us in a preliminary precursor analysis, indicating its potential role in pheromone biosynthesis.

In an earlier study on pheromone biosynthesis in the turnip moth, we showed that deuterium-labeled fatty acids, as well as their corresponding acetates, could be separated from unlabeled analogs by gas chromatography on polyethylene glycol-type stationary phases (Löfstedt et al., 1986a. In the present paper pheromone biosynthesis in the codling moth is investigated by the same technique. Deuterium labeled fatty acids are applied topically to the pheromone gland and their incorporation into pheromone alcohols is studied.

METHODS AND MATERIALS

Insect Sources and Topical Application of Labeled Fatty Acids to Glands. Codling moths for the experiments with saturated fatty acids were obtained as pupae from Drs. H. Arn and E. Mani, Wädenswil, Switzerland. Moths for the experiment with labeled (*E*)-9-dodecanoic acid (*E*9–12 : acid) were obtained from a culture in our laboratory, initiated from eggs obtained from Dr. R. Bues, Avignon, France. The pupae and emerged insects were kept at a 16 : 8 hr light–dark cycle. Emerged insects were fed a 5% sucrose solution.

Labeled fatty acids in DMSO (4 μg in 0.2 μl) were applied topically to the pheromone glands of 2- to 4-day-old *Cydia pomonella* females as described earlier by Bjostad and Roelofs (1983) for other lepidoptera. After the application at the beginning of the dark period, the glands were held everted for 1 hr until they were dissected from the ovipositors and extracted for analysis.

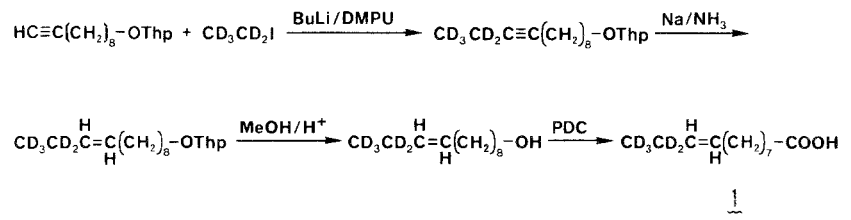
Pheromone Gland Extraction and Methylation. The pheromone glands were dissected from the ovipositors with a pair of fine forceps and then extracted with 10 μl of redistilled hexane for analysis of pheromone alcohols in the gland. After 10–30 min, the hexane extract was recovered and 10 μl of a 2 : 1 (v/v) mixture of chloroform and methanol was added for total lipid extraction (Folch et al., 1957). Under these conditions more than 90% of the acyl moieties were contained in the chloroform–methanol extract. Fatty acyl moieties in this extract were converted to methyl esters by base-catalyzed methanolysis as described in detail by Löfstedt et al. (1986b). The samples usually were analyzed immediately on the gas chromatograph or occasionally stored in the freezer at $-20°C$ until analysis.

Gas Chromatographic Analysis with Flame Ionization Detection and Selected Ion Monitoring. Capillary gas chromatography with flame ionization

detection (GC-FID) was performed on a Hewlett Packard model 5880 GC equipped with a 30-m × 0.25-mm-id DB-wax column (cross-linked polyethylene glycol) (J&W Scientific Inc., Rancho Cordova, California). Conditions of chromatography were: hydrogen carrier gas velocity 40 cm/sec at 80°C; split valve opened 1 min after injection; temperature maintained at 80°C for 2 min following injection and then programmed at 5°C/min to 230°C. Under these conditions omega-labeled ($^2H_3$) fatty acid methyl esters and acetates are resolved from the corresponding nonlabeled compounds, so that the labeled compounds elute slightly earlier (Löfstedt et al., 1986a). Synthetic samples of the deuterium-labeled alcohols [12,12,12-$^2H_3$]dodecanol, [14,14,14-$^2H_3$]tetradecanol, [16,16,16-$^2H_3$]hexadecanol, and (*E*)-[11,11,12,12,12-$^2H_5$]9-dodecenol were analyzed together with a series of homologous unlabeled straight-chain alcohols, and their equivalent chain lengths (ECLs) were calculated. ECL values of the saturated alcohols were 1197, 1396, and 1597, respectively. ECL of the labeled (*E*)-9-dodecenol was 1236 compared to 1242 for unlabeled. Mass spectrometry with electron impact ionization was performed on a Hewlett Packard model 5970B GC-MS system equipped with a 59970B computer system, and interfaced with a Hewlett Packard 5890 GC (courtesy of Dr. J. Eyem, Hewlett Packard, Sweden). The molecular ion of *E,E8, 10–12:OH* amounts to about 10% of the base peak in EI spectra of this compound and is suitable for sensitive and selective analysis by selected ion monitoring (SIM) in the EI mode (Löfstedt and Odham, 1984). An acquisition program was designed to monitor the incorporation of (*E*)- [11,11,12,12,12-$^2H_5$]9-dodecanoic acid into *E,E*8,10–12:OH. The ions *m/z* 181.20, 182.20, 185.20, 186.20, and 187.20 were monitored, being equal to $[M\text{-}1]^+$, and $[M]^+$ for the native *E,E*8,10–12:OH, and to $[M]^{+\cdot}$ for the corresponding alcohols with three, four, and five deuterium labels, respectively.

Chemicals. Deuterium-labeled saturated fatty acids were purchased from Larodan Fine Chemicals, Malmö, Sweden. The deuterium enrichment of these omega-labeled acids was 99%. The acids are abbreviated: [12,12,12-$^2H_3$]dodecanoic acid, [12-D_3]12:acid, etc.

(*E*)-[11,11,12,12,12-$^2H_5$]9-dodecenoic acid (1) [11,12-D_5]*E*9–12:acid) was prepared from 1-(2-tetrahydropyranyloxy)-decyne (1.4 g, 6 mmol), *n*-butyllithium (4.4 ml, 1.44 M in hexane) in dry THF and [$^2H_5$]ethyl iodide in 10 ml freshly distilled DMPU (1,3-dimethyloxohexahydropyrimidine) (Bengtsson and Liljefors, 1988). Reduction with sodium in liquid ammonia (Warthen and Jacobson, 1973) gave the pure *E* monoene, which was treated with *p*-toluene sulfonic acid in methanol to give the corresponding alcohol. Oxidation with pyridinium dichromate (PDC) in dry dimethylformamide (DMF) (Corey and Smidt, 1979) gave the final product (0.52 g, 40% overall yield) (Scheme 1).



SCHEME 1.

The product was purified by TLC mesh column chromatography (Taber, 1982) and argentation liquid chromatography (Houx et al., 1974). An EI mass spectrum of the acid was recorded on a Finnigan 4021 mass spectrometer and [^{1}H]NMR on a Varian XL-300 spectrometer in $CDCl_3$ solutions with Me_4Si as internal reference: *m/z* 203 (M^+, 2%), 185(2), 143(2), 123(3), 110(4), 97(15), 87(12), 83(24), 74(42), 69(45), 60(42), 55(78), 43(100), 33(6). [^{1}H]NMR ($CDCl_3$) (300 MHz); δ 1.30 (m, 8 H, CH_2CH_2), 1.56–1.67 (m, 2H, CH_2—C—COOH), 1.93–1.99 (m, 2H, CH_2—C=C), 2.34 (t, 2H, CH_2—COOH), 5.32–5.46 (m, 2H, CH=CH)

The deuterium enrichment of the acid was >95% according to FAB high-resolution mass spectroscopy on a ZAB-HF, VG Analytical, 11-250, mass spectrometer: $[M\text{-}H]^-_{calc}$ = 202.185, $[M\text{-}H]^-_{obs}$ = 202.178. [12,12,12-$^2H_3$]-Dodecanol, [14,14,14-$^2H_3$]tetradecanol, [16,16,16-$^2H_3$]hexadecanol, and (*E*)-[11,11,12,12,12-$^2H_5$]9-dodecenol were prepared from the corresponding acids by reduction with lithium aluminum hydride in dry diethyl ether.

RESULTS

In a first set of experiments, sex pheromone glands were incubated with [12-D_3]12:acid. The glands incorporated the labeled acid into the alcohols 12:OH, *E*9–12:OH, and *E*,*E*8,10–12:OH (Table 1, Figure 1A), as well as into the corresponding fatty acyl moieties 12:acyl, *E*9–12:acyl, and *E*,*E*8,10–12:acyl (Table 1, Figure 2A). The peaks, indicating 12-D_3-labeled pheromone component and pheromone precursor analogs, were all absent in chromatograms of unlabeled glands (Figures 1B, 2B). The relatively low amount of labeled methyl dodecanoate (12:Me) recovered indicates that only a fraction of the 4 μg of topically applied [12-D_3]12:acid is incorporated into the pheromone gland fatty acid metabolism. No labeled 14- and 16-carbon alcohols or acyl moieties were produced.

In a second set of experiments, pheromone glands were incubated with [14-D_3]14:acid and [16-D_3]16:acid (Table 1). The relative amounts of labeled

TABLE 1. INCORPORATION OF DEUTERIUM-LABELED FATTY ACIDS INTO SEX PHEROMONE OF CODLING MOTH, *Cydia pomonella*.[a]

Labeled precursor	No. of ♀	Native *E*,*E*8, 10–12:OH (ng/♀)	Amount of labeled compound relative to native (=100)									
			Alcohols					Acyl moieties as methyl esters				
			12:OH	*E*9–12:OH	*EE*8, 10–12:OH	14:OH	16:OH	12:Me	*E*9–12:Me	*EE*8, 10–12:Me	14:Me	16:Me
[12-D_3] 12:acid	3	0.1	58	*[b]	257	0	0	—	—	—	—	—
	7	0.1	58	30	144	0	0	95	30	140	0	0
	8	0.5	108	74	103	0	0	65	49	134	0	0
[14-D_3] 14:acid	4	2	2	0	4	10	0	—	—	—	—	—
	4	1.5	8	—[c]	8	19	0	0	0	0	25	0
[16-D_3] 16:acid	4	0.5	32	30	65	49	25	2	0	36	1	1
[11,12-D_5]-	7	0.1	0	1000	287	0	0	0	302	236	0	0
*E*9–12:acid	5	0.7	0	664	76	0	0	0	567	120	0	0

[a] List of experiments including replicates.
[b] contaminated.
[c] —not analyzed.

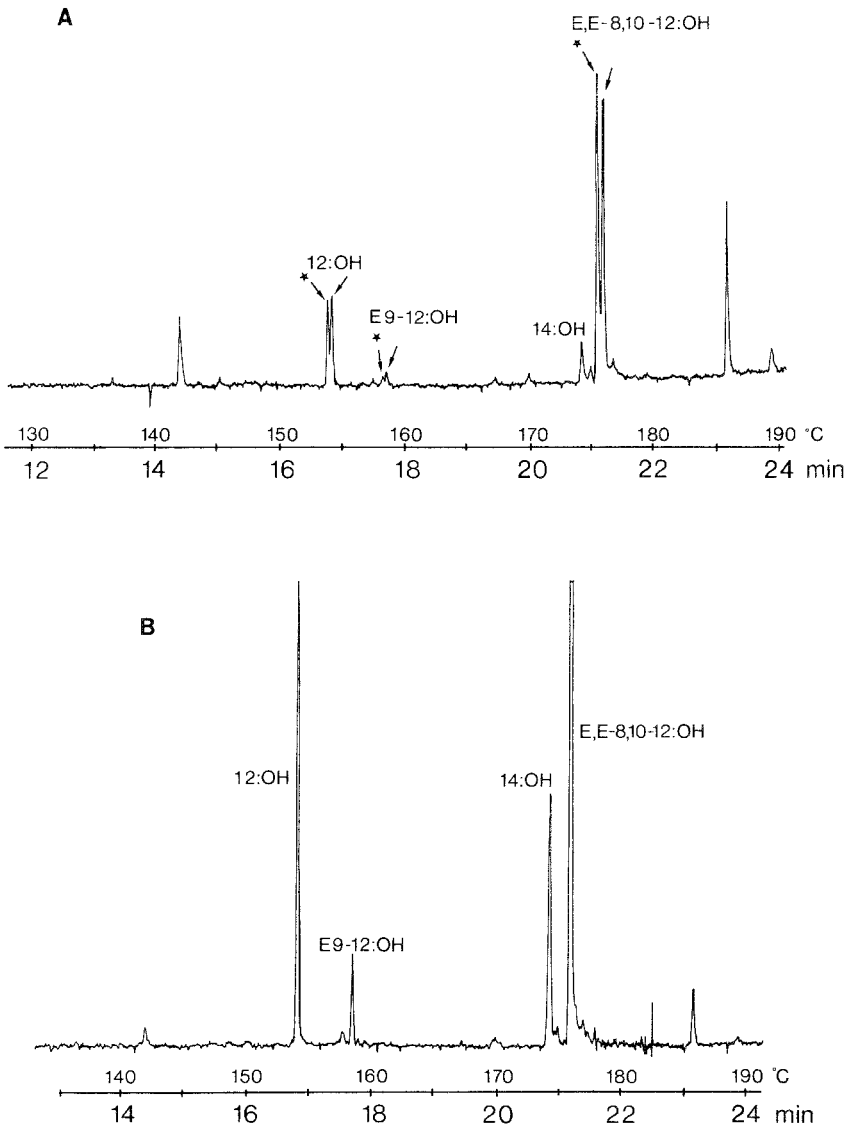


FIG. 1. Incorporation of deuterium label from topically applied [12,12,12-$^2H_3$]dodecanoic acid into pheromone components in glands of 8 female *Cydia pomonella*, monitored by capillary gas chromatography with flame ionization detection (A) and a control of two unlabeled glands (B). Peaks with (*) have the predicted retention times of omega-labeled ($^2H_3$) analogs of the pheromone components. See text for abbreviations.

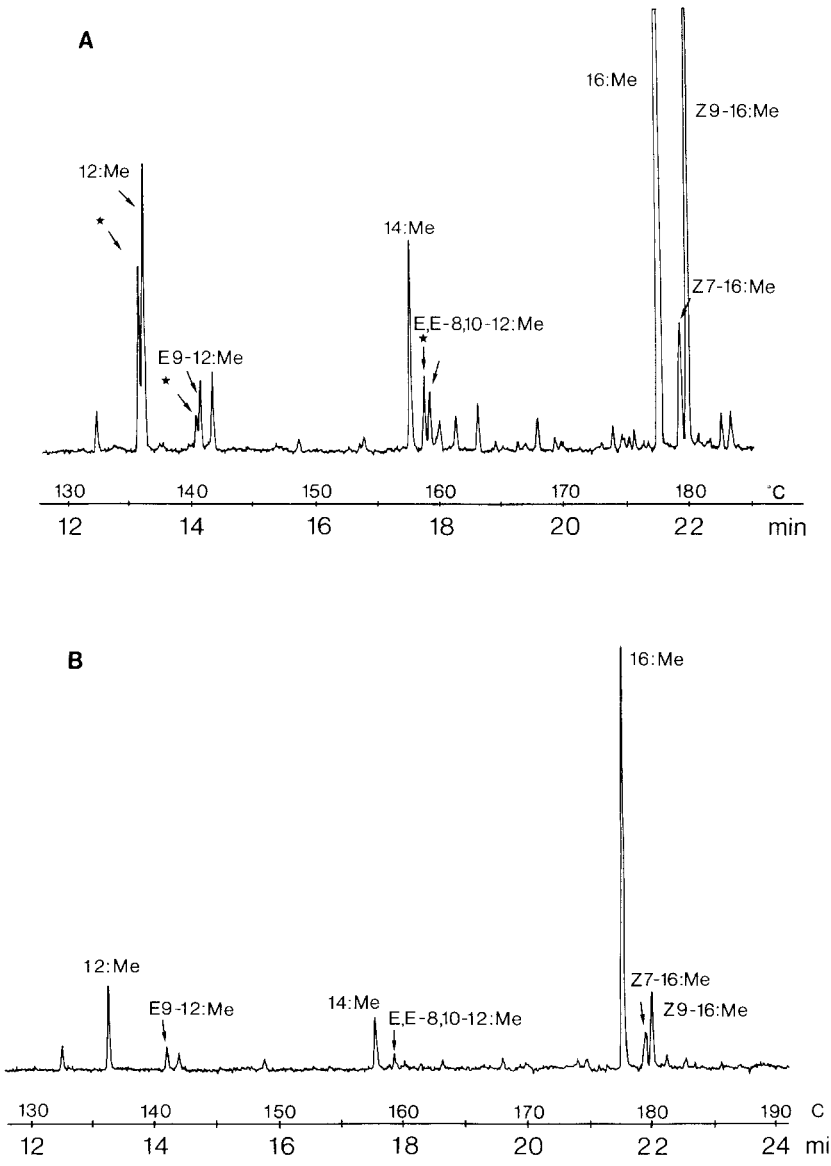


FIG. 2. Incorporation of deuterium label from topically applied [12,12,12-$^2H_3$]-dodecanoic acid into fatty acids in glands of eight female *Cydia pomonella*, monitored by capillary gas chromatography with flame ionization detection (A) and a control of two unlabeled glands (B). The first peak (*) in a pair has the predicted retention time of the omega-labeled ($^2H_3$) analog of the respective fatty acids. See text for abbreviations.

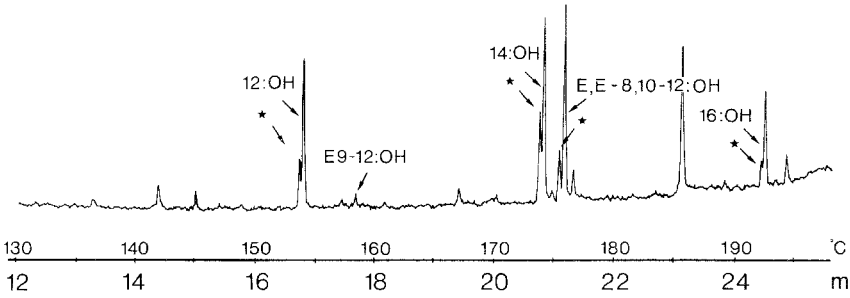


FIG. 3. Incorporation of deuterium label from topically applied [16,16,16-$^2H_3$]-hexadecanoic acid into alcohols in glands of four female *Cydia pomonella*, monitored by gas chromatography with flame ionization detection. The first peak (*) in a pair has the predicted retention time of the omega-labeled ($^2H_3$) analog of the respective alcohol. See text for abbreviations.

alcohols and acids produced with the [14-D_3]14:acid precursor were low, but still 4–8% of labeled *E,E*8,10–12:OH was obtained. This time, the label was found also in 14:OH and 14:Me. When glands were incubated with [16-D_3]16:COOH, the label was found in all the pheromone gland alcohols, including 16:OH (Figure 3).

Finally, pheromone glands were incubated with [11,12-D_5]*E*9–12:acid. These incubations produced large relative amounts of *E*9–12:OH and *E,E*8,10–12:OH, as well as their corresponding fatty acyl groups (Table 1). No label was incorporated into any of the saturated alcohols or acids studied. The incorporation of [11,12-D_5]*E*9–12:acid into *E,E*8,12:OH was further studied by mass spectrometric analysis of the products of an incubation. We found that incubation of glands with acid labeled with five deuterium atoms produced *E,E*8,10–12:OH labeled with four rather than five deuterons (Figure 4). The signal *m/z* 187 indicating [D_5]*E,E*8,10–12:OH was only 13% of *m/z* 186, indicating [D_4]*E,E*8,10–12:OH. The production of *E,E*8, 10–12:OH labeled with four deuterons should be due to metabolic (as opposed to mass spectrometric) loss of one deuterium atom as the mass spectrum of unlabeled *E,E*8,10–12:OH contained no detectable amounts of $[M-1]^+$.

DISCUSSION

We conclude that the major pheromone component of the codling moth *E,E*8,10–12:OH is biosynthesized from palmitic acid by two cycles of β-oxidation, followed by *E*9 desaturation of dodecanoic acid and a subsequent trans-

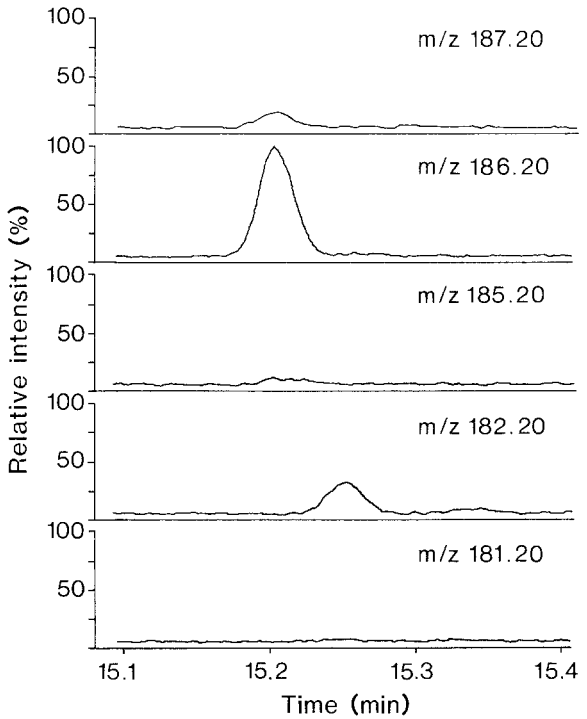


FIG. 4. Incorporation of deuterium label from topically applied (*E*)-[11,11,12,12,12-$^2H_5$]9-dodecenoic acid into (*E*,*E*)-8,10-dodecadienol in glands of seven female *Cydia pomonella*, analyzed by selected ion monitoring of ions *m/z* 181.20, 182.20, 185.20, 186.20, and 187.20. See text for details.

formation of the *E*9 double bond into the conjugated (*E*,*E*)-8,10-diene. The acid is finally reduced to produce the corresponding alcohol (Figure 5). The production of the conjugated diene before the reduction of acid to alcohol is supported by the presence of labeled *E*,*E*8,10–12 : acyl.

The fact that the unusual *E*9 desaturation of dodecanoic acid is a key step is supported by the absence of labeled chain-elongation products in the incubations with [12-D_3]12 : acid. An alternative route to the monoene could have been Δ 11 desaturation of tetradecanoic acid, a reaction widely distributed in tortricids (Roelofs and Brown, 1982), followed by chain shortening to give *E*9–12 : acyl. However, in this case [12-D_3]12 : acid should first have been elongated to labeled tetradecanoic acid (which we could not detect) and then desaturated and chain-shortened to produce the labeled *E*9–12 and *E*,*E*8,10–12 moieties.

We found that the incorporation of labeled precursors, as well as the amount of pheromone components in the glands, were quite variable from one experi-

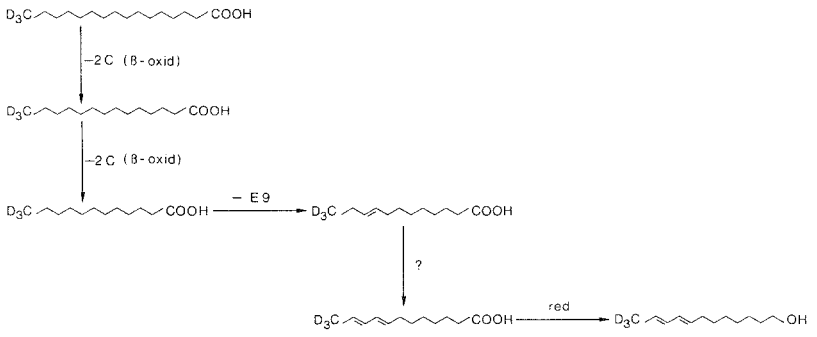
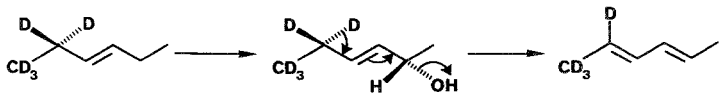


FIG. 5. Biosynthesis of sex pheromone gland constituents from hexadecanoic acid in the codling moth, *Cydia pomonella*. The proposed reactions involved are chain shortening (β-oxidation), *E*9 desaturation, conversion of the monoene into a conjugated diene, and reduction of the acids to alcohols.

ment to another (Table 1), even though usually four or more females were included in one batch. This problem is not easily overcome by merely increasing the number of females in each batch, as Bjostad and Roelofs (1986) noticed the same phenomenon with as many as 20 glands used for each extraction. For this reason, we preferred to estimate the relative, rather than absolute, amounts of labeled compounds produced. Ideally, incorporation of each compound should have been measured relative to the incorporation of an internal standard, a compound with known metabolic activity, applied simultaneously.

The similarity of pheromone biosynthesis in the codling moth with that of *E*,*Z*10,12-16:OH in the silkworm moth is interesting, as the two species are representatives of two quite different lepidopteran families, Tortricidae and Lymantriidae, respectively. The loss of one deuteron, when the dienic pheromone precursor in *C. pomonella* is formed from [11,12-D_5]*E*9-12:acid, was not investigated in detail, but we suggest that the key steps involve oxidation of one of the α positions surrounding the double bond, followed by a 1,4 elimination of water (Scheme 2). Such a reaction mechanism accounts also for the



SCHEME 2.

biosynthesis of bombykol. Yamaoka et al. (1984) report 15% of $[M]^+$ for bombykol labeled with two deuterons relative to $[M]^+$ for the native bombykol when (*Z*)-[11,12-$^2H_2$]11-hexadecenoic acid is metabolized to bombykol. There is no indication of a product with one deuterium label only. This demonstrates that

both labeled atoms are retained when [11,12-$^2H_2$]Z11-16:acyl is converted to bombykol in *B. mori*.

*E*9 desaturation has not been described before in moth pheromone biosynthesis. Roelofs and Brown (1982) used information available on pheromone production in the Tortricidae to discuss taxonomy and the possible phylogeny of this group of Lepidoptera. Our finding of an *E*9-desaturase in *C. pomonella* does not resolve the classic controversy over the phylogenetic relationship among different tortricid subfamilies or tribes. However, the novel enzyme inferred tells us that there is no close biosynthetic relationship between the production of Δ 8 and Δ 10 monounsaturated compounds by a Δ 10-desaturase, and the production of Δ 8,10 doubly unsaturated compounds involving *E*9 desaturation. Whether the *E*9-desaturase is more closely related to the *E*11-, the Z10-, or the Z11-desaturase remains an open question, as does the phylogenetic position of the Olethreutinae relative to the Tortricinae.

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IRIDOID GLYCOSIDES AS OVIPOSITION STIMULANTS FOR THE BUCKEYE BUTTERFLY, *Junonia coenia* (NYMPHALIDAE)

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Abstract—Females of *Junonia coenia* (Nymphalidae), a specialist on plants that contain iridoid glycosides, were found to use aucubin and catalpol, iridoid glycosides typical of a host plant, *Plantago lanceolata* (Plantaginaceae), as oviposition cues. Incorporating dried ground leaf material or pure iridoid glycosides into agar disks proved to be a very effective method of testing. In no-choice tests and choice tests, females laid more eggs on disks with *P. lanceolata* leaf material or iridoid glycosides, compared to agar controls. There was variation among individual females in preference for disks with *P. lanceolata* leaf material versus disks with iridoid glycosides. Females given a choice of three different concentrations of iridoid glycoside (0.2, 0.5, 1.0%) in the agar disks and a control laid more eggs on the disk with the highest concentration of iridoid glycoside.

Key Words—Iridoid glycoside, *Junonia coenia*, Lepidoptera, Nymphalidae, catalpol, aucubin, oviposition, *Plantago lanceolata*, insect–plant interaction.

INTRODUCTION

The oviposition behavior of a butterfly is a complex chain of stimuli and responses (Papaj and Rausher, 1983). It begins with the location of the host plant, where visual and olfactory cues are of primary importance. Once the butterfly has located a plant, however, contact chemoreception becomes

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involved, and in specialist species, the leaf allelochemicals may be critical stimulants for oviposition (Schoonhoven, 1972; Stanton, 1979; Feeny et al., 1983; Ohsugi et al., 1985; Honda, 1986). Although other factors are certainly involved in the decision of whether or not to oviposit on an individual host plant or leaf (see Singer, 1982, 1983), leaf chemicals have been suggested, although seldom shown, to be critical as oviposition cues (Feeny et al., 1983; Chew and Robbins, 1984). In the Lepidoptera, the best studied systems are mustard oils and pierid butterflies (David and Gardner, 1962) and the swallowtail butterflies of the Papilionidae (Feeny et al., 1983 and references therein; Ohsugi et al., 1985; Honda, 1986).

The buckeye, *Junonia coenia* Hübner (Nymphalidae), is a specialist on plants containing iridoid glycosides (Bowers, 1984), as are butterflies of the genus *Euphydryas* (Bowers, 1981, 1983). Certain of these compounds characteristic of the host plants, such as aucubin and catalpol, serve as feeding stimulants and attractants for larvae of these insects (Bowers, 1983, 1984). Iridoid glycosides are characteristic of about 50 families of plants (El-Naggar and Beal, 1980), but they are particularly prevalent in the Scrophulariaceae and Plantaginaceae, the primary host-plant families of *J. coenia* and *Euphydryas* (Jensen et al., 1975; Bowers, 1983, 1984).

The importance of iridoid glycosides as critical cues for larval feeding preferences in *J. coenia* suggested that these compounds would also be important as oviposition stimulants, but this had not been demonstrated. The relationship between adult oviposition preference and larval requirements is not always clear, and errors in oviposition are not uncommon (Dethier, 1959; Singer, 1971, 1982; Wiklund, 1974; Chew, 1977; Chew and Robbins, 1984). In addition, the role of host-plant allelochemicals in determining oviposition behavior can be extremely complex and is not always as straightforward as suggested by the work of David and Gardner (1962) on pierids and mustard oils (see discussion in Feeny et al., 1983).

The objectives of the experiments described in this paper were to determine, first, if iridoid glycosides were oviposition stimulants for *J. coenia* and, second, whether females responded to differences in the concentration of iridoid glycosides in the oviposition substrate.

METHODS AND MATERIALS

Insects, Plants, and Iridoid Glycosides

Adult *J. coenia* were obtained by rearing larvae on leaves of *Plantago lanceolata* L. (Plantaginaceae), a commonly used host plant (Shapiro, 1974; Scott, 1975; Bowers, 1984). This plant species contains the iridoid glycosides catalpol and aucubin (Duff et al., 1965; Bobbitt and Segebarth, 1969). The

colony used for these experiments originated from a strain in North Carolina. *Plantago lanceolata* leaves were obtained from plants grown in the greenhouse or outside in a garden plot.

Newly emerged males and females were put into cylindrical net bags (30 × 30 × 45 cm) for mating, for a period of six days. Preliminary experiments had shown that this period was sufficient for most of the females to have mated. They were kept in growth chambers (photoperiod 16:8; light–dark, 30°C day, 25°C night). Butterflies were fed once a day with a solution of 2 g sucrose + 1 g glucose + 1 g fructose in 50 ml water. Females were isolated on the seventh day in individual plastic boxes without any oviposition stimulus.

The pure iridoid glycosides used in these experiments were obtained from W. Berkowitz at Queens College (aucubin) or extracted from *Catalpa bignonioides* (Bignoniaceae) (catalpol). Gas chromatography showed these compounds to be very pure (at least 99%, D. Gardner, personal communication; methods in Stermitz et al., 1986).

Oviposition Tests

Preliminary experiments showed that using paper "leaves" with test solutions (as used by Stanton, 1979; Feeny et al., 1983) did not work with *J. coenia.* Search of the literature and additional tests showed that a modification of the method used by Hovanitz and Chang (1964) worked extremely well. This method used agar disks molded in 5.5-cm-diameter Petri dishes as the oviposition substrate. The basic agar disk, 0.5 g agar mixed and cooked with 15 ml water, was used as the control. To this agar solution could be added various experimental components: 0.5 g dried, ground *P. lanceolata* leaf material, or various amounts and combinations of aucubin and catalpol. Since iridoid glycosides are polar compounds, they were easily combined with water and agar.

The test disks were all white except for those containing the *P. lanceolata* leaf material, which were dark green. Since females would oviposit well on the white disks, we did not try to control for color. Although visual orientation is clearly important in approaching host plants from a distance (Papaj and Rausher, 1983), in our experimental set-up, such cues were probably much less important than short-range chemical cues.

For the oviposition tests, individual females were isolated in clear plastic boxes (13.4 × 7.1 × 8.4 cm) or clear plastic cylinders between two large Petri dishes (12 cm high × 14 cm diameter). Three different experiments were carried out, including both no-choice and choice tests. Different sets of females were used for each experiment. All females used were 8, 9, or 10 days old at the beginning of an experiment.

Experiment 1, No-Choice Test. Twenty-four females were randomly assigned to each of three test groups, eight females to each group. Individual

females in each group were isolated with one of three oviposition substrates. Group 1 (control) females received pure agar disks, group 2 females received agar disks containing *P. lanceolata* leaf material (0.5 g leaf material/0.5 g agar), and group 3 females received agar disks with iridoid glycosides. For the group 3 females, on the first day, we used 0.01 g iridoid glycoside per disk (0.5 g agar), or 2.0% dry weight. The iridoid complement was 0.007 g catalpol + 0.003 g aucubin. This amount of pure iridoid was equivalent to 2% of the weight of *P. lanceolata* leaves used in those disks. Although the amount of iridoid glycosides in plants can vary dramatically (Stermitz et al., 1986; Harris et al., 1986; Fajer and Bowers, unpublished data), 1–2% of the dry weight of a leaf is common (Stermitz, personal communication) and is well within the range of what has been found in *P. lanceolata* (Duff, 1965). Because we were directly comparing leaf material and pure iridoid glycoside, we wanted the amounts of iridoid glycosides in disks with *P. lanceolata* leaves and pure iridoid glycoside to be roughly equivalent. On the second day, we used 1.0% dry weight (0.005 g) iridoid (1% of the weight of the leaf material).

We compared the number of eggs laid by individual females on each of the three groups over two consecutive 24-hr periods. Disks were collected after the first day and the eggs counted. Fresh disks of the same kind were given to each female and they were again allowed to oviposit for 24 hr.

The Kruskal-Wallis test (Conover, 1980) was used to compare the number of eggs laid on the three substrates.

Experiment 2, Paired Choice Test. Twelve females were presented with three different choice tests over three days: Agar versus *P. lanceolata* leaf material in agar (0.5 g leaf/0.5 g agar), agar versus catalpol in agar (0.005 g/0.5 g agar, 1% dry weight), and *P. lanceolata* leaf material versus catalpol (amounts as above). On the first day, four females were randomly assigned to each of the treatments. On the second day, each group of four females was given a different choice test, and on the third day, each group of four females was again given a different choice. Thus each of the 12 females was exposed to each of the three possible choices. Females were allowed to oviposit from 8:00 AM until 1:00 PM each day. After this, females were isolated without oviposition substrate until the next morning when they were given another choice. The females were 10 days old on the first day of the test. The number of eggs laid by each female on each test disk was counted after the 5-hr test.

Comparisons were made by one-way ANOVA on the arcsine-transformed percentages of the number of eggs laid on each substrate.

Experiment 3, Concentration Test. Four concentrations of catalpol were presented to individual females at the same time to determine, first, whether there was a threshold concentration necessary to elicit oviposition, and second, whether females discriminated among different concentrations. Each of 18

females was placed into a transparent cylinder and presented with four disks: 0% catalpol, 0.2% of the weight of the leaf material (0.001 g/0.5 g agar), 0.5% (0.0025 g/0.5 g agar), 1.0% (0.005 g/0.5 g agar). The females were allowed to oviposit for 48 hr and the number of eggs on each disk counted for each female.

The results were analyzed by one-way ANOVA on the arcsine-transformed percentages of the total number of eggs laid on each of the test substrates, followed by linear comparisons (Sokal and Rohlf, 1969).

RESULTS

No-Choice Test. Five females that did not lay any eggs at all during the two days were excluded from the analysis (two from the control group, two from the group receiving agar disks with *P. lanceolata* leaf material, and one from the group receiving agar disks with iridoid glycosides). We have no idea why they refused to lay eggs, when the others did so readily, and all the females used in these experiments subsequently laid fertile eggs and so were known to be inseminated. One female in the pure agar treatment died after the first day, having laid no eggs, and was also excluded.

Our results show that dried, ground *P. lanceolata* leaf material or pure iridoid glycoside stimulated oviposition, while the agar control did not (Figure 1). The total number of eggs laid by the females varied significantly with oviposition substrate (Figure 1) (Kruskal-Wallis test, $T = 8.19$, $df = 2$, $P < 0.025$). There was no significant difference in the number of eggs laid on disks with *P. lanceolata* leaf powder and pure iridoid glycosides (Kruskal-Wallis test, $P > 0.20$). The number of eggs laid on substrates with *P. lanceolata* leaf powder or pure iridoid glycosides was significantly different from the number laid on the control agar disks (Kruskal-Wallis test, *P. lanceolata* leaves—$P < 0.05$, pure iridoid—$P < 0.05$). Thus pure iridoid glycosides are sufficient to stimulate oviposition in *J. coenia* and at a level similar to that of *P. lanceolata* leaves.

Paired Choice Test. Females significantly preferred to oviposit on the substrates containing ground *P. lanceolata* leaf material or catalpol over the agar control (Figure 2). Although the mean for the percentage of eggs laid on the substrate with *P. lanceolata* leaf material was twice that on the substrate with catalpol, this was not significantly different, probably due to the variation among individual females (Figures 2 and 3). Although eight females laid more eggs on the disks with *P. lanceolata* leaf material, three preferred the substrate with catalpol, while one laid equal numbers of eggs on the two substrates (Figure 3).

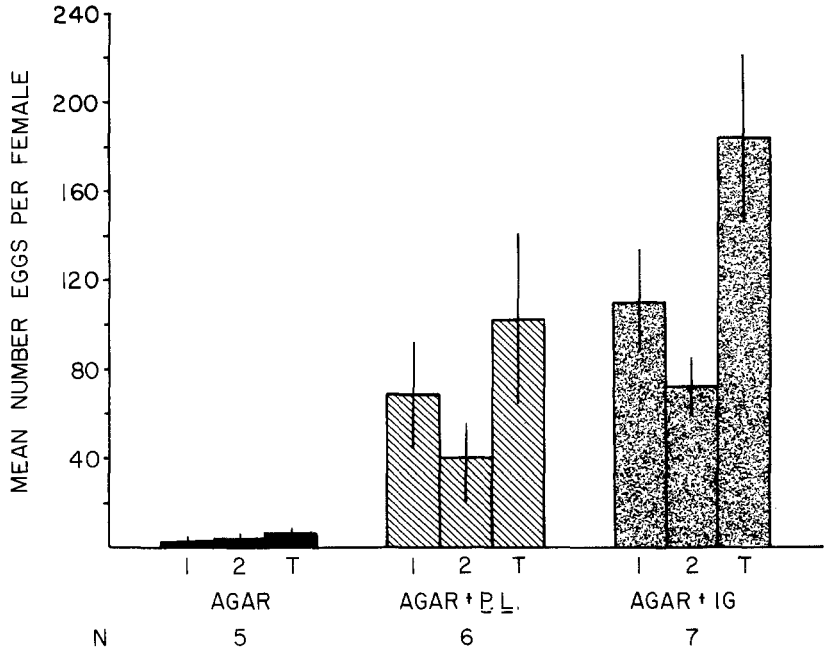

FIG. 1. Mean number of eggs laid by female *Junonia coenia* in a no-choice experiment. Results are shown for day 1, day 2, and the pooled data for days 1 and 2. Values are means ± standard error. N = number of females. AGAR = pure agar (control), AGAR + P.L. = agar + *P. lanceolata* leaf material (0.5 g/0.5 g agar), AGAR + IG = agar + catalpol and aucubin (0.007 g catalpol + 0.003 g aucubin/0.5 g agar).

Concentration Test. There was a highly significant difference in the number of eggs laid on the four test substrates (Figure 4) ($F = 7.853$, $df = 3,68$, $P < 0.001$). Linear comparisons (Sokal and Rohlf, 1969) showed that the number of eggs laid on the agar control was significantly less than that laid on any of the substrates with the iridoid glycoside, catalpol (Figure 4) ($F = 16.669$, $df = 1,68$, $P < 0.001$), as predicted by the results of the previous experiments. In addition, the females laid significantly more eggs on the substrate with 1% catalpol than on the other substrates with lower concentrations of the iridoid glycoside (Figure 4) ($F = 6.861$, $df = 1,68$, $P < 0.001$). Thus females were able to recognize the substrate containing the highest concentration of catalpol and laid most of their eggs there. There was no difference in the number of eggs laid on the substrates with 0.2% and 0.5% catalpol ($F = 0.027$, $df = 1,68$, NS).

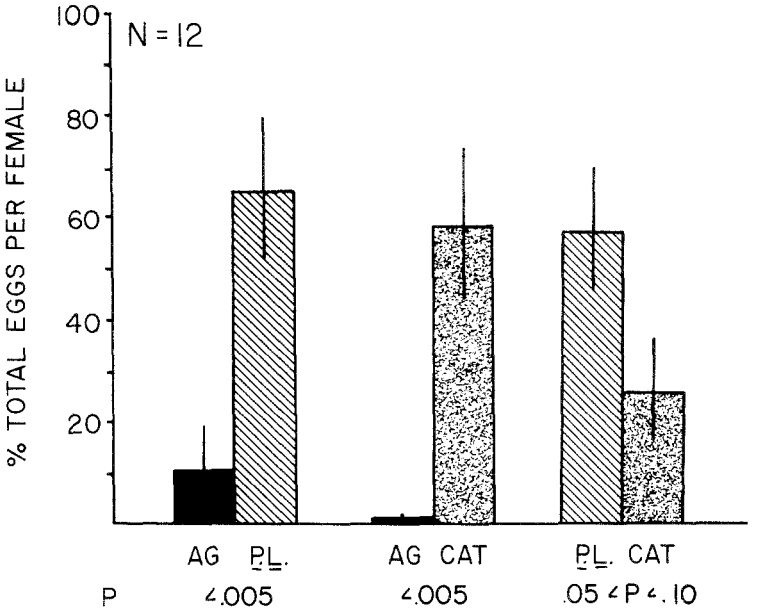

FIG. 2. Mean percent of eggs laid per female in a choice experiment. Values are means ± standard error. A total of 12 females were used. AG = pure agar (control), P.L. = agar + *P. lanceolata* leaf material (0.5 g/0.5 g agar), CAT = agar + catalpol (0.005 g/.5 g agar).

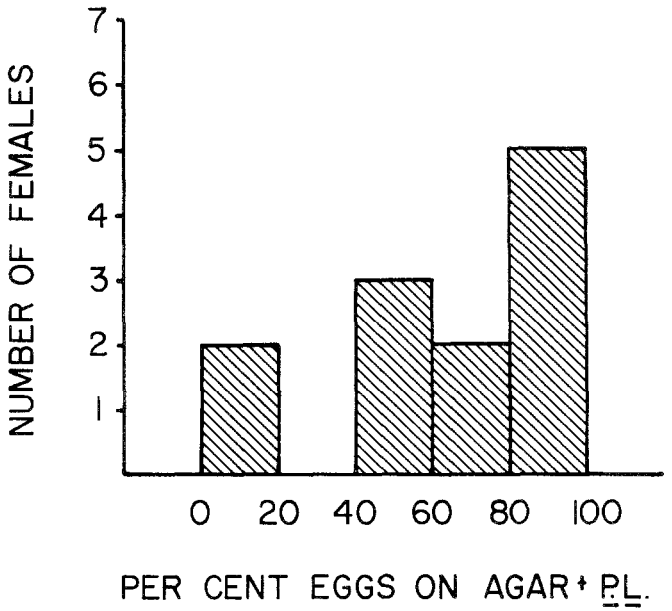

FIG. 3. Variation in the number of eggs laid by individual female *J. coenia* when given a choice of substrates containing *P. lanceolata* leaf material or pure catalpol. AGAR + P.L. = agar + *P. lanceolata* leaf material (0.5 g/0.5 g agar).

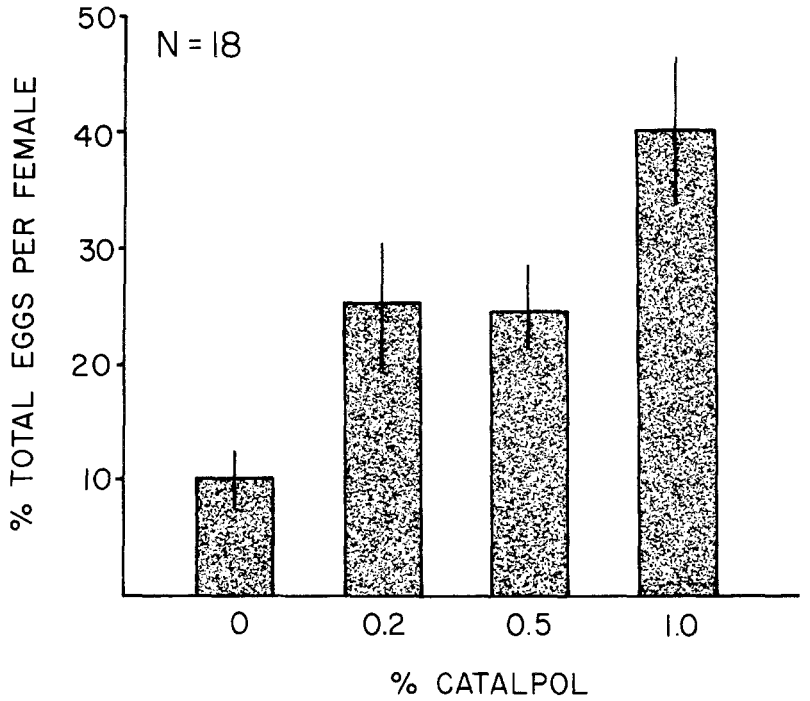

FIG. 4. Mean percent of eggs laid per female in a choice test of a control disk (pure agar) and disks with three different concentrations of catalpol. Values are means ± standard error.

DISCUSSION

Our results showed that *J. coenia* females used iridoid glycosides found in a typical host-plant species *P. lanceolata*, as oviposition cues. They responded positively to either a mixture of aucubin and catalpol, which are found in *P. lanceolata*, or to pure catalpol. Similar results were found in both choice and no-choice tests. In addition, females were able to discriminate among different concentrations and, when given a choice, laid significantly more eggs on the substrate with the highest concentration of catalpol.

The method of using agar disks to test compounds and plant material for oviposition activity (Hovanitz and Chang, 1964) proved to be very effective. Iridoid glycosides are generally polar compounds, and this method should work well with other polar compounds as well. Work with various species of *Papilio* has shown that the fractions of host-plant material eliciting oviposition behavior

are the polar ones (Nishida, 1977; Ichinose and Honda, 1978; Feeny et al., 1983). In addition, the presence of moisture appears to be essential for a positive contact response (Ichinose and Honda, 1978; Saxena and Goyal, 1978; Feeny et al., 1983), and this method provides that without the problems associated with evaporation from paper test substrates. Although we did not attempt to reuse disks, this should be possible. Disks begin to mold after 48 hr or so, but refrigeration or freezing would retard this process. Addition of mold inhibitors, such as those used in caterpillar artificial diets, might also aid in preventing mold growth. Drying out of the agar disks can also be a problem, but our use of plastic boxes or cylinders helped prevent this. This method of testing plant extracts, plant material, and pure polar chemical compounds has much potential.

Research on the chemical basis of oviposition behavior in butterflies has concentrated on two groups: the genus *Pieris* (Pieridae), which use cruciferous plants containing glucosinolates as hosts, and the Papilionidae. The classic example of a particular compound or group of compounds acting as oviposition (and larval feeding) stimulants is sinigrin and other glucosinolates for butterflies in the genus *Pieris* (David and Gardner, 1962). Attempts to find a similarly critical sign stimulus for oviposition in other butterfly species, such as the swallowtails which have been extensively investigated (Ichinose and Honda, 1978; Nishida, 1977; Feeny et al., 1983), have met with frustration (see discussion in Feeny et al., 1983). However, recent work by Honda (1986) has resulted in identification of flavanone glycosides as oviposition stimulants for a citrus-feeding swallowtail, *Papilio protenor* (Papilionidae). Work on *Pieris rapae* has shown that the chemical basis for oviposition in *Pieris* may be more complicated than previously thought (Renwick, 1985). In *J. coenia*, however, a single pure iridoid glycoside is sufficient to stimulate oviposition at levels similar to that of leaf material.

In conjunction with the demonstrated importance of other factors such as thermal requirements, environmental variation, host-plant demography, and previous experience of the ovipositing female, the importance of plant allelochemistry in the control of oviposition behavior is certainly complex and may vary from one system to another. Yet work on chemosensory systems of female butterflies has often shown that chemical cues are necessary for oviposition. The mechanisms of integration of chemical cues with other environmental and experiential cues to determine patterns of oviposition remain a mystery (Dethier, 1982). A first step, however, is to determine what chemical cues are involved. In *J. coenia* these cues are iridoid glycosides.

The behavioral response of the females to the agar disks containing ground leaves of *P. lanceolata* or pure iridoid glycosides was immediate: they began "drumming" (Ilse, 1937) immediately with the forelegs, curled the abdomen,

and oviposited. This response appeared to occur more quickly on these substrates than on intact leaves of *P. lanceolata.* Drumming behavior is believed to serve as a means of penetrating the plant cuticle to "taste" the leaf and obtain chemical information (Fox, 1966; Calvert and Hanson, 1983) via chemoreceptors on the foretarsi (Calvert, 1974). The agar disks appear to make such chemical information available more quickly than it would be in an intact leaf. If both foretarsi of *J. coenia* are cut, the number of eggs laid is reduced by 42% compared with females with foretarsi intact but with tarsi removed from one of the walking legs ($N = 18$ females) (Pereyra, unpublished). Similar results have been found in other butterfly species (Feeny et al., 1983).

Individual females given a choice of disks with *P. lanceolata* or catalpol varied in the percentage of eggs laid on these substrates (Figure 3)—some preferred *P. lanceolata* and others catalpol. Variation among individual females has been documented in other systems (Tabashnik et al., 1981; Singer, 1982; Papaj and Rausher, 1983) and is important in determining host-plant utilization patterns in nature (Stanton and Cook, 1984). Variation such as we observed among the females in the lab may be important in determining patterns of oviposition in the field.

The ability of *J. coenia* females to discriminate the disk with the highest concentration of catalpol (Figure 4) is intriguing. We used concentrations of iridoid glycoside that are well within the range of iridoid glycoside concentrations found in *P. lanceolata* (Duff et al., 1965) and other plants containing iridoid glycosides (Stermitz et al., 1986; Harris et al., 1986). Our results suggest that in nature, females might search for and prefer to oviposit on plants with a higher concentration of iridoid glycosides. Although *J. coenia* adults do not contain iridoid glycosides, the larvae do (Bowers and Puttick, 1986) [they are expelled in the meconium upon adult emergence (Bowers and Puttick, 1986)]. A higher concentration of iridoid glycosides in the larvae due to feeding on a host plant higher in iridoid glycosides may aid in protecting them from predation, as suggested generally for Lepidoptera feeding on toxic plants (Brower, 1984, p. 114). Thus female *J. coenia* able to discriminate and prefer higher concentrations of iridoid glycosides in the host plant might produce larval offspring protected by containing a higher concentration of iridoid glycosides.

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COMPONENTS OF FEMALE SEX PHEROMONE OF SPOTTED BOLLWORM, *Earias vittella* F. (LEPIDOPTERA: NOCTUIDAE): Identification and Field Evaluation in Pakistan

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Abstract—Ovipositor washings from virgin female *Earias vittella* (F.) (Lepidoptera: Noctuidae) moths were examined by gas chromatography (GC) linked to electroantennography (EAG). Six components were detected by the male moth. These were identified by comparison of their retention times with those of a range of synthetic standards on fused silica capillary GC columns as hexadecanal, (*Z*)-11-hexadecenal, (*E,E*)-10,12-hexadecadienal, octadecanal, (*Z*)-11-octadecenal, and (*E,E*)-10,12-hexadecadien-1-ol in 1:2:10:2:4:1 ratio. Field testing in Pakistan showed that a 2:10:2 mixture of (*Z*)-11-hexadecenal, (*E,E*)-10,12-hexadecadienal, and (*Z*)-11-octadecenal was as attractive to male *E. vittella* moths as the six-component mixture and equal in attractiveness to a virgin female moth. Omitting (*Z*)-11-hexadecenal or (*Z*)-11-octadecenal greatly reduced this attractiveness. It was found that synthetic lures must be protected from sunlight to prevent loss of attractiveness caused by isomerization of the conjugated diene aldehyde, and addition of (*E,Z*)-10,12-hexadecadienal, one of the products of isomerization, was shown to reduce attractiveness significantly. During this work, a 10:1 mixture of (*E,E*)-10,12-hexadecadienal and (*Z*)-11-hexadecenal was shown to be as attractive to *E. insulana* (Boisd.) male moths as a virgin female moth, and the attractiveness of this mixture was further increased by addition of (*E,Z*)-10,12-hexadecadienal.

Key Words—Spotted bollworm, *Earias vittella*, spiny bollworm, *Earias insulana*, Lepidoptera, Noctuidae, gas chromatography, electroantennography, pheromone traps, hexadecanal; (*Z*)-11-hexadecenal, (*E*,*E*)-10,12-hexadecadienal, (*Z*,*E*)-10,12-hexadecadienal, (*E*,*Z*)-10,12-hexadecadienal, octadecanal, (*Z*)-11-octadecenal, (*E*,*E*)-10,12-hexadecadien-1-ol.

INTRODUCTION

The spotted bollworm, *Earias vittella* (F.) (Lepidoptera: Noctuidae) is an important pest throughout the cotton-growing areas of the Indian subcontinent, southeast Asia, Indonesia, and northern Australia. The female moth lays eggs singly on the cotton bolls, and the larvae develop entirely within the cotton boll, reducing the quantity and quality of the cotton lint. Control with conventional insecticides is made difficult by the inaccessibility of the larvae. Control by novel methods, such as mating disruption with sex pheromones, necessitates the development of a sensitive, species-specific monitoring tool. This is particularly important in Pakistan where *E. vittella* coexists with the closely related spiny bollworm, *E. insulana* (Boisd.) (Lepidoptera: Noctuidae), and the pink bollworm, *Pectinophora gossypiella* (Saund.) (Lepidoptera: Gelechiidae). The sex pheromone of *P. gossypiella*, (*Z*,*E*)- and (*Z*,*Z*)-7,11-hexadecadienyl acetates, is well known (Bierl et al., 1974), and the major component of the sex pheromone of *E. insulana* was identified as (*E*,*E*)-10,12-hexadecadienal by Hall et al. (1980). This paper describes the identification and field evaluation of the female sex pheromone of *E. vittella*. Preliminary results were reported briefly by Cork et al. (1985a).

METHODS AND MATERIALS

Insect Material. For chemical analyses, pupae were sent from India by air to London. The pupae were left in their cocoons and placed in individual tubes held in an environmental cabinet on a reversed 12-hr/12-hr light–dark cycle with temperature alternating between 27°C and 22°C and relative humidity 85%. Adult moths were sexed on emergence and provided with 10% sucrose solution under the same conditions.

For field work in Pakistan, larvae were collected from cotton bolls, separated from *E. insulana* larvae, and reared on fresh cotton seeds. The resulting pupae were removed from their cocoons, sexed, and maintained singly in tubes on moist cotton wool until emergence.

Pheromone Collection. Ovipositor washings in carbon disulfide or heptane were prepared from virgin female moths up to 3 days old and between 4 and 12 hr into the dark period. Volatiles from virgin female moths were collected

on filters containing 5 mg of activated charcoal, as described previously (Grob and Zurcher, 1976; Nesbitt et al., 1979a; Tumlinson et al., 1982).

Gas Chromatography (GC). Analyses were carried out on a Carlo Erba Fractovap 4160 instrument equipped with Grob splitless injector at 200°C and flame ionization detector at 220°C. Fused silica capillary columns (25 m × 0.32 mm ID) were used with helium carrier gas (0.4 kg/cm^2) as follows: column (A), polar CP Wax 57CB (0.11 μm film thickness; chemically bonded Carbowax 20 M; Chrompack), oven temperature held at 70°C for 2 min, then programmed at 20°C/min to 90°C, then at 1°C/min to 190°C; Column (B), nonpolar CP Sil 5CB (0.15 μm film thickness; chemically bonded methylsilicone; Chrompack), oven temperature held at 70°C for 2 min, then programmed at 20°C/min to 100°C, then at 1°C/min to 190°C.

Retention times for the pheromone components are given in equivalent chain length (ECL) units relative to the retention times of straight-chain acetates. Retention times of series of synthetic isomers are given in minutes.

Electroantennography (EAG). Recording of male moth antennal responses to GC column effluent (GC-EAG) was carried out as described by Beevor et al. (1986), except that the electrodes were inserted into the interstitial membranes of the intact antenna. To record the EAG response profile to hexadecenal isomers, a solution of the test compound in hexane (2 μl) was syringed onto the inner wall of a disposable Pasteur pipet and solvent evaporated under a stream of nitrogen (500 ml/min for 6 sec). The pipet tip was then positioned 1 cm above the recording antenna and a pulse of nitrogen (500 ml/min for 3 sec) directed through the pipet. A 3-min interval was allowed between successive stimuli for the preparation to recover fully.

Synthetic Chemicals. Monounsaturated aldehydes were prepared by standard acetylenic routes and purified by kugelrohr distillation to give products containing less than 3% of the opposite geometric isomer.

(*Z*,*E*)-10,12-Hexadecadienol was prepared by Wittig reaction between (*E*)-2-hexenal (Aldrich) and the triphenylphosphonium salt from 9-bromo-1-decanol in tetrahydrofuran at −20°C with potassium *t*-butoxide as base. The product contained 91.8% *Z*,*E*, 0.7% *Z*,*Z*, and 7.5% *E*,*E* isomers and less than 0.1% of the *E*,*Z* isomer (cf. Klug et al., 1982).

(*E*,*Z*)-10,12-Hexadecadienol was prepared by Wittig reaction under the same conditions between (*E*)-11-(2-tetrahydropyranyloxy)-2-dodecenal and butyl(triphenyl)-phosphonium bromide (Hall et al., 1980). After deprotection, the product alcohol contained 85.8% *E*,*Z*, 3.2% *Z*,*Z*, 11.0% *E*,*E* isomers and less than 0.2% *Z*,*E* isomer.

(*E*,*E*)-10,12-Hexadecadienol was prepared by isomerization of the *Z*,*E* isomer, prepared as above, with thiophenol (Henrick et al., 1975; Hall et al., 1980), or, better, by exposing a solution in petroleum spirit containing a catalytic amount of iodine to sunlight (Klug, personal communication, 1983). The

isomeric mixture, containing 65–70% of the *E,E* isomer, was then recrystallized three times from petroleum spirit at −20°C to give the *E,E* isomer with less than 0.3% each of the *Z,E* and *E,Z* isomers and a negligible amount of the *Z,Z* isomer. The mother liquors could be isomerized again and recrystallized to give more product.

The diene alcohols were oxidized to the corresponding aldehydes with pyridinium chlorochromate in dichloromethane, and the products purified by fractional distillation (*E,E*) or kugelrohr distillation (*Z,E* and *E,Z*). No change in isomeric composition was detected on oxidation or distillation. Base-washed glassware was used for distillation and storage of the aldehydes to discourage trimerization (Dunkelblum et al., 1984).

Isomers of 9,11-, 11,13-, and 12,14-hexadecadienal and the corresponding alcohols were available from previous work (Hall et al., 1980).

Field Tests. Field testing was carried out in cotton fields near Multan, Pakistan, between 1982 and 1985. Pheromone dispensers were polyethylene vials (35 mm × 8 mm × 1.5 mm thick) loaded by adding the appropriate amount of a hexane solution of each pheromone component and 2,6-di-*tert*-butyl-4-methylphenol (BHT) as antioxidant (10 mg/ml each), allowing the solvent to evaporate, and closing the lid. Sticky disk traps (60 cm diameter) were used in initial field tests carried out in 1982–1983. In subsequent tests, covered, yellow plastic funnel traps (10 cm diameter; Biological Control Systems Ltd., Treforest, U.K.) were used, and the pheromone dispensers were protected from sunlight by aluminium sleeves (55 mm × 12 mm), open at the lower end. Traps were positioned just above crop height (approx. 1.5 m) on wooden poles. Synthetic lures were hung just below the roof of the trap at the center, and they were not renewed during a trial unless they were stolen. For tests with virgin female moths as bait, single moths, 2, 3, or 4 days old, were housed in tubes (50 mm × 30 mm) covered with muslin at each end and suspended horizontally under the roof of the trap. Mortality was minimized by providing the moths with cotton wool soaked in sucrose solution, and by placing them in the traps as late in the afternoon as possible and recording catches early the following morning.

The experiments carried out in 1984 were set out in Latin square or completely randomized block designs with at least 30 m spacing between traps. There were up to four replicates of each treatment, and experiments were run for eight nights without renewing the pheromone dispensers or changing treatment positions. Catches of *E. vittella* and *E. insulana* male moths were recorded each day. The catches were transformed to $\log(x + 1)$ in order to stabilize the variance and then subjected to analysis of variance. Differences between mean catches for each treatment were assessed for significance by Duncan's multiple-range test (DMRT).

In 1985, six synthetic blends were tested with six replicates in 1-hectare plots at least 100 m apart. In each replicate, the treatments were set out randomly around the plot with at least 30 m between traps. Catches of the two species were recorded each day when the treatments were moved on one position within each replicate. Each replicate was run for 36 nights, i.e., six complete rotations, without renewing the pheromone dispensers unless one was stolen. Total catches over the 36 nights for each treatment in each replicate were transformed to $\log(x + 1)$ and subjected to analysis of variance. Differences between catches by the different treatments were tested for significance by DMRT.

In other experiments carried out during 1985, the attractiveness of the synthetic pheromone for *E. vittella* and for *E. insulana* was compared with that of 2-, 3-, and 4-day-old virgin female moths of the corresponding species. Three replicates of each treatment were run simultaneously for seven nights. Catches were recorded each day when female moths were renewed and each treatment moved on one position within a replicate. Data from traps found to contain dead female moths or no pheromone dispenser were discarded, and differences between mean catches in the female-baited traps and those baited with the synthetic lure were tested for significance by *t* tests.

RESULTS

The female sex pheromone of *E. vittella* was identified on the basis of GC retention times of components in female ovipositor washings that elicited EAG responses from the male moth in linked GC-EAG analyses, comparison of EAG responses of the male moth to synthetic compounds, and extensive field testing of synthetic mixtures.

Structure Determination. Ovipositor washings from virgin female moths were analyzed by linked GC-EAG on both polar (A) and nonpolar (B) fused silica capillary columns. Six components were detected that caused an EAG response from the male moth at least 10% greater than the background response in two or more analyses. These components were numbered I to VI in their order of elution on polar column A. Retention times on columns A and B are given in Table 1, along with the average relative proportions of the components from analyses of a total of 75 female equivalents.

The major component, III, had retention times consistent with those of conjugated hexadecadienals. These retention times were compared with those of isomers of 9,11-, 10,12-, 11,13-, and 12,14-hexadecadienal, using hexadecanal as internal standard (Table 2). Component III could not be distinguished from (*E*,*E*)-10,12-hexadecadienal and (*E*,*E*)-11,13-hexadecadienal on nonpo-

TABLE 1. COMPONENTS OF SEX PHEROMONE OF *Earias vittella* AND THEIR RELATIVE ABUNDANCE IN OVIPOSITOR WASHINGS

	Component	Retention time[a]		Composition (%)		
		Column A	Column B	Column A	Column B	Relative composition
I	16:Ald	14.31	14.00	7.0	5.0	1
II	Z11-16:Ald	14.59	13.83	18.0	9.5	2
III	*E,E*10,12-16:Ald	16.18	14.47	51.0	53.0	10
IV	18:Ald	16.34	16.02	11.0	19.5	2
V	Z11-18:Ald	16.51	15.75	9.0	8.5	2
VI	*E,E*10,12-16:OH	18.48	15.17	4.0	4.5	1

[a]Retention times in equivalent chain length units relative to the retention times of straight-chain acetates.

TABLE 2. RETENTION DATA FOR HEXADECADIENAL AND HEXADECADIEN-1-OL PHEROMONE COMPONENTS AND SYNTHETIC COMPOUNDS

Compound		Retention time (min)			
		16:Ald		16:OH	
		Column A	Column B	Column A	Column B
Response III		63.44	55.86		
Response VI				84.50	62.70
9,11	*Z,E*	60.36	53.00	81.65	59.80
	E,Z	61.30	53.70	82.33	60.50
	Z,Z	62.20	54.90	83.75	61.80
	E,E	64.24	56.20	85.10	63.04
10,12	*Z,E*	60.50	53.30	81.80	60.08
	E,Z	61.65	54.30	82.80	61.02
	Z,Z	62.55	55.00	83.80	61.94
	E,E	63.60	55.86	84.50	62.64
11,13	*Z,E*	61.80	53.96	83.02	60.90
	E,Z	62.60	54.50	83.60	61.66
	Z,Z	63.40	55.30	84.46	62.20
	E,E	64.30	55.90	85.30	62.80
12,14	*Z,E*	63.40	55.30		
	E,Z	65.60	56.76		
	Z,Z	67.40	57.40		
	E,E	65.10	56.40		

lar column B, but these two isomers were readily separated on polar column A, and the retention times of component III were consistent only with those of (*E*,*E*)-10,12-hexadecadienal (*E*,*E*10,12–16 : Ald) on both columns.

The ratio of the amplitudes of the GC peaks corresponding to components I and II was approximately 1 : 2, and the EAG responses to component I were much less than those to component II. Using these criteria, it was deduced that the order of elution of components I and II was reversed on nonpolar column B relative to their order of elution on polar column A. Component I cochromatographed with hexadecanal (16 : Ald) on both columns. The retention times of component II suggested it was a hexadecenal isomer, and comparison with the retention times of the available hexadecenal isomers showed that the retention times of component II matched only those of (*Z*)-11-hexadecenal (Z11–16 : Ald) on both columns (Table 3).

The retention times of components IV and V indicated that they were two-carbon homologs of components I and II (Table 1), and they showed the same reversal of elution order on the two columns. Component IV cochromatographed with octadecanal (18 : Ald) and component V had the same retention time as (*Z*)-11-octadecenal (Z11–18 : Ald) on both column A and column B. Only a limited number of octadecenal isomers was available for comparison (Table 4), but, by analogy with the hexadecenal isomers (Cork et al., 1985b),

TABLE 3. RETENTION DATA FOR 16-CARBON ALDEHYDE PHEROMONE COMPONENTS AND SYNTHETIC COMPOUNDS

Compound	Retention time (min)	
	Column A	Column B
Response I	47.00	51.30
Response II	49.60	49.66
16 : Ald	47.00	51.30
*Z*13–16 : Ald	51.10	50.90
*E*13–16 : Ald	50.30	50.64
*Z*12–16 : Ald	50.30	50.36
*E*12–16 : Ald	49.44	50.14
*Z*11–16 : Ald	49.54	49.66
*E*11–16 : Ald	49.30	49.88
*Z*10–16 : Ald	49.02	49.16
*E*10–16 : Ald	48.90	49.46
*Z*9–16 : Ald	48.50	48.76
*E*9–16 : Ald	48.50	49.30
*Z*7–16 : Ald	48.32	48.60
*E*7–16 : Ald	48.32	49.20

TABLE 4. RETENTION DATA FOR 18-CARBON ALDEHYDE PHEROMONE COMPONENTS AND SYNTHETIC COMPOUNDS

Compound	Retention time (min)	
	Column A	Column B
Response IV	65.00	69.20
Response V	66.40	61.10
18:Ald	64.92	69.20
*Z*9–18:Ald	66.10	59.58
*Z*10–18:Ald	66.19	60.14
*E*10–18:Ald	66.19	62.60
*Z*11–18:Ald	66.40	61.20
*E*11–18:Ald	66.32	62.78
*Z*13–18:Ald	67.30	64.22
*E*13–18:Ald	66.88	64.71

*Z*11–18:Ald should have had unique retention times under the conditions used. Other possible candidates for component V were thought to be the hexadecenyl acetates. However, the retention time of component V on polar column A was shown to be different from those of the *Z* and *E* isomers of 10-, 11-, 12-, 13-, and 14-hexadecenyl acetate.

In some analyses on polar column A, a significant EAG response was recorded to a component eluting between components IV and V at ECL 16.42 with a magnitude approximately 10% that of the major component III. A corresponding response was never recorded during analyses on nonpolar column B, and no further work was done to verify the existence of this component or identify it.

The GC retention times of component VI were consistent with those of a conjugated hexadecadien-1-ol. Only the isomers of 9,11-, 10,12-, and 11,13-hexadecadien-1-ol were available for comparison, but their retention times relative to each other were similar to those of the corresponding hexadecadienals (Table 2). The retention times of component VI matched only those of (*E*,*E*)-10,12-hexadecadien-1-ol (*E*,*E*10,12–16:OH) on both columns (Table 2).

Pheromone yields were much higher than those reported for *E. insulana* by Hall et al. (1980). Typically, approximately 25 ng of the major component III per female was obtained in ovipositor washings prepared 11–12 hr into the dark period. Relative abundances of the pheromone components were reasonably consistent in different batches of ovipositor washings, although the presence of impurity peaks gave high assays for *Z*11–16:Ald on column A and for 18:Ald on column B (Table 1). Entrainment of volatiles from virgin female

moths gave more EAG-active material during the first 6 hr of the dark period than during the second 6 hr. However, yields were poor, and only the major component was consistently detected in linked GC-EAG analyses.

EAG responses of a male moth to the available hexadecenal isomers were recorded at the 20ng level (Figure 1). The *E* isomers were consistently more active than the corresponding *Z* isomers, and, of the isomers tested, the (*E*)-12-, (*E*)-11-, and (*Z*)-11- isomers elicited the largest EAG responses. This pattern of responses to the monounsaturated hexadecenal isomers is consistent with the (*E*,*E*)-10,12 unsaturation assigned to the major, diunsaturated hexadecadienal pheromone component (Hall et al., 1980, and references therein). However, these responses were much lower than that to *E*,*E*10,12–16:Ald, 5 ng of which caused a response of 4.8 mV.

Field Tests. In 1982, the total mixture of six components in their naturally occurring ratios was tested in different trap types, including sticky disk traps, dry funnel traps, and delta sticky traps. In all cases, the lures remained attractive

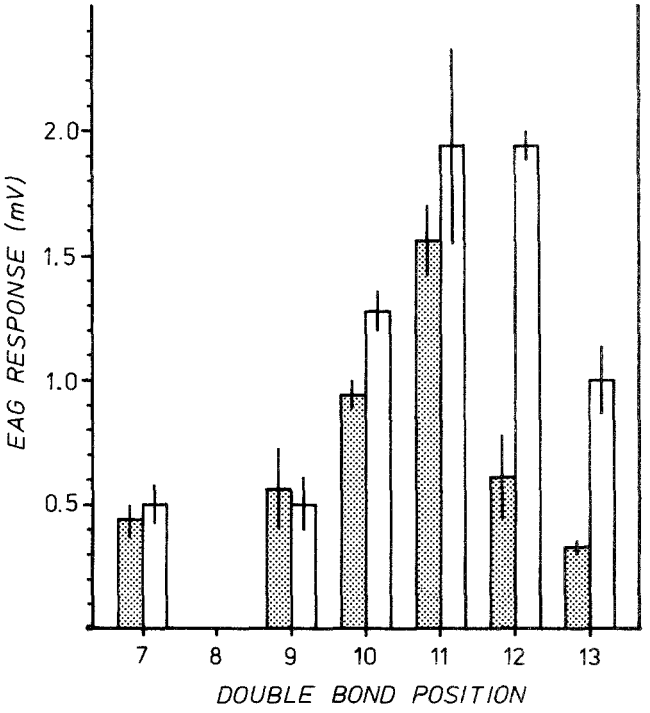

FIG. 1. EAG responses of a male *E. vittella* moth to hexadecenal isomers (20 ng); ▨ *Z* isomers; □ *E* isomers; bars indicate the spread of duplicate results; (*Z*)- and (*E*)-8-hexadecenal were not tested.

to male *E. vittella* moths for only a few days at best. It was thought that changes in the pheromone on exposure to sunlight might be responsible for this loss of attractiveness, and experiments were set up in 1983 to test this.

Using unprotected lures on white sticky disk traps, catches of *E. vittella* and *E. insulana* male moths in one representative experiment were 122 and 44, respectively, on the first night. On the second night, the catch of *E. vittella* was reduced to zero while that of *E. insulana* was 23. Subsequent GC analysis of these lures showed that the *E,E*10,12–16:Ald had been isomerized to the thermodynamic equilibrium mixture containing approximately 65% *E,E*, 15% *Z,E*, 15% *E,Z*, and 5% *Z,Z* isomers. In a parallel experiment, the lures remained attractive to *E. vittella* for at least 11 days when they were removed from the field during daylight hours. All subsequent experiments were carried out with the polythene vial dispensers shielded from sunlight with aluminium sleeves open at the lower end.

In 1984, tests were run to determine the effect on attractiveness of removing components from the six-component mixture. Removal of either or both of the saturated aldehydes, 16:Ald and 18:Ald, did not affect the catch significantly (Table 5, experiment 1), and these were omitted from most of the subsequent tests. If either of the monosaturated aldehydes, Z11–16:Ald or Z11–18:Ald, was subsequently removed, the catch was very significantly reduced. There was also an indication that omitting the diene alcohol, *E,E*10, 12–16:OH, increased the catch, but this was not statistically significant (Table 5, experiment 2).

In view of the importance of the monounsaturated aldehydes and possibly the *E,E*10,12–16:OH, experiments were carried out to examine the effects of changing the proportions of these components while keeping the others constant. Over the ranges of levels tested, there were no significant differences in catches between any of the treatments (Table 6). Variability in catches at different positions and from day to day was very high during these experiments, and this may have obscured small differences between the different treatments.

Previous results had suggested that isomerization of the conjugated diene aldehyde, *E,E*10,12–16:Ald, caused loss of attractiveness to *E. vittella* male moths. In order to study this further, the effect on catches of adding known amounts of isomerization products was studied. Addition of 10% *Z,E*10,12–16:Ald had no effect on catches, but a similar amount of *E,Z*10,12–16:Ald significantly reduced the catch (Table 7).

During 1984, populations of *E. insulana* were very low and very few moths of this species were caught in the pheromone traps.

In 1985, the results obtained above were consolidated in a highly replicated series of experiments run when populations of both *E. vittella* and *E. insulana* were high (Table 8). Omitting either of the monounsaturated aldehydes caused a very significant reduction in attractiveness to *E. vittella*, but the presence of

TABLE 5. CATCHES OF MALE *E. vittella* MOTHS IN TRAPS BAITED WITH COMBINATIONS OF PHEROMONE COMPONENTS IN THEIR NATURALLY OCCURRING RATIOS

Pheromone component (μg)						Mean catch/trap/night	
*E,E*10,12-16:Ald	Z11-16:Ald	Z11-18:Ald	*E,E*10,12-16:OH	16:Ald	18:Ald	Actual	Detransformed[a]
Experiment 1 (4 replicates; 8 nights)							
1000	200	200	100	100	400	23.8	7.9 a
1000	200	200	100	—	—	11.1	6.4 a
1000	200	200	100	100	—	17.9	8.2 a
1000	200	200	100	—	400	20.0	6.9 a
Experiment 2 (3 replicates; 8 nights)							
1000	200	200	100	—	—	20.5	8.1 a
1000	—	200	100	—	—	0.3	0.2 b
1000	200	—	100	—	—	1.5	0.8 b
1000	200	200	—	—	—	41.0	13.3 a

[a]Trap catches transformed to log $(x + 1)$; means followed by different letters are significantly different at the 5% level by DMRT in each experiment.

TABLE 6. CATCHES OF MALE *E. vittella* MOTHS IN TRAPS BAITED WITH FOUR-COMPONENT MIXTURES IN WHICH PROPORTION OF ONE COMPONENT IS VARIED (4 replicates; 8 nights)

Pheromone component (μg)				Mean catch/trap/night	
*E,E*10,12-16:Ald	*Z*11-16:Ald	*Z*11-18:Ald	*E,E*10,12-16:OH	Actual	Detransformed[a]
Experiment 1					
1000	100	200	100	41.5	32.7
1000	200	200	100	72.6	44.6
1000	300	200	100	44.1	21.8
1000	500	200	100	54.5	40.6
Experiment 2					
1000	200	100	100	42.3	18.2
1000	200	200	100	38.0	18.1
1000	200	300	100	58.9	36.6
1000	200	500	100	45.7	26.4
Experiment 3					
1000	200	200	20	93.7	66.1
1000	200	200	50	56.1	35.3
1000	200	200	100	51.5	40.4
1000	200	200	200	72.2	36.3

[a] Trap catches transformed to log $(x + 1)$; no significant differences between means in each experiment at the 5% level.

*E,E*10,12-16:OH at 10% of the major component had no effect. As was found in 1984, addition of *E,Z*10,12-16:Ald drastically reduced catches of *E. vittella*, but addition of *Z,E*10,12-16:Ald had no effect. Interestingly, *E,Z*10,12-16:Ald not only reduced the catches of *E. vittella* but also significantly increased the catches of *E. insulana* (Table 8).

Each replicate in this experiment was run for 36 nights without renewing the pheromone dispensers. Analyses of the pheromone remaining in vials containing the three main components were carried out after 30 days' exposure in traps in Pakistan. These analyses showed that approximately half the initial loading of pheromone remained and that little isomerization of the *E,E*10,12-16:Ald had occurred (*E,E*10,12-16:Ald, 0.50 mg; *Z*11-16:Ald, 0.09 mg; *Z*11-18:Ald, 0.09 mg; and 10,12-16:Ald, 94.1% *E,E* isomer).

In a final series of experiments, the attractiveness of synthetic lures for *E. vittella* and for *E. insulana* were compared with that of a caged virgin female moth of the corresponding species. The *E. vittella* lure used was the four component mixture of *E,E*10,12-16:Ald, *Z*11-16:Ald, *Z*11-18:Ald, and *E,E*10,12-16:OH. The *E. insulana* lure contained a binary mixture of

TABLE 7. CATCHES OF MALE *E. vittella* MOTHS IN TRAPS BAITED WITH SIX-COMPONENT MIXTURE CONTAINING ISOMERS OF 10,12–16:Ald (8 nights)

Pheromone component (μg)										
10,12-16:Ald									Mean catch/trap/night	
E,E	*Z,E*	*E,Z*	*Z*11-16:Ald	*Z*11-18:Ald	*E,E*10,12-16:OH	16:Ald	18:Ald	No. Replicate	Actual	Detransformed[a]
1000	—	—	200	200	100	100	400	3	27.8	12.4 a
1000	100	—	200	200	100	100	400	4	24.2	5.5 a
1000	—	100	200	200	100	100	400	2	0.1	0.1 b

[a]Trap catches transformed to log $(x + 1)$; means followed by a different letter are significantly different at the 5% level by DMRT.

TABLE 8. CATCHES OF *E. vittella* AND *E. insulana* MALE MOTHS IN TRAPS BAITED WITH VARIOUS SYNTHETIC MIXTURES (6 replicates; 36 nights)

Pheromone component (μg)						Mean catch/trap/night			
10,12-16:Ald			Z11-16:Ald	Z11-18:Ald	E,E10,12-16:OH	*E. vittella*		*E. insulana*	
E,E	Z,E	E,Z				Actual	Detransformed[a]	Actual	Detransformed[a]
1000	—	—	200	200	100	75.5	70.4 a	6.6	8.6 ab
1000	—	—	200	200	—	75.3	71.7 a	5.9	3.4 b
1000	—	—	—	200	—	3.9	2.7 c	3.4	1.9 b
1000	—	—	200	—	—	7.6	6.8 b	7.5	4.4 b
1000	100	—	200	200	—	77.3	68.8 a	8.7	4.8 b
1000	—	100	200	200	—	13.4	11.3 b	29.5	25.8 a

[a]Trap catch over 36 nights in each replicate transformed to log $(x + 1)$; means followed by a different letter are significantly different at the 5% level by DMRT for each species.

TABLE 9. COMPARISON OF CATCHES BY VIRGIN FEMALE MOTHS AND SYNTHETIC LURES (3 replicates; 7 nights)

Lure	No. Replicates[a]	Mean catch/trap/night[b]	
		E. vittella	*E. insulana*
Experiment 1			
E. vittella			
Virgin female			
2 days old	14	135.9	0
3 days old	16	175.1	0
4 days old	15	167.4	0
Synthetic lure[c]	20	176.5	3.4
Experiment 2			
E. insulana			
Virgin female			
2 days old	15	0	79.1
3 days old	15	0	80.3
4 days old	15	0	102.7
Synthetic lure[d]	21	0.1	99.1

[a] Data were discarded from traps found to contain dead female moths or no pheromone dispenser on the following morning.
[b] In each experiment, there were no significant differences at the 5% level between the catch by the synthetic lure and the catches by any of the virgin female lures, using *t* tests.
[c] 1000 μg *E,E*10,12-16:Ald + 200 μg Z11-16:Ald + 200 μg Z11-18:Ald + 100 μg *E,E*10,12-16:OH.
[d] 1000 μg *E,E*10,12-16:Ald + 100 μg Z11-16:Ald.

*E,E*10,12-16:Ald and Z11-16:Ald in a 10:1 ratio. At the time, this was thought to be the most attractive lure for *E. insulana* since the results of the previous experiment were not available. For both species, there were no significant differences between the number of male moths caught in traps baited with the synthetic lures and those baited with a 2-, 3-, or 4-day-old virgin female moth (Table 9).

DISCUSSION

The results of analytical studies and field testing of synthetic mixtures indicate that the female sex pheromone of *E. vittella* is composed of six components: 16:Ald, Z11-16:Ald, *E,E*10,12-16:Ald, 18:Ald, Z11-18:Ald and *E,E*10,12-16:OH in the ratio of 1:2:10:4:2:1. A synthetic mixture based on the natural ratio of these components without the saturated aldehydes, 16:Ald and 18:Ald, was shown to be as attractive to the male moths as was a caged

virgin female moth, although the addition of *E,E*10,12–16:OH was probably not necessary. Removing either of the monounsaturated aldehydes, Z11–16:Ald or Z11–18:Ald, reduced the catch of male moths significantly. Thus, Z11–16:Ald and Z11–18:Ald together with the major component, *E,E*10,12–16:Ald, in the naturally occurring ratio of 2:2:10 constitute a highly effective synthetic attractant for this species.

Early indications that the synthetic lures lost their attractiveness through photochemically induced isomerization of the *E,E*10,12–16:Ald were supported by the finding that addition of *E,Z*10,12–16:Ald dramatically reduced catches of *E. vittella*. The other isomer that is formed in any quantity, *Z,E*10,12–16:Ald, had no effect on catches. Work is in progress to find dispensers that completely prevent isomerization of the conjugated diene aldehyde, but at present lures should be protected from sunlight with sleeves of aluminium tubing or foil.

*E,E*10,12–16:Ald was reported to be the major component of the female sex pheromone of *E. insulana* by Hall et al. (1980). Subsequent results (unpublished) suggested that addition of Z11–16:Ald increased the attractiveness of the diene aldehyde to *E. insulana*, and in this work a binary mixture of *E,E*10,12–16:Ald with 10% Z11–16:Ald was shown to be as attractive as a virgin female moth. This synthetic mixture is currently being used to monitor *E. insulana* in Pakistan and Egypt.

Whereas *E,Z*10,12–16:Ald decreased the attractiveness of synthetic lures to *E. vittella*, it increased the attractiveness of *E. insulana*. It remains to be seen whether Z11–16:Ald or *E,Z*10,12–16:Ald is produced by the virgin female *E. insulana* moth, but the presence of the latter component could help to ensure species specificity in mating by these sympatric species. Night observation work in Pakistan by Murlis and Bettany (personal communication) showed that male *E. insulana* are active during the first half of the night, while male *E. vittella* are only active much later, so that species specificity may not be entirely chemical in origin. This observation was in agreement with the laboratory finding that ovipositor washings from female *E. vittella* moths contained more pheromone when they were prepared during the latter part of the scotophase. Volatiles collected from female moths were apparently more EAG-active when collected during the first half of the dark period, but amounts of pheromone obtained by this method were very small, and quantification by GC analysis was not possible. If species specificity is achieved by a combination of chemical and temporal separation, then a certain degree of cross-attraction to pheromone traps might be expected because the synthetic lures emit pheromone throughout the night.

Z11–16:Ald has been reported to be a pheromone component of many moth species (Arn et al., 1986), and it was reported to attract *E. biplaga* (Wlk.) by Nesbitt et al. (1979b). To our knowledge, Z11–18:Ald has not been found in any other lepidopteran sex pheromone, although Z13–18:Ald is produced by

several species in the Pyralidae, Crambinae, and Phycitinae families (Arn et al., 1986).

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IDENTIFICATION OF FEMALE SEX PHEROMONE IN ALFALFA BLOTCH LEAFMINER, *Agromyza frontella* (RONDANI)(DIPTERA: AGROMYZIDAE)

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Abstract—Gas chromatography of hexane extracts of 3-day-old virgin *A. frontella* adults revealed a branched saturated hydrocarbon present only in females. This compound was isolated by argentation chromotography and preparative GC, identified by GC and GC-MS as 3,7-dimethylnonadecane, and subsequently synthesized. Three-day-old virgin females contained 54.1 ± 3.5 ng (±SEM; $N = 48$) of 3,7-dimethylnonadecane. Male cadavers do not elicit male courtship behavior, but when treated with ~ 18 ng of 3,7-dimethylnonadecane they were as attractive as cadavers of 3-day-old virgin females. Black cotton knots were less attractive than male cadavers when treated with 3,7-dimethylnonadecane, but all bioassays indicated that this compound is an important semiochemical modulating male mating behavior.

Key Words—Alfalfa blotch leafminer, *Agromyza frontella*, Diptera, Agromyzidae, 3,7-dimethylnonadecane, female sex pheromone, male response, synthesis.

INTRODUCTION

The alfalfa blotch leafminer (ABL), *Agromyza frontella* (Rondani), a species recently introduced to North America from Europe (Miller and Jensen, 1970), has spread throughout the eastern United States and Canada (Harcourt and Binns, 1980; Hendrickson and Plummer, 1983). Concern about the pest potential of *A. frontella* to alfalfa production stimulated considerable research of both an

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applied and basic nature (e.g., Suzuki and Thomson, 1981; MacCollom et al., 1982; Tauber et al., 1982; McNeil and Quiring, 1983; Mellors and Helgesen, 1983; Quiring and McNeil, 1984a–e; Daley and McNeil, 1987; Harcourt et al., 1987).

In a recent study on the mating behavior of the alfalfa blotch leafminer (Carrière and McNeil, in press), it became clear that while the presence of the host plant was essential for mating, there was evidence of a female sex pheromone. Male cadavers, which are normally ignored, elicited the entire repertoire of male mating behavior when treated with a virgin female hexane extract. In this paper we report on the isolation, identification, and synthesis of this female sex pheromone.

METHODS AND MATERIALS

Insect Rearing. All *A. frontella* used in this study came from a laboratory colony, restocked annually and maintained on potted alfalfa (Saranac variety) in a greenhouse at 20 ± 2°C, 50 ± 10% relative humidity under a 16:8 light–dark photoperiodic regime. Newly formed pupae, <24 hr, were transferred from the colony and maintained, as well as the resultant flies, at 20 ± 1°C, 60 ± 5% relative humidity under a 14:10 light–dark regime. Upon emergence, adults were sexed, and virgin males and females were held in separate cages (ca. 40 per cage), in presence of alfalfa, until needed. Three-day-old virgin adults were killed, 3 hr after the onset of the photophase, by a 15-min exposure to a temperature of −16°C. Groups of ca. 40 males or females were soaked in hexane (5 μl per fly) for 3 hr, the extract collected with a Pasteur pipet and used for chromatographic analyses or bioassays within 8 hr. All bioassays were carried out using plants grown in isolation from the leafminer.

Bioassay Methods. The bioassay developed by Carrière and McNeil (in press) was used to test all extracts and synthetic material. Three freshly killed male cadavers were treated with 5 μl of the extract or product under consideration. They were then glued on the undersurface of each of the three leaflets of a single alfalfa leaf and placed in a 16-dram transparent plastic vial (10 cm high, 4.5 cm diameter) fitted with plastic screening at one end to permit air circulation. After 15 min, to allow for solvent evaporation, five virgin 1-day-old males were introduced and observed for 30 min. Bioassays were carried out at 25°C, 65% relative humidity, and males were transferred to these conditions 15 min before being bioassayed. The number of attempted copulations and the total number of contacts with the treated cadavers were recorded. The frequency and intensity of wing vibration, a male–male behavior exhibited when males are in the presence of the female hexane extract (Carrière and McNeil, in press), was also noted. Time spent by males on the plant was recorded using an event

recorder (Richardson and McNeil, 1987). In each bioassay, dead 3-day-old virgin female and male cadavers treated with hexane were used as controls. Ten replicates for each treatment in the bioassay were carried out.

Isolation and Identification. Hexane extracts of male and female flies were initially analyzed on DB-1 (25 m × 0.25 mm) and Carbowax 20 M (15 m × 0.25 mm) capillary columns (J & W Scientific), using a Hewlett Packard 5890 gas chromatograph equipped with a flame-ionization detector. The column temperature program used was 80°C for 1 min, increased to 175°C at 28°/min, maintained at 175°C for 1.5 min, and then increased to a final temperature of 275°C, at 20°/min. The detector was at 300°C, the splitless injector temperature was 275°C, and the carrier gas (H_2) flow was maintained at ca. 1 ml/min.

Argentation chromatography of the behaviorally active female extract was performed on silver nitrate-coated Florisil 60/200 mesh (Applied Science Laboratories). The active component was isolated from the hexane fraction by preparative GC, using the same instruments and temperature program mentioned above.

The purified biologically active compound was identified by EI (70 eV) and CI (isobutane) GC-MS, using a DB-1 capillary column (60 m × 0.32 mm ID) in a Finnigan 4000E GC-MS, with an Incos 2300 data system. The initial temperature was 50°C, increasing at 25°C/min to 100°C, then increasing to 280°C at 4°/min; He carrier gas was used.

Synthesis of 3,7-Dimethylnonadecane (1). Compound 1 was synthesized in 38% overall yield from dodecyltriphenylphosphonium bromide 2, as shown in Figure 1. The compound was obtained as a mixture of four stereoisomers, which were not resolved by capillary GC on DB-1, DB-5, or DB-1701 columns; bp ~ 150°C (0.1 torr) by Kugelrohr distillation. [^{1}H]NMR (CDC13): 1.35–1.2 (m, 32H, methylenes), 1.1–1.0 (m, 2H, methines), 0.89–0.81 (m, 12H, methyls). MS: see Figure 2. Anal. calcd. for $C_{21}H_{44}$:C, 85.04; H, 14.96. Found: C, 84.98; H, 14.86.

RESULTS AND DISCUSSION

The hexane extract of females contained a compound, having a retention time of 9.38 min, that was absent in male extracts. The compound was obtained by argentation chromatography followed by preparative GC. Male's responses to the isolated fraction were the same as those elicited by the crude extract. The compound was then identified by a combination of GC and GC-MS techniques.

The highest mass ion in the EI mass spectrum was at *m/z* 296, for a possible molecular formula of $C_{21}H_{44}$, corresponding to a saturated hydrocarbon. The isobutane CI mass spectrum gave a strong peak at *m/z* 295 (M − 1), confirming the molecular weight. In addition, there was no M + 1 peak at *m/z* 297

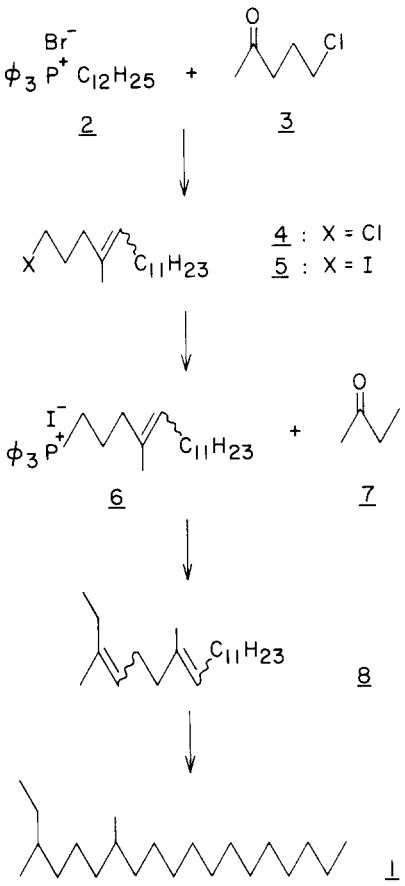

FIG. 1. Synthetic route to 3,7-dimethylnonadecane. Reaction conditions as follows: 2 → 4: i. *n*-BuLi, ether, −30°C; ii. 3, −78° →20°C. 4 → 5: NaI, acetone, reflux. 5 → 6: Ph_3P, 90°C. 6 → 8: i. *n*-BuLi, −30°C, THF; ii. 7, −78° → 20°C. 8 → 1: 5% Pd on carbon, H_2, hexane.

in the CI spectrum, confirming that the compound was a saturated hydrocarbon. The compound had a retention index relative to the *n*-alkanes of 2013 and 1994 on the DB-1 and Carbowax columns, respectively. This suggested a branched alkane.

Comparison of the EI spectrum of the unknown with that of the straight-chain C_{21} compound showed several fragments of enhanced intensity, indicative of cleavages at branch points. An ion of enhanced intensity at *m/z* 127 (Figure 2) indicated a cleavage into a C_9H_{19} and a $C_{12}H_{25}$ fragment, with the charge remaining on the C_9 fragment, thus locating the secondary carbonium ion from

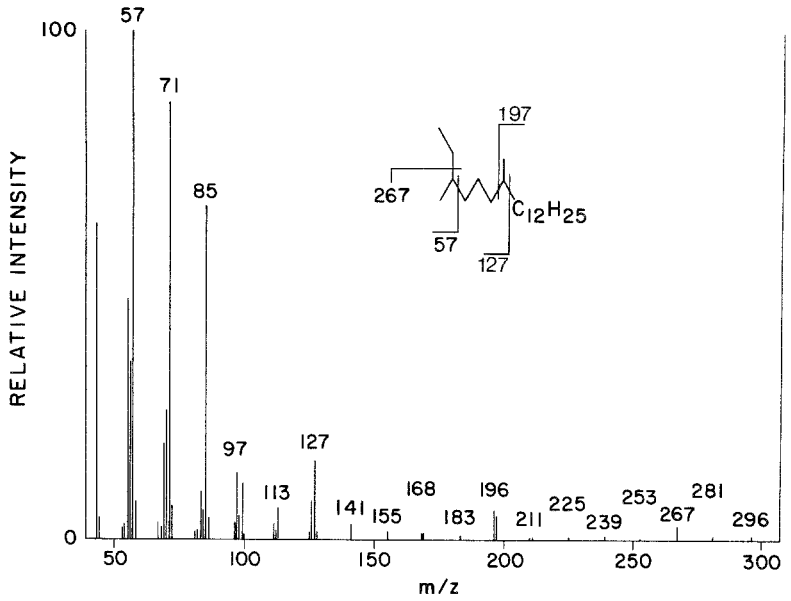

FIG. 2. EI mass spectrum of the behaviorally active compound found in *A. frontella* females.

the branch point cleavage on this fragment. Similarly, an enhanced ion at *m/z* 197 was indicative of a second cleavage into a C_7H_{15} and a $C_{14}H_{29}$ fragment, with the charge remaining on the C_{14} fragment. Taken together, the two sets of fragments indicate cleavage on either side of a methyl branch point, that is, a tertiary carbon substituted with a methyl group, a C_7 group, and a C_{12} group. The cleavage of the methyl group from this tertiary center is not favored, so only a very small ion at *m/z* 281 was seen.

There was also a slightly enhanced peak at *m/z* 267 (M-29), indicating a second branch point. This branch point was probably near one end of the alkyl chain, as would be found in a 3-methylalkane. If the ethyl branch were further along the chain, fragments of increased intensity from cleavages on the other two sides of the branch point would be expected: these were not seen. For a 3-methylalkane, an enhanced fragment at *m/z* 57 would be expected. However, as this ion is the base peak in the spectrum of the straight-chain hydrocarbon, it was impossible to tell whether this was the case.

From the above information, two possible structures were postulated, 3,12-dimethylnonadecane and 3,7-dimethylnonadecane, 1. The latter structure was favored due to consideration of possible biosynthetic pathways. That is, the 3,7-dimethyl fragment nicely fits the biosynthetic template of a head-to-tail fusion of two isoprene units.

On the strength of these observations, 3,7-dimethylnonadecane was synthesized as shown in Figure 1 and shown to have identical mass spectra (EI and CI) and retention times to the unknown compound. It should be noted that the synthetic material was a chromatographically inseparable mixture of four stereoisomers (two diastereomeric pairs of enantiomers), as there are two chiral centers in the molecule. The stereochemistry of the natural material is presently being investigated.

Male cadavers treated with 5 μl of the 3,7-dimethylnonadecane in hexane (17.6 ng/μl) elicited the same level of responses as either freshly killed 3-day-old virgin females or males treated with a female hexane extract, all of which were significantly higher than the responses to male cadavers treated with hexane only (Table 1). Males that had been treated with 88 ng of 3,7-dimethylnonadecane were soaked in 50 μl of hexane containing 127 ng of tetradecane as an internal standard. After 48 hr, extracts were analyzed by GC. Only 20 $\pm$ 5% of the 3,7-dimethylnonadecane was recovered, a loss undoubtedly due to runoff at the time of application. Three-day-old virgin females were weighed and their level of 3,7-dimethylnonadecane was determined using the same method as for males. The mean 3,7-dimethylnonadecane content per female was 54.1 $\pm$ 3.5 ng ($\pm$SEM; $N = 48$), with a positive correlation between female weight and the quantity of 3,7-dimethylnonadecane recovered ($R^2 = 0.25$, $F_{1,47} = 15.81$, $P = 0.001$). Thus, in bioassays, males had, $\sim$ 18 ng of 3,7-dimethylnonadecane, a quantity somewhat lower than levels found in 3-day-old virgin females.

To test that other cuticular chemicals, possibly present in both sexes of *A. frontella*, may be necessary together with 3,7-dimethylnonadecane to initiate male mating behavior, two concentrations (88 and 176 ng) of 3,7-dimethylnonadecane were applied to knots of black cotton. These knots were approximately the size of alfalfa blotch leafminer females and were tested using the standard bioassay technique. It is evident that while the time spent on the plants and the number of contacts with the treated knots (Table 2) were similar to values obtained when 88 ng of 3,7-dimethylnonadecane was applied to male cadavers (Table 1), the occurrence and intensity of males wing vibrating and especially the number of copulation attempts were lower. Furthermore, while there was a tendency for the behavioral responses to increase with a doubling of the concentration of 3,7-dimethylnonadecane, only the time spent on plant increased significantly (Table 2).

Based on the results obtained (Table 2), the possibility that other chemicals act synergistically at short range to influence the male mating behaviors cannot be ruled out. However, Carrière and McNeil (in press) presented evidence suggesting that while the female sex pheromone was important in bringing the male close to the female, there was also a short-range visual component involved in the mating behavior of *A. frontella*. It is therefore conceivable that the shape

TABLE 1. RESPONSES OF FIVE 1-DAY-OLD *Agromyza frontalla* MALES IN PRESENCE OF THREE DEAD VIRGIN FEMALES, THREE DEAD MALES TREATED WITH 5 μl OF VIRGIN FEMALE EXTRACT, 3,7-DIMETHYLNONADECANE, OR HEXANE

Treatment	*N*	Mean number (±SEM)[a]		Replicates (%) with male wing displays[b]	Mean number (±SEM) of wing vibration sequences[a]	Time (± SEM) spent on plant (min)[c]
		Attempted copulation	Total contacts			
Hexane	10	0 a	1.00 ± 0.33 a	0 a	0 a	10.7 ± 3.4 a
3-day-old virgin female extract	10	1.80 ± 0.88 b	3.60 ± 1.22 ab	50 b	6.70 ± 3.24 b	37.9 ± 8.2 b
88 ng of 3,7-dimethylnonadecane	10	4.30 ± 1.12 bc	5.40 ± 1.17 b	60 b	19.40 ± 7.92 b	27.5 ± 4.0 b
Whole 3-day-old virgin female	10	5.50 ± 1.45 c	5.90 ± 1.43 b	60 b	17.90 ± 6.13 b	37.5 ± 2.6 b

[a] Means followed by the same letter are not significantly different (Kruskal-Wallis followed by multiple comparisons test, $P > 0.05$).
[b] Percentages followed by the same letter are not significantly different (G test, $P > 0.05$).
[c] Means (in each replicate time is the summed total for all five males) followed by the same letter are not significantly different (Tukey, $P > 0.01$).

TABLE 2. RESPONSES OF FIVE 1-DAY-OLD *Agromyza frontella* MALES IN PRESENCE OF THREE BLACK COTTON KNOTS TREATED WITH 5 μl OF VIRGIN FEMALE EXTRACT, 3,7-DIMETHYLNONADECANE, OR HEXANE

Treatment	*N*	Mean number (±SEM) Attempted copulation[a]	Mean number (±SEM) Total contacts[b]	Replicates (%) with male wing displays[c]	Mean number (±SEM) of wing vibration sequences[b]	Time (±SEM) spent on plant (min)[d]
Hexane	10	0 a	0.42 ± 0.13 a	0 a	0 a	10.1 ± 3.2 a
3-day-old virgin female extract	10	0 a	2.90 ± 0.69 b	10 ab	4.40 ± 4.40 a	14.9 ± 2.8 ab
88 ng of 3,7-dimethylnonadecane	10	0.50 ± 0.27 a	6.30 ± 0.96 c	30 b	3.30 ± 2.59 ab	23.5 ± 1.8 b
176 ng of 3,7-dimethylnonadecane	10	0.20 ± 0.13 a	4.30 ± 1.04 c	50 b	10.60 ± 4.05 b	35.4 ± 3.2 c

[a] Means followed by the same letter are not significantly different (Kruskal-Wallis analysis of variance, $P = 0.1029$).
[b] Means followed by the same letter are not significantly different (Kruskal-Wallis followed by multiple comparisons test, $P > 0.05$).
[c] Percentages followed by the same letter are not significantly different (G test, $P > 0.05$).
[d] Means (in each replicate time is the summed total for all five males) followed by the same letter are not significantly different (Tukey, $P > 0.01$).

of the knots did not sufficiently resemble an *A. frontella* adult to provide the correct visual stimulation, thus explaining the decrease in certain behaviors. It has been demonstrated for the crane fly *Tipula oleracea* that morphologic characteristics serve as one of the obligatory signals, in a stimulus–response behavioral reaction chain, for execution of male copulatory behavior (Stich, 1963; Matthews and Matthews, 1978). Furthermore, the necessity for the correct shape and size of the model to elicit male copulatory behavior has also been demonstrated for *Glossina morsitans morsitans* (Huyton et al., 1980). Another potential cause for the observed reductions in male copulatory response could be the absence of appropriate mechanical stimuli, given that the texture of the cotton knot is markedly different to the insect exoskeleton. However, regardless of the cause(s) of the reduction in certain parameters relating to male mating when artificial models were used, there is little doubt that 3,7-dimethylnonadecane is important in mediating mating behavior of *A. frontella* males.

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SYNTHESIS OF CARRIER-FREE TRITIUM-LABELED QUEEN BEE PHEROMONE

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Abstract—A short synthesis of [4,5-$^3H_2$] (*E*)-9-oxo-2-decenoic acid (ODA), a high-specific-activity tritium-containing isotopomer of the queen bee pheromone, is described. Catalytic tritiation of the ketal of ethyl 9-oxo-4-decenoate introduces tritium into two positions, one of which is completely unactivated. Subsequent transformation by selenation, oxidation, and hydrolysis affords the labeled 9-ODA at >60 Ci/mmol. The material is suitable for biochemical studies of binding and catabolism in ovarian, antennal, and other target tissues.

Key Words—(*E*)-9-oxo-2-decenoic acid, tritium labeling, *Apis mellifera*, primer pheromone, radiosynthesis, Hymenoptera, Apidae.

INTRODUCTION

The chemical nature of the queen bee pheromone (Butler et al., 1961) and its dual role in the physiology of worker ovarian suppression (Butler, 1957; Butler and Gibbons, 1959) and in drone attraction (Gary, 1962) are well documented. Futhermore, the metabolic reduction of both the ketone and the alkene functionalities were reported two decades ago (Johnston et al., 1965). In stark contrast, there are no reported biochemical studies on the catabolic, transport, and receptor proteins which mediate the effects of this primer/sex pheromone on a

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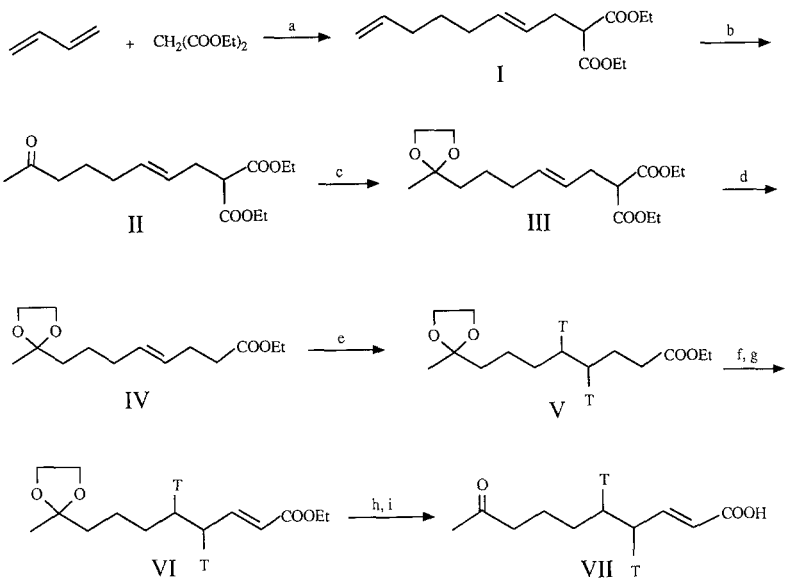

FIG. 1. Synthesis of radiolabeled (*E*)-9-oxo-2-decenoic acid. (a) Ph_3P, $Pd(OAc)_2$, 80°C, 16 hr; (b) $PdCl_2$, CuCl, DMF, H_2O, O_2, 20°C, 30 hr; (c) $HOCH_2CH_2OH$, *p*-TsOH, C_6H_6, 85°C, 4 hr; (d) 1 equiv 1 N KOH, CH_3OH; then H_3^+O; then 180°C; (e) $^3H_2$, 10% Pd/C, THF; (f) LiN(Cy)iPr, THF; then PhSeCl; (g) H_2O_2, CH_3COOH; (h) 2 N KOH, CH_3OH; (i) 6 N HCl, ether.

molecular level. The ^{14}C-labeled (*E*)-9-oxo-2-decenoic acid used by the Law group (Johnston et al., 1965) has too low a specific activity to detect proteins with submicromolar binding constants, and the tritiated material described by Butler et al. (1974) is unusable because the tritium is in exchangeable (enolizable) positions. We describe here a short radiosynthesis (Figure 1) of a high-specific-activity (>60 Ci/mmol) tritium-labeled queen bee pheromone isotopomer, in which at least one tritium atom is in an unactivated position.

METHODS AND MATERIALS

General. Anhydrous THF and ether were distilled from benzophenone ketyl. Flash chromatographic purifications were carried out on Woelm Silica 32–63 μm. TLC was performed using MN Polygram Sil G/UV 254 (4 x 8 cm) TLC plates. All products were homogeneous by TLC (R_fs are reported for 20% EtOAc–hexane). [^{1}H]NMR spectra were determined at 300 MHz on an NT-300 spectrometer or at 80 MHz on an CFT-20 instrument. Gas chromatography was carried out on a Varian 3700 equipped with a fused silica capillary column (DB-

5, 30 m x 0.263 mm). Radioactive samples were counted in an LKB 1218 RackBeta liquid scintillation counter using a PPO–POPOP–toluene or Scintiverse II scintillation cocktails. The efficiency for ^3H was 55–60%, as determined by an automatic external standardization ratio method.

Ethyl 2-carbethoxy-4, 9-decadienoate (I). Diethyl malonate (8.0 g, 50 mmol), triphenylphosphine (52.4 mg, 0.2 mmol), and palladium acetate (22.5 mg, 0.1 mmol) were heated while stirring until a homogeneous solution was obtained. This solution was transferred to a 50-ml stainless-steel bomb, butadiene (8.1 g, 150 mmol) was added, and the bomb was sealed and heated to 80° C for 16 hr. Distillation gave 10.32 g (77%) of the desired diene–diester I (Tsuji et al., 1977): bp 109–112°C/0.1 mm Hg.

Ethyl 2-carbethoxy-9-oxo-4-decenoate (II). A mixture of 2.97 g (30 mmol) of CuCl, 0.53 g (3 mmol) of $PdCl_2$, 1 ml of water, 8 ml of DMF, and 7.95 g (30 mmol) of the above malonate ester I were stirred under an atmosphere of oxygen for 30 hr. After this period, TLC indicated that the reaction was complete. The methyl ketone II (Tsuji et al., 1977) was isolated by distillation (bp 128–131°C/0.1 mm Hg) in 72% yield (5.86 g).

Ethyl 2-carbethoxy-9, 9-ethylenedioxy-4-decenoate (III). A 250-ml flask was charged with 5.52 g (20 mmol) of the methyl ketone II, 1.55 g (25 mmol) of ethylene glycol, and a catalytic amount of *p*-toluenesulfonic acid in 100 ml of benzene. The solution was heated under reflux with continuous removal of water for 4 hr. Usual work-up gave 5.12 g (80%) of the desired ketal which was used without purification.

Ethyl 9,9-ethylenedioxy-4-decenoate (IV). In 10 ml of 1 N KOH in MeOH was refluxed 3.18 g (10 mmol) of the ketal diester III for 1 hr. After cooling, the MeOH was evaporated, and the residue was dissolved in 10 ml of water. The aqueous solution was extracted with ether, acidified, and again extracted with ether. The ether solution containing the acid fraction was washed with brine, dried (Na_2SO_4), and evaporated. The oily residue was heated to 180°C in an oil bath under N_2 for 1 hr. The material was purified by flash chromatography on silica gel (Still et al., 1978) using 15% ethyl acetate–hexane. The unsaturated ketal ester IV was obtained in 52% yield (1.16 g): [^{1}H]NMR δ1.25 (t, J = 9 Hz, 3H), 1.31 (s, 3H), 1.46 (m, 2H), 1.61 (m, 2H), 2.00 (m, 2H), 2.36 (m, 4H), 3.92 (m, 4H), 4.24 (q, J = 9 Hz, 2H), 5.43 (m, 2H): [^{13}C]NMR δ15.2, 24.7, 24.8, 28.9, 33.5, 35.3, 39.5, 61.2, 65.5, 111.0, 129.3, 132.2, 174.1.

Ethyl [4,5-$^3H_2$]9,9-ethylenedioxydecanoate (V). The tritiation reaction was carried out at the National Tritium Labeling Facility at Lawrence Berkeley Laboratory. To 20 mg (0.078 mmol) of the unsaturated ketal ester IV in 3 ml of THF was suspended 7.1 mg of 10% Pd/C. The reaction was freeze–degassed and stirred for 1 hr under one atmosphere of carrier-free T_2 gas. The tritium gas and volatiles were removed *in vacuo* until the volume was reduced to 1 ml,

and then the solution was filtered and lyophilized. The residue was chromatographed on flash silica gel with 10% ethyl acetate–hexane to give homogeneous ketal ester V. This material was frozen in benzene and shipped to Stony Brook for futher transformations. All radiochemicals were kept cold ($< -10°C$) and stored below 500 mCi/ml in degassed, distilled 1:1 heptane–toluene to minimize radiolytic decomposition.

Ethyl [4,5-$^3H_2$]-9,9-ethylenedioxy-2-decenoate (VI). A solution of lithium isopropylcyclohexylamide (made from 16.9 mg, 0.12 mmol of isopropylcyclohexylamine, and 0.12 mmol of *n*-butyllithium in hexane) was prepared in 1 ml of dry THF and cooled to $-78°C$. To this stirred solution was added approximately 15 mg (0.06 mmol, 3.7 Ci) of the tritiated ketal ester in a minimum of THF. The solution was stirred for 30 min at $-78°C$ and quenched with 34.5 mg (0.18 mmol) of phenylselenenyl chloride. The reaction mixture was allowed to warm to room temperature and was treated with 50 μl of acetic acid and 250 μL of 30% H_2O_2. The mixture was stirred for an additional 45 min; the reaction was worked up and chromatographed on flash silica gel with 15% ethyl acetate–hexane to give 2.34 Ci (62%) of the unsaturated ester VI which was radiochemically pure by TLC.

(E)-[4,5-$^3H_2$]9-Oxo-2-decenoic acid (VII). The ketal ester was heated in 1 ml of 2 N methanolic KOH for 30 min. The MeOH was evaporated, and the residue was acidified with 2 ml of 6 N HCl. Five milliliters of ether were added and the two-phase solution was stirred overnight to cleave the ketal. The ether was separated, washed with brine, and dried to give 2.12 Ci of the "hot" queen bee substance. The material was only 90% pure by TLC of the derived methyl ester (from diazomethane). The material can be purified by converting to the methyl ester and purifying by flash chromatography on silica gel (15% ethyl acetate–hexane). The free acid is regenerated by heating with aqueous KOH for 30 min with continuous shaking. Acidification gives radiochemically pure acid.

RESULTS AND DISCUSSION

High-specific-activity, tritium-labeled pheromones are becoming an indispensable adjuvant in probing the biochemical machinery of pheromone transport, reception, and metabolism (Prestwich, 1987a,b; Prestwich et al., 1987a). Detailed insights have already been obtained in some Lepidopteran species, e.g., *Antheraea polyphemus* (Vogt *et al.*, 1985; Prestwich et al., 1986), *Heliothis virescens* (Ding and Prestwich, 1986), *Plutella xylostella* (Prestwich and Streinz, 1988), and *Lymantria dispar* (Prestwich et al., 1987b). To date, however, there have been no reports on studies involving proteins that mediate transport, reception, or metabolism of pheromones in the Hymenoptera. The

availability of tritium-labeled, high-specific-activity queen bee pheromone will allow us to begin these important biochemical studies in *Apis mellifera*.

The primary consideration in designing this synthesis was to allow for the incorporation of two tritons, at least one of which is nonexchangeable in aqueous solution and in positions that were unlikely to be initially metabolized. Thus, we felt that it was imperative to place the tritium atoms in the middle of the chain. For these reasons, we chose the unsaturated ketal IV as our target for tritiation.

The synthesis of IV was accomplished by modifying another queen bee pheromone synthesis (Tsuji et al., 1977). Palladium-catalyzed telomerization of butadiene with diethyl malonate has been reported previously (Hata et al., 1971). Oxidation of the terminal double bond to the corresponding methyl ketone was carried out in good yield using CuCl and catalytic $PdCl_2$ (Clement and Selwitz, 1964). The ketone moiety was protected as the ethylene ketal to avoid unwanted exchange during the tritiation reaction. Furthermore, protection of the ketone carbonyl was necessary during the ester–enolate formation to introduce the (*E*)-alkene. The synthesis of IV was completed by converting the substituted diethyl malonate to the half ester using one equivalent of KOH in methanol; acidification and decarboxylation gave the unsaturated ketal IV in moderate yield.

Reduction of the double bond in IV with carrier-free T_2 gas using 10% Pd/C as catalyst afforded material with a specific activity greater than the theoretical 58 Ci/mmol because of vinylic and allylic exchange reactions (cf. Evans et al., 1985) The conjugated *E*-double bond was introduced using a selenation–selenoxide elimination sequence (Sharpless et al, 1973). Saponification and acid catalyzed cleavage of the ketal gave material containing 10–15% of an impurity (estimated from TLC). The impurity apparently arises from saponification in alcoholic solvent. The pheromone can be purified by reesterification with diazomethane, flash chromatography, and saponification in aqueous KOH.

We are currently pursuing biochemical studies using this high-specific-activity pheromone isotopomer.

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CUTICULAR HYDROCARBONS OF TSETSE FLIES II: *Glossina fuscipes fuscipes, G. palpalis palpalis, G. p. gambiensis, G. tachinoides,* and *G. brevipalpis*

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Abstract—The alkanes (methylalkanes) were analyzed from both sexes of four members of the *palpalis* group of tsetse flies, *Glossina fuscipes fuscipes, G. palpalis gambiensis, G. palpalis palpalis, G. tachinoides* plus one *fusca* group member, *G. brevipalpis*, to determine structures, abundances, and the presence of unique or specific methylalkanes. These insects are unique in that trimethylalkanes were major components except in female *G. tachinoides* and both sexes of *G. brevipalpis* where 2-methylalkanes were the major components. The identification of novel long-chain tetramethylalkanes, including 11,15,19,23-tetramethylpentatriacontane, a minor component of female *G. f. fuscipes, G. p. gambiensis*, and *G. p. palpalis*, is reported here. Tetramethylalkanes were significant components of both sexes of *G. brevipalpis*. The major tetramethylalkane in *G. brevipalpis* is 3,7,11,15-tetramethylhentriacontane. The use of the methylalkanes as taxonomic indicators in tsetse is discussed.

Key Words—Cuticular hydrocarbons, alkanes, methylalkanes, trimethylalkanes, tetramethylalkanes, *Glossina* spp., Diptera, Glossinidae.

INTRODUCTION

The cuticular hydrocarbons of several species of tsetse flies have been investigated for their biological activity as sexual stimulants for males. In a previous study, only tsetse of the *morsitans* group were examined (Nelson and Carlson,

1986). Methylalkanes were identified by combined gas chromatography–mass spectrometry (GS-MS) in the surface lipids of three species of female tsetse flies, including several compounds that had new sequences of methyl branching with five or seven methylenes between the branch points of the trimethylalkanes. Sequences of 3,3-, 3,5-, 5,3- and 7,3-methylenes between adjacent methyl branch points were identified. There was some variation in isomeric composition of dimethyltritriacontane in female *G. pallidipes:* Those from England (laboratory reared), Kenya, Mozambique, and Zambia had 11,15-dimethyltritriacontane as the major 33B isomer, whereas females from Uganda and Zimbabwe had 13,21-dimethyltritriacontane as the major component of 33B.

Previously, tsetse fly females (*G. morsitans*) were shown to produce a trimethylalkane, 15,19,23-trimethylheptatriacontane, that stimulated the male to mate with a treated decoy, as did two less active dimethyl analogs, 15,19- and 17,21-dimethylheptatriacontane (Carlson et al., 1978). Synthetic 15,19-dimethyltritriacontane was stimulatory to male *G. austeni* (Huyton et al., 1980). Cuticular components were also implicated as sexual stimulants in *G. pallidipes* (Austen) (Langley et al., 1982) and *G. palpalis palpalis* Robineau-Desvoidy (Offor et al., 1981). Carlson et al. (1983, 1984) identified the active component of *G. pallidipes* as 13,23-dimethylpentatriacontane and confirmed the activity with the racemic synthetic compound, whereas McDowell et al. (1981) reported the aphrodisiac as a mixture of 13,17- and 15,19-dimethylpentatriacontane.

The species and subspecies of African *Glossina* are placed in three groups on the basis of morphological differences. The *palpalis* group consists of *G. caliginea* Austen, *G. tachinoides* Westwood, two subspecies of *palpalis* called *G. palpalis palpalis* and *G. palpalis gambiensis* Vanderplank; three subspecies of *fuscipes* called *G. fuscipes fuscipes* Newstead, *G. fuscipes martinii* Zumpt, and *G. fuscipes quanzensis* Pires; and two subspecies of *pallicera* called *G. pallicera pallicera* Bigot and *G. pallicera newsteadi* Austen. The *palpalis* group includes species that are the most important vectors of tsetse-borne diseases in West Africa, with the addition of *G. m. submorsitans*, the latter from the *morsitans* group that contains seven species and subspecies. The *fusca* group contains 14 more species and subspecies including *G. brevipalpis* Newstead (Dame, 1978).

Carlson (1982, 1983) reported that males of the *palpalis* group contained major alkanes that were unusual homologous methylalkanes with 27, 28, and/or 29 carbon backbones. These materials were not detected by GC in males of the *morsitans* group. Patterns of methylalkanes were shown to be species specific in other *palpalis* group females.

Some long-chain, methyl-branched hydrocarbons act as contact sexual stimulants in tsetse, and it appears that these materials are likely to be species specific. Therefore, we have examined the methylalkanes of four species of the

palpalis group of tsetse flies, *G. fuscipes fuscipes, G. palpalis gambiensis, G. palpalis palpalis*, and *G. tachinoides*, plus one *fusca* group member *G. brevipalpis*, to determine structures, abundances, and the presence of unique or specific methylalkanes. Several components could not be unequivocally identified from their mass spectra although suggested structures are indicated, but the spectra are included for the benefit of the reader and future investigators. The mass spectra of novel tetramethylalkanes have been included as well as mass spectra of trimethylalkanes from males.

METHODS AND MATERIALS

Samples for analysis were obtained as dried pinned specimens or as dried intact specimens shipped in capped vials. Neutral lipids from each sample were extracted in hexane and separated by liquid chromatography on silica gel, then argentation liquid and thin-layer chromatography to obtain the alkanes (Carlson et al., 1978). Further analysis and quantitation were conducted by temperature programmed capillary gas chromatography (CGC) on a 15-m × 0.23-mm ID column of DB-1 using a Nelson Analytical 3000 series data system (Carlson and Langley, 1986).

Most mass spectra (MS) were from pooled samples of several insects because of the larger quantities needed for packed column GC-MS. The EI-MS were obtained with a Varian/MAT SS-200 data system and a MAT 112S mass spectrometer coupled via a jet separator to a 3-m × 3.2-mm ID glass column of 3.5% OV-101 on 100–120 mesh Gas Chrom Q. The carrier gas was helium, and the temperature was programmed from 180° to 324°C at 2°/min. Scan time was 8 sec, and a new scan was started every 8.5 sec. Capillary GC-MS (CGC-MS) was conducted on a 12.5-m × 0.2-mm fused silica column coated with a 0.33-μm thick film of cross-linked methyl silicone using cool on-column injection without a splitter and ramping from 60° to 180°C at 28°/min. The temperature was then raised from 180° to 320°C at 3°/min. Scan time was 6 sec with 0.1 sec return. Separate GC runs were made to obtain the traces shown in Figure 1. The MS were interpreted as previously described (Carlson et al., 1984; Nelson and Sukkestad, 1970; Nelson et al., 1980, 1981; Nelson and Carlson, 1986).

RESULTS AND DISCUSSION

The CGC composition of alkanes from *G. f. fuscipes, G. p. palpalis, G. p. gambiensis, G. tachinoides*, and *G. brevipalpis* are presented in Figure 1 and tabulated in Table 1. An obvious feature is that the methylalkanes of the females consisted of longer chain-length components than did those of the males. The

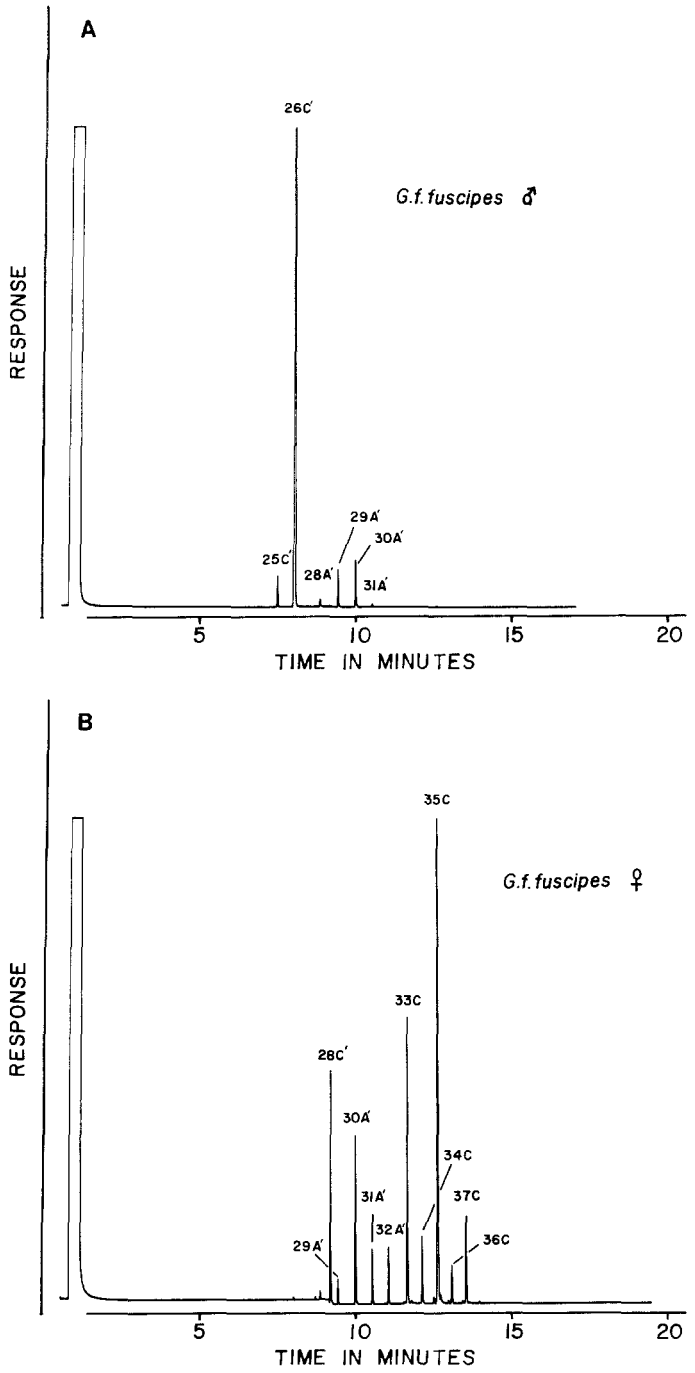


FIG. 1. (A–J) Capillary GC traces of methylalkanes from the surface lipids of male and female *G. f. fuscipes*, *G. p. gambiensis*, *G. p. palpalis*, *G. tachinoides*, and *G. brevipalpis*.

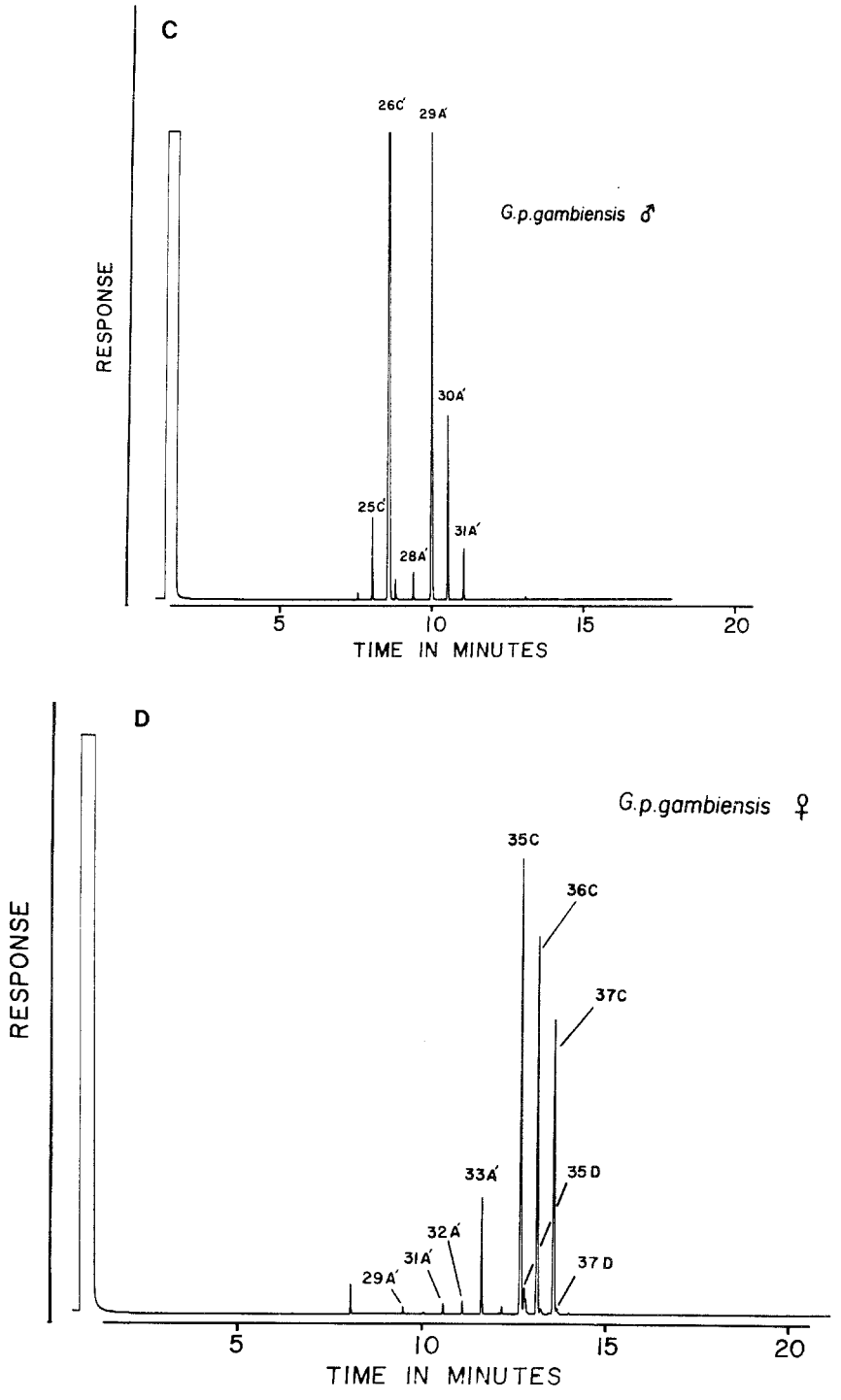


FIG. 1. Continued.

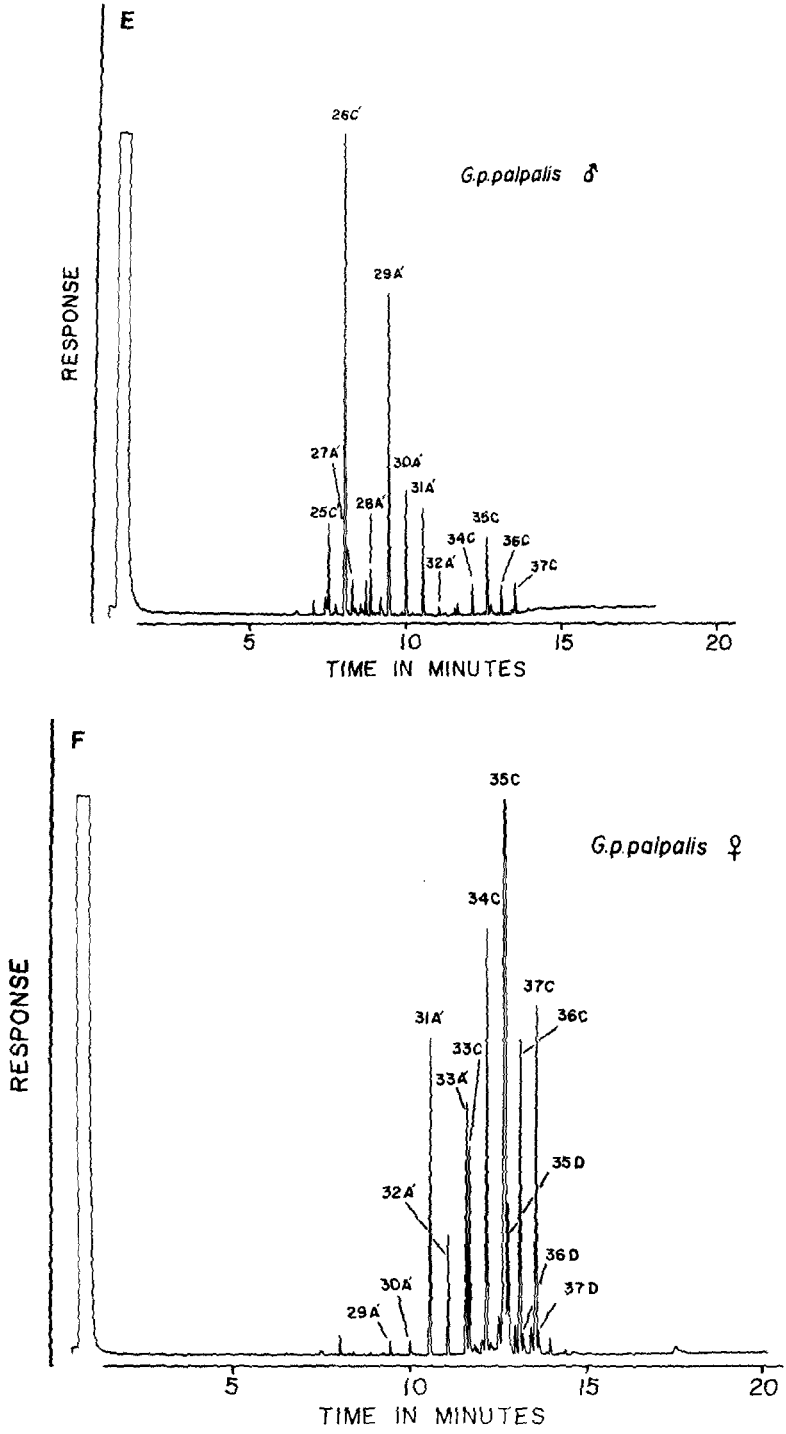
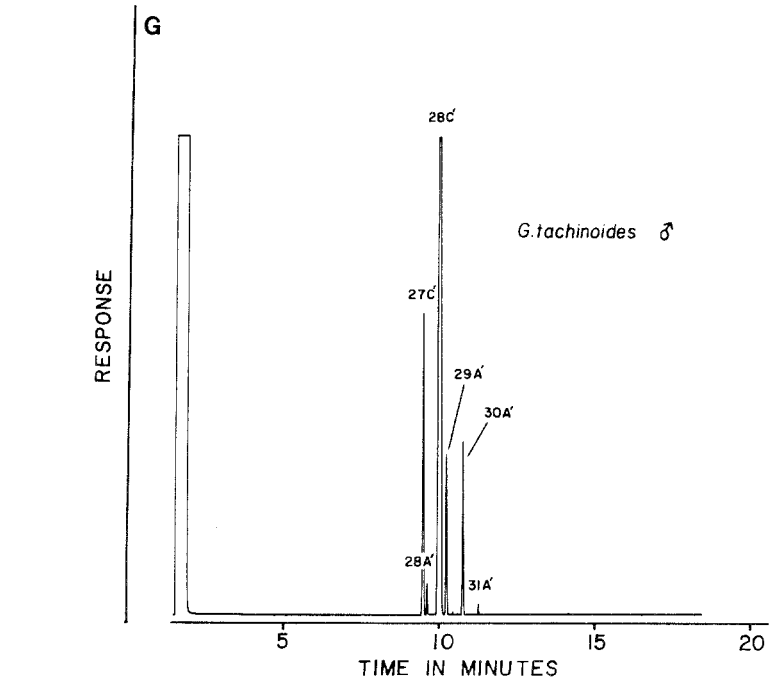


FIG. 1. Continued.



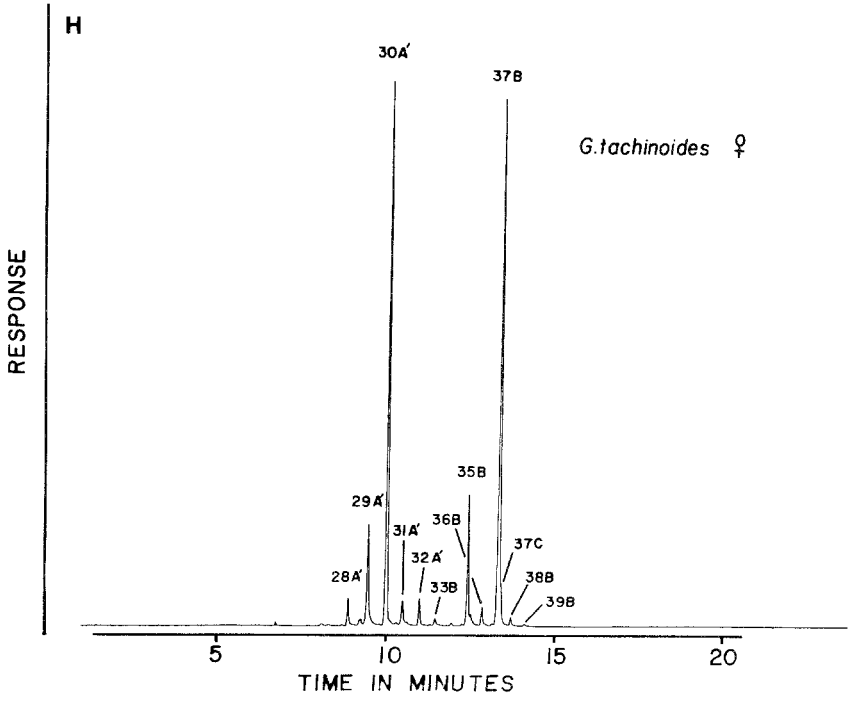


FIG. 1. Continued.

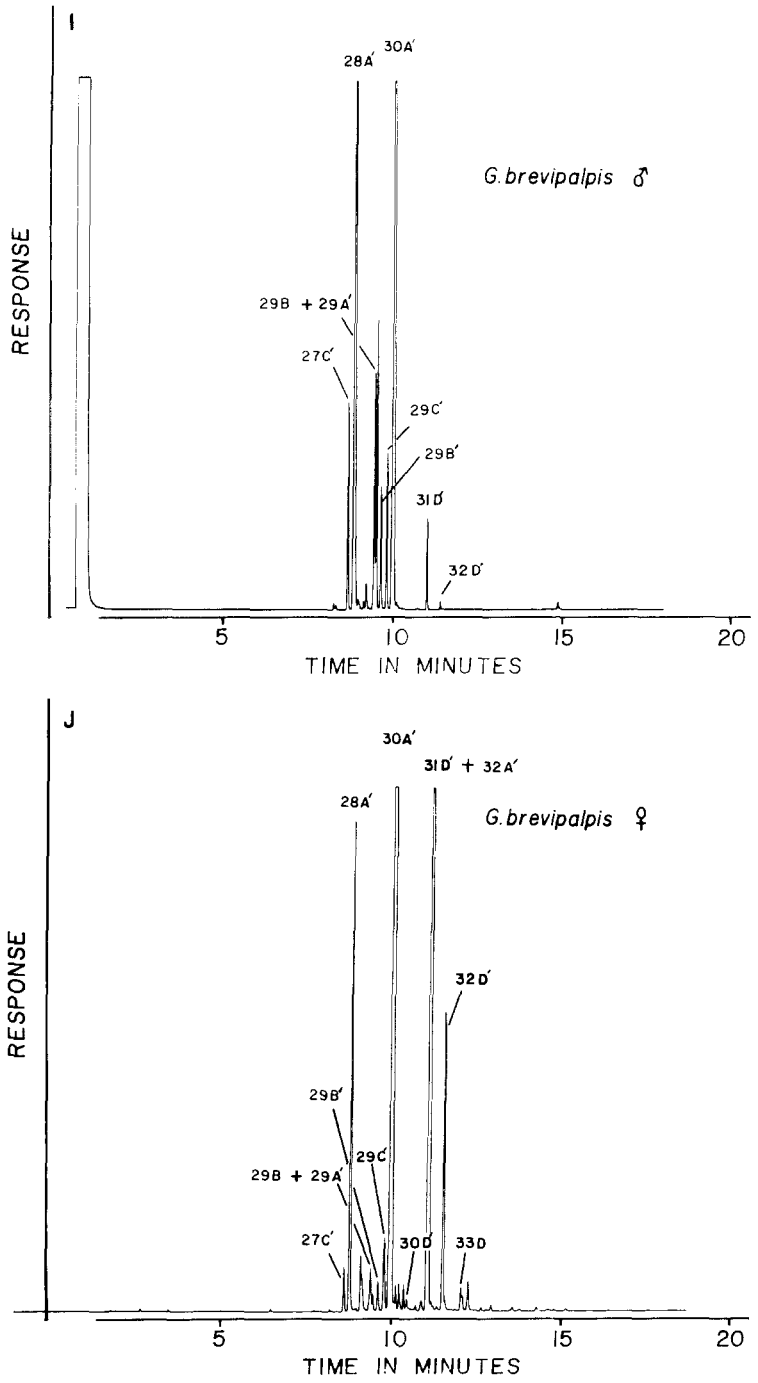


FIG. 1. Continued.

composition of each GC peak as determined by GC-MS or CGC-MS is tabulated in Table 2. The paraffins of these five species of tsetse flies are composed of methyl-branched alkanes, as *n*-alkanes are essentially absent. The GC peak number corresponds to the number of carbon atoms in the backbone of the methylalkane. The letters A, B, C, and D indicate one, two, three, and four methyl branches, respectively. The "prime" symbol indicates that one of the branches is near the end of the carbon chain, i.e., on carbon 2, 3, or 4. Generally, the closer a methyl branch is located to the end of the carbon chain, the slower the compound elutes, except that 2- and 4-methylalkanes elute together and before a 3-methylalkane (Mold et al., 1966; Nelson et al., 1981). This is also the situation when additional internal branches are present, as 4,8,12-trimethyloctacosane elutes earlier and is partially resolved from 3,7,11-trimethyloctacosane even on a packed column. In general, a mixture of different methylalkanes (plus *n*-alkane standards) elutes from a packed column in the following order: (31), 31A, 31A′ and 31B, 31C, 31D, 31B′ and (32), 31C′, 32A, 31D′. The components in 31A′ plus 31B may or may not separate, depending on the isomers present. GC peak 31B′ elutes at nearly the same time as 32-carbon *n*-alkane. Also, it is unlikely that 32A would be resolved from 31C′. Fortunately, all types of methylalkanes are not present in any one sample, and *n*-alkanes are virtually absent from the tsetse flies examined here, making interpretation of the mass spectra possible.

The methylalkanes mainly consisted of homologous series of 2-methylalkanes, and internally branched di-, tri-, and tetramethylalkanes. Few internally branched monomethylalkanes were present. Unique to these insects, except *G. tachinoides*, was the presence of significant amounts of tetramethylalkanes. Male tsetse of the *palpalis* group have a simpler methylalkane composition than the females [a similar sex difference was found in *morsitans* (Nelson and Carlson, 1986)]. Branched alkanes ranged in size from 28 carbons (25C′) to 35 carbons (33B) in males, and from 28 carbons (25C′) to 42 carbons (39C) in females.

The MS of the monomethylalkanes are readily interpreted and are not illustrated here. In those instances where 3-methylalkanes were found, they were only present as a trace component of the 2-methylalkane GC peak.

Dimethylalkanes from Female G. tachinoides *and* G. F. fuscipes. Dimethylalkanes were identified which had 3, 7, 9, or 11 methylenes between the branch points. The longest series of internally branched dimethylalkanes was found in female *G. tachinoides*. Because this series exhibited unusual variation in the separation of the methyl branch points, and because this insect was the only one in this study to have a significant amount of dimethylalkanes, the data are presented here in detail. The shorter-chain dimethylalkanes, 29B through 35B, had nine methylenes between the branch points except that GC peak 33B, a minor component, was a mixture of the isomers 11,15- and 11,21-dimethyl-

TABLE 1. COMPOSITION OF CUTICULAR PARAFFINS IN TSETSE FLIES (*Glossina* spp.).

Species	Average percent composition of characteristic peaks (by KI values and peak numbers)								
	2620 25C′	2720 26C′	2820 27C′	2865 28A′	2920 28C′	2965 29A′	3065 30A′	3165 31A′	3265 32A′
Male									
G. f. fuscipes	9.5	49.0	1.3	4.2	1.5	10.2	7.9	3.6	0.4
G. p. palpalis	6.9	41.5	0.8	3.7	0.5	22.1	8.8	6.5	0.5
G. p. gambiensis	7.0	37.5	—	4.2	0.6	21.6	10.7	5.7	—
G. tachinoides	—	—	12.2	8.8	37.7	7.8	20.6	1.9	0.8
G. brevipalpis[a]	—	—	—	24.3	7.0[b]	10.2	27.0	—	4.8[c]
Female									
G. f. fuscipes	—	—	—	—	—	—	5.5	10.2	7.3
G. p. palpalis	—	—	—	—	—	—	1.1	7.9	5.3
G. p. gambiensis	—	—	—	—	—	—	1.4	3.7	4.4
G. tachinoides	—	—	—	3.5	2.2	5.7	28.5	2.6	8.5
G. brevipalpis[a]	—	—	2.8	9.1	—	1.8	33.6	—	21.4[c]

[a] Member of *fusca* group.
[b] GC peak 29A (KI2932).
[c] GC peak 31D′ + 32A′.

tritriacontanes with three and nine methylenes between the branch points, respectively (Figure 2A). This was the only three-methylene interrupted dimethylalkane found in *G. tachinoides*. The mass spectrum of 35B was due to a mixture of two nine-methylene interrupted isomers, 11,21- and 13,23-dimethylpentatriacontanes (Figure 2B).

GC peak 36B was composed of 12,22- and 13,23-dimethylhexatriacontanes (Figure 2C). Although the intensity of *m/z* 210 from the 13,23 isomer seems too small relative to the intensity of *m/z* 196, it must be kept in mind that the intensity of *m/z* 196 is increased by the 14-carbon primary ion from the 12,22 isomer. A small amount of a 10,20 isomer is supported by the ions at *m/z* 309 and 407, although there is no ion of significant intensity at *m/z* 252 as expected for this isomer. The ion at *m/z* 168 is derived from the 13,23 isomer rather than from an 11,21 isomer. Expected ions of significant intensity for an 11,21 isomer at *m/z* 393 and 323 are not present.

The MS of the major peak, GC peak 37B, from female *G. tachinoides* were interpreted as consisting of two major isomers with 11 methylenes between the branch points, 11,23- and the symmetrical 13,25-dimethylheptatriacontanes, in the ratio of 1.4:1 (Figure 2D). The correlation of the fragmentations with the diagnostic ions of major intensity is obvious. However, the ions of low intensity at *m/z* 252 and 323 indicated that a third isomer must be present, i.e., the 11,21 isomer is compatible with the mass spectrum.

Average percent composition of characteristic peaks (by KI values and peak numbers)												
3365 33A′	3375 33C	3465 34C	3550 35B	3570 35C	3590 35D	3650 36B	3670 36C	3690 36D	3750 37B	3770 37C	3790 37D	Total (%)
—	—	—	—	—	—	—	—	—	—	—	—	87.6
0.5	—	—	—	—	—	—	—	—	—	—	—	91.8
—	—	—	—	—	—	—	—	—	—	—	—	87.3
—	—	—	—	—	—	—	—	—	—	—	—	89.8
—	—	—	—	—	—	—	—	—	—	—	—	73.3
0.5	15.3	—	1.3	21.1	5.4	0.9	4.9	1.6	1.0	9.2	1.5	85.7
8.9	11.4	—	1.2	28.6	3.4	0.8	12.9	0.5	0.7	11.2	0.3	94.2
7.3	0.8	5.9	—	30.4	7.2	—	16.5	1.5	—	13.5	1.5	94.1
0.9	1.2	1.0	6.6	tr	—	3.8	tr	—	29.7	tr	—	94.2
7.2[d]	1.9[e]	1.7[f]	—	—	—	—	—	—	—	—	—	79.5

[d]GC peak 32D′.
[e]GC peak 33D + 33D′.
[f]0.5% of trailing peak (KI 3790 = 37D).

The rationale for the interpretation of the MS of GC peak 38B (Figure 2E) was similar to that used above for GC peak 37B. The mass spectrum of GC peak 38 B corresponded to that of a mixture of 12,22- and 12,24-dimethyloctatriacontanes. The low intensity of the ions at *m/z* 252 and 337 relative to those at 182 and 407 indicated that two isomers must be present with the first methyl branch on carbon 12. The minor 12,24 isomer accounts for the intensity of ions at *m/z* 182 and 407 and for those at *m/z* 224 and 365. A comparison of the intensities of the ions at *m/z* 337 and 365 indicates that about twice as much of the 11-methylene interrupted isomer is present as the nine-methylene interrupted isomer. This is almost an exact reversal of isomer ratios as is observed in the MS of GC peak 36B (Figure 2C). The ion at *m/z* 196 is mainly from the 12,24 isomer rather than from a 13,?? isomer because no ion of significant intensity is present at *m/z* 393.

GC peak 39B from female *G. tachinoides* was concluded to be a mixture of dimethylisomers with nine and 13 methylenes between the branch points; 11,25- and 13,23-dimethylnonatriacontanes (Figure 2F). GC peak 39B was only a minor component of the methyalkanes, and good MS without column bleed could not be obtained. Initial inspection of the mass spectrum leads to the conclusion that the major (keeping in mind that the straight-chain tail of the isomer yielding the secondary ion at *m/z* 196 contributes to the intensity of the

TABLE 2. METHYLALKANES FROM TSETSE FLIES[a]

GLC peak	Methyl branch points	Composition									
		Fuscipes		*Palpalis*		*Gambiensis*		*Tachinoides*		*Brevipalpis*	
		M	F	M	F	M	F	M	F	M	F
25C′	4,8,12-, 3,7,11-	t+	−−	−+	t?	−+	−−	−−	−−	−−	−−
26A′	2-	−	t	−	−	t?	−	−	−	−	−
26B	6,10-	t	−	−	−	t?	−	−	−	−	−
26C′	4,8,12-	+	+	+	+	+	−	−	−	t	−
27A′	2-	t	+	t	+	+	−	−	−	−	t
27B	5,9- and 7,11-	−−	−−	−−	−−	−−	−−	−−	−−	++	−+
27B′	3,9- and 3,11-	−−	−−	−−	−−	−−	−−	−−	−−	++	−−
27C′	4,8,12-, 3,7,11-	++	−+	t?	−−	−+	−−	−+	−−	−+	−+
28A′	2- and 3-	+−	+−	+−	+−	+−	+−	+−	+−	++	+−
28B		−	−	t?	−	t?	−	−	−	t?	
28C′	4,8,12-	+	+	t	−	+	−	+	t	+	+
29A	9-, 11- and 13-	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−t−	−++	−+−
29A′	2- and 3-	+−	++	+−	++	+−	+−	+−	+−	+−	+−
29B	5,9-, 7,11-, 9,19-	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−+	++−	−+−
29B′	3,7-, 3,9- and 3,11-	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	+++	t?
29C′	5,9,13- and 3,7,11-	−+	t?	−−	−−	−−	−−	++	−−	−+	−+
30A′	2-	+	+	+	+	+	+	+	+	+	+
30C′	6,10,14- and 4,8,12-	−t	−+	−−	−−	−−	−−	++	−−	t?	+−
30D′	4,8,12,16-	−	−	−	−	−	−	−	−	t	+
31A	11-, 13- and 15-	−−−	ttt	−−−	−−−	−−−	−−−	−−−	tt−	−−−	−−−
31A′	2- and 3-	++	+−	+−	+−	+−	+−	+−	+−	t−	++
31B	9,19- and 11,21-	−−	−−	−−	−−	−−	−−	−−	++	−−	−−
31D′	3,7,11,15-	−	−	−	−	−	−	−	−	+	+

32A′	2-	+	+	t?	+	−	+	+	+	−	+
32D′	4,8,12,16-	−	−	−	−	−	−	−	−	+	+
33A′	2-	t?	+	t?	+	−	+	−	+	−	−
33B	11,15- and 11,21-	−−	−−	−−	−−	−−	−−	t?	++	t−	−−
33C	11,15,19-	−	+	−	+	−	?	−	−	−	−
33D	7,11,15,19-	−	?	−	+	−	−	−	−	−	+
	5,9,13,17										+
	3,7,11,15-										+
34A′	2-	−	+	−	+	−	+	−	+	−	−
34B	12,16-	−	?	−	−	−	−	t?	t?	−	−
34C	12,16,20-	−	+	−	+	−	+	−	−	−	−
34D	8,12,16,20	−	t?	−	?	−	−	−	−	−	t?
35A′	2-	−	−	−	t?	−	t?	−	−	−	−
35B	11,19-, 13,21-	t?	++	−−	t?	−−	−−	−−	−−		
	11,21- and 13,23-								++	−	−
35C	11,15,19-	t?	+	−	+	−	+	−	−	−	−
	13,17,21-		+		+		+				
35D	7,11,15,19-	−	t?	−	t?	−	?	−	−	−	?
	11,15,19,23-		+		+		+				
36B	15,19-, 12,20-	−−	+?	−−	−?	−−	t?	−−	−−	−−	−−
	12,22-, 13,23-								++		
36C	12,16,20-	−	+	−	+	−	+	−	−	−	−
	12,16,22-[b]		?		?		?				
	12,18,22-[b]		?		?		?				
36D	8,12,18,22-[b]	−	t?	−	?	−	t?	−	−	−	?
37B	11,19-, 13,21-	−−	++	−−	++	−−	t?	−−	−−	−	−
	15,23-		+		+						
	11,21-								+		
	11,23-, 13,25-							t?	++		
37C	11,15,??-	−	+	−	+	−	?	−	−	−	−
	11,17,21-		+		+		+				

TABLE 2. Continued.

GLC peak	Methyl branch points	Composition									
		Fuscipes		*Palpalis*		*Gambiensis*		*Tachinoides*		*Brevipalpis*	
		M	F	M	F	M	F	M	F	M	F
	15,19,23-		+		+		?		t?		
37D	11,15,19,23-	–	t?	–	t	–	t?	–	–	–	t?
38B	12,22- and 12,24-	– –	t?	– –	t?	– –	– –	– –	+ +		
38C	12,16,20-	–	+	–	+	–	t?	–	–		
39B	13,23- and 11,25-	– –	– –	– –	– –	– –	– –	– –	+ +	– –	– –
39C	11,15,19-	–	t?	–	t	–	–	–	–	–	–
	13,17,21-				t						

[a] Plus and minus signs indicate the presence or absence of each isomer, as determined by GC-MS. Although listed separately, the A′ and B components usually elute as an unresolved GC peak. A string of minuses and/or a blank space means that no GC peak was observed. A "t" means that a trace amount was identified. A question mark means that that particular isomer was not positively identified. A "t?" means that a trace amount of a component was indicated on GC-MS but identification was not possible nor was confirmation that it belongs to the indicated series.

[b] A novel branching sequence is proposed.

ion at *m/z* 168) isomer is nine-methylene interrupted 13,23-dimethylnonatriacontane. However, the entire mass spectrum cannot be interpreted using this structure unless the second isomer is a 13-methylene interrupted dimethylalkane. In this study, the only species with nine- or 11-methylene interrupted dimethylalkanes were female *G. tachinoides*, and now it appears they also have

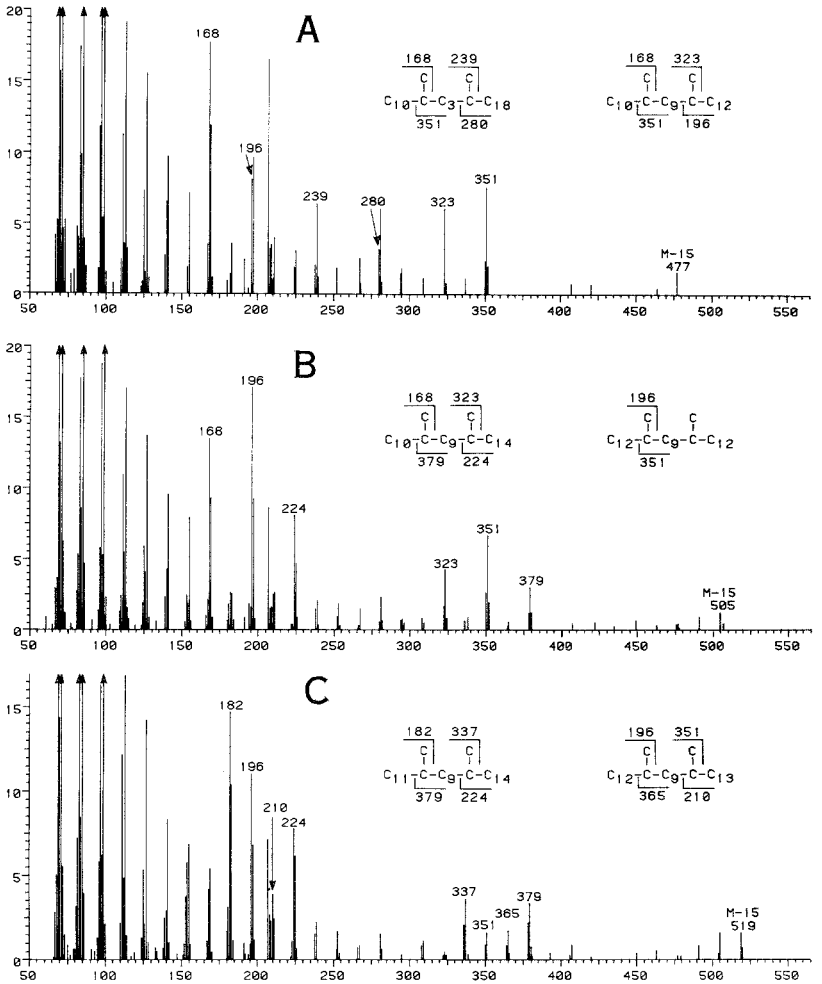


FIG. 2. CGC-MS spectra of dimethylalkanes from female *G. tachinoides*. (A) CGC peak 33B, 11,15- and 11,21-dimethyltritriacontanes; (B) CGC peak 35B, 11,21- and 13,23-dimethylpentatriacontanes; (C) CGC peak 36B, mainly 12,22- and 13,23-dimethylhexatriacontanes; (D) CGC peak 37B, 11,21-, 11,23-, and 13,25-dimethylheptatriacontanes; (E) CGC peak 38B, 12,22- and 12,24-dimethyloctatriacontanes; and (F) CGC peak 39B, 11,25- and 13,23-dimethylnonatriacontanes.

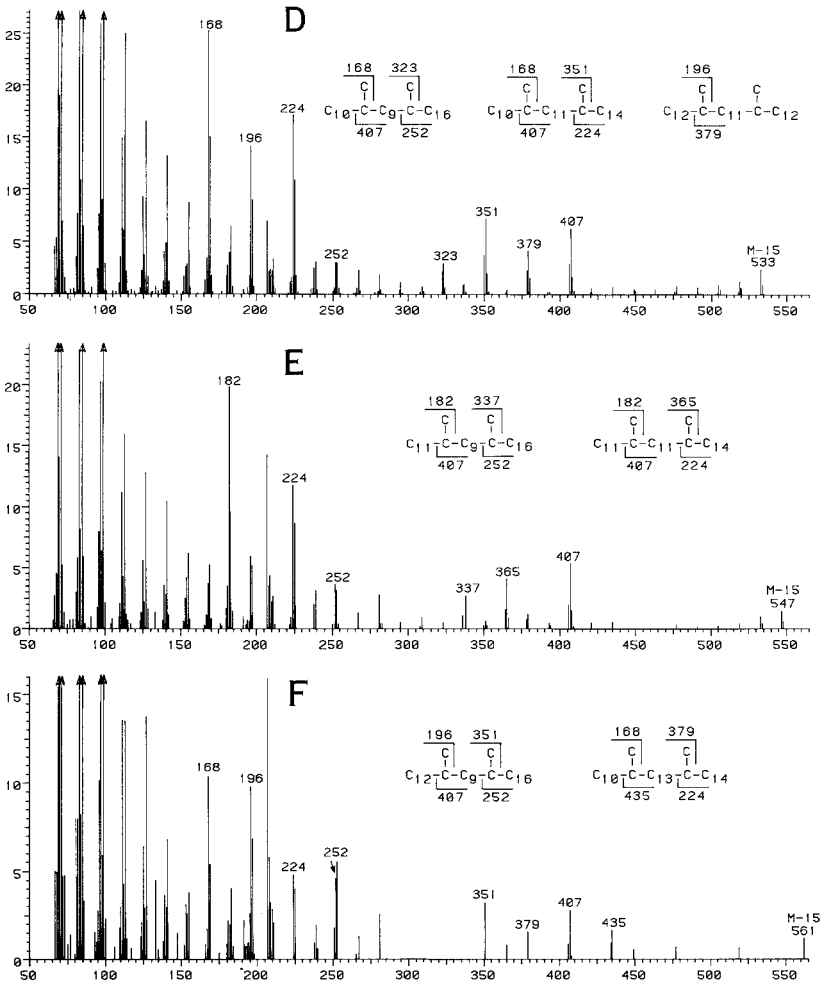


FIG. 2. Continued.

a 13-methylene interrupted dimethylalkane. How the biosynthetic mechanisms go from adding four two-carbon units before the second methyl branch is added in 35B, to adding six two-carbon units before the second methyl branch is added in 39B is an intriguing question.

GC peak 35B was of significant intensity only in female *G. tachinoides*. However, we were able to identify this peak in female *G. f. fuscipes* as being a mixture of 11,19- and 13,21-dimethylpentatriacontanes with seven methylenes between the branch points. The only other dimethylalkanes in female *G. f. fuscipes* were three-methylene interrupted (GC peak 36B) and seven-methylene interrupted (GC peak 37B).

Trimethylalkanes in Females. The methylalkane composition of the females was more complex and included longer chain length components than were present in the males. The major component in female *G. fuscipes, G. palpalis*, and *G. p. gambiensis* was GC peak 35C. The MS were similar for the three species and were interrupted as being a 1.4:1 mixture of 11,15,19- and 13,17,21-trimethylpentatriacontanes (Figure 3A). These isomers have a 3,3 sequence of methyl branching, and neither isomer is symmetrical. On the other hand, GC peak 35C was not identified in female *G. tachinoides*, although the CGC trace (Figure 1G) indicates that a trace amount may be present. The major components in female *G. tachinoides* were GC peaks 30A′ and 37B.

The occurrence of trimethylalkanes in which the number of methylenes between the first and second branch points was different from the number of methylenes between the second and third branch points was first described in *G. pallidipes* and *G. austeni* (Nelson and Carlson, 1986). In the present study, similar variations in branching sequences were not as obvious but may exist

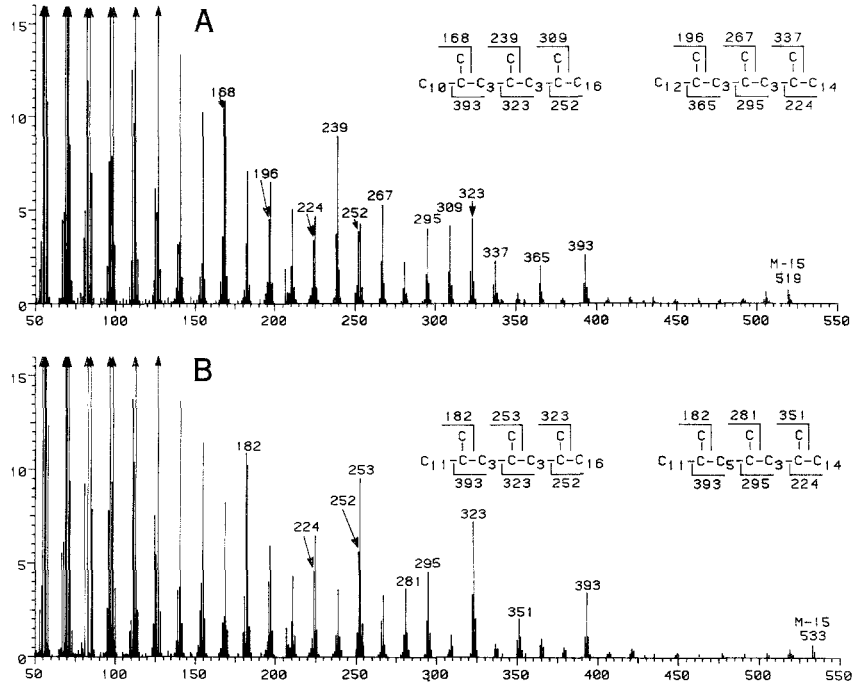


FIG. 3. GC-MS spectra of trimethylalkanes from female. (A) CGC peak 35C, from female *G. p. gambiensis*, 11,15,19- and 13,17,21-trimethylpentatriacontanes; (B) CGC peak 36C from female *G. p. palpalis*, predominantly 12,16,20-trimethylhexatriacontane; (C) GC peak 37C from *G. p. gambiensis*, predominatly 13,17,21-trimethylheptatriacontane; (D) CGC peak 37C from *G. p. palpalis*, a mixture with 11,15,21-, 11,17.21-, and 13,19,23-trimethylheptatriacontanes.

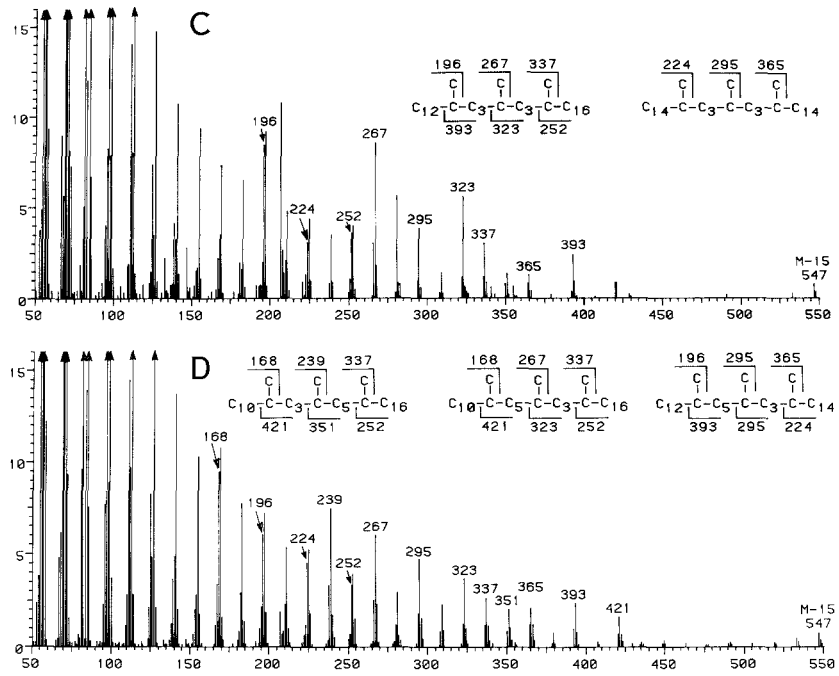


FIG. 3. Continued.

among the components of GC peaks 36C and 37C from female *G. f. fuscipes*, *G. p. palpalis*, and *G. gambiensis*. The MS of GC peaks 36C and 37C were similar for all three species.

The problem was to determine the positions of the methyl branches from the MS of a mixture of isomers. The major isomer in GC peak 36C was clearly 12,16,20-trimethylhexatriacontane (Figure 3B). However, the increased intensity of the ions at *m/z* 224, 281, 295, and 351 indicated that a second isomer was present. The lack of diagnostic ions at *m/z* 239, 309, 337, and 379 eliminated the 13,17,21 isomer from consideration, and the lack of diagnostic ions at *m/z* 210 and 365 ruled out the 14,18,22 isomer. We concluded that the second isomer could be 12,18,22-trimethylhexatriacontane with a 5,3 sequence of methyl branching.

The major isomer in GC peak 37C from female *G. gambiensis* was clearly 13,17,21-trimethylheptatriacontane (Figure 3C) with a 3,3 sequence of methyl branches. A second isomer is indicated by the ions at *m/z* 224, 295, and 365. These ions could be accounted for by the symmetrical 15,19,23 isomer. In addition, the less intense ions at *m/z* 168 and 421 show the presence of an 11,??,?? isomer. The low intensity of the ions at *m/z* 280 and 309 indicated

that the third methyl branch is not on carbon 19 as would be expected for a 3,3 branching sequence. However, the ions at *m/z* 239 and 351 indicate the second methyl branch may be on carbon 15.

GC peaks 37C from female *G. f. fuscipes* and *G. p. palpalis* had similar MS (Figure 3D) but were different to the MS of GC peak 37C from female *G. p. gambiensis* (Figure 3C). An isomer with a 3,3 branching sequence in these two species could not be clearly identified from the MS in Figure 3D. The ions at *m/z* 168/169 and 421 show the presence of an isomer with the first methyl branch on carbon 11, and the ions at *m/z* 239 and 351 establish a second methyl branch on carbon 15, and the ions at *m/z* 267 and 323 establish a second methyl branch on carbon 17. The low intensity of the ions at *m/z* 280 and 309 showed that a third methyl branch is not on carbon 19. Possible structures are 11,15,21-trimethylheptatriacontane with a 3,5 branching sequence and 11,17,21-trimethylheptatriacontane with a 5,3 branching sequence. The diagnostic ions at *m/z* 196, 224, 295, 365, and 393 could be accounted for by the presence of a third isomer, 13,19,23-trimethylheptatriacontane, with a 5,3 branching sequence. Considering the relative intensities of the ions, these three isomers could account for the diagnostic ions in the mass spectrum. However, the possibility of other isomers forming this spectrum or contributing to it cannot be ruled out.

Trimethylalkanes in Males. Males usually have simpler methylalkane composition and structures than females, but sometimes have higher alkanes of female origin from physical contact. Males of the *palpalis* group differ from other tsetse males because of the predominance of trimethylalkanes rather than 2-methylalkanes. The male methylalkanes ranged in size from 28 total carbons (25C′) to 35 carbons (33B) (compared to 28 carbons (25C′) to 42 carbons (39C) in females). The major methylalkanes in male *G. f. fuscipes* were 4,8,12-trimethylhexacosane (26%) and 4,8,12-trimethyloctacosane (31%). The major methylalkanes were 4,8,12-trimethylhexacosane (Figure 4A) and 2-methylnonacosane in male *G. p. palpalis*, 41% and 22%, respectively, and in male *G. p. gambiensis*, 37% and 22%, respectively. The major component (38%) in male *G. tachinoides* was 4,8,12-trimethyloctacosane.

Interestingly, the major trimethylalkane in males all had even-numbered backbones and the first methyl branch on carbon 4. Trimethylalkane with odd-numbered backbones had the first methyl branch on carbon 3, i.e., 3,7,11-trimethylheptacosane from male *G. tachinoides* (Figure 4B). However, in male *G. f. fuscipes*, the odd-numbered backbone trimethylalkanes, 25C′ and 27C′, had both isomers present, resolved by GC-MS, in which the first methyl branch occured on carbons 4 and 3 (Table 2).

Tetramethylalkanes. The tetramethylalkanes with branch sequences starting near the end of the molecule elute at about the same time as the internally branched dimethylalkanes with a backbone one carbon longer, i.e., 31D′ elutes

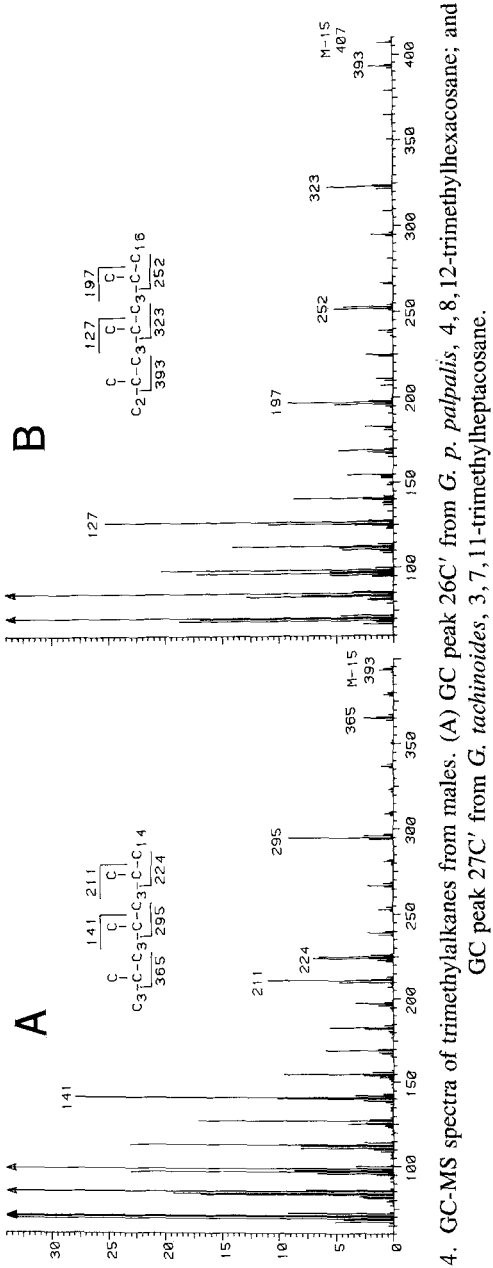


FIG. 4. GC-MS spectra of trimethylalkanes from males. (A) GC peak 26C′ from *G. p. palpalis*, 4, 8, 12-trimethylhexacosane; and (B) GC peak 27C′ from *G. tachinoides*, 3, 7, 11-trimethylheptacosane.

near 32A′ [Kovats index (KI) 3265, see Table 1], whereas 31D (KI 3190) elutes near the 32-carbon *n*-alkane. The presence of tetramethylalkanes in this group of insects made them unique. They were present only in females of *G. f. fuscipes*, *G. p. palpalis*, and *G. gambiensis* and were not identified in *G. tachinoides*, or in flies of the *morsitans* group in our previous study (Nelson and Carlson, 1986). Tetramethylalkanes were minor components except in *G. brevipalpis* where they were present in significant amounts in both sexes; the female having 31D′, 32D′, and 33D and the male, 31D′ and 32D′.

The smallest tetramethylalkane found in this study was 31D′, 3,7,11,15-tetramethylhentriacontane from both male and female *G. brevipalpis* (Figure 5A and Table 2). The ion at *m/z* 421 is from coeluting 2-methyldotriacontane present at about 20% of 31D′. Those tetramethylalkanes with an even number of carbons in the backbone had the first branch point on an even-numbered carbon, either 4 or 8 (Table 2). The mass spectrum (Figure 5B) of 4,8,12,16-tetramethyldotriacontane (32D′) depicts the fragmentation obtained. The *n*-propyl end of the molecule forms a diagnostic ion at *m/z* 463 (M-43). Cleavage internal to the first branch point to give the 5-carbon secondary ion did not increase the intensity of the ion at *m/z* 70, relative to those at *m/z* 69 and 71, to make it of diagnostic value.

In contrast to *G. brevipalpis*, the tetramethylalkanes from the other *palpalis* species were internally branched. For example, 35D, 11,15,19,23-tetramethylpentatriacontane (Figure 5C), and 35D, 7,11,15,19-tetramethylpentatriacontane (Figure 5D) were identified from sequential mass spectra recorded 8 sec apart and were found in female *G. p. palpalis* as a single peak. Although GC peak 35D was the major tetramethylalkane, it was only a minor component of the total methylalkanes. Interestingly, these compounds were homologs of trimethylalkanes and also characteristically contained three-methylene interrupted branch points.

Use of GC Composition for Species/Sex Identification. After identification of components from their mass spectra, it is intriguing to look for unique or characteristic components in each species and to correlate them with GC peak intensities (Table 1). The percent composition values are averages of two to five chromatograms of individual insects and so do not exactly match the gas chromatographic traces (Figure 1). Males of the two *G. palpalis* subspecies, *G. p. palpalis* and *G. p. gambiensis*, were distinguished by their 26C′ major peaks (KI 2720), which also appeared as the major component in *G. f. fuscipes* but were absent in *G. tachinoides*. There were traces of 27C′, 32A′, and 33A′ in *G. p. palpalis* that were undetectable in *G. p. gambiensis*; these are markers that separate the two *palpalis* subspecies. Both *G. f. fuscipes* and *G. tachinoides* males had major amounts of 28C′, which distinguished them from the two *palpalis* subspecies that contained only trace quantities. The absence of 26C′ in male *G. tachinoides* was sufficient to separate them from the other three *palpalis* group males and was noted earlier (Carlson, 1983).

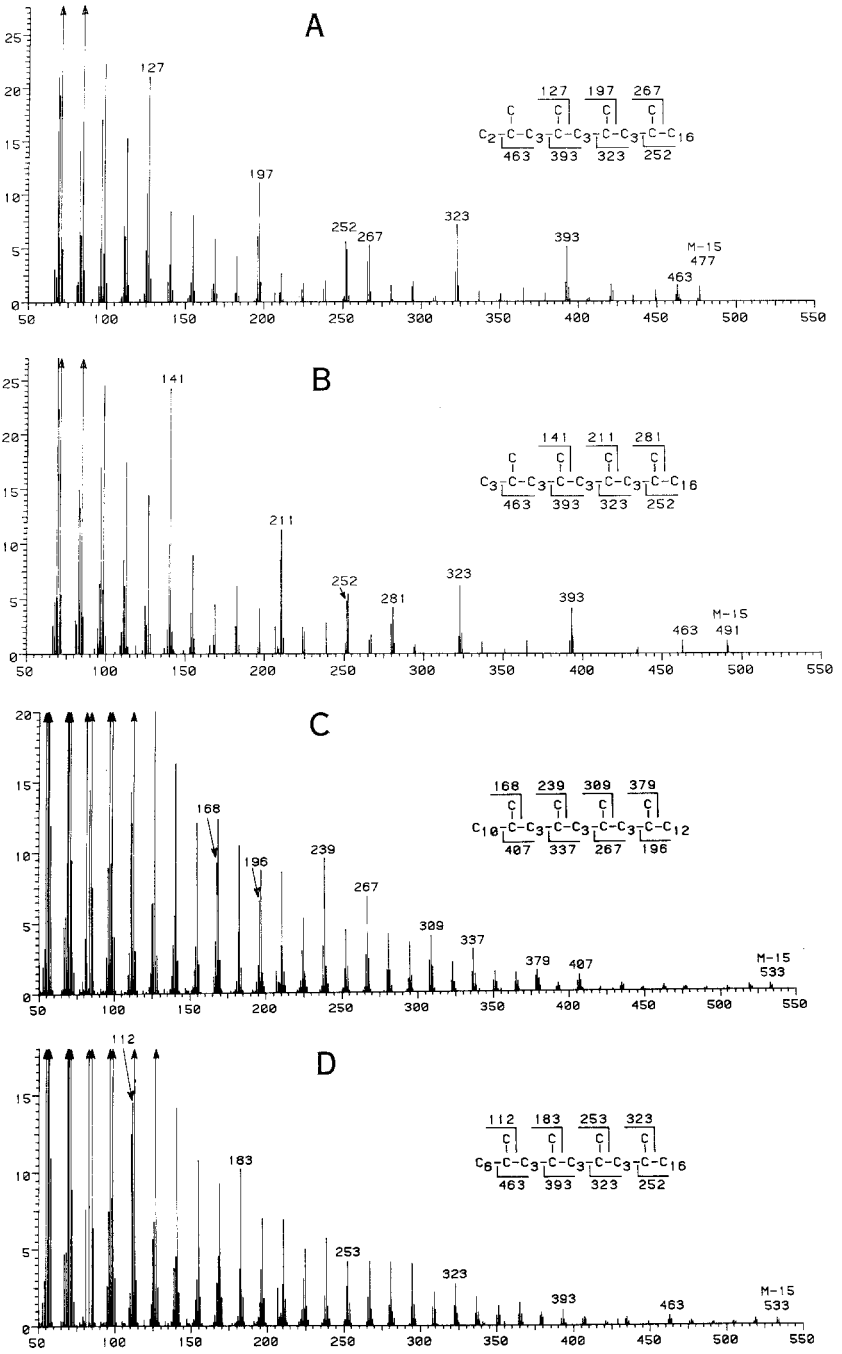


FIG. 5. CGC-MS spectra of tetramethylalkanes from females. (A) CGC peak 31D′ from *G. brevipalpis*, 3,7,11,15-tetramethylhentriacontane; (B) GC peak 32D′ from *G. brevipalpis*, 4,8,12,16-tetramethyldotriacontane; (C) GC peak 35D from *G. p. palpalis*, 11,15,19,23-tetramethylpentatriacontane; and a later scan from GC peak 35D, 7,11,15,19-tetramethylpentatriacontane.

The *G. f. fuscipes* females were readily distinguished from those of the *palpalis* subspecies by the smaller 33A′, 35D, 36C, and 37C peaks, whereas the *G. p. palpalis* and *G. p. gambiensis* females differed from each other by the presence of nearly equal quantities of 33A′ and 33C in the former and the lack of 33C and 34C in the latter. Also, *G. p. gambiensis* and *G. p. palpalis* were conspicuously different from *G. f. fuscipes* because of a very small 30A′ peak compared to a medium 30A′ peak in *G. f. fuscipes*, while all three had large 35C peaks. The *G. tachinoides* female profile was different from the other three species in this group because of its large 30A′, very small 31A′, conspicuous lack of major trimethylalkanes of 35C, 36C, and 37C, and large 37B.

Variation in the separation of the methyl branch points has been observed in dimethylalkanes of the Japanese beetle, *Popillia japonica* (Nelson, et al., 1977), and in the housefly, *Musca domestica* (Nelson, et al., 1981), with the transition occurring about 31B to 33B. However, the methylalkanes smaller than 31B always had the methyl branch points separated by three methylenes, and those larger than 33B had the methyl branch points separated by seven or nine methylenes. In *G. pallidipes* females, the smaller dimethylalkanes were variable (Nelson and Carlson, 1986) as GC peak 25B was a mixture of 11,15- and 5,13-dimethylpentacosanes (this was indicated incorrectly in Table 2 in that paper), 26B was 11,15-dimethylhexacosane, 27B was a mixture of 11,15- and 5,15-dimethylheptacosanes, 29B was 7,17-dimethylnonacosane, and 30B was 10,14-dimethyltriacontane.

Dimethylalkanes with 11 methylenes between the branch points have been reported in *Lasioderma serricorne* (Baker et al., 1979); *Zootermopsis augusticollis* (Blomquist et al., 1979), *Trichopsenius frosti* and *Reticulitermes flavipes* (Howard et al., 1980), *Onymacris marginipennis* (Breme) (Lockey, 1982), *Iridomyrmex nitidiceps* (Brophy et al., 1983), *Oncopeltus fasciatus* (Jackson, 1983), *Melanoplus packardii* and *M. sanguinipes* (Nelson et al., 1984), *Sitophilus zeamais* (Baker et al., 1984), *Cylindrocopturus adspersus* (LeConte) (Pomonis and Hakk, 1984), *Renatiella scrobipennis* (Lockey, 1984), *Periplaneta americana* (Lockey and Dularay, 1986); and 13-methylene interrupted dimethylalkanes have been identified in *O. fasciatus* (Jackson, 1983), *R. scrobipennis* (Lockey, 1984), and in the maize weevil, *S. oryzae* (Baker et al., 1984).

It will be an interesting study to elucidate the biosynthetic mechanisms involved in controlling the separation of the branch points as the chain length increases and how these mechanisms vary between species and orders. These insects synthesize major amounts of trimethylalkanes and the unique tetramethylalkanes from their only food source, animal blood. Langley and Carlson (1983) showed that succinate was incorporated into the methylalkanes of *G. morsitans* by the abdominal cuticle. The fact that di- and trimethylalkanes are known sex pheromones in several species of tsetse make further study of these insects worthwhile, especially since it appears that the 35C peak contains the

major sex pheromone components of *G. p. palpalis* and *G. p. gambiensis* (D.A. Carlson, submitted). Also, it is tempting to suggest that the unusual 37B components in *G. tachinoides* may comprise the sex stimulant pheromone in this species. Either 13,25- or 11,23-dimethylheptatriacontane might be biologically active. The former structure is symmetrical, a type of structure previously shown to be active in *G. morsitans* and *G. pallidipes*. Particularly interesting in this regard are the novel, potentially synergistic, potentially bioactive, tetramethylalkanes.

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TOXICITY AND TOXICOKINETICS OF 6-METHOXYBENZOXAZOLINONE (MBOA) IN THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (HÜBNER).

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Abstract—The maize-derived secondary chemical 6-methyoxybenzoxazolinone (MBOA) and a tritiated derivative were prepared synthetically for a detailed examination of their toxicity and toxicokinetics in the European corn borer (ECB), *Ostrinia nubilalis*. During feeding trials with MBOA incorporated into meridic diets (at 0, 0.5, 1.5, 2.5, and 4.0 mg/g diet), the mean time to pupation and adult emergence was significantly lengthened at concentrations of 1.5 mg MBOA/g diet and above. Increased mortality occurred at concentrations at 1.5 mg/g and above. A decrease in the sex ratio (female/total) and fecundity was observed at concentrations of 0.5 mg/g and above. The latter observations represent new biological effects related to MBOA. In tracer studies, both uptake and excretion of MBOA administered in diets to larvae increased linearly with concentration. Body burden values indicated that the ECB larvae were capable of excreting enough compound to maintain total tissue levels at approximately 50% of the dietary concentration. Total amount of label increased with larval stage, but decreased in adults due to a large amount of label eliminated in the pupal case. In topical application studies, elimination of the label in the frass was rapid, reaching 60% by 6 hr and 82% of applied dose by 24 hr. Accumulation of label in tissues other than hemolymph was small. The results show that MBOA is toxic to ECB, but the insect has efficient methods for minimizing these effects.

Key Words—MBOA, tritiated MBOA, 6-methoxybenzoxazolinone, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, European corn borer, toxicokinetics, life-cycle parameters, body burden values, topical application, corn, maize, feeding resistance.

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INTRODUCTION

Leaf feeding resistance to the ECB (European corn borer, *Ostrinia nubilalis*) in some corn genotypes (*Zea mays*) has been strongly correlated with levels of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) present in the whorl of the corn plant (Klun and Brindley, 1966; Klun et al., 1967, 1970; Reed et al., 1972; Robinson et al., 1982). Development of inbred lines with high levels of DIMBOA has provided one of the principal means of control of the ECB (Lynch, 1980). Its glucoside form is present in uninjured corn tissue. Injury to the plant tissue, as would occur during larval feeding, results in the enzymatic conversion of the glucoside (1) to the aglycone, DIMBOA (2), by the hydrolytic enzyme β-glucosidase (Figure 1). DIMBOA decomposes in a reaction which is pH and temperature dependent (Woodward et al., 1978a; Perez and Niemeyer, 1986), through intermediates 3 and 4, to yield 6-MBOA (5) (6-methoxybenzoxazolinone) and formic acid (6). As the pH of the reacting medium is lowered, there is a decrease in the yield of MBOA from DIMBOA which has been attributed to the conversion of intermediate 3 to intermediates 7 and 8, resulting in the formation of 3,4-dihydroxy-7-methoxy-1,4-benzoxazin-2-one (9) (an isomer of DIMBOA) (Figure 1) (Bravo and Niemeyer, 1986).

DIMBOA and other hydroxamic acids (HA), present in several species of Poceae (Graminae), have been shown to play a role in the defense of the plants against several other insect species (Long et al., 1975; Argandoña et al., 1980, 1981), fungi (Long et al., 1975), and bacteria (Woodward et al., 1978b). DIMBOA, at concentrations corresponding to levels in resistant cultivars of maize

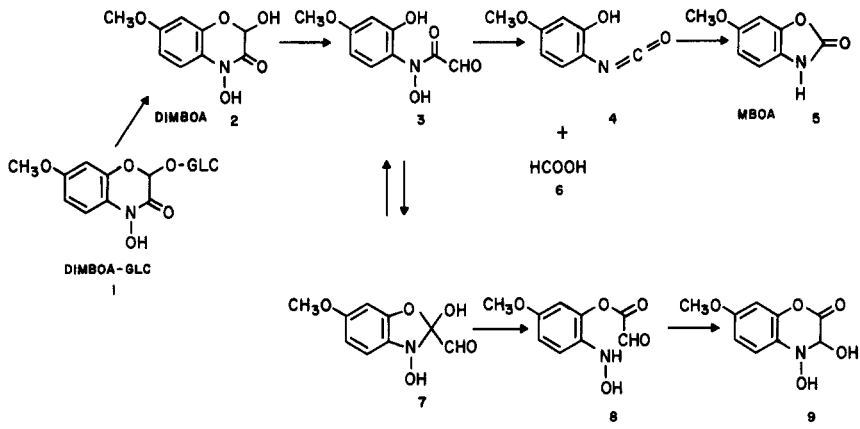


FIG. 1. Chemical degradation of DIMBOA-Glc. (Adapted from Bravo and Niemeyer, 1986.)

and wheat, reduce the rate of larval development of aphids (Corcuera et al., 1982) and greenbugs (Argandoña et al., 1983), as well as of ECB (Robinson et al., 1982). The reproductive capabilities of these adults is reduced, since the number of egg masses produced is lower. In addition, DIMBOA has also been shown to act as a feeding deterrent at high concentrations in the above species (Argandoña et al., 1983; Corcuera et al., 1985). Although DIMBOA is generally more active than its degradation product, MBOA is known to elicit biological effects on some plant pathogens. In the ECB, MBOA was shown to lengthen both larval and pupal periods (Klun and Brindley, 1966), while in aphids, MBOA had deleterious effects when incorporated in the diet, increasing the mortality of the insects (Argandoña et al., 1980).

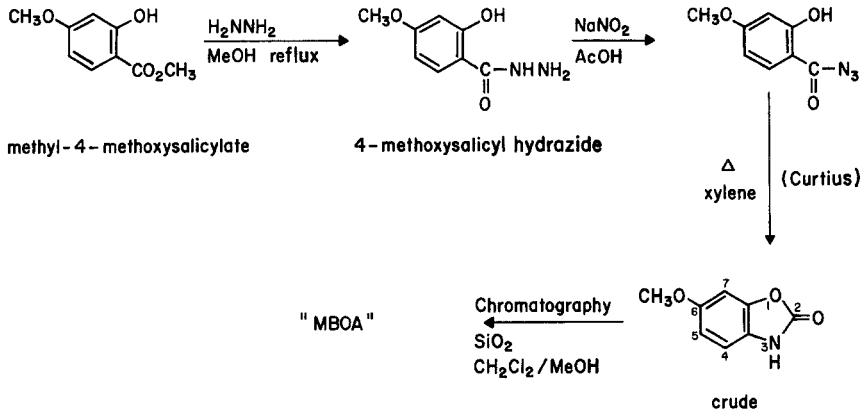
The yield of MBOA from the decomposition of DIMBOA increases with pH (Bravo and Niemeyer, 1986). It is important to note that the midgut (the principal site of digestion and assimilation) of most lepidopteran larvae has a pH of approximately 8.5 (Berenbaum, 1980), an environment which is suitable for the production of MBOA from the decomposition of DIMBOA, thus confounding the contribution of each compound to toxicity.

The differences in response to feeding dietary DIMBOA or MBOA suggest that there may be differential penetration, distribution, and metabolism, as well as a dissimilar mode of action of the compounds. The toxicokinetics of both substances are unknown at present; thus it is difficult to determine their relative role in insect deterrence. This report, the first of a two-part study on the pharmacokinetics of MBOA and DIMBOA, examines in detail the biological activity of MBOA on the ECB and the fate of labeled MBOA in feeding trials and topical applications to the insect. The object of this study was to determine the effect of MBOA on the ECB and the response of the insect to the plant compound. The doses of MBOA used represent a range of levels produced naturally in corn (Klun et al., 1970; Long et al., 1975; Tang et al., 1975), except the highest value (4 mg/g), which represents a value above what has normally been found, but is still potentially attainable in a breeding program.

METHODS AND MATERIALS

ECB Rearing. The bivoltine strain of ECB was originally obtained from the Agriculture Canada Research Station (London, Ontario). The culture was maintained in our laboratory according to the procedures and techniques of Guthrie et al. (1972). The MBOA-treated diets and control diets used in the experiments lacked corn cob grits so as to maintain textural uniformity.

6-Methoxybenzoxazolinone. MBOA was synthesized by the method of Kubo and Kamikawa (1983). These authors did not provide a detailed experimental procedure. Following is a description of our experimental method to



SCHEME 1. Synthesis of MBOA.

prepare MBOA (Scheme 1) based on the sequence of reactions used by Kubo and Kamikawa (1983). To 10.1 g of methyl-4-methoxysalicylate in 25 ml of methanol was added 16 ml of 95% hydrazine (Aldrich). The mixture was dissolved by warming and set to reflux for 8 hr. When cooled, the product 4-methoxysalicylhydrazide crystalized and was filtered and washed with cold methanol. Yield was 8.48 g (84%), mp 172.5–173.5°C. MS: *m/z* (%) 182 (28.8), 151 (100), 108 (21.4), 95 (23.7), 63 (10.5), 53 (10.6), 52 (14.2), 51 (12.1).

In a 500-ml round-bottom flask, 5.3 g of the hydrazide was disolved on 100 ml of glacial acetic acid. To this was added 100 ml of ether giving two phases. The stirred mixture was cooled in an ice bath to 10–12°C, and 5.3 g $NaNO_2$ in 20 ml of water was added dropwise over 10 min. After 30 min, the reaction was diluted with water and extracted with ether. Drying and evaporation of ethereals gave 5.3 g of azide (94%) as a peach-colored solid. MS: *m/z* (%) 193 (100), 165 (12.5), 151 (68.1), 150 (97.5), 122 (53.3), 109 (33.1), 108 (24.3), 107 (20.6), 106 (61.7), 95 (18.3), 94 (21.6).

The crude azide was immediately added to 500 ml of dry xylene and refluxed for 2 hr, whereupon TLC showed no azide remained. On cooling, a solid precipitated. This was collected and washed with hexane yielding 4.6 g crude MBOA.

Silica gel flash chromatography of this material provided varying amounts of pure MBOA with each reaction. Sufficient quantities of analytically pure MBOA (GC, MS, NMR) were obtained to perform the feeding trials.

The melting point of MBOA was 152.5–153.5°C (EtOH) [lit. 154°C (Nachman and Ronald, 1982), 168–170°C (Kubo and Kamikawa, 1983)]. [^{1}H]NMR (300 MHz), H_5 6.71 (dd), H_7 6.88 (d), H_4 7.01 (d), 3.78 (s). $J_{5,4}$

= 8.9 Hz, $J_{7,5}$ = 2.2 Hz, $J_{4,5}$ = 9 Hz, $J_{5,7}$ = 2.2 Hz. [^{13}C]NMR 156.6, 155.1, 145.3, 124.3, 110.3, 109.6, 97.5, 55.9. IR (C=O) 1750 cm^{-1}.

Tritiated MBOA was prepared by a modified high-temperature, dilute-acid method (Werstiuk and Timmins, 1981). The method was optimized with D_2O prior to the labeling experiments with tritiated water, thus establishing that the label was located at C-5. The specific activity was 2.0 mCi/mmol.

Partition Coefficient (Log P). The octan-1-ol–water partition coefficient for MBOA was determined using the shake flask method (Leo et al., 1971). [^{3}H]MBOA was utilized to facilitate the determination of the concentration of MBOA in each phase.

[^{3}H]MBOA as Tracer for MBOA. To determine if the uptake of the tritiated material by the insect was representative of the cold MBOA present in the diet and topical solution (i.e., that there was no isotope effect), third-instar larvae were fed diets containing 9.0, 27.1, 40.6, or 54.1 μg [^{3}H]MBOA/g diet to which was added sufficient unlabeled MBOA to bring the total to 60 μg/g. Thus the specific activity of the isotope varied, but the total dose remained constant. The larvae were maintained on these diets until fifth instar. Larvae and frass were recovered and burned in a sample oxidizer (Packard Tricarb model 306 using Permafluor and Monophase, Packard Instrument Company Inc.) to determine the total content of ^{3}H in each sample. ^{3}H content was measured using a Beckman scintillation counter and Scintilene scintilation fluid (Fisher Scientific Inc.)

Topical Application. Early fifth-instar larvae (five replicates with five larvae each) were topically applied with [^{3}H]MBOA (83.3 ng/mg insect) dissolved in 5 μl of dimethyl sulfoxide (DMSO). Larvae were allowed to feed on control diet until sampling time (1, 2, 6, 12, 24, and 48 hr). At these times and prior to dissection, larvae were rinsed in methanol to remove any of the compound from the surface of the cuticle that had not been absorbed. The dissection was performed on filter paper so as to trap the hemolymph at the time of cutting. The samples collected were: hemolymph, gut (with contents), fat bodies, cuticle, and frass. Tritium content of tissues were analyzed as before. Cross-contamination from hemolymph remaining on the tissues was evaluated by repeated rinsing of body tissues with saline and estimated to be 6.9 ± 1% of the total hemolymph content.

Dose-Related Uptake and Excretion of MBOA in Feeding Trials. MBOA and [^{3}H]MBOA were dissolved in ethanol and incorporated into freshly prepared meridic diets when the diet had cooled to approximately 40°C prior to solidifying. Final concentration of ethanol was 1% for all diets prepared including control diet. For each concentration of MBOA diet prepared (0.5, 1.5, 2.5, and 4.0 mg/g diet), 7 μg [^{3}H]MBOA/g diet (110,000 dpm/g diet) were added.

For each of the above concentrations, 100 eight-day-old larvae (second instar), fed from the neonate stage on the corresponding concentration, were

placed individually in vials plugged with cotton and provided with an appropriately treated diet cube. Twenty larvae for each concentration, plus 20 for control, were used for measuring growth parameters, while the remaining 80 larvae for each concentration were used for measuring the concentration of MBOA at various stages in development (fourth and fifth instar, pupal, and adult). For the 1.5 mg/g concentration, fourth and fifth instar, pupal, and adult stages were sampled to determine the body burden of MBOA through the development of the insect. For the remaining concentrations, 0.5, 2.5, and 4.0 mg/g, fifth instars, pupae, and adults were sampled to determine the body burden of MBOA under varying doses of MBOA in the diet. The fifth instar was dissected and the concentration of MBOA determined for various tissues (hemolymph, gut plus contents, fat body, and cuticle). Frass samples were also collected. The experiment measuring growth parameters was repeated twice.

RESULTS AND DISCUSSION

Growth Studies. When larvae were fed on varying concentrations of MBOA (0, 0.5, 1.5, 2.5, and 4.0 mg/g), the biological effects were clearly evident in the mortality data (Table 1). An increase in the mortality of the larvae, which occurred throughout larval development, was observed with an increase in concentration and ranged from 3.9% for controls to 22.5% for the highest concentration, 4.0 mg/g. Mortality occurring throughout the larval period has also been reported for ECB reared on a high DIMBOA line of corn (Reed et al., 1972). In the present study, an increase in concentration resulted in an increase in the duration of the larval period, which was significant at $P \leq 0.05$ (from 27.2 days for controls to 34.9 days for the 4.0 mg/g diet). Such a retardation in development was also reported by Klun and Brindley (1966) when they fed ECB on a diet of 0.5 mg MBOA/g diet as compared to control diet. Although there was a significant increase in the development time of the larvae, the final weight of the fifth-instar larvae was not affected.

A prolongation of development was not as clearly defined in the pupal instar as in the larval period, although the trend of increasing duration of this period is still present (Table 2). As with larval weights, pupal weights do not seem to be affected by the presence of MBOA in the diet. Pupal mortality, which ranged from 6.1 to 18.5%, appeared to be higher in the treated groups as compared to controls, but was not correlated to the concentration of MBOA.

The total duration of the development of the ECB was significantly increased with an increase in concentration of MBOA (Table 3), for both males and females, although the mean weight of the adults was not affected. A decrease in the sex ratio (females/total) was consistently observed when MBOA was present in the diet, although not well correlated to the concentration of MBOA. This suggests that ECB females are probably more susceptible to the effects of

TABLE 1. EFFECT OF MBOA (0.5, 1.5, 2.5, 4.0 mg/g) ON DEVELOPMENTAL PARAMETERS OF *Ostrinia nubilalis* LARVAE (SE in parentheses)

MBOA conc. (mg/g)	Mean wt of fifth instar (g)	Mortality of larvae (%)	Days to pupation
0	0.102a[a]	3.9	27.2*a*
	(0.003)		(0.322)
	(N = 51)		(N = 50)
0.5	0.097a	5.0	28.3ab
	(0.004)		(0.611)
	(N = 38)		(N = 37)
1.5	0.094a	20.6	30.4bc
	(0.005)		(0.898)
	(N = 28)		(N = 25)
2.5	0.096a	17.5	31.8c
	(0.004)		(0.771)
	(N = 30)		(N = 28)
4.0	0.099a	22.5	34.9d
	(0.004)		(0.774)
	(N = 30)		(N = 28)

[a]Means followed by the same letters within columns indicate no significant different ($P \leq 0.05$) in Tukey's Studentized range test. N = sample size.

TABLE 2. EFFECT OF MBOA (0.5, 1.5, 2.5, 4.0 mg/g) ON PUPAL INSTAR OF *Ostrinia nubilalis* (SE in parentheses)

MBOA conc. (mg/g)	Pupal period (days)		Pupal mortality (%)	Pupal weight (g)	
	F	M		F	M
0	7.4a[a]	8.2a	6.1	0.096a	0.071ab
	(0.319)	(0.345)		(0.003)	(0.001)
	(N = 26)	(N = 18)		(N = 27)	(N = 18)
0.5	7.6ab	9.0ab	10.5	0.097a	0.073a
	(0.528)	(0.340)		(0.006)	(0.001)
	(N = 7)	(N = 27)		(N = 7)	(N = 27)
1.5	8.7ab	9.5ab	18.5	0.092a	0.070ab
	(0.423)	(0.340)		(0.005)	(0.002)
	(N = 10)	(N = 11)		(N = 10)	(N = 11)
2.5	9.2b	9.7b	6.1	0.089a	0.071ab
	(0.464)	(0.270)		(0.003)	(0.002)
	(N = 11)	(N = 16)		(N = 11)	(N = 17)
4.0	9.2b	9.5ab	12.9	0.086a	0.068b
	(0.297)	(0.386)		(0.002)	(0.002)
	(N = 12)	(N = 13)		(N = 13)	(N = 14)

[a]Means followed by the same letters within columns indicate no significant difference ($P \leq 0.05$) in Tukey's Studentized range test.

TABLE 3. EFFECT OF MBOA (0.5, 1.5, 2.5, 4.0 mg/g) ON ADULT STAGE of *Osrtrinia nubilalis* (SE in parentheses)

MBOA conc. (mg/g)	Days to adult emergence		Mean wt. of adult (g)		Sex ratio (F/total)
	F	M	F	M	
0	36.6a[a]	35.1a	0.058a	0.034a	0.61
	(0.458)	(0.419)	(0.002)	(0.002)	
	(N = 28)	(N = 17)	(N = 27)	(N = 18)	
0.5	36.1ab	37.0ab	0.059a	0.034a	0.21
	(1.29)	(0.582)	(0.002)	(0.002)	
	(N = 8)	(N = 26)	(N = 7)	(N = 16)	
1.5	39.7bc	37.9bc	0.050a	0.033a	0.48
	(0.870)	(0.456)	(0.003)	(0.002)	
	(N = 10)	(N = 11)	(N = 10)	(N = 11)	
2.5	42.6cd	39.9cd	0.049a	0.030a	0.42
	(1.81)	(0.601)	(0.002)	(0.002)	
	(N = 11)	(N = 15)	(N = 12)	(N = 15)	
4.0	45.8d	42.6d	0.050a	0.029a	0.35
	(1.36)	(1.25)	(0.004)	(0.002)	
	(N = 12)	(N = 13)	(N = 13)	(N = 9)	

[a] Means followed by the same letters within columns indicate no significant difference ($P \leq 0.05$) in Tukey's Studentized range test.

MBOA during their development. Along with this decrease in the sex ratio, there was also a decrease in the number of egg masses per female (Table 4), although the number of eggs per egg mass was not affected. A small experiment on the effect of sex ratio indicated that this decrease in the number of eggs per female cannot be accounted for by the different sex ratios of adults in the dif-

TABLE 4. EFFECT OF MBOA (0.5, 1.5, 2.5, 4.0 mg/g) ON FERTILITY OF *Ostrinia nubilalis* (SE in parentheses)

MBOA conc. (mg/g)	Egg masses/F[a]	No. eggs/egg mass	Eggs/F
0	5.9	28.9	170.5
0.5	2.0	25.8	51.6
1.5	1.0	24.0	24.0
2.5	2.9	32.3	93.7
4.0	3.0	26.9	80.6

[a] Obtained by placing all emerging males and females in mating chambers.

ferent MBOA-treated trials. This decrease in fertility has been observed with ECB fed on varieties of resistant corn, although the effects were attributed to DIMBOA (Reed et al., 1972). Since the effects of MBOA are observed at the F_1 generation as well as throughout development, even at doses of 0.5–2.5 mg/g, which are relavent to maize varieties, it may be concluded that MBOA does have biological effects on the ECB, contrary to previous reports (Klun et al., 1967).

Validation of [^{3}H]MBOA as Biological Tracer. The amount of ^{3}H isotope found in the insect and frass of the fifth-instar larvae, after feeding on diets with varying levels of [^{3}H]MBOA but the same total amount of MBOA, was directly related to the amount of ^{3}H isotope in the diet ($r = 0.993$, slope $= 0.26$, $P < 0.01$, and $r = 0.986$, slope $= 0.60$, $P < 0.01$ for the insect and frass samples, respectively) (Figure 2). This linear relationship suggests that there was no preferential uptake or excretion of the tritiated compound over the cold compound. Thus, the [^{3}H]MBOA used for these experiments was acting as a reliable tracer for the cold MBOA.

Uptake and excretion of labeled compound, in feeding trials using varying concentrations of the compound, was affected by concentration. The levels of [^{3}H]compound in all stages of development and in the frass increased approximately linearly with an increase in concentration, leading to a constant body burden (defined as the concentration in the tissues/concentration in the frass) (Figure 3, inset) ($r > 0.95$). This indicates that the ECB is unable to react to the presence of MBOA by increasing the excretion rate of the compound when

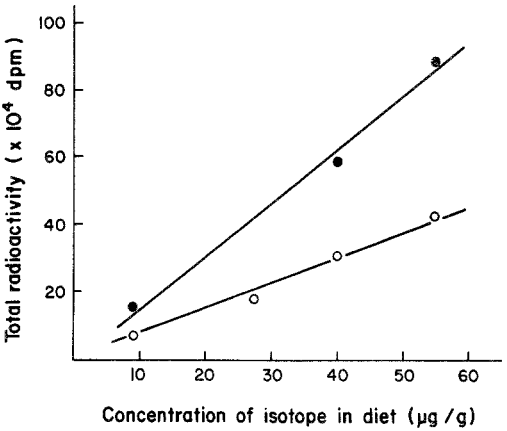


FIG. 2. Relationship between the concentration of labeled MBOA in the diet and that found in the fifth-instar larvae (○) of *Ostrinia nubilalis* and the frass (●) of the larvae. Total MBOA concentration was maintained at 60 mg/g diet by addition of cold compound. Total radioactivity in insect and frass are expressed on a per gram basis. The specific activity of MBOA was 2.0 mCi/mmole.

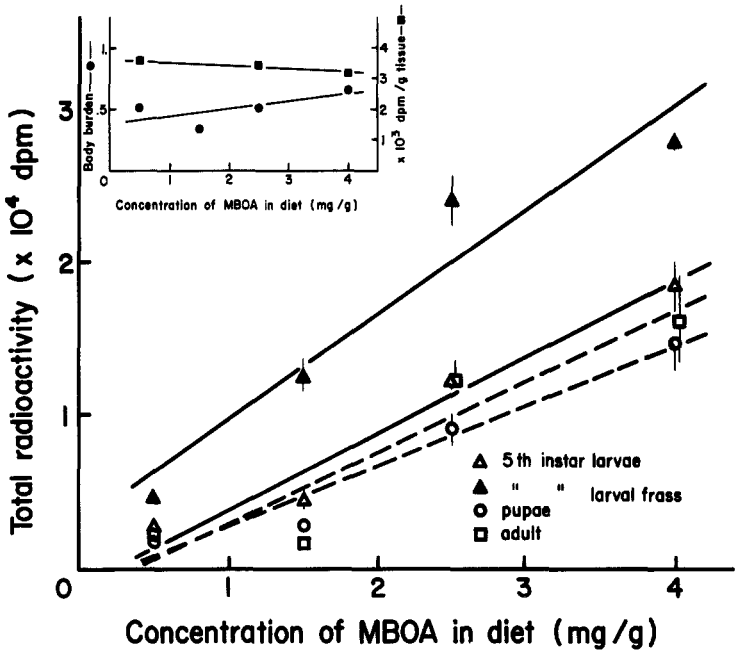


FIG. 3. Dose-related uptake and excretion of labeled compounds by the ECB, *Ostrinia nubilalis*, in feeding trials. Total radioactivity expressed on a per gram basis. The diets contained 1.0×10^4 dpm/mg MBOA. Inset shows the relationship between the concentration of MBOA in the diet and body burden (●) and the relationship between the concentration of MBOA in the diet and the total dpm present in the tissue (■).

a higher content is present in the diet. The body burden values, for the concentrations studied, showed that ECB is capable of excreting enough compound to maintain tissue levels at approximately 50% of the dietary concentration. The accumulation of [^{3}H]compound in the insect tissues increased during larval development (Figure 4) but declined when the insect pupated. The levels from the pupae to the adult further declined due to the transfer into the pupal case.

The [^{3}H]compounds were not sequestered in any specific tissue, as the data from Table 5 indicate. MBOA is a relatively hydrophilic molecule (log *P* value was calculated to be approximately 1.2), and thus it would not tend to accumulate in any specific tissue. The higher levels in the hemolymph indicate that any absorbed MBOA was readily transported to all tissues as well as rapidly excreted.

Since the same level (7 μg/g diet) of tritiated MBOA was used in all concentrations of diet, consumption can be inferred from these results. From Figure 3 (inset), the levels of [^{3}H]compound/g tissue did not change with an increase

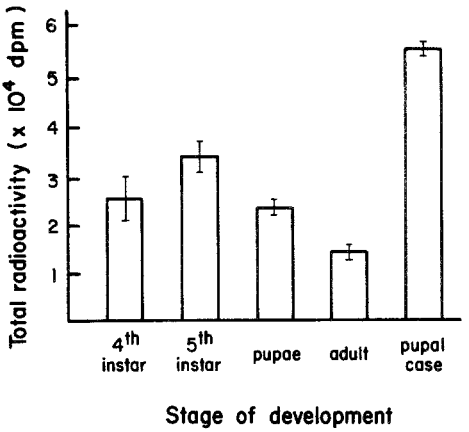


FIG. 4. The level of tritium label (dpm/g tissue) in ECB during its development when fed on a diet of 1.5 mg MBOA/g diet containing 1.19×10^5 dpm/g diet. Bars indicate SE.

in the concentration of MBOA in the diet (Tukey's Studentized test, $P \leq 0.05$), suggesting that all insect groups consumed the same amount of diet. We observed previously (Arnason et al., 1985) that the ECB has a greatly reduced antifeedant response to plant chemicals in artificial diets as compared to leaf disk. This result suggests that the effects observed with MBOA in artificial diets are due to toxic rather than antifeedant properties of this compound.

Some metabolites, as well as the parent compound, were found following TLC and HPLC analysis of insect and frass samples. These are currently being identified and will be reported in a subsequent article.

In fifth-instar larvae, topically applied [^{3}H]MBOA was rapidly absorbed

TABLE 5. DISTRIBUTION OF TOTAL RECOVERED [^{3}H]COMPOUNDS IN FIFTH INSTAR OF ECB *Ostrinia nubilalis*, FROM FEEDING TRIALS (SE in Parentheses)

MBOA conc. (mg/g)	Hemolymph	Gut (with contents)	Fat body	Cuticle
0.5	32.2	22.7	23.8	21.5
	(2.13)	(1.67)	(1.04)	(0.75)
2.5	40.5	16.4	22.6	20.3
	(1.06)	(1.37)	(1.60)	(1.18)
4.0	35.8	21.1	19.5	23.7
	(2.4)	(0.60)	(0.50)	(2.71)

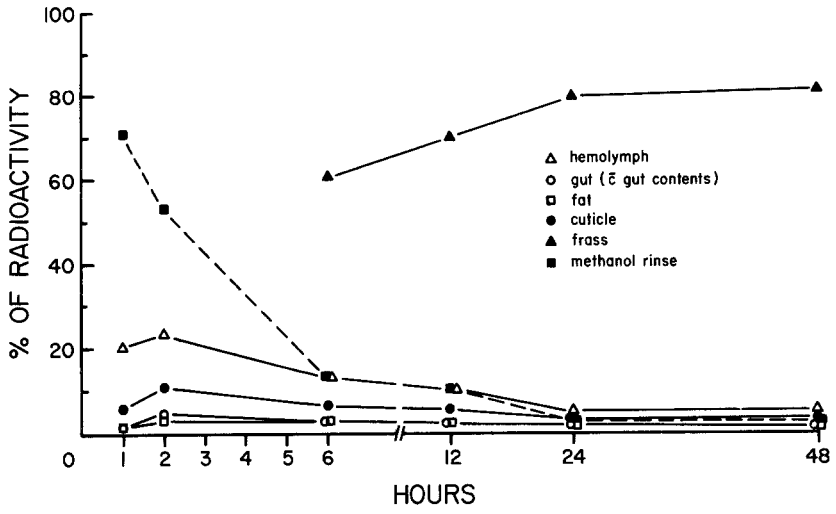


FIG. 5. Toxicokinetics of topically applied [^{3}H]MBOA on the fifth instar of the ECB, *Ostrinia nubilalis*.

through the cuticle as is demonstrated in Figure 5 by a sharp decrease in the percent of radioactivity in the methanol rinse (nonabsorbed compound from the surface of the cuticle). Six hours following application, only 13.8% of the ^{3}H isotope remained on the surface of the cuticle. Although MBOA was readily absorbed, elimination of the compound in the frass by the fifth instar was also rapid, reaching 60% by 6 hr and leveling off at 82% by 24 hr. The percent of ^{3}H isotope in other tissues (gut plus its contents, fat bodies, and cuticle) remained relatively low (the standard error for data points is less than 5%). The rapid rise in levels of ^{3}H isotope observed in the hemolymph during the first 2 hr, followed by its decline over 24 hr, indicates that transport and excretion of the compound occurred relatively quickly.

Previous studies by Olson and O'Brien (1963), and Chio (1976) have demonstrated that compounds having higher water solubility penetrate the insect cuticle faster than more lipophilic substances. The rapid decline in counts in the methanol rinse, which in this case indicated rapid penetration of MBOA, may be accounted for by the low log P value of this compound.

In conclusion, the growth and development data suggest that MBOA does act as a toxic substance, since a variety of effects were observed from mortality of larvae to reduction in fecundity at the F_1 level. The rapid uptake of the compound into the insect and its subsequent excretion may be as much a function of the hydrophilic nature of MBOA, as an adapative mechanism in handling the phytochemical. However, the insect does successfully minimize levels in

tissues other than hemolymph and eliminates a large amount of material in the pupal case. Further experiments with the parent compound, DIMBOA, should give a better indication as to the relative importance of MBOA in the resistance of corn against the ECB.

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HALOACETATE ANALOGS OF PHEROMONES: Effects on Catabolism and Electrophysiology in *Plutella xylostella*

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Abstract—A series of mono-, di-, and trihalogenated acetate analogs of Z11-16:Ac were prepared and examined for electrophysiological activity in antennae of males of the diamondback moth, *Plutella xylostella*. In addition, two potential affinity labels, a diazoacetate (Dza) and a trifluoromethyl ketone (Tfp), were evaluated for EAG activity. The Z11-16:Ac showed the highest activity in EAG assays, followed by the fluorinated acetates, but other haloacetates were essentially inactive. The polar diazoacetate and the trifluoromethyl ketone were also very weak EAG stimulants. The effects of these analogs on the hydrolysis of [^{3}H]Z11-16:Ac to [^{3}H]Z11-16:OH by antennal esterases was also examined. The three fluorinated acetates showed the greatest activity as inhibitors in competition assays, with rank order F_2Ac > F_3Ac > FAc > Ac > Cl_2Ac > ClAc > Dza > Br_2Ac > BrAc > Tfp > I > Cl_3Ac > Br_3Ac > OH. The relative polarities of the haloacetates, as determined by TLC mobility, are in the order mono- > di- > trihalo, but F, Cl, Br, and I all confer similar polarities within a substitution group. Thus, the steric size appears to be the predominant parameter affecting the interactions of the haloacetate analogs with both receptor and catabolic proteins in *P. xylostella* males.

Key Words—Haloacetate, pheromone analog, catabolic protein, EAG, *Plutella xylostella*, Lepidoptera, Yponomeutidae, inhibition.

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INTRODUCTION

One approach to the study of the molecular mechanisms by which pheromones activate transductory processes in insect sensory neurons involves the use of radioligands to characterize pheromone binding and catabolic proteins in male olfactory sensillae (Prestwich, 1987a,b; Prestwich et al., 1987). A second approach involves the synthesis of pheromone analogs and then determination of their relative activities by electrophysiological methods (Roelofs, 1984; Bestmann, 1986; Priesner, 1979). To our knowledge, there are no reports for which both electrophysiological and biochemical studies were conducted with the same set of analogs using the same insect. Moreover, to our surprise, there has never been a systematic evaluation of the relative potency of the mono-, di, and trihalogenated acetate analogs of any acetate component of a pheromone blend.

Thus, we report the synthesis of haloacetate, diazoacetate, and trifluoromethyl ketone analogs of Z11-16:Ac (Figure 1) and their EAG activity for intact male antennae of the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). Furthermore, we present data on the relative potency of the haloacetates as competitive inhibitors of the hydrolysis of [^{3}H]Z11-16:Ac to [^{3}H]Z11-16:OH by soluble antennal esterases.

METHODS AND MATERIALS

Insects

Plutella xylostella were obtained from continuous laboratory cultures from three locations. EAG experiments employed insects reared in Saskatoon (see

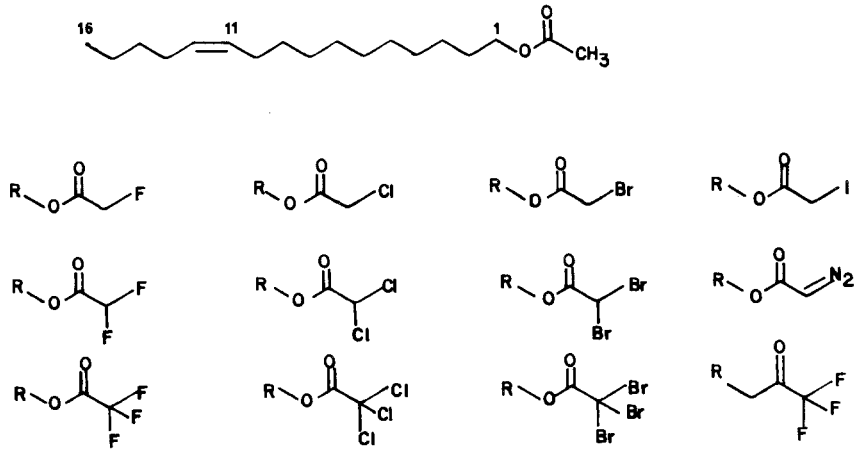

FIG. 1. Analogs of (Z)-11-hexadecenyl acetate prepared and evaluated *in vitro* and *in vivo*.

below); enzyme assays were performed with day 1 or 2 adults emerging from pupae obtained from Dr. B. Tabashnik and N. Cushing, University of Hawaii, and from pupae provided by Dr. K. D. Biever (USDA, Yakima, Washington).

Electroantennograms

EAG responses to synthetic compounds were measured using intact antennae of 2- to 4-day-old male diamondback moths by Drs. E. M. Giblin and E. W. Underhill, National Research Institute–Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada. Responses were elicited according to the procedure of Chisholm et al. (1975) in which a 1-ml "puff" of purified air, passed first over a filter paper disk containing 1 μg of compound, was admixed with an air stream (2.5 liters/min^{-1}) directed at the antennae. Alternating stimuli of Z11–16:Ac and each analog were administered at 15-sec intervals, and each response was corrected for background (mechanical) stimulation. Relative antennal responses were calculated by dividing the analog-elicited response by the mean of the Z11–16:Ac responses (3.59 $\pm$ 0.25 mV) recorded prior to and following that of the analog. The complete set of analogs, including the Z11–16:Ac stimuli, was administered to 11 male moths, the relative response data were analyzed by an analysis of variance test, and significant differences among means (5% level) were determined by Duncan's (1955) multiple-range test.

Tissue Preparations

Day 1 or day 2 emerged adults were sexed during normal photophase under CO_2 narcosis and were held below 8°C prior to processing. Antennae and legs from chilled, narcotized male and female insects were obtained using forceps and collected in a porcelain dish at 4°C. Tissues were homogenized at this temperature in either 10 mM Tris HCl, pH 7.0, or in 76 mM sodium phosphate buffer, pH 7.4, using a hand-operated ground-glass homogenizer. Typically, 100 pairs of antennae or 50 pairs of legs were homogenized in 400 μl of buffer, the homogenate was sonicated at 4°C in an ultrasonic cleaning bath for 10 min (Ferkovich, 1981), the homogenate was rinsed into a 1.5-ml polypropylene tube with two washes with 400 μl buffer, and the suspension was centrifuged (12,000 *g*) for 10 min to give a pellet and a supernatant. The supernatant was divided into portions for a single day's assays and frozen at −80°C.

Enzyme Assays

A typical enzyme assay is described. To each assay tube, first 90 μl of buffer was added, followed by 1.0 μl of an ethanol solution of inhibitor (100-fold the desired final concentration). The tubes were vortexed gently, placed in ice, and the homogenate (10 μl) was added. Using a repeating dispenser, 1.0 μl of 50 μM solution of [^{3}H]Z11–16:Ac (synthesis described below) in ethanol

was added. The substrate solution was prepared for a week's use from a 4 mM stock solution of tritiated pheromone (see below) in 1 : 1 heptane–toluene stored at −80°C to retard autoradiolysis (Prestwich, 1987a). The reaction mixtures were incubated at 26°C for 30 min, the reaction tubes were cooled in an ice bath, 100 μl of ethyl acetate was added, and the tubes were vigorously mixed on a vortex mixer for 10 sec. For each compound, a series of nine concentrations from 0.1 μM to 100 μM was performed, and IC_{50} values were obtained graphically.

A 3-μl aliquot of the organic layer was taken for direct liquid scintillation counting (LSC) to confirm the total extractable radioactivity, and duplicate 3-μl aliquots were spotted onto TLC plates which had been marked into zones and prespotted with unlabeled Z11-16:Ac and Z11-16:OH. The plates were developed in 10% EtOAc–hexane, visualized with iodine, and the alcohol (product) and acetate (substrate) zones were cut into LSC vials containing 4 ml of Scintiverse II (Fisher). Liquid scintillation counting was performed on an LKB RackBeta Model 1217 instrument with automatic external ratio standardization. For many assays, the plates were also analyzed using a Bioscan Imaging Analyzer to reveal the overall distribution of radioactivity in a given zone on the plate. The percentage conversion was calculated as alcohol/(alcohol + acetate), which corrects for errors in sampling and chromatography. The results are most conveniently compared as relative percent hydrolysis, by normalizing a single day's assay results to the uninhibited control with correction for the nonenzymatic hydrolysis in a blank.

Synthesis of Radioligand

[11, 12-$^3H_2$](Z)-11-Hexadecenyl acetate was prepared by reductive tritiation of the corresponding 11-alkyne (see Ding and Prestwich, 1986), prepared in turn by alkylation of the acetylide of 1-tetrahydropyranyloxy-11-dodecyne with 1-bromobutane in the THF/HMPA (Henrick, 1977). Acid hydrolysis of the THP ether (HOAc–THF–H_2O, 4:2:1), acetylation (Ac_2O, pyridine), and flash chromatography provided the necessary starting material.

The tritiation was carried out (May 15, 1985) at the National Tritium Labeling Facility at Lawrence Berkeley Laboratory under the supervision of Dr. Hiromi Morimoto (see Prestwich, 1987a). A flask containing 30.8 mg (0.11 mmol) of alkyne, 2 mg of quinoline, and 5.7 mg of 5% Pd/$BaSO_4$ in 5 ml of methanol was vacuum degassed three times with nitrogen flushing and then stirred at room temperature under 0.9 atmospheres of carrier-free tritium gas for 30 min. The specific activity obtained from this procedure is about 25–30 Ci/mmol, due to vinylic exchange from the protic solvent; this has been minimized in later partial tritiations by using dry THF as solvent to give >45 Ci/mmol specific activities (Prestwich, 1987a). Approximately 40% radiodecom-

position had occurred when the heptane–toluene stored samples were repurified in November 1986. The labeled acetate used for enzyme assays was >98% radiochemically pure as assessed both by LSC and scanning analysis.

Synthesis of Analogs (Figure 2)

Haloacetates. The haloacetates were synthesized from (Z)-11-hexadecen-1-ol prepared in our laboratories according to Vinczer et al. (1984) and shown to possess less than 6% of the *E* isomer. [^{1}H]NMR spectra were obtained in $CDCl_3$ solutions with Varian HFT-80 spectrometer. Chemical shifts are expressed in δ (ppm) relative to δ (TMS) = 0 ppm. IR spectra were obtained on Perkin Elmer 727 spectrometer. Products were purified on a simple column chromatography 30-fold excess silica gel (Merck) by elution with 9:1 hexane–ethyl acetate. Products were dried at 70°C and 0.1 torr for 1 hr. Each haloacetate was repurified by flash chromatography on Woelm 30-63 μm silica prior to use for EAG or esterase inhibition assays. Capillary GC (30 m × 0.25 mm DB-5 fused silica column) analysis indicated that none of the Z11-16:OH was present; however, thermal decomposition of the pure haloesters was observed for the bromo-, iodo-, and diazoacetates. There were two methods used for preparation of haloacetates:

In method A, to a cooled, stirred solution of the haloacetyl chloride (0.166 mmol) in dry ether (1 ml) under N_2 was added a solution of (Z)-11-hexadecen-1-ol (0.1 mmol) and pyridine (0.166 mmol) in ether (2 ml). The reaction mixture was allowed to warm up to room temperature and then worked up by evaporation of solvent to dryness, dissolution of the solid in 5 ml of water, extraction with three portions of ether (5 ml), and drying the combined organic phases with $MgSO_4$.

In method B (Neises and Steglich, 1978), to the solution of (Z)-11-hexadecen-1-ol (0.068 mmol) and α-halogenated acetic acid (0.102 mmol) in dry ether was added 4-(*N*, *N*-dimethylamino) pyridine (0.01 mmol) followed by dicyclohexylcarbodiimide (0.102 mmol). The reaction mixture was stirred at room temperature for 15 hr and then worked up by evaporation to dryness, addition of 2 ml of water, and extraction with three 2-ml portions of hexane. Table 1 summarizes the yields, diagnostic proton NMR resonances, and carbonyl stretching frequencies (IR) for each compound.

(Z)-11-Hexadecenyl-diazoacetate. The procedure used for *A. polyphemus* diazoacetate (Prestwich et al., 1984), *E*6, Z11-16:Dza, was modified to minimize the formation of a sulfinate ester by-product (Corey and Myers, 1984). To a stirred 0°C solution of (Z)-11-hexadecen-1-ol (14.6 mg, 0.061 mmol) in 1 ml of dry methylene chloride under Ar was added glyoxylic acid chloride *p*-toluenesulfonyl hydrazone (29.6 mg, 0.113 mmol) in 1 ml of methylene chloride. Then, 14 μl of *N*, *N*-dimethylaniline was injected, and the mixture was

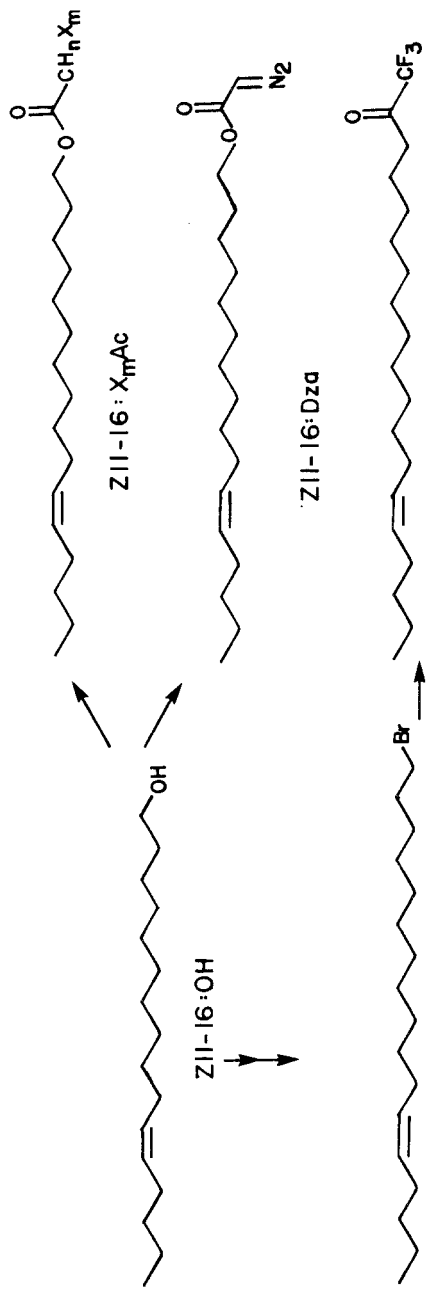

FIG. 2. Synthesis of haloacetates (Z11–16:X_mAc), diazoacetate (Z11–16:Dza), and trifluoromethyl ketone analogs (Z11–16:Tfp) of (Z)-11-hexadecen-1-yl. Reagents and conditions are described in the text.

TABLE 1. SPECTRAL AND CHROMATOGRAPHIC DATA FOR HALOACETATE DERIVATIVES OF Z11-16:OH [R = (Z)-11-hexadecen-1-yl, R' = (Z)-12-heptadecen-1-yl]

Formula	$\delta(X_mC\underline{H}_nCO_2)$	$\delta(X_mCH_nCO_2C\underline{H}_2)$	IR(cm^{-1})	R_f[a]	Method used	Yield (%)[b]
Monohaloacetates						
FCH_2CO_2R	4.53	4.12	1780, 1750	0.33	B	50
$ClCH_2CO_2R$	4.04	4.18	1770, 1755	0.33	A	72
$BrCH_2CO_2R$	3.75	4.09	1750	0.33	B	48
ICH_2CO_2R	3.68	4.13	1740	0.34	B	35
Dihaloacetates						
F_2CHCO_2R	5.88	4.28	1780, 1740	0.38	B	69
Cl_2CHCO_2R	5.93	4.26	1770, 1750	0.40	A	71
Br_2CHCO_2R	5.80	4.25	1780, 1750	0.40	B	40
Trihaloacetates						
CF_3CO_2R		4.33	1780, 1730	0.55	A	49
CCl_3CO_2R		4.35	1760	0.48	A	82
CBr_3CO_2R		4.35	1760	0.45	B	52
Diazoacetate						
N_2CHCO_2R	4.71	4.14	1700	0.25		89
Trifluoromethyl ketone						
CF_3COR'		2.70	1770			29

[a] Polygram TLC plates 40 × 80 mm Machery-Nagel (West Germany), hexane with 10% ethyl acetate. R_f(Z11-16:Ac) = 0.34.
[b] Based on (Z)-11-hexadecen-1-ol.

stirred 15 min. Next, 40 μl of triethylamine was added and stirring was continued for additional 10 min at 0°C and 15 min at room temperature. The reaction mixture was extracted with five 3-ml portions of 10% solution of citric acid in water, dried ($MgSO_4$), and concentrated *in vacuo*. Chromatography on 50-fold excess of silica gel with 9:1 hexane–ethyl acetate afforded the desired product. IR (CCl_4): 3110, 2850–2950, 2112, 1700 cm^{-1}. [^{1}H] NMR: δ 0.89 (br t, J = 6 Hz, 3H, CH_3), 1.99 (m, 4H, $C\underline{H}_2CH$=), 4.14 (t, J = 6 Hz, 2H, $OC\underline{H}_2$), 4.71 (s, 1H, $N_2C\underline{H}CO_2$), 5.34 (m, 2H, $-C\underline{H}$=).

1,1,1-Trifluoro-(Z)-14-nonadecen-2-one (Z11-16:Tfp). A solution of (Z)-12-heptadecenyl magnesium bromide, prepared from (Z)-12-heptadecenyl bromide (0.212 g, 0.667 mmol) and magnesium turnings (0.024g, 1.0 g-at.) in 5 ml of dry ether was cooled (0°), and a solution of *N*-methoxy, *N*-methyl trifluoroacetamide (0.158 g, 1.0 mmol) (Shaw and Tuominen, 1985) in 3 ml of ether was added slowly. The reaction mixture was then stirred for 60 min at room temperature and then decomposed with ice and 3 ml of 5% hydrochloric acid, extracted with three 10-ml portions of hexane–methylene chloride (1:1), and dried ($MgSO_4$). Distillation afforded 63.5 mg of desired product (bath tem-

perature 120–170°C, vacuum 0.1 torr). IR (CCl_4): 3110, 2850–2950, 1770 cm^{-1}. [^{1}H]NMR: δ 0.89 (br t, J = 6 Hz, 3H), 2.05 (m, 4H, $C\underline{H}_2CH=$), 2.70 (m, 2H, $-C\underline{H}_2CO$), 5.34 (m, 2H, $-C\underline{H}=$).

RESULTS AND DISCUSSION

The diamondback moth, *Plutella xylostella* (L.), is a major, worldwide pest of cruciferous oil-seed and vegetable crops. A potent and highly species-specific field attractant has been reported (Chisolm et al., 1983) comprised of Z11-16:Al (70 μg) + Z11-16:Ac (30 μg) + Z11-16:OH (1 μg) + Z9-14:OH (10 μg). Three of the four compounds have been identified as components of the sex pheromone of this pest and include Z11-16:Al, Z11-16:Ac (Chow et al., 1977; Tamaki et al., 1977), and Z11-16:OH (Underhill, unpublished); Z9-14:OH, which has not been reported as a pheromone component, increased male captures and improved lure specificity (Chisholm et al., 1983).

Studies at Stony Brook have been focused on the chemical interactions between catalytic proteins and binding proteins found in insect antennae, with a practical goal being the design of novel mating disruptants (Prestwich, 1986, 1987a, b; Prestwich et al., 1987). In order to achieve this goal, we have prepared chemically reactive analogs for a number of pheromones, and we have sought to evaluate their biological activity in behavioral and electrophysiological assays in parallel with biochemical assays. The biochemical assays employ radiolabeled pheromones at very high specific activity and allow monitoring of binding and catabolism at physiological (nanomolar to micromolar) concentrations (Prestwich, 1987a, b). In this study we wished to explore the interactions of halogenated acetates with acetate-recognizing proteins in male antennae, with the hope that we could correlate analogs active in signal transduction with the recognition of these analogs by catabolic proteins.

Synthesis

The pheromone analogs were prepared from known precursors as shown in Figure 2. In the synthesis of the haloacetates, either the haloacetyl chloride or the haloacetic acid was condensed with (Z)-11-hexadecen-1-ol under standard conditions. The mono-, di-, and trihaloacetates were thus prepared for X = F, Br, and Cl. Only the monoiodoacetate was readily prepared. Comparison of the polarities of these 10 haloacetates revealed a striking correlation between number of halogens and TLC mobility on silica gel, while there was little difference in polarity among the different halogens. Monohaloacetates are more polar than dihaloacetates, and trihaloacetates are the least polar. Surprisingly, the monohaloacetates showed essentially the same mobility as the parent acetate, Z11-16:Ac.

The diazoacetate was prepared using the Corey-Myers modification (Corey

and Myers, 1984) to minimize formation of the unwanted and difficult to remove *p*-toluenesulfinate by using *N*, *N*-dimethylaniline as the first base in the condensation of glyoxylic acid chloride tosylhydrazone with Z11–16:OH and triethylamine as the second base to produce the diazoester. The diazoacetate can, in principle, function as either a photoaffinity label or as an enzyme inhibitor. Precedent for the first role is available from our studies with the sensillar pheromone-binding protein and dendritic membrane proteins of *Antheraea polyphemus*, successfully employing [^{3}H]*E*6, Z11–16:Ac as a photoaffinity label (Prestwich et al., 1987; Prestwich, 1987a, b; Vogt et al., 1988). In contrast, the pheromonal diazoacetate and several geometrical isomers were found to be generally weak competitive inhibitors of the sensillar esterase from the same tissues (Vogt et al., 1985; Prestwich et al., 1986b).

The final analog, trifluoromethyl ketone Z11–16:Tfp (where Tfp is shorthand for 1,1,1-trifluoropropanone), was prepared as a steric and electronic mimic of the enzyme-bound intermediate in ester hydrolysis (Hammock et al., 1982) (see below). These long-chain aliphatic analogs are not readily prepared in high yields by conventional methods. All methods using trifluoroacetic acid, trifluoroacetic anhydride, lithium trifluoroacetate, and magnesium trifluoroacetate as the acceptor for the Grignard component gave complex mixtures with large proportions of the alkene (protonolysis) and the tertiary alcohol (second addition of Grignard to regenerated carbonyl) products. The cleanest and highest-yield method employed the *N*-methoxy, *N*-methyl trifluoromethylacetamide as the acceptor (Shaw and Tuominen, 1985) and is strongly recommended for future work on aliphatic trifluoromethyl ketones where the aliphatic moiety is precious and functionalized.

Electrophysiology

The responses of antennae of male *P. xylostella* to the haloacetates, the diazoacetate, and the trifluoromethyl ketone were determined relative to the response to the natural Z11–16:Ac component. Alternating stimuli of Z11–16:Ac and each analog of the complete set of 12 were administered at 15-sec intervals to 11 replicated single antennal preparations. The average relative responses, obtained from the ratios of blank-corrected millivolt response for the analog to millivolt response for the standard (Z11–16:Ac), are shown in Figure 3. The fluorinated acetates possess potent EAG activity, with the monofluoroacetate response significantly lower than Z11–16:Ac but significantly higher than the di- and trifluoroacetate responses. While the trichloroacetate showed some activity, the remaining chlorinated, brominated, and iodinated acetates produced much lower responses (or no response in most insects tested). Similarly, the diazoacetate showed negligible activity, and the fluoroketone showed low activity similar to the monochloroacetate.

While this is the first examination of a complete series of haloacetates for

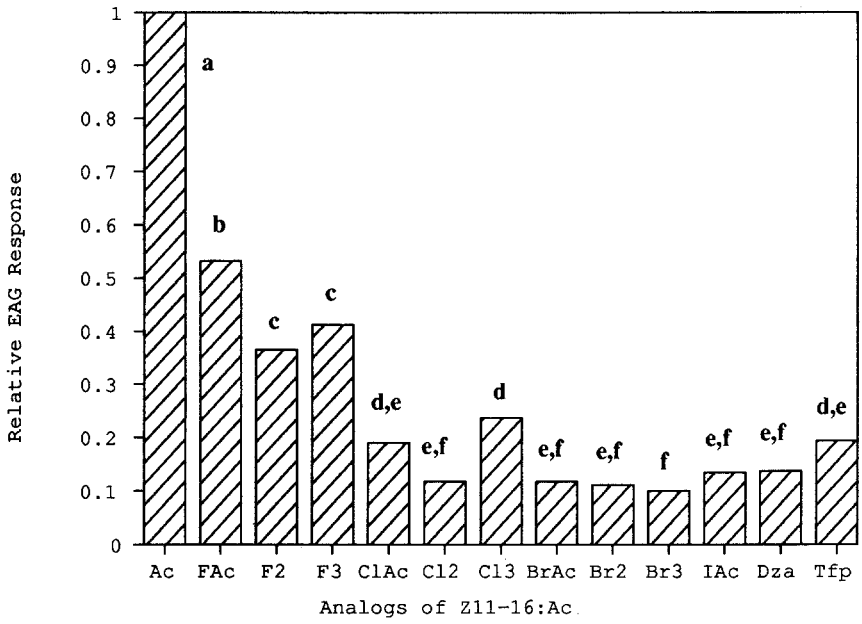

FIG. 3. Average electroantennogram responses to haloacetates and two other analogs of Z11–16 : Ac by antennae of male *Plutella xylostella*. Bars represent the mean responses of 11 separate trials, and bars with different letters are significantly different at $P < 0.05$. Numerical relative response values (±SE): FAc, 0.53 (0.05); F_2Ac, 0.36 (0.04); F_3Ac 0.41 (0.04); ClAc, 0.19 (0.04); Cl_2Ac, 0.12 (0.03); Cl_3Ac, 0.24 (0.03); BrAc, 0.12 (0.03); Br_2Ac 0.11 (0.04); Br_3Ac, 0.10 (0.03); IAc, 0.13 (0.03); Dza, 0.14 (0.03); Tfp, 0.19 (0.02).

electrophysiological activity, there are several reports of selected acetate analogs in other species. Baker et al. (1981) and Albans et al. (1984) have patented several reactive "antipheromones" related to Z9–14 : Ac and Z9–16 : Al for use against *Heliothis virescens* (Figure 4A). The *N*-methylcarbamate analog (Z9–14 : Nmc) reduced oviposition by this insect on cotton in field trials. In lab tests using preexposure to 100 mg (!) of test compound, the carbamate compound, Z9–14 : F_3Ac, Z9–14 : Cl_3Ac, and (Z)-12-heptadecan-2-one (replacement of oxygen by methylene in Z9–14 : Ac) all acted as competitive antipheromones for *H. virescens*, producing reversible inhibition of behavioral (flight activation) responses. Unfortunately, EAG assays were not reported by these workers, so it is unclear whether the Z9 analogs described mimic Z9–14 : Ac, Z9–14 : Al, Z11–16 : Ac, or Z11–16 : Al. In contrast, they found that a variety of saturated analogs, alkenyl regioisomers, shorter or longer unsaturated ketones, mono-

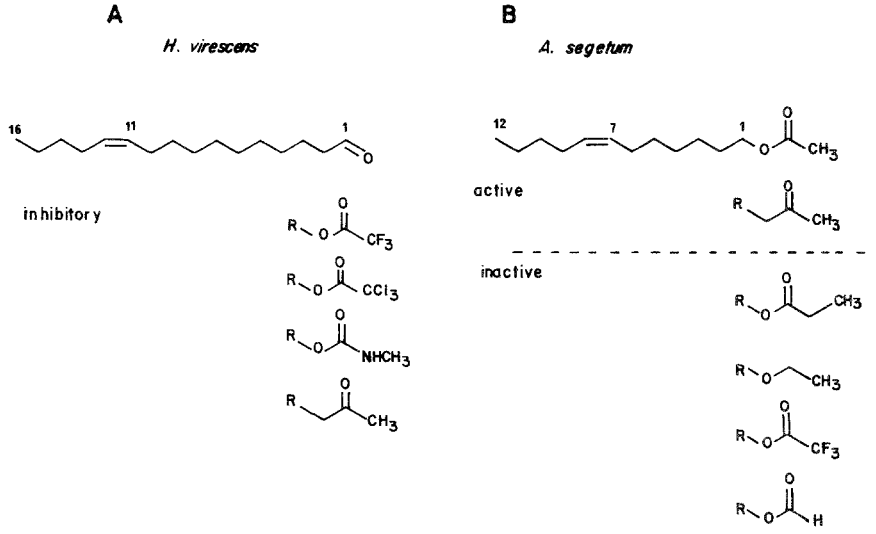

FIG. 4. (A) Structures of antipheromones for *H. virescens* based on Z9–14:Al (Prestwich et al., 1986a) and Z9–14:Ac (Albans et al., 1984). (B) Structural analogs of Z7–12:Ac, the pheromone of *A. segetum* (Liljefors et al., 1984).

chloroacetates, alcohols, alkenes, acrylate esters, dimethylcarbamates, and methyl carbonates were essentially inactive as antipheromones. In a related study, the acyl fluoride Z9–14:Acf produced unusual aphrodisiac-like responses in male *H. virescens* (Prestwich et al., 1986a).

The *N*-methylcarbamate could, in principle, be an irreversible inhibitor of the esterase by carbamoylation of the active site. I. Ujvary and M. Toth (personal communication, cited in Prestwich, 1987a) prepared the Z11–16:Nmc and saturated C_8, C_{12}, and C_{16} *N*-methylcarbamates. In preliminary EAG assays with male *Mamestra brassicae* antennae, the Z11–16:Nmc elicited a response slightly less than that of Z11–16:Ac, while the saturated carbamates produced no response. Furthermore, subsequent responses to Z11–16:Ac were unaffected by a preexposure to the carbamate analog. We found this compound is only a weak inhibitor of the antennal esterase of *P. xylostella* (L. Streinz, I. Ujvary, and G. D. Prestwich, unpublished results), in the same range as the Z11–16:Tfp analog.

The responses of sensory cells from male antennae of the turnip moth *Agrotis segetum* (Noctuidae) were measured during stimulation with analogs of Z7–12:Ac (Figure 4B), the major pheromone component of this species (Liljefors et al., 1984). Replacement of the alcohol oxygen with a methylene to

obtain the methyl ketone afforded a weakly active stimulus, as in the higher homolog reported by Albans et al. (1984) for *H. virescens*. In contrast, the propionate, the ethyl ether (methylene replaces carbonyl), the trifluoroacetate, and the formate analogs of Z7–12 : Ac were all essentially inactive. All portions of the acetate group, electronic and steric, are apparently important to pheromonal activity for this moth.

Finally, the diazoacetate *E*6, Z11–16 : Dza was an excellent stimulant for single-cell responses of the *E*6, Z11–16 : Ac sensillae of the wild silkmoth, *Antheraea polyphemus* and its sister species, *A. perneyi* (Saturnidae) (Ganjian et al., 1978; Kaissling, 1986). About 10% of the normal acetate response was produced from equal quantities in filter paper, and in view of the lower volatility of the polar diazoacetate, the activity is likely to be underestimated. In addition, R. G. Vogt and G. D. Prestwich have determined that the diazoacetate binds effectively to both soluble and membrane associated proteins in *A. polyphemus* male sensillar homogenates (Vogt et al., 1988; Prestwich 1987a, b; Prestwich et al., 1987).

The importance of the electronic effects found for *A. segetum* deserves further comment. The straight-chain olefinic acetates are attractants of numerous lepidopterous species (cf. Steck et al., 1982; Tamaki, 1985). The models used to explain pheromone–receptor interactions focus on the acetate functionality, the olefin, and the terminal methyl group. Binding may occur by a static three-point mechanism (Kafka and Neuwirth, 1975) or by a dynamic, stepwise association invoked by Bestmann (Bestmann, 1986; Bestmann and Vostrowsky, 1982), or by other mechanisms.

Liljefors et al. (1984) calculated van der Waals surfaces of methyl acetate, formate, propionate, trifluoroacetate, as well as the ethyl ether and the ketone analog 2-butanone in a homogeneous medium. The trifluoroacetate, methyl ketone, and ethyl ether are quite similar in size and shape to the acetate. On the other hand, while the dipole moments of the methyl ketone and acetate are of similar direction and magnitude, the trifluoroacetate dipole vector is orthogonal in this simple example. The Swedish group inferred that the dipole moment of the acetate functionality may be important in the receptor interactions. However, these interactions may be formed by additional orientational effects due to the lipid bilayer membrane in which the putative receptor protein(s) reside.

For *P. xylostella*, this solution model fails to explain the high EAG activity of all three fluorinated acetate analogs and the low activity of other halogenated acetates of similar polarities. These analogs have similar molecular geometries but very different dipole moments. Thus, it appears that for *P. xylostella*, the steric size of the halogenated acetate group dominates over compound polarity in determining the summed electroantennogram responses. Based on our data, we cannot exclude the possibility that the haloacetate analogs are stimulating sensory cells other than the Z11–16 : Ac-specific cell(s).

Esterase Activity

The removal of an olfactory stimulus is crucial to the normal functioning of the insect pheromone-sensing system (Ferkovich, 1981). Various models have been suggested to account for the translocation of the stimulus from the air to the dendritic membrane of the responsive neuron (Vogt and Riddiford, 1986b; Kaissling, 1986; reviewed by Vogt, 1987), and approaches to understanding the electrical transduction have been made (de Kramer and Hemberger, 1987; Kaissling, 1986). It has become clear that the biochemical properties, i.e., maximal velocity, substrate specificity, and *in vivo* concentrations, of the antenna-specific pheromone processing enzymes fully account for their role as the primary modulators of stimulus concentration in the lumen of the receptor hair (Vogt, 1987; Vogt et al., 1985). This is now well documented for acetate esterases and aldehyde dehydrogenases in *Choristoneura fumiferana* (Morse and Meighen, 1984, 1986, 1987; Lonergan, 1986), for aldehyde dehydrogenases in *Heliothis virescens* (Ding and Prestwich, 1986; Prestwich, 1987a,b), for acetate esterases in *Antheraea polyphemus* (Prestwich et al., 1986b; Vogt et al., 1985; Vogt, 1987; Klein, 1987), for acetate esterases in *Trichoplusia ni* (Ferkovich, 1981; Ferkovich et al., 1982a, b), and for epoxide hydrolases in *Lymantria dispar* (Prestwich et al., 1987; Prestwich, 1987a, b). Other tissues of both sexes possess enzymatic activity for pheromone degradation in these insects as well (Ferkovich et al., 1982a, b; Vogt and Riddiford, 1986a), and this presumably reflects the need to prevent becoming one's own slow-release dispenser.

Thus, we wished to examine the effects of these haloacetate analogs on the hydrolysis of [^{3}H]Z11–16:Ac by *P. xylostella* male antennae (Figure 5). Indeed, we have demonstrated electrophoretically unique esterase bands in *P. xylostella* antennal tissues, and we have shown tissue specificity for hydrolysis of [^{3}H]Z11–16:Ac by tissues (antennae, legs, and abdominal tips) of both sexes (G. D. Prestwich, R. G. Vogt, and L. Streinz, unpublished results). Biochemical characterization of the leg and antennal enzymes will be reported in full elsewhere. For this study, we used the esterase assay to measure the ability of the haloacetates and the other two analogs to reduce the rate of hydrolysis of the labeled pheromone *in vitro*.

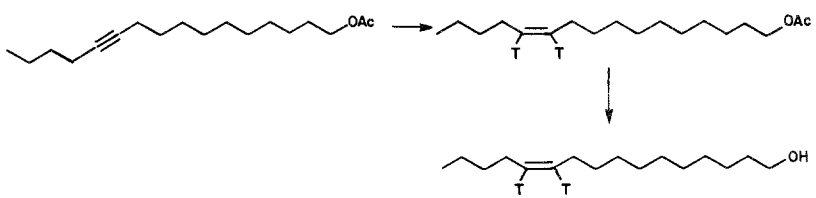

FIG. 5. Radiosynthesis and enzymatic hydrolysis of [11, 12-$^3H_2$]Z11–16:Ac.

Antennae were removed from anesthetized male moths one or two days posteclosion, homogenized in an ice bath, sonicated for 10 min in an ultrasonic bath to more thoroughly fracture the short sensory hairs (Ferkovich, 1981), and then centrifuged to remove cuticular and cellular debris. The antennae obtained in this fashion contain significant quantities of hemolymph proteins, but this is the only practical approach to the initial study of antennal proteins. In insects with plumose antennae adorned with abundant long sensory hairs, purified sensory hair preparations can be obtained in which blood proteins are essentially absent (Klein and Keil, 1984; Vogt et al., 1985). The antennae of *P. xylostella* males are also rich in cuticle and annular scales, and the esterase activity remaining in the low-speed pellet is due to scale esterases associated with these tissues (Vogt and Riddiford, 1986a; Ferkovich, 1982; Klein, 1987). A dilution series is performed on the supernatant, and an aliquot of this solution is used such that 30–50% hydrolysis occurs at 26°C with 0.5 μm [^{3}H]Z11-16:Ac. For male antennae, this level of activity was obtained with about 1.6 antennae/100 μl assay; each antenna contained 1.2 μg of soluble total protein. The assay mixture contains up to 4% ethanol (used to deliver competitors and inhibitors), but we have not observed any significant diminution of activity compared to controls with less than 0.5% ethanol. The enzymic hydrolysis is not affected by preincubation with 0.5 mM diisopropylfluorophosphonate, but boiling the enzyme solution for 5 min prior to addition of substrate completely destroys hydrolytic activity. Recently, we have kinetically resolved two distinct enzymes activities (slow, tight-binding and fast, loose-binding) in antennal tissues (G.D. Prestwich and L. Streinz, unpublished results).

In contrast to the potent inhibition of the sensillar esterase of *A. polyphemus* by 1,1,1-trifluoro-2-tetradecanone (Vogt et al., 1985), the trifluoromethyl ketone (Z11-16:Tfp), which should sterically mimic Z11-16:Ac, shows only very modest inhibition. For convenience in shorthand notation and to emphasize the isosteric substitutions involved, we introduce Tfp, or 1,1,1-trifluoropropyl, for the $CH_2C(O)CF_3$ analog to $OC(O)CH_3$. These compounds are so-called "transition state analogs" for ester hydrolysis (cf. Hammock et al., 1982); in reality, those which are highly active esterase inhibitors form stable tetrahedral hemiketals with serine hydroxyl groups, and these stable adducts are steric and electronic mimics of the tetrahedral intermediate in normal ester hydrolysis. Figure 6 illustrates this analogy for the present example. We observed that the trifluoroketones react rapidly with Tris buffer (a primary amine) and slowly with hydroxylic solvents to produce adducts inactive as esterase inhibitors.

Figure 7 illustrates the effects of the haloacetates, diazoacetate, and fluoroketone on the hydrolysis of 0.5 μM [^{3}H]Z11-16:Ac. In this assay, we have essentially measured competition for both binding and hydrolysis. Binding involves both the hydrophobic chain and the ester functionality. Hydrolysis involves only the ester group and can be susceptible to electronic effects (e.g.,

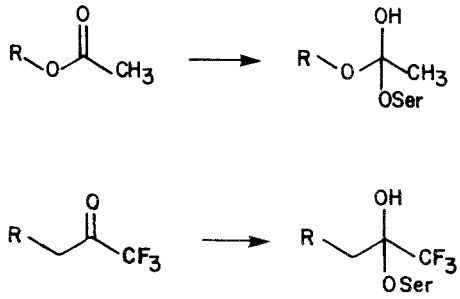

FIG. 6. Mimicry proposed for the tetrahedral intermediate involved in hydrolysis of Z11-16:Ac and the stable tetrahedral hemihydrate of a trifluoromethyl ketone which corresponds sterically to the pheromone.

the basicity of the leaving haloacetate anion) as well as steric (size) effects. As we have demonstrated in *A. polyphemus*, competition for binding can slow hydrolysis even when the steric parameters of the ester preclude its hydrolytic removal in the active site (Vogt et al., 1985; Prestwich et al., 1986b).

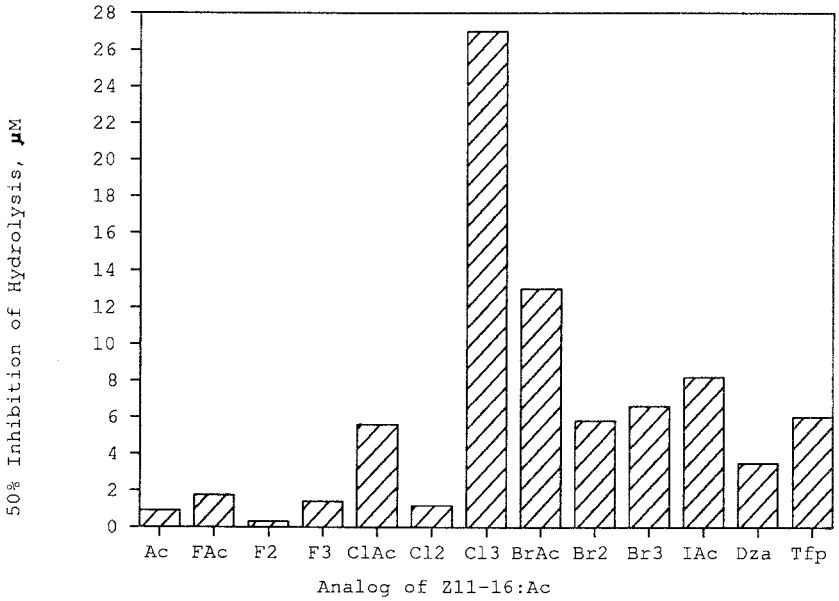

FIG. 7. Inhibition of male antennal esterase activity by analogs of Z11-16:Ac. Bars show the concentrations required for 50% reduction in hydrolysis of a 0.5 μM solution of labeled Z11-16:Ac by the supernatant of a homogenate containing 1.6 antennal equivalents/100 μl assay.

Addition of unlabeled Z11-16:Ac reduces hydrolysis effectively with an apparent I_{50} of 1 μM. [This turns out to be quite close to the K_m of the high affinity enzyme (Prestwich and Streinz, unpublished results).] The product, Z11-16:OH is 20–50 times less potent, and the saturated 16:Ac is less potent by a factor of 3 to 5. Of the halogenated acetates, the fluorinated acetates all showed strong competitive inhibition, with the difluoroacetate showing the greatest reduction in substrate hydrolysis. The diazoacetate, iodoacetate, bromoacetates, and trichloroacetates were poor inhibitors in this assay. The mono- and dichloroacetates were modest inhibitors. The enzyme kinetics will be described in detail elsewhere. We interpret these results to suggest that there is a limiting steric size which can be accommodated by the hydrolytic site. When an analog has too bulky an ester group, then only the weaker competitive hydrophobic effects are possible. Apparently, the maximum van der Waals radius tolerated by this hydrolytic site corresponds to two chlorines on the acetate methyl.

CONCLUSION

It is clear that the steric size of a haloacetate analog is of primary importance in both recognition by the postulated dendritic receptor protein involved in transduction and by the pheromone-degrading esterase responsible for maintaining a low stimulus noise level in the sensilla. In both assays, the differences in relative polarity of the fluorinated acetates and relative basicity of the fluorinated acetate anion were overshadowed by the difference in size relative to the chlorinated, brominated, and halogenated acetates regardless of polarity. It is gratifying to observe this agreement between *in vitro* and *in vivo* assays with *P. xylostella*.

Note Added in Proof—Electrophoretic studies of antennal esterases of *Plutella xylostella* have also been reported by Maa and Lin (1985) [MAA, W.C.-J. and LIN, Y.-M. 1985. Esterase of diamondback moth (*Plutella xylostella* L.). II. The antennal carboxylesterase of adult male with reference on male response to synthetic female sex pheromone. *Bull. Inst. Zool. Acad. Sinica* 24:165-176]. Also, an elegant molecular mechanics approach to pheromone-receptor interactions has been reported by the Liljefors group [LILJEFORS, T., BENGTSSON, M. and HANSSON, B.S. (1987) Effects of double bond configuration on interaction between a moth sex pheromone component and its receptor: A receptor-interaction model based on molecular mechanics. *J. Chem. Ecol.* 13:2023–2040.

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COMPOSITION AND ROLE OF VOLATILE SUBSTANCES IN ATMOSPHERE SURROUNDING TWO GREGARIOUS LOCUSTS, *Locusta migratoria* AND *Schistocerca gregaria*

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Abstract—Volatile substances in the atmosphere surrounding gregarious locusts *Schistocerca gregaria* and *Locusta migratoria* were captured and investigated by combined gas chromatography–mass spectrometry. Three aromatic derivates have been identified: phenol, guaiacol, and veratrole. Their relative percentages differ for different ages and species. Behavioral tests show that essentially phenol, guaiacol, and the mixture of the three products tend to increase the aggregation behavior in both species and thus act as "cohesion pheromones."

Key Words—Locusts, Orthoptera, Locustidae, *Schistocerca gregaria*, *Locusta migratoria*, pheromones, phenol, guaiacol, veratrole, aggregation, gregarious locusts.

INTRODUCTION

The great predatory locusts are well known for their ability to change their kind of life from isolated or "solitary" to crowded or "gregarious" phases (Uvarov, 1921), which differ in all their morphological, behavioral, and physiological characteristics. Visual or tactile stimuli play a part in these changes (Chauvin, 1941; Ellis, 1959). Chemical stimuli can also come into play (Loher, 1960; Norris, 1970; Gillett, 1975), but they have not been clearly identified until now.

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A gregarization pheromone, which seems to turn solitary locusts into gregarious ones, was postulated by Nolte (1968) as a result of the study of some pigmentary and morphometric tests, and in particular, an important new analysis was made of the frequency of chiasmata in adult males (Nolte et al., 1970), which seems to increase with gregarization. Nolte et al. (1973) also isolated ethyl guaiacol, called "locustol," from extracts of 1 kg of feces from a gregarious population. These investigators concluded that other substances may exist, and in a complementary paper locustol was compared with chemical analoges (Nolte, 1976). Later Dearn (1974) showed that the chiasmata test is not reliable, and thus doubts were cast upon the efficacy of ethyl guaiacol in the natural situation of gregarious populations. It is difficult to know if a substance from the feces is effective in the surrounding atmosphere of living locusts. Gillett (1975) agrees with the existence of a chemical volatile factor that causes *Schistocerca gregaria* individuals to turn into gregarious insects in some of their characteristics. However, the breeding conditions were not natural (no nycthemeral changes of light and temperature) and thus may have given artificial results. Moreover, Gillett (1983) described later the occurrence in young adult populations of an unknown atmospheric factor from adult feces that caused the insect to change to the solitary type.

In this study, we have analyzed the chemical composition of the environmental atmosphere in gregarious populations with gas chromatography and mass spectrometry, looking for the behavioral effect of the substances isolated from this atmosphere on gregarious insects.

METHODS AND MATERIALS

Strains and Insects. Two species were used in this work: the desert locust, *Schistocerca gregaria*, and the migratory locust, *Locusta migratoria*. For the latter, we principally used the subspecies *migratorioides* from Africa, but a comparison was also made with the subspecies *cinerascens* from Sardinia. All were bred during their gregarious phase in cages 60 × 60 × 60 cm that contained 500–200 individuals of both sexes. They were reared in a special room with a nycthemeral temperature and light cycle of 12 : 12 hr light–dark and 33–23 °C; they received fresh corn shoots and bran daily.

Trapping the Atmosphere. To collect the volatile products of the atmosphere contained in a cage as described above, a current of air was sucked out by a vacuum pump through a burrow in one lateral part of the cage at a rate of 10 liters/hr. A glass container, maintained in a Dewar flask of crushed ice, was connected in one direction through the burrow into the cage, and in the other to the vacuum pump, so that the volatile products were condensed in the con-

tainer with the atmospheric water vapor. (If the atmosphere was too dry, we inserted a damp piece of cotton into the cage). The products were collected during the photophase over a period of 50 hr. Between two photoperiods, the condensate was put in a freezer at −15°C. At the end of the collection period, the condensate (10–20 ml) was immediately extracted three times with 15 ml of Freon 11 which was previously distilled. After drying with $MgSO_4$, the Freon was distilled by slight heating at 25°C. Evaporation was completed in a small fine tube into which we introduced 5–15 μl of pure hexane. The solution obtained was kept in a freezer, ready to be analyzed.

Analyses. The analyses were carried out by gas chromatography (GC) and combined gas chromatography–mass spectrometry (GC-MS). Mass spectra were obtained on a Nermag R10-10 spectrometer associated with a PDP8 calculator (Digital Equipment Instrument) and coupled to a Girdel 31 chromatograph with a splitless injector. Fractions to be analyzed were temperature programmed from 40°C to 300°C, at 4°C/min, on a capillary column (25 m × 0.32 mm) coated with CpSil 5 CB Chrompack. The integrations of chromatographic peaks were made with a Hewlett-Packard 3390A integrator associated with a Varian 3700 chromatograph which was equipped with a column already described, programmed from 40°C to 300°C at 4°C/min with a delivery of 18 cm/sec, and an on column injector.

Behavioral Tests. The tests were carried out on both sexes of fifth-instar nymphs, young immature adults two days after ecdysis, copulating mature adults, and mature adults at the time of laying the eggpods, for each strain in the gregarious state. For this first study, we avoided the use of the solitary phase. The work was based on an olfactometric method similar to that described by Siddiqi and Khan (1982).

Four 1-liter compartments (numbered 1, 2, 3 and 4) were arranged in the form of a cross joined by a middle compartment, "M," with four tunnels. An equal current of air was passed through lateral compartments 2, 3, and 4 and drawn into number 1 by a vacuum pump with delivery of 85 liters/hr, so that the middle space, "M," received air coming from the three lateral ones. The air arrived from outside (the campus is in the middle of large gardens in the country) and was preheated to the same temperature (33°C) as the apparatus. The apparatus was made of glass to allow observation of the insects and proper cleaning between each experiment. Correct functioning was tested by many preliminary assays using the attractive smell of fresh corn shoots with fasting animals. We found that the best results were obtained with compartment 1 as the starting point for the animals, and the smell in one lateral compartment, number 4, so that the locusts could also go to number 3 (straight on) or 2 (90° angle) with pure air. The light was uniform. In assays without any smell, we confirmed that there were no subtle factors which gave asymmetric behavior.

Only compartment 4 showed a few animals in controls because of the presence of white filter paper which we had to introduce to carry out the experiments. Since this phenomenon was consistent, we consider it had no influence on our calculations and conclusions. Numeric observations were made every 3 min over a period of 15 min after the release of 10 fifth-instar larvae or five adults. We did not work with single insects because the response is too long and we wanted to study interferences between identified substances and gregarism in conditions close to the natural ones.

Each substance detected by analyses was tested: hexane solutions of 10^{-3} M were prepared, either pure or as a mixture with percentages given by the previous analyses corresponding to the ages of the animals tested. Whenever possible, we took the results of atmosphere analyses as the basis for calculation of mixture solutions (see Results). A 5-μl solution was deposited on a filter paper on which a drop of 10 μl of paraffin oil had been previously deposited to moderate the evaporation of the very volatile components involved. Controls were made with filter paper where hexane and oil had been deposited without the phenols. All of this was done in a separate room. The paper was quickly put into compartment 4, which was taken into the olfactory room and placed in the apparatus. Compartment 1 with the locusts to be tested was immediately put in place (time = 0).

To interpret the results, preliminary experiments were done with a special compartment 4 where crowded locusts (10–15) were kept contained by a special partial glass stopper which permitted the air with locust odor to pass normally. It was observed that insects tested in these experimental conditions always tend to assemble in the middle M, so we decided to study statistically the general distribution by the χ^2 test and look particularly at the number of individuals in M.

For these tests, we used only the strains *Schistocerca gregaria* and *Locusta migratoria migratorioides* (because the subspecies *cinerascens* was found to be too poor in the chemical analyses) in a first series with fifth-instar larvae and in a second series, only a group of *Locusta migratoria migratorioides* that had been followed from fifth instar to mature adults in order to see if any change could be detected for different ages of the same animals.

RESULTS

Analyses. All chromatograms show four major substances (Figure 1), which are the same for both species but which differ in each case in their relative quantities. Three of the four products are identified by mass spectrometry; they

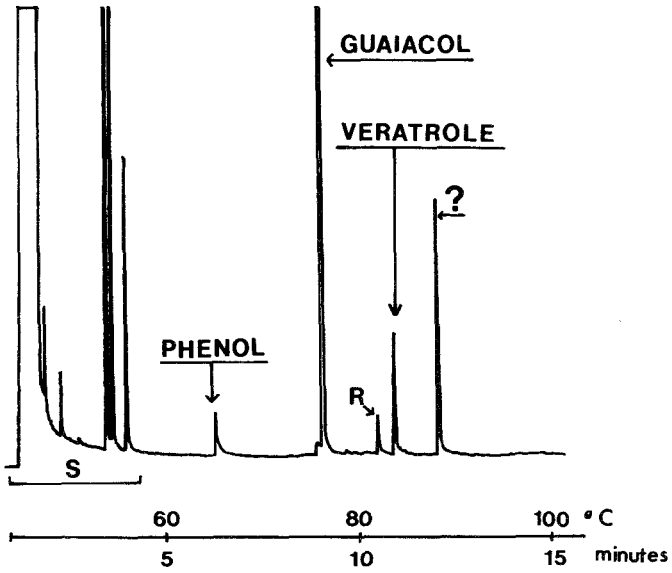
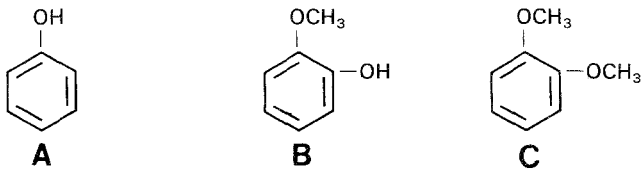


FIG. 1. An example of the chromatogram obtained with the mixture of substances in a capturing liquid: *Schistocerca gregaria* young adult. Three substances are identified: phenol, guaiacol, and veratrole, the fourth (?) is unknown. R = product used as reference (*o*-methyl acetophenone). S = solvent and impurities.

are all aromatic derivatives (Scheme 1): phenol (A), guaiacol (B), and veratrole (C). We are not yet able to identify the product corresponding to the fourth peak.

The mass spectra of these products agree with data in previous studies, *m/z* (intensity): phenol 94 (100), 66 (56), 65 (45), 39 (32), 55 (14), 50 (11); guaiacol 109 (100), 124 (74), 81 (55), 39 (21), 53 (22), 52 and 51 (17); veratrole 138 (100), 95 (50), 77 (44.2), 123 (36), 52 (32.7) (Barnes and Occlowitz, 1963; Cornut and Massot, 1966). Identity and retention times are in agreement



SCHEME 1.

with those of the three commercial products assayed and used in behavioral tests.

Chromatograms and mass spectra are obtained for the atmosphere trapped for each of the three strains described, and for each strain, the four described states of the same animals which have been followed. Results are presented in Table 1. For all insects, the percentages of each products are calculated by integration of peak surfaces obtained in gas chromatography except in the case of fifth-instar larvae of *Locusta migratoria cinerascens*, where the quantities of products are smaller, so that we obtain the percentages by integration of the ionic current areas in mass spectrometry. In two cases, the young adults of *Locusta migratoria migratorioides* and *cinerascens*, we cannot do any calculation because of the small amounts of material trapped. The quantities of trapped products in all other cases are only a few micrograms, but sufficient for experiments.

We see that the percentages differ from one sample to another, but in the three strains guaiacol is always the major product. Both minor products, phenol and veratrole, vary greatly from one strain to the other and also among the different ages of the same animals. *Schistocerca gregaria* give the best results

TABLE 1. PRODUCTS DETECTED IN CAPTURING MIXTURES FOR FOUR DIFFERENT AGES OF *Schistocerca gregaria*, AND *Locusta migratoria* (subspecies *migratorioides* and *cinerascens*).[a]

Insects	Phenol (%)	Guaiacol (%)	Veratrole (%)	Unknown product (%)
Schistocerca gregaria				
Fifth-instar larvae	3.70	94.40	1.95	Trace
Young adults	4.45	71.00	8.50	16.00
Copulating adults	5.05	56.50	22.80	15.55
Laying adults	8.40	62.00	24.70	4.90
Locusta migratoria migratorioides				
Fifth-instar larvae	9.85	63.60	3.50	23.00
Young adults		Traces of products		
Copulating adults	5.50	86.00	4.10	4.40
Laying adults	5.30	84.80	9.70	Tr.
Locusta migratoria cinerascens				
Fifth-instar larvae	13.10	65.00	2.00	19.80
Young adults		Traces of products		
Copulating adults	5.60	86.20	4.60	3.50
Laying adults	5.00	80.60	7.60	6.80

[a] Integration of surfaces of chromatographic peaks.

where all ages provide enough substance for easy calculation. It seems that the young immature adults always correspond to a lower concentration of volatile products. In the same species, *Locusta migratoria*, both subspecies present approximately the same atmosphere composition, although that of *cinerascens* seems to be less concentrated. The percentages of phenol and veratrole increase between larvae and laying adults for *Schistocerca*. In contrast, in *Locusta*, phenol decreases and veratrole increases. It is difficult to discuss the importance of the unidentified product.

Behavioral Tests. As described before, it was necessary to know the molecular concentrations of the solutions. Response coefficients of phenol, guaiacol, and veratrole in GC, based on normal solutions of them, were calculated. The reference was veratrole. The relative areas calculated from an equimolecular mixture of the three identified products are 1 for veratrole and 0.7 for guaiacol and phenol; thus it is possible to deduce the molar concentrations from the respective areas of the products in the chromatograms (Table 2) for both strains and each age. Since we know the percentages of molar concentrations of the mixtures (Table 2), it becomes easy to prepare suitably diluted solutions (Table 3).

The distribution of larvae in each compartment is presented in Table 4. The most marked response was an aggregation in the middle, M, where the products or a mixture of them statistically affect the distribution of the insects in the different experiments. However, the responses differ basically between *Schistocerca* and *Locusta*: guaiacol is very active on *Schistocerca* and not on *Locusta*. Veratrole alone has no effect under our conditions. Phenol is active in

TABLE 2. MOLAR CONCENTRATIONS OF THREE IDENTIFIED PRODUCTS DETECTED IN CAPTURING MIXTURES FOR DIFFERENT AGES OF *Schistocerca gregaria* AND *Locusta migratoria migratorioides*[a]

Insects	Molar concentration (%)		
	Phenol	Guaiacol	Veratrole
Schistocerca gregaria			
Fifth-instar larvae	3.70	94.90	1.40
Young adults	5.45	87.20	7.30
Copulating adults	6.50	72.90	20.60
Laying adults	9.50	70.75	19.70
Locusta migratoria migratorioides			
Fifth-instar larvae	13.00	83.80	3.20
Copulating adults	5.90	91.05	3.05
Laying adults	5.50	87.50	7.00

[a] For calculations, see the text.

TABLE 3. CONCENTRATIONS OF SOLUTIONS USED FOR BEHAVIORAL TESTS (PURE IDENTIFIED PRODUCTS AND THEIR MIXTURE)

Solutions for behavioral tests	Concentration (10^{-3} M)				
	Phenol		Guaiacol		Veratrole
1. 3 initial solutions (pure products)	25.3		20.8		15.7
2. *Locusta migratoria m.* (fifth-instar larvae)	1.3	+	8.3	+	0.4
3. *Locusta migratoria m.* (copulating adults)	0.6	+	9.1	+	0.3
4. *Schistocerca gregaria* (fifth-instar larvae)	0.4	+	9.5	+	0.1

TABLE 4. DISTRIBUTION (PERCENTAGE) OF *Schistocerca gregaria* AND *Locusta migratoria (migratorioides)* FIFTH-INSTAR LARVAE IN OLFACTOMETER WITH PHENOL, GUAIACOL, AND VERATROLE, AND THEIR MIXTURE[a]

Substances		*N*	Compartments					*P*
			1	2	3	4	M	
Schistocerca gregaria								
Phenol	c	200	40	16	15	1	28	
	s	200	44	8	5	1	42	<0.01
Guaiacol	c	200	56	13	6	1	24	
	s	200	33	17	4	1	45	<0.001
Veratrole	c	200	47	17	12	1	23	
	s	200	52	15	15	1	17	<0.9
Mixture (phenol, guaiacol, veratrole)	c	200	37	23	21	1	18	
	s	200	28	15	12	2	48	<0.001
Locusta migratoria migratorioides								
Phenol	c	200	21	25	23	15	16	
	s	200	20	10	24	8	38	<0.001
Guaiacol	c	200	21	7	16	4	52	
	s	200	26	15	18	6	35	<0.01
Veratrole	c	200	22	17	21	8	32	
	s	200	19	21	12	14	34	<0.05
Mixture (phenol, guaiacol, veratrole)	c	200	20	21	20	5	34	
	s	200	14	12	13	4	57	<0.001

[a] N = number of individuals; P = probabilities under chi-squared tests on row data; c = control without volatile products, with filter paper, oil, and hexane; s = with volatile substances (solution number 1 for pure products and numbers 2 and 4 for their mixture, see Table 3).

TABLE 5. CHANGE IN BEHAVIORAL RESPONSE TO MIXTURE OF PHENOL, GUAIACOL AND VERATROLE IN A GROUP OF GREGARIOUS *Locusta migratoria migratorioides*[a]

		Compartments						
	N	1	2	3	4	M	*P*	
Fifth-instar	c	200	20	21	20	5	34	
larvae	s	200	14	12	13	4	57	<0.001
Young adults								
2 days after ecdysis	c	200	26	39	15	2	18	
(females and	s	200	26	18	12	6	36	<0.001
males)								
Just before	c	150	38	12.4	13.2	16.4	20	
maturation								
(females and	s	200	28	12	5	14	41	<0.001
males)								
Mature males	c	100	52	8	0	4	12	
	s	100	28	4	8	0	60	<0.001
Mature females	c	100	32	16	24	16	12	
	s	100	20	0	4	8	68	<0.001

[a] Distribution (percentage) of locusts in olfactometer. N = number of individuals; P = probability under chi-squared tests on row data; c = control; s = with volatile substances (see Table 3), first and second experiments with solution number 2, others with solution number 3 (see Table 3).

both species, but more strongly on *Locusta*. It is also interesting to note that a mixture of the three products always has a marked effect on both species.

Table 5 shows the change in the same *Locusta migratoria* in response to the mixture of products at the four different ages. In all cases, males and females were tested together, except when they were mature. It is clear that the mixture always tends to aggregate the animals. In no cases are the substances attractive: animals never assemble in compartment 4 as they do in preliminary assays with the volatiles of fresh corn shoots. We also emphasize that controls, too, have a few animals in compartment 4.

DISCUSSION

Guaiacol was found in 1973 by Nolte et al. (1973) with 5-ethylguaiacol in the products obtained through extraction and purification of mixtures of substances extracted by hot water steam from 1 kg of feces of *Locusta migratoria* larvae. In the present investigations, based on extraction from the atmosphere in which the locusts are living, we never found the 5-ethylguaiacol described by Nolte et al. (1973) as the gregarization pheromone and called "locustol."

It is possible that this substance exists in the feces in derived form, which may be hydrolyzed through extraction by steam. In contrast, we always found phenol and veratrole, which had not been detected by Nolte. Thus, it does not seem possible to call 5-ethylguaiacol, an active biological volatile pheromone, Locustol, even if it is present in the feces. It is evident that, in the field, only volatile substances in the air may potentially act as a pheromone.

It is to be emphasized that both species of locusts have the same volatile products in their breeding atmosphere during the gregarious phase, but the relative percentages are variable from one species to the other and from one age to the other. Both subspecies, *migratorioides* and *cinerascens*, are similar to each other, but the atmosphere for *cinerascens* seems to be less rich in products, and this may be related to the fact that *cinerascens* is less apt to crowd in its higher geographic latitude. Further assays will allow to statistical comparison of the different ages of each strains.

It is also possible that variations obtained in the relative quantities of products through different analyses lead one to conclude that some of these products play another pheromonal role for which further special research is necessary.

Behavioral tests clearly show, under our conditions, that the mixture of the three products and also pure guaiacol or phenol have the effect of keeping more animals together and seem to have the properties of a "cohesion pheromone," which may act in the field and contribute to the maintenance of the large migratory groups. But these substances that act as a cohesion pheromone are not acting as an attractant pheromone at our concentrations (compartment 4 shows only a few animals responding to phenols cases). It is possible that these pheromones act only when other stimuli, visual and tactile, already play a part in the formation of crowds (Gillett et al., 1976).

It is also interesting, even without special studies here, to note that the volatiles may come from the animals themselves (see preliminary assays) without feces. Since Nolte et al. (1973) has worked only with feces, this is perhaps the reason for the difference between our results; trapping the surrounding atmosphere is of greater interest in relation to the problem of substances that may act as true natural pheromones.

Since we have completed only short-term behavioral tests, we do not know yet if these volatile products have direct long-term effects during ontogenesis and when they might have a true gregarisation effect. Further research is required for this second problem.

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CUTICULAR HYDROCARBONS OF DAMPWOOD TERMITES, *Zootermopsis:* Intra- and Intercolony Variation and Potential as Taxonomic Characters[1]

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Abstract—Colonies of *Zootermopsis* were collected from the central Sierra Nevada and the Monterey Penninsula in California, and from southern Arizona. Cuticular hydrocarbons were identified by gas chromatography–mass spectrometry (GC-MS) and quantified by gas–liquid chromatography (GLC) for each caste of all colonies. Four consistent and distinct cuticular hydrocarbon patterns, or chemical phenotypes, were identified. Unique and abundant monomethyl- and dimethylalkanes, and an *n*-alkene provided easy separation of the various phenotypes. Significant differences in the proportions of the various components were found among castes within a colony and colonies within phenotypes from California. Differences in the hydrocarbon proportions for castes were not consistent between colonies. The current taxonomy of the genus *Zootermopsis* recognizes three species. Our identification of four consistent, unique cuticular hydrocarbon phenotypes from the three described species should alert systematists and others to a major concern. If there are truly only three extant species, then the hypothesis that cuticular hydrocarbon profiles in this genus are species specific is not acceptable. Conversely, if cuticular hydrocarbon profiles are truly species specific, then there is at least one new, undescribed species of *Zootermopsis*.

Key Words—Chemotaxonomy, Isoptera, Termopsidae, termites, methyl-branched hydrocarbons, lipids, agonisitic behavior, cuticular hydro-carbons.

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INTRODUCTION

The surface of all terrestrial insects is covered with a complex mixture of aliphatic material. This surface lipid plays a key role in survival of the insect by providing protection from desiccation, as well as serving as a barrier to abrasion, microorganisms, and chemicals. Hydrocarbons are ubiquitous components in insect cuticular lipids (Blomquist and Dillwith, 1985; Jackson and Blomquist, 1976; Hadley, 1980, 1985). They have been shown to be important in chemical communication as sex attractants and aphrodisiacs; as territory-marking, recruitment, and alarm pheromones; as defense secretions; and as kairomones, and they have been postulated as species and caste recognition cues (Howard and Blomquist, 1982).

Moore (1969) was the first to report the composition of cuticular hydrocarbons from a termite, *Nasutitermes exitiosus* (Hill). He found the majority of the hydrocarbons to be paraffins from C_{24} to C_{47} with the odd-carbon-numbered compounds predominating. Blomquist et al. (1979) and Howard et al. (1978, 1980, 1982) have completely characterized the cuticular hydrocarbons of a *Zootermposis* species and two *Reticulitermes* species. Because the mixtures of hydrocarbons of these three species were markedly different from one another and *N. exitiosus*, they postulated that these compounds might serve as semiochemical cues for caste and species recognition. Furthermore, Howard and Blomquist (1982) noted that there are extremely few reports of two insect species having identical hydrocarbon mixtures. Because of this, we were curious about their usefulness as taxonomic characters for separating morphologically similar species.

In preliminary work, we collected what we tentatively identified as colonies of *Z. angusticollis* (Hagen) from the vicinity of Burney, California, on the Lassen National Forest, and extracted their cuticular hydrocarbons. Our chemical analyses identified the same hydrocarbon components as published by Blomquist et al. (1979) but in markedly different proportions. This was our first clue that considerable intraspecific variation might exist in the mixtures of cuticular hydrocarbons of *Zootermopsis* and prompted us to examine this genus more thoroughly.

Zootermopsis is a genus of primitive termites in the family Termopsidae, which is restricted to western North America. Three species are recognized: *Z. angusticollis*, *Z. nevadensis* (Hagen), and *Z. laticeps* (Banks). The former two species occur in the forested areas along the Pacific Coast, in the Cascade Mountains and Sierra Nevada, and in some of the inland ranges west of the Continental Divide. The latter species occurs east and southeast of the Sonoran Desert in trees along water courses and around ponds (Weesner, 1970). Present keys to the species, written decades ago, are based on a limited number of specimens and use morphological features and measurements of the head, pronotum, and/or wings of either soldiers or imagoes (Emerson, 1933; Sumner,

1933). A thorough morphological analysis of *Zootermopsis* has never been done. The material studied by these early taxonomists was collected from a narrow geographic range, and we now recognize that this genus may be in need of revision.

With only three extant species, we felt that this genus would be ideal to test the hypothesis that cuticular hydrocarbon profiles are species specific. To date, no one has satisfactorily examined the qualitative or quantitative variation in cuticular hydrocarbons of castes within a termite colony or species, among different colonies in a species, or, most important of all, among all species within a genus. In this paper we report: (1) identification of all cuticular hydrocarbons from each recognized species of *Zootermopsis*, and (2) intercaste, intracolony, and intercolony variation in hydrocarbon mixtures within two species.

METHODS AND MATERIALS

Zootermopsis colonies or portions of colonies were collected in spring and summer of 1985 from the following two locations: near Placerville, California, on the Eldorado National Forest (eight colonies), and from Cypress Point near Pacific Grove, California, on the Monterey Peninsula (seven colonies). In the spring of 1986 additional collections were made at Cypress Point (nine colonies) and on the Eldorado National Forest (10 colonies). Also in the spring of 1986 samples from five colonies of *Z. laticeps* were obtained from an area along the Santa Cruz River near Rio Rico, Santa Cruz County, Arizona.

Two sets of chromatographic analyses were completed. Termites were removed from their galleries and placed in plastic dishes containing moist host wood. In the first analysis, one to five individuals of each caste from each of the colonies collected in 1985 were placed in separate numbered vials (one termite/vial) and kept at −20°C until extracted for hydrocarbon analysis. Individual termites were coded by number; thus the species or location was unknown before gas chromatographic analysis. In the second analysis, groups of five individuals from each caste from each of the colonies collected in 1985 and 1986 were placed in numbered vials as were the individual termites. Termites used in both the first and second analysis were removed from their host wood and frozen within days of collection.

Individual termites or groups of individuals were removed from the freezer and allowed to come to ambient temperature. Cuticular lipids were extracted by immersion of termites in 10 ml of hexane for 10 min. After extraction, hydrocarbons were separated from other nonpolar components by pipetting the 10-ml extract through a minicolumn packed with 100–200 mesh BioSil A activated overnight at 70°C in a drying oven. This hydrocarbom extract was then evaporated to dryness under a stream of nitrogen and redissolved in 30 μl of hexane for GLC analysis. After extraction, the insects

were stored in 70% ethanol for later identification by morphological features and to serve as voucher specimens. All voucher specimens will be deposited in the U.S. National Museum, Smithsonian Institution, Washington, D.C.

The first analysis of cuticular hydrocarbons was performed on a Hewlett Packard 5790A gas chromatograph using a wide-bore capillary column (30 m × 0.75 mm ID SPB-1) with a flame ionization detector (FID). Peak resolution was adequate with this system to observe obvious qualitative differences in cuticular hydrocarbon mixtures and allowed us to separate the California *Zootermopsis* into three obvious chemical phenotypes (see Results and Discussion). Individual termites were used for these analyses, which permitted us to quantify each hydrocarbon with the exception of a few which coeluted in the same peak. Peaks were characterized by retention time, and areas under curves (= quantity of a particular component) were automatically quantified by a Hewlett Packard 3390A electronic integrator. The total hydrocarbon was calculated by summing the area for each component and proportion of each component then calculated. For each of the three California chemical phenotypes, proportions of each hydrocarbon were transformed to the arcsine of the square root of the proportion and subjected to analysis of variance to evaluate differences among castes and within and among colonies, and to provide summary statistics.

Better hydrocarbon separation was obtained with the acquisition of a Hewlett Packard 5890 gas chromatograph equipped with a fused silica capillary column (30 m × 0.32 mm ID, SPB-1), FID, and operated in the split mode (with a split ratio of 33:1). The temperature program in each case had a starting temperature of 200°C. Temperature was increased at 3°C/min to 300°C, with a final holding time of 12 min. Helium carrier gas flow was 25 cc/min on the HP 5790A and 3 cc/min on the HP 5890. Hydrocarbons were quantified as in the first analysis.

Routine identification of all hydrocarbons was based on external standards or retention times from hydrocarbons identified by GC-MS. Equivalent chain length (ECL) for each hydrocarbon was calculated by comparing the retention time of a given peak to known *n*-alkanes in external standards. GC-MS analyses were performed on a Finnigan 4023 mass spectrometer interfaced with an INCOS data system. Electron impact (EI) mass spectrometry was performed at 70 eV. Mass spectra of methylakanes were interpreted according to the criteria of Nelson et al. (1972), Nelson (1978), and Pomonis et al. (1978). *n*-Alkenes were isolated by silver nitrate chromatography (column and/or TLC), and the positions of double bonds were determined by GC-MS analysis of the monomethoxy and dimethoxy derivatives of the parent compounds after methoxymercuration–demercuration (Blomquist et al., 1980).

To assure correct species identification and to correlate cuticular hydrocarbon mixtures with species, preserved, extracted specimens (imagoes and soldiers, if both were present) were shipped in numbered vials, one individual per vial, to termite specialists for identification. The termites were identified by

these specialists solely on the basis of external morphology without knowledge of location or host. The only information provided was the association of individuals from the same colony. The keys used were from Banks and Snyder (1920), Snyder (1954), Sumner (1933), and Weesner (1965).

RESULTS AND DISCUSSION

All of the abundant hydrocarbon components (mean percent $\geq 0.5\%$ of the total hydrocarbon component) and most of the less abundant components were identified for each location and/or species (Tables 1 and 2, Figure 1). All col-

TABLE 1. HYDROCARBONS IDENTIFIED FOR PHENOTYPES OF *Zootermopsis*.[a]

Peak	Hydrocarbon	ECL[b]	CN[b]	Diagnostic MS ions
1	*n*-Nonadecane	19.00	19	268
2	*n*-Eicosane	20.00	20	282
3	5-Methyleicosane	20.50	21	84/85, 238/239, 296
4	2-Methyleicosane	20.62	21	280/281, 296
5	*n*-Heneicosane	21.00	21	296
6	9-; 11-Methylheneicosane	21.37	22	140/141, 196/197, 310; 168/169, 310
7	7-Methylheneicosane	21.41	22	112/113, 224/225, 310
8	5-Methylheneicosane	21.50	22	84/85, 252/253, 310
9	2- or 4-Methylheneicosane	21.62	22	266/267, 310
10	3-Methylheneicosane	21.71	22	252/253, 280/281, 310
11	*n*-Docosane	22.00	22	310
12	7,15-Dimethylheneicosane	22.00	23	112/113, 238/239
13	5,17-Dimethylheneicosane	22.04	23	84/85, 266/267
14	3,11-; 3,13-Dimethylheneicosane	22.11	23	168/169, 182/183, 196/197, 210/211, 294/295
15	7-Methyldocosane	22.37	23	112/113, 238/239, 324
16	2- or 4-Methyldocosane	22.63	23	252/253, 280/281, 294/295, 308/309, 324
17	*n*-Tricosene	22.70	23	322
18	Unknown 1	22.77		
19	*n*-Tricosane	23.00	23	324
20	9-; 11-Methyltricosane	23.36	24	140/141, 224/255, 338; 168/169, 196/197, 338
21	7-Methyltricosane	23.43	24	112/113, 252/253, 338
22	5-Methyltricosane	23.50	24	84/85, 280/281, 338
23	3-Methyltricosane	23.73	24	280/281, 308/309, 338
24	5,13-; 5,17-Dimethyltricosane	23.95	25	84/85, 168/169, 210/211, 294/295; 84/85, 112/113, 266/267, 294/295
25	*n*-Tetracosane	24.00	24	338
26	3,11-; 3,13-Dimethyltricosane	24.10	25	168/169, 182/183, 196/197, 210/211, 322/232, 352
27	2-Methyltetracosane	24.72	25	308/309, 336/337, 352

TABLE 1. Continued

Peak	Hydrocarbon	ECL[b]	CN[b]	Diagnostic MS ions
28	*n*-Pentacosane	25.00	25	352
29	9-; 11-; 13-Methylpentacosane	25.38	26	140/141, 252/253, 366; 168/169, 224/225, 366; 196/197, 366
30	5-Methylpentacosane	25.52	26	85/86, 308/309, 366
31	3-Methylpentacosane	25.73	26	336/337, 366
32	5,17-Dimethylpentacosane	25.90	27	84/85, 140/141, 266/267, 322/323
33	*n*-Hexacosane	26.00	26	366
34	2-or 4-Methylheptacosane	26.59	27	308/309, 336/337, 364/365
35	*n*-Heptacosane	27.00	27	380
36	11-Methylheptacosane	27.35	28	168/169, 252/253, 394
37	5-Methylheptacosane	27.53	28	84/85, 336/337, 394
38	5,17-Dimethylheptacosane	27.88	29	84/85, 168/169, 266/267, 350/351
39	*n*-Octacosane	28.00	28	394
40	2- or 4-Methyloctacosane	28.60	29	336/337, 364/365, 392/393, 408
41	*n*-Nonacosane	29.00	29	408
42	11-; 13-; 15-Methylnonacosane	29.32	30	168/169, 280/281, 422; 196/197, 252/253, 422; 196/197, 224/225, 422
43	7-Methylnonacosane	29.42	30	112/113, 336/337, 422
44	5-Methylnonacosane	29.51	30	84/85, 364/365, 422
45	5,17-Dimethylnonacosane	29.83	31	84/85, 196/197, 266/267, 378/379
46	*n*-Triacontane[c]	29.99	30	
47	Unknown 2	30.05		
48	3,7-Dimethylnonacosane	30.13	31	126/127, 336/337, 406/407
49	Unknown 3	30.45		
50	*n*-Hentriacotene	30.60	31	434
51	Unknown 4	30.70		
52	13-; 15-Methylhentriacontane	30.87	32	196/197, 280/281, 450; 224/225, 252/253, 450
53	Unknown 5	30.96		
54	7-; 9-Methylhentriacontane	31.37	32	112/113, 140/141, 336/337, 364/365, 392/393
55	5-Methylhentriacontane	31.46	32	84/85, 364/365, 392/393
56	2- or 4-Methylhentriacontane	31.75	32	406/407, 434/435, 450
57	*n*-Dotriacontane[c]	31.98		
58	Unknown 6	32.26		
59	Unknown 7	32.61		
60	5,13-; 5,15-; 5,17-Dimethyltritriacontane	33.60	35	84/85, 210/211, 308/309, 434/435; 238/239, 280/281, 434/435; 252/253, 266/267, 434/435
61	5,17-Dimethylpentatriacontane	35.70	37	84/85, 266/267, 280/281, 462/463
62	7,11-Dimethylpentatriacontane	35.70	37	112/113, 182/183, 364/365, 434/435
63	7,13-Dimethylheptatriacontane	38.53	39	112/113, 210/211, 364/365, 463/464

[a] Peak numbers refer to peaks identified in Figure 1.
[b] ECL = equivalent chain length; CN = carbon number.
[c] Hydrocarbon determination made by ECL, not GC-MS.

TABLE 2. HYDROCARBONS IDENTIFIED FROM FOUR PHENOTYPES OF *Zootermopsis*.[a]

Peak	Hydrocarbon	ECL[b]	Phenotype[c] I	II	III	IV
1	*n*-Nonadecane	19.00	+	0	0	0
2	*n*-Eicosane	20.00	+	0	0	tr
3	5-Methyleicosane	20.50	tr	0	0	0
4	2-Methyleicosane	20.62	tr	0	0	tr
5	*n*-Heneicosane	21.00	+++	+++	+++	+++
6	9-; 11-Methylheneicosane	21.37	0	++	0	tr
7	7-Methylheneicosane	21.41	+	++	++	tr
8	5-Methylheneicosane	21.50	+++	++	0	0
9	2- or 4-Methylheneicosane	21.62	0	0	0	++
10	3-Methylheneicosane	21.71	++	++	++	++
11	*n*-Docosane	22.00	0	++	++	++
12	7,15-Dimethylheneicosane	22.00	++	0	0	0
13	5,17-Dimethylheneicosane	22.04	+++	0	0	0
14	3,11-; 3,13-Dimethylheneicosane	22.11	0	+++	0	0
15	7-Methyldocosane	22.37	0	+	0	0
16	2- or 4-Methyldocosane	22.63	0	++	+++	+++
17	*n*-Tricosene	22.70	0	0	++	++
18	Unknown 1	22.77	0	0	0	++
19	*n*-Tricosane	23.00	+++	+++	+++	+++
20	9-; 11-Methyltricosane	23.36	0	+	0	tr
21	7-Methyltricosane	23.43	+	++	0	tr
22	5-Methyltricosane	23.50	++	0	0	0
23	3-Methyltricosane	23.73	+	+	+++	++
24	5,13-; 5,17-Dimethyltricosane	23.95	tr	0	0	0
25	*n*-Tetracosane	24.00	+	0	++	+
26	3,11-; 3,13-Dimethyltricosane	24.10	0	++	0	0
27	2-Methyltetracosane	24.72	0	0	tr	+
28	*n*-Pentacosane	25.00	+++	++	+++	+++
29	9-; 11-; 13-Methylpentacosane	25.38	tr	tr	tr	0
30	5-Methylpentacosane	25.52	tr	0	0	0
31	3-Methylpentacosane	25.73	0	0	+	0
32	5,17-Dimethypentacosane	25.90	tr	0	0	0
33	*n*-Hexacosane	26.00	tr	tr	+	tr
34	2- or 4-Methylheptacosane	26.59	0	0	tr	0
35	*n*-Heptacosane	27.00	+	++	tr	++
36	11-Methylheptacosane	27.35	0	tr	0	0
37	5-Methylheptacosane	27.53	tr	tr	0	0
38	5,17-Dimethylheptacosane	27.88	tr	0	0	0
39	*n*-Octacosane	28.00	tr	+	0	0
40	2- or 4-Methyloctacosane	28.60	0	++	0	0
41	*n*-Nonacosane	29.00	++	++	0	tr
42	11-; 13-; 15-Methylnonacosane	29.32	0	+	0	0
43	7-Methylnonacosane	29.42	tr	0	0	tr
44	5-Methylnonacosane	29.51	++	++	0	tr
45	5,17-Dimethylnonacosane	29.83	tr	++	0	0

TABLE 2. Continued

Peak	Hydrocarbon	ECL[b]	Phenotype[c] I	II	III	IV
46	*n*-Triacontane	29.99	tr	0	0	0
47	Unknown 2	30.05	0	tr	0	0
48	3,7-Dimethylnonacosane	30.13	tr	+	tr	0
49	Unknown 3	30.45	tr	0	0	0
50	*n*-Hentriacontene	30.60	0	+	0	0
51	Unknown 4	30.70	0	0	tr	0
52	13-; 15-Methylhentriacontane	30.87	0	+	0	0
53	Unknown 5	30.96	tr	0	0	0
54	7-, 9-Methylhentriacontane	31.37	tr	tr	0	0
55	5-Methylhentriacontane	31.46	tr	++	0	0
56	2- or 4-Methylhentriacontane	31.75	0	++	0	0
57	*n*-Dotriacontane	31.98	0	tr	tr	0
58	Unknown 6	32.26	0	0	+	0
59	Unknown 7	32.61	0	0	tr	0
60	5,13-; 5-15-; 5,17-Dimethyltricontane	33.60	+	++	0	0
61	5,17-Dimethylpentatriacontane	35.70	++	++	0	0
62	7,11-Dimethylpentatriacontane	35.70	0	0	0	++
63	7,13-Dimethylpentatriacontane	38.53	0	0	0	++

[a] A triple + indicates ≥5.0% of the total, ++ from 1.0 to 5.0% of the total, and + from 0.5 to 1.0% of the total hydrocarbon component. Some trace (tr.) components appear infrequently or consistently in very small quantities (<0.5% of the total). A zero indicates the hydrocarbon was never identified for the phenotype.

[b] Hydrocarbon identifications by GC-MS (see Table 1 and Figure 1).

[c] Phenotypes and collection locations: I = Eldorado NF, California, *Z. nevadensis*; II and III = Pacific Grove, California, *Z. angusticollis* and *Z. nevadensis*, respectively; IV = Rio Rico, Santa Cruz County, Arizonia, *Z. laticeps*.

onies from the Eldorado National Forest possessed qualitatively identical mixtures of hydrocarbons, which we will call phenotype I. Ninety-one percent of the taxonomic determinations for this phenotype were *Z. nevadensis*; the remaining 9% were determined to be *Z. angusticollis*. Two different hydrocarbon phenotypes (II and III) were recognized from the Pacific Grove area; both were qualitatively different from phenotype I. All colonies in either phenotypes II or III had the same hydrocarbon components. All of the taxonomic determinations for phenotype II were *Z. angusticollis*; 84% of the determinations for phenotype III were *Z. nevadensis*. Colonies collected from Rio Rico in southern Arizona were identified as *Z. laticeps*. These termites all had qualitatively identical hydrocarbon profiles that were qualitatively different from phenotypes I, II, and III. *Zootermopsis laticeps* constitutes phenotype IV.

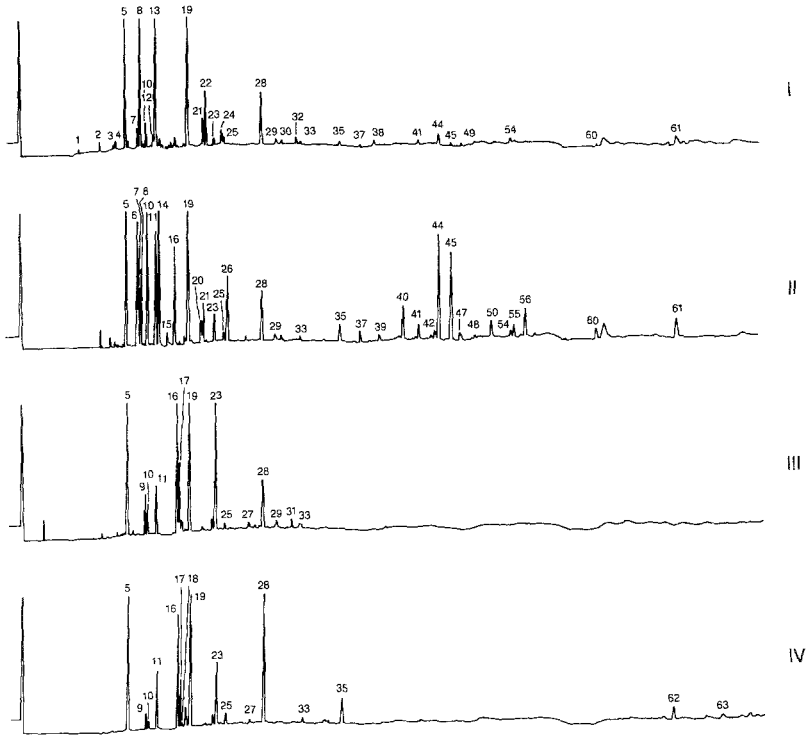

FIG. 1. Gas chromatograms of the surface hydrocarbons of four phenotypes of *Zootermopsis*. Numbers identify peaks whose compositions are listed in Table 1.

GC-MS was used to characterize each of the major hydrocarbon components of each phenotype, and the mass spectra of some of the components unique to certain phenotypes are presented in Figures 2–6. The mass spectrum of peak 13 (Figure 1), which elutes about one equivalent chain length (ECL = 22.04) in front of the corresponding *n*-alkane and is identified as the symetrical 5, 17-dimethylheneicosane, is presented in Figure 2. The strong *m/z* 84/85 ion (Figure 2) is interpreted as arising from cleavage internal to the methyl group on carbons 5 and 17 giving rise to a six-carbon fragment ion. Cleavage external to the methyl groups gives rise to ions at *m/z* 266/267, in which the much greater intensity of the odd-numbered ion is consistent with the fragment containing a second methyl group.

Peak 16 from phenotype IV is interpreted as 2-methyldocosane based on its retention time and mass spectrum (Figure 3). The ECL = 22.63 and strong M − 15 ion at *m/z* 309 and M − 43 ion at *m/z* 281 are consistent with a 2-methylalkane. In some spectra of components identified as 2- or 4-methylal-

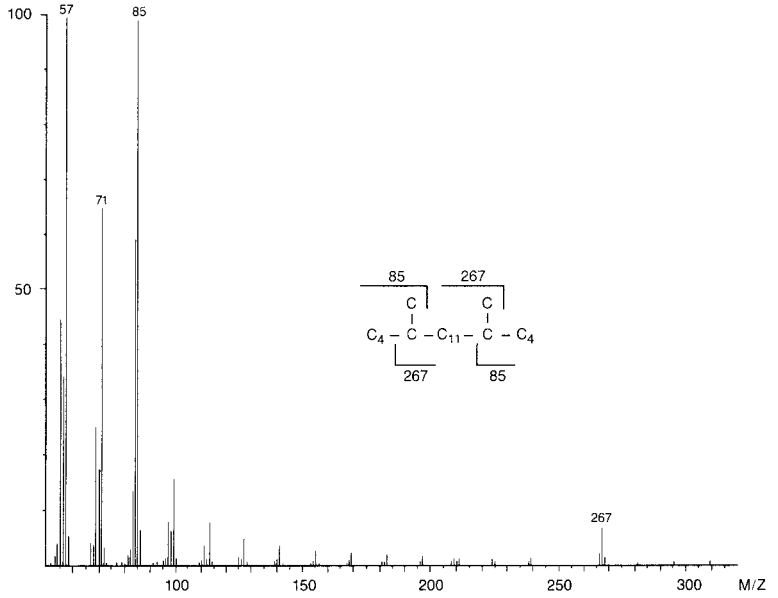

FIG. 2. EI mass spectrum of peak 13, Figure 1, identified as 5,17-dimethylheneicosane.

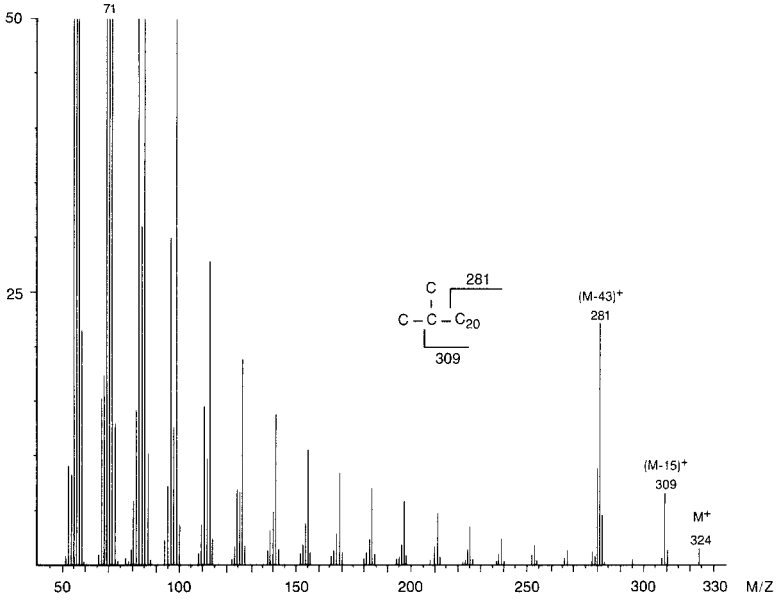

FIG. 3. EI mass spectrum of peak 16, Figure 1, identified as 2-methyldocosane.

kanes, there is a relatively strong ion pair at M-71:M-72, indicating that 4-methylalkanes may also be present. The M-71:M-72 ion from the alkanes identified as 2- or 4-methylalkanes varied in intensity among different spectra and therefore are designated as either 2- or 4-methylalkanes. Even when the M-71:M-72 ions are relatively strong, we cannot rule out the possibility that the peak also contains a 2-methylalkane.

Phenotype II also contains peaks identified as a mixture of 3,11- and 3,13-dimethylalkanes. In Figure 4 we show the mass spectrum of peak 26, which is identified as a mixture of 3,11- and 3,13-dimethyltetracosanes. The major isomer appears to be the 3,11- component, which gives rise to ions at *m/z* 182/183, 196/197, and 322/323. The 3,13- isomer gives rise to ions at *m/z* 168/169, 210/211, and 322/323. This phenotype appears to be the only one that contains 3,11- and 3,13-dimethylalkanes (peaks 14 and 26, Figure 1, Table 2).

Phenotypes III and IV contain an alkene (peak 17), which we identify as 9-tricosene from the mass spectra of its mono- and dimethyoxy derivatives. The spectra (Figures 5A and 5B) show the expected fragment ions (*m/z* 157, 171, 227, and 241 for the monomethoxy derivative and 157 and 227 for the dimethoxy derivative). Phenotype IV also contains two unique dimethylalkanes, 7,11-dimethylpentatriacontane and 7,13-dimethylheptatriacontane (peaks 62 and

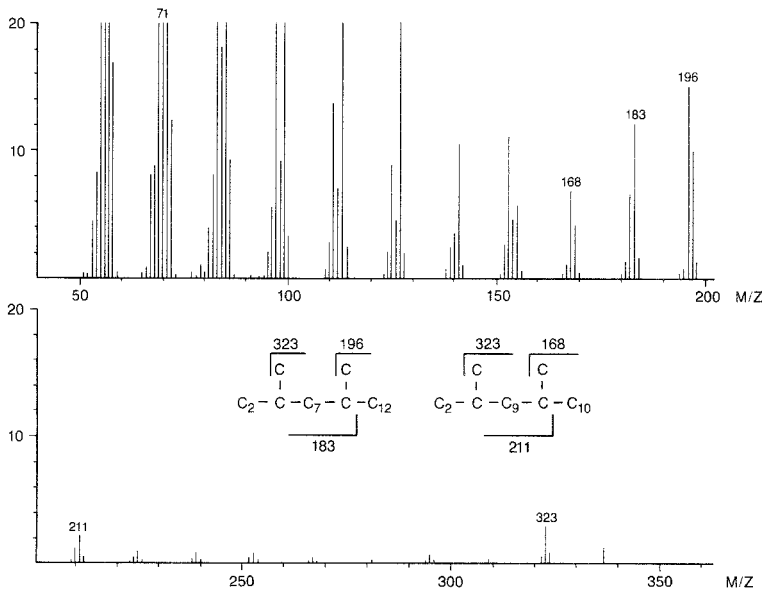

FIG. 4. EI mass spectrum of peak 26, Figure 1, identified as 3,11- and 3,13-dimethyltricosane.

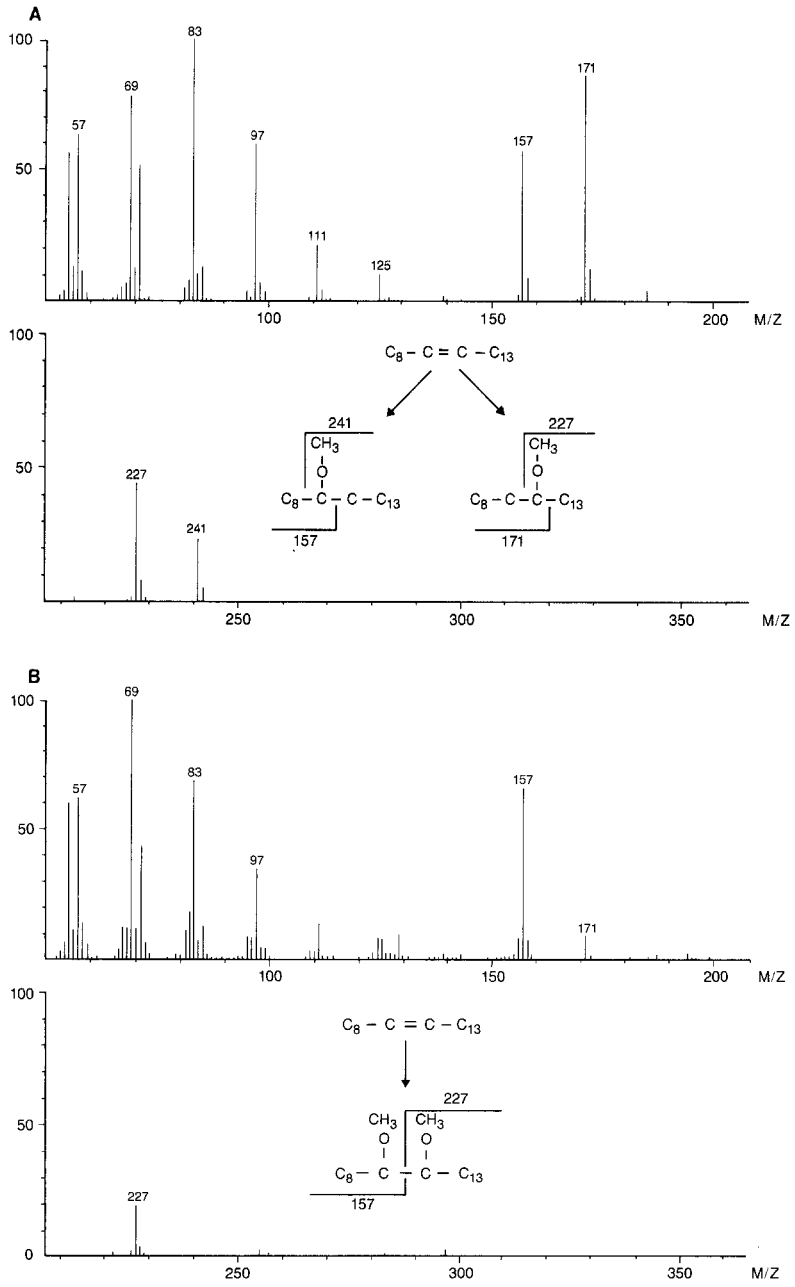

FIG. 5. EI mass spectra of (A) monomethoxy and (B) dimethoxy derivatives of 9-tricosene.

63). One of these (peak 63) elutes much later than any of the other components in any of the *Zootermopsis* phenotypes. The mass spectrum of 7,11-dimethylpentatriacontane is presented in Figure 6. Cleavage internal to one or the other of the branching methyl groups gives rise to fragments at *m/z* 112/113 and 364/365. Cleavage external to the branching methyl groups gives rise to fragments that contain a second methyl branch and are at *m/z* 183 and 435.

Mass spectra of each of the other major components for all four phenotypes were interpreted in a similar manner to that described above, and each component and its diagnostic ions are presented in Table 1.

Phenotype I contains major amounts (ca. 10% of the total hydrocarbon) of the symmetrical dimethylalkane 5,17-dimethylheneicosane (peak 13, Figure 1 and Table 3). This dimethylalkane is not shared with any other phenotype (Table 2). Phenotype I is rich in minor 5,17-dimethyl components including a homologous series of 5,17-dimethyl C_{25}, C_{27}, C_{29}, C_{31}, C_{35}, and C_{37}. With the exception of 5,17-dimethyl C_{31}, C_{35}, and C_{37}, these components of this homologous series are unique to phenotype I (Table 2).

Phenotype II is the most complex of the *Zootermopsis* chemical phenotypes, sharing many of the components eluting before *n*-nonacosane with at least one of the other phenotypes. This phenotype possesses many unique compounds in major quantities (>1.0%, Table 4): an isomeric mixture of 9- and

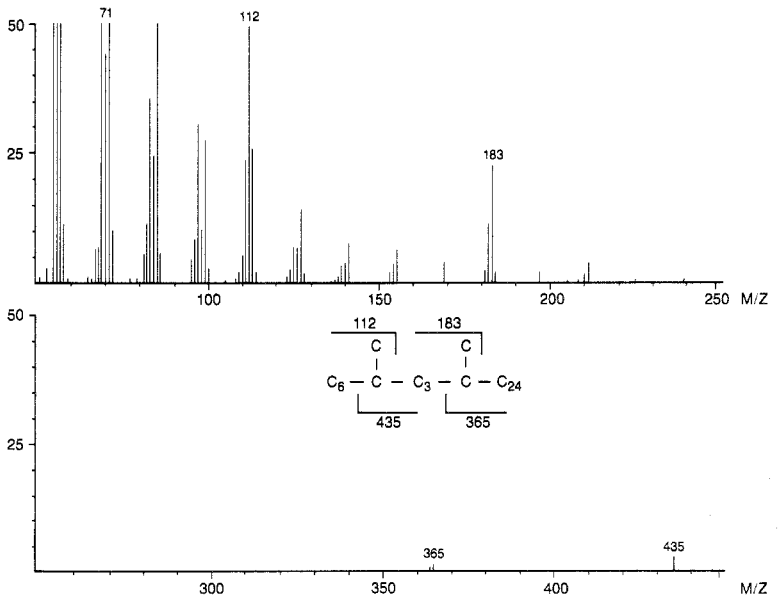

FIG. 6. EI mass spectrum of peak 62, Figure 1, identified as 7,11-dimethylpentatriacontane.

TABLE 3. STATISTICS FOR PERCENT HYDROCARBON FROM FOUR CASTES FROM EIGHTEEN COLONIES

		Overall (N = 53)				
Peak No.[a]	Hydrocarbon[a]	X	SD	Minimum value[b]	Maximum Value	Proportion ≤0.1% of total[c]
1	n-C_{19}	0.6	0.8	0.0	3.3	0.55
2	n-C_{20}	0.9	1.3	0.0	9.3	0.15
3	5-MeC_{21}	0.0	0.0	0.0	0.2	0.98
4	2-MeC_{21}	0.2	0.2	0.0	0.7	0.41
5	n-C_{21}	36.1	8.6	16.5	51.2	0.00
7	7-MeC_{22}	0.6	0.2	0.3	1.3	0.00
8	5-MeC_{22}	12.6	3.1	6.9	22.2	0.00
10	3-MeC_{22}	1.1	0.2	0.6	1.8	0.00
12	7,15-DimeC_{23}	1.9	0.6	0.3	3.1	0.00
13	5,17-DimeC_{23}	9.9	5.4	3.8	27.9	0.00
19	n-C_{23}	16.3	3.6	10.1	27.6	0.00
21	7-MeC_{24}	0.9	0.4	0.4	2.2	0.00
22	5-MeC_{24}	2.9	1.2	1.3	5.8	0.00
23	3-MeC_{24}	0.6	0.2	0.4	1.3	0.00
24	5,17-DimeC_{25}	0.2	0.3	0.0	1.0	0.43
25	n-C_{24}	0.7	0.2	0.4	1.5	0.00
28	n-C_{25}	6.3	3.5	2.4	19.7	0.00
29	9-; 11-; 13-MeC_{26}	0.2	0.2	0.0	0.9	0.47
30	5-MeC_{26}	0.3	0.2	0.0	0.8	0.23
32	5,17-DimeC_{27}	0.3	0.2	0.0	0.7	0.28
33	n-C_{26}	0.4	0.3	0.0	1.6	0.15
35	n-C_{27}	0.7	0.6	0.0	3.1	0.02
37	5-MeC_{28}	0.3	0.3	0.0	1.3	0.34
38	5,17-DimeC_{29}	0.4	0.6	0.0	3.0	0.30
39	n-C_{28}	0.1	0.1	0.0	0.7	0.75
41	n-C_{29}	1.0	1.4	0.0	6.5	0.11
43	7-MeC_{30}	0.2	0.4	0.0	2.2	0.71
44	5-MeC_{30}	1.3	0.9	0.0	5.8	0.06
45	5,17-DimeC_{31}	0.4	0.6	0.0	3.2	0.32
46	n-C_{30}	0.1	0.3	0.0	1.6	0.81
48	3,7-DimeC_{31}	0.3	0.4	0.0	1.2	0.30
49	Unknown 3	0.1	0.1	0.0	0.5	0.70
53	Unknown 5	0.3	0.3	0.0	1.2	0.34
54	7-,9-MeC_{32}	0.4	0.2	0.0	1.1	0.09
55	5-MeC_{32}	0.1	0.2	0.0	0.5	0.64
60	5,17-DimeC_{35}	0.5	1.0	0.0	7.4	0.42
61	5,17-DimeC_{37}	1.1	0.5	0.0	2.9	0.02

[a] Hydrocarbons and peak numbers are the same as those reported in Table 1 and Figure 1. Carbon number is the total number of carbons, including methyl groups.

[b] Values not necessarily zero but may have had a very low quantity (<0.1% of total hydrocarbon) or were simply not detectable for a given GLC analysis.

[c] Proportion of the 53 chromatograms where a given hydrocarbon was not detectable.

[d] Castes are SO = soldier, PS = pseudergate, NY = nymph, and AL = alate (imago).

OF *Zootermopsis* COLLECTED FROM ELDORADO NATIONAL FOREST, CALIFORNIA (PHENOTYPE I)

Castes[d]						
SO (N = 17)	PS (N = 18)	NY (N = 11)	AL (N = 7)	Statistical differences[e]		
X SD	X SD	X SD	X SD	Colony	Caste	Interaction
1.0 (1.0)	0.6 (0.7)	0.6 (0.9)	0.0 (0.1)			
1.5 (2.1)	0.8 (0.5)	0.6 (0.5)	0.2 (0.2)			
0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.1)			
0.3 (0.2)	0.2 (0.2)	0.1 (0.2)	0.1 (0.2)			
37.9 (4.7)	39.5 (6.7)	37.4 (9.0)	21.0 (3.4)	*	*	*
0.5 (0.2)	0.5 (0.2)	0.6 (0.2)	1.0 (0.2)	*	*	*
12.7 (2.5)	12.9 (2.8)	11.4 (3.1)	13.0 (5.0)	*	*	*
1.2 (0.2)	1.0 (0.2)	0.9 (0.2)	1.1 (0.3)	*	*	NS
1.8 (0.2)	2.2 (0.4)	2.0 (0.8)	1.0 (0.4)	*	*	*
8.3 (1.7)	7.6 (2.1)	8.6 (3.6)	21.7 (4.5)	*	*	*
15.3 (2.6)	15.8 (2.9)	16.9 (4.1)	18.6 (5.7)	*	*	*
0.8 (0.3)	0.7 (0.2)	0.8 (0.3)	1.7 (0.3)	NS	*	*
3.0 (1.1)	2.3 (0.8)	2.6 (0.8)	4.7 (0.8)	*	*	*
0.6 (0.2)	0.5 (0.1)	0.6 (0.1)	1.0 (0.2)	NS	*	NS
0.3 (0.2)	0.1 (0.1)	0.1 (0.2)	0.7 (0.2)			
0.7 (0.1)	0.7 (0.2)	0.8 (0.3)	0.8 (0.2)	*	*	NS
5.5 (2.0)	5.9 (4.1)	7.6 (4.4)	7.1 (2.5)	*	*	NS
0.2 (0.2)	0.1 (0.2)	0.2 (0.2)	0.2 (0.1)	*	*	NS
0.3 (0.2)	0.2 (0.2)	0.3 (0.2)	0.4 (0.2)	*	*	NS
0.4 (0.2)	0.2 (0.2)	0.2 (0.2)	0.3 (0.2)	*	NS	NS
0.3 (0.1)	0.4 (0.4)	0.4 (0.4)	0.3 (0.2)	*	NS	NS
0.5 (0.3)	0.7 (0.7)	0.9 (0.9)	0.4 (0.2)	*	NS	NS
0.2 (0.3)	0.2 (0.3)	0.5 (0.4)	0.4 (0.4)	*	*	NS
0.5 (0.8)	0.4 (0.5)	0.4 (0.3)	0.5 (0.5)	*	*	*
0.0 (0.1)	0.1 (0.2)	0.0 (0.1)	0.0 (0.0)	*	*	*
1.0 (1.4)	1.3 (1.7)	0.9 (0.8)	0.3 (0.2)	*	*	NS
0.2 (0.5)	0.2 (0.4)	0.2 (0.3)	0.0 (0.0)	*	*	*
1.3 (0.5)	1.3 (1.2)	1.4 (0.8)	0.8 (0.6)	*	*	*
0.5 (0.8)	0.5 (0.7)	0.2 (0.3)	0.1 (0.2)			
0.1 (0.1)	0.2 (0.4)	0.1 (0.3)	0.0 (0.0)			
0.2 (0.2)	0.2 (0.2)	0.5 (0.4)	0.7 (0.5)			
0.1 (0.2)	0.1 (0.1)	0.1 (0.1)	0.1 (0.2)			
0.3 (0.3)	0.3 (0.3)	0.2 (0.2)	0.1 (0.1)			
0.5 (0.2)	0.4 (0.2)	0.5 (0.4)	0.3 (0.2)			
0.1 (0.2)	0.1 (0.1)	0.1 (0.2)	0.2 (0.1)			
0.4 (0.3)	0.7 (1.7)	0.3 (0.4)	0.3 (0.3)			
1.2 (0.5)	1.0 (0.3)	1.1 (0.7)	0.9 (0.5)			

[e] Proportions of each hydrocarbon were transformed to the arcsine of the square root of the proportion and subjected to analysis of variance. Asterisk indicates significant difference (α = 0.05) between colony, caste, or interaction term for a given hydrocarbon. Analysis of variance was done only for hydrocarbon components detectable from individual termites from collections made in 1985. The gas chromatograph with a 0.75-mm-ID capillary column was used.

TABLE 4. STATISTICS FOR PERCENT HYDROCARBON FROM FOUR CASTES FROM SEVEN COLONIES OF *Zootermopsis* COLLECTED FROM PACIFIC GROVE, CALIFORNIA (PHENOTYPE II)

		Overall ($N = 20$)					Castes[d]				Statistical differences[e]		
Peak No.[a]	Hydrocarbon[a]	X	SD	Minimum value[b]	Maximum value	Proportion ≤0.1% of total[c]	SO ($N = 7$) X SD	PS ($N = 6$) X SD	NY ($N = 6$) X SD	AL ($N = 1$) X SD	Colony	Caste	Interaction
5	*n*-C_{21}	23.9	5.4	12.8	32.2	0.00	20.7 (4.8)	27.7 (4.8)	25.4 (3.0)	14.2 —	*	*	NS
6	9-; 11-MeC_{22}	3.8	0.6	2.4	5.1	0.00	3.9 (0.6)	3.7 (0.3)	3.8 (0.9)	4.0 —	*	*	*
7	7-MeC_{22}	1.6	0.3	1.0	2.1	0.00	1.8 (0.2)	1.5 (0.2)	1.4 (0.3)	2.1 —	*	*	*
8	5-MeC_{22}	2.6	0.6	1.6	4.9	0.00	2.6 (0.3)	2.5 (0.3)	2.6 (1.2)	2.9 —	*	*	*
10	3-MeC_{22}	8.5	1.2	6.9	11.0	0.00	9.3 (1.3)	8.3 (0.9)	7.6 (0.8)	8.7 —	*	*	*
11	*n*-C_{22}	3.0	0.7	1.9	4.4	0.00	2.5 (0.3)	3.7 (0.6)	3.2 (0.5)	1.9 —	*	*	NS
14	3,11-; 3,13-DimeC_{24}	5.2	1.4	2.2	7.7	0.00	6.6 (0.7)	3.9 (0.5)	4.8 (1.5)	6.0 —	*	*	NS
15	7-MeC_{23}	0.5	0.4	0.2	2.2	0.00	0.5 (0.1)	0.7 (0.7)	0.4 (0.1)	0.5 —	NS	*	NS
16	2- or 4-MeC_{23}	2.3	0.6	0.5	3.6	0.00	2.7 (0.6)	2.0 (0.8)	2.2 (0.3)	2.5 —	*	*	*
19	*n*-C_{23}	17.3	2.6	14.1	23.0	0.00	16.5 (2.4)	18.0 (2.6)	18.0 (3.1)	15.3 —	*	*	*
20	9-; 11-MeC_{24}	0.6	0.3	0.3	1.3	0.00	0.7 (0.2)	0.5 (0.2)	0.5 (0.1)	1.3 —	*	*	*
21	7-MeC_{24}	1.2	1.1	0.5	5.2	0.00	1.1 (0.6)	0.9 (0.6)	0.9 (0.4)	5.2 —	*	*	*
23	3-MeC_{24}	0.8	0.2	0.5	1.0	0.00	0.9 (0.1)	0.8 (0.1)	0.7 (0.2)	0.6 —	NS	NS	NS
25	*n*-C_{24}	0.4	0.1	0.3	0.7	0.00	0.4 (0.2)	0.4 (0.1)	0.4 (0.1)	0.4 —	*	*	NS
26	3,11-; 3,13-DimeC_{25}	1.2	0.4	0.6	2.1	0.00	1.5 (0.3)	0.9 (0.2)	1.0 (0.2)	2.0 —	*	*	NS
28	*n*-C_{25}	3.3	1.2	1.9	6.1	0.00	3.4 (1.5)	3.0 (1.0)	3.6 (1.3)	3.8 —	*	*	*
29	9-; 11-MeC_{26}	0.2	0.1	0.0	0.5	0.25	0.2 (0.2)	0.1 (0.2)	0.1 (0.1)	0.5 —	*	*	NS
33	*n*-C_{26}	0.3	0.1	0.0	0.7	0.05	0.4 (0.2)	0.3 (0.2)	0.4 (0.1)	0.3 —	*	NS	NS

35	*n*-C_{27}	2.6	0.8	1.7	5.1	0.00	2.9 (1.1)	2.3 (0.5)	2.7 (0.5)	2.2 —	*	*	*
36	11-MeC_{28}	0.3	0.2	0.0	0.7	0.00	0.3 (0.2)	0.3 (0.2)	0.4 (0.1)	0.2 —	*	*	NS
37	5-MeC_{28}	0.2	0.2	0.0	1.0	0.10	0.2 (0.1)	0.3 (0.4)	0.2 (0.1)	0.3 —	*	NS	NS
39	*n*-C_{28}	0.5	0.4	0.3	2.4	0.00	0.4 (0.1)	0.7 (0.8)	0.4 (0.1)	0.3 —	*	NS	*
40	2- or 4-MeC_{29}	1.9	1.3	0.5	6.6	0.00	2.7 (1.9)	1.7 (0.8)	1.5 (0.4)	0.6 —	*	NS	NS
41	*n*-C_{29}	1.6	0.3	1.0	2.2	0.00	1.8 (0.4)	1.4 (0.4)	1.6 (0.2)	1.6 —	*	*	*
42	11-; 13-; 15-MeC_{30}	0.6	0.3	0.0	1.1	0.10	0.5 (0.4)	0.5 (0.4)	0.7 (0.2)	0.7 —	*	*	*
44	5-MeC_{30}	3.6	0.7	1.8	5.0	0.00	3.8 (0.7)	3.6 (0.5)	3.5 (0.6)	1.8 —	*	*	NS
45	5,17-$DimeC_{31}$	2.7	0.7	1.7	4.0	0.00	2.8 (0.7)	2.4 (0.8)	2.5 (0.5)	4.0 —	*	*	*
47	Unknown 2	0.2	0.3	0.0	1.1	0.60	0.3 (0.4)	0.1 (0.1)	0.1 (0.1)	0.0 —	*	NS	NS
48	3,7-$DimeC_{31}$	0.6	0.3	0.4	1.4	0.00	0.5 (0.1)	0.7 (0.5)	0.7 (0.3)	0.8 —	*	NS	NS
50	*n*-$C_{31:1}$	0.5	0.4	0.0	1.4	0.20	0.5 (0.5)	0.4 (0.4)	0.5 (0.3)	0.0 —	*	*	NS
52	13-; 15-MeC_{32}	0.6	0.3	0.0	1.4	0.05	0.8 (0.4)	0.4 (0.2)	0.5 (0.1)	0.4 —	*	*	*
54	7-; 9-MeC_{32}	0.2	0.1	0.0	0.4	0.30	0.3 (0.1)	0.1 (0.1)	0.2 (0.1)	0.0 —	*	*	NS
55	5-MeC_{32}	1.0	0.2	0.7	1.2	0.00	1.0 (0.2)	1.0 (0.2)	1.0 (0.1)	0.8 —	*	*	NS
56	2- or 4-MeC_{32}	1.5	0.6	1.0	3.5	0.00	1.7 (0.5)	1.3 (0.3)	1.3 (0.1)	3.5 —	*	*	*
57	*n*-C_{32}	0.1	0.2	0.0	0.6	0.55	0.2 (0.2)	0.1 (0.1)	0.1 (0.1)	0.0 —	*	*	*
60	5,13-; 5,17-$DimeC_{35}$	3.2	2.8	0.4	8.9	0.00	2.4 (2.8)	2.7 (2.2)	3.6 (2.7)	8.9 —	NS	NS	NS
61	5,17-$DimeC_{37}$	1.4	0.4	0.8	2.3	0.00	1.4 (0.4)	1.3 (0.5)	1.4 (0.3)	2.0 —	NS	*	NS

[a] Hydrocarbons and peak numbers are the same as those reported in Table 1 and Figure 1. Carbon number is the total number of carbons, including methyl groups.

[b] Values not necessarily zero but may have had a very low quantity (<0.1% of total hydrocarbon) or were simply not detectable for a given GLC analysis.

[c] Proportion of the 20 chromatograms where a given hydrocarbon was not detectable.

[d] Castes are SO = soldier, PS = pseudergate, NY = nymph, and AL = alate (imago).

[e] Proportions of each hydrocarbon were transformed to the arcsine of the square root of the proportion and subjected to analysis of variance. Asterisk indicates significant difference (α = 0.05) between colony, caste, or interaction term for a given hydrocarbon. Analysis of variance was done only for hydrocarbon components detectable from individual termites from collections made in 1985. The gas chromatograph with a 0.75-mm-ID capillary column was used.

TABLE 5. STATISTICS FOR PERCENT HYDROCARBON FROM FOUR CASTES FROM EIGHT COLONIES

		Overall (N = 27)				
Peak No.[a]	Hydrocarbon[a]	$\bar{X}$	SD	Minimum value[b]	Maximum value	Proportion ≤0.1% of total[c]
5	n-C_{21}	12.7	3.6	5.5	19.4	0.00
9	2- or 4-MeC_{22}	1.0	0.3	0.4	1.7	0.00
10	3-MeC_{22}	1.0	0.4	0.3	1.9	0.00
11	n-C_{22}	2.6	0.6	1.8	3.6	0.00
16	2- or 4-MeC_{23}	15.1	5.5	6.1	25.2	0.00
17	n-$C_{23:1}$	3.0	2.0	0.8	8.2	0.00
19	n-C_{23}	33.3	6.2	21.6	43.3	0.00
23	3-MeC_{24}	8.5	2.4	3.8	14.2	0.00
25	n-C_{24}	1.4	0.4	0.1	2.4	0.00
27	2-MeC_{25}	0.3	0.3	0.0	0.7	0.41
28	n-C_{25}	18.7	8.2	10.3	42.7	0.00
29	9-; 11-MeC_{26}	0.2	0.3	0.0	1.0	0.67
31	3-MeC_{26}	0.8	0.2	0.6	1.2	0.00
33	n-C_{26}	0.5	0.5	0.0	2.0	0.30
34	2-MeC_{27}	0.0	0.2	0.0	0.1	0.96
35	n-C_{27}	0.1	0.2	0.0	0.9	0.70
48	3,7-DimeC_{31}	0.1	0.2	0.0	1.0	0.78
51	Unknown 4	0.1	0.2	0.0	0.7	0.70
57	n-C_{32}	0.1	0.1	0.0	0.4	0.78
58	Unknown 6	0.5	0.7	0.0	3.4	0.48
59	Unknown 7	0.0	0.1	0.0	0.5	0.85

[a] Hydrocarbons and peak numbers are the same as those reported in Table 1 and Figure 1. Carbon number is the total number of carbons, including methyl groups.
[b] Values not necessarily zero but may have had a very low quantity (<0.1% of total hydrocarbon) or were simply not detectable for a given GLC analysis.
[c] Proportion of the 27 chromatograms where a given hydrocarbon was not detectable.
[d] Castes are SO = soldier, PS = pseudergate, NY = nymph, and AL = alate (imago).

11-methylheneicosane (peak 6), 3,11- and 3,13-dimethylheneicosane (peak 14, which elutes at nearly the same time as the unique peak 13 found in phenotype I), 3,11- and 3,13-dimethyltricosane (peak 26), 2- or 4-methyloctacosane (peak 40), and 2- or 4-methyhentriacontane (peak 56).

Phenotype III clearly contains the simplest hydrocarbon profile of the phenotypes from California. Only 10 hydrocarbon components comprise greater than 1.0% of the total hydrocarbon fraction (Table 5). This is the only phenotype we collected from California with an olefin (peak 17). All of the other

OF *Zootermopsis* COLLECTED FROM PACIFIC GROVE, CALIFORNIA (PHENOTYPE III)

Castes[d]				*Statistical differences*[e]		
SO (N = 9)	*PS* (N = 8)	NY (N = 8)	AL (N = 2)			
$\bar{X}$ *SD*	$\bar{X}$ *SD*	$\bar{X}$ *SD*	$\bar{X}$ *SD*	Colony	Caste	Inter-action
11.7 (2.5)	13.6 (4.6)	12.4 (4.1)	4.3 (2.0)	*	*	*
1.2 (0.2)	0.9 (0.2)	0.8 (0.3)	1.4 (0.1)	*	*	*
1.3 (0.3)	0.9 (0.3)	0.8 (0.5)	1.2 (0.3)	*	*	*
2.3 (0.4)	2.8 (0.5)	2.8 (0.7)	2.8 (0.3)	NS	*	NS
18.6 (3.0)	14.0 (3.5)	12.3 (7.0)	15.0 (9.6)	*	*	*
4.6 (1.9)	2.0 (0.7)	2.1 (1.9)	3.9 (1.4)	*	*	*
29.1 (4.3)	35.5 (5.0)	36.0 (7.5)	31.7 (4.5)	*	*	*
9.7 (2.4)	7.9 (1.7)	7.3 (2.6)	10.6 (0.4)	*	*	*
1.3 (0.2)	1.5 (0.3)	1.7 (0.4)	0.7 (0.9)	NS	*	*
0.4 (0.2)	0.3 (0.3)	0.3 (0.3)	0.0 (0.0)			
17.5 (5.0)	18.1 (9.5)	21.2 (10.8)	16.3 (4.3)	*	*	*
0.2 (0.2)	0.1 (0.2)	0.1 (0.2)	0.5 (0.7)			
0.9 (0.2)	0.7 (0.1)	0.8 (0.2)	0.8 (0.0)			
0.4 (0.4)	0.6 (0.5)	0.6 (0.7)	0.3 (0.4)			
0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)			
0.1 (0.1)	0.2 (0.3)	0.1 (0.2)	0.0 (0.0)			
0.1 (0.2)	0.0 (0.1)	0.0 (0.1)	0.5 (0.7)			
0.1 (0.2)	0.2 (0.3)	0.2 (0.2)	0.0 (0.0)			
0.1 (0.2)	0.0 (0.1)	0.0 (0.1)	0.0 (0.0)			
0.3 (0.5)	0.8 (1.1)	0.4 (0.4)	0.0 (0.0)			
0.0 (0.1)	0.0 (0.1)	0.1 (0.2)	0.0 (0.0)			

[e] Proportions of each hydrocarbon were transformed to the arcsine of the square root of the proportion and subjected to analysis of variance. Asterisk indicates significant difference (α = 0.05) between colony, caste, or interaction term for a given hydrocarbon. Analysis of variance was done only for hydrocarbon components detectable from individual termites from collections made in 1985. The gas chromatograph with a 0.75-mm-ID capillary column was used.

major (>5.0%) or significant (>1.0%) components of phenotype III are shared with at least one of the other phenotypes. Phenotype IV is also fairly simple, with only 13 significant components (Table 6), and is quite similar to phenotype III. The major differences are the four unique compounds: 2- or 4-methylheneicosane (peak 9), unknown 1 (peak 18) eluting between *n*-tricosene and *n*-tricosane, 7,11-dimethylpentatriacontane (peak 62), and 7,13-dimethylheptatriacontane (peak 63).

Variation in the proportions of hydrocarbons among individuals of each

TABLE 6. STATISTICS FOR PERCENT HYDROCARBON FROM FOUR CASTES FROM FIVE COLONIES OF *Zootermopsis laticeps* COLLECTED FROM RIO RICO, SANTA CRUZ COUNTY, ARIZONA (PHENOTYPE IV)

		Overall (n = 8)[b]					Castes[e]					
						Proportion ≤0.1% of total[d]	SO (*N* = 2)		*PS* (*N* = 4)		*NY* (*N* = 1)	
Peak No.[a]	Hydrocarbon[a]	$\bar{X}$	SD	Minimum value[c]	Maximum value		$\bar{X}$	*SD*	$\bar{X}$	*SD*	$\bar{X}$	*SD*
5	*n*-C_{21}	16.3	7.9	2.5	31.2	0.00	8.6	(8.7)	20.3	(7.7)	15.4	—
9	2- or 4-MeC_{22}	1.9	0.5	1.3	2.5	0.00	2.2	(0.1)	1.7	(0.4)	2.5	—
10	3-MeC_{22}	1.6	0.4	1.1	2.2	0.00	1.8	(0.0)	1.4	(0.3)	2.2	—
11	*n*-C_{22}	2.9	0.4	2.3	3.5	0.00	3.1	(0.2)	2.9	(0.5)	3.2	—
16	2- or 4-MeC_{23}	30.3	4.9	19.3	35.7	0.00	33.4	(3.2)	29.0	(6.6)	30.2	—
17	*n*-$C_{23:1}$	3.0	1.3	1.0	5.2	0.00	3.8	(2.0)	2.4	(1.0)	2.5	—
18	Unknown 1	1.1	0.3	0.5	1.5	0.00	1.0	(0.2)	1.0	(0.5)	1.3	—
19	*n*-C_{23}	23.2	2.4	20.1	27.9	0.00	22.5	(1.1)	24.1	(2.8)	23.7	—
23	3-MeC_{24}	2.3	0.6	1.5	3.2	0.00	3.0	(0.3)	2.0	(0.4)	1.9	—
25	*n*-C_{24}	0.6	0.1	0.5	0.7	0.00	0.6	(0.0)	0.5	(0.0)	0.7	—
27	2-MeC_{25}	0.7	0.2	0.3	0.9	0.00	0.8	(0.1)	0.7	(0.3)	0.8	—
28	*n*-C_{25}	7.8	1.1	5.5	8.7	0.00	8.5	(0.2)	7.0	(1.2)	8.6	—
33	*n*-C_{26}	0.4	0.2	0.0	0.8	0.13	0.7	(0.2)	0.3	(0.2)	0.5	—
35	*n*-C_{27}	2.0	0.8	1.0	3.2	0.00	2.8	(0.6)	1.5	(0.4)	1.5	—
62	7,11-DimeC_{37}	1.5	0.7	1.0	3.2	0.00	2.3	(1.3)	1.2	(0.3)	1.2	—
63	7,13-DimeC_{39}	4.3	1.2	3.5	6.3	0.00	5.0	(1.9)	3.8	(0.3)	3.7	—

[a] Hydrocarbons and peak numbers are the same as those reported in Table 1 and Figure 1. Carbon number is the total number of carbons, including methyl groups.
[b] Includes one analysis of a group composed of four pseudergates and one soldier.
[c] Values not necessarily zero but may have had a very low quantity (<0.1% of total hydrocarbon) or were simply not detectable for a given GLC analysis.
[d] Proportion of the eight chromatograms where a given hydrocarbon was not detectable.
[e] Castes are SO = soldier, PS = pseudergate, and NY = nymph.

caste, among castes within a colony, and among colonies within phenotypes I, II, and III was examined during the first hydrocarbon analyses with individual termites. By using individual termites and the wide-bore (0.75 mm ID) capillary column, we were not able to separate and quantify all hydrocarbon components. Furthermore, in many of the individual chromatograms, one or more of the components were not detected, probably because of insufficient sample. In any case, we conducted analyses of variance of the transformed proportions of each hydrocarbon component (or coeluting components) for phenotypes I, II, and III and found numerous statistically significant differences (Table 3–5). For many of the hydrocarbon components, we observed statistically significant interactions among colonies and castes. This implies that caste-specific blends of hydrocarbons within a phenotype are not consistent from colony to colony. Furthermore, the minimum and maximum values for any given hydrocarbon component for colonies or castes were quite distant.

Much better hydrocarbon resolution and more consistent quantification were achieved with the 0.32-mm-ID capillary column and extraction of five individual termites (of the same caste and from the same colony). Quantification of the percentage of each hydrocarbon component for the four phenotypes of *Zootermopsis* are presented in Tables 3–6. The data for the California phenotypes are more precise because of the increased sample size: 18 colonies for phenotype I, seven colonies for II, and eight colonies for III. Although we did not statistically analyze these data, from the range of the colony and caste means it appears that colony-to-colony differences account for most of the variation. With the exception of the underrepresented alates, variation among the castes was small. Many more minor components ($<0.5\%$ of the total) were detected with the 0.32-mm-ID capillary column. The minimum value for nearly all of the minor components is zero because the component was very low in quantity or was simply not detected for a given GLC analysis (Tables 3–6).

These data suggest caution in assuming that cuticular hydrocarbons serve as semiochemical cues for caste recognition (Howard and Blomquist, 1982). None of the previous studies involved bioassays to test the caste-recognition hypothesis (Howard et al., 1978, 1982; Blomquist et al., 1979), and such bioassays will be very difficult to execute. The information content in these cuticular hydrocarbon mixtures is great. However, if members of a colony are, in fact, cognizant of the caste of each of the other members that they encounter, a chemical cue could involve many classes of compounds other than or in addition to hydrocarbons. This question remains to be resolved, and more data are needed to prove or disprove the hypothesis that hydrocarbons act as caste-recognition cues.

The results of this study clearly identify four distinct, consistent cuticular hydrocarbon phenotypes. Thus far, we (M.I.H., M.P., and L.J.N.) have collected 143 additional *Zootermopsis* spp. colonies throughout much of northern

California to study the geographic distribution of these phenotypes. We have also received live samples from Washington, Oregon, the San Francisco Bay area, and southern California, and have yet to see an additional hydrocarbon phenotype.

Agonistic bioassays pairing a soldier of one phenotype and three pseudergates or nymphs of the same phenotype or a different phenotype indicate that these cuticular hydrocarbon phenotypes are capable of recognizing one another (B.L. Thorne, personal communication). Preliminary results indicate that an encounter between a soldier from one colony and nymphs from a second colony of the same chemical phenotype very rarely produces a highly aggressive response (mandible snapping or biting). Similar encounters between chemical phenotypes II and III virtually always evoke an immediate soldier biting response, as do encounters between soldiers of phenotypes I, II, or III and nymphs of phenotype IV. Finally, encounters between soldiers of phenotype I and nymphs of either phenotype II or III or between soldiers of phenotype II or III and nymphs of phenotype I produce occasional highly aggressive responses that seem to depend on the individual soldier involved and perhaps on the nymphs' parental colony. Agonistic experiments never evoke intraphenotype aggression. All other pairwise interchemical phenotype interactions elicit highly aggressive responses all or part of the time. These results are potentially consistent with the hypothesis that individual termite recognition is based, at least in part, on cuticular hydrocarbon composition. Further evaluation of this behavior is obviously necessary and is the subject of continuing studies (Haverty and Thorne, unpublished).

To summarize the results of this study, identification of four consistent, unique cuticular hydrocarbon phenotypes from three extant species, and inconsistent identification of species using existing morphological keys, should alert insect biochemists, systematists interested in chemotaxonomy with cuticular hydrocarbons, and termite biologists to a major concern. If there are truly only three extant species, then we cannot accept the hypothesis that cuticular hydrocarbon profiles in this genus are species specific. Conversely, if cuticular hydrocarbon profiles are truly species specific, then there is at least one new, undescribed species of *Zootermopsis*. It would appear that *Z. angusticollis* and *Z. laticeps* are valid species, but *Z. nevadensis* is a complex of at least two species or one species with polymorphic hydrocarbon chemistry. We feel that it is extremely important to clarify the taxonomic status of this genus, as it has been frequently used as a model for behavioral and caste-determination studies. Determination of specific status of each phenotype may take many different approaches, including bioassays of aggressive behavior (in progress), breeding experiments (in progress), correlation of hydrocarbon chemistry and external morphology from an extensive geographic collection (in progress), enzyme analysis, DNA hybridization, etc. These studies will probably result in a revision of the genus.

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PRECISE CHARACTERIZATION OF CUTICULAR COMPOUNDS IN YOUNG *Drosophila* BY MASS SPECTROMETRY

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Abstract—Cuticular hydrocarbons of young *Drosophila* flies are singular with very long chains and complex diene mixtures. A precise characterization of these substances was carried out by epoxidation and analysis of the products by GC-MS with negative chemical ionization. In *D. melanogaster*, double bonds of dienes are more probable at carbon positions 11 or 13 and 21 or 23. In *D. simulans*, double bonds are shifted more towards the interior of the chain. Such a difference is also found among monoenes of both species. The analyses of monoenes and dienes confirm the similarity of cuticular compounds of young flies of both sexes in both species. A main cuticular compound of *D. erecta* females, 9, 23-tritriacontadiene, is also presented.

Key Words—*Drosophila melanogaster, Drosophila simulans*, Drosophilidae, Diptera, aphrodisiac pheromone, cuticular hydrocarbon, double bonds, epoxidation, chemical ionization, mass spectrometry, (Z,Z)-7,11-heptacosadiene, alkenes, olefins.

INTRODUCTION

The behavioral effects of long-chain unsaturated hydrocarbons in *Drosophila melanogaster* have been clearly demonstrated. Cuticular extracts of these substances from mature female flies can induce wing vibration in males. This is one of the most characteristic precopulatory behaviors in these insects (Jallon and Hotta, 1979). Using such a bioassay, we were able to identify the molecules responsible for this response as being between 25 and 29 carbons long in a complex mixture, the principal component of which is (Z,Z)-7,11-heptacosa-

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diene, a substance that is characteristic of mature females (Antony et al., 1985). (Z)-7-tricosene has been shown to be an aphrodisiac for males of the sympatric sibling species, *Drosophila simulans* (Jallon, 1984).

Other behavioral analyses have shown that in *D. melanogaster*, young flies of both sexes are courted by mature males, suggesting that young flies produce signals that mimic those of mature females (Cook and Cook, 1975; Jallon and Hotta, 1979; Tompkins et al., 1980). The exact nature of the chemical stimuli produced by these young flies is therefore of interest. Preliminary chromatographic analyses of young *D. melanogaster* revealed a very particular hydrocarbon pattern with similar profiles for the two sexes: the major components were long chains of 29 to 35 carbons. Branched alkanes were abundant, and unsaturated hydrocarbons seemed to consist of a complex mixture of position isomers (Antony and Jallon, 1981; Antony, 1984).

The localization of double bonds in olefins is a classic problem of analytical chemistry. Direct methods involving GC-MS with chemical ionization using gases like isobutane, methylamine, or methyl vinyl ether, have been established. However, extrapolation of such methods to high-molecular-weight dienes and polyenes was not simple (Budzikiewics and Busker, 1980; Einhorn et al., 1985; Doolittle et al., 1985). Many methods involve first a derivatization (Baker et al., 1963; Aplin and Coles, 1967; Suzuki et al., 1981) but the analysis of the products is always more or less laborious. Mass spectrometry with electron impact has been helpful in the analysis of several of these substances, although a significant amount of the product was necessary in the determination (Capella and Zorzut, 1968; Blomquist et al., 1980; Suzuki et al., 1981; Dunkelblum et al., 1985).

For the study of the structure of mixtures, chemical ionization is better than electron impact as it minimizes background and therefore the diagnostic fragment peaks are more obvious (Budzikiewics and Busker, 1980). Tumlinson et al. (1974) have analyzed a series of epoxydecanes in this way, with methane and isobutane as reagent gases, but the mass spectra were not so simple.

In the past we have used methoxymercuration–demercuration coupled with CI mass spectrography analysis to describe the monoenes of mature *D. melanogaster* males, and mature flies of both sexes in *D. simulans* (Pechiné et al., 1985). This method revealed that in mature *D. melanogaster* females the double bonds were mainly in positions 7 and 11 (Antony et al., 1985). However, there was an inherent difficulty in this method because the derived methoxy products which correspond to a given isomer produce two pairs of two fragmentation peaks separated by 14 mass units. Such a method was therefore not sufficiently precise for the study of complex mixtures.

Bouchoux et al. (1987) have recently developed a new method to localize the epoxide ring position in short aliphatic chains by negative ion chemical ionization–mass spectrometry. Abundant pseudomolecular anions $(M-H)^-$ are

produced. Moreover each isomer yields only two major characteristic peaks: one with a higher mass and one with a lower mass.

After checking the applicability of such a method to heavy unsaturated hydrocarbons already characterized by other methods, we have used it to compare monoenes and dienes present as a complex mixture in the cuticles of young males and females of both *D. melanogaster* and *D. simulans.*

METHODS AND MATERIALS

GC-MS Analysis. Raw products were injected into a Girdel 31 chromatograph. The machine contained an all-glass solid injector, and separation took place in a CP Sil 5 (Chrompack) capillary column, 25 m × 0.34 mm, programmed between 200 and 300°C at 5°C/min. which well separated monoenes and dienes of a given chain length. The chromatograph may be coupled to a Nermag R10-10 mass spectrometer. Either electron impact ionization (70 eV) or chemical ionization was used. In the latter case, negative chemical ionization took place by placing into the source 0.1 torr of a mixture containing 90% methane and 10% nitrous oxide (N_2O). The ionizing agent was therefore the OH^- ion. The temperature of the source was maintained at 200°C. This technique allowed us to characterize quantities of dienes of as little as 10^{-10} mol.

Cuticular Extracts of Insects. Pools of young flies ($N = 250$), of each sex and each species (*D. melanogaster* Canton S and *D. simulans* Seychelles) were collected 3–6 hr after eclosion and washed with hexane as previously described (Antony and Jallon, 1982).

Their compositions were deduced from direct GC-MS analysis with electron impact (70 eV) (Table 1). In all cases they consisted of long chains, mainly with 27–37 carbons. Branched alkanes, with methyl mainly on position 2 (as in mature flies) were specially abundant for 29 carbons as were 31-carbon monoenes. Dienes with 31–37 carbons were more abundant in young *D. simulans* flies

Epoxidation of Double Bonds. Cuticular extracts containing on average 10^{-8} mol/μl of unsaturated hydrocarbons were dissolved in hexane. Ten microliters of this extract were mixed with a 10-μl solution of methylene chloride, containing 3×10^{-6} mol of metachloroperbenzoic acid together with 1 μl of a solution of sodium bicarbonate in water (5×10^{-4} mol). The mixture was agitated for 3 hr at ambient temperature. The products were analyzed immediately and without prior separation using GC-MS. In such conditions all double bonds are epoxidized and dienes are completely transformed into diepoxydes. It is possible to keep the products for 24 hr in a freezer before analysis without significant degradation.

Chemical Compounds. 9-Tricosene was obtained commercially (Sigma).

TABLE 1. CALCULATED PERCENTAGES (%) OF MAIN HYDROCARBON COMPOSITIONS IN CUTICLE OF YOUNG FLIES OF EITHER SEX AND EITHER SPECIES (*D. melanogaster* CANTON S AND *D. simulans* SEYCHELLES)[a,b]

	me C F	me C M	si S M	si S F
27 Carbon				
Diene				
2-Methyl-alkane	4	3	2	1
Monoene				
n-Alkane				
29 Carbon				
Diene				
2-Methyl-alkane	35	33	30	24
Monoene	5	11	10	6
n-Alkane	1	3	5	1
31 Carbon				
Diene	2	3	tr	tr
2-Methyl-alkane	7	13	2	7
Monoene	27	20	24	20
n-Alkane				
33 Carbon				
Diene	8	5	5	13
2-Methyl-alkane			15	16
Monoene	7	3		
n-Alkane		4	1	
35 Carbon				
Diene	4	2	5	12
Monoene	1		2	
n-Alkane				

[a] GC-MS analysis with EI ionization.
[b] meC: *D. melanogaster* Canton S; si S: *D. simulans* Seychelles; F: female; M: male.

Diepoxides were prepared according to the method described above, using the following dienes: 7,11-heptacosadiene present in mature *D. melanogaster* females (Antony et al., 1985) and a tritriacontadiene present in mature *D. erecta* females (Jallon and David, 1987).

RESULTS

Application of the Method to Analysis of Known Simple Compounds. Commercial 9-tricosene produces a mono epoxy compound, 9,10-epoxytricosane, the spectrum of which is presented in Figure 1A. The molecular mass can be

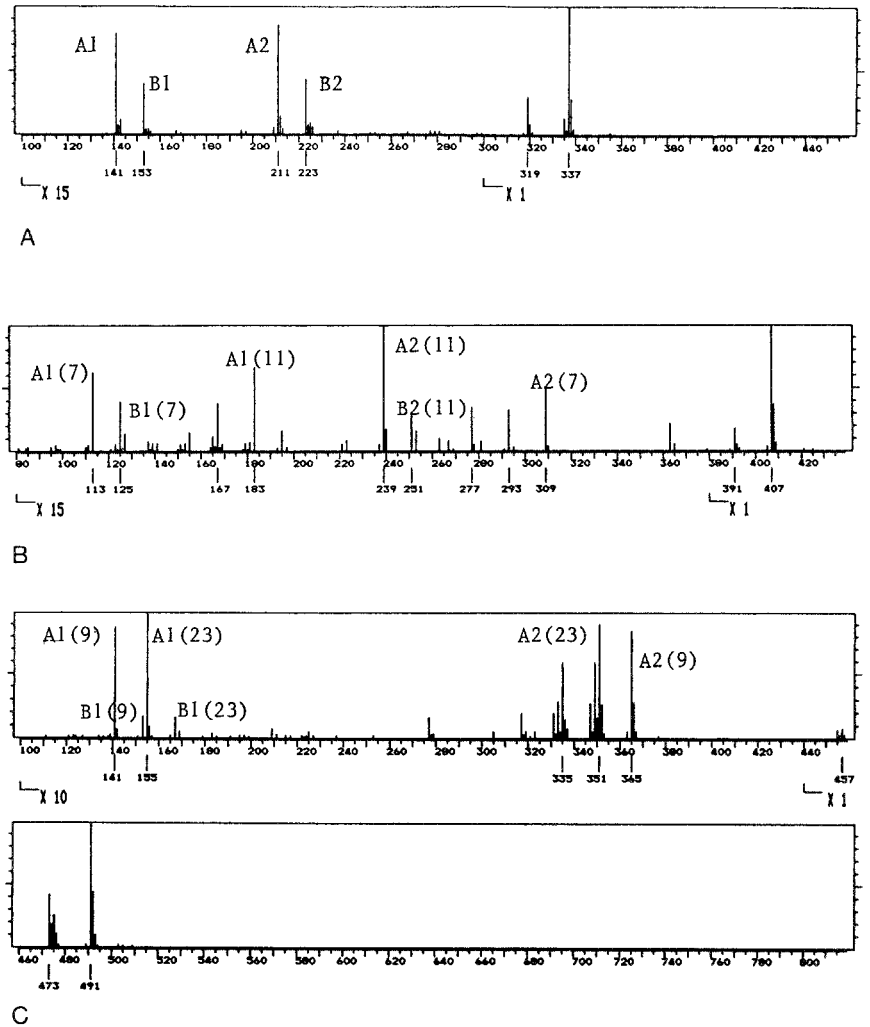
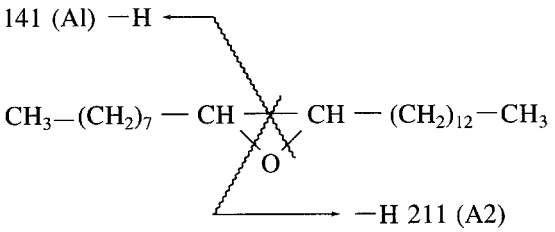
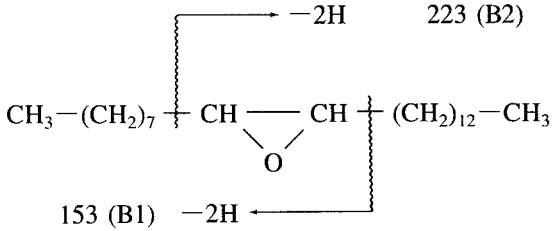


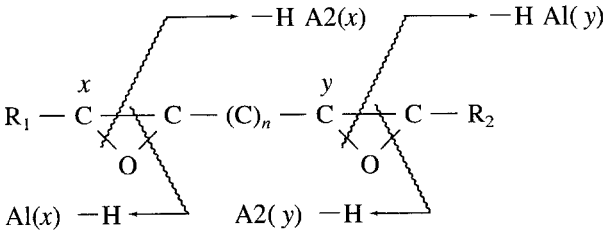
FIG. 1. Mass spectra of epoxyderivatives of various model compounds, negative chemical ionization (OH^-). (A) 9-epoxy-tricosane; (B) 7,11-diepoxy-heptacosane; (C) 9,23-diepoxy-tritriacontane.

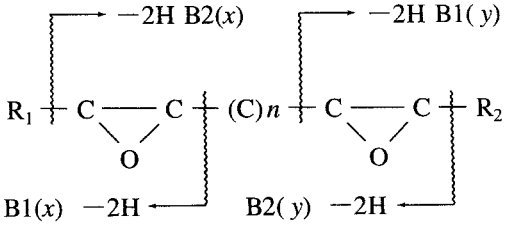
deduced as being *m/z* 338 from *m/z* 337 (for the M−H ions) and from *m/z* 319 (for $M-H-H_2O$). Two other fragmentation types (A, B) are associated with the epoxide group. Type B produces low intensity peaks; type A produces peaks that are higher than B, but still relatively low.





Figures 1B shows the mass spectrum of the diepoxide derived from 7,11 heptacosadiene, the main cuticular component produced by mature *D. melanogaster* females Canton S and already described (Antony et al., 1985). Two main fragments, A and B, were produced for each of the two epoxy groups situated at carbon positions *x* and *y*.





For this 7,11-diepoxy heptacosane, where the positions of the two epoxide groups are relatively close, the fragmentation produced the main following peaks: A1(7) = 113, A2(7) = 309, B1(7) = 125, A1(11) = 183, A2(11) = 239, and B2(11) = 251. Secondary ions from type A dioxygenated ions were produced by elimination of oxygen, water, and formaldehyde (ions *m/z* 137, 167, 263, 277, and 293).

Figure 1C shows the mass spectrum of the diepoxide of a major dienic component of the cuticle of *D. erecta* females. This member of the *Drosophila melanogaster* species subgroup is characterized by a marked sexual dimorphism, with mature females having hydrocarbons of a much greater length than in the other seven species of the subgroup (Jallon and David, 1987). In this diepoxide, a simpler mass spectrum is obtained where type A fragments are predominant. The A ions containing two oxygen atoms, at *m/z* 351 and 365, after loss of oxygen produce ions at *m/z* 335 and 349, and after loss of water produce ions at *m/z* 333 and 347; subsequent losses of oxygen and water produce ions at *m/z* 317 and 331. In this *D. erecta* female-specific $C_{33}H_{64}$, it is possible to localize the epoxide groups which are relatively far from each other, at positions 9 and 23.

Spectra 1B and also 1C show that negative chemical ionization with OH^- enables the two epoxide groups to be easily localized even in a long carbon chain. Two main fragment ions are visible, corresponding to each epoxide function, and the resultant masses are characteristic of their respective positions. Although these two substances have the same degradation scheme, certain fragmentations are favored in either isomer, and they produce characteristic and markedly different mass spectra.

Analysis of Cuticular Monoenes of Young D. melanogaster Males and Females. The same method was used to study the mixtures of unsaturated compounds present in the cuticles of young *D. melanogaster* flies. In both sexes, the cuticle of the young flies includes long chains, mainly of 27–35 carbons consisting of branched alkanes, mainly 2 methyl, and linear monoenes and dienes.

The monoenes with 29–31 and 33 carbons show a few quantitative differences between males and females (Table 1). Moreover, among monoenes, mixtures of position isomers were found in both sexes. The double bond may exist at any carbon between positions 5 and 16. Figure 2 (A,B) shows two examples of double bond distributions in monoene chains with 31 and 33 carbons of young *D. melanogaster* C5 males. Uneven carbon positions are preferred for the double bonds as against even carbon positions. Position 9 is more favored in C_{31} (and also in C_{29}) than in C_{33} where position 11 is more probable. Whatever the chain length, the probability for one double bond being in position 13 is always

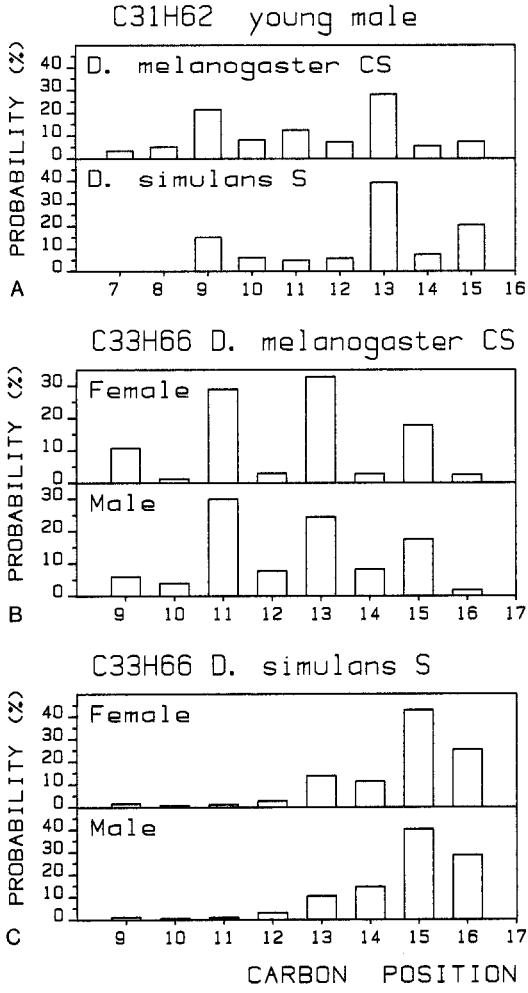


FIG. 2. Examples of double bond distributions in young flies' cuticular monoenes. The probability for a double bond being present on a given carbon as deduced from the analysis of negative chemical ionization of monoepoxyderivatives of monoenes and the distribution histograms are shown. (A) Hentriacontenes of either *D. melanogaster* (Canton S) or *D. simulans* (Seychelles) young males. (B) Tritriacontenes of young *D. melanogaster* Canton S of either sex. (C) Tritriacontenes of young *D. simulans* Seychelles of either sex.

more than 25%. The same situation was observed in young *D. melanogaster* females with small quantitative differences (Figure 2B).

Analysis of Cuticular Dienes Extracted from Young D. melanogaster CS Males and Females. Cuticular dienes of young *D. melanogaster* CS females were analyzed first. Figure 3A shows the spectrum corresponding to the diepoxides produced for the most abundant dienes: tritriacontadienes. The fragmentation pattern is complex and seems to be produced by several isomers. A similar result was found for dienes of 31, 35, and 37 carbons. The ions produced by the A fragmentations (113, 127, 141, 155, etc.) are present in a greater proportion than those produced by the B ruptures (125, 139, 153, 167, etc). This mass spectrum is substantially different from that of 7,11-diepoxide heptacosane reported above (Figure 1*B*) but seems closer to that of 9,23-diepoxy tritriacontane (Figure 1*C*). It seems, therefore, that in these compounds the major epoxide groups are far apart. The major ions (127–211 and 295–379) therefore correspond, respectively to type A monoxygen ions [A1(x) and A2(y)] and dioxygen ions [A2(x) and A1(y)].

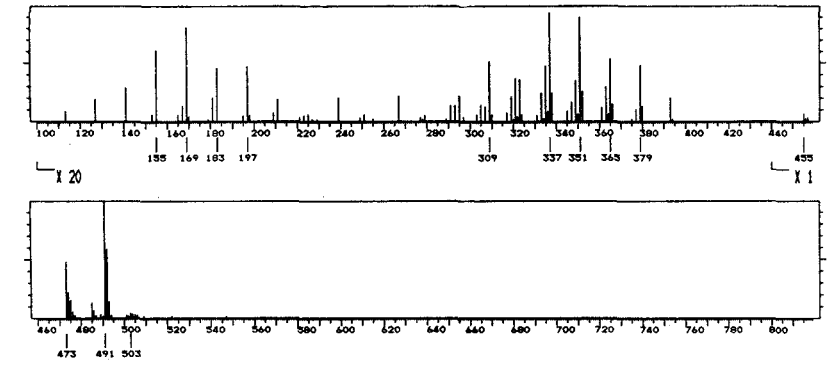
By measuring the intensities of the characterized ions, it is possible to construct a histogram showing the probabilities of the double bond being found at each carbon position. Figure 3B shows such a histogram for the tritriacontadienes of young female flies, which show a clear bimodal distribution.

Figure 4 shows the position probabilities for the other dienic compounds in young males and young females. These figures show that the probability distribution is symmetrical in either half of the chain, with a marked preference for the center of each half (for example, positions 11 and 22, or 10 and 23, are equivalent for tritriacontadienes). The results for the young males and young females are rather similar: There seems to be little dimorphism in the composition of dienic cuticular compounds in young *D. melanogaster* CS flies.

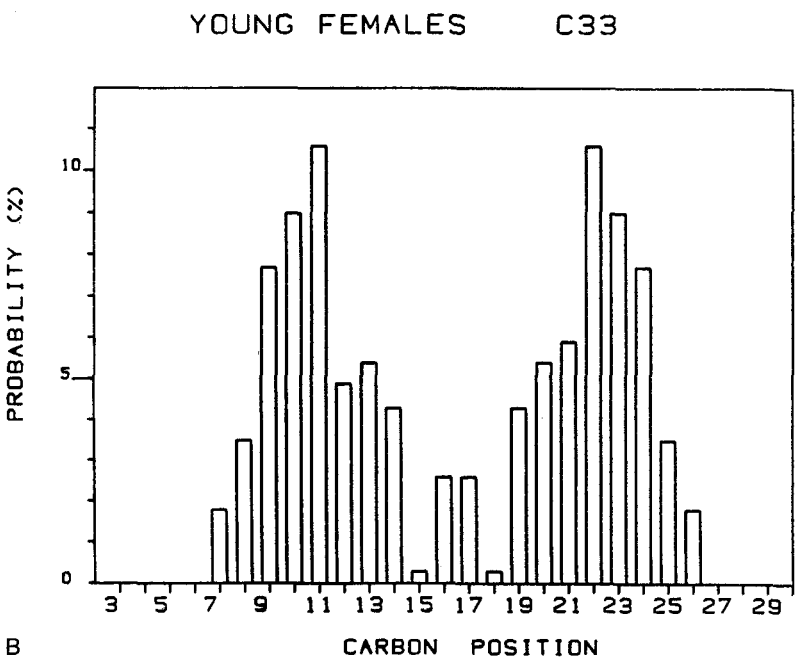
The first double bond in the hentriacontadienes ($C_{31}H_{60}$) is mainly situated between carbons 9 and 12, and the second between carbons 21 and 24; for the tritriacontadienes ($C_{33}H_{64}$), the distribution is similarly between 9–12 and 21–24. For the pentatriacontadienes ($C_{35}H_{68}$), the double bonds are mainly found between carbons 11–14 and 21–24. The double bonds in the heptatriacontadienes ($C_{37}H_{72}$) are generally situated between carbons 11–16 and 21–26.

Analysis of Monoenes and Dienes in Cuticular Washes of Young D. simulans. We performed similar studies on cuticular compounds of young male and female flies of the species *D. simulans* (Seychelles strain). (Table 1).

Monoenes showed a mixture of position isomers less rich than in *D. melanogaster*, with little difference between the sexes. Figure 2 (A,C) shows that the double bond distributions are less different between both species for chains of 31 carbons than for chains of 33 carbons. In the former case, position 13 is



A



B

FIG. 3. Cuticular tritriacontadienes of young females of *D. melanogaster* Canton S. (A) Negative chemical ionization mass spectra of diepoxide derivatives of these tritriacontadienes. (B) Histogram of double bond distribution in these tritriacontadienes.

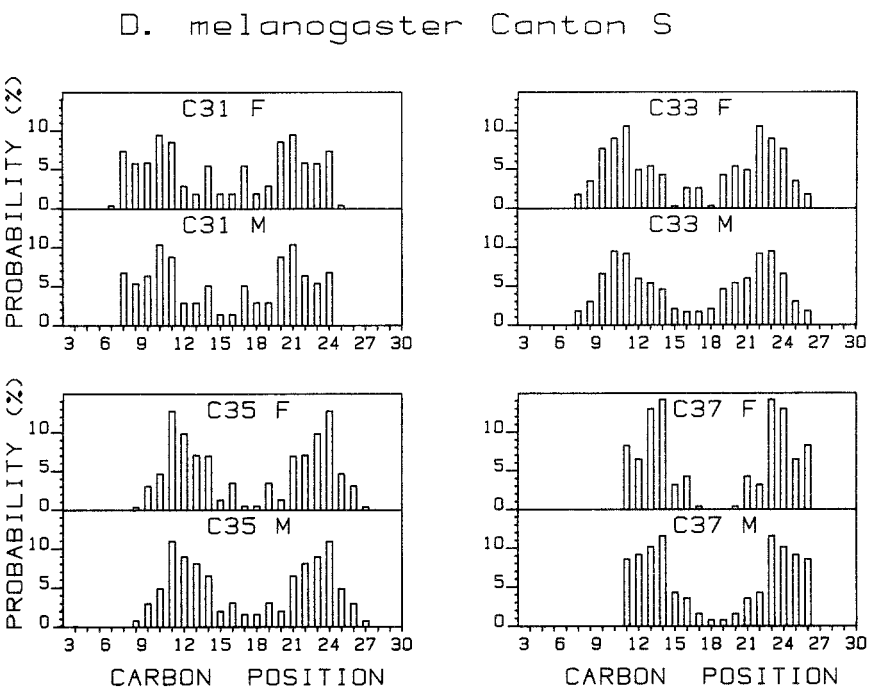


FIG. 4. Distributions are compared of double bonds in cuticular dienes of both young females and young males of *D. melanogaster* (strain Canton S) deduced from the mass spectral analyses of diepoxyderivatives. Respective chain lengths are 31, 33, 35, and 37 carbons.

preferred in both species but position 15 is more represented in *D. simulans*. In the latter case, position 15 is favored in *D. simulans* and positions 11–13 in *D. melanogaster*.

Distributions of double bonds in dienes of young *D. simulans* deduced in a similar way from epoxidation of the dienes and analysis of diepoxy products by negative chemical ionization GC-MS are presented in Figure 5. These distributions are clearly different from the bimodal distributions of *D. melanogaster* CS. The simulans distributions appear trimodal for chains with 33 and 35 carbons, with an obvious maximum in the middle. There is a relatively high probability of finding a double bond between carbons 15 and 19. It should be recalled here that in monoenes position 15 was also favored among tritriacontenes. The distributions obtained for the two sexes were again not very different.

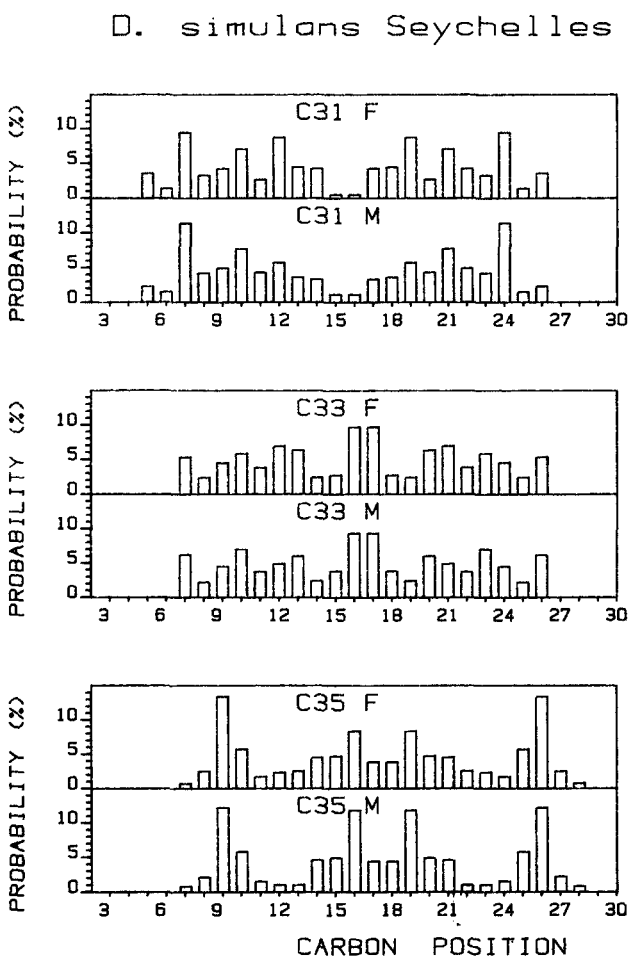


FIG. 5. Distribution is compared of double bonds in cuticular dienes of both young females and young males of the species *D. simulans* (strain Seychelles) deduced from the mass spectra analyses of diepoxyderivatives. Respective chain lengths are 31, 33, and 35 carbons.

DISCUSSION

We have used a recently developed method combining a chemical derivatization with negative chemical ionization analysis of its products which has good sensitivity and does not require any intermediate purification (Bouchoux et al., 1987). Using this method we have characterized the complex mixtures

of long chain unsaturated hydrocarbons present in pools of young *Drosophila*. We were able to establish the probability of a double bond being on a given carbon, but we were not able to determine with certainty which two double bonds were present in a given diene molecule.

In *D. melanogaster* small quantitative differences and no qualitative differences were detected among cuticular hydrocarbons of young males and females 3–6 hr after eclosion. They were all longer than those of mature flies, 27–37 carbons instead of 23–29 carbons. Like mature females, young flies possess dienes. However, their double bonds are not in discrete positions (7, 11) but are scattered over several carbons. These double bonds are farther apart than in mature females, one tending to be on carbon 11 or 13, the other on carbon 23 or 21. Among major monoenes, double bonds are also scattered among various positions, especially carbons 13, 11, and 9, and not the position 7 favored in mature flies. There is one more structural similarity between monoenes and dienes of young flies: there is a tendency for the more probable position of double bonds to shift towards the inside of the chain when this chain becomes longer. It is strongly suggested that double bonds are widely separated in the dienes of these young flies, a marked difference from the 2 CH_2 distance in 7,11-heptacosadiene, characteristic of the mature female.

The existence of singular long chain dienes in young flies has been recently reported in two other species of *Drosophila*: *Drosophila virilis* (Jackson and Bartelt, 1986) and *Drosophila pseudoobscura* (Blomquist et al., 1985). Moreover, in *Drosophila virilis*, the position of double bonds among monoenes and dienes was observed to change with age (Jackson and Bartelt, 1986).

Although dienes are completely absent in mature females of the species *D. simulans*, young flies of this species do produce such compounds, as in the case of young *D. melanogaster* flies. The dienes of young flies of both species appear to share some structural features: double bond distributions in the spectrum of isomers are similar between sexes, but not similar according to chain length. However, the detected isomers in *D. simulans* are different from those of homologous hydrocarbons of young *D. melanogaster* as a trimodal distribution was observed in *D. simulans* and a bimodal one in *D. melanogaster*. There were also obvious differences among monoenes of both species.

We have clearly shown that the heavy hydrocarbons specific to young flies also show a species specificity. It remains unclear whether this wide heterogeneity of isomers reflects variations between young individuals, between the different parts of each fly from which the components are unequally extracted, or actually reflects the complexity of the mixture borne by a single individual. Indeed, although sensitive, the method requires pools of flies.

Live young flies producing such complex mixtures of unsaturated hydrocarbons have been shown to stimulate wing vibrations from mature tester males

as much as mature females do (Jallon and Hotta, 1979). However, bioassays of hexane washes of young flies showed a poor reproducibility and were much less stimulatory than those for mature female washes. We cannot be certain if we extracted only the efficient cuticular hydrocarbons, or if we mixed in the extract inhibitors derived from internal tissues. Another possibility is that the poor results obtained with bioassays involving these heavy hydrocarbons might be linked to any defect in reconstituting the hydrocarbon layer of the epicuticle.

These singular compounds of young flies, whose biological role remains unclear, tend to disappear during the first two days, so after eclosion, together with young males attractivity, while sex-specific compounds of mature flies appear and become more and more abundant (Antony and Jallon, 1981). We have also shown that such long chain dienes with distant double bonds (9–23) are present in the cuticle of mature females (and not in mature males) of the related species *D. erecta*.

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EFFECT OF JUVENILE HORMONE ANALOG, FENOXYCARB, ON PHEROMONE PRODUCTION BY *Ips paraconfusus* (COLEOPTERA: SCOLYTIDAE)

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Abstract—Topical application of the juvenile hormone analog, fenoxycarb, in acetone induced newly emerged male California five-spined ips, *Ips paraconfusus* Lanier, to become attractive to females, as measured by positive responses to male abdominal extracts in a laboratory bioassay. Two pheromones, ipsdienol and ipsenol, were detected by gas chromatography in the abdominal extracts of fenoxycarb-treated males. Pheromone production was minimal at a dose of 0.1 μg/insect of fenoxycarb, maximal at 10 μg, and was reduced to unmeasurable amounts at a dose of 100 μg. In comparison, peak production of pheromones was induced at a dose of 0.1 μg/insect of natural juvenile hormone (JH III). Treatment with 10 μg of fenoxycarb resulted in the occurrence of pheromones 12 hr after exposure, maximal pheromone content between 16 and 20 hr, and undetectable amounts after 36 hr. The demonstration that fenoxycarb is an active juvenile hormone analog for a bark beetle suggests that it may have practical utility in managing these insects.

Key Words—Juvenile hormone analog, juvenile hormone, JH III, *Ips paraconfusus*, Coleoptera, Scolytidae, bark beetles, aggregation pheromones, pheromones, ipsenol, ipsdienol.

INTRODUCTION

Encouraging prospects for the practical use of juvenile hormone analogs (JHAs) has arisen from recent tests with fenoxycarb, ethyl[2-(*p*-phenoxyphenoxy)ethyl]carbamate, a nonneurotoxic insecticide that exhibits strong juvenile hormone activity against numerous insect pests in various orders (Anonymous,

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1983; Dorn et al., 1981; Masner et al., 1981). This compound achieved satisfactory control of a broad spectrum of lepidopterous and coleopterous pests in stored products (Kramer et al., 1981). It was also efficacious against leafrollers in orchards (Reede et al., 1984, 1985). Fenoxycarb is highly persistent in various environmental conditions, with residual activity up to one year in stored wheat (Edwards and Short, 1984) and at least four weeks on apple leaves (Dorn et al., 1981; Reede et al., 1984). On the other hand, fenoxycarb demonstrates a very low mammalian toxicity (acute oral LD_{50}, rat, $>10{,}000$ mg/kg; Anonymous, 1983). Trials under laboratory and various field conditions have shown that fenoxycarb appears to be innocuous against beneficial arthropods (Dorn et al., 1981; Parrella et al., 1983; Reede et al., 1984). Furthermore, it is effective against insects which have developed resistance to conventional insecticides (Edwards and Short, 1984). Finally, the compound is easy to synthesize and is therefore relatively inexpensive (Karrer and Farooq, 1981). It would be highly desirable if fenoxycarb could be used for control of bark beetles, which are major forest insect pests throughout much of the Northern Hemisphere.

Aggregation pheromones play a vital role in the reproductive biology of scolytid beetles (Borden, 1982, 1985). Research has demonstrated that pheromone production by bark beetles is under the influence of juvenile hormone (Borden et al., 1969; Hughes and Renwick, 1977a,b; Harring, 1978; Bridges, 1982). It is significant, therefore, to investigate the effect of a JHA on pheromone biosynthesis of a bark beetle as an indicator of its bioactivity and potential use. This paper reports the effect of fenoxycarb on pheromone production by the California five-spined ips, *Ips paraconfusus* Lanier. We chose *I. paraconfusus* because it is established that pheromone biosynthesis in this beetle is under neuroendocrine control (Hughes and Renwick, 1977a) and that it is inducible by exogenous JH treatments (Borden et al., 1969; Hughes and Renwick, 1977a). Moreover, it is easily cultured in the laboratory and readily responds to its pheromones in laboratory olfactometers (Wood and Bushing, 1963; Borden, 1967).

METHODS AND MATERIALS

Insect Rearing. I. paraconfusus were obtained from a continuously reared, laboratory culture maintained on logs of ponderosa pine, *Pinus ponderosa* Laws., in laboratory cages at $25 \pm 1°C$ and variable relative humidity. Emergent brood beetles were collected daily and sexed by the presence of the pars stridens on the posterior dorsal area of the head of females, but not males (Wood, 1961). Beetles were stored on moistened paper towels in glass jars at 4°C until used for experiments.

Treatment of Beetles. Fenoxycarb (96.6% purity, Elanco Division, Eli Lilly Canada Inc., Winnipeg, Manitoba) and JH III (96.9%, Sigma Chemical Co., St. Louis, Missouri), respectively, were dissolved in acetone to give 0.1, 1, 10, and 100 μg/μl of solution. Newly emerged male beetles were immobilized by cooling at 4°C and stuck on scotch tape with their abdominal venter upward. Each beetle received two topical applications, made to the abdominal venter, of 0.5 μl of a given solution approximately 1 min apart administrated with a 25-μl Hamilton syringe attached to a 50-stop Hamilton repeating dispenser. Treated beetles were incubated individually for a specific length of time at room temperature in glass cylinders (1.78 cm ID) placed in Petri dishes (Conn, 1981). All Petri dishes contained a water-saturated cotton wick and were sealed with parafilm to minimize loss of moisture.

Preparation of Abdominal Extracts. Groups of 12 or 24 male beetles were either: (1) treated topically with acetone extracts of fenoxycarb or JH III at 0.1, 1, 10, or 100 μg/insect and incubated at room temperature for 20 hr; (2) treated with 1 μl acetone applied in the same manner and held as above; (3) left untreated and held as above; or (4) introduced into preformed holes in ponderosa pine logs (Miller and Borden, 1985) and allowed to bore in the inner bark for 20 hr. For analysis by gas chromatography (GC), additional groups of 12 males were topically treated with 10 μg of fenoxycarb and incubated for 12, 16, 28, or 36 hr. All insects were taken from Petri dishes or excised from the bark, and their abdomens (containing hindgut and Malpighian tubules) were removed and immediately immersed in double-distilled pentane in 2 ml glass vials in Dry Ice.

For bioassays, 24 beetle abdomens from each treatment group were pooled and crushed with a glass rod in 200 μl of pentane. After crushing, samples were allowed to warm up slightly at room temperature and the clear extracts were transferred to clean 2-ml glass vials with one pentane rinse to give a 240-μl extract at a concentration of 1 abdomen/10 μl of solution. Vials containing extracts were covered with Teflon-lined lids and stored at −44°C until used in bioassays.

For analysis by GC the same extraction procedure was followed except that: (1) single abdomens were extracted with 100 μl of pentane without a rinse; and (2) solutions of two internal standards, 3- and 2-octanol, each at a concentration of 2.5 μl (2 mg) in 10 ml of pentane, were added before and after crushing, respectively, at the rate of 2.5 μl/sample (Conn, 1981).

Bioassay Procedure. The attractiveness of male abdominal extracts was bioassayed with recently emerged female *I. paraconfusus* in an open arena olfactometer (Conn, 1981). The arena was a 24-cm-diam. disposable filter paper. A glass tube (7 cm long × 1 cm ID) containing a rolled-up filter paper (4.25 cm diam.) impregnated with a stimulus was placed to allow an air stream

to flow at a rate of 1500 ml/min through it toward the insect release point at the center of the arena. Bioassays were conducted at room temperature under red light.

Three types of stimuli were tested: (1) beetle abdominal extracts; (2) a pentane control; and (3) a pheromone standard consisting of racemic ipsenol, racemic ipsdienol, and *cis*-verbenol (Borregaard, A.S., Sarpsborg, Norway) at concentrations of 2, 1.5, and 1 ng/μl in pentane, respectively. Each stimulus was assayed at the rate of 20 μl, or two male equivalents for abdominal extracts, for each group of test females. A fresh stimulus tube was used for each test, and the arena was changed for each new stimulus.

Beetles that walked normally and had no missing appendages were held in groups of 20 in 35-mm-diam. Petri dishes lined with moistened filter paper at 2–4°C. They were brought to room temperature about 2 min prior to use. Each group of insects was released in the center of the arena and given 2 min to respond. An insect was counted as a positive responder if it walked upwind and stopped within 1 cm of the stimulus source or circled around the edge of the airstream more than once. It was classified as a nonresponder if it walked off the arena or remained in the arena after 2 min without responding. Once its status was classified, an insect was returned to the Petri dish, which was placed at the end of a rotation of 13 other Petri dishes. No group of insects was reused for at least 60 min. There was no evidence that this procedure resulted in a change in response through time in this or in studies with other scolytids (Conn, 1981; Stock and Borden, 1983).

The bioassay experiment was completed in two sessions of 4 hr each in the same day. Assays were conducted before each session with pentane and the pheromone standard to ensure that the insects were responding consistently.

Gas Chromatography. GC analysis was conducted on a Hewlett-Packard 5880A gas chromatograph fitted with a flame-ionization detector and a glass capillary column (30 m × 0.50 mm ID) coated with SP-1000 (Supelco Inc., Bellefonte, Pennsylvania). The oven temperature was programmed from 120 to 180°C at 4°C/min, with injector and detector temperatures of 260 and 275°C, respectively.

Samples which were kept in Dry Ice were warmed to room temperature, and 2 μl of each sample was injected manually into the injection port. A standard sample consisting of the synthetic pheromones, ipsenol, ipsdienol, and *cis*-verbenol was recurrently analyzed under identical conditions as the beetle extracts. Determination of pheromone content in the samples was based on comparison of retention times with those of the standard sample. Quantification of ipsenol and ipsdienol was achieved by using the area under the 3-octanol peak as a reference. The 2-octanol peak was used to check for possible loss of volatiles during processing of the samples.

Statistical Analysis. Proportional data, including percentages of respond-

ing females in bioassays and males containing pheromones in GC analyses were converted using an arcsin transformation and compared using a Newman-Kuels test modified for testing proportions (Zar, 1984).

A maximum likelihood method[3] was used to adjust the mean amounts of pheromone produced by males treated with fenoxycarb or JH III, and to estimate trace amounts of pheromone when they were below the measurable threshold (8.0 ng) of the GC equipment. By replacing zero values with estimated values, this method greatly reduced sample variances. Differences among estimated means were then assessed by the Newman-Kuels test, and differences from zero by *t* tests.

RESULTS AND DISCUSSION

Within 20 hr after topical treatments of male beetles with fenoxycarb or JH III their abdominal extracts became attractive to females, when compared with the responses of females to pentane or extracts of either untreated or acetone-treated males (Table 1). Response was maximal to extracts of males treated with 10 μg of fenoxycarb, but significantly less than maximal to extracts of males subjected to the 100-μg treatment. Abdominal extracts from males treated with 0.1 μg of JH III were as attractive to females as those from males treated with 10 μg of fenoxycarb (Table 1). However, there was a progressively weaker response as the dose of JH III increased. The pheromone standard and extracts from males fed in logs (and thus exposed to the pheromone precursor, myrcene) elicited the strongest responses by females (Table 1).

The bioassay results were supported by gas chromatographic analysis (Table 2). Treatment of male beetles with fenoxycarb or JH III resulted in the occurrence of ipsenol and ipsdienol in their abdominal extracts (as determined by identical retention times as those of synthetic ipsenol and ipsdienol). In agreement with the bioassay results (Table 1), the optimal dosages for fenoxycarb and JH III were 10 and 0.1 μg, respectively. However, there was no measurable pheromone (threshold = 8.0 ng) in males treated with 100 μg of either fenoxycarb or JH III. On the other hand, treatment with fenoxycarb at a dose of 0.1 μg resulted in substantial increases in percentages of treated males containing pheromone and in amount of pheromone content (Table 2).

The results of both bioassays (Table 1) and GC analysis (Table 2) corroborate the conclusions of Borden et al. (1969) and Hughes and Renwick (1977a) that JH stimulates pheromone biosynthesis in *I. paraconfusus*, even though the

[3] Based on an exponential model developed by Drs. M.A. Stephens, K.L. Weldon, and T. Swartz and Mrs. S. Ng, Department of Mathematics and Statistics, Simon Fraser University, Burnaby, B.C., Canada, V5A 1S6.

TABLE 1. RESPONSE BY *I. paraconfusus* FEMALES IN LABORATORY BIOASSAYS TO PENTANE EXTRACTS OF ABDOMENS FROM MALES EXTRACTED 20 HR AFTER TREATMENT WITH FENOXYCARB OR JH III

Stimulus source	Dosage	No. females tested	Response (%)[a]
Pentane	20 μl	120	3.3a
Pheromone standards[b]	20 μl	121	66.1e
Abdominal extracts from			
Males in logs 20 hr	2 male equiv.	140	90.7f
Untreated males	2 male equiv.	121	9.0ab
Acetone-treated males	2 male equiv.	140	7.9ab
Abdominal extracts from fenoxycarb-treated males			
0.1 μg fenoxycarb	2 male equiv.	140	14.3bc
1.0 μg fenoxycarb	2 male equiv.	140	22.9c
10.0 μg fenoxycarb	2 male equiv.	121	48.3d
100.0 μg fenoxycarb	2 male equiv.	121	18.2bc
Abdominal extracts from JH III-treated males			
0.1 μg JH III	2 male equiv.	140	41.4d
1.0 μg JH III	2 male equiv.	119	16.8bc
10.0 μg JH III	2 male equiv.	140	15.0bc
100.0 μg JH III	2 male equiv.	120	13.3bc

[a] Percentages followed by the same letter are not significantly different, Newman-Kuels test (α = 0.05) modified for proportional data (Zar, 1984).
[b] (±)-Ipsenol + (±)-ipsdienol + *cis*-verbenol at 40, 30, and 20 ng/20 μl, respectively.

potency of JH III was found to be at least 100× higher than in the experiment conducted by Borden et al. (1969). The results also clearly indicate that fenoxycarb mimics the function of juvenile hormone in stimulating pheromone biogenesis in *I. paraconfusus* and suggest that it possesses high biological activity for scolytid beetles.

The diminished pheromone activity (Table 1) or detectable content (Table 2) with treatment dosages higher than optimal has not been observed before in *I. paraconfusus*. However, inhibitory effects on pheromone production with exogenous JHs or JHAs has been observed in a number of insects, and several hypotheses have been proposed to account for this phenomenon. Hedin et al. (1982) found that JH III incorporated at 1.0 ppm in the diet of male boll weevils, *Anthonomus grandis* Boheman, caused a threefold increase in the biosynthesis of four pheromone compounds. However, at dosages of 10 and 100 ppm, there was a decrease in pheromone synthesis. The results obtained by Hedin et

TABLE 2. EFFECTS OF TOPICAL TREATMENT WITH FENOXYCARB OR JH III ON PHEROMONE PRODUCTION BY MALE *I. paraconfusus* EXTRACTED AND ANALYZED 20 HR AFTER TREATMENT

Treatment (N = 12 males/treatment)	Percentages of treated males containing pheromones[a]	Amount (ng) of pheromone/male ($\bar{X} \pm$ SE)[b]	
		Ipsdienol	Ipsenol
Controls			
In logs 20 hr	83.3c	129.9 ± 28.5	816.5 ± 191.0
Untreated	0.0a	0.0	0.0
Acetone-treated (1 μl)	0.0a	0.0	0.0
Treated with fenoxycarb in 1 μl acetone			
0.1 μg	25.0b	3.3 ± 1.0a	10.6 ± 3.0b
1.0 μg	25.0b	7.3 ± 2.1b	26.0 ± 7.5c
10.0 μg	50.0bc	12.7 ± 3.7c	45.1 ± 13.0d
100.0 μg	0.0a	0.0	0.0
Treated with JH III in 1 μl acetone			
0.1 μg	33.3b	6.4 ± 1.8b	21.0 ± 6.1c
1.0 μg	16.7ab	3.8 ± 1.1a	12.6 ± 3.6b
10.0 μg	8.3ab	0.0	4.5 ± 1.3a
100.0 μg	0.0a	0.0	

[a] Percentages followed by the same letter are not significantly different, Newman-Kuels test (α = 0.05) modified for testing proportional data (Zar, 1984).

[b] Zero values indicate no detectable amount (< 8.0 ng) of pheromone. Where mean amounts > 0.0, original data (excluding data from males in logs 20 hr) have been replaced by maximum likelihood estimates of means from an exponential model derived from the original data. Exponentiality is assumed because by the Anderson-Darling statistic, there is no evidence that the data do not follow an exponential distribution. Adjusted means within a column followed by the same letter are not significantly different, Newman-Kuels test (α = 0.05). All adjusted means are significantly different from zero, t test, $p < 0.05$.

al. (1982) are consistent with Staal's (1975) hypothesis that the failure of increasing amounts of JH to elicit increasingly greater effects may be due to a cellular sensitivity which limits the capacity of a cell to respond to a given stimulus.

Decreases in pheromone content following application of JHA in southern pine beetles, *D. frontalis* Zimmerman, were observed by Bridges (1982), who suggested that JH might stimulate release of pheromones. Treatment with JHAs resulting in a significant reduction in pheromone activity was also observed in female yellow mealworms, *Tenebrio molitor* L. (Menon and Nair, 1972), and in female cockroaches, *Byrsotria fumigata* (Guerin) (Bell and Barth, 1970). For

both insects, the reduced activity was attributed to stimulation of yolk deposition by an excess of JHA, which might in some way have inhibited pheromone production.

Fockler and Borden (1973) reported that a high dosage (50 μg) of a JHA inhibited mating activity of the striped ambrosia beetle, *Trypodendron lineatum* (Olivier), whereas a lower dosage (0.05 μg) accelerated the activity. They attributed this phenomenon to a toxic effect of high dose of JHA since there was some mortality in treated insects. However, such a toxic effect was not evident in our experiments; there was no mortality or apparent loss of vigor of *I. paraconfusus* treated with any dose of fenoxycarb or JH III. Therefore, the failure to detect pheromone following treatment with high dosages of either material suggests an inhibitory effect. Such an effect could result from negative feedback on the release of brain hormone, which, as demonstrated by Hughes and Renwick (1977a), is essential for normal pheromone biogenesis in *I. paraconfusus*. Inhibition of brain hormone release by high JH titer has been reported for certain diapausing larval lepidopterans, such as the Asiatic rice borer, *Chilo suppressalis* (Walker) (Yagi and Fukaya, 1974), and the tobacco hornworm, *Manduca sexta* (L.) (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1984). Thus, the optimal (noninhibitory) effect of 100 μg of JH III found by Borden et al. (1969) may have been due to the nonvolatile solvent, peanut oil, which might have served as a slow release medium. The same dosage of the same compound carried by the highly active solvent acetone may have caused a rapid saturation of JH receptors on the corpora cardiaca (Hughes and Renwick, 1977a) or may have activated some other negative feedback mechanism that inhibited pheromone biosynthesis.

When male beetles were treated with 10 μg of fenoxycarb and then extracted and analyzed at various posttreatment times, pheromone was first detected 12 hr after treatment (Figure 1). The highest proportions of individuals contained pheromone at 16 and 20 hr; pheromone-containing males were fewer after 28 hr, and none contained detectable pheromone after 36 hr. The proportions of beetles containing pheromone were reflected by the amount of ipsenol, and to a lesser extent ipsdienol, content per male (Figure 1B).

These results agree in part with those of Borden et al. (1969), who concluded that it required at least 12 hr for JH-induced pheromone synthesis to produce sufficient pheromone to elicit a positive response by females, whereas it required only 3 hr for beetles boring and feeding on fresh ponderosa pine bark to possess highly attractive guts. The lag between pheromone synthesis induced by stimuli associated with boring and feeding activity and by exogenous JH treatment may provide indirect evidence for Hughes and Renwick's (1977a) hypothesis regarding neural and hormonal control mechanisms of pheromone biosynthesis in *I. paraconfusus*. The production of JH and subsequent release of brain hormone in response to the removal of neural inhibition, e.g., as a

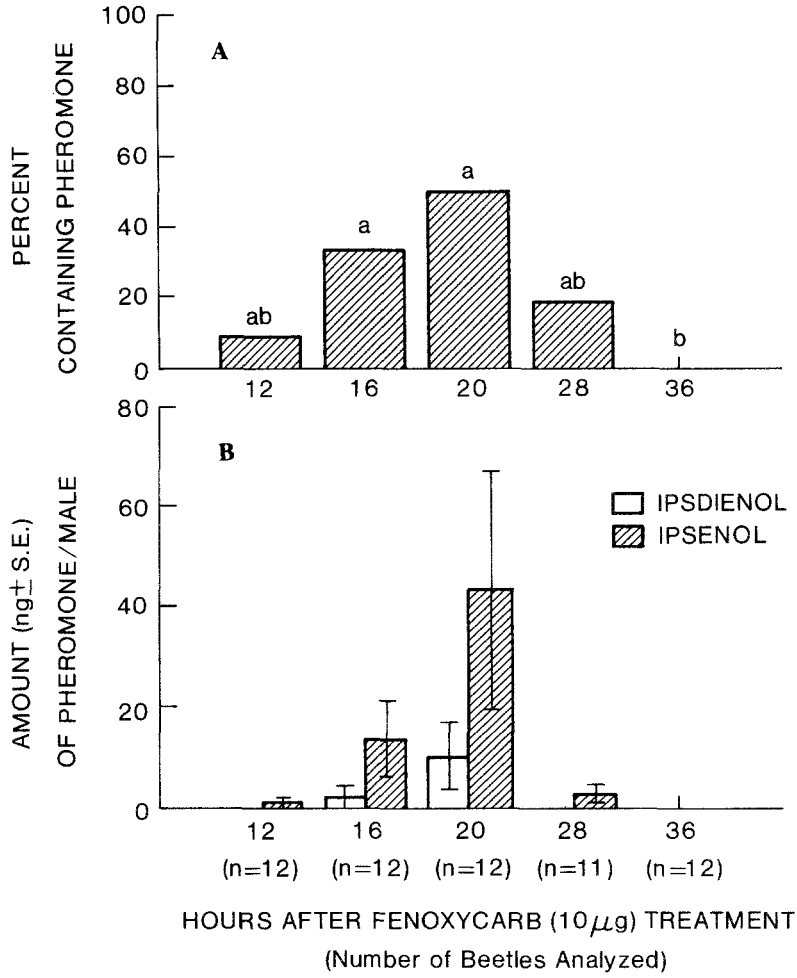

FIG. 1. Relationship between time after treatment of male *Ips paraconfusus* with fenoxycarb and percent of treated males containing ipsenol, ipsdienol, or both (A), and ng ($\bar{X} \pm$ SE) of pheromone content per male (B). Bars in A with same letter above are not significantly different, Newman-Kuels test ($\alpha = 0.05$) modified for proportional data.

result of gut distention by feeding (Hughes and Renwick, 1977a; Harring, 1978), might occur quite rapidly. On the other hand, subtle changes in hemolymph JH titers resulting from slow rates of penetration of topically applied material through the cuticle and transport to the target tissues could limit the rate of pheromone production. Possibly, however, beetles in fresh host material simply may have abundant supplies of ingested myrcene as a pheromone precursor

(Byers, 1981), and thus produce detectable levels of ipsdienol and ipsenol in a shorter time than JH- or JHA-treated beetles, which must dederivatize limited amounts of sequestered precursor (Hughes, 1975) prior to converting it to ipsdienol and ipsenol. Borden et al. (1969) and Hughes and Renwick (1977a) monitored the pheromone content in treated male *I. paraconfusus* for only 24 and 22 hr, respectively. As JH III- or fenoxycarb-treated beetles were denied food, their pheromone production must be constrained by the amount of endogenous sequestered precursor (Borden, 1985). It is likely, therefore, that this supply was exhausted within about 20 hr after treatment and that the volatile pheromones were lost eventually by emission from the hindgut (Pitman et al., 1965; Renwick et al., 1966) to the external environment and/or by diffusion into the hemocele, resulting in undetectable levels by 36 hr (Figure 1). The observation that pheromone production induced by exogenous JH or JHA varies not only with dosage but also with time may be of concern in future studies.

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BUTENOLIDES IN SMALL ERMINE MOTHS, *Yponomeuta* spp. (LEPIDOPTERA: YPONOMEUTIDAE), AND SPINDLE-TREE, *Euonymus europaeus* (CELASTRACEAE).

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Abstract—A new butenolide, isosiphonodin [3-hydroxymethyl-2(5H)-furanone], along with a trace of siphonodin [4-hydroxymethyl-2(5H)-furanone], was isolated from fifth-instar larvae of the small ermine moth *Yponomeuta cagnagellus*. Leaves of its host plant spindle-tree, *Euonymus europaeus*, were found to contain the same two butenolides with siphonodin being present as the major compound. TLC showed that isosiphonodin was also present in larvae or pupae of six other small ermine moths which did not feed on spindle-tree. In *Y. cagnagellus* butenolides might be plant derived, while isosiphonodin in the other investigated small ermine moths is probably synthesized by the insect. The possible role of butenolides in the chemical defense of small ermine moths is discussed.

Key Words—*Yponomeuta* spp., Lepidoptera, Yponomeutidae, host plants, *Euonymus europaeus*, Celastraceae, butenolides, siphonodin, isosiphonodin, plant–insect relationship, chemical defense, small ermine moth, spindle-tree.

INTRODUCTION

Nine species of small ermine moth, *Yponomeuta* spp., are native to Europe. Except for members of *Y. padellus*, European small ermine moths are mono-

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phagous and their larvae restrict their feeding to one plant genus or species only (Figure 1). In preliminary trials, we noticed that birds were reluctant to eat small ermine moths when offered as prey. Certain lepidopterous species are known to store secondary metabolites from their food plants (Rothschild, 1973; Brower, 1984). They benefit from this sequestration as these compounds are toxic to predators.

Larvae of three Euopean species of small ermine moths feed on leaves of

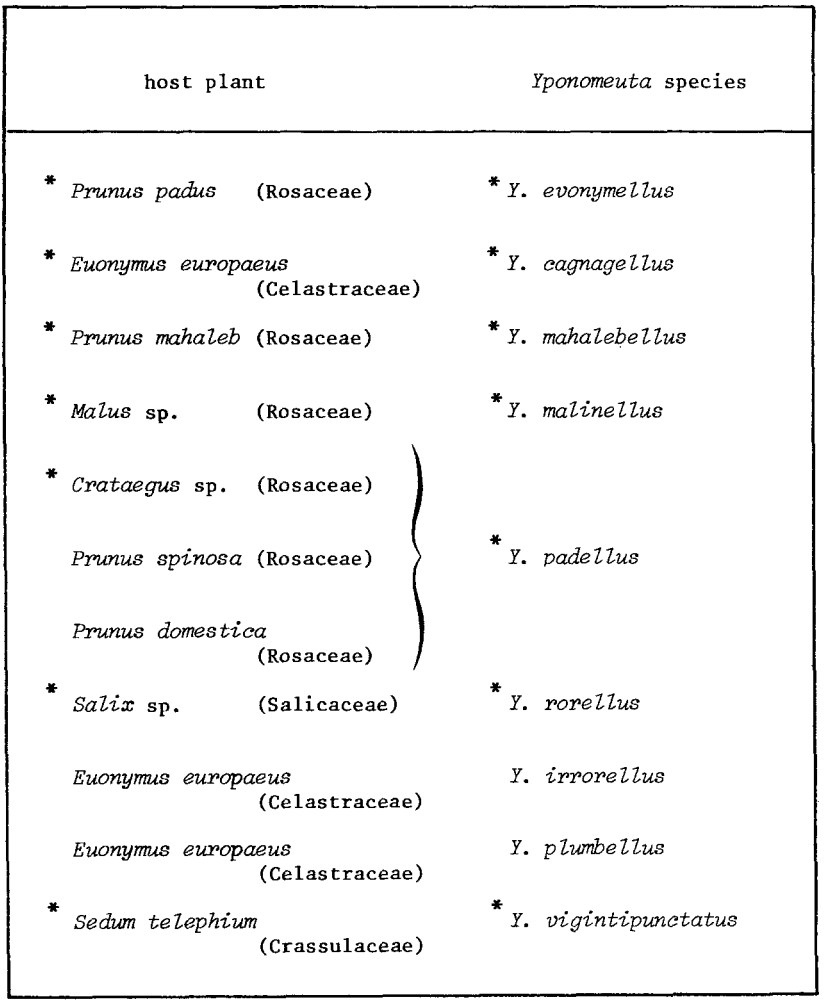

FIG. 1. The respective host plants of the European representatives of the genus *Yponomeuta*. *Species analyzed in this study.

spindle-tree, *Euonymus* sp. A screening has revealed that toxic secondary substances are present in leaves of 16 *Euonymus* taxa; all tested material contained cardenolides and, in some instances, also alkaloids (Fung, 1986). Sequestration of cardiac glycosides from food plants is known to occurt in Lepidoptera (Rothschild and Reichstein, 1976; Brower, 1984). The assumed unpalatability of small ermine moths prompted us to investigate whether these insects and their host plants contained any cardenolides.

Extracts of seven small ermine moth species and the leaves on which the larvae were found feeding were screened by TLC and a pharmacological test. Since all tested insect's extracts were colored by reagents specific for the butenolide ring in cardiac glycosides, we decided to isolate these unknown principles from *E. europaeus* and *Y. cagnagellus* in order to determine their structure.

METHODS AND MATERIALS

Insect and Plant Material. For the screening of cardenolides, larvae of all *Yponomeuta* spp., except for *Y. vigintipunctatus*, and leaves on which they were feeding were collected in the field in May and June 1985. Pupae and female and male adults of *Y. cagnagellus* were obtained by rearing fifth-instar larvae on leaves of *E. europaeus* in the laboratory (20°C). Third-instar larvae of *Y. vigintipunctatus* and flowers of *Sedum telephium* were collected in September 1985. In the laboratory, these larvae were reared until the fifth instar on leaves and flowers of *S. telephium* from our experimental garden. Prior to freezing, larvae were kept without food for one day so that gut contents would not be reflected in the assays. Insect and plant material were freeze-dried, ground, and stored in a desiccator until use. For the extraction of the active principles, bulk amounts of plant and insect were gathered. Late fifth-instar larvae of *Y. cagnagellus* were collected in June 1985 from the dunes of the Amsterdamse Waterleiding Duinen at Vogelenzang and subsequently killed by freezing at −25°C. Twigs with leaves of *E. europaeus* were collected from the dunes of the Pan van Persijn at Katwijk in August 1985. Plant and insect material was dried for 24 hr at 60°C. TLC showed no substantial loss of butenolides as a consequence of this heating process as compared to freeze-drying. After drying, the material was kept at −25°C until extraction.

Extraction and Purification for Screening. A purification with lead acetate, developed after the procedure of Rowson (1952), was employed. First insect material was defatted by extraction with petroleum ether 40–60°C (1 g/10 ml, 30 min, 40–50°C). Insect and plant material was then treated as follows: 1 g was extracted with 25 ml 70% ethanol (1 hr, 75–85°C); after filtration the residue was washed with 2 × 10 ml 70% ethanol. The filtrate was concentrated under reduced pressure at 45°C, and 2.5 ml 10% lead acetate solution was

added to the aqueous suspension. After 10–15 min, 2.5 ml 4% disodium hydrogen phosphate was added to precipitate excess lead, the suspension was centrifuged, and the precipitate rinsed with 2 × 2.5 ml 30% ethanol. The combined aqueous solution was extracted with 3 × 12.5 ml chloroform and 3 × 12.5 ml chloroform–ethanol (3:2). The combined organic layers were washed with 5 ml saturated sodium sulfate solution and dried over anhydrous sodium sulfate. Then the solution was filtered, washed with 5 ml chloroform, and the filtrate was dried under reduced pressure.

Thin-Layer Chromatography. After purification, the dried extract of 1 g material was dissolved in 400 μl methanol. This solution (20 μl) was applied on the chromatographic plates (silica gel 60 F_{254} plates, Merck); chromatograms were developed in saturated chambers. Digitoxin was applied as a reference compound for cardiac glycosides. Two solvent systems were used: (1) ethylacetate–methanol–water (81:11:8) (Wagner et al., 1983) and (2) chloroform–methanol–formamide (90:6:1); chromatograms were developed four times in system 2 (Roeske et al., 1976). Spray reagents employed were: (1) 2% 3,5-dinitrobenzoic acid solution in methanol; after air drying chromatograms were sprayed with 17% benzyltrimethylammonium hydroxide solution in 33% methanol (Doelker et al., 1969) (the use of the organic base stabilized the colors developed); (2) 0.4% 2,2′,4,4′-tetranitrodiphenyl in toluene, followed by spraying with the same base as under 1; and (3) 25% antimony(III) chloride solution in chloroform, after spraying, chromatograms were heated at 100°C for 6 min and inspected in daylight and UV light (365 nm). Reagents 1 and 2 are spray reagents for the butenolide ring in cardenolides, while reagent 3, a general reagent for several classes of natural products, gives colored products with the steroid part of cardiac glycosides.

Pharmacological Test. The purified and dried extract of 1 g material was dissolved in 1–2 ml 0.9% sodium chloride solution (5% ethanol), the appropriate volume of ethanol being first added to the dried extracts to facilitate dissolution. Precipitates formed were removed by centrifugation. Inotropic action of the extract was determined in a rat Langendorff preparation. Male or female white rats were decapitated, and the heart was excised and transferred to the Langendorff perfusion apparatus. Tyrode solution, maintained at 37°C and gased with a mixture of 95% oxygen and 5% carbon dioxide, perfused the heart. Cardiac contractile force was monitored on a Hewlett-Packard Sandborn 780-6A visoscope; 0.1 ml of each sample was injected. Ouabain (2.5 mg/ml) was used as standard.

Extraction and Purification of Butenolides from E. europaeus *and* Y. cagnagellus. Ground twigs and leaves of *E. europaeus* and fifth-instar larvae of *Y. cagnagellus* were extracted, and the extracts were purified by partition in different solvents as outlined in Figure 2. The purification steps were followed by

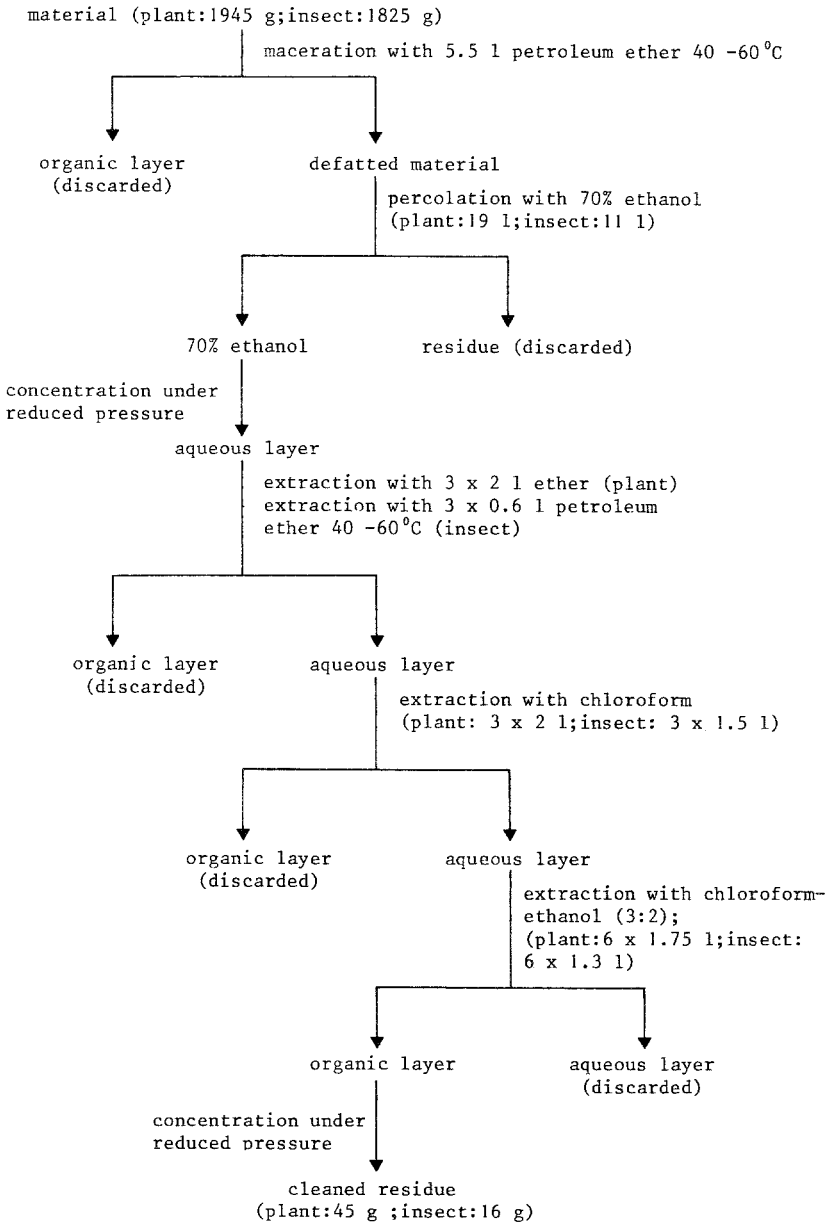

FIG. 2. Scheme of extraction and purification by solvent partition of extracts of *E. europaeus* and *Y. cagnagellus*.

TLC; fractions which gave no or slight coloring with Kedde's reagent (2% 3,5-dinitrobenzoic acid in methanol and 1 N methanolic potassium hydroxide) were not treated further and were discarded. Residues after solvent partitioning were further cleaned by vacuum liquid chromatography (Pelletier et al., 1985) on silica gel (plant: 422 g; insect: 168 g). The residues were first suspended in methanol and mixed with diatomaceous earth. After drying, aliquots of the mixture were applied on the silica gel columns. Ethylacetate–methanol–water (81:11:8) was used as eluent. Fractions with positive reactions on TLC with Kedde's reagent were concentrated under reduced pressure. The residues were then fractionated by repetitive silica gel column chromatography (Lobar Lichroprep Si 60 columns, Merck). Elution was performed with ethylacetate–methanol (9:1 and subsequently 8:2). Fractions with butenolides were pooled and concentrated. Separation of butenolides was achieved by preparative TLC (PSC-Fertigplatten silica gel 60 F_{254}, Merck). Plates were developed four times in chloroform–methanol–formamide (90:6:1). Bands with butenolides, detected by spraying a small strip of the partially uncovered plate with Kedde's reagent, were scraped off, ground and extracted with 95% ethanol. Both the plant and insect extracts yielded two butenolides (plant: E3 and E4; insect: Y1 and Y2). Final purification of the four fractions was achieved by reverse-phase column chromatography (Lobar Lichroprep RP-18 column, Merck) with 30% or 40% methanol as eluent. Yield: E3, 4 mg; E4, 18 mg; Y1, 46 mg; and Y2, 3 mg.

Spectral Data. The UV spectra were recorded on a Beckman DU-50 spectrophotometer, IR spectra on a Pye Unicam SP3-200 infrared spectrophotometer, [^{1}H]NMR spectra on a Bruker WM 300 at 300 MHz, and [^{13}C]NMR APT spectra on a Jeol JNM-FX 200 at 50.1 MHz. EI-MS was performed by direct introduction at 75 or 100°C in a Kratos MS 9/50 or a LKB-2091 mass spectrometer (70 eV). FAB-MS was carried out using a VG Micromass ZAB-2F mass spectrometer, coupled to a VG 11-250 data system. Samples were loaded in thioglycerol onto a stainless-steel probe and bombarded with xenon atoms having 8 keV energy. During the high resolution FAB-MS measurements a resolving power of 25,000 (10% valley definition) was used.

RESULTS

Screening of Extracts. TLC screening indicated that leaves of *E. europaeus* and all investigated stages of *Y. cagnagellus* contained compounds which were colored by spray reagents 1 and 2 (see Methods and Materials) (Figure 3). The colors developed, purple with reagent 1 and blue with reagent 2, are specific for a butenolide, a five-membered α,β-unsaturated γ-lactone. In natural products, this lactone ring is often part of a cardenolide. TLC in solvent system

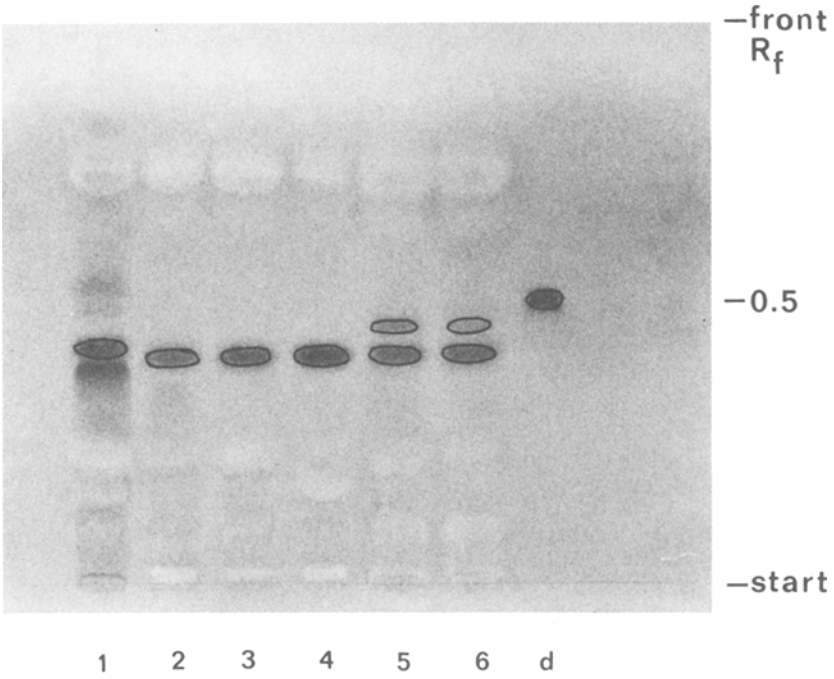

FIG. 3. TLC butenolides in *E. europaeus* and *Y. cagnagellus*. The plate was developed in solvent system 1, encircled spots are butenolides visualized with spray reagent 1. d: digitoxin (cardenolide), 1: leaf *E. europaeus*, 2: third– and fourth-instar larvae *Y. cagnagellus*, 3: fifth-instar larvae *Y. cagnagellus*, 4: pupae *Y. cagnagellus*, 5: male moths *Y. cagnagellus*, 6: female moths *Y. cagnagellus*.

2 showed that the main compound in the insect was not identical to the major butenolide in the plant.

Pupae or larvae from six other *Yponomeuta* species also contained butenolides (Figure 4). The major substance in these six small ermine moths was identical to the main butenolide in *Y. cagnagellus*. Butenolides could not be detected in extracts of the host plants *Prunus mahaleb*, *P. padus*, *Malus* sp., *Crataegus* sp., and *Salix alba*. In flower heads of *Sedum telephium* on which *Y. vigintipunctatus* larvae were collected, unidentified substances were detected which reacted with spray reagents 1 and 2. Leaves and flower heads of *S. telephium* from our experimental garden contained less of these compounds. Future studies have to reveal whether these substances are butenolides.

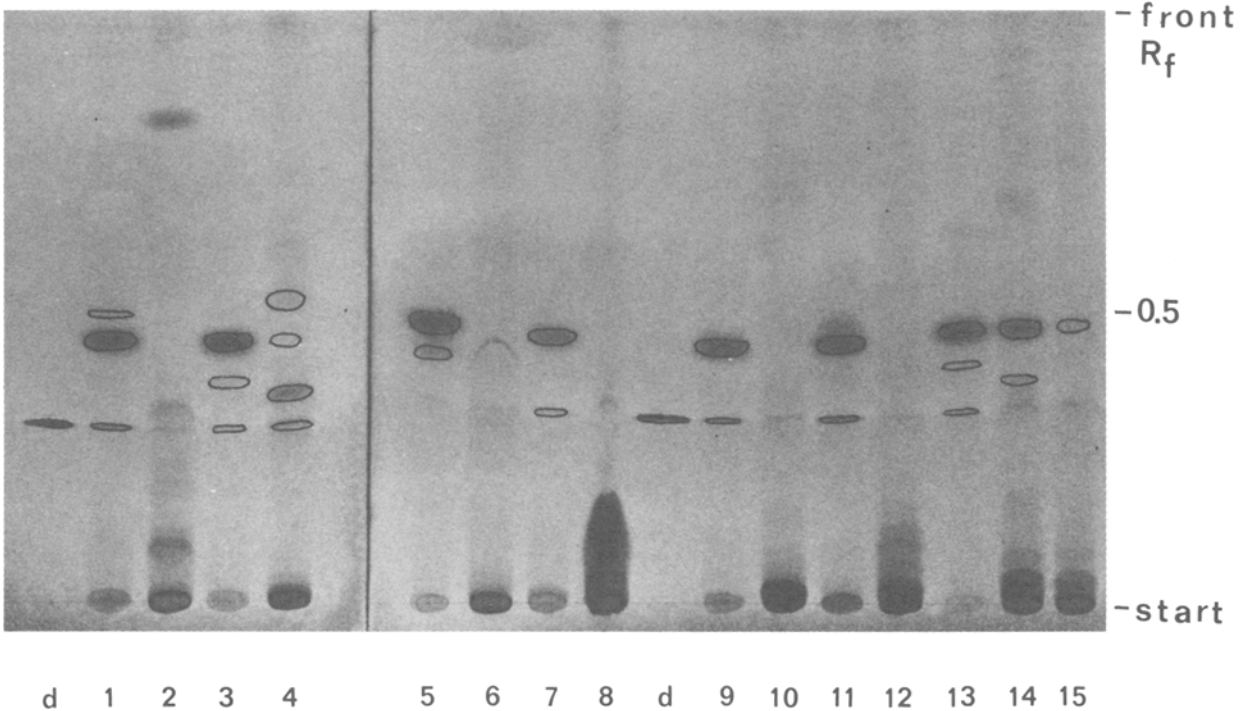

FIG. 4. TLC butenolides in *Yponomeuta* spp. and their host plants. The plate was developed in solvent system 2, encircled spots are butenolides visualized with spray reagent 2. d: digitoxin (cardenolide), 1: fifth-instar larvae *Y. evonymellus*, 2: leaf *P. padus*, 3: fifth-instar larvae *Y. cagnagellus*, 4: leaf *E. europaeus*, 5: pupae *Y. mahalebellus*, 6: leaf *P. mahaleb*, 7: fourth- and fifth-instar larvae *Y. malinellus*, 8: leaf *Malus* sp., 9: fifth-instar larvae *Y. padellus*, 10: leaf *Crataegus* sp., 11: fifth-instar larvae *Y. rorellus*, 12: leaf *S. alba*, 13: fourth- and fifth-instar larvae *Y. vigintipunctatus* 14: flower heads *S. telephium*, 15: leaf and flower heads *S. telephium*.

In testing the effect of the extracts on the rat Langendorff preparation, a small pharmacological activity was ascertained for the extracts of the seven ermine moths and of spindle-tree. This activity was different from the reaction of the standard ouabain, and it seemed that the pharmacological effect of the tested plant and insect extracts was not identical to the inotropic action of a cardenolide.

Structures of Butenolides from E. europaeus *and* Y. cagnagellus. During the isolation of butenolides from *E. europaeus* and *Y. cagnagellus*, it became clear that coloring of the extracts by antimony(III) chloride on TLC was caused by interfering substances and not the butenolides themselves. The pure compounds E3, E4, Y1, and Y2 did not react with this agent even after prolonged heating of the chromatograms. [^{13}C]NMR spectra of both E4 and Y1 showed the presence of only five carbon atoms; thus the pharmacological action and coloring with antimony(III) chloride were different from cardenolides as the steroid part is lacking in these unknown molecules.

Compound E4. $C_5H_6O_3$. Yellow oil. On the basis of similar UV, IR, and [^{1}H]NMR spectra, this main butenolide from *E. europaeus* was identified as siphonodin [4-hydroxymethyl-2(5H)-furanone] (Figure 5). This is the second record of the occurrence of this structurally simple butenolide in Celastraceae; previously siphonodin was isolated from *Siphonodon australe* (Wagner and Flitsch, 1981).

UV (nm): 207(methanol); IR (neat, cm^{-1}): 3450–3200, 2910, 1775(sh), 1735, 1635, 1440, 1270, 1175, 1135, 1060, 1035, 1010, 930, 895, and 845; EI-MS (*m/e*, % relative intensity): 115[$(M+H)^+$, 100], 85(72), 55(48), 84(45), 39(31), 70(29) and 97(16); FAB-MS (*m/e*, % relative intensity): 115]$(M+H)^+$, 100], 137[$(M+Na)^+$, 71], 251[$(2M+Na)^+$, 19], 153[$(M+K)^+$, 13], and 229[$(2M+H)^+$, 12]. NMR spectra were recorded in $CDCl_3$–CD_3OD (3:1) with TMS as internal standard. [^{1}H]NMR (ppm):δ 6.03(H_3, 1H, quintet, $J_{H3\text{-}H5}$ = $J_{H3\text{-}H6}$ = 1.8 Hz), 4.90 (H_5, 2H, doublet, $J_{H3\text{-}H5}$ = 1.8 Hz), and 4.52 (H_6, 2H, doublet, $J_{H3\text{-}H6}$ = 1.8 Hz); [^{13}C]NMR(ppm: δ 175.8(C_2), 172.9(C_4), 114.5(C_3), 72.4(C_5), and 58.7(C_6). The [^{13}C]NMR spectrum was assigned by comparison

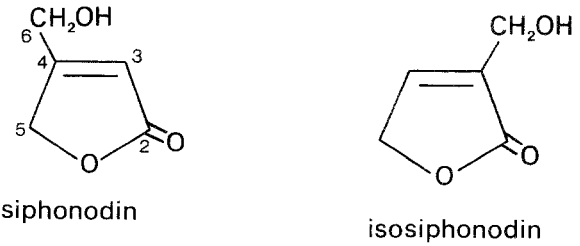

FIG. 5. Butenolides in *E. europaeus* and *Y. cagnagellus*.

with values reported for cardenolides since the same α,β-unsaturated γ-lactone ring is present in siphonodin and cardenolides (Tori et al., 1973).

Compound Y1. $C_5H_6O_3$. Yellow oil. The chromatographic behavior of this major substance from *Y. cagnagellus* and of siphonodin is much alike. Several TLC solvent systems were tested in order to achieve a separation of Y1 and the minor compound Y2 (which is identical to siphonodin, see Compound Y2 later in this section), but only the application of solvent system 2 was successful. Y1 has the same maximum in its UV spectrum as siphonodin, indicating a conjugation of the double bond and the carbonyl in the lactone ring. The NMR spectra show that Y1 is an isomer of siphonodin with signals corresponding to five carbon atoms and five protons (due to proton exchange with CD_3OD no chemical shift was recorded for the hydroxyl group). There is a close resemblance in the [^{13}C]NMR spectra of Y1 and siphonodin. Two differences were noted: in siphonodin the chemical shift of the tertiary C_3 is observed at 114.5 ppm and of the quaternary C_4 at 172.9 ppm, while Y1 showed signals at 133.9 ppm, corresponding to a quaternary carbon atom, and at 146.8 ppm, corresponding to a tertiary carbon atom. This suggests that the hydroxymethyl is attached to C_3 instead to C_4 as in siphonodin. This suggestion is confirmed by the [^{1}H]NMR spectrum. In Y1 the chemical shift of the olifinic proton is 7.47 ppm, while in siphonodin the signal of H_3 is observed at 6.03 ppm. In 5-hydroxymethyl-2(5H)-furanone, the chemical shifts of the olifinic protons H_3 and H_4 are reported at, respectively, 6.20 and 7.60 ppm (Camps et al., 1982). On the basis of this evidence, we suggest that Y1 is the butenolide 3-hydroxymethyl-2(5H)-furanone, a new product for which we propose the name isosiphonodin (Figure 5). The IR spectrum is in accordance with the proposed structure: next to the carbonyl stretching band at 1740 cm^{-1}, a second band is observed at 1690 cm^{-1}; the lower band can be attributed to the presence of intramolecular hydrogen bonding between the hydroxymethyl and the adjacent carbonyl. Fermi resonance of the carbonyl stretching mode, present in siphonodin at 1775 cm^{-1}, is absent in isosiphonodin.

UV (nm): 207(methanol); IR (neat, cm^{-1}): 3440–3190, 2915, 2880, 1740, 1690, 1610, 1445, 1390, 1350, 1310, 1205, 1090, 1050, 1020, 940, and 835; EI−MS (*m/e*, % relative intensity): 85(100), 39(70), 31(62), 96(60), 55(58), 57(53), 68(51), 45(24), and 113[$(M-H)^+$, 22)]; FAB−MS (*m/e*, % relative intensity); 57(100), 115[$(M+H)^+$, 75], 131(69), 237(45), 73(45), 89(40), and 61(26). NMR spectra were recorded in $CDCL_3$–CD_3OD (3 : 1) with TMS as internal standard. [^{1}H]NMR (ppm): δ 7.47(H_4, 1H, quintet, $J_{H4-H5} = J_{H4-H6} = 1.8$ Hz), 4.88(H_5, 2H, double doublet, $J_{H4-H5} = 1.8$ Hz, $J_{H5-H6} = 2.3$ Hz), and 4.35(H_6, 2H, double doublet, $J_{H4-H6} = 1.8$ Hz, $J_{H5-H6} = 2.3$ Hz); [^{13}C]NMR (ppm): δ 173.9(C_2), 146.8(C_4), 133.9(C_3), 71.2(C_5), and 56.3(C_6).

Compound E3. $C_5H_6O_3$. Its chromatographic behavior and EI−MS are

similar to Y1; therefore this minor butenolide from *E. europaeus* is identified as isosiphonodin.

Compound Y2. $C_5H_6O_3$. Its chromatographic behavior and EI−MS show that Y2 from *Y. cagnagellus* is identical to siphonodin. The butenolides Y1 and Y2 were isolated from fifth instar larvae which probably still contained some leaf material in their guts. Since siphonodin is the main butenolide in *E. europaeus*, siphonodin from the insect probably stems from plant material in the gut. TLC shows that siphonodin is absent in the pupal and adult stages of *Y. cagnagellus*.

DISCUSSION

In a previous work, screening for alkaloids and cardenolides in leaves of 16 *Euonymus* taxa was undertaken. It was concluded that cardenolides were present in all leaves tested (Fung, 1986). In the leaf extract of *E. europaeus*, six butenolides can be detected on TLC. Two of them we now identify as structurally simple butenolides. The major butenolide in dried leaves of *E. europaeus* is siphonodin. TLC shows that this same compound also occurs in several other *Euonymus* taxa (Fung, 1986). It is likely that *Euonymus* spp. contain both cardenolides and simple butenolides, as, in some investigated leaves, six to eight compounds are present, which are colored not only by Kedde's reagent but also by antimony(III) chloride, indicating the presence of both an α, β-unsaturated γ-lactone and a steroid part in these unidentified substances.

Next to the major substance siphonodin, a trace amount of the new compound isosiphonodin is isolated from *E. europaeus*. Siphonodin and isosiphonodin are isomers, formed probably by the same biosynthetic pathway. As these two products are not interconvertible by a single reaction, an early branching must have taken place in the biosynthesis of these butenolides.

From leaves of *Siphonodon australe*, siphonodin and its glucoside siphonoside were isolated (Wagner and Flitsch, 1981). In this plant, siphonodin was present as the major compound, the yields of siphonodin and siphonoside being 1.12 g and 45 mg, respectively. Since we restricted our isolation to major compounds in the extracts and did not consider minor components, we only isolated the aglucons siphonodin and isosiphonodin from *E. europaeus*. We need further analysis to ascertain whether glucosides of these butenolides are also present in *E. europaeus*.

Isosiphonodin is found to be the major butenolide in small ermine moths. Since the investigated *Yponomeuta* spp., with the exception of *Y. cagnagellus* and possibly *Y. vigintipunctatus*, do not feed on plants containing butenolides, isosiphonodin is probably synthesized by these insects. In the case of *Y. cag-*

nagellus however, selective uptake of isosphonodin from its host plant is possible. Secondary substances in insects are sometimes sequestered from their food plants, but *de novo* synthesis has also been demonstrated in several cases. Defensive secretions of leaf beetles from the genera *Chrysolina*, *Chrysochloa*, and *Dlochrysa* contain cardiac glycosides; evidence indicates that these cardenolides are probably synthesized by the insect from plants sterols (Pasteels and Daloze, 1977). Recently, it was shown that both sequestration and synthesis of cyanogenic glycosides are possible in the moth *Zygaena trifolii* (Nahrstedt and Davis, 1986).

We started this study with the aim of characterizing unknown compounds in small ermine moths, which could account for their repellent taste. Early in our work, we assumed that cardiac glycosides were involved in the chemical defense of *Y. cagnagellus* (Rothschild et al., 1986). As our results have shown, we did not find cardenolides but did isolate the butenolides siphonodin and isosiphonodin from this moth. Isosiphonodin was also detected in six other small ermine moth species. Since the palatability of small ermine moths has not been extensively studied, and the deterrency and/or toxicity of simple butenolides to predators of insects are not known, we cannot state at this stage whether the presence of isosiphonodin in small ermine moths will be of any use in their chemical defense. It would not be surprising if future studies reveal that this is indeed the case, as several simple butenolides are physiologically active compounds. Siphonodin and its derivatives, especially siphonoside, are reported to possess cytotoxic activity (Wagner et al., 1981). Tulipalin from tulip bulbs is shown to be a fungitoxic substance and the cause of the allergic skin reaction "tulip fingers" (Bergman et al., 1967; Brongersma-Oosterhoff, 1967). (±)-4-Hydroxy-3-methoxy-2-buten-4-olide from *Narthecium ossifragum* produces antibiotic activity against *Bacillus subtilis* (Tschesche and Hoppe, 1971). Protoanemonine, identified in extracts of several species of the Ranunculaceae, causes skin irritation and is an antibiotic agent (Ruijgrok, 1967).

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DAMAGE-INDUCED ALKALOIDS IN TOBACCO: Pot-Bound Plants Are Not Inducible

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Abstract—Field-grown wild tobacco plants (*Nicotiana sylvestris*) were subjected to a defoliation regime designed to mimic the rate and amount of leaf mass removed by one tobacco hornworm per plant. Undamaged leaves on these plants undergo a dramatic (457% for leaf position 5, 410% for leaf position 8) increase in total leaf alkaloids compared to same-age and positioned control leaves on undamaged control plants. However, potted greenhouse-grown plants fail to exhibit the same damage-induced increase in alkaloid content. The greenhouse environment differs from the field environment in factors known to influence leaf alkaloid content, particularly soil N, P, K, near-UV radiation, and relative humidity. However, altering these environmental factors does not make potted plants able to increase their leaf alkaloid levels in response to defoliation. Transplanting plants into larger pots with more soil does allow the plants to respond to defoliation. Thirty days after transplanting, the plants are again unresponsive to damage, probably as a result of becoming "pot-bound." This result suggests a mechanism for the induction response, specifically that leaf damage triggers synthesis of these alkaloids in the roots, and offers a potentially valuable experimental tool for the study of induced-plant defenses in tobacco and other plants that synthesize alkaloids in their root tissues.

Key Words—*Nicotiana sylvestris*, leaf alkaloids, defoliation, induced defense, pot-binding, nicotine.

INTRODUCTION

The herbivore-induced increases in plant secondary metabolites have received much attention in the ecological literature (Rhoades, 1979; Schultz and Baldwin, 1982; Haukioja, 1982) and have lent support to the hypothesis that plant

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secondary metabolites are not just "waste products" of plant metabolism, but, rather, a component of plants' evolved defense responses against herbivory and pathogen attack. These responses have received little attention from plant physiologists. The mechanisms of only two herbivore-induced defense responses are understood in any detail: the induction of proteinase inhibitors in tomato leaves (Ryan, 1983) and quinolizidine alkaloid in lupine leaves (Wink, 1983). Understanding the mechanistic details of the induction response would greatly augment research into the ecological significance and evolutionary origins of such plant defensive responses. The first step in a mechanistic quest is to reproduce the response in the laboratory under controlled conditions.

Members of the genus *Nicotiana* produce pyridine-containing alkaloids that are toxic to most herbivores (Gordon, 1961; Hassall, 1969; Schmetz, 1971) and even, if present in adequate concentrations, deleterious to insects that feed primarily on these alkaloid-containing plants. In artificial diet experiments, Parr and Thurston (1972) showed that tobacco hornworms—despite their ability to efficiently excrete ingested tobacco alkaloids (Self et al., 1964)—reared on diets containing 2% or higher concentrations of nicotine had significantly reduced survivorship and larval performance.

Decapitation—topping—at the onset of flowering is standard practice in the production of cultivated tobacco (*N. tabacum*); it increases the size, weight, and alkaloid content of leaves (Woetz, 1955). Although it has long been known that damage to the flowering top of a tobacco plant increases the total alkaloid content of the plant (Reuter, 1957), the possible ecological significance of this response, namely, that a plant may increase its leaf alkaloid content after damage as a defensive response to herbivore attack, has gone unnoticed.

Here I demonstrate substantial increases in the alkaloid content of undamaged leaves of field-grown wild tobacco plants suffering damage not to their flowering tops, or apical or lateral buds, but only to their fully expanded leaves. However, plants grown under normal greenhouse conditions are not responsive to damage. Unrestrained root growth appears to be necessary for the response to occur.

METHODS AND MATERIALS

Alkaloid Quantification. The four principal alkaloids of *Nicotiana* species (Saitoh et al., 1985)—nicotine, anabasine, nornicotine, and anatabine—were separated by isocratic high-pressure liquid chromatography; detected by absorbance at 254 nm; quantified with a reporting integrator using the techniques of Saunders and Blume (1981); and modified by the use of (−)-scopolamine hydrochloride as an internal standard. The retention times (minutes) of standard alkaloids were: nornicotine (11.05), anabasine (12.30), anatabine (13.99), sco-

polamine (20.68), and nicotine (23.35). All samples were injected twice and the values expressed as a percentage of dry weight leaf (mean ± SD).

Alkaloid Extraction. Fresh leaf samples, rather than air-dried samples, were used to determine alkaloid contents. Replicate extractions ($N = 10$ per group; mean = ± SD % alkaloid content) of a pooled homogeneous leaf sample extracted after 24 hr (1.243 ± 0.188), 48 hr (1.115 ± 0.531), and 72 hr (0.969 ± 0.515) of air drying at 50°C produced lower ($P < 0.0001$) and more variable ($P < 0.0005$) results than an analysis of fresh leaf material (1.924 ± 0.011). Fresh leaf samples were weighed to 0.2 mg, frozen in liquid N_2, ground to a fine powder with a glass rod, and extracted in a single extraction step in 40% methanol 0.1% N HCl (Saunders and Blume, 1981) containing the internal standard at a concentration of 50 μg/ml. Four sequential extractions of 10 replicate leaf samples revealed that 97.86% ± 0.42 of the total alkaloids was removed in the first extraction when the concentration of leaf material in the extraction solvent was 10 mg fresh wt/ml extraction solution. The alkaloid extraction and quantification technique proved to be both precise—30 samples of tobacco leaves containing a wide range of alkaloid concentrations (0.03% minimum, 2.46% maximum) extracted in duplicate had a mean percentage diffference between replicates of 0.04—and accurate—when 10 leaf samples were spiked with known amounts of nicotine, better than 95% of the alkaloid was detected.

Plant Cultivation and Leaf Sampling for Alkaloids. Seeds of *Nicotiana sylvestris* Spegazzini and Comes were originally obtained from the USDA Beltsville Agricultural Research Center, Beltsville, Maryland. Seeds from one greenhouse plant (thus plants were full sibs) were germinated in Cornell Mix A (Boodley and Sheldrake, 1977) under greenhouse conditions and either planted in a field plot at Cornell University or under greenhouse conditions in 18-cm fiber pots with 3.5 g Osmocote 14-14-14 fertilizer. The field plot was a roto-tilled old field consisting of four rows of 10 plants with 61 cm between plants and rows. The plot was neither fertilized nor watered and was manually weeded only during the first two weeks after planting. In order to minimize possible edge effects, only the two middle rows were used in this study. All plants were grown to the buttoning stage before experimentation began. The eighth fully expanded leaf from the top was designated the sample leaf and labeled with a twist-tie. The fifth leaf of each field-grown plant was also designated a sample leaf. Leaf samples were removed in a 1-cm band (Rosa, 1973) one half the leaf length from the top, across the leaf but excluding the midrib; leaf samples were then immediately weighed and extracted. A comparable band adjacent to the sample band was removed, weighed, dried at 50°C for 48 hr, and reweighed to obtain a percent dry weight calculation.

Experimental Treatments. Plants were grouped by height (±5 cm to the developing flower button) and randomly assigned to a treatment with 10 plants per treatment. Plants were damaged in order to simulate the feeding of one

Manduca sexta larva, a common lepidopteran pest of cultivated tobacco. During its larval life, a caterpillar will eat approximately 2100 cm^2 of leaf area, of which 90% will be consumed in the last larval instar (Madden, 1945). Greenhouse-grown plants are smaller at the buttoning stage than are field-grown plants. For field-grown plants, 2000 cm^2 of leaf area represents approximately 50% of the shoot weight. Thus, instead of removing the same amount of leaf area as in the field-defoliation treatment, the same proportion of leaf area was removed. Fully expanded leaves from positions 3 through 12, excluding positions 5 and 8 (the sample positions), were cut daily with scissors over an eight-day period. Cuttings was placed in labeled bags, air-dried at 50°C, and weighed. Plants assigned to a fertilizer treatment were watered daily with 100 ml/pot of a fertilizer solution consisting of 1 kg of Peter's 20-20-20 per 400 liters of water for eight days prior to the initiation of the damage regime. Plants assigned to a transplant treatment were removed from their pots and placed in 30-cm fiber pots containing 4.2 times the volume of Cornell Mix A contained in the 18-cm pots. Control plants in the transplant experiment were removed from their 18-cm pots and returned to these pots.

Statistical Treatment. Alkaloid values were N-scored (Ryan et al., 1982) and found to be normally distributed. Alkaloid values were compared with the Student's *t* test modified for unequal variances; values from field-grown plants were compared with a paired *t* test for between-treatment comparisons (Sokal and Rohlf, 1981). Percentages were arcsin-transformed.

RESULTS AND DISCUSSION

The alkaloid composition of leaves in positions 5 and 8 of *N. sylvestris* was dominated by a single compound: nicotine. The percent composition of the alkaloid profile of the 130 leaves sampled in this study was 90.3 $\pm$ 8.6% nicotine, 9.2 $\pm$ 4.2% nornicotine, 0.6 $\pm$ 0.5% anabasine, and 0.1 $\pm$ 0.1 anatabine, which is consistent with the previous work on this species (Saitoh et al., 1985). Nicotine was the only alkaloid significantly ($P < 0.05$) influenced by any of the treatments.

Field-grown plants subjected to the eight-day defoliation regime lost 50.13 $\pm$ 13.34% of their shoot mass and had more than four times the total alkaloid content in their undamaged sample leaves (Table 1). The alkaloid content of sample leaves higher on the stalk (position 5) was not significantly different ($P = 0.60$) from that of leaves on the lower stalk position (8) on undamaged plants, but tended to be higher on damaged plants ($P = 0.057$).

Greenhouse-grown plants subjected to the defoliation treatment lost 45.2 $\pm$ 7.2% of their shoot mass over the eight-day cutting period. However, unlike the experiment with field plants, no difference ($P = 0.28$, $DF = 19$) was found

TABLE 1. TOTAL ALKALOIDS OF SAMPLE LEAVES IN TWO LEAF POSITIONS FROM DAMAGED AND UNDAMAGED FIELD-GROWN WILD TOBACCO PLANTS

	% of dry wt sample leaf total alkaloid			
	N	Mean	SD	*P*
Leaf position 5				
Damaged plant	10	2.527	1.210	0.00001
Undamaged plant	10	0.553	0.308	
Leaf position 8				
Damaged plant	10	1.985	0.791	0.00001
Undamaged plant	10	0.484	0.163	

between the alkaloid contents of undamaged sample leaves from damaged (0.189 $\pm$ 0.053) and undamaged (0.196 $\pm$ 0.049) plants.

Soil nutrients, particularly B, Ca, and N, are known to substantially affect leaf nicotine contents (Tso, 1972). Deficiency in soil B and Ca increases leaf nicotine contents but also destroys the plants' apical buds (Tso, 1972). Because the apical buds of greenhouse-grown plants appeared healthy, an experiment was conducted with an N-P-K fertilization treatment. Fertilizer increased the alkaloid contents of both damaged (0.578 $\pm$ 0.135) and undamaged (0.595 $\pm$ 0.151) plants, but the damage had no effect on leaf alkaloid contents ($P = 0.80$, $DF = 19$).

The greenhouse environment differs from the field environment in that the glass of the greenhouse substantially decreases the amount of near-UV radiation and the intensity of visible light. Both alterations of the light environment are known to decrease leaf alkaloid concentration (Anderson and Kasperbauer, 1973). Plotted plants placed outdoors for 10 days prior to the defoliation treatment had elevated alkaloid levels, but sample leaves did not differ ($P = 0.33$, $DF = 19$) between damaged (0.584 $\pm$ 0.216) and undamaged (0.492 $\pm$ 0.194) plants.

Potted plants transplanted into larger pots with more soil prior to a one-day defoliation treatment (removing 35.0 $\pm$ 6.0% of the total leaf dry weight with a single cutting) had substantially more nicotine in undamaged sample leaves of undamaged plants (Table 2). The transfer of a plotted plant into a larger pot with more soil apparently enables the plant to increase its leaf alkaloid level after leaf damage. The ability to respond to damage wanes 30 days after the transfer to the larger pot.

The increase in leaf alkaloid content in cultivated tobacco (*N. tabacum*) after topping is largely due to increased nicotine synthesis in the roots (Mizusaki et al., 1973). However, decreased leaf nicotine degradation rates in the leaves

TABLE 2. TOTAL ALKALOIDS OF SAMPLE LEAVES FROM DAMAGED AND CONTROL GREENHOUSE-GROWN WILD TOBACCO PLANTS

	Days after transplant		% of dry wt sample leaf total alkaloid			
Treatment	Damaged	Sampled	Mean	SD	*N*	*P*
Damage	7	10	0.669	0.091	10	0.012
Control		10	0.385	0.126	10	
Damage	18	20	0.985	0.076	10	0.00001
Control		20	0.266	0.057	10	
Damage	30	33	0.143	0.040	10	0.22
Control		33	0.171	0.055	10	

(Yoshida, 1962) and leaf nicotine synthesis (Bose et al., 1956) may play a role in the topping-induced increase in leaf alkaloid levels. Most nicotine synthesis is thought to occur in young dividing root tips (Dewey et al., 1955). Although the hypothesis needs to be experimentally demonstrated, the increased leaf alkaloid content after leaf damage is likely due to a similar physiological process. Pot-bound plants, those growing in a pot for 30 days or more, may be unable to initiate new root growth after leaf damage, and be hence unable to synthesize more alkaloids. This hypothesis is consistent with the observation that soil depth is positively correlated with constitutive leaf nicotine concentrations (Wolf and Bates, 1964).

The observation that pot-bound plants are not responsive to leaf damage may prove to be an important experimental tool for demonstrating the ecological consequences of altered leaf chemistry on herbivorous insects. Herbivore damage has dramatic effects on leaf secondary chemistry, as well as on primary metabolites such as protein (Wagner and Evans, 1985) and sugars (Valentine et al., 1983). In some studies leaf damage apparently increased the nutritional quality of the leaves to herbivores (Williams and Myers, 1984). Hence a plant cultivation technique that allows for manipulation of a damage-induced alteration in a secondary metabolite, against a background of damage-induced alterations in primary metabolities, would be an exciting experimental tool in the study of induced plant defenses. This phenomenon may also be applicable to other plant alkaloids that are synthesized in the roots, such as hyoscyamine in *Datura*, *Atropa*, and *Hyoscyamus*; and betaines in *Beta* (Mothes, 1960).

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IDENTIFICATION OF A MALE-PRODUCED PHEROMONE OF *Anticarsia gemmatalis* (HÜBNER) (LEPIDOPTERA; NOCTUIDAE) ATTRACTIVE TO CONSPECIFIC MALES[2]

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Abstract—Observations in the laboratory and in the field indicated that male *Anticarsia gemmatalis* (Hübner), the velvetbean caterpillar (VBC), are attracted to conspecific courting males. Male VBC subsequently were found to be attracted to extracts of male abdominal tips including the extrudable hairpencils. The active chemical in these extracts was identified as (*Z,Z,Z*)-3,6,9-heneicosatriene, which is also one of the major components of the female VBC sex pheromone. Male VBC in a wind tunnel and in the field exhibited a bimodal response distribution to a range of ratios of the (*Z,Z,Z*)-3,6,9-heneicosatriene and (*Z,Z,Z*)-3,6,9-eicosatriene, with one maximum at the pure heneicosatriene alone and the other at the 60:40 female blend. This demonstrates that the male response to the male hairpencil component is distinct from that to the female sex pheromone.

Key Words—*Anticarsia gemmatalis*, velvetbean caterpillar, Lepidoptera, Noctuidae, attractant, pheromone, hairpencils, (*Z,Z,Z*)-3,6,9-heneicosatriene, male-produced pheromone.

INTRODUCTION

During field studies of the sex pheromone behavior of the male velvetbean caterpillar (VBC), *Anticarsia gemmatalis* (Hübner) (Heath et al., 1983), males

[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by USDA.

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were observed orienting to other males that were responding at close range to synthetic female sex pheromone [40:60 ratio of (*Z*,*Z*,*Z*)-3,6,9-eicosatriene (C-20 triene) and (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene) (C-21 triene)]. Similar behavior had been observed during studies of VBC sex pheromone behavior in a flight tunnel, involving calling females and multiple males (unpublished data). Whether these were visual responses of males to the shape of a moth or possibly a response to male-produced chemicals was not apparent.

Male-produced sex pheromones have been reported or suggested for a number of moth species, based on chemical analyses, behavioral studies of attraction and courtship, or hairpencil or brush morphology. Overt responses by females to male-produced pheromones have been demonstrated in few cases. Baker et al. (1981) demonstrated short-range attraction of female *Grapholitha molesta* (Busck) to a male sex pheromone released during courtship from abdominal hairpencils. *Ephestia elutella* (Hübner) females flex the abdomen ventrally in response to male sex pheromone (Krasnoff and Vick, 1984). Other studies suggest an aphrodisiac role for many male sex pheromones (Birch, 1974), and intrasexual responses to male pheromone have been reported in a few cases. Baker (1983) demonstrated that displaying *G. molesta* males attract other males at close range. Male attraction to males, resulting in aggregations in the field, is known for *Estigmene acrea* (Drury) (Willis and Birch, 1982). Also, male pheromone may reduce the attractiveness of calling females in *Heliothis virescens* (Boddie) (Hendricks and Shaver, 1975) and *Pseudaletia unipuncta* (Haworth) (Hirai et al., 1978).

Like many other species of Lepidoptera, male VBC have sexually dimorphic brushes and hairpencils. Greene (1974) described long scales on the prothoracic femorae and mesathoracic tibiae of VBC males. Male VBC also possess terminal abdominal hairpencils and scent brushes on the venter of the eighth abdominal segment. While it is presumed that these brushes and hairpencils are involved in courtship interactions possibly for the dispersal of pheromones affecting female behavior, their functions and the products of any associated exocrine glands are unknown.

We report here the results of experiments demonstrating attraction of male VBC to extracts of the male abdominal tips, including the hairpencils, and the identification of a chemical from those extracts that was attractive to other male VBC in a flight-tunnel bioassay and in field trapping tests.

METHODS AND MATERIALS

Insect Rearing and Maintenance. All insects were obtained as pupae from a colony maintained as described by Leppla (1985) at the USDA Insect Attrac-

tants, Behavior, and Basic Biology Research Laboratory, Gainesville, Florida. Pupae were sorted by sex, placed in 24 × 24 × 24-cm plastic screen and Plexiglas cages, and supplied with a sucrose solution on cotton. Males and females were held in separate environmental chambers on a 14:10 light–dark cycle at 24°C and 65% relative humidity. Pupae were transferred daily to new cages to provide adult moths of known age. Males were tested when 4–7 days old. Bioassays were conducted during third to fifth hours of the scotophase. Pheromone extracts from males were obtained during the first hour of the scotophase.

Bioassays of Male VBC Extracts. Preliminary bioassays of extracts of abdominal tips, whole insects, and leg scales indicated the attractant activity was confined to the abdominal tip. Male VBC abdominal tips were extruded by squeezing the abdomens between the thumb and index finger, extruding the terminal hairpencils, and exposing the ventral brushes. The tip was cut between the more anterior ventral brushes and hairpencils. Tips were placed in vials that contained 1 ml hexane in 50 or 25 tip batches. After several hours, the solvent extract was transferred to a new vial.

Tip extracts were tested for attractiveness to male VBC in a flight-tunnel bioassay in comparison to hexane blanks and female abdominal tip extracts [two female equivalents (FE)]. Extract dosages (as male equivalents) were placed on filter papers and, after 2 min of air drying under a fume hood, were hung on a stand near the center of the upwind end of the flight tunnel. Males were released singly at the middle of the length of the tunnel, ca. 2 m from the upwind end. Moths were scored during a 2-min period for upwind-oriented flight to the filter paper.

Abdominal tip extracts of male VBC were tested at dosages of 0.2, 1.0, and 5.0 male equivalents (ME). Either 5 or 10 males were tested (assayed) individually per treatment on each of seven different days to provide data sets composed of male responses to the female pheromone, hexane blanks, and the three dosages of male extracts.

Isolation and Identification. An initial purification of the male abdominal tip extracts was obtained on a gravity flow glass column (5 × 0.62 cm ID) prepared by slurry packing 60–100 mesh silica (J.T. Baker Chemical Co.) in hexane. Solvents used to elute the column were hexane, 10% ether–hexane, 50% ether–hexane, and 100% ether. The active fraction from the gravity flow was further purified using a Varian 1400 gas chromatograph (GC) equipped with a flame ionization detector. A glass column (2 m × 2.3 mm ID) packed with 4.4% OV-101 on 80–100 mesh Chromosorb G-HP was used, and 2% of the effluent was routed to the detector. The remaining 98% of the effluent was collected in a cooled 30-cm glass capillary tube according to the method described by Brownlee and Silverstein (1968). Helium was used as the carrier

gas (28 ml/min), and the column was temperature programmed from 80 to 240°C at 10°/min. Analyses of the active fraction eluted from the packed GC column and of the synthesized chemicals used in this study were obtained using a Varian 3700 GC equipped with splitless capillary injector and flame ionization detector. The output of the detector was interfaced with a Nelson 4000 data system. Helium was used as the carrier gas for all analyses at a linear flow velocity of 18 cm/sec. Columns used for analyses (from Quadrex Corp., New Haven, Connecticut) were a 50 m × 0.25mm ID fused silica column coated with a 0.25-μm film of methyl silicone and a 30 × 0.25 mm ID fused silica column coated with a 0.25-μm film of Carbowax 20 M. Columns were held at an initial temperature of 60°C for 2 min and then temperature programmed to 200°C at 32°/min. Additionally, a 30 m × 0.25 mm ID cholesterol para-chlorocinnamate liquid crystal capillary column was used isothermally at 160°C (Heath et al., 1979). Confirmation of the identity of a compound was made by cochromatography with a synthetic standard. Additionally, mass spectral data (methane chemical ionization and electron impact using a Nermag model R1010) were obtained on the active material from abdominal tip extracts. Mass spectrometry samples were introduced with an HP 5790 gas chromotograph equipped with a splitless injector and the 50-m methyl silicone column described previously.

Bioassays of Synthetic Pheromone. In a flight-tunnel bioassay, male VBC were tested for response to a range of ratios of the two components identified as the female sex pheromone and the active material obtained from male abdominal tip extracts and over a range of dosages. The pheromone was formulated in rubber septa at combined loads of 1, 2, 20, or 200 μg/septum dissolved in 100 μl of hexane and air dried for 2 days prior to use. Load ratios tested were 0:100, 2:98, 6:94, 10:90, 18:82, 45:55, 64:36, 85:15, and 100:0 of the C-20 triene–C-21 triene, respectively, with predicted release ratios (Heath et al., 1986) of 0:100, 6:94, 15:85, 22:78, 33:67, 66:34, 81:19, 90:10, and 100:0 of C-20 triene–C-21 triene. Release ratios were confirmed by analysis of volatiles collected from septa using the system described previously (Mitchell and Heath, 1986).

At each dosage, 40 male VBC were tested as four sets of 10 moths flown one at a time to each ratio. Males were scored for attraction to within 5 cm of the source and for reorientation to the source. All bioassay conditions were as described above.

Field Test of Synthetic Pheromone. The attractiveness of a range of ratios of the female sex pheromone and male pheromone component was evaluated in the field in a trapping test at 200-μg and 1-mg dosages on rubber septa. Screen cone traps (Hartstack et al., 1979) were mounted on steel poles (with the septa

positioned at the base of the cone) just above the vegetation. The test was conducted in a soybean field near Alachua, Alachua County, Florida, during September 1984. Two sets of nine traps were placed in rows perpendicular to the prevailing wind (north to south), with 10 m between traps and 90 m between rows of traps. The traps were checked daily and captured moths were identified to species, sexed, and counted. The positions of the traps were randomized daily. Daily trap catch data were converted to ranks by treatment for analysis of variance. Treatment rank means were separated using Duncan's new multiple-range test at the $P = 0.05$ level (Duncan, 1955).

RESULTS

Extracts of male abdominal tips were attractive to other males in the flight-tunnel bioassay at all dosages tested. At 0.2 ME, 36.7% of males tested responded (oriented flight upwind to the filter paper), 44.6% to the 1.0 ME samples, and 63.8% to the 5.0 ME samples. No males responded to the hexane blanks, and 94% of those tested responded to the 2 FE female abdominal tip extract standard. All of the activity from the tip extracts was recovered in the hexane fraction of the gravity-flow silica column. Male response was not increased by the addition of fractions obtained by further elution of the column using 10% and 50% ether–hexane and the 100% ether as the solvent. The active hexane fraction from the gravity-flow column was further purified by GC using the packed OV-101 column. All the activity was obtained in one peak that had a Kovats index of 2071. Further analysis of this peak on the 30-m polar Carbowax and 50-m apolar methyl silicone capillary columns resulted in a single peak.

Mass spectra obtained using electron impact and methane chemical ionization of the active peak from the packed column were identical to that obtained for the (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene reported previously as one of the pheromone components obtained from female VBC (Heath et al., 1983). Further analysis on a cholesterol para-chlorocinnamate column capable of resolving all eight possible isomers confirmed that the geometry was *Z*,*Z*,*Z*.

Using GC analysis on both polar and apolar capillary columns, it was determined that 4- to 7-day-old males contained an average of 5.9 ng/male (SD = 5.09, $N = 24$) of the (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene.

Bioassays of Synthetic Pheromone. At all dosages tested, C-21 triene was attractive to male VBC when tested alone, and at most ratios with C-20 triene (Figure 1). The greatest percentages were observed to load ratios which were

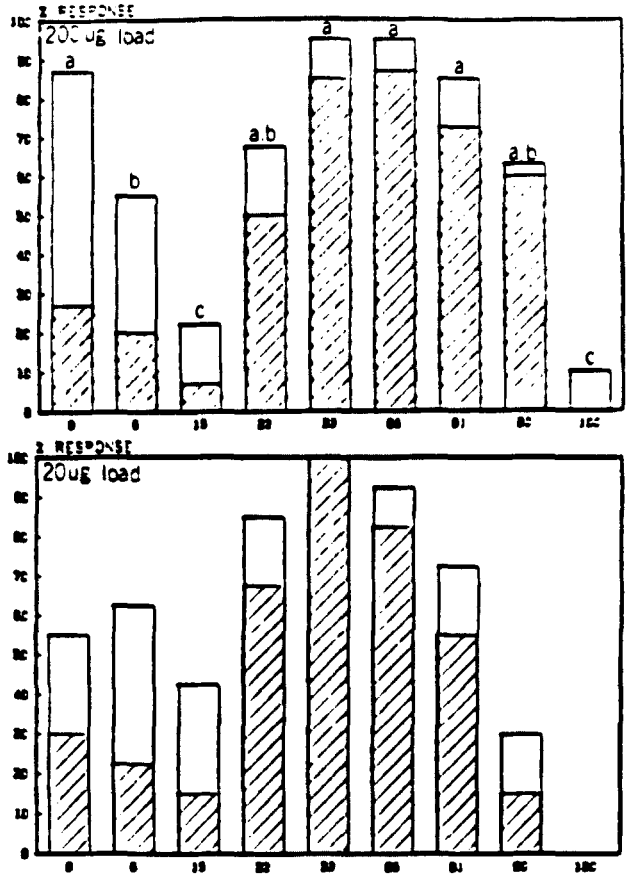

FIG. 1 Mean percentages of male *A. gemmatalis* attracted (open bar) and then reoriented (slash bar) to various release ratios of (Z,Z,Z)-3,6,9-eicosatriene and (Z,Z,Z)-3,6,9-heneicosatriene in a flight tunnel bioassay at 1-, 2-, 20-, and 200-μg loads on rubber septa. At the 200-μg load, columns having the same letter are not significantly different (Duncan, 1955).

similar to the 40:60 ratio (equivalent to a 60:40 release ratio) found in female VBC abdominal tips (Heath et al., 1983). At higher release rates, an unexpected bimodal distribution of male response was observed with minima at the 15:85% ratio of C-20–C-21 triene hydrocarbons and 100% C-20 triene hydrocarbon. At the 200-μg load, the mean percentages of males attracted to the source for the

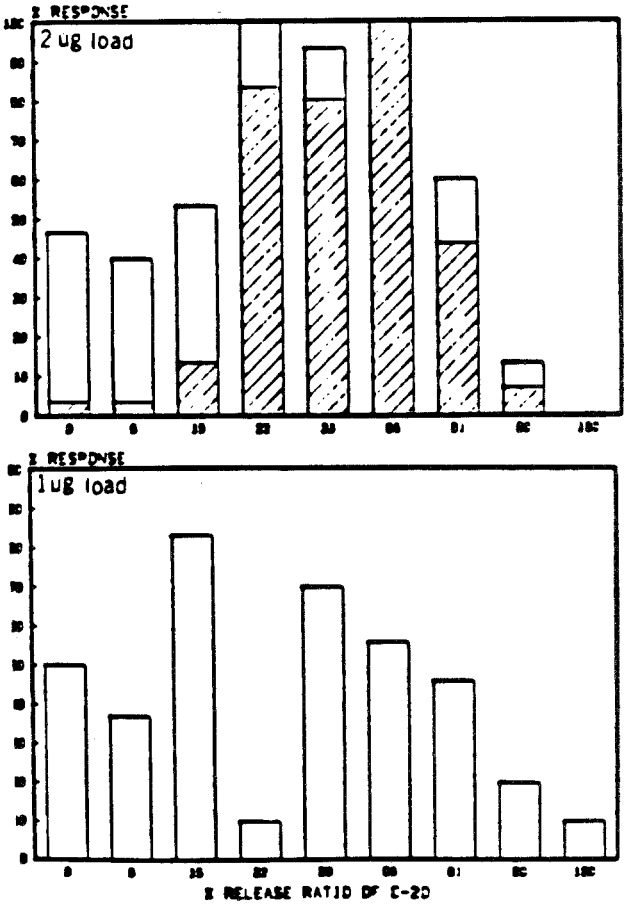

FIG. 1. Continued.

100% C-21 triene septa and the septa that released the 22, 33, 66, 81, and 90% ratios of the C-20–C-21 trienes were not significantly different.

Field Tests. At the 200-μg dose, a unimodal distribution of male response with a maximum at the female pheromone ratio (66% C-20 and 34% C-21 triene) occurred (Figure 2). However, at the 1-mg dose, a pattern similar to that observed in the wind tunnel using the 200-μg loaded septa was obtained in the field (Figure 2). That is, males were captured in a bimodal distribution, where the C-21 triene alone attracted significantly more males than septa releasing the 6:94 ratio of C-20–C21 triene.

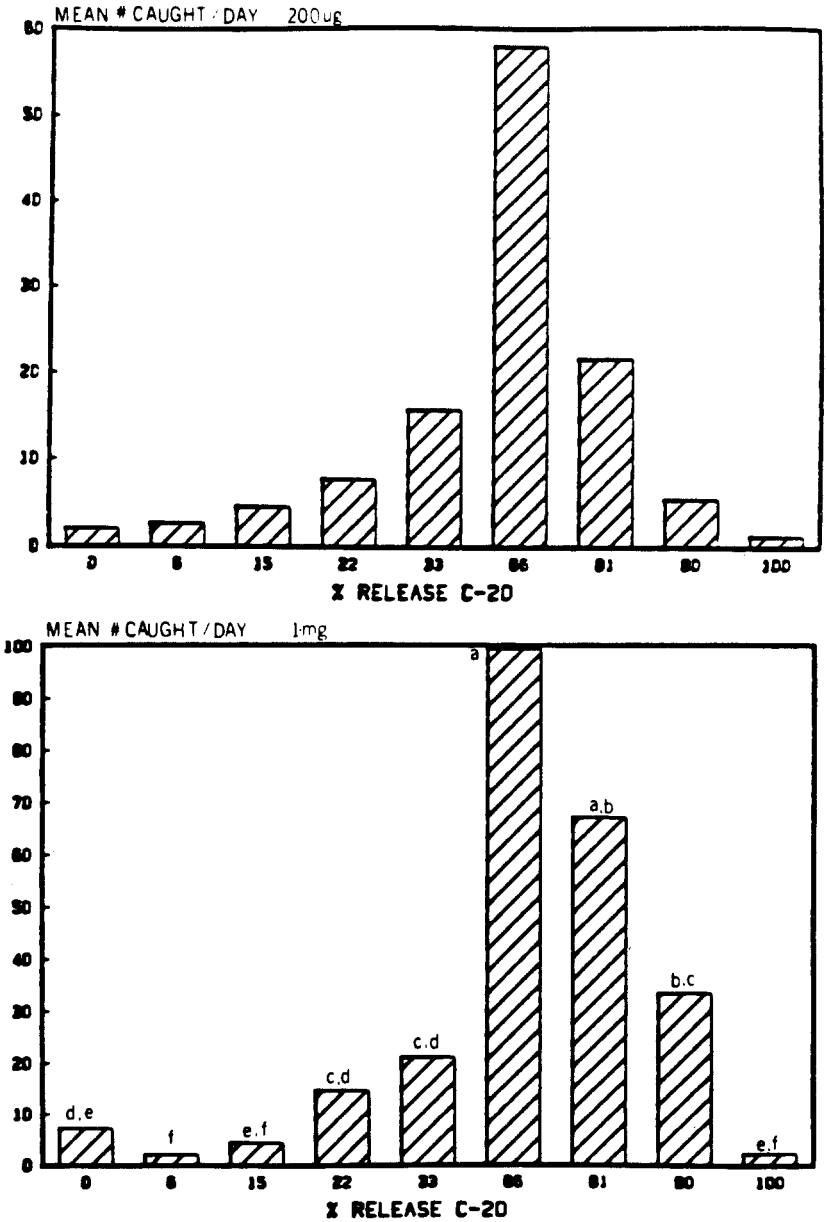

FIG. 2. Mean percentage of total trap catches of male *A. gemmatalis* in cone traps baited with ratios of (Z,Z,Z)-3,6,9-eicosatriene and (Z,Z,Z)-3,6,9-heneicosatriene at 200-μg and 1-mg loads on rubber septa. At the 1-mg load, columns having the same letter are not significantly different (Duncan, 1955).

DISCUSSION

The results of these studies demonstrate the presence of (Z,Z,Z)-3,6,9-heneicosatriene in abdominal tips of male VBC. This compound also is found as one of the sex pheromone components produced by female VBC. Male response to the male component alone and in combination with the C-20 triene produced by females resulted in a bimodal distribution.

It is doubtful that males release pheromone specifically as an attractant signal to conspecific males. According to Leppla et al. (1987), male VBC display the abdominal hairpencils and abdominal scent brushes when very near the female, as they approach, and during courtship interactions preceding mating. This suggests a courtship function for the male pheromone, directed at a calling female, possibly as an aphrodisiac. Male attraction to this signal possibly then is a secondary phenomenon resulting from male–male competition for females. Baker (1983) found that the mating success of courting *G. molesta* was reduced greatly by late-arriving competitors, which succeeded in mating 21% of the time. Behavior exhibited by late-arrivals was attraction to the first-arrivals' display, which caused the first male to misdirect a copulatory thrust, thereby disrupting normal courtship (Baker, 1983). Male VBC may be similarly able to interrupt courtship and successfully mate if they arrive before coupling. In other words, as in *G. molesta*, VBC males close by may be attracted to other courtship signals because they can sometimes interfere with an ongoing male–female courtship and gain opportunities for mating with that female.

The results from the field experiments supported in part the observations of the laboratory experiments. At the 200-μg load, no significant response (trap catch) was obtained with the male VBC pheromone. However, when septa were loaded with 1-mg amounts of pheromone, the trap catch pattern for the ratios of C-20 and C-21 triene tested resulted in a bimodal distribution similar to that observed in the wind-tunnel experiments: The male pheromone, a blend distinct from the female pheromone, was significantly more attractive than were other ratios differing from the female blend. One possible explanation of the trap catch data at the higher dose is that males release their component only during courtship and over a very short time period, perhaps at a relatively high release rate. Thus, only males in close proximity to males releasing the C-21 triene may be responsive to this male chemical cue.

We have presented in this study the identification of a compound produced by male VBC, which is also found as one of the components of the sex pheromone of female VBC. Additionally, we have demonstrated this male compound is attractive to other males in wind-tunnel and in field tests. We suspect that the primary function of this compound is as a courtship pheromone to evoke female acceptance, although this remains to be demonstrated.

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PHEROMONE-DEPENDENT SPECIES RECOGNITION MECHANISMS BETWEEN *Neodiprion pinetum* AND *Diprion similis* ON WHITE PINE

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Abstract—The sex pheromones of *Neodiprion pinetum* (Norton) and *Diprion similis* (Hartig) consist of two isomers, 2*S*,3*S*,7*S* and 2*S*,3*R*,7*R*, in either the acetate or propionate forms of 3,7-dimethylpentadecan-2-ol, respectively. The 2*S*,3*S*,7*S* acetate isomer is utilized by *N. pinetum* as the major pheromone component and the 2*S*,3*R*,7*R* acetate as a synergist. Conversely *D. similis* utilizes 2*S*,3*R*,7*R* as propionate the major pheromone component and 2*S*,3*S*,7*S* propionate as a synergist. This was confirmed in the field in both Michigan and Wisconsin. Capillary gas–liquid chromatographic analyses revealed that these two isomers are present in the natural pheromones of both species at the ratios close to those predicted by artificial blending of the two optical isomers.

Key Words—Sawflies, *Neodiprion pinetum*, *Diprion similis*, Hymenoptera, Diprionidae, 3,7-dimethylpentadecan-2-ol, *Pinus strobus*, sex pheromone.

INTRODUCTION

The sex pheromones of diprionid pine sawflies were described as the acetate or propionate of 3,7-dimethylpentadecan-2-ol (diprionol) (Jewett et al., 1976). However, when tested in the field against three diprionid sawfly species, the

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racemic synthetic compounds were not as active as the natural pheromone. When *Neodiprion lecontei* (Fitch) showed a significant response to (2*S*,3*S*,7*R/S*)-3,7-dimethylpentadecan-2-ylacetate (2*S*,3*S*,7*R/S*-A) in the field (Matsumura et al., 1979), it became clear that only certain optical isomers of diprionol esters were active. This was also confirmed in *N. pinetum* (Kraemer et al., 1979), which showed a response to 2*S*,3*S*,7*S*-A, but not to 2*R*,7*R*,7*R*-A, and 2*R*,3*R*,7*S*-A. The *N. pinetum* study was the first to show that a *Neodiprion* species responded to the 2*S*,3*S*,7*S*-isomer, although it had been predicted when 2*S*,3*S*,7*R/S*-A-baited traps attracted male *N. lecontei* in the field (Matsumura et al., 1979). Further investigations revealed that *N. lecontei, N. nanulus nanulus* Schedl, *N. sertifer* (Geoffroy), and *N. taedae linearis* Ross responded mainly to the 2*S*,3*S*,7*S* isomer (Kraemer et al., 1983), whereas two Old World species, *Diprion similis* (Hartig) and *Gilpinia frutetorum* (Fabricius), preferred the 2*S*,3*R*,7*R* isomer (Kikukawa et al., 1982a). A field study in 1980, at Higgins Lake, Michigan, by Kikukawa and Matsumura (unpublished) established that *N. pinetum responded mainly to* 2*S*,3*S*,7*S*-A and that males responded better to the acetate than to the propionate isomers. However, no definite conclusion was made from either their data or those of Kraemer et al. (1979) as to the effect of isomer blends.

The conclusion that *D. similis* responded to 2*S*,3*R*,7*R*-P isomer was based on the field response of males to the partially racemic compounds 2*S*,3*R*,7*R/S*-P; 2*S*,3*R*,7*R/S*–2*R*,3*S*,7*R/S*-P; and 2*R/S*,3*R*,7*R*-P isomers. Of these racemates, 2*R/S*,3*R*,7*R*-P was the most active, but not significantly above the others. In the same study, the natural pheromone was far superior, approximately 2500-fold. The discrepancy between the natural and synthetic was originally attributed to the racemic nature of the synthetics. There is evidence for enantiomeric specificity in the pheomones of many scolytid Coleoptera (Hedden et al., 1976; Wood et al., 1976; Borden et al., 1976, 1980). It has also been hypothesized that some racemates elicit response-inhibitory effects on the receptor system of insects that naturally respond to one enantiomeric form only (Vité et al., 1976). Experience with other *Neodiprion* sawflies showed that species respond mainly to one enantiomeric form (2*S*,3*S*,7*S*-A), but such response may be synergized by another enantiomeric form, either 2*S*,3*R*,7*R*-A or 2*S*,3*S*,7*S*-A depending on species (Kikukawa et al., 1983; Olaifa et al., 1984). This study reports on the interaction of optical isomers in field response and capillary gas-liquid chromatographic (GLC) analyses of the natural pheromones of *N. pinetum* and *D. similis*, two important defoliators of *Pinus strobus* Linneaus in North America.

METHODS AND MATERIALS

Field Tests. Field trapping of *N. pinetum* was carried out in Michigan and Wisconsin. In Michigan, two tests areas, Sec. 25 and 30, Higgins Lake State

Forest, Roscommon County, and Sec. 33, McGee on Highway M66, Kalkaska County, were used. Both consisted of pure white pine stands, each about 1.6 km square. The ground cover consisted of grasses and fern, *Pteridium aquilium* (L.) Kuhn. In Wisconsin, pure white pine stands 2 miles west of Siren were used. The larval colonies of *N. pinetum* were collected by M. Kraemer of University of Wisconsin and reared in the laboratory. They were morphologically identical to the field-trapped *N. pinetum* from Michigan.

Most of the field trapping for *D. similis* was in Kalkaska County, Michigan. All the white pine at this location were shrubby and fully exposed to the sun. Undergrowth was mainly grasses and sweet fern, *Comptonia peregrina* (L.) Coult. Also, but infrequently, used were the Higgins Lake site and the Rose Lake Wildlife Experimental Station at Bath Township in Ingham County, Michigan. At all locations, eggs, larvae, cocoons, and adults of *D. similis* were collected. Methods of bioassay have been described (Kikukawa et al., 1982a).

Pherocon II traps were used throughout, and trap preparations have been described (Kraemer et al., 1979). A randomized complete block design was used with the block choice based on rows of clearings at the two locations. At least one rerandomization of treatments within and between blocks was done, and the record of trap catch taken weekly. The data so collected were evaluated with analysis of variance and the differences among means graded at $P = 0.05$ according to Duncan's new multiple-range test. Trapped males were removed, washed with *n*-hexane to remove the sticky material, and stored in 70% alcohol. Each trapped male was examined and compared to laboratory-reared specimens. All field data were analyzed by Duncan's multiple-range test. Significant differences among samples were recognized at $P \leq 0.05$.

Natural Pheromone. Pheromone extracts (Kikukawa et al., 1982a) were obtained from adult females of *D. similis* and *N. pinetum*, reared from field-collected larvae. However, extracts used for the GLC were not treated with KOH. For capillary GLC analyses, the natural pheromone preparations from *D. similis* were hydrolyzed and reesterified to form acetate by the method of Kikukawa et al. (1982a).

Synthetic Pheromones. The propionate esters of 2*S*,3*S*,7*S*-; 2*R*,3*R*,7*R*-; 2*R*,3*R*,7*S*-; and 2*S*,3*S*,7*R*-diprionol were synthesized by Mori et al. (1978). The same group also synthesized 2*S*,3*S*,7*S*-A. Synthesis of the racemic compounds 2*S*,3*R*,7*R*/*S*-P and 2*S*,3*R*,7*R*/*S*-A; and 2*S*,3*R*,7*R*/*S*-P and 2*R*,3*S*,7*R*/*S*-P was by Tai et al. (1978). By the method described by Kikukawa et al. (1982a), the 2*R*/*S*,3*R*,7*R*-P and 2*R*/*S*,3*S*,7*S*-P were modified from one enantiomeric form from Mori et al. (1978). Kikukawa et al. (1982b) recently synthesized 2*S*,3*S*,7*S*-P; 2*S*,3*R*,7*S*-A; 2*S*,3*R*,7*R*-P; and 2*S*,3*R*,7*R*-A. As judged by NMR analyses, none of erythro preparations were contaminated by threo enantiomers and vice versa (detection limit, approx. 1%). GLC analyses (see below) on DB5 and Carbowax 20 M columns revealed no detectable enantiomer contamination (detection limit 1 ng, or about 1%). These compounds are pre-

pared in desired concentrations in 1 ml *n*-hexane sealed in glass ampules until used in the field.

Purification of Synthetic Isomers by Charcoal–Celite Column. Thirty-six grams of charcoal (Norit A., J. T. Baker Chemical Co., New Jersey) preheated for 2 hr at 180°C, were mixed thoroughly with 9 g Celite (Fisher Scientific Co.) and washed with 20 ml acetone in a funnel with Whatman No. 2 filter paper. The mixture was stirred into a 200-ml beaker with 140 ml 10% ether in hexane using a glass rod. The slurry was packed in a 50-cm × 2-cm-ID glass column. A 20-ml solvent was first used to wash the slurry down the walls of the column, followed by 80 ml in two equal volumes to stabilize the column. A maximum of 7 mg sample was introduced to the column and was eluted with 10% of ether in *n*-hexane at 0.2 ml/min of flow rate. Recovery was about 60%, but with samples less than 500 μg it was less. Sixty-three 10-ml fractions were collected using a microfractionator.

A small aliquot from each fraction was injected into a gas–liquid chromatograph (GLC) equipped with a flame ionization detector (FID). Fractions 33–63 contained the sample, but fractions 33–42 and 61–63 contained the sample and impurities while fractions 43–60 contained the pure sample. Columns less than 35 cm failed to purify the samples.

Preparative GLC Fraction Collection. A Varian 1700 preparative gas chromatograph fitted with stainless-steel column 6 m × 3 mm packed with 10% Carbowax 20 M on 80–100 mesh Chrom Q was used for fraction collection of seven synthetic isomers and the pheromone peaks of *N. pinetum* and *D. similis*. The fractions of the synthetics consisted of the front and back portions each of the GLC peaks of 2*S*,3*R*,7*R*-P; 2*S*,3*R*,7*S*-P; 2*S*,3*S*,7*S*-P; 2*S*,3*S*,7*R*-P; 2*R*,3*R*,7*R*-P; 2*R*,3*R*,7*S*-P; and 2*R*/*S*,3*S*,7*S*-P (Figure 1). The natural pheromone peaks of *N. pinetum* and *D. similis* were collected at two column temperatures: first at 200°C, then at 220°C. A 9:1 postcolumn splitter, which directed 90% of the injected material for collection and 10% to the detector, was used with a 15-cm Pyrex collection tubing bent into a U at the middle. Hexane-moistened glass wool was used to plug the distal end of the tubing, and the bent portion of the tubing contained a small amount of hexane. Trapping efficiency was about 80%. The front and back fractions were bioassayed separately in the field. The natural pheromones were concentrated for further capillary GLC analysis.

Capillary GLC Analysis. A Varian 3700 capillary GLC equipped with two FID was used. Synthetic standards containing a 1 mg/ml mixture of 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*R*-A at ratios of 1:1, 1:2, and 2:1 were used. Also used were 1 mg/ml mixtures of 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*S*-A prepared in the same ratios as above. The 2*S*,3*S*,7*S*-A was used in both species to spike the natural pheromones. Natural extracts derived from the purification were used. Concentration of natural pheromone was in the order of 5000 female equivalents/ml. Two

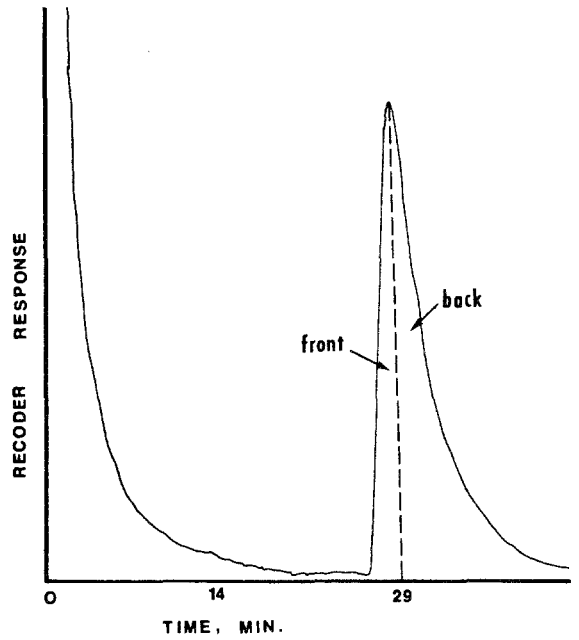

FIG. 1. GLC pattern of the 2*S*,3*R*,7*R*-P on 5% carbowax 20M column. The fractionation pattern into the front and back is shown by arrows.

fused silica capillary columns, Carbowax 20 M, 30 m × 0.25 mm ID, and DB5, 40 m × 0.25 mm ID, were used. These systems were capable of resolving threo diastereomers from erythro counterparts. In addition, in spiked samples it was possible to separate 3*S*,3*R*,7*S*-A and 2*S*,3*R*,7*R*-A. Runs were made isothermally at 180°C and temperature programmed from 130 to 190°C at 4°/min, with initial hold at zero minute, and a final hold of 18 min. The total analysis time for one injection was 32 min.

RESULTS

N. pinetum Studies. Previous studies (Kraemer et al., 1979; Kikukawa et al., 1982a) indicated that the acetate of 2*S*,3*S*,7*S*-diprionol was more attractive potent than the corresponding propionate. Therefore, various combinations of enantiomers were tested along with 2*S*,3*S*,7*S*-A.

Among those traps baited with mixtures of 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*R*-A showed a significant trap catch (Table 1). This experiment compared two pure isomers, 2*S*,3*R*,7*S*-A and 2*S*,3*R*,7*R*-A, with the racemic 2*S*,3*R*,7*R/S*-A, which

was synergistic in the earlier results. The synergistic effect of 2*S*,3*R*,7*R*-A was manifested when mixed either with 2*S*,3*S*,7*S*-A from both Mori et al. (1978) or Kikukawa et al. (unpublished). The 2*S*,3*S*,7*S*-A sample from Kikukawa et al. was utilized in all subsequent experiments. Essentially identical results were obtained at Siren, Wisconsin (May 15 to June 30, 1981) and at Higgins Lake, Michigan (May 15 to June 20, 1981). In Kalkaska, the males of *N. pinetum* were also attracted to a mixture of 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*R*-A at 1:1 and 1:2 ratios, but the latter ratio was superior (Table 2). The 2*S*,3*R*,7*S*-A and 2*R*/*S*,3*R*,7*S*-A were not active as synergists. By holding the concentration of 2*S*,3*S*,7*S*-A constant at 5 μg and varying the concentration of the synergist isomer 2*S*,3*R*,7*R*-A from 0.001 to 20 μg, it was established that maximum effectiveness of the mixture was achieved at a concentration of 10 μg of the synergist (Table 3). This implies that a 1:2 ratio gave the most active blend. When the ratio was increased to 1:4, a significant reduction in catch was recorded. Using the 2*S*,3*R*,7*R*/*S*-A as the synergist of the 2*S*,3*S*,7*S*-A isomer in Siren, Wisconsin, we again demonstrated significant effectiveness of the mixture at a blend ratio of 1:3 of 2*S*,3*S*,7*S*-A to 2*S*,3*R*,7*R*/*S*-A (data not shown).

The 1984 field tests compared the effectiveness of 2*S*,3*R*,7*R*-A and 2*S*,3*R*,7*S*-A in synergizing 2*S*,3*S*,7*S*-A in view of the results of the GLC analysis below. It was clear (Table 4) that: (1) 2*S*,3*R*,7*R*-A was indeed synergistic to 2*S*,3*S*,7*S*-A in attracting male *N. pinetum*; (2) 2*S*,3*R*,7*S*-A was not a good synergist; (3) male catches were dose related; and (4) male catch with the nat-

TABLE 1. COMPARISON OF THREE THREO ISOMERS 2*S*,3*R*,7*R*-A, 2*S*,3*R*,7*S*-A AND 2*S*,3*R*,7*R*/*S*-A IN SYNERGIZING THE EFFECTIVENESS OF 2*S*,3*S*,7*S*-A ISOMER AGAINST *N. pinetum* MALES[a]

Preparation	Amount (μg/trap)	Mean catch/ trap ± SE[b]
2*S*,3*S*,7*S*-A[c]	5	0.0 ± 0.0
2*S*,3*S*,7*S*-A[d]	5	1.3 ± 0.6
2*S*,3*R*,7*R*-A	5	0.0 ± 0.0
2*S*,3*R*,7*S*-A	5	0.0 ± 0.0
2*S*,3*R*,7*R*/*S*-A	5	0.0 ± 0.0
2*S*,3*S*,7*S*-A[c]/2*S*,3*R*,7*R*-A	5/10	5.0 ± 1.0[e]
2*S*,3*S*,7*S*-A[d]/2*S*,3*R*,7*R*-A	5/10	6.3 ± 1.1[e]
2*S*,3*S*,7*S*-A[d]/2*S*,3*R*,7*S*-A	5/10	1.0 ± 0.6
2*S*,3*S*,7*S*-A[d]/2*S*,3*R*,7*R*/*S*-A	5/10	0.3 ± 0.4
Control	0	0.0 ± 0.0

[a] Test conducted at Higgins Lake, Michigan, May 7–June 10, 1982.
[b] The results of triplicate tests.
[c] 2*S*,3*S*,7*S*-A from Mori et al. (1978).
[d] 2*S*,3*S*,7*S*-A from Kikukawa et al. (unpublished).
[e] Means significantly different from others at 5% level.

TABLE 2. COMPARISON OF THREE THREO ISOMERS 2*S*,3*R*,7*R*-A; 2*S*,3*R*,7*S*-A AND 2*R*/*S*,3*R*,7*S*-A IN SYNERGIZING THE EFFECTIVENESS OF 2*S*,3*S*,7*S*-A ISOMER AGAINST *N. pinetum* MALES[a]

Preparation	Amount (μg/trap)	Mean catch/ trap ± SE[b]
2*S*,3*S*,7*S*-A	5	0.0 ± 0.0[b]
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A	5/5	0.0 ± 0.0[b]
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A	5/10	0.0 ± 0.0[b]
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A	5/5	2.7 ± 1.1[a]
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A	5/10	4.7 ± 1.4[a]
2*S*,3*S*,7*S*-A/2*R*/*S*,3*R*,7*S*-A	5/5	0.0 ± 0.0[b]
2*S*,3*S*,7*S*-A/2*R*/*S*,3*R*,7*S*-A	5/10	0.0 ± 0.0[b]
Control	0	0.0 ± 0.0[b]

[a]Test conducted in Kalkaska, Michigan, May 5–June 10, 1982.
[b]Mean of three replicates. Means followed by same letter not significantly different at 5%.

ural pheromones was less than catches with 10 μg synthetic pheromones. Lower concentrations of synthetic pheromones were not tested.

D. similis Studies. Using crude synthetic pheromones, a series of investigations was undertaken at various locations in Michigan. The results confirmed earlier reports by Kikukawa et al. (1982a) that 2*R*/*S*,3*R*,7*R*-P; 2*S*,3*R*,7*R*/*S*-P; and 2*S*,3*R*,7*R*-P were active against *D. similis*. The 2*R*/*S*,3*R*,7*R*-P was the most active in most instances (the data not shown). In one test, crude 2*S*,3*R*,7*R*/*S*-P was more effective than pure 2*S*,3*R*,7*R*/*S*-P (purified through charcoal-celite fractions 43–60 and purity confirmed by FID GLC) which a total catch

TABLE 3. DETERMINATION OF OPTIMUM BLEND OF MIXTURE OF 2*S*,3*S*,7*S*-A AND 2*S*,3*R*,7*R*-A ISOMERS IN ATTRACTING *N. pinetum* MALES AT KALKASKA, MICHIGAN, JUNE 29–JULY 7, 1982

Preparation	Amount (μg/trap)	Mean male catch/ trap ± SE[a]
2*S*,3*S*,7*S*-A	5	0.0 ± 0.0[c]
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A	5/0.001	0.0 ± 0.0[c]
	5/0.01	0.0 ± 0.0[c]
	5/0.1	0.0 ± 0.0[c]
	5/1	0.0 ± 0.0[c]
	5/5	10.3 ± 0.9[b]
	5/10	21.0 ± 1.6[a]
	5/20	18.3 ± 1.6[ab]

[a]Means followed by the same letter not significantly different at 5% level. Means of three replicates.

TABLE 4. SYNERGISTIC INTERACTIONS OF 2*S*,3*R*,7*R*-A ISOMER AND COMPARISON OF 2*S*,3*R*,7*R*-A AND 2*S*,3*R*,7*S*-A AS SYNERGISTS FOR 2*S*,3*S*,7*S*-A IN ATTRACTING MALE *N. pinetum* IN THE FIELD[a]

Amount	2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A[b]	2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A[b]
100/0	0.7 ± 0.6[c]	0.7 ± 0.6[b]
0/100	0.0 ± 0.0[c]	0.0 ± 0.0[b]
3.3/6.7	1.6 ± 0.4[b]	0.0 ± 0.0[b]
5/10	4.3 ± 1.2[b]	0.0 ± 0.0[b]
10/20	9.0 ± 1.5[b]	0.7 ± 0.6[b]
50/100	15.3 ± 1.3[b]	2.3 ± 0.7[b]
100/200	55.7 ± 2.5[a]	7.0 ± 1.4[a]
Natural 1 FE[c]	0.3 ± 0.4[c]	0.3 ± 0.4[b]

[a] Test conducted at Kalkaska, Michigan, June 1–16, 1984. Three replicates randomized three times.
[b] Means ± SE. Means followed by the same letter not significantly different at 5% level.
[c] Female equivalent.

of 31 males for the former and five for the latter in an experiment replicated seven times. The 2*S*,3*R*,7*R*-P was purified and tested with similar results. There could be two possibilities for the poor performance of the pure 2*S*,3*R*,7*R*-P preparation. First, the inhibitory substance is one of the stereoisomers of 2*S*,3*R*,7*R*-P. Second, it is possible that a synthetic inhibitor that is not a stereoisomer, but has the same retention time as 2*S*,3*R*,7*R*-P on the chromatographic systems (GLC, TLC, and column chromatograph), is present. Testing the first possibility was not easy because there is no convenient method to measure the optical purity of each active carbon in the final synthetic product. Even the NMR spectroscopic analysis, which gives information on the diastereomeric arrangement on the 2-carbon and 3-carbon (Jewett et al. 1976), is reliable only to about 99%. However, it is known that the erythro and threo isomers could be separated by a Carbowax 20 M column (Mori et al., 1978). An attempt was made to collect the front and back portion of the peak of 2*S*,3*R*,7*R*-P (Figure 1). The same approach could also test the second possibility that the 2*S*,3*R*,7*R*-P peak contained other inhibitors. When that was done and field tested, it was found that the front part was much more active than the back part total trap catch, being 40 for the former and 12 for the latter (for nine traps).

We expected all erythro isomers to come at the front part, and therefore this excludes the possibility that any erythro enantiomers are the inhibitors. The observation, consequently, favors the second possibility that contaminants which align with the back part of the peak inhibit the effectiveness of 2*S*,3*R*,7*R*-P. Indeed for the first time, we could show that a 2*S*,3*R*,7*R*-P preparation (GLC front) was more effective than 2*R*/*S*,3*R*,7*R*-P. In view of the discovery of synergistic enantiomers for the major pheromone in many *Neodiprion* sawfly spe-

TABLE 5. SYNERGISTIC INTERACTIONS OF THREE ISOMERS ON 2*S*,3*R*,7*R*-P ISOMER IN ATTRACTING MALE *D. similis*[a]

Preparation	Amount (μg)	Mean catch/ trap ± SE[b]
2*S*,3*R*,7*R*-P	80	8.3 ± 0.7[b]
2*S*,3*R*,7*R*-P/2(*R/S*),3*S*,7*R*-P	80/0.02	14.3 ± 1.2[a]
	80/2	10.0 ± 0.8[b]
2*S*,3*R*,7*R*-P/2*S*,3*R*,7*S*-P	80/0.006	14.7 ± 0.9[a]
	80/0.06	21.0 ± 1.7[a]
2*S*,3*R*,7*R*-P/2*S*,3*R*,7*S*-P	80/0.006	8.3 ± 0.7[b]
	80/0.06	13.3 ± 1.4[a]
2*S*,3*R*,7*S*-P	80	1.3 ± 0.4[b]

[a] Test conducted at Kalkaska, Michigan, June 1–16, 1984.
[b] Means of three replicates. Means followed by same letter not significantly different at 5% level.

cies, combinations of the 2*S*,3*R*,7*R*-P GLC front with six candidate synergists were made. The results showed that 2*S*,3*S*,7*R*-P; 2*S*,3*R*,7*S*-P; 2*S*,3*S*,7*S*-P; and 2*R/S*,3*S*,7*S*-P showed some promise as synergists. The 2*S*,3*R*,7*R*-P GLC back preparation, as well as the back fraction of all the candidate synergist isomers, when mixed with the 2*S*,3*R*,7*R*-P GLC front, was not active. It is thus clear that there is a synthetic inhibitor whose retention time aligns with the back fraction.

In 1984, we pursued further the aspect of synergistic interaction of some of the isomers with 2*S*,3*R*,7*R*-P and found that the 2*R/S*,3*S*,7*R*-P; 2*S*,3*S*,7*R*-P; and 2*S*,3*R*,7*S*-P isomers were indeed synergistic to 2*S*,3*R*,7*R*-P in attracting male *D. similis* in the field (Table 5). The 2*S*,3*S*,7*S*-P isomer was the best synergist of the three at blend ratios of around 80:0.06 and 80:0.006 of 2*S*,3*R*,7*R*-P to 2*S*,3*S*,7*R*-P. The poorer performance of 2*R/S*,3*S*,7*R*-P as a synergist indicated that interaction on the 2-carbon is not important. That both 2*S*,3*S*,7*R*-P and 2*S*,3*R*,7*S*-P isomers were synergistic suggested that the synergist interaction occurred mainly on the 3-carbon and to some extent on the 7-carbon. The synergistic effect of 2*S*,3*S*,7*R*-P was again proved and the optimum blend ratio of 80:0.006 of 2*S*,3*R*,7*R*-P to 2*S*,3*S*,7*R*-P confirmed (Table 6). Concentration as low as 0.001 μg of this mixture attracted males. At the same location and time, the 2*S*,3*S*,7*S*-P isomer, at a much higher concentration than the 2*S*,3*S*,7*R*-P isomer, gave superior synergism to 2*S*,3*R*,7*R*-P isomer (Table 7), confirming synergistic interactions on the 3- and 7-carbons. The optimum blend ratio is 80:16 for 2*S*,3*R*,7*R*-P to 2*S*,3*S*,7*S*-P.

Capillary GLC Analysis. At 180°C (isothermal), the retention time of 2*S*,3*S*,7*S*-A was 12.17 min and 20.00 min on Carbowax 20 M (30 m × 0.25 mm ID) and DB5 (40 m × 0.25 mm ID), respectively. The retention time for

TABLE 6. SYNERGISTIC INTERACTION OF 2*S*,3*S*,7*R*-P ISOMER WITH 2*S*,3*R*,7*R*-P ISOMER IN ATTRACTING MALE *D. similis* IN THE FIELD[a]

Preparation	Amount (μg)	Mean catch/ trap ± SE[b]
2*S*,3*R*,7*R*-P/2*S*,3*S*,7*R*-P	80/0	8.3 ± 6.7[b]
	80/0.006	35.0 ± 2.1[a]
	80/0.06	26.0 ± 2.3[a]
	80/0.6	27.7 ± 3.0[a]
	80/6	14.3 ± 1.5[b]
	80/25	18.0 ± 1.4[a]
	80/80	0.3 ± 0.4[b]
	0/0.006	0 ± 0[b]
	0/25	0 ± 0[b]
	0/80	0 ± 0[b]
Control	0	0 ± 0[b]

[a]Test conducted at Kalkaska, Michigan, June 7–16, 1984. Three replicates randomized twice.
[b]Means followed by same letters not significantly different at 5% level.

2*S*,3*R*,7*R*-A in the same systems was 12.22 and 20.33 min, respectively. Thus, there was better resolution by the longer DB5 column. The DB5 column also separated 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*S*-A with mean retention times of 20.00 and 20.20 min, respectively. While the acetate can be separated, none of these systems could separate the alcohol preparations of 2*S*,3*S*,7*S* and 2*S*,3*R*,7*R*. However, injected singly, 2*S*,3*S*,7*S*-OH has a retention of 26.42 min, whereas 2*S*,3*R*,7*R*-OH has a retention time of 27.07 min on the DB5 temperature programmed 130–190°C at 4°/min. Also, when temperature programmed, the syn-

TABLE 7. SYNERGISTIC INTERACTION OF 2*S*,3*S*,7*S*-P ISOMER WITH 2*S*,3*R*,7*R*-P ISOMER IN ATTRACTING MALE *D. similis* IN THE FIELD[a]

Preparation	Amount (μg)	Mean catch/ trap ± SE[b]
2*S*,3*R*,7*R*-P/2*S*,3*S*,7*S*-P	80/0	8.3 ± 0.7[c]
	80/4	45.3 ± 1.7[a]
	80/16	51.0 ± 1.9[a]
	80/20	17.0 ± 1.7[b]
	80/40	20.7 ± 1.3[b]
	80/80	1.7 ± 0.8[c]
	0/16	0 ± 0[c]
	0/80	0 ± 0[c]
Control	0	0 ± 0[c]

[a]Test conducted at Kalkaska, Michigan, June 7–16, 1984. Three replicates randomized twice.
[b]Means followed by same letters not significantly different at 5% level.

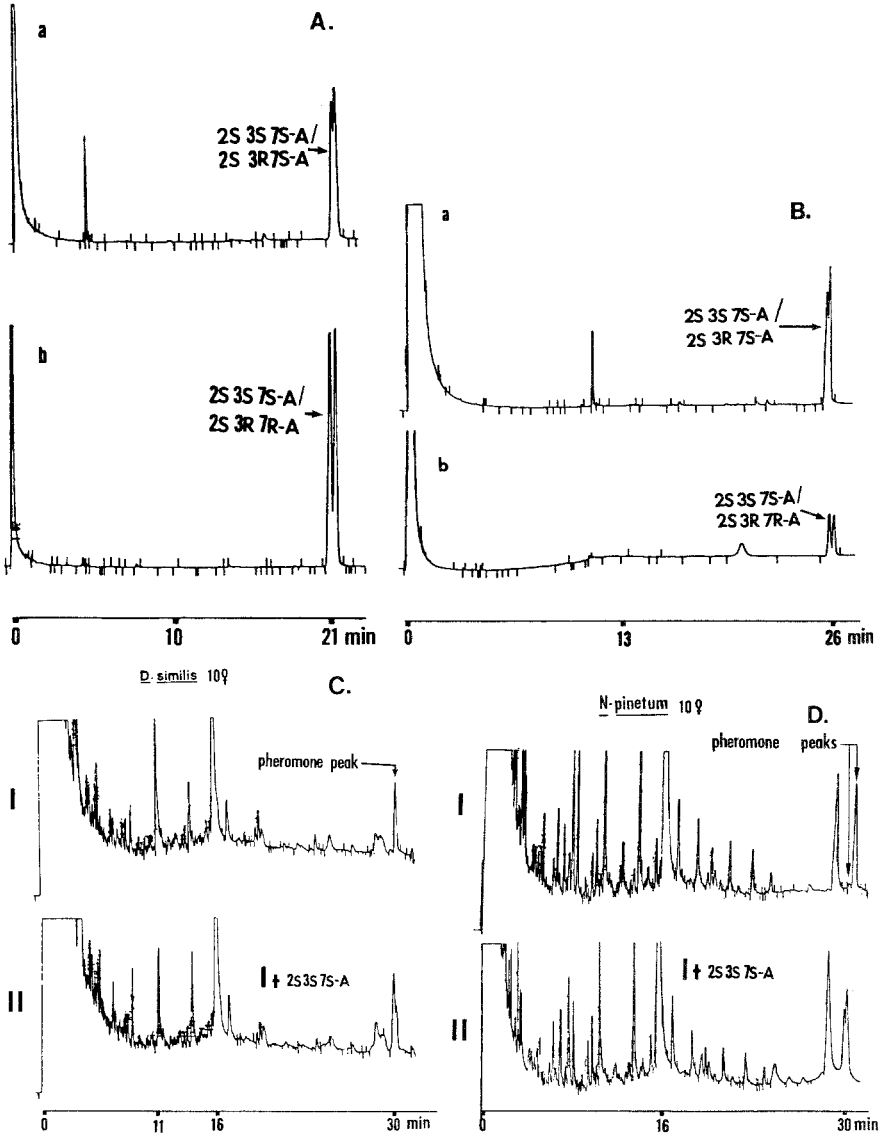

FIG. 2. (A) Capillary GLC separation on DB5 column at isothermal 180°C: (a) 2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A mixture, 100 ng; (b) 2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A mixture, 100 ng. (B) Capillary GLC separation on DB5 column temperature programmed 130–190° at 4°/min: (a) 2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A mixture, 100 ng; (b) 2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A mixture, 50 ng. (C) (I) Capillary GLC recordings of acetylated natural pheromone of *D. similis*, 10 FE, (II) Capillary GLC recordings of acetylated natural pheromone of *D. similis*, 10 FE (female equivalent) spiked with 100 ng of 2*S*,3*S*,7*S*-A. (D) (I) Capillary GLC recordings of acetylated natural pheromone of *N. pinetum*, 10 FE, (II) Capillary GLC recordings of acetylated natural pheromone of *N. pinetum*, 10 FE spiked with 100 ng of 2*S*,3*S*,7*S*-A.

thetic 2*S*,3*S*,7*S*-A has a retention time on the average of 30.20 min, whereas 2*S*,3*R*,7*R*-A was retained for 30.53 min. They were thus separated by 0.33 min. Under the same conditions, 2*S*,3*R*,7*S*-A had a retention time of 30.40 min, being separated from 2*S*,3*S*,7*S*-A by 0.20 min and from 2*S*,3*R*,7*R*-A by 0.13 min. This shows for the first time that 2*S*,3*S*,7*S*-A; 2*S*,3*R*,7*S*-A; and 2*S*,3*R*,7*R*-A can be separated and recognized by using retention time. Also, separations of 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*R*-A as well as 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*S*-A were possible on the DB5 column either when run at isothermal 180°C (Figure 2A) or temperature-programmed 130–190°C at 4°/min (Figure 2B). However, the mixture of the 2*S*,3*R*,7*R*-A/2*S*,3*R*,7*S*-A could not be separated in our system. Separation occurs only when either of the two is in mixture with 2*S*,3*S*,7*S*-A. When the acetate of the natural pheromone extract of *D. similis* was injected, two pheromone peaks were observed. The major peak corresponds to 2*S*,3*R*,7*R*-A, whereas the minor peak corresponds to 2*S*,3*S*,7*S*-A (Figure 2C). Also, the natural pheromone of *N. pinetum* indicated two peaks (Figure 2D). On spiking the sample with 2*S*,3*S*,7*S*-A, one large peak was observed which was separated by the computer into peaks corresponding to retention times of 20.06 and 20.28 min, respectively. The larger peak corresponds to 2*S*,3*S*,7*S*-A and the second peak could be aligned with either 2*S*,3*R*,7*R*-A or 2*S*,3*R*,7*S*-A. In both *D. similis* and *N. pinetum*, one virgin female contained 10 ng pheromone. This agrees with earlier reports on virgin *N. sertifer* with 10 ng/female (Kikukawa et al., 1983).

DISCUSSION

D. similis and *N. pinetum* are sympatric, sharing the same host, time span, and foliage zones. Their host specificity is strictly limited to *Pinus strobus*. One common observation about *D. similis* in the field is that the males are more discriminating than the *Neodiprion* species in response to sex pheromone regarding optical and nonoptical impurities, correctness of the chirality of the diastereomers, and choice of ester moiety. For example, while species like *Neodiprion sertifer* (Geoffroy) (Kikukawa et al., 1983) and *N. nanulus nanulus* Schedl (Kraemer et al., 1983), two normally acetate species, would respond actively to nonpurified pheromone and propionate ester of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol (2*S*,3*S*,7*S*-P), males of *D. similis* strictly prefer propionate and any impurity in the pheromone will result in little or no response.

The most important aspect of our current findings is that 2*S*,3*S*,7*R*-P interacts synergistically with 2*S*,3*R*,7*R*-P in attracting male *D. similis* in the field. This constitutes novel information as it is the first time among diprionid sawflies that an isomer which is *R* on the 3-carbon and 7-carbon will be synergized by one which is *S* on 3-carbon and 7-carbon. It is directly opposite to the situation

among pheromone systems of *N. pinetum* and other *Neodiprion* species in general where the 2*S*,3*S*,7*S* isomer is the major component and 2*S*,3*R*,7*R* or 2*S*,3*R*,7*S* is the synergist (Kikukawa et al., 1983; Olaifa et al., 1984). However, one similarity is evident: in all instances synergism occurs at a specific optimum combination, response is dose related up to that optimum, and above the optimum response inhibition sets in. In *D. similis*, the optimum blend appeared at a concentration where 2*S*,3*S*,7*R*-P constituted about 0.06–0.2% of the mixture and when 2*S*,3*S*,7*S*-P constituted about 4.8–16% of the mixture.

The evolutionary significance of this type of pheromone system in *D. similis* may be reproductive isolation from the *Neodiprion* species and *N. pinetum* in particular. These two spring-flying species share white pine as the preferred host plant. Apart from being widely divergent with respect to choice of ester moiety, acetate for *N. pinetum* and propionate for *D. similis*, the males of the former do not respond to the 2*S*,3*R*,7*R* isomer alone, even though this isomer constitutes about 66% of its optimum pheromone mixture (Table 5). It may be concluded from our experience with the pheromone systems in these two species that the sex pheromones constitute effective reproductive isolation as no male *N. pinetum* were ever caught in traps baited with pheromone of *D. similis*, be it synthetic or natural.

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HIMACHALOL AND β-HIMACHALENE: Insecticidal Principles of Himalayan Cedarwood Oil[1]

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Abstract—Chromatographic fractions of Himalayan cedarwood oil (*Cedrus deodara*) were bioassayed against the pulse beetle (*Callosobruchus analis* F.) and the housefly (*Musca domestica* L.). Almost all fractions showed insecticidal activity against both test species. Fractions I and V led to the highest mortality and also produced a quick knockdown effect. Fractions I and V, after rechromatography and purification, yielded himachalol (3%) and β-himachalene (31%), based on essential oil weight, respectively. Further evaluation of these two naturally occurring sesquiterpenes indicated 97.5% mortality at 0.56 μmol/insect against the pulse beetle. These biologically active natural products of plant origin may serve as a suitable prototypes for development of commercial insecticides.

Key Words—Himalayan cedarwood oil, *Cedrus deodara*, himachalol, β-himachalene, pulse beetle, housefly, Coleoptera, Diptera, Muscidae, insecticide, *Musca domestica*, *Callosobruchus analis*.

INTRODUCTION

Over 2000 species of plants are known to possess insecticidal properties. However, only a few of these plants containing pyrethrins, rotenoids, and alkaloids have been used commercially to meet the growing worldwide demand for natural pesticides (Balandrin et al., 1985). Among these, pyrethrum is the most commonly used. Total world production of pyrethrum is, however, not enough to satisfy the world demand for mosquito control alone. In addition, many products containing pyrethrum have an unpleasant odor (Singh et al., 1984), and

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some insects have already been reported to have developed resistance against this material (Lloyd and Parkin, 1963).

Some secondary plant metabolites, the essential oils, are known to possess numerous biological activities for pest control in addition to their perfumery and cosmetic values. The insecticidal action of essential oils was first noted in 1939, when the ovicidal effect of volatile oils was recorded against *Cochliomyia americana* (Bushland, 1939). Essential oils of some plant species have been screened in search of plant-based natural pesticides (Saxena and Koul, 1978). To date, none has been commercialized owing to inadequate efficacy, chemical complexity, and/or high cost of production.

The Himalayan cedarwood, *Cedrus deodara* Roxb. Loud. (Pinaceae) is naturally distributed in an average estimated area of 0.2 million hectares, yielding 0.75 million m^3 annual production of wood (Anonymous, 1950). Sizable quantities of wood are used for distillation of essential oils which are used worldwide in the soap industry as an inexpensive source of perfume. The toxicity and resistant properties of wood against termites (Sen-Sharma, 1963) and protection to cloth and carpeting against moths and beetles by the oil (Atwal, 1976) have been reported.

From our screening program of natural products for insecticidal properties, we have already found a knockdown property in Himalayan cedarwood oil against adult Indian mosquitoes, *Anopheles stephensi*, at low concentrations (KD_{50} 0.4452% in acetone) (Singh et al., 1984; Anonymous, 1985). The abundant availability of raw material, pleasant odor, low cost, and high potency against mosquitoes prompted us to investigate further the insecticidal principle of Himalayan cedarwood oil.

METHODS AND MATERIALS

Collection of Essential Oil. The fresh wood chips of Himalayan cedarwood (*Cedrus deodara*) were steam distilled to yield 5.0% essential oil.

Chromatography and Isolation of Active Principles. The Himalayan cedarwood oil was chromatographed on neutral alumina using cyclohexane, hexane, hexane–chloroform, chloroform, and chloroform–methanol mixtures as eluents. The various eluates were combined according to visual comparison following thin-layer chromatography on silica gel, resulting in nine chromatographic fractions. Fractions VI and VIII contained little material and thus only fractions I–V, VII, and IX were bioassayed. Because of the quick knockdown (KD) activity and high mortality in fractions I and V, these were rechromatographed on neutral alumina yielding a partially purified fraction and himachalol (I) (yield 3%), respectively. The partially purified fraction showed two spots (one major, the other minor) on $AgNO_3$ (10%) –silica gel TLC plates. Column chromatography

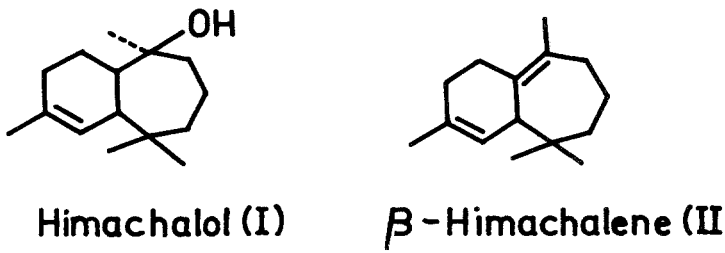

FIG. 1.

over silica gel–10% $AgNO_3$ yielded β-himachalene (II) (yield 31%). Both himachalol and β-himachalene (Figure 1) were identified by MS, NMR, GLC, and compared with authentic samples (Joseph and Dev, 1968). These purified isolates were bioassayed at different dosages by topical application.

Insects and Bioassay. Adults of the pulse beetle (*Callosobruchus analis* F.) and the housefly (*Musca domestica* L.) were used for bioassays. Pulse beetles were reared on gram (chick-pea) seeds under laboratory conditions. Adult houseflies were collected from the field. Both the test animals were starved (12 hr for *C. analis* and 2 hr for *M. domestica*) and anesthetized with diethyl ether before treatment with different fractions of Himalayan cedarwood oil or isolates of himachalol and β-himachalene.

Various chromatographic fractions of Himalayan cedarwood oil were dissolved in acetone (1 : 1 ratio v/v) and insects were treated individually at 1 μl/insect applied to the thoracic notum using an Arnold Hand Microapplicator (M/s Burkard Manufacturing Co. Ltd., London, U.K.). Controls were treated with 1 μl of acetone. For each treatment, four replicates of 10 insects were tested.

Efficacy of various chromatographic fractions was recorded in terms of mortality, i.e., adults could no longer stand (Briggs et al., 1974). Activity of different fractions was recorded 2 and 24 hr after application in the case of *C. analis*, whereas in the case of *M. domestica* the counts were taken after 2 hr only. The insecticidal action of himachalol and β-himachalene on the pulse beetle was recorded 2 hr after application.

Data are reported as percent mortality of treated insects, corrected using Abbott's formula (Abbott, 1925).

RESULTS

Data on corrected percent mortality (Table 1) indicate that, except for fraction IX against the housefly, all other fractions of Himalayan cedarwood oil exhibit some insecticidal activity against both test species. Two hours after

TABLE 1. EFFICACY OF VARIOUS CHAROMATOGRAPHIC FRACTIONS OF HIMALAYAN CEDARWOOD OIL AGAINST PULSE BEETLE AND HOUSEFLY ADULT BY TOPICAL APPLICATION

Chromatographic fraction[a]	Mean[b] % mortality (corrected)[c]		
	Pulse beetle adult (*C. analis*)		House fly adult (*M. domestica*)
	2 hr	24 hr	2 hr
I	100.0[d]	100.00	97.5[d]
II	77.5	91.7	86.7
III	32.5	83.3	12.5
IV	90.0	100.0	NT
V	95.0[d]	100.0	25.0
VII	92.5	97.2	10.0
IX	65.0	94.4	00.0
Acetone (control)	00.0	—	00.0

[a] Fractions VI and VIII not tested due to less recovery during chromatography.
[b] Mean of four replications, 10 insects per set.
[c] Abbott (1925).
[d] Quick knockdown effect. NT, not tested due to small amount of fraction IV.

application, mortality ranged from 32.5 to 100% for the pulse beetle and from 0 to 97.5% for the housefly. In the case of the pulse beetle, mortality was also recorded 24 hr after application. We found that fractions III and IX, which were less potent at 2 hr (32.5 and 65% mortality, respectively), resulted in 83.3 and 94.4% mortality, respectively, after 24 hr.

Of the chromatographic fractions, fraction I had the highest activity and rapid KD effect against both test species, while similar activity of fraction V was observed only against the pulse beetle. Other fractions that showed some insecticidal activity were fractions II, IV, and VII against the pulse beetle and fraction II against the housefly. However, all these fractions were devoid of KD effect. Fraction IX was inactive against the housefly.

Fractions I and V, which showed the highest biological activities (quick KD and mortality), were rechromatographed to purify the active principles. Rechromatography of fractions I and V on neutral alumina resulted into the isolation of a partially purified fraction containing chiefly β-himachalene and himachalol, respectively.

Biological activities of these two compounds were assessed at three different dosages (0.56, 1.12, and 2.24 μmol/insect) against the pulse beetle (*C. analis*). At the 0.56 μmol/insect dose, mortality from both compounds was 97.5%, with complete lethality at 1.12 μmol/insect (Table 2). Although both

TABLE 2. COMPARATIVE INSECTICIDAL ACTIVITY OF HIMACHALOL AND PARTIALLY PURIFIED FRACTION FROM HIMALAYAN CEDARWOOD OIL AGAINST PULSE BEETLE

Treatment	Mean mortality % after 24 hr[a]		
	0.56 μmol dose	1.12 μmol dose	2.24 μmol dose
Himachalol[b]	97.5	100.0	100.0
Partially purified chromatographic fraction I (95% β-himachalene)	97.5	100.0	100.0
Control (acetone)	00.0	00.0	00.0

[a] Test procedure as in Table 1, replicated four times.
[b] Quick knockdown effect just after treatment.

the isolates were found to possess similar insecticidal potency, only himachalol exhibited the rapid KD effect.

DISCUSSION

An earlier study (Singh et al., 1984) reported the insecticidal potential of Himalayan cedarwood (*Cedrus deodara*) oil against Indian mosquitoes, *Anopheles stephensi* under laboratory conditions. In the present communication, insecticidal activity of different fractions of Himalayan cedarwood oil against insect pests of agriculture (*Callosobruchus analis*) and human health (*Musca domestica*) is reported.

Of the different fractions of Himalayan cedarwood oil, fraction I produced the highest mortality with quick KD effect against both test species, while similar activity of fraction V was observed against the pulse beetle only. Alternatively, adult houseflies may be able to metabolize the active principle of fraction V. Species-specific activity of naturally occurring products of plant origin has also been observed by Isman et al. (1986), who noted precocene-II to be more toxic against grasshopper nymphs than encecalin, the latter compound having better activity against cutworms.

The KD activity is thought to result from rapid penetration of the chemical into the insect's body (Briggs et al., 1974; Chen et al., 1985). Similar biological activity in insects has also been noted previously for other naturally occurring botanical insecticides, such as pyrethrins. The primary target of the pyrethrins is thought to be the nervous system, judging from their quick actions (Matsumura, 1980). The quick KD activity in fractions I and V could result from a similar action. Lack of KD activity in fraction V against the housefly adults

may be due to limited penetration into the body or enhanced metabolic detoxification. However, the major component of this fraction is inactive, thus the KD activity in the crude fraction V must result from some minor components.

Wood chips of *Cedrus deodara* have been reported to possess juvenile hormone-like activity against the red cotton bug, *Dysdercus koenigii* (Bhan et al., 1984), and wood extracts have been shown to reduce the total protein content of the hemolymph, silk glands, and eggs, as well as viability of larvae of silk worm, *Bombyx mori* (Gigolashvili et al., 1983). It is also repellent to termites (Sen-Sharma, 1963) and toxic to white ants (Akhtar, 1981). Essential oils obtained from Himalayan cedarwood have been reported to be toxic to Indian mosquitoes, *Anopheles stephensi* (Singh et al., 1984; Anonymous, 1985), and to reduce fecundity of the red cotton bug, *D. koenigii* (Singh and Rao, 1986).

Two insecticidal sesquiterpenes, himachalol and β-himachalene, have been isolated as active principles of Himalayan cedarwood oil. These compounds could possibly be modified to enhance their biological activity, which could give rise to new pest control products for commercial use.

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IDENTIFICATION OF THORACIC GLAND CONSTITUENTS FROM MALE *Xylocopa* spp. LATREILLE (Hymenoptera: Anthophoridae) FROM ARIZONA[1]

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Abstract—Some male carpenter bees (*Xylocopa* spp.) possess massive propodeal exocrine glands that produce copious quantities of multicomponent blends of lipoidal material. Some of these compounds are volatile, producing a "flowery" aroma that can be easily detected several meters downwind from a territorial male. Chemical characterization of these secretions showed them to be blends of terpenoid compounds or fatty acid derivatives. In *X. varipuncta*, the mixture is composed of all *trans*-geranylgeraniol, all *trans*-farnesal, and an isomer of 3,7,11-trimethyl-2,7,10-dodecatrienal in an approximate 9:6:1 ratio. The secretion of *X. micheneri* contains isopropyl oleate, (Z)-11-eicosen-1-ol, oleyl alcohol, and methyl palmitate at approximately 63:24:11:1. We hypothesize that these compounds act as phero-

[1] Mention of a proprietary product does not constitute an endorsement or a recommendation for its use by USDA.

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mones that aid in attracting and holding conspecific females to symbolic nonresource encounter sites where mating occurs. They also appear to have utility as chemotaxonomic markers at the subgeneric level.

Key Words—Thoracic glands, secretion, *Xylocopa varipuncta*, *Xylocopa micheneri*, Hymenoptera, Anthophoridae, terpenoid, fatty acid ester, fatty alcohol.

INTRODUCTION

Carpenter bees in the genus *Xylocopa* Latreille comprise a group of more than 400 species with worldwide distribution. They are part of the tribe Xylocopini within the large family Anthophoridae. These are characterized by their habit of nesting in dead wood, with the exception of the Old World genus *Proxylocopa*, which nests in the soil.

Arizona is rich in bee species, including eight species of *Xylocopa* in five subgenera. *X. varipuncta* Patton (subgenus *Neoxylocopa*) is common in southern Arizona where it nests in old inflorescence stalks of century plants (*Agave* spp.) or in dead branches of native and ornamental plants. In Tucson, the species is easily found nesting in dead wood of chinaberry trees (*Melia azedarach* L.) and may even invade soft structural timbers such as redwood. The other species, *X. micheneri* (Hurd) (subgenus *Stenoxylocopa*), is much rarer and is not known to nest within city limits. *Xylocopa micheneri* is at the northern limits of its range and apparently inhibits only isolated localities in "island-like" desert mountains of southern Arizona (e.g., Baboquivari, Santa Catalina, and Santa Rita mountains) as reported by Hurd (1978). In Arizona, *X. varipuncta* typically has one generation per year, while *X. micheneri* may produce a second generation. Both sexes in each species are long-lived as adults, and the active flight season begins in April and usually extends until early September. Larvae metamorphose and adults of both sexes remain in their nests, or males (as in the case of *X. micheneri*) may disperse to communal "bachelor" aggregation/sleeping sites for the fall and winter to later become sexually active during the following spring.

The present investigation was stimulated by observations by Marshall and Alcock (1981) and Minckley (unpubl.) that hovering male *X. varipuncta* in lek territories emit a sweet perfume while hovering within tree crowns. An observer 2–4 m downwind from a resident male can easily detect a sweet floral fragrance emanating from the male. This odor originates in a gland in the thoracic region and is the subject of this chemical report.

During the time these behavioral observations were conducted, a paper appeared (Vinson et al., 1986) describing presumably unique thoracic glands (named dorsal mesosomal glands by them) in two Costa Rican carpenter bees.

They (Vinson et al., 1986) also described flowery aromas produced by hovering male *X. fimbriata* and *X. gualanensis* (in the subgenera *Megaxylocopa* and *Neoxylocopa*, respectively), which were further described as "terpenoid." This report was followed recently by a study of Williams et al. (1987), who determined the chemical composition of glandular contents from these two Costa Rican species. In our study, the contents of the dorsal mesosomal glands of *X. varipuncta* and *X. micheneri* are investigated and the results are compared with those from other *Xylocopa* species.

METHODS AND MATERIALS

Isolation of Glandular Components. Males of *X. varipuncta* were collected from nests in chinaberry trees within the city limits of Tucson, Pima County, Arizona, during February to April 1986. Male *X. micheneri* were collected from male overwintering clusters inside burrows in century plant (*A. palmeri*) stalks. All bees were transported back to the laboratory on ice where they were immediately dissected.

Glands from nine male *X. varipuncta* and 14 male *X. micheneri* were excised and placed in 3-ml glass-distilled hexane. After several days, the hexane solution was filtered through glass wool and reduced to a volume of 1.0 ml under a gentle stream of nitrogen.

Capillary GC was carried out on a Varian 3700 instrument equipped with a FID and a 25 m × 0.32 mm DB-1701 fused silica column (J & W). The carrier gas was helium, and the oven temperature was programmed from 100°C to 250°C at 10°/min. Injector and detector temperatures were 270°C, and 350°C, respectively. All injections were made in the split mode, with a split ratio of 70:1. Peak area measurements used to determine relative quantities were made by electronic integration, and retention indices were calculated with the homologous *n*-alkane series (Van den Dool and Kratz, 1963).

Preparative GC was carried out on the same instrument as above, equipped with a 1.7 m × 4 mm ID glass column packed with 3% OV-17 on Gas-Chrom-Q (80/100). Helium was used as the carrier gas, and the temperature was programmed from 100°C to 250°C at 4°/min for *X. varipuncta* extracts and from 150°C to 250°C at 4°/min for *X. micheneri* extracts. Injector and detector temperatures were held at 270°C and 350°C, respectively. Fractions were collected using a glass-lined effluent splitter (Scientific Glass Co., 9.5:0.5 split ratio), and a thermal gradient fraction collector cooled with a Dry Ice–methanol bath (Brownlee and Silverstein, 1968). Isolated compounds were collected in 300 mm × 0.5 mm ID capillary tubes that were flame-sealed after collection.

Spectroscopic Analysis. Electron impact–gas chromatography–mass spectrometry (EI-GC-MS) (70 eV) was performed on a Finnigan triple-stage quad-

rupole instrument interfaced with a gas chromatograph equipped with a 30 m × 0.32 mm DB-1 fused silica column. The chromatographic conditions were similar to those described above. A Varian MAT instrument was also employed coupled with a gas chromtograph, with a 30 m × 0.25 mm fused silica bonded phase of OV-1 capillary column. Chemical ionization (CI) GC-MS was performed on the Finnigan instrument using isobutane (0.3 torr) as the reagent gas and the same chromatographic conditions as above.

Proton and carbon NMR spectroscopy were performed with a Bruker WM 300 WB spectrometer using 5-mm tubes and $CDCl_3$ as the solvent. When sufficient material was available, the DEPT experiment was used to determine carbon types (CH_3, CH_2, CH, C).

Methoxymercuration–Demercuration. A solution of *X. micheneri* glandular extract (ca. 200 μg) in hexane was evaporated to near-dryness and dissolved in 500 μl methanol. To this solution, 0.5 mg mercuric acetate was added, and the mixture was left in the dark at room temperature for 16 hr. Sodium borohydride (0.5 mg) was then added, and after 1 hr the mixture was diluted with distilled water. The aqueous mixture was extracted with three volumes (1 ml) of anhydrous diethyl ether. After drying over anhydrous sodium sulfate, the ether extracts were concentrated under a stream of nitrogen and analyzed by EI-GC-MS.

Severe tailing was observed with hydroxylated methoxy derivatives, so these samples were evaporated to dryness and trimethyl silylated with Trisil (Pierce Chemical). The mixture was incubated at 60°C for 1 hr and analyzed by EI-GC-MS.

RESULTS

Xylocopa varipuncta Studies. Initial capillary analysis indicated that three components were present in large quantities (Figure 1A and Table 1), with 3 and 2 being the most abundant.

Spectral data indicated that compound 3 was an acyclic tetraunsaturated diterpene alcohol with the molecular formula $C_{20}H_{34}O$. CI-MS: *m/e* 291 (M + H), 273; EI-MS: *m/e* 290 (M), 272, 203, 161, 69; [^{1}H]NMR: δ 5.41, t (J = 6.9 Hz), 1H; 5.09, m, 3H; 4.15, d (J = 6.9 Hz), 2H; 2.04, m, 12H; 1.67, s, 6H; 1.59, s, 9H. These data are consistent with the assignment of 3 as an isomer of geranylgeraniol. Determination of the geometry of asymmetrical unsaturations was accomplished by comparison of the chemical shift values for methylene resonances in the ^{13}C spectrum with published assignments made for acyclic monoterpenoids (Bohlmann et al., 1975). A cluster of three methylene resonances at δ 39.7, 39.7, and 39.6 were assigned to the methylene groups adjacent to the methyl branched carbons and indicated an *E* configuration for

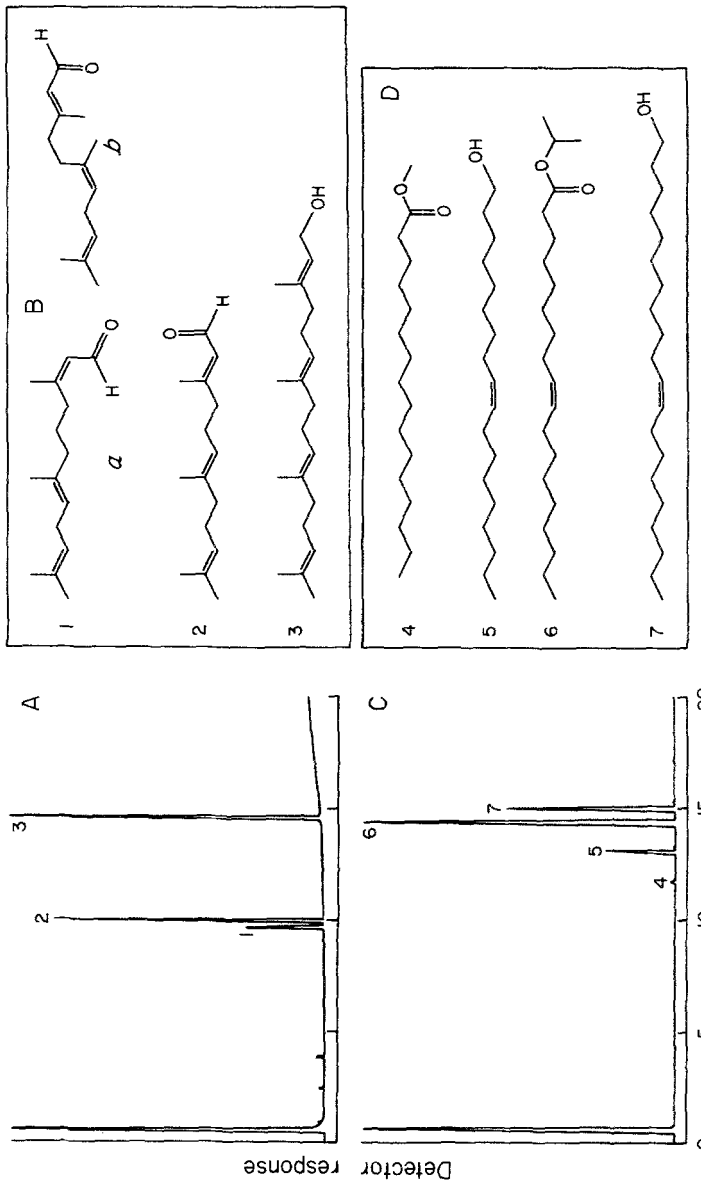

FIG. 1. (A) Gas chromatogram of *X. varipuncta* glandular mixture, with the three major components numbered. (B) Structures of glandular components from *X. varipuncta*; a and b represent two possible structures of component 1. (C) Gas chromatogram of *X. micheneri* glandular mixture. (D) Structures of glandular components from *X. micheneri*.

TABLE 1. COMPOSITION OF GLANDULAR SECRETION OF *X. varipuncta* AND *X. micheneri*

X. varipuncta			*X. micheneri*		
Compound	RI[a]	Rel. %	Compound	RI[a]	Rel. %
1a, b	1869	6.2	4	1995	1.0
2	1898	34.6	5	2186	11.1
3	2348	56.2	6	2280	62.6
			7	2401	24.8

[a] Retention indices (RI) were calculated using the DB-1701 column

all three unsaturations. Therefore, 3 was assigned as all *trans*-geranylgeraniol (Figure 1B).

Compound 2 was determined to be a geometrical isomer of farnesal ($C_{15}H_{24}O$), based on the following spectral data. CI-MS; *m/e* 221 (M + H), 203; EI-MS; *m/e* 220 (M), 205, 136, 84, 69; [^{1}H]NMR: δ 9.98, d (J = 8.0 Hz), 1H; 5.88, d (J = 8.0 Hz), 1H; 5.07, m, 2H; 2.22, m, 4H; 2.16, s, 3H; 1.67, s, 3H; 1.59, s, 6H. The location of the methylene signals (δ 40.0, 39.6, 26.6, 25.7) in the ^{13}C spectrum indicated an *E,E* configuration. Compound 2 was thus identified as all *trans*-farnesal (Figure 1B).

The mass spectra of 1 were very similar to those of 2, indicating that this compound was also an isomer of farnesal. The [^{1}H]NMR data were as follows: δ 9.91 d (J = 8.2 Hz), 1H; 5.81, d (J = 8.2 Hz), 1H; 5.11, m, 2H; 2.59, t (J = 7.5 Hz), 2H; 2.24, m, 2H; 2.02, m, 2H; 1.98, s, 3H; 1.67, s, 3H; 1.59, s, 6H; 1.53, (H_2O contaminent + CH_2). The presence of a doubly allylic methylene group (δ 2.59) showed that compound 1 was a geometrical isomer of 3,7,11-methyl-2,7,10-dodecatrienal. Because of a lack of sufficient quantities of isolated material, only a weak ^{13}C spectrum was obtained. However, the positions of four apparent methylene resonances (δ 26.6, 27.0, 32.6, 39.6) suggested that unsaturations were present in both the *E* and *Z* configurations. Since not all resonances were detected, assignment of configuration of specific bonds could not be made with certainty, and two possibilities are illustrated in Figure 1B.

Several minor components comprising less than 1% each of the glandular contents were detected by capillary GC. No attempts have been made to identify these at this time.

Xylocopa micheneri Studies. The *X. micheneri* mixture contained three major components and a single minor component comprising about 1% of the mixture (Figure 1C and Table 1). Chromatographic data indicated that the *X. micheneri* mixture had no components in common with that of *X. varipuncta*.

The spectral data for compound 7 indicated a monounsaturated linear alcohol with the molecular formula $C_{20}H_{40}O$. CI-MS: *m/e* 297(M + H), 278; EI-MS: *m/e* 123, 109, 67, 96, 82, 55. [^{1}H]NMR: δ 5.35, m, 2H; 3.63, m, 2H; 2.00, m, 4H; 1.52, m, 2H; 127, m, 26H; 0.87, t (J = 6.7 Hz), 3H. Mass spectrometry of the silylated methoxy derivative (*m/e* 157, 171) revealed a ω-9 unsaturation, and a resonance due to allylic methylene carbons at δ 27.3 in the ^{13}C spectrum showed *Z* configuration (Johnson and Jankowski, 1978). Therefore, compound 7 was determined to be (*Z*)-11-eicosen-1-ol (Figure 1D).

Compound 6 was found to be an isopropyl ester of a monounsaturated fatty acid with the molecular formula $C_{21}H_{40}O_2$. CI-MS: *m/e* 325 (M + H), 283; EI-MS: *m/e* 324 (M), 282, 265, 264; [^{1}H]NMR: δ 5.33, m, 2H; 4.99, m, 1H; 2.24, t (J = 7.4 Hz), 2H; 2.00, m, 4H; 1.60, m, 2H, 1.27, m, 20H; 1.22, d (J = 6.3 Hz), 6H; 0.87, t (J = 6.7 Hz), 3H. The mass spectrum of the methoxy derivative showed a ω-9 unsaturation, and the ^{13}C spectrum indicated the *Z* configuration. On the basis of these data, compound 6 was identified as isopropyl (*Z*)-9-octadecenoate (isopropyl oleate, Figure 1D).

The mass spectral and [^{1}H]NMR data for compound 5 indicated that it was an 18-carbon homolog of compound 7. Analysis of the methoxy derivative again indicated a ω-9 unsaturation, and comparison of capillary retention characteristics with an authentic standard verified that this compound was (*Z*)-9-octadecen-1-ol (oleyl alcohol, Figure 1D).

A fourth component (4) was detected that made up 1% of the glandular mixture. This compound had a retention index of 1995 on DB-1701, and was identified as methyl palmitate (Figure 1D) on the basis of CI and EI mass spectral data.

DISCUSSION

The exocrine gland chemistry of male and female *Xylocopa* has received relatively little attention, although these bees are natural-products chemists *par excellence*! Various secretions are used in nest construction, territorial defense, and mate attraction. Previous studies have dealt with the chemical constituents of mandibular gland secretions in *X. hirsutissima* (Wheeler et al., 1976) and *X. sulcatipes* (Hefetz, 1983), and Dufour's gland secretions in *X. micans* (Williams et al., 1983), *X. sulcatipes* (Kronenberg and Hefetz, 1984), *X. virginica texana* (Frankie and Vinson, 1977; Vinson et al., 1978), and *Proxylocopa olivieri* (Kronenberg and Hefetz, 1984). The morphological and histochemical relationships of other exocrine glands (''yellow glands'') have also been studied (Gerling et al., 1979). Only recently has information pertaining to the thoracic glands of male *Xylocopa* been presented (Vinson et al., 1986; Williams et al., 1987).

Histological studies by Buchmann (unpublished) and Vinson et al. (1986) have demonstrated the *Xylocopa* thoracic exocrine glands as massive, paired

structures occupying areas under scutellar, metanotal, and propodeal cuticles. They are composed of darkened tubules that fill the area between the cuticle and flight musculature. The glandular complex of the two Arizona species is composed of secretory and storage tubules, each leading to the exterior (not as a separate basal gland as described for *X. fimbriata* by Vinson et al., 1986). The gland is a massive structure that can produce and store large quantities (usually 10–25 μl) of secretion. This large reservior would appear to ensure the availability of volatile secretions for long periods of territorial display.

Although both *X. varipuncta* and *X. micheneri* share apparently homologous glands, each species produces a characteristic blend of glandular components, with no shared compounds. The larger *X. varipuncta* males produce a three-compound blend of terpenoids. This secretion is dominated by all *trans*-geranylgeraniol, and also contained substantial amounts of all *trans*-farnesal and its isomer, 3,7,11-methyl-2,7,10-dodecatrienal.

In marked contrast to *X. varipuncta*, males of *X. micheneri* have terpene-free secretions that contain only fatty alcohols and fatty acid esters. The dominant component in this species is isopropyl oleate, followed by (*Z*)-11-eicosen-l-ol, and oleyl alcohol. Also present was a trace quantity of methyl palmitate. Isopropyl esters have not been reported in the exocrine secretions of any hymenopterous insects but are known to occur in staphylinid beetles where they serve as surfactants for quinone defensive secretions (Dettner, 1984).

The secretions of these two species of desert carpenter bee arise by different biosynthetic pathways and could potentially serve as important phylogenetic markers. Components of the *X. varipuncta* secretion are most likely derived from mevalonic acid via the isoprenoid pathway, while the *X. micheneri* components are derived from acetate via fatty acid synthetase. These identifications and the recent identification of male thoracic gland constituents from two Costa Rican species (William et al., 1987) provide encouraging evidence for their role as phylogenetic markers. *X. varipuncta* and the Costa Rican species *X. gualanensis* Cockerell have been placed in the subgenus *Neoxylocopa* based on morphological characters. Williams et al. (1987) report that the secretion of *X. gualanensis* is very similar to that of *X. varipuncta*, being dominated by geranylgeraniol and two isomers of farnesal. Although the farnesal structures were not specifically determined, the less abundant isomer of the *X. gualanensis* mixture is possibly 3,7,11-trimethyl-2,7,10-dodecatrienal, and the more abundant may be all *trans*-farnesal.

X. micheneri is classified in a different subgenus (*Stenoxylocopa*) than *X. varipuncta* and *X. gualanensis*, and this is reflected in the vastly different composition of the thoracic gland mixture. Williams et al. (1987) report that *X. fimbriata* Fabr., a representative of a third subgenus (*Megaxylocopa*), contains a mixture dominated by 2-heptadecanone, farnesol, and farnesyl acetate. This secretion is of mixed biosynthetic origin, and could represent a third biosyn-

thetic catagory of thoracic gland mixture. These data suggest that analyses of glands from additional *Xylocopa* species will provide useful information on phylogenetic relationships at the subgeneric level.

The reproduction biology, especially male mating strategies, have been studied for very few North American carpenter bees. More is known about the biology of Old World *Xylocopa* (see Gerling et al., 1983). One study (Marshall and Alcock, 1981) focused on male behavior and mating in *X. varipuncta* in Arizona. They found that males occupied territories during the late afternoon in the crowns of nonflowering trees along desert washes. They stated that females and other males occasionally visited a hovering male, "probably drawn by sex pheromone" (Marshall and Alcock, 1981). As with most territorial male bees, the *X. varipuncta* males are bellicose toward all intruders (both animate and inanimate) into their airspace, where they attempt to repel males and inseminate females. These hovering territories contain neither nests, potential nest sites, nor floral nectar or pollen resources for females or themselves. Thus, the defense of such landmark territories has been referred to as a dispersed lek mating system.

The precise role of the male thoracic gland secretion of *Xylocopa* spp. is presently unknown. We hypothesize, however, that the lipid secretions may function as pheromones in the dispersed lek mating system (Marshall and Alcock, 1981; Minkley, unpublished data, University of Arizona, Tucson, Arizona). The large quantity of volatile material liberated by the male mesosomal gland may attract females and may also provide the female with an indicator of male fitness. Information pertaining to fitness could be contained in the quantity of volatilized pheromone in the vicinity of the landing/marking site (see Marshall and Alcock, 1981, for a description of *X. varipuncta* mating behavior). Studies are now in progress to elucidate the role of the thoracic gland secretion in the mating behavior of these two Arizona *Xylocopa* species.

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PHEROMONE GLAND COMPONENTS OF SOME AUSTRALIAN TORTRICIDS IN RELATION TO THEIR TAXONOMY

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Abstract—A survey has been carried out to identify the major sex pheromone gland components in 30 species of Australian tortricid moths. While more than half the species examined have (*Z*)-11-, (*E*)-11-, and/or (*Z*)-9-tetradecenyl acetates as the principal components, these compounds were not detected in some plesiomorphic taxa where combinations of (*Z*)-5 and (*Z*)-7 or (*Z*)-8 and (*Z*)-10 isomers are present. The results are discussed in relation to tortricid classification and current theories on pheromone evolution.

Key Words—Lepidoptera, Tortricidae, sex pheromone, pheromone biosynthesis, pheromone evolution.

INTRODUCTION

Tortricids use a restricted range of chemicals as sex attractants, comprising mainly C_{12} and C_{14} alkenyl acetates, with a few C_{10} and C_{16} compounds (Roelofs and Brown, 1982; Priesner, 1984; Arn et al., 1986). A review of tortricid sex attractants and pheromones by Roelofs and Brown (1982) indicated a correlation between the chemical nature of pheromone components and the taxonomic position of tortricid moths. This correlation is not a strict direct correspondence between specific chemical compounds and taxonomic units, but rather one of trends for a predominance of certain compounds in certain higher taxa. Thus, while a particular compound may appear as a characteristic major component in congeneric species, the same compound may also be represented in taxonomically widely separate groups. Nevertheless, certain generalizations are possible. Each of the three tortricid subfamilies is characterized by the predominance of compounds with a particular chain length in their pheromones.

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Tortricine species use mostly C_{14} compounds, while C_{12} compounds predominate among the Olethreutinae. C_{16} compounds have been reported as attractants for two species of *Isotrias* Meyrick and one of *Olindia* Guenée (Priesner, 1984), the only members so far examined from the third tortricid subfamily, the Chlidanotinae. No correlations have been demonstrated below the subfamily level, except for the tribe Cnephasiini s. str. (i.e., the genera closely associated with *Cnephasia* Curtis), which is characterized by C_{12} compounds, and is thus distinctive within the Tortricinae. However, any overall analysis of tortricid pheromones is greatly hampered by the current inadequate understanding of tortricid phylogeny and, in particular, by the great number of taxa assigned on the basis of purely superficial similarity to quite unrelated groups.

Extensive studies on tortricid pheromone biosynthesis, especially of *Argyrotaenia velutinana* (Walker), have provided a fairly complete picture of the biosynthetic pathway employed by the majority of tortricines, involving Δ^{11}-desaturation and chain shortening of C_{16} and C_{18} acyl intermediates (Roelofs and Brown, 1982; Roelofs and Bjostad, 1984; Bjostad and Roelofs, 1986). This mechanism can account for the pheromone components of most but not all tortricids. Two other related mechanisms have been proposed (Roelofs and Bjostad, 1984) to explain the formation of the (*Z*)-5- and (*Z*)-7-tetradecenyl acetates (*Z*5- and *Z*7-14:OAc) and (*Z*)-8- and (*Z*)-10-tetradecenyl acetates (*Z*8- and *Z*10-14:OAc) present in some generalized (i.e., less evolved, plesiomorphic) tortricids (Young et al., 1985; Galbreath et al., 1985; Foster et al., 1986), with the suggestion that the biosynthetic pathways may reflect the evolutionary status of the groups involved.

The presence in Australia of other morphologically generalized Tortricidae prompted an investigation of the large and diverse archaic element in the Australian tortricid fauna. We have attempted no more than a preliminary survey of the pheromone gland components of representatives of typical Australian groups, with special attention to taxa which are presumed to be plesiomorphic (underived) but whose affinities are not yet understood. The aim of the work has been to provide chemotaxonomic information on endemic Australian groups, to test techniques for pheromone analysis, particularly under circumstances where little insect material is available, and to identify groups of interest for further detailed study.

METHODS AND MATERIALS

Insect Material. Virgin females used in this study were reared from field-collected eggs, larvae, or pupae, or from eggs deposited by field-collected mated females (Table 1). Most larvae were reared on the host material from which they were obtained in an air-conditioned laboratory under a natural light regime. Pupae were segregated according to sex. Moths were provisionally identified prior to analysis and retained for more detailed taxonomic examination after the

TABLE 1. NAMES AND COLLECTION DATA FOR TORTRICIDS INVESTIGATED

Moth	Locality[a]	Food plant	Collected as
Phricanthes asperana Meyrick	Guerilla Bay, NSW	*Hibbertia scandens*	larva
Proselena tenella (Meyrick)	Black Mt., ACT	*Bursaria spinosa*	larva/pupa
Arotrophora arcuatalis (Walker)	Guerilla Bay, NSW	*Banksia* cones	larva
Syllomatia pertinax (Meyrick)	Brindabella/Tinderry Ranges	*Lomatia myricoides*	larva
"*Tortrix*" *incompta* Turner	Mt. Field Nat. Pk., Tas.	*Nothofagus cunninghamii*	larva
Cryptoptila australana (Lewin)	Brindabella/Tinderry Ranges	*Polyscias sambucifolius*	larva
Cryptoptila immersana (Walker)	Black Mt., ACT	*Lonicera* sp.	larva
Cryptoptila sp.	Mt. Mistake, Q./Cambewarra Mt., NSW	*Pennantia cunninghamii*	larva
Acropolitis ergophora Meyrick	Brindabella Range, ACT	*Daviesia* sp.	larva
Acropolitis hedista (Turner)	Mt. Mistake, Q	*Pennantia cunninghamii*	larva
Acropolitis rudisana (Walker)	Cook, ACT	artificial medium	adult
Acropolitis xuthobapta Turner	Black Mt., ACT		pupa
Merophas sp.	Black Mt., ACT	*Medicago sativa*	eggs
Epiphyas xylodes (Meyrick)	Black Mt., ACT	*Lonicera* sp.	larva
Epitymbia alaudana Meyrick	Toowoomba, Q	layered eucalypt leaves	eggs
Epitymbia cosmota (Meyrick)	Brindabella Range, ACT	eucalypt leaf litter	larva
Epitymbia eudrosa (Turner)	10 km SSE Yeppoon, Q	layered eucalypt leaves	eggs
"*Capua*" *euphona* Meyrick	Brindabella Range, ACT	eucalpyt leaf litter	larva
"*Grapholita*" *decolorana* Walker	Congo, NSW	eucalypt leaf litter	larva
Meritastis laganodes (Meyrick)	Brindabella, ACT	eucalpyt leaf litter	larva
Meritastis lythrodana (Meyrick)	Brindabella, ACT	eucalypt leaf litter	larva
Meritastis pyrosemana (Meyrick)	Black Mt., ACT	eucalypt leaf litter	larva
Meritastis sp. (ANIC sp. 2)	Brindabella, ACT	eucalypt leaf litter	larva
Capnoptycha ipnitis (Meyrick)	Toowoomba, Q	layered eucalypt leaves	eggs
Asthenoptycha sp. (ANIC sp. 2)	Toowoomba, Q	layered eucalypt leaves	eggs
"*Tortrix*" *constrictana* Walker	Cook, ACT		adult
Isochorista helota Meyrick	Brindabella Range, ACT		
Taeniarchis catenata (Meyrick)	Depot Beach, NSW	*Cissus hypoglauca*	larva
Sperchia intractana Walker	Cook, ACT	layered eucalypt leaves	adult
Cryptaspasma sp.	Nambour, Q	macadamia nuts	pupa

[a]Australian Capital Territory (ACT), New South Wales (NSW), Queensland (Q), Tasmania (Tas.).

chemical results had been obtained. The numbers of insects available were often limited because of the incidental collecting inherent in a survey and high levels of parasitism. The species studied, collection sites, food plants, and sampling methods are summarized in Table 1. Voucher specimens of all species have been deposited with the Australian National Insect Collection (ANIC).

So far, no consensus has been reached on the higher classification of the Tortricidae (Roelofs and Brown, 1982; Horak, 1984), and any conclusions on pheromone evolution will partly depend on the presumed phylogeny for the family. It is necessary to ensure a reference system of strictly monophyletic groups, whatever their present taxonomic rank, in order to make meaningful comparisons. The results of this study are presented according to a tentative classification (Table 2) (Horak, 1984; Horak and Brown, 1988), reflecting our present phylogenetic understanding of the Tortricidae, except for *Isochorista* Meyrick which is here included among the Epitymbiini. Names in quotes, such as "*Capua*" *euphona* Meyrick, refer to taxa whose presently valid name does not correctly denote their taxonomic position.

Pheromone Gland Extraction. Moths were sacrificed not less than two days after eclosion and within three hours of dusk, on the assumption that most tortricid females call near to dusk. Moths were immobilized by brief refrigeration, the ovipositor extruded by gentle pressure on the abdomen, and the ovipositor excised anterior to the pheromone gland. Volatiles from single abdominal tips were either extracted by allowing the tip to stand in hexane (Waters Associates, HPLC grade, 10 μl) for 15 min, or evaporated from the tip at 135°C under helium (5 ml/min) and trapped in a glass capillary.

GLC analyses were carried out on a Varian 3700 gas chromatograph fitted with an SP1000 SCOT column (43 m × 0.5 mm), an SGE Pty Ltd Unijector in the splitless mode, and a Hewlett-Packard HP3390A integrator. Hexane

TABLE 2. TRIBES OF THE TORTRICIDAE[a]

Subfamily Tortricinae	Subfamily Chlidanotinae	Subfamily Olethreutinae
Phricanthini	Polyorthini	Gatesclarkeanini
Tortricini	Hilarographini	Bactrini
Schoenotenini	Chlidanotini	Microcorsini
Cochylini		Olethreutini
Cnephasiini		Eucosmini
Archipini		Grapholitini
Epitymbiini		
Sparganothini		
Atteriini		
Euliini		
Ceracini		

[a] Adapted from Horak and Brown (1988).

washes of single abdominal tips or gland volatiles collected by evaporation from the abdominal tips were analyzed by a method to be described in detail elsewhere (Whittle and Bellas, unpublished results), which is an extension of the method described by Klun et al. (1980). Where sufficient material was available, portions (from trapped volatiles) were reduced with lithium aluminium hydride and/or submitted to ozonolysis by techniques that are similar to those described elsewhere (Beroza and Bierl, 1976; Fieser and Fieser, 1967). Coinjection of *n*-alkanes as standards allowed the calculation of Kovats indices (KI) (Bellas, 1975) with an accuracy of ± 0.5 KI units. The limit of detectability was of the order of 200 pg. Pheromone gland components and derivatives were identified by comparison of KI values with those of known compounds. With a combination of these methods, it was possible to distinguish all the possible geometric and positional isomers of the tetradecenyl acetates.

Electroantennograms (EAGs). Live male insects were immobilized by waxing them down by the wings and head. The tip of the antenna (about 10 segments) was cut off and a Ag–AgCl–0.5% NaCl glass microelectrode inserted into the exposed segment. A cut in the abdomen was covered with 0.5% NaCl–1% agar gel and a ground connection made with a chloridized silver wire. To elicit the EAG response, 1-ml puffs of air from a syringe were blown through a Pasteur pipet, containing 10 μg of compound on paper, and over the antennae. A continuous flow of 500 ml/min of humidified air was maintained around the pipet. The EAG signal was amplified by a GRASS P16B microelectrode amplifier and digitized with a microcomputer system at 12-bits resolution and 200 readings a second. A maximum of 12 sources (selected from *Z* and *E* unsaturated acetates) including a blank, were used for each set of measurements. Sources were chosen at random from the set and the whole set repeated four times, whenever the lifetime of the insect preparation permitted. Readings were normalized by dividing by the mean amplitude for all sources, excluding the blank, within each set.

Field Trapping. Some pheromone blends were tested in sticky traps in the field. Mixtures with 100 μg of the major component were placed on rubber baits in delta traps in the appropriate biotopes.

RESULTS

In order to survey as many species as possible, analyses were carried out to determine only the major components in the pheromone glands. Components obtained from single abdominal tips were identified from KI values and, where possible, by the GLC of products from lithium aluminium hydride reduction and ozonolysis reactions. In some cases, supporting evidence was obtained from EAG measurements and from use of the synthetic compounds as lures in traps in the field. Except for one species, *Phricanthes asperana*, which contained Z9–12:OAc, the predominant components were found to be tetradecenyl acetates (Table 3).

TABLE 3. PRINCIPAL PHEROMONE GLAND COMPONENTS

Moth	Unsaturated acetates (ratio)	No. ♀ moths	Method[a]
Phricanthini			
1. *Phricanthes asperana* Meyrick	Z9-12:OAc	4	a, b, c
Schoenotenini			
2. *Proselena tenella* (Meyrick)	Z11-14:OAc	1	f
"Cnephasiini"			
3. *Arotrophora arcuatalis* (Walker)	*E*11-, Z11-14:OAc (3:97)	1	a
4. *Syllomatia pertinax* (Meyrick)	Z9-14:OAc	4	a, d
Archipini			
5. *"Tortrix" incompta* Turner	Z11-14:OAc		d
6. *Cryptoptila australana* (Lewin)	*E*11-, Z11-14:OAc (5:95)	15	a, b, c, d
7. *Cryptoptila immersana* (Walker)	Z9-, Z11-14:OAc (9:91)	1	a, d
8. *Cryptoptila*			
sp. 8a [Mt. Mistake]	Z9-, Z11-14:OAc, Z11-16:OAc (50:39:11)	2	a
sp. 8b [Cambewarra Mt.]	Z9-, *E*11-, Z11-14:OAc, Z11-16:OAc (38:7:49:6)	3	a
9. *Acropolitis ergophora* Meyrick	Z11-14:OAc	4	a, b, c
10. *Acropolitis hedista* (Turner)	14:OAc, *E*11-14:OAc (65:35)	1	a
11. *Acropolitis rudisana* (Walker)	*E*11-, Z11-14:OAc (3:97)	4	a, d
12. *Acropolitis xuthobapta* Turner	*E*11-, Z11-14:OAc (11:89)	4	a, b, c, d, e

13. *Merophyas* sp.	*E*11–, Z11–14:OAc	(17:83)	11	a, b, c, d
14. *Epiphyas xylodes* (Meyrick)	*E*11–, Z11–14:OAc	(40:60)	3	a, c, d, e
Epitymbiini s. lat.				
(Epitymbiini s. str.: 15–25)				
15. *Epitymbia alaudana* Meyrick	*Z*5–, *Z*7–14:OAc	(66:33)	7	a, b, c, d
16. *Epitymbia cosmota* (Meyrick)	*Z*5–, *Z*7–14:OAc	(20:80)	5	a, d
17. *Epitymbia eudrosa* (Turner)	*Z*7–14:OAc		7	a, b, c, d
18. *"Capua" euphona* Meyrick	*Z*5–, *Z*7–14:OAc	(40:60)	6	a, b, c, e
19. *"Grapholita" decolorana* Walker	*Z*5–14:OAc		1	a
20. *Meritastis laganodes* (Meyrick)	*Z*9–14:OAc		1	a
21. *Meritastis lythrodana* (Meyrick)	*Z*9–14:OAc		2	a, d
22. *Meritastis pyrosemana* (Meyrick)	*Z*9–14:OAc		1	a
23. *Meritastis* sp. (ANIC sp. 2)	*Z*9–14:OAc		1	a, d
24. *Capnoptycha ipnitis* (Meyrick)	*Z*8–, Z10–14:OAc	(83:17)	5	a, b, c
25. *Asthenoptycha* sp. (ANIC sp. 2)	*Z*8–, Z10–14:OAc	(90:10)	4	a, d
26. *"Tortrix" constrictana* Walker	*Z*9–, Z11–14:OAc	(12:88)	6	a, d
27. *Isochorista helota* Meyrick	*Z*5–, *Z*7–14:OAc			e
incertae sedis				
28. *Taeniarchis catenata* (Meyrick)	*E*11–, Z11–14:OAc	(44:56)	1	a
29. *Sperchia intractana* Walker	*E*11–, Z11–14:OAc	(69:31)	8	a, d
Microcorsini				
30. *Cryptaspasma* sp.	*Z*9–14:OAc		3	a

[a] a, GLC of gland extract; b, ozonolysis/GLC; c, $LiAlH_4$ redn./GLC; d, electroantennogram; e, sticky trap; f, GC-MS.

Individual moths generally contained at least 5 ng of the major component, although there were some exceptions. Nine extracts from *Proselena tenella* failed to show significant peaks on GLC. For one extract, GC-MS, with selected ion monitoring, was able to detect 70 pg of Z11-14:OAc. For the three *Meritastis* species other than *M. laganodes*, a GLC peak consistent with less than 1.0 ng of Z9-14:OAc was observed. Strong EAG responses were recorded for this compound for the two species for which males were available.

For many of the species examined, GLC data showed the unsaturated acetates to be accompanied by the corresponding saturated acetate. In one case, *Acropolitis hedista*, tetradecyl acetate was consistently the major peak in the GLC trace in a ratio of 65:35 with *E*11-14:OAc. The average amount of saturated acetate was less than 5% of the major unsaturated acetate for the species 1, 4, 7, 9, 11, 12, 16–18, and between 8% and 85% for species 8, 13–15, 24, 26, 28, and 29, but the proportion of the saturated acetate in the blend often varied greatly between individuals of the same species.

Other peaks that often appeared at levels of less than 10% of the major component were consistent with the corresponding unsaturated alcohol (species 1, 8, 10, 13–16, 18, 24–26, 29, 30). The C_{19}, C_{21}, and C_{23} normal hydrocarbons were also usually detected in small amounts.

Two species for which GLC data could not be obtained have also been included in Table 3. "*Tortrix*" *incompta* showed a marked response to Z11-14:OAc in EAG measurements, and *Isochorista helota* was captured in large numbers in a trap baited with a 1:4 mixture of Z5-14:OAc and Z7-14:OAc.

DISCUSSION

The present work shows that current analytical techniques permit the characterization of candidate pheromone components from very few insects, often just one moth. Results obtained from such analyses can be of assistance in distinguishing species that cannot be readily separated on morphological grounds. For example, the *Cryptoptila* sp. collected on the same food plant in two different localities (species 8a, 8b, Table 3) warrants a closer morphological study. However, the most salient result of our survey is the frequent occurrence in the pheromone glands of Australian Tortricinae of tetradecenyl acetates with other than Δ^9 or Δ^{11} unsaturation.

The large majority of known tortricine pheromones contain combinations of (*Z*)-11- and (*E*)-11-tetradecenyl acetates and less commonly (*Z*)-11- and (*Z*)-9-tetradecenyl acetates (Roelofs and Brown, 1982). In fact (*Z*)-11-tetradecenyl acetate has been described as a "key component" of the Tortricinae (Tamaki, 1985). Tortricine pheromones based on a C_{14} compound with a double bond in other than the 11 or 9 position have only recently been found in morphologically

generalized New Zealand archipines (Roelofs and Brown, 1982; Galbreath et al., 1985; Young et al., 1985; Foster et al., 1986). Presence of the same (*Z*)-5-, (*Z*)-7-, (*Z*)-8-, and (*Z*)-10-tetradecenyl acetates is reported here for several groups of the Australian Epitymbiini s. lat. Whereas both the *Z* and *E* isomers of tetradecenyl acetates with a double bond in the 9 or 11 position have been identified as components of tortricine pheromones, as well as the corresponding aldehydes and alcohols, so far only the *Z* isomer of the tetradecenyl acetates with a double bond in the 5, 7, 8, or 10 position has been found in both Australian and New Zealand tortricines.

The different positional isomers observed in our survey strongly reflect the taxonomic groupings at the generic level. *Epitymbia* and its undescribed sister genus (represented by "*Capua*" *euphona* and "*Grapholita*" *decolorana*) are characterized by *Z*5–14 : OAc and/or *Z*7–14 : OAc. Only *Z*9–14 : OAc has been found for all the *Meritastis* species examined so far. *Capnoptycha ipnitis* and *Asthenoptycha* sp., which are closely related if not congeneric, both contain *Z*8–14 : OAc and *Z*10–14 : OAc in their pheromone blends. This is in obvious contrast to the wide spectrum of positional isomers found for probable sibling species within the genera *Planotortrix* Dugdale and *Ctenopseustis* Meyrick in New Zealand (Foster et al., 1986). It remains to be seen whether a larger sample of the Australian fauna will confirm this correlation of pheromone compounds with generic groupings.

Attempts to correlate the distribution pattern of pheromone components with tortricid classification at a tribal level are promising for certain groups. The three tortricid subfamilies are each broadly characterized by the predominance of compounds with a particular chain length in their pheromone blends. Some of the exceptions to this generalization form morphologically very distinct groups.

Whereas exclusively C_{12} compounds are found among the Cnephasiini s. str., the presence of C_{14} and C_{16} compounds among some taxa of the Cnephasiini s. lat. (Priesner, 1984) is strong evidence for the latter being a polyphyletic assemblage of taxa with only superficial similarity. If *Isotrias* and *Olindia*, referred to the tribe Polyorthini of the Chlidanotinae by Razowski (1979), and *Eulia ministrana* L., which shares only plesiomorphies with the Cnephsiini s. str., are removed, then there remains a morphologically well-defined group which is characterized by pheromones based on C_{12} compounds. On this basis, the tetradecenyl acetates identified in *Arotrophora arcuatalis* and *Syllomatia pertinax* are evidence for the Australian "Cnephasiini" not being genuine members of this tribe, as already suggested on morphological grounds by Powell and Common (1985). As for the tribe Phricanthini, further taxa will have to be analyzed to establish whether C_{12} compounds, as found in *Phricanthes asperana*, are characteristic at a tribal level.

The few data available from the small olethreutine tribes Gatesclarkeanini,

Bactrini, and Microcorsini indicate that all three preferentially use C_{14} derivates in their pheromones rather than the C_{12} compounds typical for the majority of the subfamily. *Z*10–14:OAc is an attractant for several species of *Endothenia* Stephens (Arn et al., 1986) in the tribe Gatesclarkeanini and is the main component in the pheromone of *Bactra verutana* Zeller (Bactrini) (Roelofs and Bjostad, 1984), whereas *E*10–14:OAc is an attractant for *Bactra lancealana* (Hübner) (Arn et al., 1986). The major gland components of an Australian species of *Cryptaspasma* Walsingham (Microcorsini) are *Z*9–14:OAc and *Z*9–14:OH. This compares with *Z*9–14:OAc and *Z*7–12:OAc reported as an attractant mixture for another species of the same tribe (Roelofs and Brown, 1982).

Exclusive correlations cannot be made between the presence of pheromones based on tetradecenyl acetates with double bonds in the 5, 7, 8, or 10 position and the tribal affinity of the respective species. All four compounds, as the *Z* isomers, have been identified from New Zealand archipines as well as from Australian Epitymbiini s. lat. Moreover, Δ^{10} unsaturated tetradecenyl acetates have been reported as pheromones or attractants for the olethreutine Gatesclarkeanini and Bactrini. These groups are not closely related, but they are all rich in morphological plesiomorphies. The New Zealand *Ctenopseustis* Meyrick and relatives are demonstrably the most generalized Archipini s. str. (Horak, 1984), and there are numerous indications that the Epitymbiini s. str. and *Isochorista* also are overall rather generalized Tortricinae (Horak, 1984; Horak and Common, 1985). Gatesclarkeanini, Bactrini, and Microcorsini are the three least evolved olethreutine tribes (Horak and Brown, 1988). Our results thus support the hypothesis that pheromones based on a C_{14} compound, with double bonds in the 5, 7, 8, or 10 positions, represent a plesiomorphy for tortricids (Roelofs and Bjostad, 1984) found in plesiomorphic members of Tortricinae as well as Olethreutinae.

Roelofs and Bjostad (1984) proposed three biosynthetic schemes for the formation of certain lepidopteran pheromones, suggesting a possible evolution from Δ^9 through Δ^{10} to Δ^{11} desaturation of C_{16} and C_{18} acyl intermediates. The ultimate proof of the validity of this scheme may well rest upon isolation and characterization of the particular enzymes involved. Nevertheless, circumstantial evidence can be obtained from the detection of appropriate precursors in the pheromone gland and also from the particular combinations of products formed. It can be seen from Figure 1 that various combinations of components are suggestive of one or another of the proposed pathways. The combinations found in this survey and their distribution in relation to tortricine classification is compatible with such a scheme.

Within the frame-work of the above hypotheses (Figure 1), the presence of *Z*5–14:OAc in some Epitymbiini can be explained by simple chain shortening of a Δ^9 C_{18} acyl intermediate, following the action of a general Δ^9 desa-

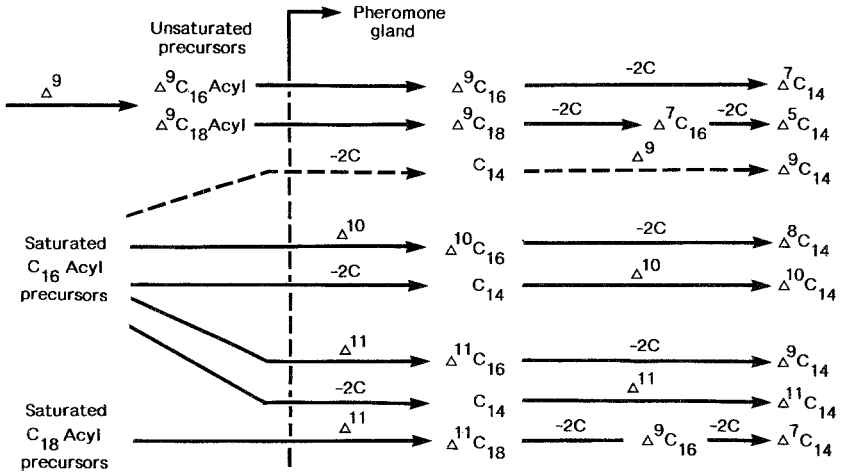

FIG. 1. Possible pathways to acyl intermediates in the biosynthesis of tetradecenyl acetates as pheromone components in Tortricinae: "-2C" indicates chain shortening by two carbon atoms; "Δ^n" indicates the action of a Δ^n desaturase, i.e., the introduction of a double bond between carbon atoms n and $n + 1$ (adapted from Roelofs and Bjostad, 1984).

turase. The occurrence of Z10–14:OAc in other Epitymbiini and the two generalized olethreutine tribes Gatesclarkeanini and Bactrini suggests the presence of a Δ^{10} desaturase. Z7–14:OAc and Z9–14:OAc could be derived with or without the action of a Δ^{11} desaturase, and only a detailed analysis can identify the underlying biosynthetic pathway. Absence of a Δ^{11} desaturase in *Meritastis* and in the olethreutine Microcorsini, both with Z9–14:OAc or derivatives thereof as the only identified pheromone gland components, would further underline the plesiomorphic nature of the two groups.

Although our results lend support to the hypothesis of three successively evolved pathways for tortricid pheromone biosynthesis, it must be pointed out that more evolved pheromones have been found for a number of morphologically very archaic Tortricidae. The Δ^{11} tetradecenyl acetates identified for *Cryptoptila* and *Taeniarchis catenata* and indicated for "*Tortrix*" *incompta* and *Eulia ministrana* L. (Priesner, 1984), as well as the Δ^{11} hexadecenyl acetates reported as attractants for the chlidanotine *Olindia* and *Isotrias* (Priesner, 1984), indicate that a Δ^{11} desaturase must have been present very early in tortricid evolution. Furthermore, the presence of the various positional isomers in several clearly monophyletic groups such as the Olethreutinae, the Epitymbiini s. str., the Cnephasiini s. str., and *Ctenopseustis* and relatives suggests repeated, parallel appearance of both the Δ^{10} and the Δ^{11} desaturase in several tortricid lineages.

If this trend towards pheromones associated with a Δ^{11} desaturase in the more evolved members of tortricid lineages is further confirmed, explanations will have to consider possible selective mechanisms. Pheromones based on C_{14} compounds with a double bond in the 11 position are clearly dominant among tortricines, with blends of *Z*11–14:OAc and *E*11–14:OAc apparently the most common combination. Mechanisms for pheromone biosynthesis, dissemination, and perception will all have to be taken into account for consideration of possible adaptive advantages of a particular compound or blend.

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RESPONSES OF NORTHERN AND WESTERN CORN ROOTWORMS[1] TO SEMIOCHEMICAL ATTRACTANTS IN CORN FIELDS[2]

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Abstract—Small plots (18 × 18 m) were treated with grids of cotton wicks that contained semiochemicals for adult *Diabrotica barberi* Smith and Lawrence, the northern corn rootworm (NCR). In plots treated with eugenol (350 g/hectare), NCR were attracted to point sources of the compound, but there were no significant changes in numbers of either NCR or *D. virgifera virgifera* LeConte, the western corn rootworm (WCR), found on plants in the plots. In plots treated with 12.5 mg/hectare of 8*R*-methyl-2*R*-decyl propanoate (2*R*,8*R*-MDP, the apparent female-produced sex pheromone of NCR and WCR), males of both species were attracted to point sources, but beetles did not congregate within treated plots. With racemic 2,8-MDP at 1.0 g/hectare, male WCR were attracted into plots, but NCR of both sexes were strongly repelled. In a separate study, capture of beetles at pheromone-baited traps declined when the surrounding area contained wicks that emitted racemic 2,8-MDP. In treated plots, male WCR were relatively inefficient at finding pheromone sources. With NCR, emigration from plots could account totally for the observed 3- to 10-fold reduction in catch at 0.01–1.0 g/hectare.

Key Words—Coleoptera, Chrysomelidae, *Diabrotica virgifera virgifera*, *Diabrotica barberi*, sex pheromone, 8-methyl-2-decyl propanoate, eugenol, attractant, repellant, disruption, diastereomers.

INTRODUCTION

Numerous semiochemicals have recently been identified for the northern corn rootworm (NCR), *Diabrotica barberi* Smith and Lawrence, and the western

[1] Coleoptera: Chrysomelidae.
[2] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

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corn rootworm (WCR), *D. virgifera virgifera* LeConte. The work of Guss et al. (1984, 1985) indicates that females of NCR and WCR produce the same single-component sex pheromone, 8*R*-methyl-2*R*-decyl propanoate (2*R*,8*R*-MDP). Eugenol, a plant-derived volatile, attracts NCR to traps (Ladd et al., 1985); indole and several phenyl propanoids are attractants for WCR (Andersen and Metcalf, 1986; Lampman et al., 1987). Cucurbitacins, bitter principles found in many Cucurbitaceae, are potent arrestants and feeding stimulants for *Diabrotica* beetles in general (Metcalf et al., 1980).

Several researchers have suggested using these semiochemicals to aid in the control of adult beetles (Rhodes et al., 1980; Johnson et al., 1985). In theory, beetles could be attracted from untreated portions of corn fields into treated areas, lured to toxic baits within treated areas, and induced to feed selectively upon toxins. Very small amounts of actual insecticide could yield effective, specific control. In this study, NCR and WCR were monitored in small plots to determine whether various semiochemicals may be useful for attracting beetles from untreated portions of fields into treated areas, luring beetles to point sources of insecticide, or disrupting mating communication.

METHODS AND MATERIALS

Influence of Attractants on Distribution of Beetles. Tests were run in a commercial cornfield near Brookings, South Dakota. The following treatments were applied to a series of 18 × 18-m plots: (1) 8*R*-methyl-2*R*-decyl propanoate (2*R*,8*R*-MDP) at 0.0125 g/hectare, (2) racemic 2,8-MDP at 0.05 g/hectare (= 0.0125 g/hectare 2*R*,8*R*-MDP), (3) racemic 2,8-MDP at 1.0 g/hectare, (4) eugenol at 330 g/hectare, (5) 1.0 g/hectare 2,8-MDP plus 330 g/hectare eugenol, and (6) untreated control. The 2*R*,8*R*-MDP was from the same lot used by Guss et al. (1984) and thus contained ca. 0.3% 2*S*,8*R* and 1.9% 2*R*,8*S*. In each treated plot, the chemicals were deployed on cotton wicks that were arranged in a 6 × 6 grid with a 3-m spacing. Each wick (1-cm-diameter × 3-cm dental roll) was loaded with neat eugenol or with 2,8-MDP in 50 μl 4:1 hexane–trioctanoin; wicks were then attached (paper clips) to corn leaves at ear height. Treatments were assigned to plots in a complete, randomized-block design with four replicates. There were 100 m between blocks and 35 m between plots within blocks. Wicks were placed August 14, 1986, and were renewed four days later. During this period, corn in the field was in the early dent stage.

White sticky traps (Guss et al., 1985) were wrapped around corn plants at ear height to monitor populations of beetles. These traps were not baited with semiochemicals and thus provided a "passive" indication of beetle populations and activity within the plots. Two traps were placed 6 m apart near the center of each plot. All NCR and WCR were removed from traps every second day

from August 16 to 22; beetles were returned to the laboratory where they were counted and sexed. When traps were checked, beetles were also counted on eight plants in each plot.

Attraction to Point Sources of Semiochemicals. Capture at point sources of attractant was evaluated using a trap similar to that of Shaw et al. (1984). Ten 0.5-cm holes were drilled at arbitrary locations around the upper 3/4 of the perimeter of a 16-dram, amber plastic medicine vial. A piece of synthetic sponge (ca. 0.125 cm^3) was glued inside the vial and was loaded with a toxic bait. The bait consisted of a feeding stimulant that contained cucurbitacin (chloroform extract of 0.2 ml of dried, powdered root of *Cucurbita foetidissima* H.B.K.) in 0.1 ml 19:1 olive oil–Sevin XLR. Using garden ties, traps were attached to corn plants at ear height. Four traps were placed at 1-m intervals along a row near the center of each treated plot; two of the traps contained toxic bait plus wicks identical to those in the surrounding plot, and two contained only toxic bait. These traps were checked concurrently with sticky traps.

Disruption of Orientation to 2R,8R-MDP. Plots for this study were similar to those above, but were only 15 × 15 m with 5 × 5 grids of cotton wicks. Wicks were loaded with racemic 2,8-MDP as described above to give effective treatment rates of 0, 0.01, 0.1, and 1.0 g/hectare. Unlike the previous study, the two sticky traps in each plot were baited with rubber septa (A.H. Thomas No. 8753-D22) that were loaded with 1.0 μg of 2*R*,8*R*-MDP in 50 μl hexane; no vial traps were used. Plots were arranged in a randomized complete block design with three replicates, and there were 125 m between adjacent plots. Wicks were placed September 11, 1986, and, on each of the following two days, NCR and WCR were counted on traps and on 20 plants in each plot. The corn on this field was planted late and was in the 12- to 14-leaf stage (i.e., just prior to tasseling) when the study was conducted.

RESULTS

Distributions of Beetles. The two species of beetles responded very differently to 2,8-MDP in the plots. In plots treated with 1.0 g/hectare 2,8-MDP, capture of male WCR on unbaited sticky traps was ca. 10 times higher than in control plots, but counts of WCR on plants were only two to three times higher (Table 1). In plots treated with low levels of 2,8-MDP, capture of male WCR on sticky traps was 1.7–2.2 times higher than in untreated plots. Numbers of female WCR on traps were not significantly affected by 2,8-MDP. In contrast, racemic 2,8-MDP repelled NCR from plots (Table 2). As with WCR, the effect of 2,8-MDP on trap catch (81–91% reduction relative to controls) was greater than its effect on plant counts (25–60% reduction); unlike WCR, both sexes of NCR responded to the 2,8-MDP. Also, capture of male NCR was reduced sig-

TABLE 1. *Diabrotica virgifera virgifera* (WCR) ON PLANTS AND UNBAITED STICKY TRAPS IN 18 × 18-m PLOTS TREATED WITH VARIOUS SEMIOCHEMICALS[a]

Semiochemical	Rate (g/hectare)	Beetles per 8 plants	$\bar{X}$ WCR on traps[b]	
			Male	Female
2*R*,8*R*-MDP	0.0125	3.31 ± 0.45 b	8.06 ± 1.12 b	4.25 ± 1.00 a
(±)-2,8-MDP	0.05	3.44 ± 0.45 bc	6.27 ± 1.02 b	2.23 ± 0.62 a
(±)-2,8-MDP	1.0	5.94 ± 0.94 c	37.33 ± 7.32 c	4.05 ± 1.45 a
Eugenol	350	2.00 ± 0.43 a	5.44 ± 1.32 ab	3.50 ± 0.58 a
Eugenol + 2,8-MDP	350 + 1.0	4.00 ± 0.42 bc	23.81 ± 3.42 c	5.50 ± 1.05 a
Control		2.56 ± 0.39 ab	3.63 ± 0.81 a	3.25 ± 0.50 a

[a] Means (±SE) from four plots on four sampling dates; data from two traps within each plot were pooled. Within a column, means followed by the same letter are not significantly different (Student-Newman-Keuls test, $\alpha = 0.05$; data were transformed to ln $(n + 1)$ and analyzed using a repeated measures design; actual means are shown).
[b] Modified milk-carton trap (Guss et al., 1985).

nificantly in plots treated with 2*R*,8*R*-MDP. Eugenol had no measurable effect on the distribution of either species (Tables 1 and 2).

Attraction to Point Sources of Semiochemicals. Within plots, WCR were attracted to point sources of 2,8-MDP, although the difference was not significant when traps were baited with 0.9 mg (= 1.0 g/hectare) (Table 3). Eugenol alone did not significantly affect the numbers of WCR captured, but traps baited

TABLE 2. *Diabrotica barberi* (NCR) ON PLANTS AND STICKY UNBAITED TRAPS IN 18 × 18-m PLOTS TREATED WITH VARIOUS SEMIOCHEMICALS[a]

Semiochemical	Rate (g/hectare)	Beetles per 8 plants	$\bar{X}$ NCR on traps[b]	
			Male	Female
2*R*,8*R*-MDP	0.0125	6.69 ± 0.93 ab	10.00 ± 1.44 b	8.25 ± 0.76 b
(±)-2,8-MDP	0.05	6.50 ± 1.41 ab	3.19 ± 0.45 a	2.56 ± 0.53 a
(±)-2,8-MDP	1.0	3.50 ± 0.61 a	1.56 ± 0.30 a	2.88 ± 0.65 a
Eugenol	350	8.13 ± 1.17 b	20.63 ± 3.18 c	15.94 ± 2.29 b
Eugenol + 2,8-MDP	350 + 1.0	6.00 ± 1.24 ab	3.25 ± 0.96 a	3.00 ± 0.51 a
Control		8.69 ± 1.44 b	18.01 ± 3.46 c	15.99 ± 3.67 b

[a] Means (±SE) from four plots on four sampling dates; data from two traps within each plot were pooled. Within a column, means followed by the same letter are not significantly different (Student-Newman-Keuls test, $\alpha = 0.05$; data were transformed to ln $(n + 1)$ and analyzed using a repeated measures design; actual means are shown).
[b] Modified milk-carton trap (Guss et al., 1985).

TABLE 3. CAPTURE OF *Diabrotica barberi* (NCR) AND *Diabrotica virgifera virgifera* (WCR) IN VIAL TRAPS WITHIN 18 × 18-m PLOTS TREATED WITH 36 COTTON WICKS CONTAINING VARIOUS SEMIOCHEMICALS[a]

Semiochemical	Amount per wick (mg)	WCR per plot per date			NCR per plot per date		
		Traps without wicks	Traps containing wicks	*P*	Traps without wicks	Traps containing wicks	*P*
2*R*,8*R*-MDP	0.011	2.94 ± 0.81	20.25 ± 4.37	0.003	2.31 ± 0.51	9.43 ± 1.15	0.004
(±)-2,8-MDP	0.045	2.94 ± 0.46	25.75 ± 4.84	0.004	2.00 ± 0.35	0.69 ± 0.27	0.012
(±)-2,8-MDP	0.90	5.69 ± 1.06	13.98 ± 3.41	0.336	2.75 ± 0.25	0.50 ± 0.16	0.008
Eugenol	320	4.56 ± 0.82	3.00 ± 0.74	0.367	7.63 ± 1.16	29.88 ± 4.83	0.028
Eugenol + 2,8-MDP	320 + 0.9	7.56 ± 1.58	3.44 ± 1.01	0.019	1.44 ± 0.30	4.31 ± 1.10	0.055

[a]Means (±SE) from four plots on four sampling dates; data from two traps within each plot were pooled. *P* values are for within-semiochemical comparisons of traps that did or did not contain wicks; *F* test of transformed data [ln (n + 1)] using a repeated measures design.

TABLE 4. CAPTURE OF *Diabrotica* BEETLES AT PHEROMONE SOURCES[a] IN PLOTS TREATED WITH 8-METHYL-2-DECYL PROPANOATE ON COTTON WICKS[b]

Treatment (g/hectare MDP)	Beetles within plots[c]		Beetles per trap per day[d]	
	NCR/20 plants	WCR/20 plants	NCR	WCR
1.0	0.5 ± 0.3 a	20.5 ± 2.9 b	2.9 ± 0.8 a	39.3 ± 7.9 a
0.1	1.8 ± 0.6 ab	13.7 ± 1.6 ab	4.8 ± 0.9 b	36.8 ± 7.8 a
0.01	2.7 ± 0.7 ab	8.8 ± 1.3 a	10.9 ± 2.0 c	36.4 ± 5.5 a
0.0	6.5 ± 2.8 b	8.0 ± 2.0 a	30.4 ± 8.2 d	121.7 ± 17.5 b

[a] Modified milk-carton trap (Guss et al., 1985) baited with 1.0 μg 8*R*-methyl-2*R*-decyl propanoate on a rubber septum.
[b] Within a column, means (+SE) followed by the same letter are not significantly different (Student-Newman-Keuls test, $\alpha = 0.05$, repeated measures design used for ANOVA).
[c] Each means represents two samples in each of three plots.
[d] For each mean, two traps in each of three plots were sampled twice; data were transformed to ln $(x + 1)$ prior to ANOVA (actual means shown).

with eugenol plus 2,8-MDP captured significantly fewer beetles than unbaited traps. With NCR, 2*R*,8*R*-MDP and eugenol both attracted beetles to vial traps, whereas racemic 2,8-MDP inhibited trap catch (Table 3). When eugenol and racemic 2,8-MDP were combined in vial traps, their effects on capture of NCR appeared somewhat counterbalanced.

Disruption of Orientation to 2R,8R-*MDP.* Capture of WCR on pheromone-baited sticky traps was significantly higher in untreated plots than in plots treated with racemic 2,8-MDP (Table 4). Capture was relatively constant over treatment rates of 0.01, 0.1, or 1.0 g/hectare, but larger amounts attracted greater numbers of WCR into plots. With NCR, capture on traps declined significantly as the amount of 2,8-MDP was increased in the surrounding plot (Table 4). Racemic 2,8-MDP apparently repelled NCR from plots in a dose-dependent fashion; in plots with higher treatment rates, fewer NCR were found on plants.

DISCUSSION

For both WCR and NCR, 2,8-MDP had less relative effect on plant counts than on capture at unbaited sticky traps. With WCR, 2,8-MDP not only attracted males into plots but apparently increased their level of activity, making the beetles prone to capture on sticky traps. Similarly, 2,8-MDP may have inhibited activity of both male and female NCR. Also, beetles of both species were often

found feeding on tips of ears under husks and thus may have been shielded from the semiochemicals. If beetles often remained in individual ears for several days, those that were feeding on ear tips could have kept plant counts relatively stable in plots where numbers of active beetles changed greatly. Accordingly, 1.0 g/hectare of 2,8-MDP caused counts of NCR to decline only ca. 60% in plots where ears were available vs. >90% in plots (disruption study) with corn that had not yet tasseled.

With both WCR and NCR, numbers of beetles captured on pheromone-baited traps were reduced if the traps were surrounded by sources, of 2,8-MDP. Reasons for this reduction in capture, however, differed for the two species. With WCR, the mechanism appeared to be analogous to "false trail following" of Bartell (1982): rather than being attracted to the traps, numerous beetles congregated on and around plants with wicks (DRL, personal observation). With NCR, 2,8-MDP did not appear to affect the efficiency with which males located sources of pheromone. Instead, NCR simply emigrated from treated plots. The potential effect of this response on mating success is unknown, especially since both males and females were repelled.

The repellency of racemic 2,8-MDP to NCR is probably caused by the 2*S*,8*R* diastereomer. Guss et al. (1985) caught two to eight times more NCR males on unbaited traps than on traps baited with rubber septa that contained 1.0 μg 2*R*,8*R* plus 0.5–2.5 μg 2*S*,8*R*; on traps baited with 2*R*,8*R* plus only 0.1 μg 2*S*,8*R*, capture was similar to that on unbaited traps. Responses of NCR to 2*S*,8*R* also may explain the low capture NCR on sticky traps in plots treated with 2*R*,8*R*. Due to impurities (see Guss et al., 1984), each wick in these plots contained 0.03–0.04 μg of 2*S*,8*R*. This was not enough to negate attraction of NCR to point sources of 2*R*,8*R* but may have caused some NCR to emigrate or reduce their activity. Although it is not certain that female NCR were responding solely or primarily to 2*S*,8*R* when they avoided the racemate, contamination by 2*S*,8*R* could have influenced the data of Ladd et al. (1985). They caught more female NCR on traps baited with eugenol alone than on traps that were baited with eugenol plus a rubber septum containing 10 μg 2*R*,8*R*. Their 2*R*,8*R* came from the source of Guss et al. (1984), so each septum contained ca. 0.03 μg 2*S*,8*R*.

To date, repellency of 2*S*,8*R*-MDP to NCR appears to be a unique phenomenon. Certainly, it is relatively common for efficiency of pheromone-baited traps to decline when enantiomers of pheromone components are added to lures (response category 5 of Silverstein, 1979). Also, in pheromone systems of some bark beetles, two enantiomers can have synergistic and inhibitory effects on the activity of a second compound (Vité et al., 1985). I know of no instance, however, in which an enantiomer of a sex pheromone actually elicits different (in this case, functionally opposite) behavior from that produced in response to

pheromone alone. Indeed, Silverstein (1979) did not even include this scenario among his categories of possible responses of insects to enantiomers of their pheromone components.

NCR may be used 2*S*,8*R*-MDP as a kairomone to ensure reproductive isolation. The 2*S*,8*R* isomer is the apparent pheromone for several members of the *Diabrotica virgifera* group, including the NCR's sibling species, *D. longicornis* (Say) (Krysan et al., 1986, and references therein). Krysan et al. (1986) also found that 2*R*,8*R* inhibits orientation of *D. longicornis* to 2*S*,8*R*, although no repellent effect was evident. It is hoped future studies will clarify the roles of these isomers in mating and reproductive isolation in the *virgifera* group.

In contrast to the current practice of prophylactically applying soil insecticides, semiochemical-based technology for control of corn rootworms will have to be tailored to individual *Diabrotica* species. For example, racemic 2,8-MDP could be used to attract male WCR to sources of toxic bait, and this tactic might have the added benefit of delaying mating. Where NCR predominate, however, racemic 2,8-MDP would repel beetles from the toxin (although the repellency, in itself, may be of some practical value). The 2*R*,8*R* isomer attracts males of both species, but individual isomers of 2,8-MDP are currently too expensive for use in commercial preparations.

For semichemical–toxicant mixtures, plant-derived volatiles appear to be the attractants of choice. Eugenol could be used to bring NCR of both sexes to point sources of toxin; other plant volatiles are similarly effective against WCR (D.R.L., unpublished data). Although interactions of various volatiles with the different *Diabrotica* must be worked out, use of plant-derived attractants may allow for highly effective control while using very small amounts of actual toxin.

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MULTIVARIATE ANALYSIS OF THE CORRELATION BETWEEN NOCTUIDAE SUBFAMILIES AND THE CHEMICAL STRUCTURE OF THEIR SEX PHEROMONES OR MALE ATTRACTANTS

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Abstract—Female-emitted pheromones and sex attractants of Noctuidae were investigated using a specific computer procedure to analyze data collected from the literature. Correspondence analysis was used to survey the structure–activity relationships of sex pheromones in seven subfamilies. Structural, stereochemical, and functional features of active molecules were related to taxonomy. This multidimensional analysis revealed that the prevalent chemical frame of noctuid moth pheromones was a monounsaturated acetate with *Z* stereochemistry and a double bond on the fifth carbon closest to the nonfunctional branch of the molecule. Possible phylogenetic relationships within Noctuidae and between Noctuidae and other families are discussed in light of the sex pheromone biochemistry. Female sex pheromones appeared to be an additional character to be considered in the classification of noctuid moths.

Key Words—Lepidoptera, Noctuidae, sex attractants, sex pheromones, correspondence analysis, chemotaxonomy, multidimensional analysis.

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INTRODUCTION

The mating behavior of moths is mediated by female-emitted sex pheromones that attract males from a distance, then induce courtship behavior. Although courtship behavior is quite variable, even in related species, attraction of males to females is present in almost all families.

The specificity of attraction, disregarding the differences in rhythm of activity or ecological factors, is supported by the well-defined chemical structure of these pheromones. Accordingly, this feature is expected to provide a basis for a chemotaxonomic classification to be compared to the existing classifications.

Roelofs and Brown (1982) first demonstrated that the chemical structure of the sex pheromones emitted by female Tortricidae moths is clearly correlated with the taxonomic position of the species. Furthermore, a multidimensional analysis of data from the literature revealed that eight tribes of Tortricidae and structural features of their sex pheromones are closely related. (Doré et al., 1986b). In this paper we used the same method as Doré et al (1986b) to investigate the relationships between taxonomic status and chemical structure of the sex pheromones of Noctuidae. Noctuidae are one of the largest families in Lepidoptera, including more than 25,000 known species, among which are numerous pests. The size of the family and its worldwide distribution have brought about great difficulties in constructing the higher classification of Noctuidae and their division in subfamilies (Kitching, 1984).

Our aim was to assess the value of the chemical structure of sex pheromones as a new character for the classification of Noctuidae. Through a multidimensional approach of the chemical structures of their pheromones, we investigated the following questions: (1) Does a relationship exist between the chemistry of sex pheromones and the taxonomic position of the noctuid moths (chemotaxonomic standpoint)? (2) Can the structural features of the pheromones be used to develop phylogenetic relationships between superior taxa (evolutionary standpoint)? (3) Could a taxon be characterized by a chemical structure and could the grouping of given genera be reconsidered on the basis of the chemistry of their sex pheromone (analytic and taxonomic standpoint)?

METHODS AND MATERIALS

Data Collection. The data were compiled from the literature using sources up to 1984, including review articles (Inscoe, 1982; Tamaki, 1984; Steck et al., 1982). Some references deal with the identification of the constituents of the natural sex pheromone of a given species. Other sources deal with the attrac-

tive effect of synthetic compounds, used either alone or mixed with other compounds, during field trapping experiments.

Thus, data included both natural components of pheromone blends, isolated and identified from female secretions, and synthetic sex attractants, discovered through field screening of laboratory produced compounds. The latter sources included a few dubious reports of attractivity based on trap captures of only a few individuals. However, in a statistical approach they bring useful information. One must admit that if the majority of the species of a taxonomic group tends to respond positively to the same molecules their pheromones are, at least, closely related compounds. Moreover, factorial correspondence analysis (FCA) minimizes the effect of faulty data on the final result by categorizing atypical data into minor factors.

In addition to field trapping experiments, chemical analyses of pheromone gland constituents have established that most lepidopteran pheromones consist of blends of two or more compounds. In this paper, we considered each constituent of the pheromone blend separately. Data concerning 44 molecules of pheromones and 200 species of Noctuidae were collected.

The 467 insect–molecule connections were used to construct a frequency table indicating how many times (variable k) a given molecule, i, was a component of the natural pheromone blend or synthetic attractant for a species of subfamily, j. The citation frequency for molecule, i, in subfamily, j, was used as the numerical variable, k, in the mathematical analysis (Table 1).

Formulae of pheromone or sex attractant molecules mentioned in this article have been assigned according to a mode commonly utilized in the pheromone literature. This designates each molecule according to distinctive structural features, i.e., stereochemistry of the double bond (Z or E), location of the unsaturations (mainly 5, 7, 9, 11, or 12), length of the hydrocarbon chain (10, 12, 14 or 16 carbon atoms), terminal functional groups (primary alcohol, OH; aldehyde, Ald; or acetate, Ac). Thus, Z7–12 : Ac, Z11–16 : Ald, and Z9–14 : OH designate Z7-dodecenyl acetate, Z11-hexadecenal and Z9-tetradecenol, respectively.

The selection of subfamilies as the basic analytical unit was obviously very restrictive. Although a lower taxonomic level such as tribe or genus would have been more desirable, this proved to be impossible because: (1) A major decrease in the number of citations within inferior systematic groups would produce a multidimensional analysis of poor reliability. (2) It would have been difficult to classify species belonging to the world fauna into tribes, because tribes are often described for local fauna and differ among the authors.

The division of Noctuidae into subfamilies as defined by Franclemont and Todd (1983) was adopted. Of the 19 subfamilies they described, only seven contained enough species with pheromones or sex attractants described well

TABLE 1. FREQUENCY OF CORRELATION BETWEEN NOCTUIDAE SUBFAMILIES AND PHEROMONES (MALE ATTRACTANTS)[a]

Molecules of pheromones and attractants	Noctuidae subfamilies						
	Noctuinae	Amphipyrinae	Plusiinae	Cuculliinae	Hadeninae	Heliothinae	Acontiinae
Z7-12:OH	5	—	2	—	—	—	—
Z7-14:OH	1	—	1	1	—	—	—
Z9-14:OH	2	3	—	—	—	—	—
Z11-16:OH	1	4	—	1	11	2	—
10:Ac	3	—	—	—	—	—	—
Z5-10:Ac	14	1	—	—	1	—	1
Z7-10:Ac	1	—	—	—	—	—	—
12:Ac	5	—	2	—	—	—	—
Z5-12:Ac	6	1	3	—	—	—	1
*E*5-12:Ac	2	—	—	—	—	—	—
Z7-12:Ac	16	3	27	4	4	—	1
*E*7-12:Ac	6	5	2	1	—	—	—
Z9-12:Ac	4	2	1	2	—	—	—
*E*9,11-12:Ac	2	1	—	—	—	—	—
11-12:Ac	1	—	—	—	—	—	—
*E*9-12:Ac	1	—	—	—	—	—	—
14:Ac	2	1	1	—	1	—	—
Z5-14:Ac	8	—	—	1	—	—	—
Z7-14:Ac	10	1	2	1	5	—	1
*E*7-14:Ac	1	—	—	—	—	—	—

Z9-14:Ac	29	19	3	15	17	1	1
E9-14:Ac	—	1	—	—	2	—	—
Z11-14:Ac	2	3	—	1	10	—	—
E11-14:Ac	—	1	—	—	—	—	—
Z9,E11-14:Ac	—	2	—	—	—	—	—
Z9,E12-14:Ac	—	9	—	2	1	—	—
16:Ac	2	1	2	2	2	1	—
Z7-16:Ac	3	1	—	—	—	1	—
Z9-16:Ac	—	—	—	—	1	1	1
Z11-16:Ac	16	17	—	10	29	3	1
E11-16:Ac	1	—	—	1	—	—	—
Z7-12:Ald	2	—	—	—	—	—	—
14:Ald	—	—	—	—	—	1	—
Z7-14:Ald	2	—	—	—	—	—	—
Z9-14:Ald	2	—	—	1	5	2	—
Z11-14:Ald	—	—	—	—	3	—	—
16:Ald	—	1	—	—	—	2	—
Z9-16:Ald	—	1	—	—	1	4	—
Z11-16:Ald	2	6	—	4	6	8	—
Z9,E12-14:Ald	—	—	—	—	1	—	—
Z5-16:Ac	1	—	—	—	—	—	—
Z9,Z12-14:Ac	—	1	—	—	—	—	—
Z7-16:Ald	—	—	—	—	—	1	—
Z5-12:OH	2	—	—	—	—	—	—

[a] This frequency matrix indicates how many species, k, of subfamily, j, are attracted by a pheromone or a synthetic attractant blend comprising a given molecule, i,.

enough to be included in our study: Acontiinae, Amphipyrinae, Cuculliinae, Hadeninae, Heliothinae, Noctuinae, and Plusiinae.

The chemical structure of attractants of subfamily Catocalinae seem to be unique in Noctuidae. Therefore, this subfamily was not included in our analysis.

Data on a few other subfamilies (Acronictinae, Chloephorinae, Herminiinae, and Hypeninae) are too scarce to permit statistical analysis and were not included.

The sex pheromones of the remaining subfamilies (Agaristinae, Eutelinae, Hypenodinae, Nolinae, Pantheinae, Rivulinae, Sarrothripinae, and Stictopterinae) have never been reported. Male moths of these subfamilies have never been trapped by Noctuidae attractants, and male antennae of some species are not sensitive to the pheromones of noctuid moths (Renou et al., 1986). It has been suggested that these subfamilies produce pheromones whose chemical structures differ from those of other Noctuidae.

Mathematical Analysis. The frequency matrix (44 × 7) (Table 1) was subjected to factorial correspondence analysis (FCA). This multidimensional data reduction procedure was developed by Benzécri et al. (1973) and fits specifically the contingency table. Unlike principal component analysis (PCA), FCA employs chi^2 metrics, and hence does not require handling of an exclusive covariance matrix. The following gives a brief outline of this approach as used herein.

The (n) molecules ($n = 44$ sex attractants) were distributed in a multidimensional subfamily space R^p ($p = 7$ dimensions). The position of each pheromone within this space is defined by ratio $f_{ij}/f_{i\cdot}$ where: f_{ij} is the relative frequency of occurrence of molecule (i) and subfamily (j): $f_{ij} = k_{ij}/\Sigma_{ij}k_{ij}$; and $f_{i\cdot}$ is the marginal relative frequency of pheromone (i) in the various subfamilies: $f_{i\cdot} = \Sigma_j f_{ij}$.

Each point corresponding to a pheromone defines its distribution among the seven subfamilies of noctuid moths (subfamily profile). Two pheromones are close neighbors in this space, if they occur as many times in the same subfamilies.

In the pheromone space R^n, the distribution of the p subfamily points is symmetrically defined by formula $f_{ij}/f_{\cdot j}$; the equivalence between the row and the column spaces is then the main characteristic of factorial correspondence analysis. To represent these two sets of points with minimum loss of information, principal projection axes (factorial axes) are established, as in principal component analysis, by determining eigenvalues (λ) and eigenvectors (V_x). A symmetrical matrix is constituted by distances $S_{jj'}$ between subfamily pairs (chi^2 distance):

$$R = \{S_{jj'}\} = \left[\sum_{i=1}^{n} \frac{1}{f_{i\cdot}} \frac{f_{ij} f_{ij'}}{\sqrt{f_{\cdot j} f_{\cdot j'}}} \right]$$

Calculation is performed by solving the equations: $[R] - \lambda[I] = 0$ and $[R]\cdot[V_x] = \lambda_x[V_x]$ (diagonalization of the symmetrical matrix) where I is the identity matrix. In FCA this is simplified by the fact that one of the sets of points is given by matrix $[R] = [M]\cdot[M']$. Permuting the indices is thus equivalent to transposing the matrix to the other set of points $[M']\cdot[M]$ with the same eigenvalues as R.

The coordinates of the various subfamily points on factorial axes are easily derived from these parameters by the equation:

$$\varphi_{\alpha j} = \lambda_\alpha^{1/2} V_{\alpha j} / f_{\cdot j}^{1/2}$$

where φ is the coordinate of subfamily for the factorial axis; λ_α, the corresponding non trivial eigenvalue; $V_{\alpha j}$ the corresponding eigenvector; $f_{\cdot j}$ the marginal relative frequency of subfamily j for the various pheromones.

Transition formulae, whose symmetry indicates that the representations for the two spaces are equivalent, give the coordinates of the pheromones for the different factors.

$$\varphi_{\alpha i} = \left(\frac{1}{\lambda_\alpha^{1/2}}\right) \sum_{j=1}^{p} (f_{ij}/f_{i\cdot})\varphi_{\alpha j}$$

$$\varphi_{\alpha j} = \left(\frac{1}{\lambda_\alpha^{1/2}}\right) \sum_{i=1}^{n} (f_{ij}/f_{\cdot j})\varphi_{\alpha i}$$

The result is a projection of the different points onto the factorial planes that have been constituted by taking the axes two by two. Several authors have developed the principles and the applications of FCA. Among the books published in English that more fully discuss FCA are those of Mardia et al. (1979) and Greenacre (1983).

In the last few years, we have used the FCA method for analysis of the multiple relationships between chemical structures and biological activities in various fields: pharmacology (Doré and Miquel, 1981; Doré et al., 1986a), toxicology (Doré et al., 1983), and biochemistry (Doré et al., 1982; Labia et al., 1983a, b). This method was also used to investigate the mechanisms of some psychophysiological responses (chemical classification of olfactive nuances, Doré et al., 1984). Similar attempts led us to define some correlations affecting pheromones and electroantennographic responses (Doré and Renou, 1985).

The calculations were made on a Hewlett-Packard 9836 microcomputer (16/32 bits of 655 kbytes RAM) using a program of correspondence analysis rewritten by us into BASIC from Fortran ANACOR program (Lebart et al., 1979). The factorial maps were drawn on a HP 7225A plotter under the direct control of the microcomputer with a precision of 0.25 mm.

RESULTS

Developed from an initial six-dimension system, the FCA method extracted 96% of the total inertia from the five first axes (Figure 1). The chi^2 metrics allowed us to consider row space (pheromone molecules) and column space (Noctuidae subfamilies) symmetrically. Therefore, the space of molecules and the space of insects could be evenly considered according to the Euclidian planes designated by factors φ_1 to φ_5. This technique provided a reciprocal structuring and also a close comparison of the elements of the two spaces charted on the factorial maps.

Each factor was characterized by its own rate of inertia. In FCA, inertia is equivalent to variance as defined in PCA and represents the percentage participation of each factor to the system structure.

The absolute contribution (AC) of each item i (molecule) or j (subfamily) to each factor represents the share of this item in the structure of this factor.

The relative contribution [RC = $\cos^2(\varphi_n)$] of each item of both spaces assessed the level of explanation of its location by factor φ_n.

Lastly, the coordinates of each item allowed us to consider each factor as an axis and to draw up positive or negative correlations between items. The two-by-two combination of these factorial axes provided the fractional view of the system structure in the form of Euclidian planes or factorial maps.

The use of these maps is closely related to that of topographic ones: the closer items are associated, the nearer their location on the factorial map. In the same way, with a few precautions, items of each space could be compared to define their correlation ratio. Finally, the further from the axis origin the various points representing the studied elements are, the more reliable are the correlations between the elements belonging to the two spaces.

Relative and absolute contributions and coordinates are displayed in Tables 2 to 4 and will be considered sequentially.

Factor φ_1. The first factor, φ_1, accounted for about one third (38%) of the total system inertia (Figure 1). This factor related to four subfamilies in the

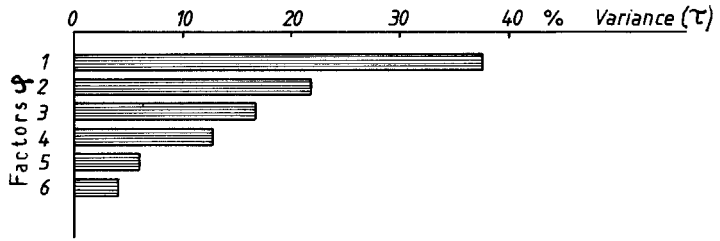


FIG. 1. FCA of the correlation between seven Noctuidae subfamilies and their sex pheromones: percent inertia (τ or variance) explained by FCA factors (φ_1 to φ_6).

TABLE 2. COORDINATES OF PROJECTIONS OF SEVEN NOCTUIDAE SUBFAMILIES AND 44 PHEROMONE MOLECULES FOR NOCTUIDAE ON FIRST FIVE FACTORIAL AXES

	AFC factors				
	φ_1	φ_2	φ_3	φ_4	φ_5
A. Subfamilies					
Noctuinae	−0.511	−0.051	−0.511	+0.083	+0.043
Amphipyrinae	+0.367	−0.355	+0.127	−0.686	−0.153
Plusiinae	−1.154	+0.778	+0.878	−0.061	+0.014
Cuculliinae	+0.135	−0.192	+0.148	−0.167	+0.342
Hadeninae	+0.544	−0.346	+0.336	+0.534	+0.007
Heliothinae	+1.574	+1.625	−0.415	−0.047	+0.058
Acontiinae	−0.307	+0.312	−0.198	+0.557	−1.817
B. Molecules of pheromones and attractants					
Z7-12:OH	−1.033	+0.359	−0.254	+0.106	+0.135
Z7-14:OH	−0.758	+0.344	+0.381	−0.122	+0.516
Z9-14:OH	+0.023	−0.452	−0.284	−0.953	−0.289
Z11-16:OH	+0.800	−0.226	+0.353	+0.392	−0.005
10:Ac	−0.760	−0.099	−1.135	+0.211	+0.167
Z5-10:Ac	−0.573	−0.126	−0.900	+0.234	−0.310
Z7-10:Ac	−0.760	−0.099	−1.135	+0.211	+0.167
12:Ac	−1.033	+0.359	−0.254	+0.106	+0.135
Z5-12:Ac	−0.874	+0.348	−0.102	+0.043	−0.588
E5-12:Ac	−0.760	−0.099	−1.135	+0.211	+0.167
Z7-12:Ac	−0.968	+0.608	+0.712	−0.016	+0.013
E7-12:Ac	−0.361	−0.099	−0.083	−0.580	−0.037
Z9-12:Ac	−0.362	−0.112	−0.151	−0.401	+0.243
E9,11-12:Ac	−0.324	−0.295	−0.662	−0.435	−0.086
11-12:Ac	−0.760	−0.099	−1.135	+0.211	+0.167
E9-12:Ac	−0.760	−0.099	−1.135	+0.211	+0.167
14:Ac	−0.376	−0.010	+0.141	−0.023	−0.035
Z5-14:Ac	−0.653	−0.130	−0.972	+0.141	+0.296
Z7-14:Ac	−0.334	−0.089	−0.177	+0.389	−0.219
E7-14:Ac	−0.760	−0.099	−1.135	+0.211	+0.167
Z9-14:Ac	+0.021	−0.290	−0.063	−0.110	+0.086
E9-14:Ac	+0.721	−0.675	+0.592	+0.321	−0.178
Z11-14:Ac	+0.525	−0.583	+0.399	+0.517	+0.010
E11-14:Ac	+0.546	−0.687	+0.282	−1.730	−0.594
Z9,E11-14:Ac	+0.546	−0.687	+0.282	−1.730	−0.594
Z9,E12-14:Ac	+0.510	−0.633	+0.329	−1.256	−0.222
16:Ac	−0.004	+0.318	+0.314	+0.011	+0.278
Z7-16:Ac	+0.120	+0.431	−0.809	−0.243	+0.026
Z9-16:Ac	+0.897	+1.026	−0.205	+0.877	−2.265
Z11-16:Ac	+0.383	−0.347	+0.110	+0.129	+0.004
E11-16:Ac	−0.279	−0.236	−0.402	−0.105	+0.747
Z7-12:Ald	−0.760	−0.099	−1.135	+0.211	+0.167

TABLE 2. Continued

	AFC factors				
	φ_1	φ_2	φ_3	φ_4	φ_5
14:Ald	+2.339	+3.144	−0.922	−0.119	+0.224
Z7-14:Ald	−0.760	−0.099	−1.135	+0.211	+0.167
Z9-14:Ald	+0.740	+0.236	−0.004	+0.650	+0.225
Z11-14:Ald	+0.809	−0.670	+0.747	+1.347	+0.029
16:Ald	+1.742	+1.867	−0.520	−0.656	−0.048
Z9-16:Ald	+1.785	+1.869	−0.443	−0.143	+0.055
Z11-16:Ald	+1.005	+0.589	−0.082	−0.173	+0.156
Z9,E12-14:Ald	+0.809	−0.670	+0.747	+1.347	+0.029
Z5-16:Ac	−0.760	−0.099	−1.135	+0.211	+0.167
Z9,Z12-14:Ac	+0.546	−0.687	+0.282	−1.730	−0.594
Z7-16:Ald	+2.339	+3.144	−0.922	−0.119	+0.224
Z5-12:OH	−0.760	−0.099	−1.135	+0.211	+0.167

TABLE 3. ABSOLUTE CONTRIBUTIONS (ACs) OF SEVEN NOCTUIDAE SUBFAMILIES AND 44 PHEROMONE AND ATTRACTANT MOLECULES FOR NOCTUIDAE ON FIRST FIVE FACTORIAL AXES

	AFC factors				
	φ_1	φ_2	φ_3	φ_4	φ_5
A. Subfamilies					
Noctuinae	19.20	0.33	42.81	1.48	0.93
Amphipyrinae	5.43	8.60	1.45	54.53	6.43
Plusiinae	28.96	22.34	37.43	0.23	0.02
Cuculliinae	0.40	1.39	1.09	1.80	17.75
Hadeninae	14.02	9.61	11.97	38.89	0.01
Heliothinae	31.65	57.16	4.92	0.08	0.29
Acontiinae	0.31	0.54	0.29	2.95	74.54
B. Molecules of pheromones and attractants					
Z7-12:OH	3.53	0.72	0.47	0.10	0.41
Z7-14:OH	0.81	0.28	0.46	0.06	2.58
Z9-14:OH	0.00	0.82	0.42	6.18	1.35
Z11-16:OH	5.75	0.78	2.49	3.97	0.00
10:Ac	0.82	0.02	4.08	0.18	0.27
Z5-10:Ac	2.64	0.21	14.54	1.27	5.26
Z7-10:Ac	0.27	0.00	1.36	0.06	0.09
12:Ac	3.53	0.72	0.47	0.10	0.41
Z5-12:Ac	3.97	1.07	0.12	0.02	12.29

TABLE 3. Continued

	AFC factors				
	φ_1	φ_2	φ_3	φ_4	φ_5
*E*5-12:Ac	0.54	0.01	2.72	0.12	0.18
*Z*7-12:Ac	24.37	16.30	29.45	0.02	0.03
*E*7-12:Ac	0.86	0.11	0.10	6.40	0.06
*Z*9-12:Ac	0.55	0.09	0.21	1.97	1.72
*E*9,11-12:Ac	0.14	0.21	1.39	0.77	0.07
11-12:Ac	0.27	0.00	1.36	0.06	0.09
*E*9-12:Ac	0.27	0.00	1.36	0.06	0.09
14:Ac	0.33	0.00	0.10	0.00	0.01
*Z*5-14:Ac	1.81	0.12	8.98	0.24	2.54
*Z*7-14:Ac	1.06	0.12	0.66	4.13	3.10
*E*7-14:Ac	0.27	0.00	1.36	0.06	0.09
*Z*9-14:Ac	0.01	5.73	0.36	1.40	2.03
*E*9-14:Ac	0.73	1.09	1.11	0.42	0.30
*Z*11-14:Ac	2.09	4.36	2.69	5.83	0.00
*E*11-14:Ac	0.14	0.37	0.08	4.07	1.13
*Z*9,*E*11-14:Ac	0.28	0.75	0.16	8.15	2.27
*Z*9,*E*12-14:Ac	1.47	3.86	1.37	25.77	1.90
16:Ac	0.00	0.81	1.04	0.00	2.50
*Z*7-16:Ac	0.03	0.74	3.45	0.40	0.01
*Z*9-16:Ac	1.14	2.53	0.13	3.14	49.63
*Z*11-16:Ac	5.28	7.35	0.98	1.74	0.00
*E*11-16:Ac	0.07	0.08	0.34	0.03	3.60
*Z*7-12:Ald	0.54	0.01	2.72	0.12	0.18
14:Ald	2.58	7.92	0.89	0.01	0.16
*Z*7-14:Ald	0.54	0.01	2.72	0.12	0.18
*Z*9-14:Ald	2.59	0.44	0.00	5.74	1.64
*Z*11-14:Ald	0.92	1.07	1.77	7.41	0.00
16:Ald	4.30	8.38	0.85	1.75	0.02
*Z*9-16:Ald	9.04	16.81	1.24	0.16	0.06
*Z*11-16:Ald	12.41	7.23	0.18	1.06	2.03
*Z*9,*E*12-14:Ald	0.30	0.35	0.59	2.47	0.00
*Z*5-16:Ac	0.27	0.00	1.36	0.06	0.09
*Z*9,*Z*12-14:Ac	0.14	0.37	0.08	4.07	1.13
*Z*7-16:Ald	2.58	7.92	0.89	0.01	0.16
*Z*5-12:OH	0.54	0.01	2.72	0.12	0.18

Noctuidae space: Heliothinae, Plusiinae, Noctuinae, and Hadeninae, the sum of absolute contributions reaching 94% (Table 3A).

Heliothinae and Hadeninae with positive coordinates (Table 2A) were opposite Plusiinae and Noctuinae with negative coordinates. Examination of the relative contributions (Table 4A) revealed that each of these four subfami-

TABLE 4. RELATIVE CONTRIBUTIONS (RCs) OF SEVEN NOCTUIDAE SUBFAMILIES AND 44 PHEROMONE AND ATTRACTANT MOLECULES FOR NOCTUIDAE ON FIRST FIVE FACTORIAL AXES

	AFC factors					
	φ_1	φ_2	φ_3	φ_4	φ_5	φ_6
A. Subfamilies						
Noctuinae	0.4867	0.0049	0.4862	0.0130	0.0034	0.0055
Amphipyrinae	0.1718	0.1603	0.0206	0.5991	0.0298	0.0181
Plusiinae	0.4898	0.2229	0.2836	0.0014	0.0000	0.0020
Cuculliinae	0.0332	0.0674	0.0402	0.0513	0.2134	0.5941
Hadeninae	0.3594	0.1453	0.1375	0.3464	0.0000	0.0111
Heliothinae	0.4678	0.4982	0.0326	0.0004	0.0006	0.0001
Acontiinae	0.0210	0.0216	0.0087	0.0691	0.7357	0.1435
B. Molecules of pheromones and attractants						
*Z*7-12:OH	0.7816	0.0944	0.0473	0.0083	0.0133	0.0549
*Z*7-14:OH	0.3669	0.0758	0.0927	0.0095	0.1704	0.2844
*Z*9-14:OH	0.0003	0.1401	0.0554	0.6233	0.0574	0.1232
*Z*11-16:OH	0.6181	0.0495	0.1202	0.1484	0.0000	0.0636
10:Ac	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
*Z*5-10:Ac	0.2512	0.0122	0.6194	0.0419	0.0734	0.0017
*Z*7-10:Ac	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
12:Ac	0.7816	0.0944	0.0473	0.0083	0.0133	0.0549
*Z*5-12:Ac	0.6125	0.0974	0.0083	0.0015	0.2778	0.0023
*E*5-12:Ac	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
*Z*7-12:Ac	0.5158	0.2034	0.2793	0.0001	0.0001	0.0011
*E*7-12:Ac	0.2551	0.0194	0.0136	0.6569	0.0027	0.0519
*Z*9-12:Ac	0.2724	0.0262	0.0478	0.3348	0.1229	0.1955
*E*9,11-12:Ac	0.1111	0.0920	0.4627	0.2001	0.0078	0.1260
11-12:Ac	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
*E*9-12:Ac	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
14:Ac	0.4799	0.0003	0.0682	0.0018	0.0041	0.4454
*Z*5-14:Ac	0.2843	0.0112	0.6296	0.0132	0.0584	0.0029
*Z*7-14:Ac	0.3188	0.0227	0.0893	0.4314	0.1367	0.0008
*E*7-14:Ac	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
*Z*9-14:Ac	0.0031	0.5778	0.0277	0.0833	0.0508	0.2568
*E*9-14:Ac	0.3088	0.2709	0.2085	0.0612	0.0189	0.1314
*Z*11-14:Ac	0.2504	0.3085	0.1444	0.2429	0.0001	0.0534
*E*11-14:Ac	0.0664	0.1051	0.0178	0.6667	0.0786	0.0652
*Z*9,*E*11-14:Ac	0.0664	0.1051	0.0178	0.6667	0.0786	0.0652
*Z*9,*E*12-14:Ac	0.1087	0.1673	0.0452	0.6580	0.0205	0.0000
16:Ac	0.0000	0.2994	0.2917	0.0003	0.2296	0.1787
*Z*7-16:Ac	0.0146	0.1868	0.6576	0.0592	0.0007	0.0807
*Z*9-16:Ac	0.0909	0.1189	0.0047	0.0870	0.5799	0.1183
*Z*11-16:Ac	0.4923	0.4037	0.0410	0.0563	0.0000	0.0064
*E*11-16:Ac	0.0350	0.0249	0.0725	0.0049	0.2500	0.6125
*Z*7-12:Ald	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
14:Ald	0.3359	0.6066	0.0522	0.0008	0.0031	0.0010

TABLE 4. Continued

	AFC factors					
	φ_1	φ_2	φ_3	φ_4	φ_5	φ_6
Z7-14:Ald	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
Z9-14:Ald	0.5081	0.0518	0.0000	0.3915	0.0472	0.0011
Z11-14:Ald	0.1784	0.1223	0.1524	0.4949	0.0002	0.0516
16:Ald	0.4158	0.4776	0.0371	0.0590	0.0003	0.0098
Z9-16:Ald	0.4576	0.5017	0.0282	0.0029	0.0004	0.0090
Z11-16:Ald	0.7056	0.2424	0.0047	0.0211	0.0170	0.0089
Z9,E12-14:Ald	0.1784	0.1223	0.1524	0.4949	0.0002	0.0516
Z5-16:Ac	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305
Z9,Z12-14:Ac	0.0664	0.1051	0.0178	0.6667	0.0786	0.0652
Z7-16:Ald	0.3359	0.6066	0.0522	0.0008	0.0031	0.0010
Z5-12:OH	0.2874	0.0049	0.6408	0.0222	0.0139	0.0305

lies were described very well by this factor [$\cos^2(\varphi_1)$ between 0.36 and 0.49], whereas the relative contributions to φ_1 of the last three subfamilies (Amphipyrinae, Cuculliinae, and Acontiinae) were less than 0.17.

The most involved molecules in the attractant space had 12-carbon and 16-carbon chains (Table 3B). The most striking feature was the negative correlation, or opposition, between Z7-12:Ac (AC = 24.4%, negative coordinates) and Z11-16:Ald (AC = 12.4%, positive coordinates).

On the whole, the decene or dodecene molecules were projected negatively along the φ_1 axis and were essentially correlated with Plusiinae and Noctuinae.

Hexadecenes were generally projected positively onto the φ_1 axis. The most involved molecules (Z11-16:Ald, AC = 12.4%; Z9-16:Ald, AC = 9%; 16:Ald, AC = 4.3%, on the one hand, and Z11-16:Ac, AC = 5.3%; Z11-16:OH, AC = 5.7%, on the other hand) made the hexadecene distribution bipolar.

The hexadecenals, with positive coordinates with high absolute value on φ_1, were associated with Heliothinae. Z11-16:Ald is the main component of the natural pheromone secretions of the five species studied so far: *Heliothis armigera* (Hübner) (Nesbitt et al., 1979), *H. punctigera* Wallengreen (Rothschild et al., 1982), *H. subflexa* (Guenée) (Teal et al., 1981), *H. virescens* (F.) (Klun et al., 1980a), and *H. zea* (Boddie) (Klun et al., 1980b). Furthermore, this aldehyde is a basic constituent of every attractive synthetic mixture for male *Heliothis*. Z9-16:Ald gained prominence since this compound, although uncommon in noctuid moth pheromones, has been identified in the secretions of all the species studied, except in *H. virescens*. It is an important secondary component for the attraction of male *Heliothis* as are a few other aldehydes.

Z11-16:Ald and other aldehydes were not specific to Heliothinae. They

elicit strong electroantennogram responses in various neotropical Amphipyrinae and Hadeninae species (Renou et al., 1986). However, Heliothinae show relatively exclusive responses to aldehydes in general and to Z11-16:Ald in particular; they are not attracted by acetates or alcohols. However, available data on *Heliothis* concern almost exclusively the genus *Heliothis*, although other genera would perhaps exhibit different sensitivity.

Conversely, although Z11-16:Ac and Z11-16:OH had positive coordinates, they were projected closer to the origin of the factorial axis φ_1. These features associated them with the Hadeninae.

Tetradecenes poorly contribute to the factor φ_1. 14:Ald and Z9-14:Ald have to be kept apart (AC = 2.6% for both) since their coordinates associate them with hexadecenals.

Examination of the relative contributions (Table 4B) confirmed these observations. The first factor explained between 40% and 70% of the characters of the previously mentioned dodecenes and hexadecenes. The C_{16} aldehydes and some C_{14} aldehydes were explained to the same degree by φ_1 [$\cos^2(\varphi_1)$ between 0.33 and 0.70].

Factor φ_2. The second factor, φ_2, accounted for 22% of inertia (Figure 1). In the insect space, this factor was influenced by Heliothinae (AC = 57.2%) and Plusiinae (AC = 22.3%). Both had positive coordinates on φ_2 (Table 4A). On the other hand, Hadeninae (AC = 12.4%) and, to a smaller extent, Amphipyrinae (AC = 9.8%) projected negatively onto the φ_2 axis.

In the molecule space, the 2nd factor was mainly under the influence of Z7-12:Ac (AC = 16.3%), Z9-16:Ald (AC = 16.8%), and Z11-16:Ac (AC = 7.4%).

Within the group of hexadecenes, the dualism between aldehydes 16:Ald, Z9-16:Ald and Z11-16:Ald with positive coordinates on φ_2 axis and Z11-16:Ac and Z11-16:OH with negative coordinates was confirmed. Therefore hexadecenals were associated with Heliothinae, while Z11-16:Ac and Z11-16:OH were correlated to Hadeninae.

Several tetradecenes displayed a significant absolute contribution to φ_2 (Table 3B), especially Z9-14:Ac (AC = 5.7%), Z11-14:Ac (AC = 4.4%), and Z9,E12-14:Ac (AC = 3.8%). Most of these tetradecenes projected negatively onto the φ_2 axis and were associated with the group of Hadeninae-Amphipyrinae.

At this stage of our explanation, scrutiny of the factorial map constituted by the φ_1 and φ_2 axes (Figure 2) allowed us to propound a preliminary organization of the insects–molecules system.

Chain length appeared to be important. C_{10} and C_{12} molecules gathered in a homogeneous cloud with which Noctuinae, Plusiinae, and to a lesser extent, Acontiinae were correlated. These three subfamilies preferentially displayed short chains.

The distribution of tetradecenes was more complicated; they formed an

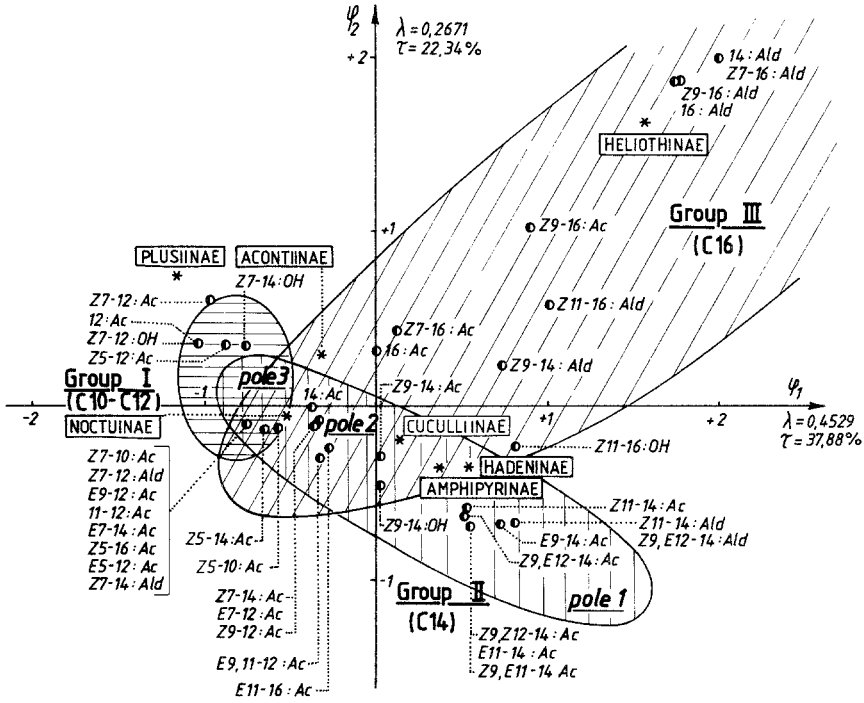


FIG. 2. FCA of the correlation between seven Noctuidae subfamilies and their sex pheromones: main factorial map ($\varphi_1\varphi_2$). λ = eigenvalue of φ_n, τ = percent inertia for φ_n.

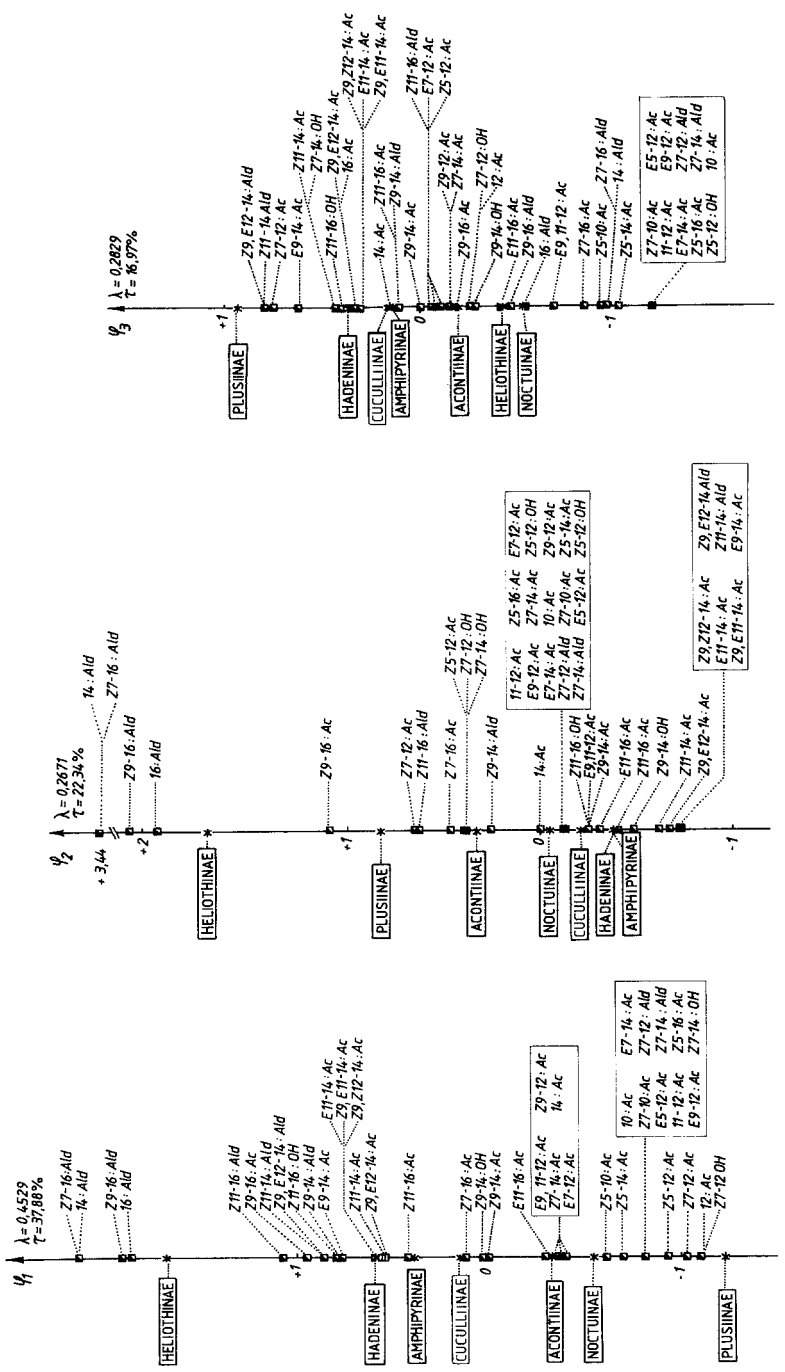
elongated cloud and were organized in three poles: first pole, Δ9, Δ12: dienes and Δ11 monoenes; second pole, Δ9 monoenes; and third pole located around the Δ5 and Δ7 monoenes. The inferior rank axes, particularly Δ3 and Δ4 allowed us to ascertain this distribution.

Hexadecenes were scattered over the factorial map φ_1 φ_2 (Figure 2). Their location is explained by the 90% ratio reached with these two factors. Their distribution was composed of an opposition between hexadecenals correlated with Heliothinae, and Z11-16:Ac—Z11-16:OH correlated with Hadeninae.

The distribution of pheromonal molecules over the factorial map φ_1 φ_2 seemed to take into account various, equally important structural features (i.e., hydrocarbon chain length, occurrence of the aldehyde functional group, and location of double bond). These features were predominant in the typological analysis of Noctuidae pheromones.

Factor φ_3. The third factor, φ_3, accounted for 17% of the system inertia; it led to the analysis of the secondary organization of the components within the poles designated by higher rank factors (Figure 3C).

In the insect space, the third factor was mainly composed of Noctuinae


λ = 0,4529
τ = 37,88%
Z7-16:Ald
14:Ald
Z9-16:Ald
16:Ald
HELIOTHINAE
Z11-16:Ald
Z9-16:Ac
Z11-14:Ald
Z9,E12-14:Ald
Z11-14:Ac
Z9,E11-14:Ac
Z9,Z12-14:Ac
HADENINAE
Z11-16:Ac
AMPHIPYRINAE
Z7-16:Ac
Z9-14:OH
Z9-14:Ac
CUCULLINAE
E11-16:Ac
E9,11-12:Ac
Z7,14:Ac
E7-12:Ac
Z9-12:Ac
14:Ac
ACONTINAE
Z5-12:Ac
Z5-14:Ac
NOCTUINAE
10:Ac
Z7-10:Ac
E5-12:Ac
11-12:Ac
E9-12:Ac
E7-14:Ac
Z7-12:Ald
Z7-14:Ald
Z5-16:Ac
Z7-14:OH
PLUSIINAE
Z5-12:Ac
Z7-12:OH
12:Ac
Z7-12OH
λ = 0,2671
τ = 22,34%
14:Ald
Z7-16:Ald
+3,44
Z9-16:Ald
16:Ald
Z9-16:Ac
Z7-12:Ac
Z11-16:Ald
Z7-16:Ac
Z9-14:Ald
11-12:Ac
E9-12:Ac
E7,14:Ac
Z7-12:Ald
Z7-14:Ald
Z5-16:Ac
Z7-14:Ac
10:Ac
Z7-10:Ac
E5-12:Ac
E7-12:Ac
Z5-12:Oh
Z9-12:Ac
Z5-14:Ac
Z5-12:Oh
Z11-16:Oh
E9,11-12:Ac
Z9-14:Ac
E11-16:Ac
Z11-16:Ac
Z9-14:Oh
Z11-14:Ac
Z9,E12-14:Ac
Z9,Z12-14:Ac
Z9,E11-14:Ac
Z11-14:Ald
E9-14:Ac
Z9,E12-14Ald
λ = 0,2829
τ = 16,97%
Z9,E12-14:Ald
Z11-14Ald
Z7-12:Ac
E9-14:Ac
Z11-14:Ac
Z7-14:Oh
Z9,E12-14:Ac
16:Ac
Z9,E11-14:Ac
Z11-16:Ac
Z9-14:Ald
Z9-14:Ac
Z11-16:Ald
E7-12:Ac
Z5-12:Ac
Z9-12:Ac
Z7-14:Ac
Z9-16:Ac
Z7-12:Oh
12:Ac
Z9-14:Oh
E11-16:Ac
Z9-16:Ac
16:Ald
E9,11-12:Ac
Z7-16:Ac
Z5-10:Ac
Z7-16:Ald
14:Ald
Z5-14:Ac
E5-12:Ac
E9-12:Ac
Z7-12:Ald
Z7-14:Ald
Z5-16:Ac
Z5-12:Oh
10:Ac
PLUSIINAE
HELIOTHINAE
HADENINAE
AMPHIPYRINAE
CUCULLINAE
ACONTINAE
NOCTUINAE

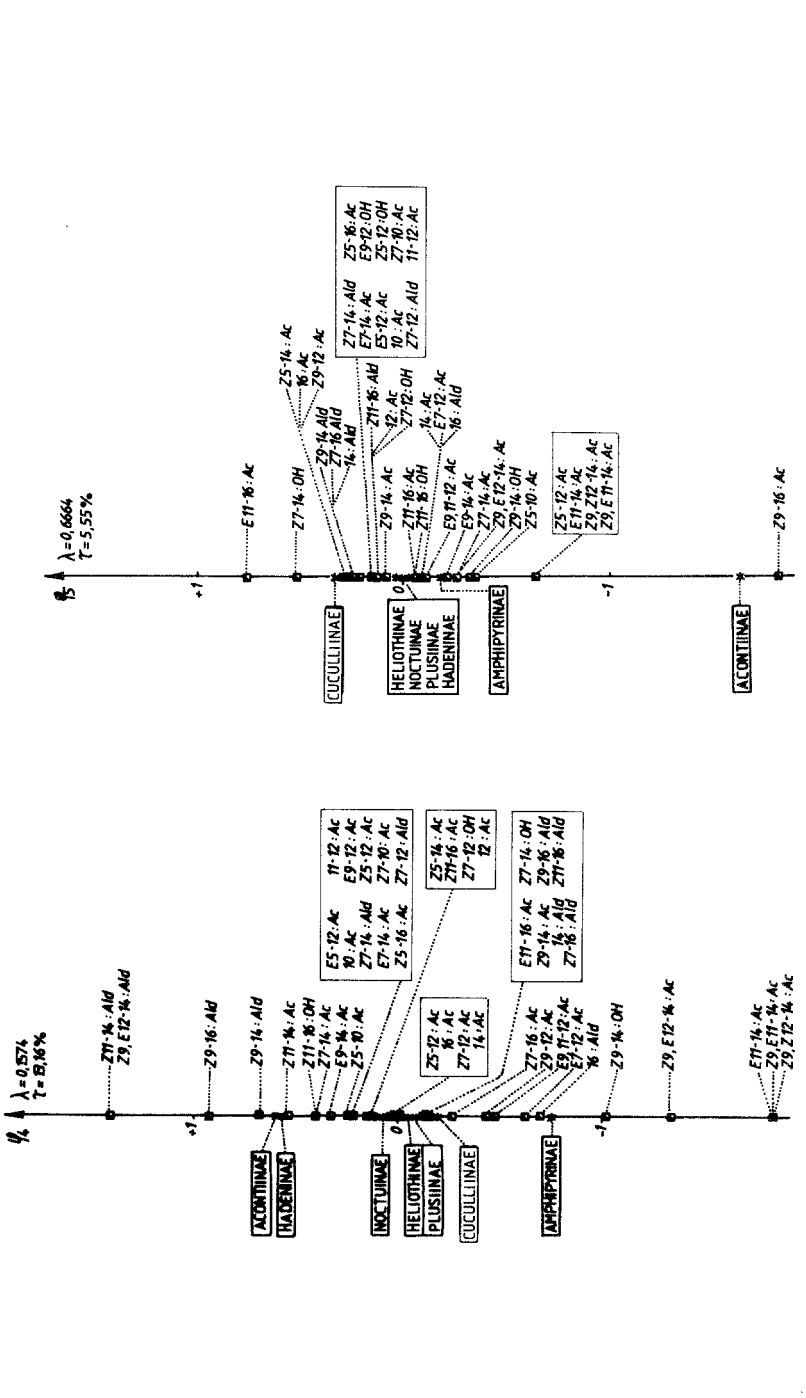


FIG. 3. Distribution of pheromone molecules and noctuid subfamilies on factorial axes φ_1 (3A), φ_2 (3B), φ_3 (3C), φ_4 (3D), and φ_5 (3E).

(AC = 42.8%), which projected positively onto the φ_3 axis, opposite Plusiinae (AC = 37.4%), which projected negatively.

In the molecule space, φ_3 accounted for the opposition between Z7–12 : Ac (AC = 29.5%, positive coordinates) and Z5–10 : Ac (AC = 14.5%, negative coordinates).

Z7–12 : Ac, with positive coordinates, was correlated with Plusiinae. As a matter of fact, the great majority of sex attractive blends for male Plusiinae contain Z7–12 : Ac.

Z5–10 : Ac, with negative coordinates, like the two other decenes studied (10 : Ac, AC = 4.1%; and Z7–10 : Ac, AC = 1.4%), was correlated with Noctuinae. This association is rather specific. Several species of genera *Agrotis* and *Euxoa* emit pheromone blends containing Z5–10 : Ac (Underhill et al., 1981; Löfstedt et al., 1982). This compound is rarely detected in the other Noctuidae, which explained that factor φ_3 displayed a strong correlation between Z5–10 : Ac and Noctuinae, despite that Z7–12 : Ac is also a powerful attractant for Noctuinae.

The φ_3 axis also had a high absolute contribution from Z5–14 : Ac (AC = 9.1%). This molecule possessed negative coordinates on φ_3 and was associated with Noctuinae. Although this attractant occurred only nine times, eight cases (Table 1) were related to Noctuinae. This attractant was atypically located with respect to the other tetradecenes. Z5–14 : Ac is identified as the main component in *Agrotis exclamationis* (L.) pheromone blend (Bestmann et al., 1980) and is present in small proportions (less than 4% of the total blend) in the pheromone blend of *Euxoa drewseni* (Staudinger) (Struble, 1983). Z5–14 : Ac is also reported to be a constituent of attractive mixtures for several other *Agrotis* or *Euxoa* species (Steck et al., 1982). Thus, Z5–14 : Ac is probably an important, specific secondary component of sex attractants for male Noctuinae.

E9, 11–12 : Ac and 11–12 : Ac poorly contributed to φ_3 (AC = 1.4% for both of them); they are located like the other dodecenes. This phenomenon is explained by their rare occurrence. They are uncommon compounds and are only identified in *Diparopsis castanea* Hampson (Nesbitt et al., 1975). This Noctuinae species is remarkable because of its unusual sex pheromone.

Factor φ_4. The fourth factor, φ_4 (Figure 3D), accounted for 13.2% of the system inertia and determined a subsidiary dualism between Amphipyrinae (AC = 54.5%) and Hadeninae (AC = 38.9%). The most meaningful molecules for φ_4 were Z9,E12–14 : Ac (AC = 25.8%) and Z9,E11–14 : Ac (AC = 8.1%). These two tetradecadienes are projected negatively onto the φ_4 axis and were correlated with Amphipyrinae. As a matter of fact, they are specific to *Spodoptera*.

Z11–14 : Ald contributed significantly to φ_4 (AC = 7.4%); it projected positively onto the axis and was associated with Hadeninae. This molecule is rarely present in Noctuidae sex attractants, and it occurred only in three species

of Hadeninae: *Melanchra picta* (Harris) (Underhill et al., 1977), *Lacanobia atlantica* (Grote), and *Orthosia hibisci* (Guenee) (Steck et al., 1982). Some other aldehydes (mainly Z11–16:Ald, and Z9–14:Ald) are components of Hadeninae sex attractants. They also attract insects of some other subfamilies (Heliothinae, etc.) and thus are less specifically emphasized by the factorial analysis.

Factor φ_5. The fifth factor, φ_5 (Figure 3E), with a 5.6% value for inertia, was under the unique influence of subfamily Acontiinae [$\cos^2(\varphi_5) = 0.74$]. Conversely, this subfamily was poorly explained by the first four factors [$\Sigma \cos^2(\varphi_1 - \varphi_4) = 0.14$].

In the molecule space, Z9–16:Ac (AC = 49.6%) almost exclusively determined the structure of φ_5. Its location implied that this hexadecene is not attractive to Heliothinae and Hadeninae, which are usually responsive to C_{16}. In contrast, Z9–16:Ac was correlated with Acontiinae. It is a constituent of the sex pheromone blend of the Acontiinae *Naranga aenescens* (Moore) (Ando et al., 1977). This fact explains its importance for a subfamily which is barely mentioned because of a lack of data.

The subfamily Cuculliinae was poorly explained by factors φ_1 to φ_5 (Σ RC < 10%). This subfamily was only moderately represented (34 citations). The chain length (C_{12}, C_{14}, or C_{16}: Z9–12:Ac, Z9–14:Ac, or Z11–16:Ac), the position of the double bond, and the functional group of their attractants seemed various and nonspecific. These observations explain why Cuculliinae contribute little to the opposition between molecules and were located close to the origin of the different factorial axes.

Factor φ_6. The sixth factorial axis, φ_6, accounted for only 4.1% of the system inertia. Cuculliinae, which were poorly explained by factors φ_1 to φ_5, were well explained by φ_6 [$\cos^2(\varphi_6) = 0.59$]. In the same way, E11–16:Ac was mainly explained by φ_6 [$\cos^2(\varphi_6) = 0.61$] and was correlated with Cuculliinae. But this correlation was based on a single occurrence and did not allow us to conclude the specificity of E11–16:Ac toward Cuculliinae.

The overall multidimensional analysis presented deals with each attractive compound individually. Because the compounds are chemically analogous, it is worth examining each of their functional and structural features, i.e., chain length, functional group, and location of the double bond. For this purpose, we calculated the barycenter of the groups of molecules containing a common feature. This barycenter corresponds to the center of gravity of the x molecules of the group in a two-dimensional space and was used as an additional variable in the factorial data analysis (Table 5). This procedure is correct since it takes into consideration the distributional equivalence concept inherent in the correspondence analysis (Fénelon, 1981). We examined the citation frequency of each group of isomers in the attractive compound list and the projection of each barycenter on the main factorial map φ_1 φ_2.

TABLE 5. COS^2 OF PROJECTIONS OF 11 BARYCENTERS (ACCORDING TO THREE STRUCTURAL CLASSES: CHAIN LENGTH, FUNCTIONAL GROUP, POSITION OF DOUBLE BOND) OF PHEROMONE AND ATTRACTANT MOLECULES FOR NOCTUIDAE ON SIX FACTORIAL AXES

Structural classes of pheromones	Axes					
	φ_1	φ_2	φ_3	φ_4	φ_5	φ_6
Chain length						
C_{10}	0.2673	0.0106	0.6440	0.3814	0.3459	0.0053
C_{12}	0.8058	0.1369	0.0409	0.0009	0.0011	0.0064
C_{14}	0.0832	0.8792	0.0111	0.0132	0.0040	0.0091
C_{16}	0.9608	0.0180	0.0022	0.0151	0.0007	0.0031
Functional group						
Ac	0.6958	0.2407	0.0112	0.0151	0.0164	0.0208
OH	0.1789	0.1004	0.0548	0.1177	0.0157	0.5325
Ald	0.6469	0.3115	0.0211	0.0058	0.0131	0.0016
Double bond						
3	0.1585	0.6727	0.0705	0.0166	0.0009	0.0807
5	0.0207	0.1628	0.6735	0.0012	0.0890	0.0529
7	0.1161	0.2922	0.2286	0.1308	0.2241	0.0080
9	0.1385	0.0048	0.8143	0.0000	0.0390	0.0032

Effect of Chain Length. Chain lengths C_{12}, C_{14} and C_{16} occurred equally, with C_{10} molecules less frequently observed (21 citations). Noctuidae produce molecules of various chain lengths and, even in the same species, pheromone blends may have components of different chain lengths. The biosynthesis of sex pheromones involves chain shortening through β-oxidation steps of the fatty acid precursor, generally hexadecanoic acid (palmitic acid) (Roelofs and Bjostad, 1984). Successive chain shortenings yield C_{14}, C_{12}, then C_{10} molecules. Changing chain length probably did not cause any important modification of the enzymatic mechanism present in the pheromone glands. This fact could explain the chain length diversity among the pheromones of Noctuidae subfamilies.

The factorial map (Figure 4) shows the opposition between short chains (C_{10} and C_{12}, with negative coordinates on φ_1 axis) and long chains (C_{14} and C_{16}, with positive coordinates). C_{10} and C_{12} are associated with Acontiinae and Plusiinae (two Quadrifine subfamilies of Noctuidae) and with Noctuinae (Trifines), while C_{14} and C_{16} are associated with all the other Trifine noctuid subfamilies.

Functional Groups. The acetates are the most frequent functional group with 376 citations out of 467 (Table 1). Alcohols and aldehydes are less common with 36 and 56 citations, respectively. Several biochemical studies have

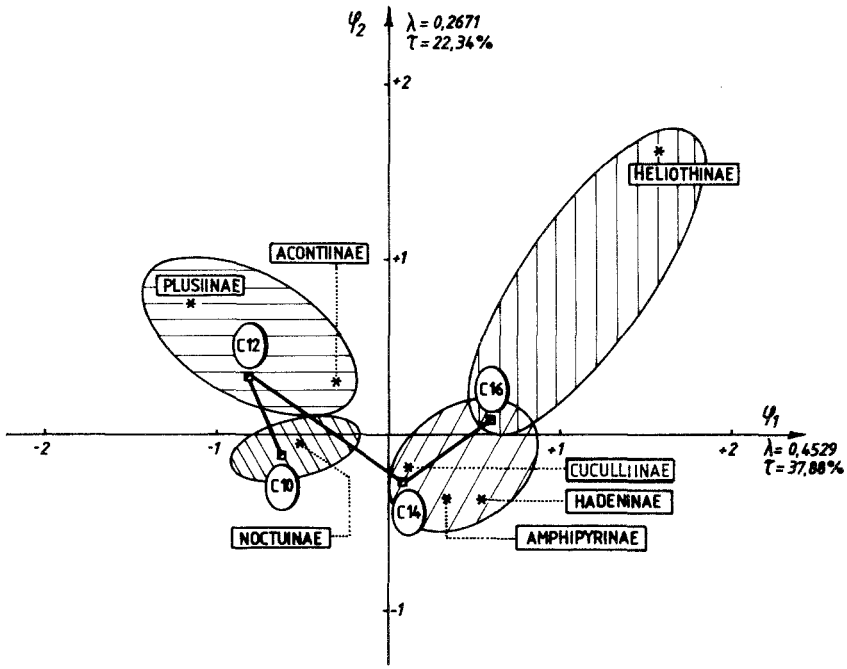


FIG. 4. Projection of noctuid subfamilies and chain length barycenters of their pheromones on the main factorial map ($\varphi_1\varphi_2$).

demonstrated that in the investigated moth species acetates are the metabolic precursors of alcohols and aldehydes (Silk et al., 1980; Morse and Meighen, 1982; Teal and Tumlinson, 1986). Thus, owing to their frequent occurrence and their biosynthetic importance, acetates may have been the ancestral pheromone components of Noctuidae. Alcohols and aldehydes would then arise in some species to provide improved pheromone specificity. The barycenters of acetates and alcohols are located close to the origin of axes φ_1 and φ_2 (Figure 5). The acetates, located near this origin, could not be specifically associated with any subfamily. Alcohols are more frequent in Hadeninae (11% of the citations) than in Cuculliinae (4.2%) and Acontiinae, but they were poorly explained by φ_1 and φ_2, and their location was not significant. The barycenter of aldehydes was strongly correlated with Heliothinae, many species of which are attracted by aldehydes.

Stereochemistry and Location of Double Bond. Most of the attractants are monoenes. There are also some saturated compounds (25 citations) and a few dienes (18 citations). The saturated compounds are never attractive in themselves but are parts of sex pheromone blends. Dienes are rare (five of 45 mol-

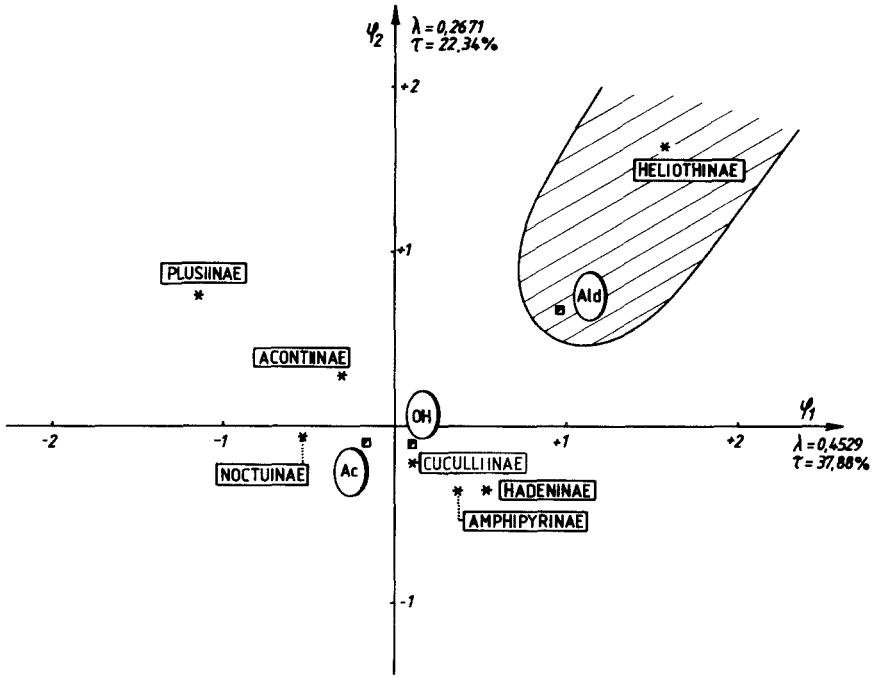


FIG. 5. Projection of noctuid subfamilies and functional group barycenters of their pheromones on the main factorial map ($\varphi_1\varphi_2$).

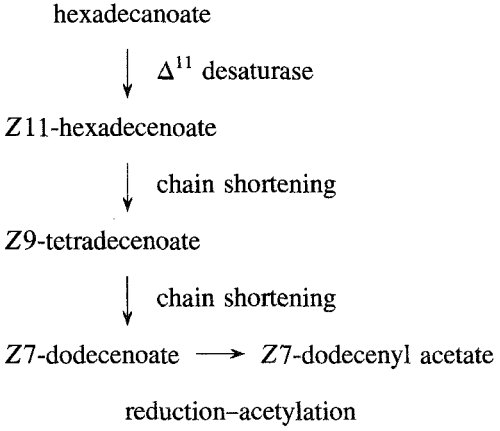
ecules). As an example, *Z*9,*E*12–14 : Ac and *Z*9,*E*11–14 : Ac are highly specific to genus *Spodoptera* (Amphipyrinae), as is *E*9,11–12 : Ac for the genus *Diparopsis* (Noctuinae; Nesbitt et al., 1975).

Almost all the monoenes (394 of 422 citations) possess the *Z* stereochemistry, while only a small number (28 citations) possess the *E* one.

The double bond of monoenes is always located on an odd-numbered carbon atom. According to the conventional numbering system, the double bond is designated by the number of its carbon atom closest to the functional group in the chain. However, β-oxidation chain-shortening steps, which play a prominent role in pheromone biosynthesis (Roelofs and Bjostad, 1984), occur on the functional group moities. This produces an apparent displacement of the considered double bond through a shift of two carbon atoms.

For example, the pheromone gland of *Trichoplusia ni* (Hübner) (Noctuinae), in which *Z*7–12 : Ac is the main pheromone component, contains the immediate fatty ester precursor, *Z*7-dodecenoate, and a large quantity of *Z*11-hexadecenoate (Bjostad and Roelofs, 1983). Labeling experiments show that

Z11-hexadecenoate is chain-shortened to Z9-tetradecenoate, which is in turn chain-shortened to Z7-dodecenoate according to the following scheme (Bjostad et al., 1985).



Thus, it appeared more adequate for the interpretation of the position of the double bond to number it starting from the carbon atom closest to the hydrocarbon end of the chain and to organize these attractive molecules into four groups corresponding to the four new locations so defined. Such a class included molecules derived from a common precursor involved in successive β-oxidation and chain-shortening steps. These new groups were classified as follows: ω3 corresponding to the Δ7–10:, Δ9–12:, and Δ11–14:; ω5 corresponding to the Δ5–10:, Δ7–12:, Δ9–14:, and Δ11–16:; ω7 corresponding to the Δ5–12:, Δ7–14:, and Δ9–16:; and ω9 corresponding to the Δ5–14: and Δ7–16: chain branches where Δ and ω mean an *E* or *Z* double bond in the corresponding position.

This additional rule of numbering emphasized a structural lineage interrelating the four most frequent attractants: Z5–10:Ac, Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac. If the alcohol and aldehyde derivatives are also included, one observe that together they collected 320 of 467 citations (68%). Their molecular skeleton was:

$$\underset{1}{CH_3}-(CH_2)-\underset{5}{CH}=\underset{6}{CH}-(CH_2)n-X$$

with $n = 3$, 5, 7, or 9 and X = CHO, CH—OH, or CHOAc.

The other locations of the double bond were less frequent, 30 citations for ω3, 45 citations for ω7, 15 citations for ω9. These molecules could be produced

either through the action of the Δ11 desaturase on fatty acids of different chain lengths, or through the action of another desaturase on a single precursor. For example, insect cells contain a Δ9 desaturase (Roelofs and Bjostad, 1984). Conversely the pheromone gland of *Trichoplusia ni* contains Z11-hexadecenoate and Z11-octadecenoate, which result from the activity of a Δ11 desaturase on the corresponding saturated fatty acids (Roelofs and Bjostad, 1984).

On the factorial map (Figure 6), we observe that the ω5 attractant molecules are positioned near the axis origin without any special association with any of the subfamilies.

The barycenter of ω3 attractants was associated with Amphipyrinae and Hadeninae (Figure 6). Z11–14 : Ac was moderately correlated with Hadeninae (10 citations for this single subfamily).

The barycenter corresponding to ω7 monoenes was associated with Noctuinae. Table 1 shows that there are 10 citations for the Z7–14 : Ac and six citations for Z5–12 : Ac.

The barycenter corresponding to ω9 was also associated with the Noctuinae (eight citations for the Z5–14 : Ac).

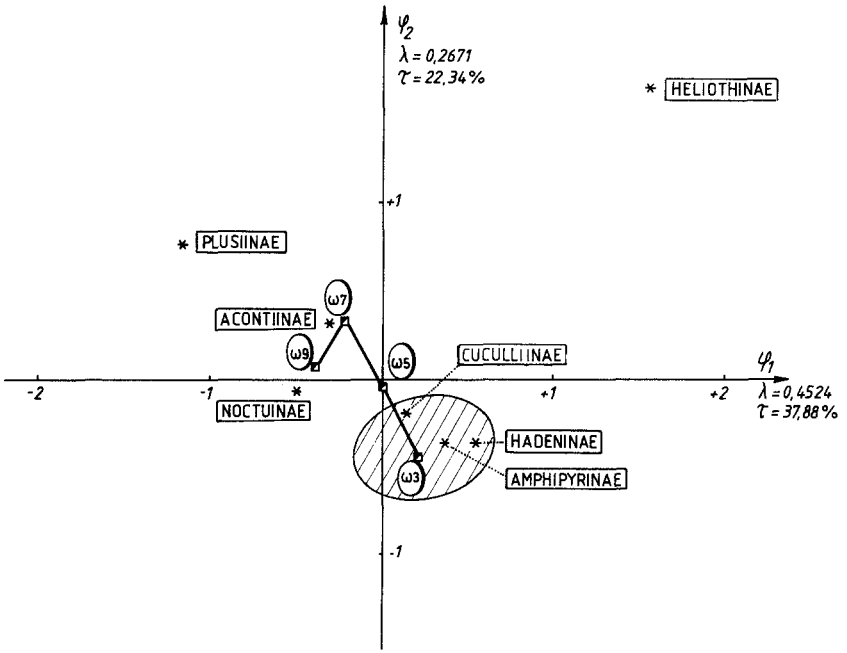


FIG. 6. Projection of noctuid subfamilies and double-bond position barycenters of their pheromones on the main factorial map ($\varphi_1\varphi_2$).

DISCUSSION

The pheromones and the sex attractants of the seven subfamilies of Noctuidae considered are linear chains containing an even number of carbons (10–16). The molecules have one or two double bonds and an acetate, alcohol, or aldehyde functional group. These chemical characteristics led to consideration of nearly 300 different homolog molecules. Of these, only 44 attract male noctuid moths. Furthermore, among these 44 molecules, four account for half of the citations while the other 40 molecules are only sparsely cited.

The prevalent chemical frame is a monounsaturated acetate with the *Z* stereochemistry and the double bond located on the fifth carbon atom closest to the nonfunctional branch of the molecule. Considering the biosynthetic pathway in the pheromone glands, the majority of pheromone constituents appear to be produced through the conversion of a restricted number of precursors, the most common being hexadecanoate. A few specific biochemical reactions then lead to the various isomers present in the sex pheromone blend.

Unlike Tortricidae, Noctuidae do not produce blends of various proportions of *Z* and *E* isomers in order to ensure the specificity of their sex pheromones. Moreover, they appear to possess a unique desaturase, whereas in Tortricidae another class of desaturase allows biosynthesis of Δ8 and Δ10 compounds (Roelofs and Brown, 1982). The basic carbon chain possesses 12, 14, or 16 carbon atoms; the chain length is a less stable structural feature, although the long carbon chains (14 and 16) are more frequent.

If we reject the hypothesis of an experimental bias related to a larger use of these latter pheromonal compounds during field screening of synthetic pheromones, we might consider Z9–14 : Ac and Z11–16 : Ac as sex pheromones of a common ancestral type of Noctuidae.

However, one must point out the lack of data for half of the noctuid moth subfamilies. Furthermore, within the subfamilies investigated, the data are focused on some tribes but ignore other tribes of possibly different phyletic origin.

The Trifines sex pheromones are the best known, and almost all the subfamilies have been investigated. Two other subfamilies, Acronictinae and Pantheinae, very likely use the same kind of compounds as the other Trifines. Dodecenyl acetates or aldehydes are attractive for *Acronicta grisea* Walker (Steck et al., 1982) and *A. sperata* Grote (Underhill et al., 1977), tetradecenal for *Simyra henrici* (Grote) (Steck et al., 1982), and Z7–12 : OH for *Raphia frater* Grote (Weatherston et al., 1974).

The female sex pheromones of most Quadrifines subfamilies are unknown. Apart from Plusiinae and Acontiinae, the structure of the sex pheromone of only one Chloephorinae, *Earias insulana* (Boisduval), has been elucidated. It

is a dienic aldehyde, *E*10,*E*12–16:Ald (Hall et al., 1980); the males of the related species *E. biplaga* Walker are attracted by *Z*11–16:Ald (Nesbitt et al., 1979).

Several recent works deal with Catocalinae sex pheromones. The active molecules are either polyenic hydrocarbons or their monoepoxyde derivatives. The monoepoxydes are sex attractants for several Herminiinae, one Hypeninae, and one Rivulinae (Wong et al., 1985). Thus, the chemical structure and the biosynthetic pathways of the sex pheromones of the deltoid group are different from those of the other Noctuidae. Their biosynthesis probably involves as precursors two essential fatty acids from food, linoleic and linolenic (Roelofs and Bjostad, 1984), instead of the de novo synthesis of saturated fatty acids, as proposed for the common pheromones. Then, one can hypothesize an early differentiation of Catocalinae, Hypeninae, and Herminiinae. These subfamilies were previously grouped in deltoids by others.

The sex pheromones of Arctiidae, Geometridae, and Catocalinae are alkenes or epoxides with double bonds on carbons 3, 6, and 9. Arctiidae are related to Noctuidae; both families belong to the Noctuidoidea superfamily and a lineage between Arctiidae and those of Noctuidae producing the same sex pheromones can be hypothesized. This is evidence for the application of pheromone science to the analysis of relationships between Noctuidae and the other Noctuidoidea. Conversely, Geometridae belong to a different superfamily and more information on their pheromones and those of related families are needed before drawing conclusions on phylogeny.

Within the seven subfamilies considered in this study, the pheromonal compounds were differentiated according to the following features: modification of the functional group, displacement of the double bond, or existence of an additional double bond (dienes). Considering the homogeneity of the basic chemical structure of the sex attractants, one can hardly characterize the subfamilies with exclusive sex pheromones criteria. At least the multidimensional analysis yields a heterogeneous statistical ordering of the chemical structures. For example, Noctuinae are strongly correlated with C_{10} molecules and Heliothinae with aldehydes.

Because of this structural homogeneity, it is difficult to explain the chemistry of the pheromone, in phylogenetic terms, since the same structural features could have arisen simultaneously in different lines. At most, discrete evolutionary trends are revealed by factorial analysis.

On the other hand, some groups (*Spodoptera* and *Diparopsis*) are individualized because of the peculiar chemical features of their pheromones. In this case, the pheromones bring an additional character to the species diagnosis.

In conclusion, the classification of the high taxa of noctuid moths is subject to reexamination and controversy (Kitching, 1984), and the contribution of a potentially new character should not be disregarded. The image of the family

Noctuidae depicted in that paper is a snapshot based on the current knowledge. This classification will certainly be reconsidered as new components are identified and new subfamilies studied, especially those of tropical fauna. Advances in pheromone biochemistry will throw new light on the science of pheromones. Male-emitted pheromones (Birch, 1972a, b) or other biochemical characters might also prove to be helpful.

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ANTENNNAL OLFACTORY AND BEHAVIORAL RESPONSE OF SOUTHERN PINE BEETLE, *Dendroctonus frontalis,* TO ANALOGS OF ITS AGGREGATION PHEROMONE FRONTALIN[1]

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Abstract—Antennal olfactory (electroantennogram) and laboratory and field behavioral tests were carried out on the response of *Dendroctonus frontalis* to its aggregation pheromone frontalin and analogs. The analogs were compounds modified by altering the position and methyl groups and/or by their deletion. Any modification to the frontalin structure significantly reduced both the antennal olfactory and behavioral response by *D. frontalis*. Beetle response, although significantly reduced, was elicited at the receptor level and in a laboratory bioassay by all analogs. However, only one analog (*endo*-5,7-dimethyl-Frontalin) elicited significant response from field populations of the beetle.

Key Words—*Dendroctonus frontalis*, Coleoptera, Scolytidae, bark beetle, pheromone, frontalin, electroantennogram, bioassay, pheromone trap, pheromone analogs.

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INTRODUCTION

Frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) is the major aggregation pheromone of the southern pine beetle, *Dendroctonus frontalis* Zimmerman (Coleoptera: Scolytidae) (Kinzer et al., 1969; Payne et al., 1978). In combination with host odor, attractiveness of frontalin to the beetle is significantly enhanced (Kinzer et al., 1969). Other pheromones and host odors, in the absence of frontalin, are significantly less attractive or totally unattractive to *D. frontalis*, indicating a high level of specificity for frontalin (Payne et al., 1978).

In an effort to gain insight into the structural activity relationships of frontalin, we conducted electrophysiological, laboratory behavioral, and field studies on the response of *D. frontalis* to analogs of the pheromone.

METHODS AND MATERIALS

Insects used in laboratory experiments were newly emerged from naturally infested loblolly pine trees.

Structure and purity of the compounds used are presented in Table I. The

TABLE 1. STRUCTURE AND PURITY OF COMPOUNDS TESTED

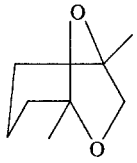
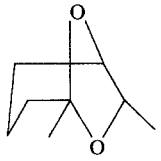
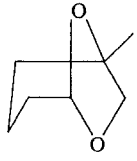
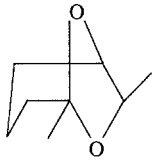
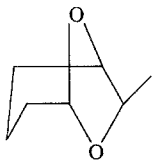
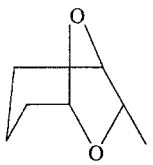
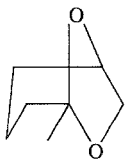
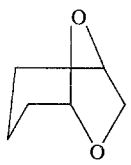
Compound number	Structure	Name	Purity (%)[a]	Field elution (mg/day)
1		1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane (frontalin)	99.7	5
2		*endo*-5,7-dimethyl-(frontalin)	99.4	4
3		1-methyl-(frontalin)	98.8	7

TABLE 1. Continued

Compound number	Structure	Name	Purity (%)[a]	Field elution (mg/day)
4		*exo*-5,7-dimethyl-(frontalin)	100	18
5		*exo*-7-methyl-(frontalin)	99.7	7
6		*endo*-7-methyl-(frontalin)	98.6	4
7		5-methyl-(frontalin)	99.2	10
8		6,8-dioxabicyclo[3.2.1]octane	100	3

[a] Determined by analytical gas chromatography.

compounds were synthesized with known and modified procedures in order to provide a systematic series of modifications to the structure of frontalin. Details on synthesis of the compounds will be published elsewhere.

The electroantennogram (EAG) technique used to measure antennal olfactory response was previously described in detail (Dickens and Payne, 1977) and was a modification of earlier techniques (Schneider, 1957; Payne, 1975). Only male beetles were used in the EAG experiment since, in a previous study, both sexes showed similar antennal olfactory responses to frontalin (Payne, 1975).

Stimuli were diluted in nanograde pentane and were delivered as 10 μl samples of 1 μg/μl placed on filter paper inserted into glass cartridges oriented toward the preparation from ca. 1 cm. Stimulus duration was 2 sec and air flow was ca. 1 liter/min. Three to 5 min were allowed between each stimulation. This time interval was found to be adequate for complete recovery of the EAG. Four replicates were obtained to each stimulus. Each preparation was exposed to nanograde pentane in air as a control, and response to the control was subtracted from subsequent responses to the compounds for a given preparation.

Frontalin at 1 μg/μl was used as the standard to which all responses were normalized in order that responses obtained from different preparations could be compared. Stimulation with the standard followed the control and every two stimulations. Responses to intervening test stimuli were represented as a percent of the mean of the two responses to the standard closest in time to a given test stimulus.

The amplitude of the EAG depolarization was considered a measure of the relative number of responding receptor sites (acceptors) (Payne, 1975; Dickens and Payne, 1977) and a relative measure of the sensitivity of *D. frontalis* to a given stimulus. Responses were compared for significant differences using the Student's *t* test.

A pedestrian bioassay (Payne et al., 1976) was used to test attraction to the compounds, which were delivered at 1 μg/μl/min in nanograde pentane in a 1 liter/min air flow. For each assay, five beetles of a single sex were used. At least 30 min were allowed to pass before a group was tested against a different stimulus. All beetles were assayed within 24 hr of emergence.

Ten replicates (50 beetles) per sex were assayed against each compound. Results were recorded as percent positive response per replicate of response to frontalin as a standard. The Student's *t* test was used to compare responses between compounds and between sexes per compound.

Field tests were carried out during May through July 1986 in the east Texas pine forest. Funnel-barrier traps (Rose et al., 1981), positioned at 20–30 m apart, were used to monitor response of *D. frontalis* to the compounds. The compounds were eluted from 2-dram ampules which had been severed at the neck (Table 1). Turpentine, as a host odor, was released separately at ca. 1440 mg/day through an 18-cm-long alcohol burner wick (7 cm exposed) from a 125-

ml amber bottle (Fisher Scientific). Host odor has been shown to be significant to the attractiveness of frontalin to field populations of *D. frontalis* (Renwick and Vité, 1969; Payne et al., 1978; Billings, 1985). Traps and compounds were repositioned within the trap line after each replicate. Data collected consisted of the total number of male and female *D. frontalis* that responded to each compound and the control traps per three- to four-day replicate. The Student's *t* test was used to compare the responses to compounds and between sexes per compound.

RESULTS AND DISCUSSION

Electrophysiology. Response to the standard was $\bar{X} = 1.04$ mV $\pm$ SE = 0.07 ($N = 36$). Response by *D. frontalis* to frontalin (compound 1, Table 1) was significantly greater ($P < 0.05$) than response to other compounds. The level of response decreased significantly as the structure of the compound was altered from that of frontalin (Figure 1). Consequently, response to compound 8 (no methyl groups) was significantly less ($P < 0.01$) than response to all other compounds.

Response to 1-methyl-frontalin (compound 3) was significantly greater (*P*

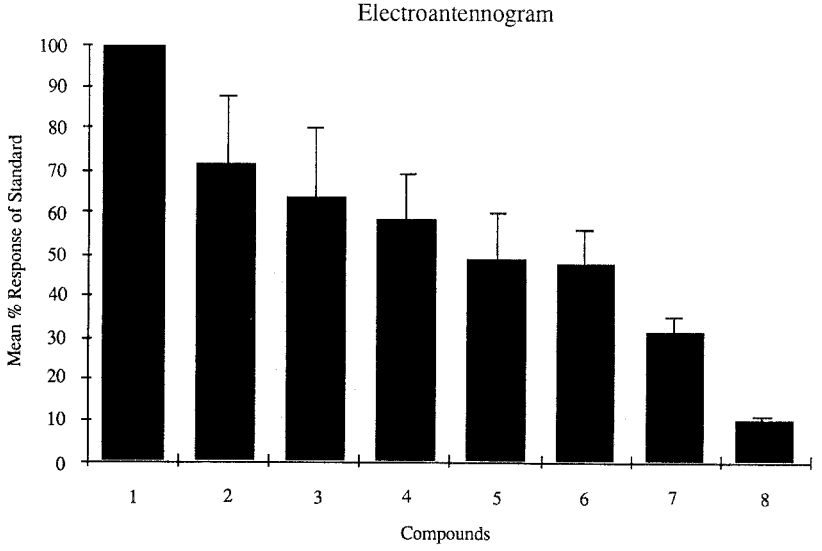


FIG. 1. Mean percent ($\pm$SE) EAGs to frontalin and analogs by male *D. frontalis*. N = 4/compound. All compounds presented as 10 μg on filter paper. Standard = frontalin. See Table 1 for compound name.

< 0.05) than response to 5-methyl-frontalin (compound 7), indicating a greater level of receptor specificity for a methyl in the 1 position than in the 5 position. By comparison, the level of response to the 7-methyl-frontalin compounds (compounds 5 and 6) was intermediate between response to compounds 3 and 7, but not significantly different.

Laboratory Bioassays. Both male and female beetles responded to all of the compounds, and there were no significant differences between the sexes for a given compound (Figure 2). As expected, response to frontalin was significantly greater ($P < 0.05$) than to any of the other compounds, and response to compound 8 was significantly less than to the other compounds. Response to compound 3 was not significantly different from response to the other methyl-

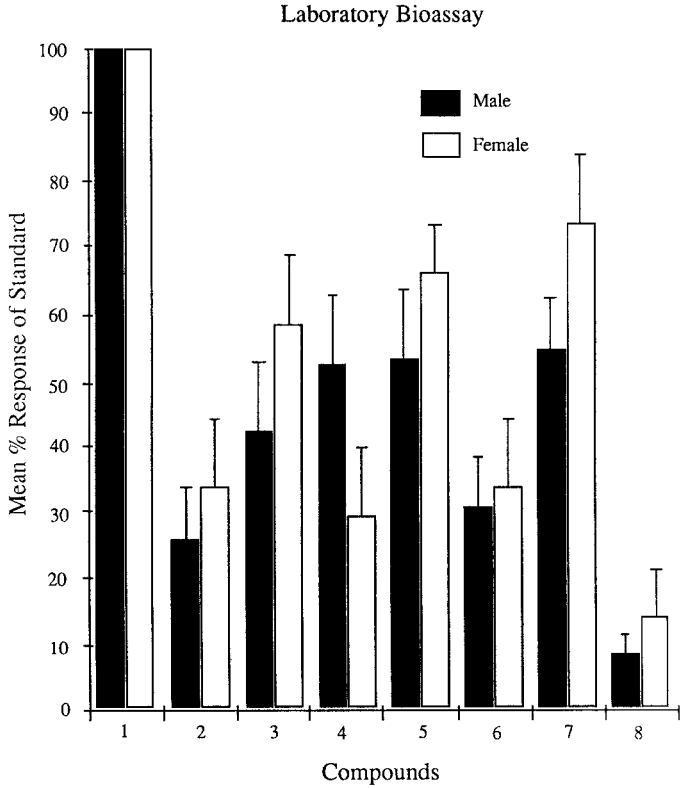


FIG. 2. Mean percent response (±SE) to frontalin and analogs by male and female *D. frontalis* in a laboratory bioassay. $N = 50$ beetles/compound/sex. All compounds presented at 1 μg/min. Standard = frontalin. See Table 1 for compound name.

ated compounds (except frontalin), indicating that a methyl in position 1 was not as significant to behavioral response in the bioassay as it appeared to be in receptor response. In fact, the absence of significant differences in response to compounds 3, 4, 5, and 7 indicate that the position and number of methyl groups (other than in the frontalin structure) are not critical to the walking response in the laboratory bioassay.

Field Tests. Significantly more *D. frontalis* were caught in response to frontalin than to any of the analogs (Figure 3). Of the analogs, only compound 2 caught numbers of beetles significantly different ($P < 0.01$) from the blank ($\bar{X} = <1$, $\bar{X} = 2$) and turpentine only ($\bar{X} = 1$, $\bar{X} = 2$), male and female, respectively. Obviously, any structural modification to frontalin greatly reduced or eliminated its attractiveness to flying populations of *D. frontalis*.

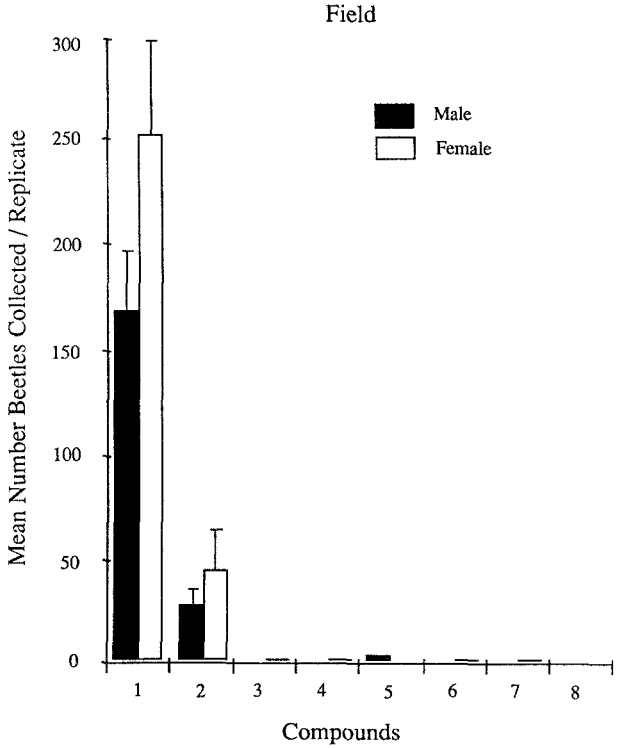


FIG. 3. Mean number (±SE) of male and female *D. frontalis* caught in traps baited with frontalin and analogs. Data from eight 3- to 4-day replicates. All compounds released with turpentine as a host odor. See Table 1 for compound name.

CONCLUSION

Any modification to the structure of frontalin significantly reduced antennal olfactory and behavioral response in *D. frontalis*. Similar findings were reported for the structural activity relationships of pheromones in several lepidopterous species (Payne, 1969; Roelofs and Comeau, 1971; Gaston et al., 1972; Roelofs, 1977).

The results show that *D. frontalis* possesses receptors capable of responding to analogs of frontalin. However, whether or not all of the analogs interact with the same receptors as frontalin is not known. Single-cell studies will be required to provide that information. It is likely that compound 2 does interact with the receptors for frontalin since the compound was attractive to natural populations of the beetle. The fact that the other analogs elicited behavioral responses in the laboratory bioassay, but not in the field, leads to the speculation that the compounds might interact with receptors that function in close range olfactory behaviors such as might occur on the host tree surface.

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ASTRINGENCY OF DOUGLAS-FIR FOLIAGE IN RELATION TO PHENOLOGY AND XYLEM PRESSURE POTENTIAL

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Abstract—Astringency (tannin content) of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] foliage was examined in relation to phenology and water status. Foliage was sampled from control trees, trees on south-facing slopes, and trenched trees prior to budbreak and at periods of two, six, and 12 weeks after budbreak. Astringency of prebudbreak foliage from untrenched trees was comparable to that of mature foliage. Foliage expansion was accompanied by dilution of the tannin content. Percent relative astringency of control trees was significantly and positively related to the absolute value of predawn xylem pressure potential, while this relationship was negative for trees in the south-facing group. Coefficients of determination for the relationship between astringency and predawn xylem pressure potential were high (0.67 and 0.79 for the control and south-facing groups, respectively). Astringency of foliage from trees in the south-facing group also was affected significantly by daytime xylem pressure potential. Astringency of foliage from trees in the control and south-facing groups was not significantly related to tissue age, while that from trenched trees was significantly related only to age. Results demonstrate that water status is a better predictor of foliage astringency than is tissue age in unperturbed trees of this species and that, depending on the magnitude and/or timing of water deficits, opposite relationships between astringency and xylem pressure potential can be observed.

Key Words—Astringency, Douglas-fir, moisture stress, phenology, polyphenols, *Pseudotsuga menziesii*, tannins, xylem pressure potential.

INTRODUCTION

Herbivores and pathogens may be affected by tannin content of foliage (Feeny, 1976; Rhoades and Cates, 1976), which in turn may be affected by both phe-

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nology and environmental stress. Tannin concentration is often higher in mature than in immature leaves (Dement and Mooney, 1974; Feeny, 1970; Feeny and Bostock, 1968; Mooney et al., 1983; Tempel, 1981). The lower concentration in immature tissue has been suggested to result from compartmentalization problems, potential for autotoxicity, physiological constraints imposed by source and sink strengths, and/or lower predictability of the tissue to herbivores (Feeny, 1976; Rhoades and Cates, 1976). In many perennial plants, leaf buds are produced months before foliage expansion and potentially are exposed to herbivores for long periods. Unexpanded foliage in leaf buds represents a large potential source of carbon gain and should be well defended. In plants with a favorable carbon balance, such as those with large reserves and/or the capacity to photosynthesize throughout the year, unexpanded foliage in buds could contain relatively high tannin contents if compartmentalization and autotoxicity problems do not prevent it. Therefore, both unexpanded and mature foliage may have higher concentrations of tannins than rapidly expanding foliage. The tannin content of foliage in buds rarely has been studied (but see Crankshaw and Langenheim, 1981).

In addition to phenological change, tannin content of plant tissues also can vary in response to such environmental stresses as water deficits. Most reports on the relationship between tannin content and water status have been implicit, with no measure of plant water status. For instance, the tannin content of cotton (*Gossypium hirsutum*) leaves has been correlated with the frequency of irrigation (Guinn and Eidenbock, 1982). Few studies have attempted to correlate foliar tannin content with indices of water status such as xylem pressure potential (see Gershenzon, 1984). Since such factors as stomatal conductance, photosynthesis, use of storage reserves, and growth are affected differentially as moisture stress progresses, the magnitude of stress may affect the response of plants to moisture stress (Salisbury and Ross, 1978; Waring and Schlesinger, 1985). In addition, prior exposure may have an effect: plants that regularly experience water deficits may exhibit physiological adaptations and respond differently to a given level of stress than plants that have not been previously stressed. Therefore, the duration as well as the magnitude of stress may affect the response of a plant. Lastly, since water relations in many habitats change seasonally, it is often difficult to separate the relative effects of tissue age and water status on tannin content of tissues (Tempel, 1981).

The objectives of this study were: (1) to examine the phenological patterns that occur in astringency of prebudbreak and expanding foliage of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco]; (2) to ascertain whether tissue age or plant water status is a better predictor of foliage astringency; and (3) to identify differences in the effects of magnitude and duration of water deficits on foliage astringency. Results indicate that xylem pressure potential is a better predictor than tissue age of foliage astringency in unperturbed trees and that opposite

relationships between astringency and xylem pressure potential can be observed depending on the magnitude and/or timing of water deficits.

METHODS AND MATERIALS

Study Site. The study site was located in a canyon in the Jemez Mountains, Santa Fe National Forest, New Mexico (35° 54′ N, 106° 42′ 30″ E). The median elevation of the canyon bottom was 2440 m (range 2375–2500 m). The dominant vegetation in the area was Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] and white fir (*Abies concolor* Lindl. and Gord.). Some Colorado blue spruce (*Picea pungens* Engelm.), ponderosa pine (*Pinus ponderosa* Laws.), quaking aspen (*Populus tremuloides* Michx.), and several oak (*Quercus*) species also occurred in the study area.

Field Methods. Twelve trees, eight in the canyon bottom and four on south-facing aspects, were selected along a 4-km section of the canyon. Study trees were similar in age, height, diameter at breast height, and increment in bole diameter over the previous five years (Table 1). Four of the bottomland trees served as controls. The other four bottomland trees were trenched at the dripline to a depth of 1 m in early June, 1980. The trenches were lined with polyethylene sheets and back-filled. The understory vegetation beneath the crown of each tree was removed, and the ground from bole to dripline was covered with polyethylene sheets. The plastic was used to prevent precipitation and throughfall from replenishing soil moisture. By so doing, it was hoped that these trees would gradually deplete the soil moisture and develop a water deficit, thus representing trees under a short-term moisture stress. It was assumed that the four trees on south-facing slopes had experienced a long-term moisture deficit.

Prior to budbreak, 55 buds were selected and tagged in the midcrown of the south-facing quarter of each tree. Selected buds were similar in length,

TABLE 1. COMPARISON OF AGE AND PHYSICAL PARAMETERS OF TREES OF EACH EXPERIMENTAL GROUP

Treatment	Age (yr)[a]	Height (m)	DBH (cm)[b]	IBD (cm)
Control	36.8 ± 0.5	11.4 ± 0.4	22.4 ± 0.4	1.70 ± 0.1
South-facing	37.8 ± 1.7	12.1 ± 1.5	25.7 ± 3.1	1.28 ± 0.3
Trenched	35.0 ± 1.6	11.6 ± 1.7	21.4 ± 2.5	2.08 ± 0.3

[a] Values are mean ± SE. $N = 4$ in each case. Numbers in columns are not significantly different ($P > 0.05$).

[b] Abbreviations: DBH = diameter at breast height; IBD = increment in bole diameter over the previous five years.

amount of swelling (i.e., width) and distension of the bud scales. Current foliage was collected on June 13, June 26, July 25, and September 7, 1981. These dates corresponded to about three days prior to budbreak and periods of two, six, and 12 weeks after budbreak. Mature tissue produced during the previous year also was collected on June 13. On each sampling date, nine tagged buds (or the shoots developing from these buds) were clipped from each tree. The tissue was placed on ice in airtight plastic bags for transport to the laboratory. Tissue samples were frozen upon arrival at the laboratory.

Predawn xylem pressure potentials represent the minimum to which the water deficit can be reduced under conditions of minimum transpiration and are less variable than those measured during daylight hours. On the other hand, xylem pressure potential measurements obtained during the daylight hours can reveal the maximum water deficit experienced in the course of a day. It seems reasonable that either or both of these parameters might affect the physiology of trees. Xylem pressure potentials were measured on the distal 10 cm of a branch originating at about 3 m height using a Scholander pressure bomb. Daytime xylem pressure potentials were measured between 1300 and 1600 hr; only shaded sprigs were used. Predawn xylem pressure potentials were measured between 0100 and 0500 hr. Xylem pressure potentials were measured on all postbudbreak collection dates. Because of logistical problems, the data set from the first postbudbreak collection was very unbalanced. Therefore, only measurements from the last two collection dates were included in the analysis.

Laboratory Methods. Bud scales and twigs were removed and discarded prior to analysis. Foliage from three buds (shoots) per tree from each sampling date were dried at 60°C to determine the water content and dry weight.

The suggested ecological importance of tannins has been attributed to their capacity to form recalcitrant complexes with proteins and other substrates. Since correlations between astringency and estimates of tannin content obtained by other methods are insignificant or weak both within and between species (Horner, unpublished data; Martin and Martin, 1982), relative astringency determinations seem the best analytical methods to obtain the desired information in ecological studies (Martin and Martin, 1982). Foliage astringency was analyzed by the hemanalysis method of Schultz et al. (1981), except that the stock hemoglobin solution was prepared from fresh human blood and leaf tissue was ground under liquid nitrogen. Douglas-fir (and gymnosperms in general) apparently do not produce hydrolyzable tannins (see Haslam, 1981, for a general review); the condensed tannins of Douglas-fir have been partially characterized by Stafford and Cheng (1980) and Stafford and Lester (1980, 1981). Although a condensed tannin may have been the best choice for a standard, I was unable to obtain a condensed tannin standard of adequate purity. Therefore, percent relative astringency was calculated from a standard curve generated from the action of tannic acid (a hydrolyzable tannin) solutions of known concentration and

expressed as tannic acid equivalents (TAE). Use of tannic acid rather than a condensed tannin results in differences in values obtained but not in the trends observed (Horner, unpublished data). Total relative astringency of current shoot foliage was calculated from percent TAE and total dry mass of current shoot foliage. Because of the small mass of prebudbreak foliage, foliage was composited from two unopened buds per tree for analysis of prebudbreak tissue.

Data Analysis. Physical parameters of the trees of each experimental group were compared by one-way analysis of variance (ANOVA) (Zar, 1974). Predawn and daytime xylem pressure potentials were analyzed by two-way ANOVA. The factors for the two-way ANOVAs were date (two levels) and treatment (three levels). Differences between levels of a factor were identified by Duncan's multiple-range test.

Tannin data were analyzed in terms of both percent and total relative astringency of current shoot foliage. Percentage astringency data were normalized by arcsine transformation, and foliage weight data were normalized by log transformation before statistical analysis. Astringency data were analyzed by analysis of covariance, with treatment as a fixed class variable and both age and xylem pressure potential as concomitant variables (Sokal and Rohlf, 1981). Since an objective was to determine whether foliage astringency was better explained by age or xylem pressure potential, a forward stepwise regression technique for maximal r^2 improvement was employed. Selection criteria for entering into the model were an improvement in r^2 of 0.05 and a significant ($P < 0.05$) overall model.

RESULTS AND DISCUSSION

Phenological Patterns in Astringency. In all experimental groups, foliage percent astringency showed an initial postbudbreak decrease followed by a gradual increase (Figure 1a). Decreased concentration during foliage expansion could be due to a number of factors, including reduced or halted production of tannins or a dilution effect (i.e., the allocation to primary metabolites and growth exceeding that to tannins). Total relative astringency of current shoot foliage generally increased during the first six weeks after budbreak (Figure 1b). This indicates that production continued during foliage expansion and that the decrease in percent TAE observed during foliage expansion is due at least in part to a dilution effect rather than complete cessation of tannin production.

Relative to their mass, leaf buds represent disproportionately large potential sources of carbon gain. As a result, they should be relatively valuable to the plant. Since tannins appear to be important in reducing herbivory in Douglas-fir (Walters and Stafford, 1984), it is interesting that relative astringency levels were high in prebudbreak foliage of untrenched trees. Percent relative

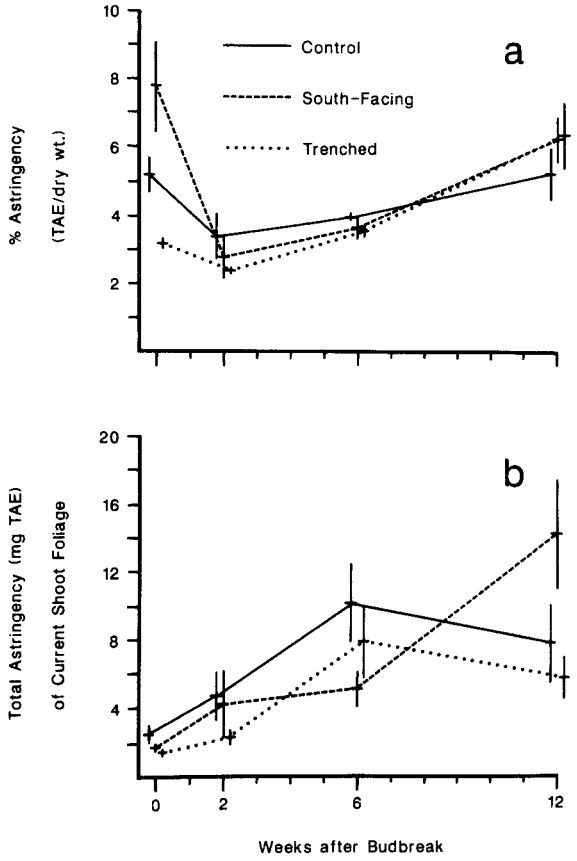

FIG. 1. Seasonal changes in (a) % relative astringency (tannic acid equivalents; TAE) and (b) total relative astringency of current shoot foliage from trees in control, south-facing, and trenched experimental groups. Horizontal bars are means and vertical bars are 2 SE. Collections at each time interval were made on the same day; data for each treatment were separated horizontally to minimize overlap.

astringency of prebudbreak foliage from trees in the control and south-facing groups (5.18 $\pm$ 0.48 and 7.83 $\pm$ 1.31, respectively; mean $\pm$ SE; Figure 1a) was comparable to that in mature foliage (5.73 $\pm$ 0.54 and 6.11 $\pm$ 0.57, respectively; $P > 0.05$ between prebudbreak and mature foliage within the control and south-facing groups). High tannin content in immature foliage may be possible in plants with a favorable carbon balance, such as those that are slow growing, capable of photosynthesizing throughout the year, or that have adequate storage reserves (Crankshaw and Langenheim, 1981; Dement and

Mooney, 1974; Mooney et al., 1983). Autotoxicity has been suggested as a reason for low tannin concentrations in immature foliage (Feeny, 1976), but this is obviously not the case in prebudbreak foliage of untrenched Douglas-fir. In contrast to unperturbed trees, tannin content of prebudbreak foliage from trenched trees was significantly lower than that of mature foliage (3.21 $\pm$ 0.15 and 7.12 $\pm$ 0.68, respectively; $P > 0.05$).

Treatment-Related Differences in Xylem Pressure Potential. ANOVA revealed significant treatment effects on both predawn ($P < 0.0001$) and daytime ($P > 0.03$) xylem pressure potentials (Table 2). However, differences between the experimental groups were not consistent throughout the season, as indicated by significant date $\times$ treatment interactions ($P > 0.0002$ and $P < 0.02$ for predawn and daytime measurements, respectively). During July, both predawn and daytime xylem pressure potentials of control trees were significantly less negative than those of trees on south-facing aspects. In September, on the other hand, the average daytime xylem pressure potential of trees on south-facing slopes was significantly less negative than that of control trees, while predawn xylem pressure potentials were not significantly different. Although the xylem pressure potentials of trenched trees were never significantly different from those of control trees, the former were consistently lower (more negative) than the latter (Table 2). It therefore appears that trenching had not yet elicited significantly decreased water potentials by the end of the first growing season following trenching. Predawn and daytime xylem pressure potentials were not significantly correlated ($P > 0.10$). This could have resulted from different seasonal patterns of soil moisture recharge in the experimental groups.

Astringency in Relation to Tissue Age and Xylem Pressure Potential. Analysis of covariance revealed significant ($P > 0.05$) treatment effects on foliage

TABLE 2. SEASONAL AND TREATMENT-RELATED VARIATION IN XYLEM PRESSURE POTENTIAL

	Xylem pressure potential (—bars)[a]		
Sampling	Control	South-facing	Trenched
Predawn			
July 25	3.8b $\pm$ 0.2 (4)	8.5a $\pm$ 1.0 (3)	5.4b $\pm$ 0.4 (4)
Sept. 7	5.0a $\pm$ 0.3 (4)	5.3a $\pm$ 0.6 (3)	5.2a $\pm$ 0.2 (4)
Daytime			
July 24	14.0b $\pm$ 0.5 (3)	17.0a $\pm$ 0.9 (3)	16.0ab $\pm$ 0.9 (4)
Sept. 6	18.8a $\pm$ 0.8 (4)	16.0b $\pm$ 0.9 (4)	19.9a $\pm$ 0.7 (4)

[a] Values are mean $\pm$ SE (N). Means in rows followed by different letters are significantly different ($P > 0.05$) between treatments.

astringency. Since xylem pressure potential was significantly affected by treatment, and since it was hypothesized that the treatments might elicit different effects on production of astringency, treatments were separated for stepwise regression analysis. Inasmuch as predawn and daytime xylem pressure potentials were not significantly correlated, the regression analysis included both predawn and daytime xylem pressure potential measurements as well as age. The percent relative astringency of foliage from trees in both the control and south-facing groups was strongly affected by predawn xylem pressure potential (Table 3). The coefficients of determination were surprisingly high. Interestingly, while the relationship between astringency and the absolute value of predawn xylem pressure potential was positive for control trees, this relationship was negative for trees in the south-facing group. The percent relative astringency of foliage from trees in the south-facing group also was affected significantly by daytime xylem pressure potential, but in this case the relationship was positive. Total astringency of current shoot foliage was not significantly related to either tissue age or xylem pressure potential in any treatment.

The differences between the control and south-facing groups in the relationship between xylem pressure potential and foliage astringency may have been due to differential effects on carbon gain versus carbon allocation patterns brought about by differences in water status during a critical growth period. Mild water deficits may reduce foliage expansion and growth, while more extreme water deficits can reduce stomatal conductance and carbon acquisition (Salisbury and Ross, 1978). Therefore, a positive relationship between the absolute value of xylem pressure potential and astringency, as observed in the control group, may indicate an effect on carbon partitioning between primary and secondary metabolites caused by a reduction in growth. An inverse relationship may indicate a reduction in stomatal conductance and carbon fixation. Both predawn and daytime xylem pressure potentials of trees in the south-facing

TABLE 3. SUMMARY OF SIGNIFICANT STEPWISE REGRESSION STATISTICS FOR ASTRINGENCY ON AGE AND XYLEM PRESSURE POTENTIAL[a]

Treatment	Step	Variable entered[b]	Total P	Total r^2	Slope[c]	Intercept
Control	1	Predawn XPP	0.01	0.67	1.06	−0.09
South-facing	1	Predawn XPP	0.003	0.79	−0.82	10.71
	2	Daytime XPP	0.0008	0.94	0.46	−2.75
Trenched	1	Age	0.02	0.61	0.30	2.41

[a] The absolute value of xylem pressure potential was used in the analysis.
[b] XPP = xylem pressure potential.
[c] For ease of interpretation, slopes and intercepts are given for untransformed regression equations using single independent variables.

group were significantly more negative than those in the control group during the middle of the growing season (July 25; Table 2). The inverse relationship between tannin content and absolute value of predawn xylem pressure potential observed in trees in the south-facing group may have been due to a reduction in carbon gain. Unfortunately, no measurements of stomatal conductance were made in this study. Wagner (1986) also reported a decrease in tannin content of *Pinus ponderosa* Dougl. ex Laws seedlings stressed with polyethylene glycol. The positive effect of daytime xylem pressure potential on tannin content observed in the south-facing group is more difficult to explain. It suggests a contribution of reduced foliage expansion related to more negative daytime xylem pressure potentials in this group. An alternative to the carbon acquisition/allocation hypothesis might be that there were unmeasured correlates to xylem pressure potential that differed between control trees and those on south-facing aspects.

Outbreaks of herbivorous insects often occur during periods of plant environmental stress, and this has been suggested to be the result of reduced defenses during these periods (Rhoades, 1979). Results from this study suggest that the timing and magnitude of water deficits may be important factors affecting the production of plant chemical defenses.

In contrast to the other two experimental groups, only age had a significant effect on relative astringency of trenched trees (Table 3). Several lines of evidence suggest that trenching produced effects other than the desired effect on water relations. First and most obviously, neither predawn nor daytime xylem pressure potentials of trenched trees were significantly different from those of controls. Second, unlike unperturbed trees, the foliar tannin content of trenched trees showed no significant relationship to xylem pressure potential. Lastly, the tannin content of prebudbreak foliage from trenched trees was significantly lower than that of mature foliage, unlike the relationships observed in the control and south-facing groups. Together, these data suggest that trenching may have had other effects. Reduced moisture and elevated temperatures beneath the plastic might have altered nutrient mineralization (Floate, 1970; Swift et al., 1979) and availability to trenched trees. Additionally, despite an effort not to cut large roots during trenching, some root cutting may have occurred and could be expected to have effects other than diminishing water uptake. Because of probable undesired side effects brought about by trenching, this study did not adequately test the effects of duration of water deficits. Wagner (1986) also found somewhat anomalous results in foliar tannin content of trenched trees, and I reiterate his caveat toward the use of trenching as a means for inducing water stress in field studies.

Final Considerations. It should be mentioned that differences in astringency may not be directly related to differences in carbon allocation to tannins. Beart et al. (1985) have shown that chain length and astringency of hydrolyzable

tannins are not necessarily directly correlated. Although a similar phenomenon has been suggested to occur with condensed tannins (Goldstein and Swain, 1963; White, 1957), this has not been directly demonstrated. Furthermore, qualitative as well as quantitative differences in tannin composition may affect astringency (Asquith and Butler, 1986; Zucker, 1983).

Conclusions. Astringency of prebudbreak foliage in unperturbed trees was comparable to that of mature tissue. The postbudbreak decrease in percent relative astringency appeared to be due at least in part to a dilution effect rather than a cessation of tannin production. Percent astringency of current shoot foliage of unperturbed trees was better explained by the absolute value of predawn xylem pressure potential than by tissue age. The relationship was positive for control trees but negative for trees on south-facing aspects, suggesting differences in carbon acquisition and allocation related to magnitude and/or timing of water deficits.

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COMPARATIVE EFFECTS OF TWO PLANT SECONDARY METABOLITES ON HOST–PARASITOID ASSOCIATION

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Abstract—Two plant-derived allelochemicals, berberine and α-terthienyl (α-T), were tested for their effects on the European corn borer, *Ostrinia nubilalis*, and its endoparasitoid *Diadegma terebrans*. The compounds were administered to the host insect in meridic diets, and the responses of the host larvae and parasitoids reared from treated hosts were measured in terms of growth parameters and survival. In *O. nubilalis*, survival to pupation and adult emergence were reduced significantly by the inclusion of berberine and α-T in larval diets at a concentration of 100 μg/g. However, in the parasitoid, adverse effects were much more apparent with the α-T treatment than with the berberine treatment. α-T and one of its metabolites were found in host larvae and in emerged adult parasitoids and their cocoons. Berberine residues were not detected. The implications of these responses to compounds of widely differing physiological properties are discussed with reference to host-plant resistance and biological control.

Key Words—Third trophic level interaction, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, *Diadegma terebrans*, Hymenoptera, Ichneumonidae, α-terthienyl, berberine, allelochemicals.

INTRODUCTION

The effects of secondary plant substances on the parasitoids of phytophagous insects have been the subject of few publications. Thurston and Fox (1972) demonstrated that nicotine in the diet of the tobacco hornworm, *Manduca sexta* (L.), reduced the emergence of *Cotesia congregata* (Say) (=*Apanteles congregatus*). Campbell and Duffey (1979, 1981) demonstrated that α-tomatine in the host

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diet had negative effects on *Hyposoter exiguae* (Viereck), a parasitoid of the tomato fruitworm, *Heliothis zea* (Boddie). Apparently, α-tomatine was not directly toxic, but rather produced these effects by imparing sterol utilization.

The effects of nicotine on two parasitoids—*C. congregata* (a parasitoid of *M. sexta*) and *Hyposoter annulipes* (the parasitoid of the fall armyworm, *Spodoptera frugiperda* [Smith],—were studied in detail by Barbosa et al. (1986). As found by Thurston and Fox (1972), they observed reduced emergence for both parasitoid species. They also reported that cocoon formation was inhibited. In *H. annulipes*, the toxic effects of nicotine were more severe; sublethal effects were also apparent, including prolonged larval development and reduced adult size.

Toxic effects on the third trophic level were also recently reported for the tomato phenolic rutin (Duffey et al., 1986). In this case, the lethal and sublethal effects of *H. exiguae* depended on the nutritional quality of the host's diet, particularly with respect to levels of protein. For each of the two noctuid hosts *H. zea* and *Spodoptera exigua* (Hbn.), specific levels of protein in host diets were found to enhance the expression of toxicity in the parasitoids.

Nicotine (Barbosa et al., 1982, 1986) and α-tomatine (Campbell and Duffey, 1979) were shown to persist in the host and were detected in emerged parasitoids reared from alkaloid-fed hosts. Carotenoids (Rothschild et al., 1977) and pyrrolizidine alkaloids (Benn et al., 1979) from food plants have also been found to persist in and travel through herbivorous insects into their parasitoids.

Investigations of three trophic level interactions have focused on the effects of one particular toxin within these host–parasitoid food chain models. We have examined the physiological effects of two secondary plant substances with different physiological properties and biological sites of action on the European corn borer, *Ostrinia nubilalis* (Hübner) (Pyralidae), and its endoparasitoid *Diadegma terebrans* (Gravenhorst) (Ichneumonidae). *O. nubilalis* is a polyphagous species (Caffrey and Worthley, 1927; Hodgson, 1928), and as such has been exposed to a wide range of allelochemicals during its evolutionary past.

The two compounds used in this study were berberine (Figure 1), an iso-

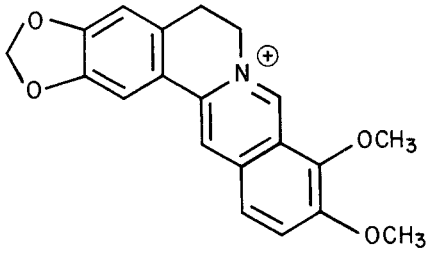

FIG. 1. Molecular structure of berberine.

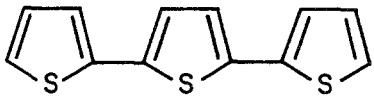

FIG. 2. Molecular structure of α-terthienyl.

quinoline alkaloid found in at least 26 genera of 10 plant families (Suffness and Cordell, 1985), and α-terthienyl (α-T) (Figure 2), a thiophene characteristic of numerous genera of the Asteraceae (Bohlmann et al., 1973), including most genera of the subtribe Pectinidae (Downum et al., 1985). The two compounds are different with respect to polarity: berberine is a quaternary ammonium compound (cationic), while α-T is highly lipophilic (McLachlan, 1984). Berberine-containing plants are not normally attacked by *O. nubilalis*, while plants containing thiophenes are among those preferentially attacked by the insect (Caffrey and Worthley, 1927; Hodgson, 1928). The very different physical properties of berberine and α-T provide a basis for studying a range of responses possible in one host–parasitoid system.

METHODS AND MATERIALS

Growth and Survival Studies with Ostrinia nubilalis. A laboratory colony of *O. nubilalis* was maintained by using the methods of Guthrie et al. (1971) except that corncob grits were added to the diet used for routine culture maintenance. The diets used in the experiments were prepared by mixing berberine (dissolved in water) or α-T (95% ethanol solution added to α-cellulose powder and solvent evaporated) to warm, unsolidified meridic diet at concentrations of 10, 31, and 100 μg/g (wet weight). These concentrations are realistic with respect to natural concentrations in plants and were selected after preliminary feeding tests in which chronic effects on *O. nubilalis* could be demonstrated. Control diets were prepared with an appropriate aliquot of water (for the berberine experiments) or α-cellulose (for the α-T experiments). Corncob grits were not included in these diets in order to ensure uniform distribution of the compounds throughout the media.

Experimental larvae were reared in a growth chamber under the following conditions: photoperiod 18:6 hr (light–dark) with a day–night temperature regime of 26.5°C/19°C and a constant relative humidity of 85%. Because both of the experimental compounds are potentially phototoxic, conditions of subdued photosensitizing ultraviolet (UV) illumination were used. Lighting within the environmental chamber was provided by four 20-W solar-simulating flu-

orescent lamps (Vitalight 48T12 U.H.O.) separated from the chamber by a 3-mm sheet of Plexiglas. The resultant light intensity was measured to be 7.6 W/m, with a UV cutoff at 390 nm.

Neonate *O. nubilalis* were placed on the appropriate treated or control diets in glass scintillation vials plugged with cotton wool. After eight days, 10 surviving larvae were randomly chosen from each treatment and placed in individual vials with fresh diet. Subsequently, every four days, vials and diets were changed. The larvae were observed daily and growth parameters were recorded. This procedure was replicated three times, such that data was generated from 30 individuals per treatment.

Growth and Survival with Diadegma terebrans. *O. nubilalis* were reared from hatching on the variously treated and control diets until they were of the instar and weight range of control larvae at age 9 days. At this time, 100 second-instar larvae ranging in weight from 2 to 7 mg were selected from each treatment and placed on 25–30 1 cm pieces of chopped corn stalk (Silver Queen, Stokes Seeds Ltd.) in a round plastic rearing box (18.3 cm diameter, 7.8 cm high). These larvae were allowed to feed and burrow into the corn overnight. The presence of host-plant tissue provided necessary visual and chemical cues for parasitization by *D. terebrans*.

The following morning, the dish with the corn and larvae was placed in the *D. terebrans* rearing cage for 1 hr, which enabled the female wasps to "saturate" the larvae and parasitize all acceptable hosts. The parasitization occurred within a controlled environment chamber (25°C and 70% relative humidity), with a high light intensity (8 W/m^2) provided by six 20-W solar-simulating fluorescent lamps (Vitalight 48T12 U.H.O.). The *O. nubilalis* larvae were then removed from the corn and returned to their appropriate diets in vials. A 5-cm square of paper towelling was placed in each vial above the diet cube. This provided a dry place within which the emerging *D. terebrans* could pupate. Pupae were held in gelatin capsules until adult emergence. Growth parameters were recorded.

As the different treatment larvae were parasitized within one week of each other, separate control groups (100 larvae each) were run for each treatment. Control groups were parasitized one day prior to the treated groups. A full day was left between treatment and control parasitizations in order to allow the female wasps to feed and replenish their ovarioles with mature eggs. In this way, at least 30 larvae were parasitized in each of the treatment and control groups.

Analysis for Body Burden of Compounds in Host Larvae and Parasitoids. The analysis for body burden of berberine and α-T was performed on second- and third-instar *O. nubilalis* larvae which had been reared since eclosion on treated diets and then allowed to feed on fresh corn stalks for 24 hr in order to clear their guts of the compounds. *D. terebrans* adults and cocoons (including the fluid meconia) were collected on the day of adult emergence. Samples of

corn borer larvae, wasps, and cocoons were weighed and preserved in sample groups of three individuals in 2 ml HPLC grad methanol and held at −4 to −6°C until extraction. Three groups of each type of sample were analyzed.

The extraction protocol was as follows. Each sample was thoroughly ground using a 15-ml Wheaton homogenizer. An additional 3 ml methanol was added, and the slurry was futher homogenized. The suspension was refrigerated for two days at 4°C to allow the compounds to dissolve from the insect material. Samples were then vortexed and centrifuged for 10 min at 500*g* to remove macromolecular debris. The supernatant was evaporated to dryness under vacuum using a rotary evaporator. HPLC grade acetonitrile (0.5 ml) was added to the dried sample, and this was then sonicated for approximately 1 min to ensure that the compounds absorbed to the interior of the vials would be dissolved. Samples were then filtered using a millipore filter (Schleicher and Schuell, 0.45 μm), and 20-μl aliquots were analyzed by HPLC.

HPLC was performed using a Beckman system (Series 332), equipped with a model 165 variable wavelength UV–visible light detector. Reversed phase (C_{18}) columns, 25 cm × 4 mm internal diameter, packed with Ultrasphere octadecylsilicate (ODS), pore size 5 μm, were used for both compounds. The ion-pairing system for berberine consisted of 50% 0.1 M trichloroacetic acid (aqueous), buffered to pH 2.15 with saturated NaOH, and 50% acetonitrile, run isocratically at 1 ml/min. Berberine was monitored at 343 nm, one of the maximum absorbance wavelengths for the compound, and the minimum level of detection for this compound was 1.8 ng in a 20-μl injection. The retention time was 11.2 min.

Alpha-T was run isocratically with 85% acetonitrile and 15% water, and monitored at 350 nm. The minimum level of detection for this compound was 0.16 ng in a 20-μl injection, and the retention time under these conditions was 7.0 min. Insect extracts run in both solvent systems yielded very clean chromatograms (no more than two peaks), and the compound (if present) was readily distinguishable. Control larvae, wasps, and pupal cases were also extracted and analyzed in both solvent systems, to ensure that peaks in treated samples were not present in the controls. Untreated insects were also homogenized after injecting them with known amounts of berberine and α-T; the extraction efficiency was 80% for both compounds.

RESULTS AND DISCUSSION

The investigations with the two compounds under study demonstrate that the biological activity of secondary plant substances may be expressed differently in the host than in the parasitoids. The growth and survival studies with *O. nubilalis* alone on diets containing berberine did not demonstrate significant changes in the duration of the developmental stages (Table 1). A significant

TABLE 1. EFFECTS OF DIETARY BERBERINE ON *Ostrinia nubilalis*[a]

Berberine concentration (μg/g)	Growth parameters								Survival	
	Mean time (days) to pupation		Mean pupal weight (mg)		Mean time (days) to adult emergence		Mean adult weight (mg)		Pupation (%)	Adult emergence (%)
	M	F	M	F	M	F	M	F		
0	31.9	35.3	77.2a	100.4a	41.7	44.1	40.8a	53.3a	85	81
10	30.9	31.4	77.3a	93.6ab	41.6	40.9	40.8a	58.2a	89	82
31	34.9	36.8	74.8a	96.3ab	45.3	45.9	41.4a	60.2a	93	83
100	39.3	40.0	70.4a	85.3b	49.7	49.4	39.8a	52.8a	53*	45*

[a]For growth parameters, males (M) and females (F) were analyzed separately. Time measurements were subjected to the Kruskal-Wallis test ($P = 0.05$). Weight measurements were subjected to Duncan's multiple-range test: Means in columns followed by the same letter are not significantly different ($P = 0.05$). The chi square test was applied to data expressed as percentages: significant differences from the controls ($\alpha = 0.05$) are denoted by an asterisk.

reduction in female pupal weight was observed in the 100 μg/g berberine treatment compared to the controls. However, the weights of surviving adults were not significantly different between any of the concentrations tested. Although sublethal effects on growth parameters were not highly pronounced with berberine in the diet, survival to pupation and adult emergence was significantly decreased in the 100 μg/g treatment.

With α-T in the diet, sublethal effects on *O. nubilalis* alone were more obvious (Table 2). Mean time to adult emergence was significantly prolonged for females fed 31 μg/g α-T. With the 100 μg/g treatment, the duration of the developmental stages was increased significantly for both males and females. In addition, pupal and adult weights were significantly reduced with this treatment. These results confirm and extend observations first made by Champagne et al. (1986): the growth rate of *O. nubilalis* larvae was significantly reduced with 100 μg/g α-T in the diet. in the present study, survival to pupation and adult emergence of *O. nubilalis* was also significantly reduced with 100 μg/g α-T in the diet.

Parasitoids reared from hosts fed diets containing berberine did not exhibit sublethal effects in their growth (Table 3), except for an increase in mean pupal weight in the 31 μg/g treatment group. Although no significant differences from the controls were apparent in survival to pupation on any of the berberine treatments, survival to adult emergence was significantly decreased with 100 μg/g berberine in the host's diet. The parasitoids reared from hosts fed 100 μ/g berberine did not incur as much mortality (significantly different by the chi square test at $\alpha = 0.05$) as did the unparasitized host larvae (Table 1), which suggests that the parasitoids were less susceptible than their hosts to the effects of berberine.

Parasitoids reared from hosts fed α-T at concentrations of 10 and 31 μg/g in the diet were similar to the controls in the growth parameters of surviving insects (Table 4). However, the male parasitoids reared from hosts fed 100 μg/g α-T were significantly smaller than the controls. No females were produced in this treatment group. Mean time to pupation and adult emergence was not significantly affected by any concentration of α-T. Survival to adult emergence was somewhat reduced with the 31 μg/g treatment ($P > 0.90$ by the chi square test). However, significant mortality occurred with 100 μg/g α-T in the host diet. The mortality caused by 100 μ/g α-T was significantly higher (by the chi square test at $\alpha = 0.05$) in *D. terebrans* than in the unparasitized hosts. The parasitoid was apparently more susceptible to the thiophene than its host.

The chromatographic analyses for berberine did not show any evidence of the compound either in host larvae (removed to corn for 24 hr) or emerged wasps and cocoons. In contrast, α-T and/or a metabolite were detected in groups of each of the sample types analyzed (Tables 5 and 6). Despite the differences between the detection efficiencies of berberine and α-T (1.8 and 0.16 ng in a

TABLE 2. EFFECTS OF DIETARY α-TERTHIENYL ON *Ostrinia nubilalis*[a]

α − T concentration (μg/g)	Growth parameters								Survival	
	Mean time (days) to pupation		Mean pupal weight (mg)		Mean time (days) to adult emergence		Mean adult weight (mg)		Pupation (%)	Adult emergence (%)
	M	F	M	F	M	F	M	F		
0	23.7	24.8	77.7a	98.3a	32.9	31.2	36.7a	60.3a	93	93
10	22.2	23.5	76.0a	100.1a	30.9	31.9	35.8a	60.2a	93	90
31	22.7	24.7	71.9a	95.2a	31.3	33.3*	34.8a	54.9a	90	83
100	30.7*	37.7*	62.2b	65.5b	39.9*	46.0*	27.7b	27.7b	66*	66*

[a] For growth parameters, males (M) and females (F) were analyzed separately. Time measurements were subjected to the Kruskal-Wallis test: significant differences from the controls ($P = 0.05$) are denoted by an asterisk. Weight measurements were subjected to Duncan's multiple-range test: means in columns followed by the same letter are not significantly different ($P = 0.05$). The chi square test was applied to data expressed as percentages: significant differences from the controls ($\alpha = 0.05$) are denoted by an asterisk.

TABLE 3. EFFECTS OF BERBERINE ADMINISTERED TO HOST LARVAE ON *Diadegma terebrans*[a]

Berberine concentration (μg/g)	Growth parameters								Survival	
	Mean time (days) to pupation		Mean pupal weight (mg)		Mean time (days) to adult emergence		Mean adult weight (mg)		Pupation (%)	Adult emergence (%)
	M	F	M	F	M	F	M	F		
10	17.6	18.2	28.3	28.5	28.1	29.5	9.0	9.6	76	52
Control	16.7	16.8	29.2	32.2	27.4	28.4	8.9	10.7	82	69
31	16.0	17.2	31.0*	31.3	26.5	27.8	9.7	10.3	88	81
Control	16.9	18.0	25.8	32.3	27.3	28.6	8.3	10.0	94	76
100	18.4	18.8	27.9	31.2	28.9	30.4	8.6	9.6	77	61*
Control	17.7	19.6	26.6	28.1	28.1	30.1	8.4	9.2	88	81

[a] For growth parameters, males (M) and females (F) were analyzed separately. Time measurements were subjected to the Kruskal-Wallis test ($P = 0.05$). Weight measurements were subjected to t tests ($P = 0.05$). The chi square test was applied to the data expressed as percentages ($\alpha = 0.05$). An asterisk indicates that the treatment value is significantly different from the control.

TABLE 4. EFFECTS OF α-TERTHIENYL ADMINISTERED TO HOST LARVAE ON *Diadegma terebrans*[a]

α − T concentration (μg/g)	Growth parameters								Survival	
	Mean time (days) to pupation		Mean pupal weight (mg)		Mean time (days) to adult emergence		Mean adult weight (mg)		Pupation (%)	Adult emergence (%)
	M	F	M	F	M	F	M	F		
10	17.6	19.6	26.8	29.8	28.2	30.6	8.1	9.8	92	77
Control	18.1	18.3	29.9	32.9	28.6	29.4	8.9	10.2	89	74
31	18.2	17.8	26.0	31.6	28.3	28.6	8.2	10.5	76	65
Control	17.5	19.1	26.9	33.3	27.6	29.9	8.4	11.4	95	84
100	20.8	ND	22.1*	ND	30.8	ND	7.3*	ND	38*	25*
Control	17.8	19.1	31.5	31.6	28.4	30.3	9.5	9.9	88	76

[a] For growth parameters, males (M) and females (F) were analyzed separately. Time measurements were subjected to the Kruskal-Wallis test ($P = 0.05$). Weight measurements were subjected to t tests ($P = 0.05$). The chi square test was applied to the data expressed as percentages ($\alpha = 0.05$). An asterisk indicates that the treatment value is significantly different from the control. ND = no data available.

TABLE 5. QUANTIFICATION OF α-TERTHIENYL IN INSECT SAMPLES[a]

	Treatment concentrations (μg/g)		
	10	31	100
Host larvae	ND	0.31	0.05
		(0.26)	(0.074)
Adult parasitoids	0.53	0.20	ND
	(0.34)	(0.29)	
Cocoons + meconia	4.17	0.23	3.19
	(2.97)	(0.023)	(1.68)

[a] Units are in μg/g insect material. Values are means of three samples. Standard deviations are in parentheses. ND = not detected at lowest level of detection.

20-μl injection, respectively), an estimated minimum body burden of 1.9 μg berberine per gram insect material would be detectable by HPLC. This was not present even in the pupal cases, where waste products are often execreted as meconia by the metamorphosing parasitoids. The absence of berberine in insect samples is not surprising, considering that berberine is a quaternary ammonium compound and likely to be rapidly excreted (Bodor et al., 1981). The concentration of berberine in the host hemolymph would probably be much less than was originally ingested by the host.

The absence of berberine in insect samples is consistent with the data pertaining to the biological effects of berberine (Tables 1 and 3). Although survival was decreased for both host and parasitoid, the mortality in *D. terebrans* was not as severe as that observed in unparasitized *O. nubilalis*. In fact, mortality during the parasitoid's internal (feeding) stages was not significantly different

TABLE 6. RELATIVE QUANTITIES OF α-TERTHIENYL METABOLITE IN INSECT SAMPLES[a]

	Treatment concentrations (μg/g)		
	10	31	100
Host larvae	1.75	0.75	1.50
	(0.82)	(0.20)	(0.32)
Adult parasitoids	3.50	2.00	3.00
	(1.07)	(0.32)	(2.80)
Cocoons + meconia	9.75	12.75	8.25
	(6.72)	(8.17)	(6.10)

[a] Values are in absorbance units $\times 10^{-5}$/mg insect material, representing the mean of three samples. Standard deviations are in parentheses.

between the berberine treatments and the controls. This suggests that berberine may have indirectly affected *D. terebrans* by reducing the quality of the host as a food source (Duffey et al., 1986), but this was only manifested during metamorphosis in the group reared from hosts fed diets containing 100 μg/g berberine. The results with α-T demonstrate that this compound is clearly capable of accumulating in insect tissues, which is to be expected of a highly lipophilic xenobiotic. Although *O. nubilalis* larvae reared on diets containing 10μg/g α-T did not appear to sequester the thiophene, α-T was present in the larvae with 31 and 100 μg/g α-T. The level of α-T in the larvae fed 100 μg/g α-T was relatively low compared to the 31 μg/g samples. Apparently, less α-T was accumulating in the 100 μg/g-fed larvae, and this may have been the result of reduced feeding with this treatment. The 100 μg/g α-T has been previously shown to effectively deter feeding in *O. nubilalis* larvae (Champagne et al., 1986).

Alpha-T was recovered from parasitoid adults reared from hosts fed 10 and 31 μg/g α-T but not from hosts feed 100 μg/g α-T. Cocoons from all three groups contained α-T. The levels of α-T were relatively high in the cocoons of the parasitoids in the 10 and 100 μg/g treatments compared to the levels of the compound in the emerged wasps. This is similar to the findings of Barbosa et al. (1986) with nicotine: the parasitoids apprently dispose of such toxins via the cocoon silk and meconium.

The presence of α-T in the emerged parasitoids and cocoons suggests that the thiophene could have had a direct toxic effect on *D. terebrans*, in contrast to the indications of the data on berberine. Although the highest parasitoid mortality occurred with 100 μg/g α-T in the host diet, the surviving wasps did not contain detectable levels of α-T. This was a small and relatively resilient segment of the experimental population, which may have been inherently superior in their ability to metabolize α-T.

In addition to detecting the parent compound in the insects treated with α-T, a polar metabolite of α-T was found (Table 6). The compound has a typical thiophene spectrum, with a retention time of 1.7 min under the conditions of this HPLC system. As the structure and extinction coefficient of the metabolite have not yet been elucidated, absolute quantification was not possible. However, relative quantities (based on absorption at 350 nm) have been determined (Table 6). Relatively higher quantities of the metabolite were found in the cocoon samples than in the adult parasitoids. This is further evidence for the suggestion of Barbosa et al. (1986) that wastes from host-ingested allelochemicals are shunted by the parasitoid into the cocoon silk and meconia.

In the present study, the adverse effects on immature *D. terebrans* were much more severe with the highly lipophilic thiophene, α-T, than with the polar alkaloid, berberine, even though the parasitoid was capable of detoxifying and possibly metabolizing α-T. This suggests that pest management startegies which

take advantage of secondary plant substances for breeding resistance could run into problems with compounds which tend to be lipophilic (reminiscent of the pesticide bioaccumulation problem). On the other hand, an amphiphilic compound such as berberine may be relatively innocuous for the parasitoid, even though it can dramatically reduce host survival.

It is clear from this and other studies that allelochemical effects on herbivores do not necessarily reflect the consequences of these substances on the third trophic level. Novel allelochemicals with potential for agricultural applications should be examined for effects on natural enemies in order to assess their real usefulness for pest management strategies involving biological control.

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α-TOMATINE AND RESISTANCE OF TOMATO CULTIVARS TOWARD THE NEMATODE, *Meloidogyne incognita*

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Abstract—Susceptible and resistant tomato cultivars were assayed for root tomatine concentrations at different developmental times. No correlation between high tomatine levels and resistance toward *Meloidogyne incognita* was apparent. In plants infected with nematodes, tomatine in the roots was the same as in uninfected controls. Tomatine concentration, as measured by bulk analysis, does not appear to play an important role in resistance toward the nematode studied.

Key Words—Tomatine, nematode resistance, *Lycopersicon esculentum*, *Meloidogyne incognita*, host-plant resistance.

INTRODUCTION

The steroidal glycoalkaloid α-tomatine (Figure 1) has been recognized as an allelochemical agent that imparts resistance in certain tomato varieties toward insect pests (Juvik and Stevens, 1982; Elliger et al., 1981) and fungal attack (Roddick, 1974). A report on the nematicidal effect of tomatine upon *Panagrellus redivivus* (Allen and Feldmesser, 1970) suggested that this alkaloid may be important in conferring nematode resistance as well. They also observed (Allen and Feldmesser, 1971) that a related glycoalkaloid, α-chaconine, which occurs in potato (*Solanum tuberosum*), was toxic to this nematode and that the pH dependence of toxic action showed the free base to be the form of the compound responsible for activity. This was also the case for tomatine; however, no attempt was made to correlate alkaloid levels with nematode resistance in vivo. A brief report (Okopnyi and Sadykin, 1976) stated that resistance to the nematode *Meloidogyne incognita* could be related to tomatine content in the

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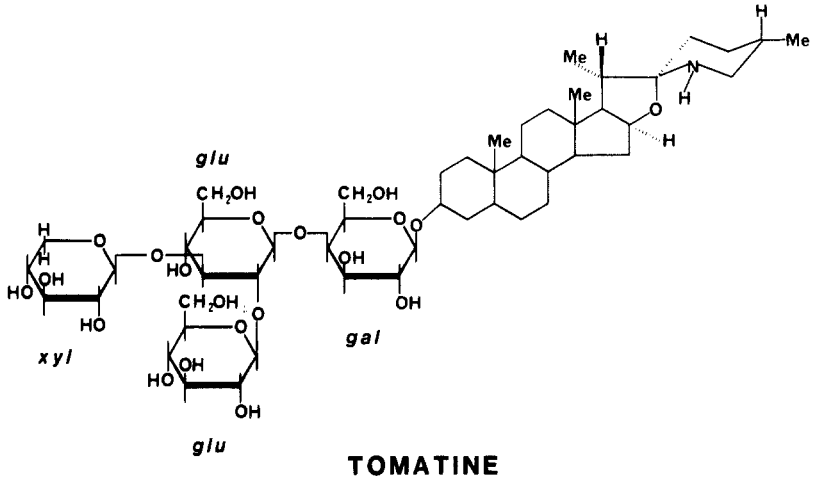

FIG. 1.

roots of resistant tomato plants. These authors found that resistant varieties had a tomatine content of ca. 11–12 mg/g in dry root material, whereas susceptible varieties contained about one third to one half that amount. No information on plant age or concentration with respect to time was provided, however. Since *M. incognita* is a significant pest of tomatoes and since a wide range of susceptibility or resistance can be obtained between numerous cultivars (Hadisoeganda and Sasser, 1982), we investigated the relationship of root tomatine with respect to resistance toward this nematode.

METHODS AND MATERIALS

Nematodes. A culture of *Meloidogyne incognita* (Kofoid and White) Chitwood was obtained from E.C. Jorgenson, U.S. Cotton Research Station, Shafter, California, and confirmed as race 3 by differential host test (Sasser, 1954; Taylor and Sasser, 1978). Nematode colonies were maintained on tomato roots (cv. Rutgers) and differentially selected on cotton (cv. Deltapine-16). The degree of nematode infestation on plant root material was determined by fixation and staining for 4 hr in 50:50 HOAc–EtOH containing 17.5 mg/liter acid fuchsin followed by 12–24 hr clearing in saturated aqueous chloral hydrate and preservation in lactophenol solution (Dropkin et al., 1969). Nematode counts were determined manually by microscopic examination. Nematode motility was assayed by counting the relative number of individuals that migrated through cellulose tissues (Kaplan et al., 1980).

Tomato Plant Material. Readily available tomato cultivars that had already

been assayed for resistance toward *M. incognita*, race 3 (Hadisoeganda and Sasser, 1982), were obtained as seed from commercial sources and were germinated and grown on beds of sand (ca. 1- to 3-mm particles) saturated with half strength Hoagland's solution (Johnson, 1980) using 150-ml beakers about one third full contained within plastic boxes (to establish 100% humidity). These plant boxes were held in a growth chamber maintained at 22°C with a 16-hr light period per 24 hr. Illumination was by six F40CW fluorescent tubes supplemented by four 100-W incandescent bulbs. At approximately weekly intervals the liquid remaining within the sand was aspirated off using a pipet inserted to the beaker bottom, and fresh nutrient was added. Root samples were taken at the cotyledon stage and at later physiological ages corresponding to the full development of two, four, and six true leaves. Roots were cut from the stem at about the level of the sand, washed free of sand grains, and freeze-dried. Dry root material was screened through a mesh smaller than the sand to ensure complete removal of extraneous material. Exudates from developing plants could be isolated from liquid surrounding the roots.

Tomatine Analyses. Qualitative analysis for tomatine in root exudates was conducted by initial passage of liquid through a Waters Associates C-18 Sep-Pak followed by water wash to remove nonabsorbed material with desorption of any tomatine being effected by methanol. TLC on material retained from the aqueous solutions was carried out on Merck Si60 plates using as solvent $CHCl_3$–MeOH–2% NH_4OH, 70:30:5 (Jellema et al., 1981) with detection by anisaldehyde spray reagent (Waldi, 1962).

Quantitative determination of tomatine in tomato roots was accomplished by initial hydrolytic removal of sugar residues to give the aglycone, tomatidine, followed by GLC determination of the latter material. Thus, 5–10 mg of dry root powder was weighed into 2-ml serum cap vials, 0.5 ml of 1 N HCl in 50:50 methanol–water was added, and the caps were crimped in place. The vials of hydrolysis mixture were warmed at 100°C (block heater) for 90 min. After cooling, they were vented with a hypodermic needle to relieve the pressure which occasionally built up, and 0.6 ml of 1 N sodium hydroxide was injected. Into each of the now slightly basic mixtures was injected 0.500 ml of ethyl acetate containing 25 μg/ml of cholesterol valerate as internal standard. The vials were thoroughly agitated by a vortex homogenizer to ensure complete mixing of contents, after which the now partly emulsified layers were separated by centrifugation. About 0.25 ml of the upper phase was taken from each vial by syringe and transferred to conical cavity vials for evaporative removal of ethyl acetate and associated water under nitrogen at 30°C. Methylene chloride, 0.1 ml, was added to all samples, and complete solution was ensured through brief immersion of the vials in an ultrasonic bath. GLC analysis of the methylene chloride solutions was conducted on a 2-ft × $\frac{1}{4}$-in. glass column containing 3% OV-101 on 80–100 mesh Chromasorb W(HP) (Pierce Chemical Co.) using a HP-5880 gas chromatograph equipped for on-column injection. At oven

temperature 255°C and carrier gas flow (N_2) of 40 ml/min, tomatidine and cholesteryl valerate had respective retention times of 4 and 6 min, and all other component peaks emerged earlier. Every set of root material analyzed included at least one tomatine standard that had been subjected to the above hydrolysis and extraction.

RESULTS AND DISCUSSION

Preliminary examination of nematode-susceptible (cv. Rutgers) tomato plants grown in close contact with resistant plants (cv. Small Fry) showed that, after artificial infestation, the degree of susceptibility was lessened for the former plants. This suggested that a diffusable material might be migrating from the roots of resistant plants and lessening the viability of nematodes in the vicinity of otherwise susceptible roots. Examination by TLC of diffusate from Small Fry roots after absorption and concentration on RP-18 Sep-Pak showed qualitatively that tomatine was present. Assay of tomatine in vitro with *M. incognita* gave results similar to those involving *P. redivivus* (Allen and Feldmesser, 1970, 1971), indicating that the free base form was the active nematistatic agent and that tomatine might reasonably be expected to play a role in resistance toward *M. incognita*.

We examined 12 cultivars of tomato for root tomatine content over the initial developmental stages of the plants (Figure 2). Although it had been pre-

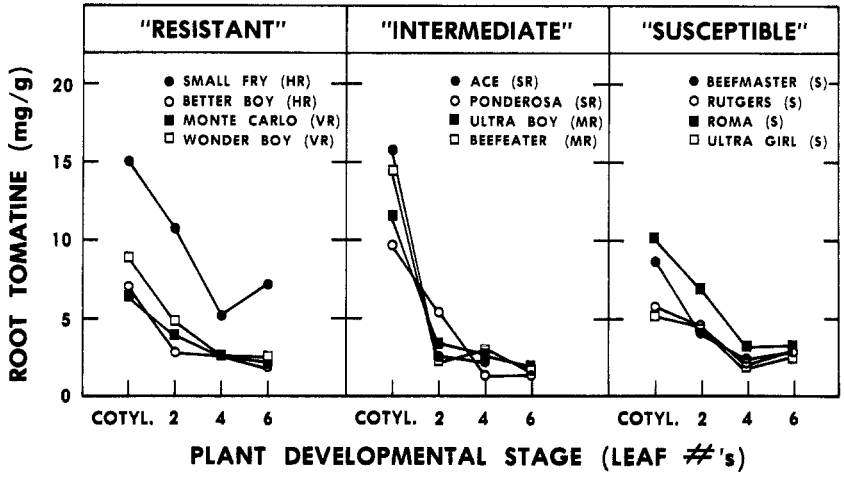

FIG. 2. Tomatine content of resistant and susceptible tomato cultivars vs. developmental age. Degree of resistance is according to Hadisoeganda and Sasser (1982). HR, VR, MR, SR are designations of highly, very, moderately, and slightly resistant, respectively. S refers to susceptible plants. Analyses were on combined root material from three to five plants.

viously reported that tomatine in root sap increased with time (Ali and Schlösser, 1977), we found that for all varieties, tomatine levels showed a general decrease with increasing plant age after germination (no tomatine is present in seeds). Initial content varied from ca. 15 mg/g to as low as one third that amount. After the plants had reached an age corresponding to the development of four to six true leaves, tomatine was present to the extent of 2–6 mg/g in the roots. Although certain tomato varieties showed high initial tomatine content, it was clear that this alone did not account for nematode resistance inasmuch as plants showing the highest content at the cotyledon stage were not necessarily the most resistant, and, additionally, at later developmental times all plants had about the same amount.

It might be suggested that tomatine could increase in amount after nematode attack has taken place. Such postpredation elevation of allelochemical content has been described as an energetically conservative means whereby a plant may preserve its chemical resources (Rhoades, 1979). Indeed, increased tomatine concentration in tomato leaves has been observed after fungal attack (Smith and MacHardy, 1982). If nematode penetration of root tissue were to stimulate tomatine production in resistant tomato cultivars, then growth and reproduction of *Meloidogyne* could still be adversely influenced. We examined the effect of nematode infestation upon root tomatine content in a resistant and a susceptible tomato cultivar to assay this possibility. The varieties, Better Boy and Rutgers characterized, respectively, as highly resistant and susceptible to *M. incognita*, race 3 (Hadisoeganda and Sasser, 1982), were allowed to grow to the point where four true leaves had developed. At this time a suspension containing about 800 nematodes was added to each container in which there were generally three plants. Roots were assayed for tomatine at three, seven, and 14 days after inoculation and compared with control samples from plants free of nematodes. In both varieties, infection of nematode-inoculated plants was confirmed by root staining and nematode counts. Both cultivars failed to show much difference in root tomatine levels between infected and control samples (Table 1), further confirming the inefficacy of tomatine as the determining agent of resistance. Rutgers (susceptible) plants exhibited an infection 2.4 times greater than that of Better Boy (highly resistant); additionally, the former cultivar supported a more robust population of nematodes. It was observed on stained roots that for Rutgers all nematodes were at least second-stage juveniles, whereas of those infecting Better Boy, only 10% had attained this growth stage. We did not attempt to explore host suitability for reproduction or extended survival of the nematodes, it being sufficient that infestation could be maintained over the time interval chosen for tomatine assay.

In conclusion, it can be stated that despite tomatine having nematistatic properties in vitro, we could find no evidence from comparisons of bulk root concentrations that it played a role in nematode resistance over the time course studied. In only one resistant variety (Small Fry) was tomatine content consist-

TABLE 1. TOMATINE CONTENT[a] OF RESISTANT AND SUSCEPTIBLE TOMATO CULTIVARS AFTER NEMATODE INFECTION

Time after inoculation (days)[c]	Variety			
	Rutgers (S)[b]		Better Boy (HR)[b]	
	Control	Infected	Control	Infected
0	2.2	2.2	2.8	2.8
3	2.7	2.0	1.5	2.0
7	2.4	2.3	1.8	1.4
14	2.1	3.0	2.1	2.2

[a] mg/g, dry root (combined material from 3–5 plants).
[b] According to Hadisoeganda and Sasser (1982).
[c] Plants initially grown to four true leaf stage.

ently high, but even in this example the difference at later times was not extremely great in comparison to more susceptible cultivars. Other comparably resistant lines did not show as high a tomatine level and were, in fact, lower in root tomatine content than completely susceptible plants. This points to the presence of some other diffusable factor, at least in the case of Small Fry, and we are investigating root diffusates of this cultivar at the present time. A possible means by which tomatine could nevertheless adversely affect nematodes and thereby impart resistance may be in conjunction with the incompatible reaction shown by resistant varieties whereby localized necrosis (Giebel, 1982) takes place at the site of nematode penetration. If tomatine buildup should occur in response only at these sites, then the demonstrated toxic effect of the alkaloid may still be important. However, such localization would be difficult to assay by methods of bulk analysis because the overall tomatine concentration may still be quite low. This may explain why no difference in postinfection tomatine content was discernable.

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INFLUENCE OF TRICHOME EXUDATES FROM SPECIES OF *Lycopersicon* ON OVIPOSITION BEHAVIOR OF *Heliothis zea* (BODDIE)

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Abstract—Cage experiments revealed that accessions of the wild tomato species *Lycopersicon hirsutum* were preferred sites for oviposition by *Heliothis zea*. Hexane extracts from the leaves of *L. hirsutum* were also preferred sites of oviposition in choice experiments among extracts from several *Lycopersicon* species. Extracts of *L. hirsutum* were still biologically active several days after application, indicating that the phytochemical(s) involved are relatively stable and of low volatility. Gas chromatographic analysis of leaf hexane extracts from 12 different accessions of the *L. hirsutum* complex and three tomato cultivars revealed substantial qualitative and quantitative variation in the chemical composition of these extracts. Comparison of these results with extract oviposition studies implicate a group of structurally related compounds as the active agents. Mass spectroscopy has tentatively identified these compounds as sesquiterpenes with the chemical formula $C_{15}H_{22}O_2$. These compounds are apparently synthesized and secreted from glandular trichomes on the leaf surface. These phytochemicals did not stimulate ovipositional behavior in females of the cabbage looper, *Trichoplusia ni*. The existence of genetic variation for the presence and amount of kairomones that serve as cues for insect orientation and oviposition could be utilized in a breeding program to develop tomato cultivars with genetically modified allelochemic profiles that would disrupt the sequential behavioral processes of insect host-plant selection.

Key Words—*Heliothis zea*, corn earworm, Lepidoptera, Noctuidae, *Trichoplusia ni*, cabbage looper, *Lycopersicon hirsutum*, tomato, allelochemics, kairomone, oviposition, preference, sesquiterpenes, trichomes, host-plant selection, host-plant resistance.

INTRODUCTION

In the past, research on the role of allelochemics in insect host-plant selection has predominantly focused on plant compounds that act as feeding stimuli (Hedin et al., 1974). Plant chemical factors that influence host choice by holometabolous female adults for oviposition have received surprisingly little attention considering that successful completion of an insect's life cycle depends on the female parent's location and oviposition on or near a plant that can supply the necessary ingredients for larval growth and development. Volatile plant chemicals that can impinge on the olfactory chemoreceptors of the insect have been implicated as agents mediating the process of host-plant selection (Kennedy, 1977). Individual plants in a cabbage field that contained higher than average amounts of volatile allyl nitriles were found to be favored sites for oviposition by females of *Pieris brassicae* (Mitchell, 1977). The complex of "green leaf" volatiles from potato plants were found to elicit a positive anemotactic response in Colorado potato beetles (Visser and Avé, 1978). The onion volatiles *n*-dipropyl disulfide and *n*-propyl mercaptan were found to act as attractants and oviposition stimulants for the onion fly, *Hylemia antiqua* (Matsumoto, 1970). As exemplified by the cucurbitacins that have been shown to operate as kairomones for a wide variety of Diabroticina and Aulacophorina beetles (Metcalf et al., 1982), insects have apparently evolved the sensory apparatus to detect and discriminate within their environment the discrete phytochemical profiles of their host plants.

Although *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) is a major insect pest of corn, tomatoes, and cotton, there is remarkably little pertinent literature concerning its oviposition behavior and oviposition stimulants. Johnson et al. (1975) have made a detailed study on the ovipositional response of *H. zea* to various phenological states of corn, cotton, tobacco, and soybean and have constructed a preference matrix. The ovipositional response decreased in the order: corn > tobacco > soybeans > cotton. In tomatoes, *H. zea* was found to oviposit preferentially on leaf surfaces rather than on blooms, fruit, or stems (Alvarado-Rodriguez et al., 1982). According to Snodderly and Lambdin (1982), the preferred ovipositional site for caged *H. zea* was on the underside of leaves in the interior of the plant canopy, while stems and petioles were less preferred. In corn (Widstrom et al., 1979) and cotton (Lukefahr et al., 1971), genotypes with heavier leaf pubescence were preferred hosts for oviposition.

Numerous volatile compounds have been isolated from corn silk (Flath et al., 1978), corn kernels and husks (Buttery et al., 1978), and corn tassels (Buttery et al., 1980) that may act as attractants to *H. zea*. These compounds include C_2–C_{12} alkanols, hexadienal, decadienal; esters such as ethyl acetate and ethyl cinnamate; ketones including pentanone, nonanone, and octadienone; and various methylated benzenes and naphthalenes. Centello and Jacobsen (1979)

reported that phenyl acetaldehyde, a volatile constituent of corn silk, acts as an attractant to *H. zea*. The corn volatile triacetin was found to be the most effective of 33 structurally related compounds in stimulating oviposition behavior in *H. zea* (Jones et al., 1973). Individually none of these compounds was competitive with corn extracts for stimulating oviposition. *H. zea* apparently responds to a complex mixture of corn volatile compounds that act as attractants or oviposition stimulants.

There have been several reports of chemical factors in tomatoes that influence host-plant selection and oviposition by other lepidipterous pests. A nitrogen-containing phenolic glycoside ($C_{17}H_{29}O_{10}N$), isolated from cultivated tomato leaves, was found to stimulate oviposition by the tobacco hornworm, *Manduca sexta* (Yamamoto and Fraenkel, 1959). Tichenor and Seigler (1980) have shown that aqueous extracts and steam distillates of cultivated tomatoes induced electroantennogram activity and stimulated oviposition by *M. sexta*. Bordner et al. (1983) have reported on the presence of an orientation factor for *M. sexta* in the steam distillate of cultivated tomato leaves. Ethanolic extracts of tomato leaves were found to elicit oviposition by the potato tuber moth, *Phthorimaea operculella* (Meisner et al., 1974). A chemical factor on the foliar surfaces of a tomato cultivar was shown by Burton and Schuster (1981) to act as an oviposition kairomone to the tomato pinworm, *Keiferia lycopersicella*.

A review of the literature has uncovered no reports on tomato phytochemicals that affect *H. zea* orientation or host plant selection for oviposition. This study was conducted to survey the genus *Lycopersicon* for the existence of allelochemicals that may influence the oviposition behavior of *H. zea*.

METHODS AND MATERIALS

Plant Material. Seeds of wild and cultivated species of the genus *Lycopersicon* were procured from Dr. C.M. Rick, Department of Vegetable Crops, University of California, Davis, and from the USDA Plant Introduction Station, Ames, Iowa. Seeds of Chico III and Jet Star were purchased from the Harris-Moran Seed Company. Seeds of the different accessions were planted into flats containing potting soil consisting of peat, vermiculite, and soil (1 : 1 : 1). Seedlings with two to four true leaves were transplanted into either 7.57-liter pots in the greenhouse or into field plots on the University of Illinois Vegetable Crops Research Farm in south Urbana, Illinois.

Insects. The gravid female moths of *Trichoplusia ni* (Hubner) and *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) used in these experiments were obtained from colonies maintained in incubators in the authors' laboratory. The *T. ni* colony was initiated from eggs provided by Ms. Paula Peters of the USDA Insect Biological Control Laboratory of the Department of Entomology, Uni-

versity of Missouri, Columbia. Dr. Gilbert Waldbauer in the Department of Entomology, University of Illinois, Champaign–Urbana, supplied eggs that were the original source of our *H. zea* colony. The larvae, pupae, and adult moths are reared in Percival incubators at 25°C and 75% relative humidity with a daily regimen of 14 hr light and 10 hr darkness. The *T. ni H. zea* populations are maintained on artificial diets following the methods described by Waldbauer et al. (1984). The same dietary mix is used to feed the larvae of both insect species. Unlike *H. zea*, the larvae of *T. ni* are not cannibalistic, allowing for the rearing of 20–30 larvae in individual 237-ml paper containers with clear plastic lids purchased from Lily-Tulip, Inc. (Augusta, Georgia). To reduce inbreeding in the insect populations, wild collections of the Illinois insect species are added to the cultures every summer. To minimize microbial diseases in the colonies, eggs and pupae of each generation are surface sterilized.

Oviposition Studies of Caged Plants. For experiment 1, three plants of the wild tomato accession LA 716 (*L. pennellii*), LA 1306 (*L. chmielewskii*), LA 1335 (*L. pimpinellifolium*), LA 1777 (*L. hirsutum*), LA 407 (*L. hirsutum* f. *glabratum*), and the processing tomato cultivar, UC82B (*L. esculentum*), were placed in a screened cage (1.8 m on a side, 1.2 m high) in a greenhouse with a 28°C/18°C ± 3°C day–night temperature regimen. The plants were four weeks old and displaying vegetative growth. The pots were randomly positioned within the cage to allow for maximum distance between each plant. Twenty-five gravid *H. zea* moths were released within the cage between 3 and 5 PM and allowed to oviposit on preferred host plants for 24 hr. After sundown, the cage was covered on all sides with black plastic to reduce any positional effect that may have occurred due to light sources in the greenhouse. Twenty-four hours after the moths were released, the plants were removed from the cage, eggs marked with a red felt-tip pen, and total egg numbers for each plant recorded. When returned to the cage, plants were randomly assigned new locations to further reduce positional bias. At 48 hr, a second egg count was conducted followed by a third and final count at 72 hr. Moths that had died during the first and second nights were replaced with new females to maintain total moth numbers. Within 48 hr of the final egg count, all plants were prepared for leaf extraction. The aboveground portion of each plant was harvested and weighed. Leaves and stems of each plant were then separated and weighed.

For experiment 2, three plants of the accessions LA 1306 (*L. chmielewskii*), LA 407 (*L. hirsutum* f. *glabratum*), LA 1777 (*L. hirsutum*), and the processing tomato cultivar Chico III were tested for *H. zea* oviposition preference using the procedures described above. To separate the variance resulting to positional effects, plants were separated into three blocks within the cages where each block contained one plant of each species. Species were randomized within the blocks. Replication was achieved by repeating the experiment four times with new plants and insects. Within 48 hr of the final egg count, all plants from

each replicate were prepared for leaf extraction. Measurements from each plant included: total leaf weight, total leaflet weight, stem weight, total plant weight, and leaf area (leaf area in cm^2 of approximately five leaflets measured on a Li-Cor leaf area meter).

Arena Leaf Extract Oviposition Studies. Preliminary studies of leaf extracts of the *L. hirsutum* accessions LA 1777 and PI 379013 indicated that in equal-weight leaf samples extracted in hexane, chloroform, ethanol, and water, the hexane extracts stimulated *H. zea* oviposition the most. For this reason, hexane was used as the solvent for our extract oviposition bioassays. A 5.00-g sample of leaflets from each plant in experiments 1 and 2 was extracted three times in hexane aliquots of 20 ml, 15 ml, and 15 ml. Hexane and leaflets were placed in 50-ml capped nalgene centrifuge tubes and shaken at 300 rpm for 15 min on a G24 Environmental Incubator Shaker (New Brunswick Scientific Co.). Each extraction aliquot was poured through Whatman No. 1 filter paper into a 150-ml Erlenmeyer flask containing approximately 8 g of sodium sulfate crystals to remove water from the extract. After the third hexane rinse, the extract was brought to total volume of 50 ml in a volumetric flask. Extracts were transferred into 20-ml glass scintillation vials and stored at −70°C for later use. Prior to the moth oviposition studies, plant extracts of the same genotype from experiment 1 were bulked and concentrated in a Buchler vortex–evaporator at 40°C at 150 rpm with a vacuum at −80 kPa. Sample volumes were manipulated so as to achieve concentrations of 0.5 g of original wet weight leaflet tissue per milliliter of concentrated extracts. While this same concentration was used in the plants from experiment 2, four separate concentrated extracts of plants of the same genotype were saved from each replicate.

The extract oviposition studies were conducted in 30-cm-diameter round cake carriers inverted to allow the bottom to serve as a lid. A 23-cm-diameter hole was cut out of the lid and fine insect netting glued over the hole to allow for air flow. Whatman 3-mm sheet chromatographic paper (0.33 mm thick) was cut to an exact fit to cover completely the inside wall of the containers. Eighteen small squares of two-sided tape were affixed at even intervals (approximately every 6.2 cm), 3.5 cm beneath the upper edge of the chromatographic paper. Whatman No. 1 filter paper disks with a diameter of 4.25 cm were permeated with 1.0 ml of the different extracts, dried under a hood, and attached to the squares of two-sided tape. In experiment 1, six filter disks were permeated with 1.0 ml of extract from each of the six *Lycopersicon* species. Two filter disks of each of the species extracts and six control filter disks previously permeated with 1.0 ml of hexane were randomly attached to the chromatographic paper fitted to the inside wall of each of the three cake carriers (hereafter called arenas). Fifteen gravid female *H. zea* were then placed within each arena. To improve moth survival and oviposition, a Petri dish containing sterile cotton fiber infused with a 10% solution of honey was positioned in the center of each

arena. The arenas were then placed within a Percival growth chamber at 25°C, 75% relative humidity, and a 14 : 10 hr light–dark cycle. A 1/4-watt night light was placed approximately 15 cm above the center of the lid of each arena to stimulate moth oviposition. At 24 and 48 hr, the arenas were removed from the incubators, the moths anesthetized with carbon dioxide, and the sheet removed for counting. After the eggs were counted and recorded for each disk, the sheets were returned to the containers, dead females replaced with living ones, and a fresh honey solution added to the Petri dish. A third and final count was conducted at 72 hr after the experiment was initiated.

In experiment 2, three filter disks were permeated with 1.0 ml of extract of each genotype for each of the four replicates. Disks from the different replicates were placed into four separate arenas. Extract disks were randomly arranged with six hexane blanks for each arena. The experiment was conducted as described above. In both experiments, extract oviposition preference values were calculated by dividing the average number of eggs per filter disk of a specific species by the mean number of eggs laid on the hexane controls. Values significantly less than one indicated nonpreference for *H. zea* oviposition, while those greater than one were preferred sites for oviposition.

Trichoplusia ni *Leaf Extract Oviposition Study.* The concentrated extracts from each replicate of the four species in experiment 2 were bulked to test for *T. ni* oviposition preference. Three filter disks permeated with 1.0 ml of the concentrated extract from leaves of each of the four species and a hexane control were randomly arranged in each of three separate arenas. Fifteen gravid *T. ni* females were released in each of the areas. The experiment was conducted similarly to the *H. zea* studies.

Variation in H. zea *Oviposition Preference—Volatile or Contact Chemical Action.* To ascertain if the chemical basis for *H. zea* oviposition preference is due to the interaction of volatile compounds with moth orientation or if physical contact of the insect with the extract disks is required for oviposition stimulation, the following experiment was conducted. Twenty-five grams of fresh leaflets from field-grown plants of *L. esculentum* cultivar, Chico III, and the wild *L. hirsutum* accessions, PI 379013 and LA 1777 were extracted in hexane and concentrated to 0.5 g of original leaf fresh weight per milliliter; 1 ml of each of the extracts was pipetted onto four filter disks and allowed to dry. These disks and six hexane blanks were randomly attached to a single sheet of chromatographic paper precut to fit inside a cake carrier as described above. The side of the sheet with the attached filter disks was then completely covered with eight layers of prewashed and sterilized cotton gauze. A fine line was drawn on the gauze indicating the position of the filter disk located directly beneath. This porous gauze layer, which was approximately 3 mm thick, prevented any direct contact between the extract disks and the 15 female moths used for the ovipo-

sition study. For replication, three arenas were prepared in this manner for *H. zea* oviposition studies.

H. zea *Oviposition Preference among Extracts of* L. hirsutum. Samples of leaf tissue from 12 different wild tomato accessions of *Lycopersicon hirsutum* and *L. hirsutum* f. *glabratum* were harvested from plots on the Department of Horticulture's Vegetable Research Farm in Urbana, Illinois. Each sample was a composite of leaves collected from 10 plants of each wild accession. Only undamaged leaves from the fifth or sixth node below shoot meristems were harvested. Leaves were carefully handled to minimize damage and disturbance to the leaf trichome vestiture. From each accession, 25.0 g of leaflets were weighed out and placed in 500-ml nalgene screw-top bottles. The samples were then extracted three times with 100-ml aliquots of hexane using the procedure previously described. Sample volumes were manipulated to achieve concentrations of 1.0 g of original leaflet tissue per milliliter of extract for each of the *L. hirsutum* accessions. One milliliter from each of the 12 accession extracts was applied to each of the four filter disks. One complete set of disks of each accession's extract and three hexane controls were randomly arranged in each of four arenas to test the *H. zea* oviposition preference among extracts.

To ascertain the volatile constituents of the leaf hexane extracts from these accessions, aliquots were diluted 10-fold and injected into a Hewlett Packard model 5790a gas chromatograph with an HP 7671a autosampler, a 12.5-m cross-linked methyl silicone capillary column (0.2 mm internal diameter and 0.33 μm film thickness), flame ionization detector, and an HP 3390a integrator. One microliter of each sample was injected by the autosampler into the injection port where the volatile constituents were loaded onto the column at a split ratio of 10:1 with helium as a carrier gas at 1.0 ml/min. Figure 1 presents a typical chromatogram of the hexane extract from leaves of the *L. hirsutum* accession LA 1777, with information on the temperature program used for these analyses. The retention time and area of each peak (greater than 1% of total peak area) of each genotype's extract was recorded. These data were then compared with the *H. zea* oviposition response to the same extracts.

To compare the chemical nature of the leaf hexane extracts with that of trichome exudates, absorbent cotton was very lightly rubbed over the surface of attached leaves of greenhouse-grown LA 1777 plants. The cotton was then extracted in hexane and the extract gas chromatographed as described above. This chromatogram was then compared to a chromatogram of a hexane extract from cut leaflets of the same plants. These two extracts of LA 1777 were compared for relative *H. zea* oviposition preference in a single arena study.

GC-Mass Spectroscopy of Hexane Extracts. For the purposes of preliminary compound identification, hexane extracts of the *L. hirsutum* accessions LA 1777 and PI 379013 were run through a Hewlett Packard 5995 gas chromatog-

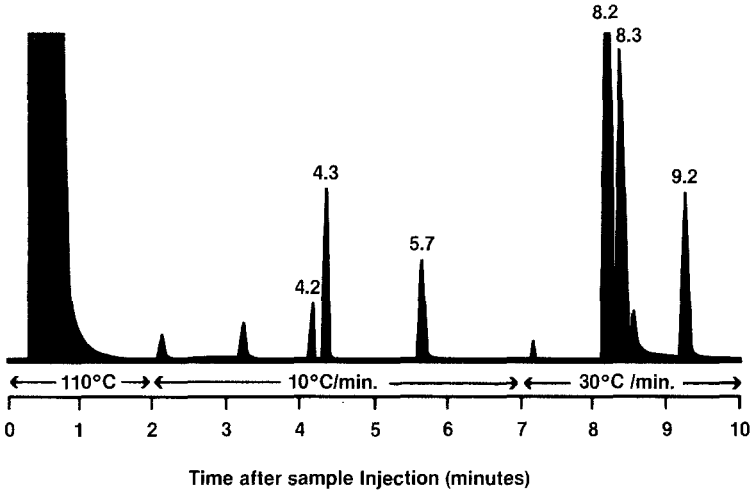

FIG. 1. Gas chromatogram of leaf hexane extract of the *Lycopersicon hirsutum* accession LA 1777.

raphy–mass spectrometer. One microliter of extract was injected in the splitless mode and loaded on to a 10-m cross-linked methyl silicone capillary column (0.2 mm internal diameter) with helium as a carrier gas at 1.0 ml/min. After 1 min at 80°C, the oven temperature was programmed to increase at 20°C/min to a final temperature of 260°C. As the individual volatile chemical constituents entered the quadrapole mass spectrometer, fragmentation was achieved by electron impact at 70 eV. Fragmentation patterns were recorded and analyzed with an HP 236 Quicksilver data processor. Individual peak fragmentation patterns were compared and matched against an MS software library of 39,000 known compounds.

RESULTS AND DISCUSSION

Table 1 presents the results of experiment 1 where six different accessions from five separate species of *Lycopersicon* were tested for both *H. zea* oviposition on plants and on extracts from leaflets of the same plants. Plants of the *L. hirsutum* accession LA 1777 were significantly preferred hosts for oviposition both on the basis of total egg numbers per plant and on eggs per 100 g of foliage. The hexane extracts from this accession were also preferred sites of oviposition in the arena study. These findings strongly suggest that a chemical factor(s) in the hexane extract of leaves of this accession possesses kairomonal properties that modify moth orientation and oviposition response. The correla-

TABLE 1. *Heliothis zea* OVIPOSITION PREFERENCE AMONG PLANTS AND LEAF EXTRACTS FROM GENUS *Lycopersicon*

Species and accession	Oviposition on plants		Oviposition on leaf extracts (Eggs on extract/eggs on blank)
	Eggs/24 hr.	Eggs/100 g foliage	
L. pennellii			
LA 716	14 a[a]	13 a[a]	1.2 a[a]
L. pimpinelliifolium			
LA 1335	25 b	16 a	1.1 a
L. chmielewskii			
LA 1306	19 ab	27 b	4.4 b
L. esculentum			
UC 82-1-8	64 c	37 c	5.3 bc
L. hirsutum f. *glabratum*			
LA 407	70 c	44 c	8.0 c
L. hirsutum			
LA 1777	111 d	75 d	31.7 d

[a]Numbers followed by different letters are significantly different at $P = 0.05$ (protected LSD test). Linear regression of *Heliothis zea* oviposition on plants per 100 g of foliage with oviposition on leaf extracts provides a highly significant correlation coefficient ($r = 0.95$, $P < 0.01$).

tion between moth oviposition per 100 g of leaf tissue and preference values from the arena studies for the six accessions was $r = 0.95$ ($P < 0.01$), implying that kairomonal phytochemicals on the leaves of *Lycopersicon* may serve as important token stimuli in the behavioral processes of *Heliothis* host-plant selection for oviposition. While extracts of the cultivated tomato stimulated oviposition, those of the *L. pennellii* and *L. pimpinellifolium* accessions were not significantly different from the control. The action of repellent or oviposition-inhibiting chemical stimuli was not observed in this study.

The results from experiment 2, presented in Table 2, tend to agree with the preceding experiment. *H. zea* oviposition on plants of LA 1777 (as determined by total egg numbers, eggs per 100 g foliage, or eggs per leaf area) and the hexane leaf extracts of these plants was significantly greater than that observed for the other accessions. The final column of this table presents the results of the response of gravid *T. ni* females to the same extracts. The moths exhibited no significant ovipositional preference for any of the extracts. While the values were less than 1.0, indicating extract nonpreference, oviposition on any of the extract disks was not significantly different from egg numbers found on control disks. This study suggests that the chemical factor(s) stimulatory to *H. zea* oviposition does not appear to induce a similar behavioral response in

TABLE 2. COMPARISON OF *Heliothis zea* AND *Trichoplusia ni* OVIPOSITIONAL RESPONSE TO PLANTS AND HEXANE EXTRACTS OF LEAVES FROM DIFFERENT SPECIES OF GENUS *Lycopersicon*

Species and accession	*H. zea* oviposition			Oviposition on leaf extracts (Eggs on extracts/ eggs on blank)	
	Eggs/plant 24 hr	Eggs/100 g foliage	Eggs/10^3 cm^2 leaf area	*H. zea*	*T. ni*
L. esculentum					
Chico III	29.9 a[a]	20.5 a[a]	8.4 a[a]	2.8 a[a]	0.92
L. chmielewskii					
LA 1306	15.4 a	42.6 ab	16.3 ab	3.2 ab	0.72
L. hirsutum f. *glabratum*					
LA 407	41.5 b	64.1 b	18.1 b	3.9 b	0.86
L. hirsutum					
LA 1777	62.8 c	84.6 c	28.4 c	13.5 c	0.80

[a]Numbers in each column followed by different letters very significantly at $P = 0.05$ (protected LSD test).

T. ni. This finding corroborates previous evidence (Schoonhoven, 1972) indicating that different insect species have evolved chemical sensory organs that show discretion to phytochemical stimuli generally associated with their hosts.

The results from the arena studies of experiments 1 and 2 indicated that the chemical factor(s) stimulatory to *H. zea* oviposition maintained their biological activity for at least three days. While the relative oviposition preference for the disks permeated with extracts of LA 1777 decreased slightly (12%) over the course of the 72-hr arena study, this change was statistically nonsignificant. In contrast, the preference displayed for extracts from tomato cultivars decreased from the first to the third day's measurement by nearly 50%. This suggests that the kairomone in LA 1777 is both relatively stable and has no or low volatility under the conditions maintained for the arena studies. It also suggests that different compounds may be operating as kairomones in the extracts from the different tomato species.

In the arena test where cotton gauze prohibited physical contact between *H. zea* and the extract discs, oviposition on the gauze overlying disks permeated with extracts from LA 1777 and PI 379013 was significantly greater than that associated with the hexane controls. The mean egg numbers per 24 hr over disks of LA 1777, PI 379016, Chico III, and the hexane controls was 62.0(a), 50.6(b), 47.4(bc), and 40.9(c), respectively (means with the same letter are not significantly different according to the protected LSD test). Moth oviposition behavior was modified without the need for direct physical contact between

chemoreceptors (on the moths tarsi, maxillae, and antennae) and the hexane soluble phytochemicals in the filter disks. Volatile constituents in the extracts are apparently serving as olfactory stimuli responsible for chemotactic orientation (possibly positive anemotaxis) by *H. zea* female moths (Kennedy, 1977). The mixing of extract volatile compounds within these comparatively small containers invalidates any conclusions from these studies on the moth chemotactic orientation for distances greater than several centimeters separating the chemical source and the insect. The close agreement between extract oviposition preferences of *H. zea* for LA 1777 plants in the large cages and extracts in the arenas does suggest, however, that the volatile chemical(s) may be operating as an attractant over longer distances. The much greater preference values associated with uncovered LA 1777 extract disks may be a result of a much greater kairomone concentration gradient very close to the disk or to the interaction of this olfactory cue with chemical and tactile stimuli detected upon physical contact of the insect with the filter disk.

Results of the gas chromatographic analysis of the hexane leaf extracts from 12 different accessions of *L. hirsutum* and *L. hirsutum* f. *glabratum* and results from *H. zea* oviposition preference for the same extracts are presented in Table 3. The chromatograms contained seven major and recurrent peaks with retention times of less than 10.0 min (250°C). Many other minor peaks (less than 1% of total peak area) were observed but not included in Table 2. There were also a number of peaks with retention times beyond 10 min that came off the column at oven temperatures between 250 and 325°C. Areas of these peaks were generally quite small (0–2% of total area) and their presence consistent among all the extracts. Table 3 indicates that there is significant qualitative and quantitative variation within the *L. hirsutum* complex for hexane-soluble leaf phytochemicals. Most of the genotypes can be discriminately "fingerprinted" by their individual chromatograms. The *L. hirsutum* accessions appear to fall into three or four "chemotypic" groups.

There were also significant differences exhibited by female *H. zea* moths for oviposition among the extracts in the arena study. When the areas of the primary peaks were compared to the relative oviposition preference of the leaf extracts, areas of the peaks with GC retention times of 8.2 and 8.3 min were highly correlated with *H. zea* oviposition preference. Regression analysis of these data revealed that the equation: Relative *H. zea* oviposition preference = 0.0289 (area of 8.2-min peak) − [0.0000461 (area of 8.3-min peak)]^2 + 2.75, describes 88% of the total variation in extract oviposition preference. This highly significant correlation coefficient ($P < 0.001$) suggests that these two peaks are the traces of compounds that stimulate *Heliothis* oviposition. In the chromatograms of LA 1777 and PI 365936 these two peaks accounted for 70–80% of the total area, suggesting that they represent the major chemical components in the hexane extracts. The y-intercept value of 2.75 indicates that after removal

TABLE 3. ASSOCIATION OF EXTRACT CHROMATOGRAMS WITH *Heliothis zea* OVIPOSITION PREFERENCE AMONG LEAF EXTRACTS OF DIFFERENT *Lycopersicon hirsutum* ACCESSIONS

L. hirsutum accessions	Chromatographic peak areas (10^3 mm^2), peak retention time (min)							Oviposition (Eggs on extracts/ eggs on blank)
	4.2	4.3	5.0	5.7	8.2[a]	8.3[a]	9.2	
LA 1738	0	0	0	0	0	0	84.5	3.23
LA 1777	12.3	102.5	0	16.7	842.8	486.1	66.7	10.72
LA 2101	11.1	0	9.8	0	0	0	0	1.29
PI 199381	0	34.7	172.7	5.4	8.8	11.9	9.1	3.89
PI 251304	5.9	0	226.4	0	0	0	10.5	3.14
PI 251305	0	6.2	83.6	0	0	0	13.4	1.89
PI 308182	7.4	21.3	4.2	0	0	0	26.4	3.58
PI 365904	2.0	0	287.9	0	0	0	18.7	2.82
PI 365936	7.8	64.9	0	10.1	545.3	302.4	19.2	12.89
PI 379013	102.3	115.2	99.9	40.5	196.9	107.5	17.6	7.00
PI 390659	0	14.3	0	0	0	0	0	2.66
PI 390662	0	18.1	0	2.5	15.5	9.4	36.7	4.50

[a] Areas of peaks with retention times of 8.2 and 8.3 were highly correlated with *H. zea* oviposition preference.

of the effects of the chemicals with retention times of 8.2 and 8.3 min there are other compounds in the extracts that stimulate oviposition. This "background" chemical stimulation is present in most of the extracts from the *L. hirsutum* complex and is not clearly associated with the presence or absence of individual peaks on the chromatograms.

The preceding studies suggest the *L. esculentum* tomato cultivars also possess similar stimulatory chemical cues. Chromatograms from extracts of *L. esculentum* cultivars displayed only trace amounts of the compounds with retention times of 8.2 and 8.3 min. These peaks are apparently not involved in the oviposition preference displayed by *Heliothis* for hexane extracts from Chico III, Jet Star, or UC 82B. Other kairomones with greater volatility are implicated.

The same peaks in roughly the same proportion were observed in chromatograms of the LA 1777 hexane leaf extract and from the cotton-wiped leaf tissue. The minor peaks with retention times greater than 10 min (250–300°C) found in the extracts were missing from the leaf surface wipe. The high boiling point, ubiquity among extracts, and the regular spacing in the chromatograms of these compounds suggest they may be cuticular waxes. The peaks with retention times of 8.2 and 8.3 min were found to account for 71% of the total peak area in the leaf hexane extracts compared to 79% in the leaf wipe chromatogram. When the concentration of the LA 1777 leaf wipe hexane solution was adjusted so that the area under the 8.2- and 8.3-min peaks were approximately equivalent to that of the leaf hexane extract, *H. zea* oviposition preference was nearly identical when extracts were tested in the arenas.

Comparison of the mass spectrum of specific GC peaks of the LA 1777 and PI 379013 hexane extracts with known spectra provided superior match quality (>0.95) for only the peaks with retention times of 5.0 and 9.2 min. The 5.0 min peak is definitely the ketone, 2-tridecanone, since the mass spectrum and GC retention time of this peak was identical to a commercially available source (Pfaltz & Bauer, Inc., Stamford, Connecticut). The peak at 9.2 min closely matched (0.98) the spectra of 9,12,15-octadecatrien-1-ol. The molecular weight of the extract peak was 278, 14 units greater than that of the matched compound, suggesting that the unknown may contain an additional methylene group (CH_2). The mass spectrum of the 4.3-min peak shared some homology with zingiberene, a sesquiterpene that is one of the principal volatile components of ginger (Moshonas and Lund, 1970). Lundgren et al. (1985) have reported on the presence of this compound in volatile collections from leaves of *L. hirsutum.*

When GC oven conditions were manipulated to provide for maximum separation of the 8.2- and 8.3-min peaks in LA 1777, three additional peaks in this range of retention times were uncovered. When the area under all these peaks was combined, what was originally the 8.2-min peak accounted for 51% of this

total and the 8.3-min peak for 35%. A third peak which preceded the 8.2-min peak by 8 sec accounted for 12%, while two very small peaks were responsible for the remainder. On the basis of similar fragment size, abundance, and the apparent molecular weight as determined from the mass spectra, the three largest of these peaks represent compounds that are structurally similar. The mass spectra of the major (8.2 min) peak is presented in Figure 2 and yielded the following major peaks: *m/e* (% relative intensity), 93(100), 41(51), 91(49), 79(43), 77(36), 119(35), 107(34), 39(26), 43(26), 94(26), 55(25), 121(24), 105(22), 100(21), and 53(21). A low-voltage scan indicated a compound molecular weight of 234. A diagnostic peak was observed at *m/e* 219 (molecular ion $-$ CH_3). This fragmentation pattern is quite similar to the mass spectrum of an essential oil fraction of a distillate from leaves of the *L. hirsutum* accession PI 251303. Patterson et al. (1975) suggested, on the basis of the mass spectrum and other analytical tests, that the major component of this distillate fraction was a sesquiterpene. The mass spectrum of the 8.2-min peak tentatively suggests the compounds of interest are sesquiterpenes with chemical formulas of $C_{15}H_{22}O_2$. Lundgren et al. (1985) also reported that one of the major volatile components from leaves of one accession of *L. hirsutum* was the sesquiterpene, α-santalene. This sesquiterpene, or a derivative of it, could well be the olfactory kairomone stimulating *H. zea* oviposition. Isolation and precise identification of the structure of these sesquiterpenoids from LA 1777 and validation of their biological activity are reported elsewhere (Coates et al., 1988).

Chromatograms of the leaf wipes suggest that these compounds are present on the leaf surface. Their presumed source is the glandular trichomes known to be found on leaf surfaces of most *Lycopersicon* species. Of the five types of glandular trichomes described by Luckwell (1943) to exist in *Lycopersicon, L. hirsutum* accessions generally display abundant densities of types I, IV, and VI. Type I trichomes have a long multicellular stalk with a very small bulbous

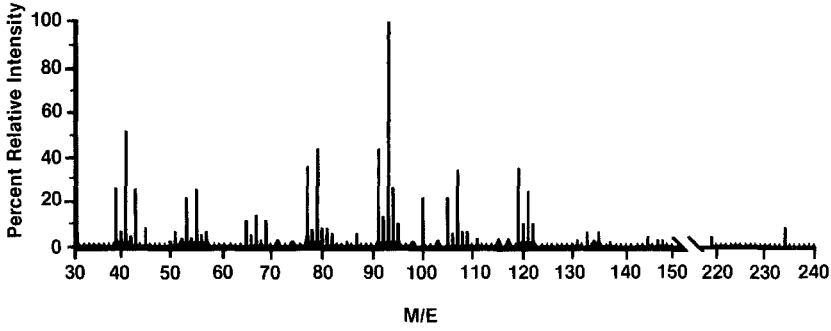

FIG. 2. Mass spectrum of the 8.2-min peak from the hexane leaf extract of LA 1777.

swelling at the tip. The smallest of these glandular hairs, type IV trichomes, have a short multicellular stalk with a droplet of exudate at the tip. Type VI trichomes hve a short stalk with a large tetralobulate glandular head at its apex. Type VI trichomes on leaves of *L. esculentum* were found to synthesize and store phenolic compounds, primarily rutin and chlorogenic acid (Duffey and Isman, 1981), while type VI trichomes in the *L. hirsutum* f. *glabratum* accession, PI 134417 were found to synthesize 2-tridecanone (Dimock and Kennedy, 1983), a compound highly toxic to the tobacco hornworm, the tomato fruitworm, and the potato aphid (Williams et al., 1980). The density of type IV trichomes in the *L. hirsutum* accession, PI 251303 has been associated with resistance to the spider mite (Snyder and Carter, 1984). This resistance was not associated with leaf sesquiterpene (zingiberene or curcumene) content, but appears to operate through mite nonpreference. Microscopic viewing of leaflets of LA 1777 and PI 365936 (*H. zea* preferred) revealed a dense vestiture of what appear to be type IV trichomes, each with a tiny droplet of exudate on their tips. The abundance of these trichomes and the apparent amount of total exudate suggest they are major contributors of the chemical components in the hexane and leaf wipe extracts.

It is difficult to understand why a volatile compound from the leaf surface of several accessions of *L. hirsutum* is acting as a putative olfactory stimulus for *H. zea* orientation and host-plant selection. Wild accessions of *L. hirsutum* inhabit a rather limited zone on the western slopes of the Andes at elevations from 500 to 3300 m from central Peru to northern Ecuador (Rick, 1973). As an ephemeral perennial, this species is usually found at very low population densities in its environment and as such represents a relatively poor resource for phytophagous insects. In addition, previous work has shown that LA 1777 foliage provides the poorest diets for survival and growth of larvae of *Spodoptera littoralis, Plusia chalcites, Phthorimaea operculella*, and *Heliothis armigera* (Juvik et al., 1982). When neonate larvae of *H. zea* were reared on the foliage of 45 different accessions of *Lycopersicon*, survival after four days was lowest on leaflets of LA 1777 (unpublished data). This tends to indicate that *H. zea*'s behavioral response to the kairomonal chemical factor on LA 1777 and other accessions does not represent an evolutionary adaptation by the insect for the location and host selection of *L. hirsutum* plants. This is supported by evidence showing that females of *Heliothis armigera*, a species related to *H. zea* but endemic to the Old World, also display ovipositional preference for LA 1777 plants (Juvik et al., 1982). A more likely explanation for the kairomonal properties of this compound or of a structural analog is that it is synthesized by one or more other sympatric host plant species that are or have been in the past a significant food resource for developing *Heliothis* larvae.

The existence of species variation in the qualitative presence and quanti-

tative amount of *Lycopersicon* allelochemics that serve as cues for *H. zea* orientation and oviposition could be utilized in a breeding program to develop tomato cultivars with genetically modified allelochemic profiles. Such as modification might disrupt the sequential behavioral processes of *H. zea* host-plant selection and colonization. Altering a tomato plant's allelochemic constitution could prevent female *H. zea* in search of oviposition sites from recognizing that plant as an acceptable host for the growth and development of its young. Such disruption in an agricultural ecosystem could result in reduced crop damage from larval feeding. The *H. zea* ovipositional kairomone in *L. hirsutum*, once isolated and identified, may be of use to trap moths for field monitoring of female population levels for effective pest management strategies. Alternatively, the compound might be usable when combined with pesticide baits for actual control.

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DETOXIFICATION OF ISOTHIOCYANATE ALLELOCHEMICALS BY GLUTATHIONE TRANSFERASE IN THREE LEPIDOPTEROUS SPECIES

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Abstract—Glutathione transferase activity towards various plant isothiocyanates was studied in larvae of the two generalists, fall armyworm [*Spodoptera frugiperda* (J.E. Smith)], and cabbage looper [*Trichoplusia ni* (Hübner)], and the specialist, velvetbean caterpillar (*Anticarsia gemmatalis* Hübner) using the midgut soluble fraction as enzyme source. The generalists, but not the specialist, are adapted to feeding on isothiocyanate-containing crucifers. Allyl and benzyl isothiocyanate were found to be metabolized by glutathione transferase from the two generalist species, but no activity was detected with the specialist. The transferase activity towards these allelochemicals in the cabbage looper was two- to sixfold higher than that in the fall armyworm. In all instances, activity was induced by various allelochemicals including indole 3-acetonitrile, indole 3-carbinol, flavone, xanthotoxin, and its own substrates. The induction ranged from 1.3- to 10.1-fold depending on the allelochemical, with the fall armyworm being more inducible. The transferase system of fall armyworm also metabolized another analog, 2-phenylethyl isothiocyanate, but activity can only be observed after induction. Bioassay results showed that these isothiocyanates were all toxic to the lepidopterans, causing acute toxicity in neonates and final-instar larvae. The results suggest that glutathione transferase plays an important role in the detoxification of isothiocyanates and hence food-plant adaptation in phytophagous insects.

Key Words—Glutathione transferase, isothiocyanate metabolism, fall armyworm, *Spodoptera frugiperda*, cabbage looper, *Trichoplusia ni*, velvetbean caterpillar, *Anticarsia gemmatalis*, Lepidoptera, Noctuidae.

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INTRODUCTION

The glutathione transferases are a group of enzymes that catalyze the conjugation of reduced glutathione to electrophilic compounds. The reaction is considered an important pathway for xenobiotic detoxification since the resulting conjugates are further transformed to more excretable metabolites such as mercapturic acids (Boyland and Chasseaud, 1969).

In insects, glutathione transferases catalyze the metabolism of organophosphorus and organochlorine insecticides and are involved in the development of resistance to these compounds (Motayama and Dauterman, 1980; Tanaka et al., 1981; Clark and Shamaan, 1984). It is also believed that glutathione transferase plays an important role in insect herbivory through the detoxification of toxic plant allelochemicals. However, unlike insecticides, knowledge about the detoxification of plant allelochemicals by this enzyme is limited. Recently, we demonstrated that toxic α,β-unsaturated carbonyl allelochemicals such as *trans*-cinnamaldehyde, *trans,trans*-2, 4-decadienal, *trans*-2-hexenal, and benzaldehyde were detoxified by glutathione transferase prepared from the midguts of fall armyworm larvae (Wadleigh and Yu, 1987). Transferase activity towards these compounds was induced by various dietary allelochemicals including, in some cases, the substrate itself.

In cruciferous plants, isothiocyanates (RNCS) are an important component of the chemical defenses against nonadapted insect herbivores, but they provide no protection against adapted herbivores that include crucifers in their normal host range (Feeny, 1977). The mechanism of isothiocyanate detoxification in the adapted herbivores is not completely understood. Previous research has shown that 2-phenylethyl isothiocyanate was a substrate for microsomal oxidases in the fall armyworm (Yu, 1987a). Detoxification by glutathione transferase is also likely since allyl and benzyl isothiocyanate were metabolized in vivo via glutathione-dependent conjugation in mammals (Brüsewitz et al., 1977; Mennicke et al., 1983). The reaction occurs as follows: RNCS + GSH $\rightarrow$ RNHC(S)SG.

The purpose of this research was to examine the detoxification of isothiocyanates by glutathione transferase in the fall armyworm, cabbage looper, and velvetbean caterpillar. The role of enzyme induction in detoxification was also investigated. The fall armyworm and cabbage looper are generalists adapted to feeding on isothiocyanate-containing crucifers. The velvetbean caterpillar is a specialist insect feeding primarily on certain species of plants in the Leguminosae.

METHODS AND MATERIALS

Insects. Larvae of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (FAW), cabbage looper, *Trichoplusia ni* (Hübner) (CL), and velvetbean cat-

erpillar, *Anticarsia gemmatalis* Hübner (VBC), were reared on an artificial diet (Burton, 1969) and maintained in environmental chambers at 24°C with a 16:8 hr light–dark photoperiod.

Chemicals. Ellagic acid, flavone, indole 3-acetonitrile, indole 3-carbinol, quercetin, juglone, and reduced glutathione were purchased from the Sigma Chemical Company, St. Louis, Missouri. Triphenyltin chloride, allyl isothiocyanate (AITC), and benzyl isothiocyanate (BITC) were from the Aldrich Chemical Company, Milwaukee, Wisconsin. 2-Phenylethyl isothiocyanate (PITC) was obtained from Fluka AG, Hauppauge, New York.

Treatments of Insects. In the induction experiments, groups of final-instar larvae of FAW and CL (newly molted) and VBC (1 day old) were individually fed on artificial diet containing allelochemicals and held at 24°C in 1-oz. plastic cups for two days before the enzyme assays were conducted. Control larvae of all species were fed the artificial diet alone.

To study the toxicity of the allelochemicals, groups of 15 one-day-old first-instar FAW larvae were fed the allelochemicals mixed in their artificial diet and reared to adulthood, as described earlier. Mortality counts were made 24 hr later. The surviving larvae were weighted at day 12 and shortly after the fifth molt. Survivorship and the time required to reach pupation and adult emergence were recorded.

The acute toxicity of AITC was also determined using larvae of the CL (two days after the final larval molt) and VBC (three days after the final larval molt) as test stages. In these experiments, groups of 15 or 20 larvae were fed an artificial diet containing the allelochemicals for 24 hr before mortality counts were taken.

Enzyme Preparation. The midguts of 24 larvae were dissected and the gut contents removed. The guts were washed in ice-cold 1.15% KCl and homogenized in 0.1 M sodium phosphate buffer (pH 6.5) followed by differential centrifugation to obtain the soluble fraction as described previously (Yu, 1982). The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Glutathione Transferase Assays. Glutathione transferase activity towards the isothiocyanate was studied using the allelochemical-dependent glutathione depletion assay (Boyland and Chasseaud, 1967). The 4-ml reaction mixture contained equimolar concentrations of allelochemicals (AITC, 3 mM; BITC and PITC, 1.5 mM) and reduced glutathione, 0.1 M sodium phosphate buffer (pH 6.5), and the soluble fraction (equivalent to 1 mg of protein per incubate). Duplicate reaction mixtures were incubated for 2 min at 25°C in a water bath with shaking. The concentration of glutathione remaining in the reaction mixture at the end of the incubation period was measured using the method of Ellman (1959). All reactions were corrected for nonenzymatic activity using boiled soluble fraction as a blank.

Statistical Analysis. Enzyme assay data were subjected to analysis of var-

iance (SAS Institute, 1985) followed by Dunnett's test (Steel and Torrie, 1960) when the ANOVA was significant at $P < 0.05$. Probit analysis was performed on acute toxicity data (SAS Institute, 1985).

RESULTS

Glutathione Transferase Activity Towards Isothiocyanates. Glutathione transferase activity towards isothiocyanates was characterized in the FAW using AITC as a model substrate. The assays were conducted at pH 6.5 to avoid the severe nonenzymatic conjugation of AITC with GSH that occurs at higher pHs. Enzyme activity was maximized by inducing newly molted sixth-instar larvae with indole 3-acetonitrile (0.2%) for two days before preparation of the soluble fraction. At pH 6.5, enzyme activity against AITC reached its maximum after 2 min of incubation and was linear up to 1.5 mg protein/sample (data not shown). The transferase activity was inhibited by the known glutathione transferase inhibitors guercetin, triphenyltin chloride (Wadleigh and Yu, 1987), ellagic acid (Das et al., 1984), and juglone (Kulkarni et al., 1978) at 10^{-4} M (Table 1). Juglone and triphenyltin chloride were the most potent inhibitors, reducing activity by 99% at this concentration.

The results summarized in Table 2 showed that glutathione transferase prepared from the midgut homogenates of noninduced FAW and CL larvae catalyzed the conjugation of GSH with AITC and BITC but not PITC. The transferase activity in the CL was two- to sixfold greater than that in the FAW. In all instances, no activity towards these allelochemicals was detected in VBC larvae.

TABLE 1. In Vitro EFFECT OF INHIBITORS ON GLUTATHIONE TRANSFERASE ACTIVITY TOWARDS ALLYL ISOTHIOCYANATE IN FALL ARMYWORM LARVAE

Inhibitor (10^{-4} M)	Glutathione transferase (μmol AITC conjugated/ 2 min/mg protein)[a]	Inhibition (%)
Control	1.47 ± 0.01	0
Triphenyltin chloride	0.02 ± 0.02[b]	99
Juglone	0.02 ± 0.02[b]	99
Ellagic acid	0.31 ± 0.08[b]	79
Quercetin	0.80 ± 0.10[b]	46

[a] Newly molted sixth-instar larvae were fed 0.2% indole 3-acetonitrile for two days before the enzyme assays were conducted. Mean ± SEM of three experiments.
[b] Value significantly different from the control ($P < 0.05$).

TABLE 2. GLUTATHIONE TRANSFERASE ACTIVITY TOWARDS ISOTHIOCYANATES IN THREE SPECIES OF LEPIDOPTEROUS LARVAE

Substrate	Glutathione transferase (μmol substrate conjugated/2 min/mg protein)[a] Velvetbean caterpillar	(N)	Fall armyworm	(N)	Cabbage looper	(N)
Allyl isothiocyanate	0	(2)	0.19 ± 0.09	(5)	1.14 ± 0.04	(4)
Benzyl isothiocyanate	0	(3)	0.16 ± 0.06	(3)	0.34 ± 0.13	(3)
2-Phenylethyl isothiocyanate	0	(3)	0	(4)	0	(3)

[a] Mean ± SEM of N experiments.

From Table 3 it is seen that various allelochemicals induced glutathione transferase activity towards AITC, BITC, and PITC in FAW larvae. Transferase activity towards AITC was induced by indole 3-acetonitrile, indole 3-carbinol, flavone, xanthotoxin, and its own substrate, AITC. The induction ranged from 5.4- to 10.9-fold, with indole 3-acetonitrile being most active. Transferase activity with BITC was induced up to 10-fold by its own substrate and nearly sixfold by indole 3-acetonitrile. In the case of PITC, conjugase activity can only be detected in induced larvae.

TABLE 3. INDUCTION OF GLUTATHIONE TRANSFERASE ACTIVITY TOWARDS ISOTHIOCYANATES IN FALL ARMYWORM LARVAE

Treatment[a]	Glutathione transferase with substrate (μmol allelochemical conjugated/2 min/mg protein)[b] Allyl isothiocyanate	Benzyl isothiocyanate	Phenylethyl isothiocyanate
Control	0.12 ± 0.07	0.16 ± 0.03	0
Indole 3-acetonitrile (0.2%)	1.31 ± 0.13[c]	0.88 ± 0.06[c]	0.33 ± 0.13
Indole 3-carbinol (0.2%)	0.66 ± 0.19[c]		
Flavone (0.2%)	0.75 ± 0.11[c]		
Xanthotoxin (0.01%)	0.67 ± 0.12[c]		
Allyl isothiocyanate (0.025%)	0.65 ± 0.15[c]		
Benzyl isothiocyanate (0.025%)		1.61 ± 0.44[c]	
2-Phenylethyl isothiocyanate (0.025%)			0.37 ± 0.03

[a] Newly molted sixth-instar larvae were fed artificial diet containing allelochemicals, at the concentrations indicated in parenthesis, for two days before the enzyme assays were conducted.
[b] Mean ± SEM of four experiments for AITC and three experiments for BITC and PITC.
[c] Value significantly different from the control ($P < 0.05$).

TABLE 4. INDUCTION OF GLUTATHIONE TRANSFERASE ACTIVITY TOWARDS ALLYL ISOTHIOCYANATE IN TWO SPECIES OF LEPIDOPTEROUS LARVAE

Treatment[a]	Glutathione transferase (μmol AITC conjugated/2 min/mg protein)[b]	
	Velvetbean caterpillar	Cabbage looper
Control	0	1.17 ± 0.04
Indole 3-acetonitrile (0.2%)	0	1.63 ± 0.15[c]
Allyl isothiocyanate (0.025%)	0	1.50 ± 0.09[c]

[a] Final-instar larvae were fed an artificial diet containing allelochemicals, at the concentrations indicated in parenthesis, for two days before the assays were conducted. VBC larvae were fed 0.1% indole 3-acetonitrile.
[b] Mean ± SEM of three experiments.
[c] Value significantly different from the control ($P < 0.05$).

The results obtained from studies of induction of glutathione transferase activity towards AITC in VBC and CL larvae are shown in Table 4. Surprisingly, the induction in CL larvae was only marginal; increases of 39% and 28% by indole 3-acetonitrile and AITC, respectively. No such transferase activity was observed in VBC larvae even though larvae were exposed to these known inducers.

Data in Table 5 show that the induction by indole 3-acetonitrile in FAW larvae was dose-dependent, causing gradual increases in activity from 0.01% to 0.2% in the diet.

Toxicity of Isothiocyanates. Table 6 shows that all of the allelochemicals were acutely toxic when fed to first-instar FAW larvae at 0.005% or greater. However, these compounds had no effect on larval weight, development time,

TABLE 5. EFFECT OF INDOLE 3-ACETONITRILE CONCENTRATION ON GLUTATHIONE TRANSFERASE ACTIVITY IN FALL ARMYWORM LARVAE

Indole 3-acetonitrile (% in diet)	Glutathione transferase (μmol AITC conjugated/2 min/mg protein)[a]
Control	0.28 ± 0.08
0.01	0.79 ± 0.12
0.05	1.35 ± 0.05
0.1	1.51 ± 0.20
0.2	2.01 ± 0.21

[a] Mean ± SEM of three experiments.

TABLE 6. TOXICITY OF ISOTHIOCYANATES TO FIRST-INSTAR FALL ARMYWORM LARVAE

Allelochemical	Mortality (%) at indicated concentration[a]		
	0.005%	0.01%	0.05%
Allyl isothiocyanate	0	23	100
Benzyl isothiocyanate	29	93	100
2-Phenylethyl isothiocyanate	29	100	100

[a] One-day-old larvae were fed an artificial diet containing the allelochemicals for 24 hr before mortality counts were made.

TABLE 7. TOXICITY OF ALLYL ISOTHIOCYANATE TO THREE SPECIES OF LEPIDOPTEROUS LARVAE

Species[a]	LC_{50} (% in diet)	95% fiducial limits of LC_{50} (%)
Fall armyworm	0.09	0.08 – 0.1
Velvetbean caterpillar	0.17	0.14 – 0.22
Cabbage looper	0.22	0.16 – 0.25

[a] Groups of 15 (VBC) or 20 (FAW and CL) final-instar larvae were fed an artificial diet containing AITC for 24 hr before mortality counts were made.

and percent pupal emergence of larvae that survived the initial 24-hr exposure (data not shown). AITC was also acutely toxic to final-instar larvae of FAW, CL, and VBC, the LC_{50} values ranging from 0.09% to 0.22% (Table 7). The FAW was most susceptible to AITC, whereas the VBC and CL were equally tolerant.

DISCUSSION

Isothiocyanates, which are the hydrolysis products of glucosinolates (mustard oil glucosides), are widely distributed in cruciferous plants. The hydrolysis is catalyzed by an enzyme known as thioglucosidase, which is stored separately in plant tissue and comes in contact with its glucosinolate substrates when the tissue is fed upon by insects or otherwise damaged (Fenwick, et al., 1982). There is much evidence to suggest that isothiocyanates are insecticidal (Lichtenstein et al., 1964; Seo et al., 1982; and this study, Tables 6 and 7) and as such are important in chemical defense against nonadapted insect herbivores. However, isothiocyanates present no barrier to insect herbivores that are adapted

to feeding on isothiocyanate-producing plants and may act even as attractants and ovipositioning and feeding stimulants (Thorsteinson, 1953; Nair and McEwen, 1976; Vincent and Stewart, 1984).

The results of the present study demonstrate that the isothiocyanates (AITC, BITC, PITC) are metabolized by glutathione transferase in the FAW and CL. In the case of PITC, activity can only be observed after FAW larvae were fed inducers. This suggests that the FAW possesses different glutathione transferase isozymes, some of which can only be detected after induction. Our results also showed that levels of glutathione transferase activity (towards isothiocyanates) in phytophagous insects were correlated with the degree of adaptation to cruciferous plants. The CL, which feeds more widely on crucifers, possessed higher transferase activity than the less crucifer-adapted fall armyworm (Tietz, 1972). On the other hand, the specialist VBC, which feeds mainly on soybeans, did not show any detectable transferase activity towards these compounds. Apparently, the glutathione transferase activity was quite different from that towards DCNB (1,2-dichloro-4-nitrobenzene), CDNB (1-chloro-2,4-dinitrobenzene), or PNPA (*p*-nitrophenyl acetate) in these insects. In a previous report (Yu, 1987b), we showed that glutathione transferase activity was active against all of these model substrates in VBC larvae, their activity with DCNB and PNPA being comparable to FAW larvae.

The data also showed that, as in the case of glutathione transferase towards the model substrates and certain α,β-unsaturated carbonyl allelochemicals (Yu, 1982, 1984; Wadleigh and Yu, 1987), glutathione transferase activity against isothiocyanates was inducible by various allelochemicals including its own substrates. We find it of interest that the CL, which contained 10-fold higher transferase activity than the FAW, was only slightly inducible by allelochemicals. Assuming the rate of feeding was similar in these two species, the differential inducibility of the transferase activity may reflect the nearly fully expressed gene activity in the CL compared to the less expressed gene in the FAW. Alternatively, the CL may have detoxified the inducers at a higher rate than the FAW after administration, resulting in less inducer available for induction. More work is needed to answer this question.

The role of enzyme induction in plant–insect interactions was further clarified by our data showing a dose-dependent response of glutathione transferase to an inducer (Table 5). These results suggest that when an insect feeds on an isothiocyanate-containing plant, the degree of induction is likely influenced by the concentration of the allelochemicals in the plant. In this connection, it was reported that factors such as variety, plant part, and seasonal variation affected levels of isothiocyanates and their parent compounds, the glucosinolates, in plants (Itoh et al., 1984; Rodman and Louda, 1984, 1985).

The bioassay results (Table 7) showed that the VBC was as tolerant of allyl isothiocyanate as the CL. The fact that the VBC contained no detectable

glutathione transferase activity (with isothiocyanates), as compared with the highly active CL, suggests that factors other than glutathione conjugation are also involved in determining the toxicity of the isothiocyanate. It is likely that the microsomal oxidase system which was found to metabolize 2-phenylethyl isothiocyanate (Yu, 1987a) plays an important role in the detoxification of isothiocyanates in the VBC. This insect has been shown to possess high microsomal oxidase activity including epoxidation and hydroxylation (Christian and Yu, 1986; Yu, 1987b).

It is now obvious that glutathione transferase can detoxify numerous plant toxins including α,β-unsaturated carbonyl compounds (e.g., *trans*-cinnamaldehyde, *trans,trans*-2,4-decadienal) and isothiocyanates (this study) in phytophagous insects. Since the transferase activity can also be induced by various allelochemicals (including its own substrates) commonly found in their food plants, glutathione transferase must play a significant role in the detoxification of plant toxins in certain insects. In addition to the compounds mentioned above, there are numerous other allelochemicals such as thiocyanates, coumarins, quinones, terpenoids, cardenolids, and alkaloids that possess the structural requirements to serve as substrates for glutathione transferases. Work is now in progress to explore this area of knowledge which is essential for future pest management.

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ANTENNAL OLFACTORY RESPONSIVENESS OF THREE SYMPATRIC *Ips* SPECIES [*Ips avulsus* (EICHHOFF), *Ips calligraphus* (GERMAR), *Ips grandicollis* (EICHHOFF)], TO INTRA- AND INTERSPECIFIC BEHAVIORAL CHEMICALS[1,2]

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Abstract—Electroantennograms (EAGs) from male and female *Ips avulsus*, *I. calligraphus*, and *I. grandicollis* to their pheromones and selected host odorants or kairomones verified the presence of antennal olfactory receptors in both sexes of each species capable of detecting ipsdienol, ipsenol, *cis*- and *trans*-verbenol, *endo*-brevicomin α-pinene, frontalin, and verbenone. Each species possesses receptors with lower thresholds and in greater abundance for the compounds they produce and to which they are behaviorally most responsive. Detection of both *Ips* and *Dendroctonus* pheromones by the three cohabiting species provides a sensory basis for olfactory interactions among the species. Differences in both threshold and saturation levels for EAGs for the various behavioral chemicals could denote differences in specific behavioral roles for each compound.

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Key Words—Electroantennogram, pheromone, bark beetles, *Ips*, *Dendroctonus*, Coleoptera, Scolytidae, olfactory receptors, intraspecific, interspecific, electrophysiology.

INTRODUCTION

The orchestration of host selection, aggregation, and colonization by scolytids cohabiting southern pine involves a chemical communication system composed of both intra- and interspecific chemical messengers. Three engraver beetle species are among the five scolytid species comprising the southern pine bark beetle guild. The *Ips* species, *I. avulsus* (Eichhoff), (four-spined engraver), *I. calligraphus* (Germar), (six-spined engraver) and *I. grandicollis* (Eichhoff), (five-spined engraver), together with *Dendroctonus frontalis* Zimmerman (southern pine beetle) and *D. terebrans* (Olivier) (black turpentine beetle), often coexist and compete for resources in the same host trees.

Chemical communication by this bark beetle guild is complicated and involves both beetle- and host-tree-produced compounds that can function as pheromones, kairomones, allomones, and possibly synomones (Kinzer et al., 1969; Vité et al., 1978; Birch et al., 1980; Svihra et al., 1980). The sequence of arrival and competitive interactions among the three *Ips* species, and sympatric *D. frontalis*, as they colonize pine trees has been investigated (Dixon and Payne, 1979; Birch and Svihra, 1979; Svihra et al., 1980). Furthermore, olfactory communication among these scolytid species may play a significant role in defining their temporal and spatial patterns of colonization and ultimate attack density (Svihra et al., 1980).

In the past, considerable attention has been given to describing the behavioral responses of the species to selected behavioral chemicals or chemical combinations in the field (Vité et al., 1964, 1976a,b, 1978; Renwick and Vité, 1972; Vité and Renwick, 1971a; Werner, 1972; Hedden et al., 1976; Richerson and Payne, 1979; Birch and Svihra, 1979; Dixon and Payne, 1980; Billings, 1985). More recently, studies have concentrated on response patterns of the beetles to trees or logs containing various species of the guild (Hedden et al., 1976; Birch and Svihra, 1979; Svihra et al., 1980; Svihra, 1982). Electrophysiological analyses of antennal olfactory response have been reported for *D. frontalis* and *D. terebrans* (Payne 1970, 1971, 1975; Payne and Dickens, 1976; Dickens and Payne, 1977, 1978; Payne et al. 1982, 1987) and in a preliminary study of *I. calligraphus* (Dickens, 1979). Therefore, the study reported here was undertaken to determine in detail antennal olfactory response of the three sympatric *Ips* species to beetle- and host-tree-produced compounds implicated in the aggregation behavior of the beetles and to provide insight into intra- and interspecific whole organism behavior of the beetles. Results from the study should afford a broader understanding of the olfactory communication systems of the beetles by providing information at the olfactory receptor level for inte-

gration with data on pheromone production, behavioral relationships, niche breadth, and resource partitioning.

METHODS AND MATERIALS

Insects. Bolts of loblolly pine (*Pinus taeda* L.), naturally infested with *I. avulsus, I. calligraphus*, and/or *I. grandicollis*, were collected in eastern Texas and placed in an emergence chamber (Browne, 1972). Upon emergence, adult beetles were sorted, sexed, and stored at 5°C on moist filter paper in Petri dishes until use.

Behavioral Chemicals. The compounds used in the study, and their source and purity, are listed in Table 1. Tenfold serial dilutions of each compound (0.00001 μg/μl to 100 μg/μl) were prepared in nanograde pentane.

Electrophysiological Technique. Olfactory responses were measured using the electroantennogram (EAG) technique, previously described in detail (Dick-

TABLE 1. BEETLE- AND HOST-TREE PRODUCED COMPOUNDS TESTED

Compound	Biological source (ref.)[a]	Source of supply	Purity (%)
Frontalin			
(+, −)	*D. frontalis* (1–4)	Chem. Samp. Co.	99
(+)	*D. frontalis*	K. Mori	98
(−)	*D. frontalis*	K. Mori	98
Verbenone			
(+, −)	*D. frontalis* (2, 5, 6)	Chem. Samp. Co.	98
(+)	?	Chem. Samp. Co.	98
(−)	?	Chem. Samp. Co.	98
endo-Brevicomin	*D. frontalis* (6, 7)	Chem. Samp. Co.	99
Ipsdienol	*I. avulsus* (8, 12) *I. calligraphus* (8, 9, 12)	Borregaard Industries	81
Ipsenol	*I. grandicollis* (8, 10, 12)	Borregaard Industries	89
cis-Verbenol	*I. avulsus* (8) *I. calligraphus* (8, 9) *I. grandicollis* (8) *D. frontalis* (2, 7)	Borregaard Industries	95
trans-Verbenol	*I. avulsus* (8) *I. calligraphus* (8, 9) *I. grandicollis* (8) *D. frontalis* (2, 5, 7, 11)	Borregaard Industries	95
α-Pinene	host tree	Aldrich Chem. Co.	97

[a] (1) Kinzer et al., 1969; (2) Pitman et al., 1969; (3) Stewart et al., 1977; (4) Renwick and Vité, 1968; (5) Renwick 1967; (6) Vité and Renwick, 1971a; (7) Hughes, 1973; (8) Vité et al., 1972; (9) Renwick and Vité, 1972; (10) Vité and Renwick, 1971b; (11) Hughes, 1975; (12) Hughes, 1974.

ens and Payne, 1977) and modified from earlier techniques (Schneider, 1957a,b; Payne, 1975). Glass capillary Ag–AgCl microelectrodes filled with 3 M KCl were used. The recording electrode was inserted into the distal end of the antennal club following prepuncture with an electrolytically sharpened tungsten needle. The indifferent electrode was implanted either in the head capsule or mouth of the beetle. EAGs were recorded on Polaroid film with a Tektronix C-27 oscilloscope camera.

Stimulation. Test stimuli were delivered as 5-μl aliquots onto a piece of Whatman No. 1 filter paper (20 × 7 mm) inserted into a glass cartridge (75 mm long × 5 mm ID). The cartridge was oriented toward the antennal preparation from ca. 1 cm for delivery of the stimulus. Odor molecules evaporating from the filter paper were carried over the preparation by a filtered air flow of ca. 1 liter/min. Stimulus duration was 1 sec. An odor delivery system previously described in detail (Payne and Dickens, 1976) was modified for a single compound.

Serial dilutions were presented in order from the lowest to the highest concentration for a given compound. Three minutes were allowed between each stimulus, except at higher concentrations when 5 min were allowed. These intervals were found to be adequate for complete recovery of the EAG. Each dilution was replicated three to eight times for each sex of each species. Five microliters of nanograde pentane served as the control. Response to the control was subtracted from responses to test stimuli.

The use of a common standard, 10 μg of ipsdienol, for all preparations allowed for normalization of data and comparisons among compounds and sexes. Stimulation with the standard either preceded or followed each test stimulus. Responses to test stimuli were represented as a percentage of the mean of the two closest responses to the standard (Dickens, 1978).

Data Analysis. Dosage–response curves plotted from mean responses to each compound were used to determine the relative sensitivity of the olfactory receptors to each compound. The threshold of response, the minimum stimulus concentration at which an EAG was detectable above background, was considered an indication of olfactory receptor sensitivity to a given compound. The maximum level of response (saturation level) was considered a measure of the relative number of responding acceptors (Payne, 1975). Responses were compared using the nonparametric Kruskal-Wallis test (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Response (mV) to the standard, ipsdienol at 10 μg, was similar for each sex of each species tested: (*I. avulsus* males = 2.56 ± 0.66, females = 2.36 ± 0.74; *I. calligraphus* males = 1.58 ± 0.55, females = 2.20 ± 0.68; *I. grandicollis* males = 1.80 ± 0.92, females = 1.37 ± 0.48). Response to the standard was not significantly different between the sexes of a given species.

All compounds tested elicited detectable EAGs from males and females of each species (Figures 1–3). Detection of the compounds suggests a possible past, present, or even potential future behavioral role for the compounds in the olfactory communication systems of the species.

Intraspecific Responses. EAGs for each species generally increased with increasing stimulus concentration above threshold for all test compounds. Similarity of the slopes and shapes of the dosage–response curves for a given compound suggest similar receptor mechanisms for both sexes of each species as was reported in single-cell studies of *Ips pini* (Mustaparta et al., 1979). There were no significant differences in responsiveness between sexes for each compound within each species ($P < 0.05$), except female *I. calligraphus* were significantly more sensitive to *endo*-brevicomin than were males ($P < 0.05$) (Figure 2f).

The threshold and saturation level for each compound varied according to sex and/or species, which suggests probable differences in sensitivity and/or acceptor population size for the compounds tested. Difference in threshold and saturation level might be indicative of the role of each compound in the aggregation process. *I. avulsus* possess larger populations of acceptors with greater sensitivity for ipsdienol, α-pinene, *endo*-brevicomin, and racemic and enantiomeric frontalin than for the other compounds tested (Figure 1). *I. calligraphus* had lower thresholds of response and larger EAGs (to the upper range of concentrations tested) for ipsdienol, α-pinene and *endo*-brevicomin than for the other compounds tested (Figure 2). For *I. grandicollis* the threshold level was lower and saturation level greater for ipsenol, ipsdienol, α-pinene, *endo*-brevicomin, and *trans*-verbenol (Figure 3). The lower thresholds for the respective compounds in each species might indicate the ability of the species to perceive the compounds in low concentrations at greater distances form its source. The higher thresholds for the other compounds (i.e., ipsenol, *cis*- and *trans*-verbenol, and racemic and enantiomeric verbenone for *I. avulsus*; ipsenol, *cis*- and *trans*-verbenol, and racemic and enantiomeric verbenone and frontalin for *I. calligraphus*; *cis*-verbenol and racemic and enantiomeric frontalin and verbenone for *I. grandicollis*) indicate that these compounds might function in short-range orientation and/or arrestment. Intermediate thresholds might be indicative of a compound that functions in intermediate or short-range host orientation or synergism (Dickens, 1981). Similarly, in EAG studies of *I. typographus*, Dickens (1981) reported that 2-methyl-3-buten-2-ol is probably a short-range orientation and/or arrestment substance. This hypothesis was based on the very high response threshold and narrow response width to saturation in EAG measurements. Birgersson et al. (1984) suggest that the adaptive value of a short-range orientation substance is its ability to orient and concentrate the beetles to a tree in the critical initial aggregation phase when the pioneering males must overcome the tree's natural defenses, primarily its heavy resin flow.

Insight into the specificity of a volatile messenger may enter all levels of the communication system, for example: pheromone production, olfactory per-

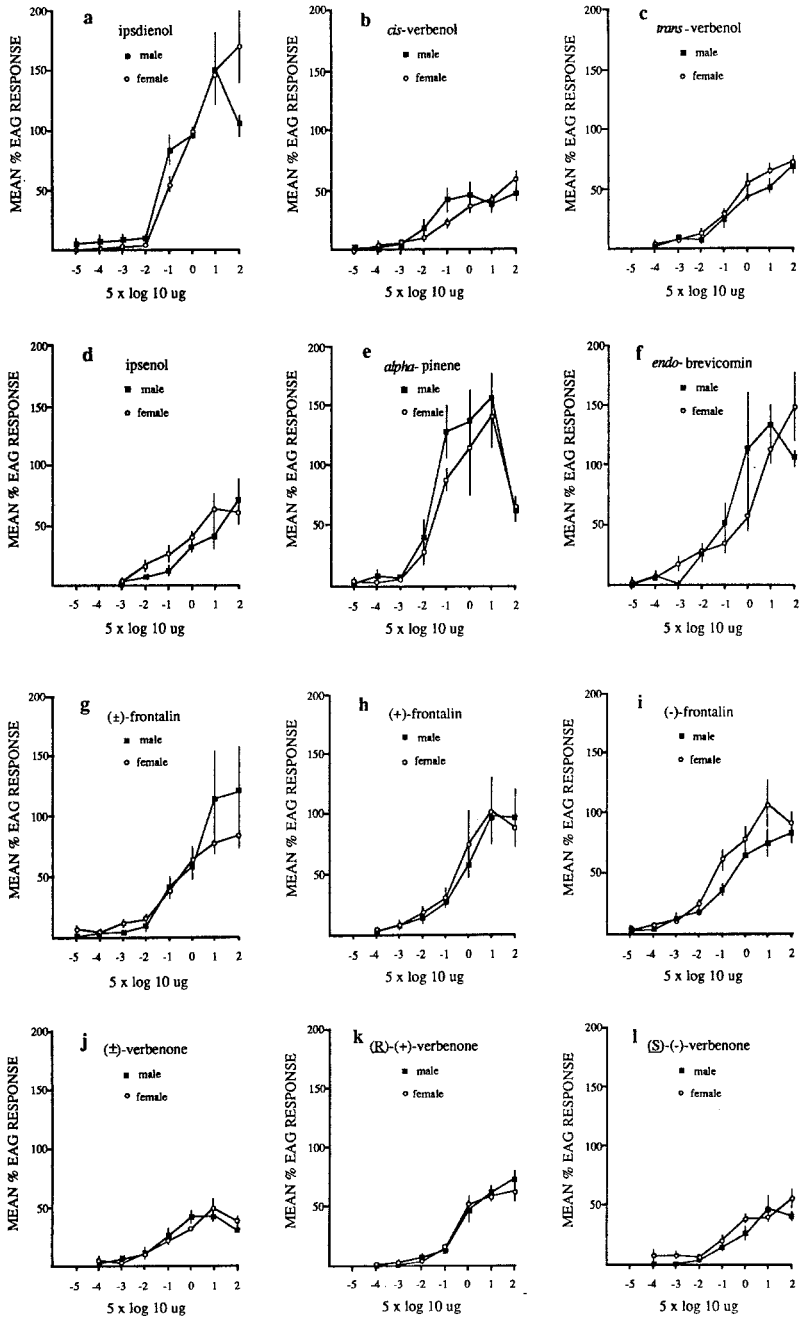

FIG. 1. Mean percent response (±SE) of *I. avulsus* to behavioral chemicals. Means are percent of EAG to the standard ipsdienol.

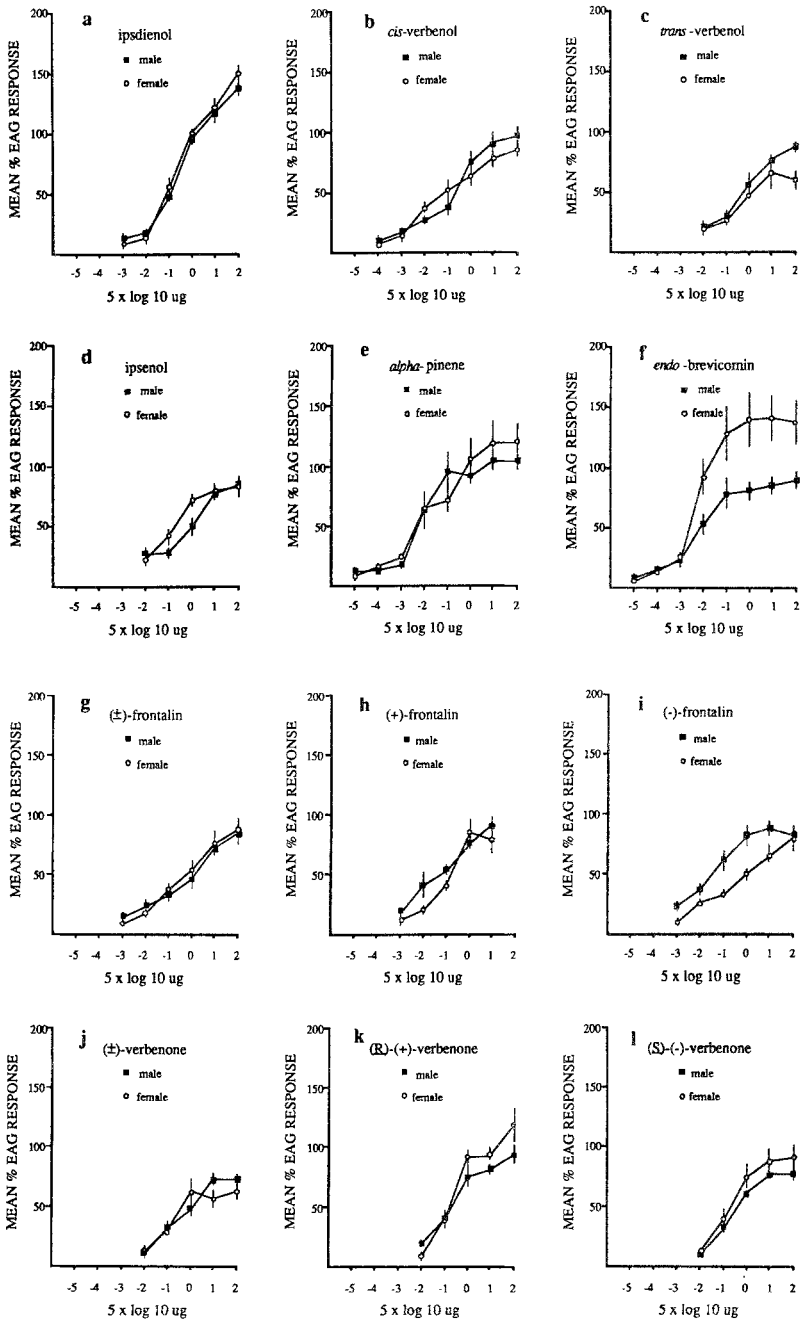

FIG. 2. Mean percent response (±SE) of *I. calligraphus* to behavioral chemicals. Means are percent of EAG to the standard ipsdienol.

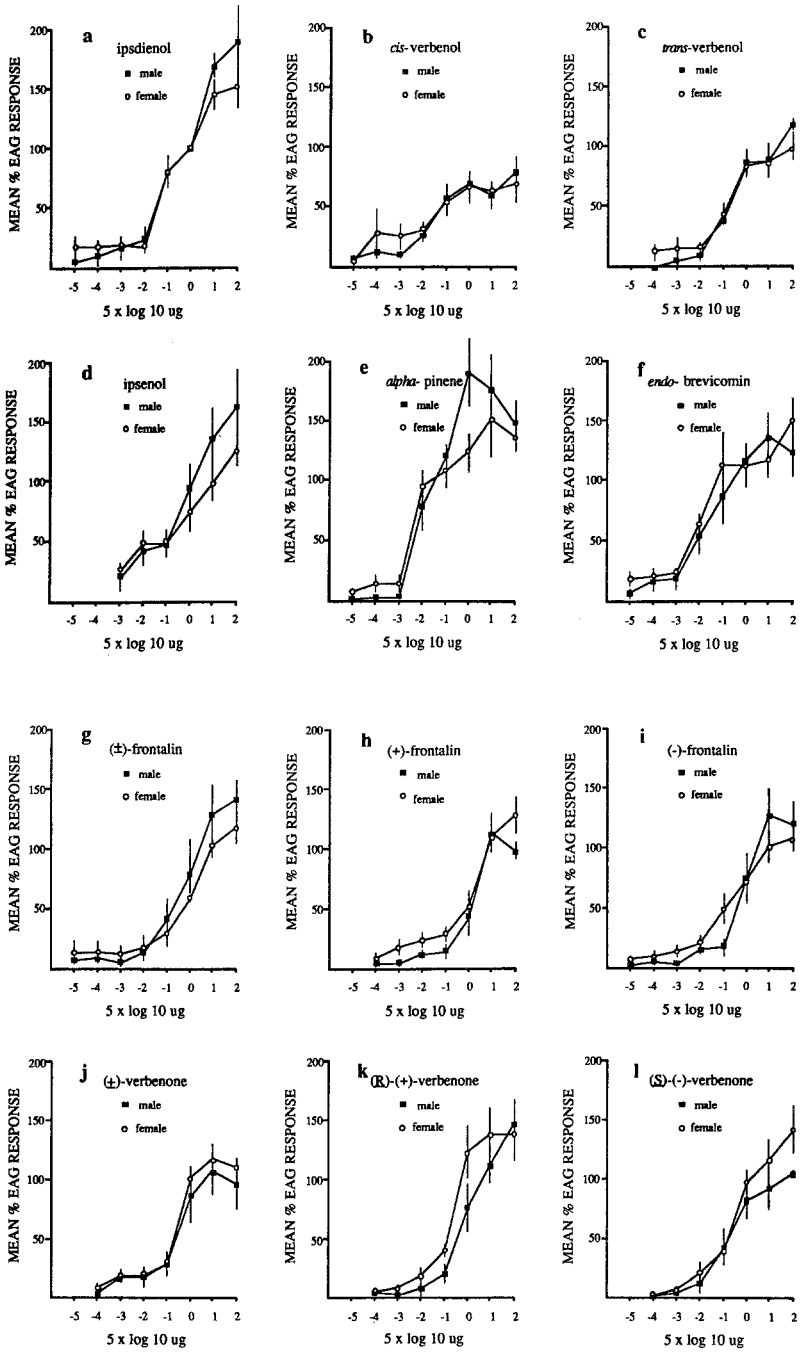

FIG. 3. Mean percent response (±SE) of *I. grandicollis* to behavioral chemicals. Means are percent of EAG to the standard ipsdienol.

ception, and behavioral response. Specificity in bark beetle species has been considered via behavioral response rather than pheromone production or perception (Pitman et al., 1969; Vité and Francke, 1976). However, pheromone production as well as behavioral response information provide supportive evidence for the olfactory perception data presented here.

The high sensitivity of *I. avulsus* and *I. calligraphus* to ipsdienol, and of *I. grandicollis* to ipsenol, is not surprising since these compounds are produced by males, and they are believed to be the primary aggregation pheromones of the respective species (Vité and Renwick, 1971b; Renwick and Vité, 1972; Vité et al., 1972; Hughes 1974). Each species had a somewhat lower sensitivity to *cis*- and *trans*-verbenol which are produced by males of each species, as well as females of *I. calligraphus* (Renwick and Vité, 1972; Vité et al., 1972).

There have been only a few studies of the olfactory behavior of *I. avulsus*, *I. calligraphus*, and *I. grandicollis*; therefore, data are limited for correlation with antennal olfactory response. Compounds tested under field conditions and shown to be attractants, synergists, and/or interruptants of *I. avulsus*, *I. calligraphus*, and *I. grandicollis* are summarized in Tables 2, 3, and 4, respectively. Additionally, based upon behavioral response of the three species to pine bolts infested with the various species of the southern pine bark beetle guild, compounds released by each species, as well as the host, are implicated as functioning in various behavioral capacities (Table 5). Collectively, these data show behavioral significance and possible correlation for the antennal olfactory responsiveness of the three *Ips* species to all compounds tested.

Interspecific Responses. Except for *cis*-verbenol, the olfactory receptors of *I. grandicollis* had lower threshold and higher saturation levels for all compounds tested than those observed for *I. avulsus* and *I. calligraphus* (Figure 4).

TABLE 2. SUGGESTED ROLE OF SPECIFIC BEHAVIORAL CHEMICALS IN OLFACTORY COMMUNICATION SYSTEM OF *Ips Avulsus*

Attractant (ref.)[a]	Synergist of attractant (ref.)[a]	Interruptant of attractant (ref.)[a]
Ipsdienol (1, 3)[b]	Ipsenol (2, 3), (*R*)-(+)ipsenol (3), (*S*)-(−)ipsenol (3)[b]	
(*R*)-(−)-Ipsdienol (*S*)-(−)Ipsenol (3)[b]	Ipsenol (2)	(*S*)-(+)-Ipsdienol (2)
Ipsdienol + ipsenol + *cis*-verbenol (4)		Turpentine (4)
endo- + *exo*-Brevicomin (5)		

[a] (1) Renwick and Vité, 1972; (2) Vité et al., 1978; (3) Hedden et al., 1976; (4) Billings, 1985; (5) Richerson and Payne, 1979.

[b] Response was significantly higher to a combination of ipsdienol and ipsenol than to either pheromone alone.

TABLE 3. SUGGESTED ROLE OF SPECIFIC BEHAVIORAL CHEMICALS IN OLFACTORY COMMUNICATION SYSTEM OF *Ips calligraphus*

Attractant (ref.)[a]	Synergist of attractant	Interruptant of attractant (ref.)[a]
Ipsdienol (1)		
cis-Verbenol (1)		
Ipsdienol + *cis*-verbenol (1)		
Ipsdienol + *trans*-Verbenol (1)		
Ipsdienol + *cis*-verbenol + *I. calligraphus* infested log (1)		
Ipsdienol + *cis*-verbenol + uninfested log (1)		
Ipsdienol + (*S*)-*cis*-verbenol (2)		(*R*)-*cis*-Verbenol (2)
(*R*)-(−)-Ipsdienol + (*S*)-*cis*-verbenol (3)		(*S*)-(+)-Ipsdienol (3)
Ipsdienol + ipsenol + *cis*-verbenol (4)		

[a]Renwick and Vité, 1972; (2) Vité et al., 1976b; (3) Vité et al., 1978; (4) Billings, 1985.

TABLE 4. SUGGESTED ROLE OF SPECIFIC BEHAVIORAL CHEMICALS IN OLFACTORY COMMUNICATION SYSTEM OF *Ips grandicollis*

Attractant (ref.)[a]	Synergist of attractant (ref.)[a]	Interruptant of attractant (ref.)
Ipsenol (1, 2)	*trans*-Verbenol (1)	
(*S*)-(−)-ipsenol (2)		(*R*)-(+)-Ipsenol (2)
Ipsdienol (2)		Frontalin (4)
Frontalin + turpentine + *trans*-verbenol (5)		
Frontalin + turpentine +*exo*-brevicomin (5)		
α-Pinene (1)	*trans*-Verbenol (1), *cis*-verbenol (1)	
trans-Verbenol (1)		
D,L-Camphene (3)	*I. grandicollis* frass extract (3)	
D-Limonene (3)	*I. grandicollis* frass extract (3)	
Methyl chavicol (3)	*I. grandicollis* frass extract (3)	
Mycrene (3)	*I. grandicollis* frass extract (3)	
Geraniol (3)		
Ipsdienol + ipsenol + *cis*-verbenol (6)	Turpentine (6)	

[a](1) Vité and Renwick, 1971b; (2) Vité et al., 1976a; (3) Werner, 1972; (4) Birch and Svihra, 1979; (5) Dixon and Payne, 1980; (6) Billings, 1985.

TABLE 5. BEHAVIORAL RESPONSE OF *I. avulsus*, *I. calligraphus*, AND *I. grandicollis* TO PINE BOLTS INFESTED WITH VARIOUS SYMPATRIC BARK BEETLE SPECIES

Species	Attracted to logs infested with (ref.)[a]	Attractancy enhanced by addition of (ref.)[a]	Attractancy inhibited by addition of (ref.)[a]
I. avulsus	*I. avulsus* (2, 7, 8)	*I. grandicollis* (3, 6, 8)	
	I. calligraphus (6–8)	*I. grandicollis* (8)	*D. frontalis* (8)
	I. grandicollis (8)		*D. frontalis* (8)
	D. frontalis (2)		
I. calligraphus	*I. calligraphus* (2, 7, 8)	*I. avulsus* (8),	*I. avulsus* (6),
		I. grandicollis (8)	*I. grandicollis* (8)
	I. avulsus (8)		
	I. grandicollis (8)		
	D. frontalis (2)		
I. grandicollis	*I. grandicollis* (1, 2, 4, 5, 7, 8)	*I. calligraphus* (5, 6)	*D. frontalis* (5, 6, 8)
	I. avulsus (8)	*I. calligraphus* (8)	
	I. calligraphus (7, 8)		*D. frontalis* (8)
	D. frontalis (2, 5, 6, 8)		

[a](1) Vité and Renwick, 1971b; (2) Vité et al., 1964; (3) Hedden et al., 1976; (4) Hertel et al., 1969; (5) Birch and Svihra, 1979; (6) Svihra et al., 1980; (7) Svihra, 1982; (8) Birch et al., 1980.

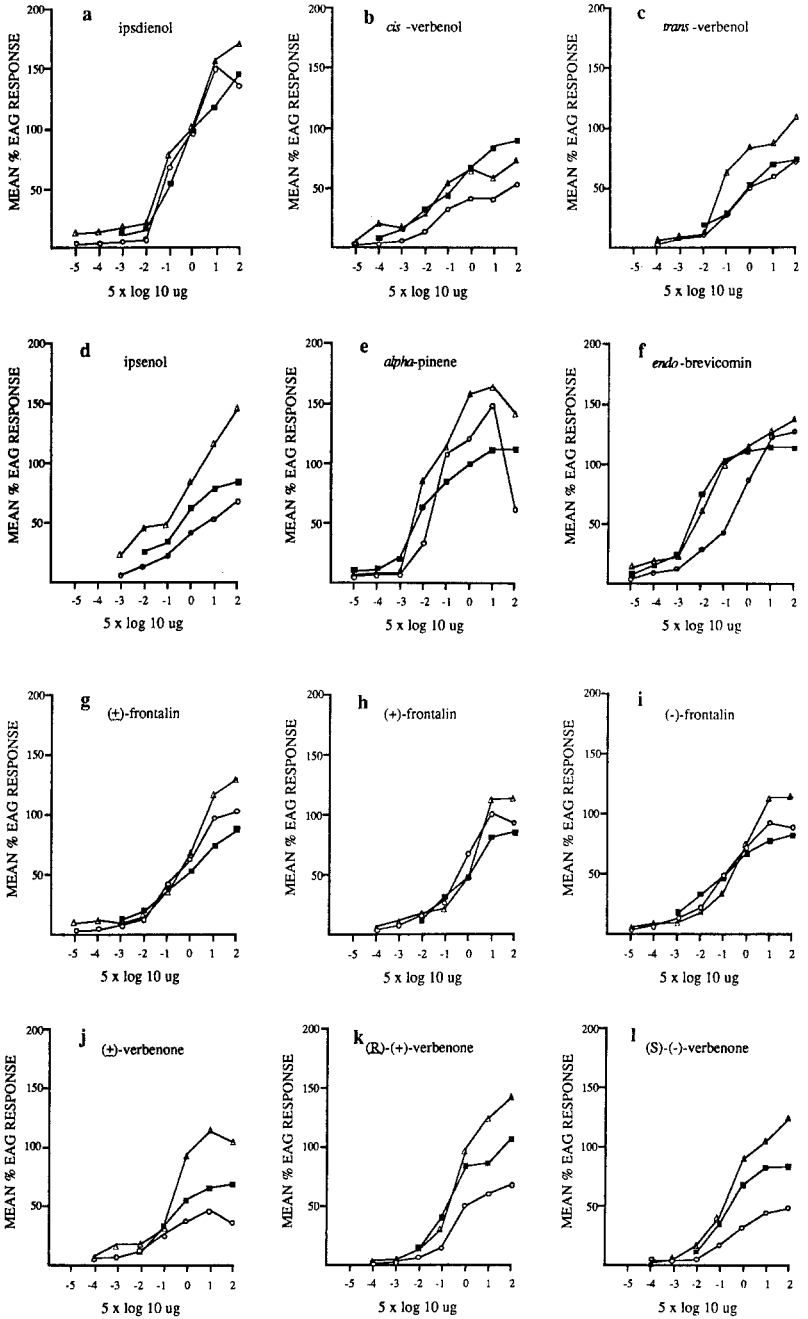

FIG. 4. Mean percent response of *I. avulsus*, *I. calligraphus* and *I. grandicollis* to behavioral chemicals. Means are percent EAG to the standard ipsdienol. ○ = *I. avulsus*; ■ = *I. calligraphus*; △ = *I. grandicollis*.

This was particularly apparent for ipsenol, α-pinene, *endo*-brevicomin, and (*S*)-(−)-verbenone. For field tests, *I. grandicollis* was more general in its response to intra- and interspecific olfactory stimuli than were the other species of the bark beetle guild (Vité et al., 1964). Birch et al. (1980) stated that, in terms of overlapping areas of colonization on a given host, *I. grandicollis* was the least interactive species among the southern pine beetle guild and was largely confined to the larger branches of the crown. Furthermore, Paine et al. (1981) found *I. grandicollis* occupied significantly less area when in the presence of any other bark beetle species or pair of species. The apparent sensitivity of *I. grandicollis* for the compounds may orchestrate this host selection and/or partitioning phenomenon. In comparison, *I. avulsus* and *I. calligraphus* were somewhat less sensitive to the compounds and are more often found with overlapping patterns of colonization.

Interestingly enough, *trans*-verbenol acceptors in *I. grandicollis* are more abundant than similar acceptors possessed by *I. avulsus* and *I. calligraphus* (Figure 4c). *trans*-Verbenol is known to be a synergist of (*S*)-(−)-ipsenol in the pheromone system of *I. grandicollis* (Vité and Renwick, 1971b). Also noteworthy, *cis*-verbenol acceptors are more abundant in *I. calligraphus* than in *I. avulsus* and *I. grandicollis* (Figure 4b). *cis*-Verbenol is not a synergist, but together with (*R*)-(−)-ipsdienol, it is the primary attractant in the apparently dual component pheromone system of *I. calligraphus* (Vité et al., 1978).

EAG results presented here correlate well with the fact that the antennal olfactory receptors of the species are relatively sensitive to compounds that have been implicated as playing a role in interspecific olfactory behavior (Tables 2–5).

CONCLUSIONS

Each *Ips* species possesses receptors with lower thresholds and in greater abundance for the compounds they produce and to which they are behaviorally most responsive. Detection of both *Ips* and *Dendroctonus* pheromonal compounds by the three cohabiting *Ips* species shows a sensory basis for interaction among the species. The fact that differences in both threshold responses and relative numbers of acceptors for the various behavioral chemicals do occur could denote differences in specific behavioral roles for each compound. Although past studies have indicated various attractancy and/or inhibitory interactions among these species (Vité and Renwick, 1971b; Vité et al., 1964; Hedden et al., 1976; Hertel et al., 1969; Birch and Svihra, 1979; Svihra et al., 1980; Svihra, 1982; Birch et al., 1980; Paine et al., 1981), future research may reveal more covert relationships of the intra- and interspecific chemical messengers among the species.

These data provide the basis for a detailed investigation of the underlying sensory mechanisms that enables interspecific olfactory communication. Single-cell analysis aimed at providing this information is currently underway in our laboratory.

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SITES OF PRODUCTION AND OCCURRENCE OF VOLATILES IN DOUGLAS-FIR BEETLE, *Dendroctonus pseudotsugae* HOPKINS

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Abstract—Volatiles were found to be distributed throughout adult Douglas-fir beetles, *Dendroctonus pseudotsugae* Hopkins, suggesting that the hindgut may not be the sole site of production. The volatile content of individual beetles increased dramatically with feeding; qualitative changes reflected the different bark habitats of newly emerged and established beetles. All detected volatiles were also found in beetle frass. Myrcene was the predominant monoterpene of emergent beetles and α-pinene of fed beetles. Linalool and bornyl acetate occurred in significant amounts after feeding, and diacetone alcohol and sulcatol were also detected for the first time. The presence of sulcatol explains the reported cross-attraction with *Gnathotrichus* spp.. *cis*-Verbenol was also found to occur in addition to *trans*-verbenol. The volatile content of male beetles was similar to that of females but amounts of individual compounds were less. Acetic acid was found in both emerged and fed beetles and in lesser amounts in frass, suggesting that microbial metabolism could be a potential source of volatile production.

Key Words—Douglas-fir beetle, *Dendroctonus pseudotsugae*, Coleoptera, Scolytidae, monoterpenes, volatiles, frass, cross-attraction.

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INTRODUCTION

The Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopkins (DFB), is an important pest of Douglas-fir, *Pseudotsuga menziesii* Mirb. (Franco), throughout its natural range (Furniss and Carolin, 1977). One approach to its control has been the use of semiochemicals either to induce attack and contain infestations around trees baited with attractive semiochemicals (Pitman, 1973; Ringold et al., 1975), or to repel beetles from trees treated with pheromone components (Furniss et al., 1972, 1974, 1977).

A number of beetle-produced compounds have been reported to act singly or in combination to aggregate DFB and include frontalin (Kinzer et al., 1969), *trans*-verbenol (Rudinsky et al., 1972), 3,2-seudenol (3,2-MCHol) (Rudinsky et al., 1973), and 1,2-seudenol (1,2-MCHol) (Libbey et al., 1983) (see Table 1 for trivial names of some of these compounds used in this paper). In addition, certain host monoterpenes, notably α-pinene, camphene (Pitman and Vité, 1970), ethanol and *trans*-3-penten-1-ol (Pitman and Vité, 1970) may act as attractive kairomones either alone or by synergizing the attractive pheromones (Pitman, 1973).

In contrast, 3,2- (Kinzer et al., 1971) and 3,3-MCH-one (Rudinsky and Ryker, 1980) and verbenone (Rudinsky et al., 1973) were found to act as antiaggregation pheromones, although both verbenone and 3,2-MCH-one were claimed to be concentration-dependent, multifunctional pheromones (Rudinsky and Ryker, 1980).

The female hindgut has been suggested as the site of production of frontalin, *trans*-verbenol, 3,2- and 1,2-MCHol, verbenone, and *trans*-3-pentenol.

TABLE 1. ABBREVIATED OR TRIVIAL AND CHEMICAL NAMES OF CERTAIN VOLATILES MENTIONED IN THIS PAPER

Trivial name[a]	Chemical name	Reference
1,2-MCHol	1-methyl-2-cyclohexen-1-ol	Libbey et al. (1983)
3,2-MCHol	3-methyl-2-cyclohexen-1-ol	Vité et al. (1972)
3,2-MCH-one	3-methyl-2-cyclohexen-1-one	Kinzer et al. (1971)
3,3-MCH-one	3-methyl-3-cyclohexen-1-one	Libbey et al. (1976)
Sulcatol	6-methyl-5-hepten-2-ol	Borden et al. (1976)
Sulcatone	6-methyl-5-hepten-2-one	Ryker et al. (1979)

[a] 3,2-MCHol was originally given the trivial name "seudenol" (Vité et al., 1972) and 3,2-MCH-one has been commonly referred to as MCH (Borden, 1982). However, the occurrence and activity of 1,2-MCHol demands either a new trivial name be given or that the trivial names seudenol and MCH be replaced by more descriptive abbreviations. We have chosen the latter option. On the other hand, we have used the trivial name sulcatone for 6-methyl-5-hepten-2-one which corresponds well with the established trivial name sulcatol.

Other volatiles include 3,2- and 3,3-MCH-one released by female DFB and 6-methyl-5-hepten-2-one released by both males and females (Ryker et al., 1979). Fresh DFB frass was found to be highly attractive to male DFB in laboratory bioassays (Borden et al., 1968), but frass volatiles have not been analyzed exhaustively.

The objectives of this investigation were to assess and compare the volatiles of unfed and fed DFB of both sexes and DFB frass.

METHODS AND MATERIALS

Beetle-infested, Douglas-fir bark was collected from different sources throughout the Fraser Valley and Canyon in southern British Columbia. The bark was stored at 4–5°C or incubated at 28°C to induce emergence. Individual beetles were excised from bark from infested stumps and felled trees at the University of British Columbia Research Forest, Maple Ridge, British Columbia and at the British Columbia Forest Service Cowichan Lake Experiment Station, Mesachie Lake, British Columbia.

Beetles were established within 24 hr of emergence in vertical, 2-month-old, Douglas-fir logs at two-day intervals by inserting a female, and 6 hr later a male, into a 0.5-cm-diameter hole cut with a cork borer. A gelatin capsule pressed into each hole prevented escape of beetles. Capsules were replaced every two days, and the collected frass was weighed and stored in screw-cap vials at −20°C.

All beetles, whether excised from bark prior to emergence, after establishment, or allowed to emerge naturally, were handled identically thereafter. Individual beetles were placed in numbered vials on Dry Ice. The frozen beetle was sexed, weighed, and sectioned transversely between the prothoracic–elytral junction and the separate sections placed into 100 μl of pentane in chilled 2-ml vials. Sectioning divided the alimentary system in the vicinity of the proventriculus so that the anterior section contained the foregut and the posterior section contained the mid- and hindguts.

Standard solutions of 2- and 3-octanol were prepared by adding 2.5 μl of the appropriate alcohol to 10 ml of redistilled pentane. The frozen sections were allowed to thaw and 2 μl of 2-octanol solution were added to each vial. The sections were refrozen, crushed with the flat head of a stainless-steel probe, and 2 μl of 3-octanol solution added. Minimal difference in the subsequent peak heights of 2- and 3-octanol indicated little loss of volatiles during maceration. Vials were permitted to thaw at room temperature and shaken to ensure solvent penetration; the tissue was refrozen and the pentane extract transferred to clean vials which were sealed with Teflon-lined caps prior to storage at −20°C.

Weighed samples of frass were covered with a known volume of pentane

containing 2-octanol marker, capped, and allowed to stand for 16 hr at 10°C. The water and frass components were frozen on Dry Ice, and the pentane extract transferred to clean vials and stored.

Thirty-gram samples of bark, containing either overwintering or attacking beetles, were steam distilled in a glass still for 1 hr, cooled, and the distilled oil collected in pentane containing octanol markers.

Individual extracts and oil samples were analyzed by splitless injection of 2 μl of extract into a Hewlett-Packard 5830A gas chromatograph programmed to run isothermally at 65°C for 8.5 min after elution of the solvent peak and thereafter to 160°C at 5°C/min. A glass capillary column (30 m $\times$ 0.66 mm ID) coated with SP-1000 (Supelco Inc., Bellefonte, Pennsylvania) was employed. The retention times of different compounds were compared to those of a standard quantitative mix of monoterpenes and compounds reported in DFB volatiles and octanol markers. The identities of all known pheromones and kairomones and additional, commonly occurring compounds were verified by mass spectrometry.

The percentage content of identified compounds was divided by that obtained for the 2-octanol marker to make separate runs comparable. Comparisons with the octanol peaks allowed conversion of amounts of each compound into micrograms per beetle segment or micrograms per milligram of frass.

The data obtained for different life-stage classes of beetles and their frass were analyzed following $\log_{10}(x + 1)$ transformation, except where otherwise stated, and comparisons of mean volatile contents of individual compounds between different classes of beetles were by the Newman-Keul's test. The untransformed means and standard errors of amounts of extracted volatiles are shown in the tables to indicate the increasing variability in the volatile status between individual beetles with time. Proportional amounts of compounds in beetles and bark extracts were compared, following arcsin transformation.

RESULTS

Twenty-one compounds were commonly found in extracts of DFBs and their frass; amounts of individual compounds varied with stage of adult development and behavior.

No difference was found between the monoterpenoid volatiles in anterior and posterior sections of individual female beetles, with the exception of *trans*-verbenol (Table 2). Emergent beetles contained less and fewer volatiles than beetles which had experienced 48 hr feeding and maturation within new host material. At emergence, the myrcene content was relatively high, but after feeding, levels of monoterpenes, with the exception of myrcene and terpinolene, increased significantly (Table 3). The α-pinene content increased 225-fold (P

TABLE 2. COMPARISON OF MONOTERPENOID VOLATILES EXTRACTED FROM ANTERIOR AND POSTERIOR SECTIONS OF 9 NEWLY EMERGED DFB FEMALES

Compound	Volatiles/section (μg, $\bar{X} \pm$ SE)	
	Anterior section	Posterior section
α-Pinene	0.117 ± 0.020	0.155 ± 0.013
Camphene	0.015 ± 0.002	0.014 ± 0.004
β-Pinene	0.101 ± 0.020	0.100 ± 0.016
Δ-3-Carene	0.140 ± 0.019	0.139 ± 0.016
Myrcene	5.741 ± 0.683	5.841 ± 0.734
Limonene	0.299 ± 0.046	0.377 ± 0.043
Terpinolene	0.009 ± 0.003	0.011 ± 0.001
cis-Verbenol	0.005 ± 0.001	0.005 ± 0.004
trans-Verbenol	0.005 ± 0.001	0.048 ± 0.023
Verbenone	0.011 ± 0.002	0.013 ± 0.004
α-Pinene/myrcene ratio	0.020 ± 0.004	0.027 ± 0.007

< 0.05), but myrcene declined 11-fold in comparison to the levels at emergence ($P < 0.05$). Levels of the monoterpene derivatives, *cis*- and *trans*-verbenol and verbenone, also increased but linalool and bornyl acetate only occurred after beetles had fed in new bark (Table 3). Acetic acid was consis-

TABLE 3. COMPARISON OF MONOTERPENOID VOLATILES EXTRACTED FROM POSTERIOR SECTIONS OF EARLY FEEDING DFB FEMALES WITH LITTLE FOOD IN MIDGUT ($N = 5$) AND FULLY FED FEMALES WITH MIDGUT FULLY DISTENDED WITH FOOD ($N = 12$)

Compound	Volatiles/section (μg, $X \pm$ SE)	
	Early feeding females	Fed, preoviposition females
α-pinene	0.335 ± 0.043	25.500 ± 8.959
Camphene	0.001	0.326 ± 0.106
β-Pinene	0.047 ± .019	2.404 ± 1.124
Δ-3-Carene	0.030 ± .026	0.070 ± 0.040
Myrcene	0.132 ± .004	0.489 ± 0.178
Limonene	0.024 ± .004	0.292 ± 0.119
Linalool	0.083 ± 0.048	0.371 ± 0.162
Bornyl acetate	0.053 ± 0.048	0.194 ± 0.058
cis-Verbenol	0.025 ± 0.010	1.214 ± 1.037
trans-Verbenol	0.036 ± 0.021	0.256 ± 0.143
Verbenone	0.016 ± 0.006	1.307 ± 0.937
α-Pinene/myrcene ratio	13.73 ± 5.01	52.77 ± 16.56

tently detected, but in differing amounts in postdiapause (active), emergent, and newly established beetles; maximum amounts were recorded at emergence (Tables 4 and 5).

The differences in the distribution of volatiles of emerged and fed beetles reflected in a general way the monoterpene composition of the respective bark habitats which differed in age, degree of infestation, and food and moisture status. In a comparison of the relative proportions of host tree volatiles within beetles and bark oils, 14 individual compounds in both beetles and bark oils were highly correlated between fed and mature beetles in fresh bark ($r = 0.972$; $N = 14$; $P < 0.005$) but less so between emergent beetles and the bark from which they emerged ($r = 0.531$; $N = 14$; $P < 0.10$).

Compounds not detected in bark oil volatiles but present in beetles on emergence were frontalin, diacetone alcohol, 1,2-MCHol, 3,2-MCHol, and 3,2-MCH-one. Levels of these compounds changed significantly after feeding in new hosts (Table 4). The same materials were recovered from the frass of beetles that had been feeding for approximately 48 hr. With the exception of myrcene and terpinolene, all monoterpenes and their derivatives increased in frass. Similarly, levels of frontalin and 1,2-MCHol increased, but 3,2-MCHol and 3,2-MCH-one declined. Sulcatol and sulcatone were also detected in mature females (Tables 4 and 5), and relatively high amounts of sulcatol were detected in 48-hr frass (Table 5). An unidentified sesquiterpene (molecular ion 204, fragments 161, 189, and retention time 14.19 min) was detected in small amounts in emergent and fully fed beetles.

Beetles established in a relatively fresh (five weeks old) log had consis-

TABLE 4. COMPARISON OF NONMONOTERPENOID VOLATILES EXTRACTED FROM POSTERIOR SECTIONS OF NEWLY EMERGED, UNFED DFB FEMALES ($N = 9$) AND FULLY FED FEMALES WITH MIDGUT DISTENDED WITH FOOD ($N = 12$)

Compound	Volatiles/section (μg, $\bar{X} \pm$ SE)	
	Newly emerged females	Fed, preoviposition females
Frontalin	0.024 ± 0.005	0.002
Diacetone alcohol	0.018 ± 0.007	0.177 ± 0.043
1,2-MCHol	0.009 ± 0.002	0.012
3,2-MCHol	0.014 ± 0.003	0.005 ± 0.002
3,2-MCH-one	0.013 ± 0.004	0
Sulcatone	0	0.011 ± 0.008
Sulcatol	0	0.003 ± 0.002
Acetic acid	0.242 ± 0.060	0.035 ± 0.003

TABLE 5. COMPARISON OF TOTAL VOLATILES EXTRACTED FROM DFB FEMALES WITH MATURING OVARIOLES ($N = 10$) AND THEIR FRASS, 2 DAYS FOLLOWING ESTABLISHMENT

Compound	Volatile content ($\bar{X} \pm$ SE) DFB females (μg/section)[a]	DFB frass (μg/mg frass)[b]
α-pinene	9.954 ± 4.457	16.578 ± 4.574
Camphene	0.072 ± 0.034	0.236 ± 0.058
β-Pinene	0.830 ± 0.344	1.585 ± 0.412
Δ-3-Carene	0.050 ± 0.020	0.073 ± 0.022
Myrcene	0.204 ± 0.073	0.209 ± 0.051
Limonene	0.070 ± 0.035	0.171 ± 0.049
Terpinolene	0.062 ± 0.026	0.100 ± 0.046
Linalool	0.190 ± 0.076	0.613 ± 0.302
Bornyl acetate	0.104 ± 0.039	0.240 ± 0.064
cis-Verbenol	0.106 ± 0.059	0.316 ± 0.126
trans-Verbenol	0.103 ± 0.045	0.644 ± 0.250
Verbenone	0.011 ± 0.005	0.197 ± 0.097
Frontalin	0.012 ± 0.005	0.028 ± 0.005
Diacetone alcohol	0.015 ± 0.006	0.014 ± 0.007
1,2-MCHol	0.007 ± 0.001	0.036 ± 0.027
3,2-MCHol	0.041 ± 0.010	0.012 ± 0.004
3,2-MCH-one	0.008 ± 0.002	0.008 ± 0.005
Sulcatone	0.001	0
Sulcatol	0.001	0.022 ± 0.007
Acetic acid	0.128 ± 0.037	0.030 ± 0.003
α-Pinene/myrcene ratio	57.05 ± 16.51	78.75 ± 10.26

[a] Mean live weight of female = 19.31 ± 0.62 mg.
[b] Mean weight of frass produced/female = 5.17 ± 0.69 mg.

tently high levels of sulcatone but little sulcatol. In contrast, beetles attacking the same log four weeks later showed a 10-fold increase in sulcatol but little change in sulcatone (Table 6).

The volatile content of male DFBs that had been resident with females in fresh bark for 48 hr was significantly greater, both qualitatively and quantitatively ($P < 0.05$), than that of diapausing or emerged males (Table 7). At this time, levels of all compounds were significantly less ($P < 0.05$) than that of their female partners, but small amounts of frontalin were detected despite its absence on emergence.

The data were examined to assess any significant correlations between the various compounds. The two forms of verbenol were detected either singly or

TABLE 6. COMPARISON OF TOTAL VOLATILES EXTRACTED FROM POSTERIOR SECTIONS OF DFB FEMALES ESTABLISHED AT DIFFERENT TIMES IN SAME LOGS FROM SAME TREES[a]

Compound	Volatiles/section (μg, $\bar{X}$ ± SE)	
	Established May 12, 1983	Established June 16, 1983
α-Pinene	0.080 ± 0.029	15.657 ± 5.075
Camphene	0.006 ± 0.004	0.117 ± 0.036
β-Pinene	0.006 ± 0.002	1.365 ± 0.447
Δ-3-Carene	0.001	0.069 ± 0.022
Myrcene	0.004 ± 0.001	0.387 ± 0.105
Limonene	0.001 ± 0.001	0.161 ± 0.047
Terpinolene	0.002 ± 0.001	0.054 ± 0.034
Linalool	0.004 ± 0.001	0.711 ± 0.393
Bornyl acetate	0.002 ± 0.001	0.253 ± 0.125
cis-Verbenol	0.001	0.196 ± 0.079
trans-Verbenol	0.002 ± 0.001	0.134 ± 0.045
Verbenone	0	0.038 ± 0.019
Frontalin	0	0.007 ± 0.004
Diacetone alcohol	0	0.011 ± 0.007
1,2-MCHol	0.015 ± 0.003	0.064 ± 0.026
3,2-MCHol	0.006 ± 0.002	
3,2-MCH-one	0.002	0.024 ± 0.011
Sulcatone	0.008 ± 0.004	0.009 ± 0.006
Sulcatol	0.002 ± 0.001	0.019 ± 0.010
Acetic acid	0.023 ± 0.005	0.151 ± 0.050
α-Pinene/myrcene ratio	16.15 ± 5.80	49.25 ± 16.04

[a]Beetles had been in the logs 3.0 ± 0.4 and 2.8 ± 0.7 days for the May 12 and June 16, 1983, establishment times, respectively. The respective gallery lengths were 1.6 ± 0.3 and 2.7 ± 0.4 cm.

in combination and total amounts (*cis* plus *trans*) were compared to α-pinene and verbenone levels. The regression of total verbenols on α-pinene was highly significant ($r = 0.678$; $N = 50$; $P << 0.0005$) with 46% of the variation being explained by the relationship. The regression of verbenone and total verbenols was not significant ($P > 0.05$), although the regressions of verbenone to either of the two compounds were significant ($r = 0.71$; $N = 26$; $P << 0.0005$ and $r = 0.50$; $N = 30$; $P < 0.01$, respectively). A significant regression was found without transformation between *cis*- and *trans*-verbenol ($r = 0.81$; $N = 29$; $P << 0.0005$) and the regressions of verbenone to the two compounds. For the comparison of total verbenol and verbenone in beetles excised from bark, the

TABLE 7. VOLATILE CONTENT IN POSTERIOR SECTION OF MALE DFB IN THREE LIFE STAGES

Compound	Volatiles/section μg, $\bar{X}$ ± SE)		
	Diapausing males ($N = 5$)[a]	Emerged males ($N = 5$)[b]	Males with females in new logs ($N = 7$)[c]
α-Pinene	0.077 ± 0.034	0.024 ± 0.005	6.584 ± 1.512
Camphene	0.001	0	0.860 ± 0.016
β-Pinene	0.025 ± 0.010	0.014 ± 0.004	0.239 ± 0.073
Δ-3-Carene	0.001	0	0.064 ± 0.048
Myrcene	0.088 ± 0.034	0.001	0.328 ± 0.201
Limonene	0.011 ± 0.006	0.001	0
Terpinolene	0.054 ± 0.017	0	0.002 ± 0.001
Linalool	0	0	0.011 ± 0.067
Bornyl acetate	0	0	0.030 ± 0.017
cis-Verbenol	0	0.003 ± 0.001	0.037 ± 0.012
trans-Verbenol	0	0.003 ± 0.001	0.013 ± 0.007
Verbenone	0.001	0.009 ± 0.001	0.046 ± 0.029
Frontalin	0	0	0.017 ± 0.008
Diacetone alcohol	0	0	0.008 ± 0.003
1,2-MCHol	0	0	0.012 ± 0.006
3,2-MCHol	0	0	0.006 ± 0.005
3,2-MCH-one	0	0	0.004 ± 0.002
Sulcatone	0	0	0.002 ± 0.001
Acetic acid	0.001	0.006 ± 0.001	0.042 ± 0.014
α-pinene/myrcene ratio	1.4 ± 0.3	0	60.0 ± 14.2

[a] Diapause beetles ex Clearwater, British Columbia.
[b] Emerged beetles ex Mission, British Columbia.
[c] New log males ex UBC forest, and Mission, British Columbia.

regression was significant for females ($r = 0.77$; $N = 6$; $P < 0.025$) but not for males ($r = 0.81$; $N = 8$; $P < 0.25$).

Of 66 females, either established in logs or collected from the field, 40% contained sulcatone but no sulcatol; 44% contained sulcatol only; and eight beetles had both compounds among their volatiles. Significant relationships between the occurrence of sulcatol and sulcatone ($r = 0.87$; $N = 8$; $P < 0.05$), 1,2-MCHol and 3,2-MCHol ($r = 0.52$; $N = 15$; $P < 0.05$), and 3,2-MCH-one and 3,2-MCHol ($r = 0.57$; $N = 14$; $P < 0.05$) were found but only in active, preoviposition beetles. Sulcatol did not occur, and there was no significant relationship between either 1,2-MCHol and 3,2-MCHol or 3,2-MCHol and 3,2-MCH-one in newly emerged beetles.

DISCUSSION

Our investigation has demonstrated for the first time the occurrence of the following volatiles in the DFB: sulcatol, *cis*-verbenol, linalool, bornyl acetate, and diacetone alcohol. Frontalin (Pitman and Vité, 1970), *trans*-verbenol (Rudinsky et al., 1972), verbenone (Rudinsky et al., 1973), 1,2-MCHol (Libbey et al., 1983), and 3,2-MCHol (Vité et al., 1972) were previously identified from dissected and extracted hindguts of female DFBs. Reference to an unpublished account of the production of 1,2-MCHol in the hindgut of DFB females had been made earlier (Renwick and Hughes, 1975). However, 3,2-MCH-one was found in the headspace above male and female beetles (Kinzer et al., 1971), while 1,2-MCH (Libbey et al., 1976) and sulcatone (Ryker et al., 1979) were identified from male volatiles only.

Our results demonstrate that many volatiles are distributed throughout the beetle, suggesting that sequestering of volatiles, in addition to their production and release, may occur in the anterior region of the beetles as well as the posterior. These findings contrast with those of Zenther-Moller and Rudinsky (1967) and Hughes (1973). The former authors considered that pheromone production was localized within the hindgut and/or the Malpighian tubules; the latter author hypothesized that attractants produced outside the alimentary tract, in response to exposure to monoterpene vapors, were secreted into the hindgut via the Malpighian tubules. Furthermore, with the exception of frontalin, the volatile spectrum of males and females was qualitatively identical.

The occurrence of frontalin in males cohabiting galleries with females is similar to that in males of other *Dendroctonus* spp., such as *D. ponderosae* Hopkins (Libbey et al., 1985), despite its absence at emergence. Two hypotheses could explain the occurrence of frontalin in these males, as well as the concurrent dramatic increases in other volatiles between emergence and after feeding: (1) the males synthesize or sequester these volatiles directly from the host, or (2) males ingest the borings of female beetles.

Monoterpene levels in fed beetles and new host bark were more closely correlated than those between emergent beetles and the bark from which they had emerged. The relatively high content of Δ-3-carene, myrcene, and limonene in emerged beetles suggests that such materials are preferentially retained, while more volatile components, e.g., α-pinene, are either voided or sequestered in a nonvolatile, nonextractable form, as has been suggested for the mountain pine beetle. Evidence of verbenol production proportional to α-pinene content was found. For the first time, *cis*-verbenol was found to occur either singly or associated with *trans*-verbenol. The occurrence of either form of the verbenols in 25% of the beetles analyzed reflected a difference in temporal production. A preferential biosynthetic production was suggested by the closer correlation of verbenone levels to those of *cis*- rather than *trans*-verbenol.

Borden et al. (1968) found that frass produced by females in the first 4–8 hr in a fresh log was most attractive to male DFBs, suggesting defecation was a major mechanism of volatile release. Furthermore, differences in aeration and microbial activity between frass and the hindgut (Brand et al., 1975, 1976) could potentiate the production of volatiles, e.g., *cis*- and *trans*-verbenol and verbenone. Neither *trans*-verbenol nor verbenone is attractive to DFB, while *cis*-verbenol has yet to be evaluated.

In addition to materials previously reported, 1,2-MCHol was also detected, and levels of this compound were correlated to those of 3,2-MCHol. However, 1,2-MCH-one (Libbey et al., 1983) was not detected and levels of 3,2-MCH-one were related significantly only to 3,2-MCHol. The low amount of variation explained by the regressions is perhaps indicative of the labile nature of these compounds, and no such relationships were found in beetles which had not fed. Similarly, linalool and bornyl acetate, which occurred in high amounts in fed beetles, were absent from both active postdiapause, emerging, and newly established, unfed beetles.

The positive correlation between sulcatone and sulcatol supports the proposition that sulcatone is either a precursor or product of sulcatol (Brand et al., 1977). The production of sulcatol by the DFB adult and its occurrence in frass undoubtedly explains the cross-attraction of *Gnathotrichus* spp. to DFB-attacked logs (Rudinsky and Daterman, 1964), as this compound is a major pheromone utilized by members of this genus (Borden, 1982).

Although acetic acid content was highest at emergence, the increase recorded after feeding and its absence in frass at this time further emphasizes the importance of both aeration and microbial activity in generating biologically active volatiles (Brand et al., 1975, 1976, 1977).

During the course of the experimentation, it was found that the practice of establishment of many beetles by manual insertion into logs of variable and unknown quality produced aberrant results. Despite extensive gallery formation, many of these beetles were found by dissection not to contain any material within the alimentary tract, and myrcene remained the dominant monoterpene. In contrast, beetles that had freely selected and attacked host material contained appreciable amounts of phloem tissue, and α-pinene was the dominant volatile. Therefore, this commonly employed practice must be approached with caution, for it may bypass changes that occur during flight, does not always permit normal host acceptance, and may result in a sequence of volatile changes that rarely occur under natural conditions.

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AGGREGATION PHEROMONES IN *Drosophila borealis* AND *Drosophila littoralis*

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Abstract—Mature males of *Drosophila borealis* and *D. littoralis* (Diptera: Drosophilidae) produce pheromones that attract both males and females in a wind-tunnel bioassay. Ethyl tiglate is a major pheromone component in both species. Isopropyl tiglate is a minor component, as active as ethyl tiglate on an equal-weight basis, but less abundant in the flies. Both species respond to (Z)-9-heneicosene, a compound they do not possess, but which is a pheromone component in closely related species. *D. borealis* and *D. littoralis* are also able to discriminate among various heneicosene isomers. These responses to hydrocarbons may represent artifacts of evolution in this group. For both species, the pheromone was strongly synergized by an extract of fermenting aspen bark, a "host" material of *D. borealis*. Benzyl alcohol was identified as one active component, although it did not account for all the activity of the extract. In nature, the flies probably respond to mixtures of fly-derived and host-derived volatiles.

Key Words—Diptera, Drosophilidae, *Drosophila borealis*, *Drosophila littoralis*, pheromone, aggregation, ethyl tiglate, heneicosene, benzyl alcohol, *virilis* group.

INTRODUCTION

The existence of aggregation pheromones has been demonstrated in a number of *Drosophila* species (Bartelt and Jackson, 1984; Bartelt et al., 1985a,b, 1986a;

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Moats et al., 1987; Schaner et al., 1987). Mature males produce compounds that attract both males and females in a laboratory wind-tunnel bioassay. Many of the taxa that have been studied belong to the *virilis* species group. Comparative research on this group was of particular interest because the phylogeny of this group is well known (Throckmorton, 1982).

The *virilis* group consists of the *virilis* and *montana* phylads (Throckmorton, 1982). In the *virilis* phylad, four of the five taxa use heneicosenes as pheromone components. *D. virilis* uses (*Z*)-10-heneicosene (Z10–21), while *D. a. americana*, *D. a. texana*, and *D. novamexicana* use (*Z*)-9-heneicosene (*Z*9–21). The fifth taxon, *D. lummei*, lacks an active hydrocarbon. In contrast to the differences with respect to hydrocarbons, all the *virilis* phylad taxa possess the same five male-specific esters: ethyl tiglate, methyl tiglate, isopropyl tiglate, methyl hexanoate, and ethyl hexanoate. Ethyl tiglate was always the most abundant of these and was active in the pheromone bioassay for all these taxa except *D. a. americana*. The esters present in smaller amounts were also active in some cases. Thus the aggregation pheromones in this phylad generally consist of hydrocarbon and ester components, but *D. lummei* uses only the esters, primarily ethyl tiglate. Curiously, *D. lummei* responds readily to *Z*9–21 and *Z*10–21 in the bioassay, although it possesses neither compound, perhaps an evolutionary artifact (Bartelt et al., 1986a).

This research continues the study of the *virilis* group by considering two species of the *montana* phylad, *D. borealis* and *D. littoralis*. *D. borealis* is a North American species, while *D. littoralis* is European. Of particular interest was whether these species shared any of the ester pheromone components present in the *virilis* phylad. Secondly, these species were tested for responsiveness to heneicosenes, even though they possessed none nor was there any male–female dimorphism in hydrocarbon profiles (Bartelt et al., 1986b). We wished to know whether these species would respond to heneicosenes as *D. lummei* does. Such information could add to the understanding of how pheromone systems evolve.

METHODS AND MATERIALS

The *D. borealis* (strain 2077) and *D. littoralis* (strain 2096.1) cultures were obtained from Dr. L.H. Throckmorton. Rearing of cultures was as described previously (Bartelt and Jackson, 1984). Bioassays were conducted in a wind-tunnel olfactometer containing ca. 1000 one- to four-day-old flies which had been starved overnight before tests began. An extract, fraction, synthetic compound, or control solvent was applied to a filter paper strip inserted around the lip of a glass vial. Each bioassay test consisted of placing two such treated vials, to be compared, into the olfactometer for 3 min. Counts of flies entering the

vials were recorded. Details of bioassays, experimental design, and statistical analysis were as presented earlier (Bartelt and Jackson, 1984; Bartelt et al., 1986a).

Chromatographic methods and identification of tiglic and hexanoic esters were described previously (Bartelt et al., 1985a, 1986a). Briefly, crude extracts were separated into nonpolar and polar bioassay fractions by column chromatography on silicic acid, the solvents for the two fractions being 10% ether in hexane and 10% methanol in methylene chloride, respectively. Alternatively, the column was eluted with hexane before 10% ether in hexane, affording the "hydrocarbon" and "ester" subfractions of the nonpolar fraction. Esters were identified by capillary gas chromatography relative to synthetic standards and were quantitated using an internal standard. Purified synthetic esters and hydrocarbons were on hand from previous work.

The aspen (*Populus tremuloides* Michx.) extract was prepared by peeling ca. 5 g of bark from field-collected, dead, water-soaked aspen branches, <1.0 cm in diameter, and immediately treating the bark with 100 ml of methylene chloride in a Soxhlet apparatus for 3 hr. This extract was used for bioassays at the rate of 10 μl per test. Portions of the extract were fractionated by column chromatography on silicic acid, and these were also bioassayed. Identification of one active compound was based upon the electron impact mass spectrum and GC retention relative to an authentic standard.

RESULTS AND DISCUSSION

Ester Pheromone Components. Bioassay experiments that demonstrated the existence of aggregation pheromones in these species and that led to the chemical identification are summarized in Table 1. Unlike the members of the *virilis* phylad (Bartelt and Jackson, 1984; Bartelt et al., 1986a), *D. borealis* and *D. littoralis* never responded clearly to the extracts of mature males (Table 1A). Although significantly over control levels in most tests, these extracts seldom attracted more than two or three flies per 3-min test period. However, the pheromone preparations were strongly synergistic with an extract of fermented aspen bark. By adding the aspen extract to all subsequent bioassay vials (treatments and controls), larger bioassay catches and clearer tests for pheromonal activity were consistently obtained. Synergism between pheromones and "host" volatiles was previously demonstrated for all species of the *virilis* phylad (Bartelt et al., 1986a). The aspen extract was chosen for the present studies because *D. borealis* is known to use aspen as a host (Throckmorton, 1982).

As in the previously studied species, the extracts of mature males were far more active than those of females (Table 1B), but both sexes responded significantly. In Table 1B, for example, the captured *D. borealis* were 68% males and the *D. littoralis*, 71% males. A male–female sex ratio of ca. 2:1 was fre-

TABLE 1. BIOASSAY EXPERIMENTS DEMONSTRATING EXISTENCE OF AGGREGATION PHEROMONE AND LEADING TO IDENTIFICATION OF ETHYL TIGLATE AS MAJOR COMPONENT

Treatment[a]	Mean catch[b]	
	D. borealis	*D. littoralis*
A. Demonstration of activity of extract from mature males and synergism with aspen extract ($N = 18$)		
Male extract	2.2b	1.8b
Aspen extract	1.5b	1.3b
Male + aspen extracts	9.5a	11.3a
Control (solvent)	0.2c	0.1c
B. Activity of extracts from mature males and females ($N = 20$)		
Female extract + aspen	2.4b	2.2b
Male extract + aspen	11.3a	11.5a
Control (aspen extract)	1.9b	1.3c
C. Activity of polar and nonpolar fraction of mature male extract ($N = 18$)		
Nonpolar fraction + aspen	8.2a	9.4a
Polar fraction + aspen	2.3b	2.1b
Crude extract + aspen	10.8a	11.2a
Control (aspen extract)	2.6b	1.9b
D. Activity of hydrocarbon and ester subfractions from mature male nonpolar fraction ($N = 18$)		
Hydrocarbon subfraction + aspen	4.9b	2.2b
Ester subfraction + aspen	10.9a	10.1a
Nonpolar fraction + aspen	14.6a	11.0a
Control (aspen extract)	2.5c	1.7b
E. Activity of synthetic ester mixture vs. nonpolar fraction from mature males ($N = 24$)		
Synthetic ester mix + aspen	9.8a	12.4a
Fly-derived nonpolar fraction + aspen	8.1a	10.5a
Control (aspen extract)	1.9b	2.4b
F. Comparison of male-specific esters on equal weight basis ($N = 12$)		
Methyl tiglate + aspen	3.6bc	4.1b
Ethyl tiglate + aspen	10.5a	10.9a
Isopropyl tiglate + aspen	10.8a	8.8a
Methyl hexanoate + aspen	4.1bc	4.6b
Ethyl hexanoate + aspen	5.2b	4.3b
Synthetic ester mixture + aspen	10.9a	10.3a
Control (aspen extract)	2.9c	3.1b
G. Cross attraction of *D. borealis* and *D. littoralis* (mature male extracts, $N = 8$)		
D. borealis nonpolar fraction + aspen	7.4a	7.9a
D. littoralis nonpolar fraction + aspen	9.1a	8.9a
Control (aspen extract)	1.8b	1.9b

[a] *D. borealis* fractions and extracts used at 2 fly equivalents per test. Those for *D. littoralis* used at 1 fly equivalent per test. Extract of fermented aspen bark added to all treatments in experiments B–G. Synthetic esters used at 10 ng/test in experiment F and in amounts comparable to fly-derived extracts in experiment E.

[b] In each experiment, means followed by the same letter are not significantly different at the 0.05 level, LSD. All statistical analysis conducted in the log $(x + 1)$ scale to stabilize variance.

quently seen in these experiments but generally reflected the sex ratio of the flies placed into the olfactometer rather than a greater propensity for males to respond. Again as with the *virilis* phylad, the active compound(s) in the extracts were relatively nonpolar (Table 1C), and the subfraction with the polarity of esters contained the majority of this activity (Table 1D). Although the hydrocarbon fraction of *D. borealis* appeared to possess slight activity also, this response was not consistent enough from day to day to allow isolation of active compounds.

The mature males of *D. borealis* and *D. littoralis* possessed the same tiglic and hexanoic esters (Table 2) that were found previously in the members of the *virilis* phylad (Bartelt et al., 1986a), except that no ethyl hexanoate was detected in *D. borealis*. Ethyl tiglate was the most abundant in both species. These esters were never detected in females or newly emerged males. Correspondingly, the nonpolar fractions derived from immature males were inactive in bioassay. For example, the mean bioassay catches for newly emerged *D. borealis* males, mature males, and controls (all with aspen extract) were 3.2b, 10.9a, and 3.2b, respectively. For *D. littoralis* these catches were 2.1b, 8.7a, and 2.5b, respectively (eight replications per treatment, different letters indicate significance at the 0.05 level).

The male-derived nonpolar fractions and corresponding synthetic ester mixtures (prepared according to Table 2) were comparable in bioassay activity (Table 1E). Thus the synthetic esters could account for all the activity in the male-derived fractions. On an individual basis, only ethyl tiglate and isopropyl tiglate were clearly active, compared to controls (Table 1F). Thus the flies can discriminate among certain individual esters. Since ethyl tiglate was the most abundant, as well as among the most active, this ester is believed to be the major pheromone component. The mixture of all esters, in the proportions found in male flies, was not significantly different from an equal weight of ethyl tiglate or isopropyl tiglate. The bioassay equivalence of ethyl tiglate and the ester mix-

TABLE 2. ESTERS ISOLATED FROM MATURE MALES BUT NOT DETECTED IN FEMALES OR NEWLY EMERGED MALES

Compound	Amount (ng/mature male ± SD)	
	D. borealis	*D. littoralis*
Methyl tiglate	1.0 (±0.1)	1.8 (±0.3)
Ethyl tiglate	6.5 (±0.5)	14.2 (±1.3)
Isopropyl tiglate	1.3 (±0.1)	0.8 (±0.1)
Methyl hexanoate	0.5 (±0.1)	0.9 (±0.1)
Ethyl hexanoate	—	0.2 (±0.1)

ture (Table 1F) suggests the minor components are not crucial to the pheromone response, and the function of these minor esters remains uncertain.

Finally, both *D. borealis* and *D. littoralis* responded clearly to the male-derived nonpolar fractions of the other species (Table 1G). Cross attraction would be expected since both possess ethyl tiglate. There is no evidence for a species-isolating mechanism involving pheromones, but since the species are allopatric, none would be anticipated.

Attractive Hydrocarbons. Both *D. borealis* and *D. littoralis* responded to Z9–21 significantly above control levels (Table 3A). In fact 10 μg of this hydrocarbon was comparable in activity to the male-derived pheromone extract. (This level of heneicosene was chosen during earlier work with the *virilis* phylad, where it produced clear responses and represented ca. 2–10 male equivalents). Not only was the hydrocarbon active by itself, but it was also synergistic with ethyl tiglate (Table 3B).

Neither *D. borealis* nor *D. littoralis* possesses any heneicosene (Bartelt et al., 1986b), but both were clearly attracted to Z9–21, a "pheromone-like" response. We suggest that the use of heneicosenes as pheromone components is a primitive trait in the *virilis* species group. Although some species no longer possess these hydrocarbons (e.g., *D. borealis*, *D. littoralis*, and *D. lummei*), they still retain the ability to respond to them. Since most of the species in this group are allopatric, and since even those with overlapping ranges use different hosts/microhabitats, present kairomonal use of these hydrocarbons is not suspected.

TABLE 3. BIOASSAY ACTIVITY OF Z9–21, A HYDROCARBON POSSESSED BY SEVERAL *virilis* GROUP SPECIES, BUT NOT BY *D. borealis* OR *D. littoralis*

Treatment[a]	Mean bioassay catch ($N = 8$)[b]	
	D. borealis	*D. littoralis*
A. Comparison of Z9–21 to nonpolar fraction from mature males		
Z9–21 + aspen	14.3a	7.1a
Male nonpolar fraction + aspen	10.3a	9.8a
Control (aspen extract)	2.4b	2.9b
B. Syngerism of ethyl tiglate and Z9–21		
Z9–21 + aspen	5.6c	6.4b
Ethyl tiglate + aspen	9.9b	9.0b
Z9–21 + ethyl tiglate + aspen	21.6a	22.5a
Control (aspen extract)	1.8d	3.2c

[a] *D. borealis* fraction used at 2 fly equivalents per test. Fraction from *D. littoralis* used at 1 fly equivalent per test. Z9–21 used at 10 μg per test, ethyl tiglate at 10 ng per test.

[b] In each experiment, means followed by the same letter not significantly different at the 0.05 level (LSD).

If the ancestral stock did use heneicosenes as pheromones, then a certain amount of structural specificity might still be evident. As shown in Table 4, both species are able to discriminate to some extent among various isomeric heneicosenes. *D. borealis*, especially, was able to distinguish between Z9–21 and Z10–21 and showed preference much like *D. americana* and *D. novamexicana* of the *virilis* phylad (Bartelt et al., 1986a). *D. littoralis* responded most strongly to Z8–21, but showed significant responses to Z7–21, Z9–21, and Z10–21 as well. Neither species responded significantly to *E* isomers, which was also true for the members of the *virilis* phylad.

Aspen Extract. The activity of the aspen extract appears to be due to a mixture of rather polar compounds. In Table 5, part A, the bioassay results indicate the activity is similar in polarity to alcohols and carboxylic acids; any hydrocarbons in the aspen extract are clearly inactive. The ether fraction consists primarily of one compound (by GC, accounting for >60% of the total peak area). This compound agrees in GC retention (DB-5 capillary column) and mass spectrum with benzyl alcohol. The seven largest mass spectral peaks had mass to charge ratios of 50 (13%), 51 (22%), 77 (63%), 79 (100%), 91 (19%), 107 (70%), and 108 (91%). (Intensities are relative to base peak.)

In Table 5, part B, it is evident that benzyl alcohol accounts for a portion of the extract's activity but not all of it. It is probable that one or more organic acids also contribute to the activity, since treatment of the extract with aqueous 5% NaOH rendered the organic layer significantly less active, but reacidifica-

TABLE 4. RESPONSIVENESS OF *D. borealis* AND *D. littoralis* TOWARD SEVEN HENEICOSENE ISOMERS

Heneicosene[a]	Relative activity[b]	
	D. borealis	*D. littoralis*
Z6-21	12	4
Z7-21	22	62*
Z8-21	69*	100*
Z9-21	100*	44*
Z10-21	3	32*
E9-21	6	12
E10-21	11	8

[a] Heneicosenes used at 10 μg per test. Each was combined with the aspen extract for bioassay. The aspen extract was used as the control.

[b] Each hydrocarbon was tested against Z9–21 and controls in a balanced incomplete block experiment. Relative activity based on mean catches. Relative activity = 100 × [(test isomer − control)/(Z9–21 − control)]. For *D. littoralis*, relative activities rescaled so that the most active isomer (Z8–21) was represented by 100. Overall means for Z9–21 and controls were 10.7 and 2.7, respectively, for *D. borealis*, and 9.9 and 3.0 for *D. littoralis*. (*) Indicates that the heneicosene differed from the control at the 0.05 level.

TABLE 5. PROGRESS TOWARD IDENTIFICATION OF ACTIVE COMPOUNDS IN ASPEN EXTRACT: BIOASSAY RESULTS FOR *D. borealis*

A. Activities of silicic acid fractions of aspen extract

Fraction	Mean bioassay catch ($N \geq 4$)[a]	
	Fraction[b]	Control[b]
Hexane	1.6	2.8
5% Ether–hexane	2.1	1.5
10% Ether–hexane	5.5*	1.5
25% Ether–hexane	13.2***	1.7
Ether	20.5***	1.5
10% Methanol–CH_2Cl_2	6.5	2.8
Crude aspen extract	21.6***	2.8

B. Additional bioassay experiments

Treatments	Mean bioassay catch ($N \geq 8$)[c]
Benzyl alcohol[d] + ethyl tiglate	20.4b
Aspen extract[d] + ethyl tiglate	41.5a
Control (ethyl tiglate)	9.5c
Aspen extract, treated with NaOH[e] + ethyl tiglate	32.5b
Aspen extract + ethyl tiglate	62.1a
Control (ethyl tiglate)	17.3c
Aspen extract treated with NaOH, then HCl[e] + ethyl tiglate	52.5a
Aspen extract + ethyl tiglate	55.6a
Control (ethyl tiglate)	12.3b

[a] Significant differences from controls at 0.05 and 0.001 levels denoted by (*) and (***), respectively.
[b] In part A of this table all treatments and controls contained 10 ng ethyl tiglate + 10 μg Z9–21.
[c] In each experiment, means followed by the same letter not significantly different (LSD, 0.05).
[d] Benzyl alcohol, identified as the major component of the ether fraction, tested at 900 ng; the aspen extract had an equivalent amount.
[e] See text for details.

tion with HCl restored most of the activity. In this series of experiments, the flies were unusually responsive. Using only ethyl tiglate as a coattractant produced some mean catches as high as 60 flies per 3-min test. Although absolute responsiveness varied throughout these experiments, synergistic effects were still obvious in a relative sense.

Conclusion. Like the other members of the *virilis* group studied previously, mature males of *D. borealis* and *D. littoralis* produce compounds that

attract both males and females in a wind-tunnel olfactometer. These species were difficult to study in that fly-derived compounds by themselves never elicited clear bioassay responses. However, using an extract from a likely host, fermenting aspen bark, as a bioassay coattractant allowed strong pheromone responses to be seen. In nature, flies would probably respond to such combinations of fly-derived and host-derived volatiles, since the flies aggregate at appropriate host sites to mate and lay eggs as well as to feed (Spieth, 1974).

Ethyl tiglate is the key attractant in both of these species. The same ester was previously found to be a pheromone component in other members of the *virilis* group and even in the more distantly related *D. hydei* (Moats et al., 1987). Both *D. borealis* and *D. littoralis* respond to Z9–21 and, in varying degrees, to other heneicosene isomers. Neither species produces heneicosenes, but these hydrocarbons were previously shown to be pheromone components in closely related species. These responses may be related to the evolution of pheromone systems in this species group.

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Announcement

The fifth annual meeting of the International Society of Chemical Ecology will take place June 24–27, 1988, in Athens, Georgia (USA). Abstracts for contributed papers and posters should be submitted to the conference host, Dr. Murray S. Blum, Laboratory of Chemical Ecology, Department of Entomology, University of Georgia, Athens, GA 30605. Dr. Blum is planning a number of symposia and these will be announced in the Newsletter of ISCE to members.

Requests for information on housing, meals, and other logistics should be sent to Mr. I. A. Little, Conference Facilitator, Georgia Center for Continuing Education, University of Georgia, Athens, GA 30602.

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ANALOGS OF SEX PHEROMONE OF PROCESSIONARY MOTH, *Thaumetopoea pityocampa*:[1] SYNTHESIS AND BIOLOGICAL ACTIVITY

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Abstract—The synthesis and biological activity of some analogs of (*Z*)-13-hexadecen-11-ynyl acetate **1**, the major component of the sex pheromone of the processionary moth *Thaumetopoea pityocampa* is described. The analogs have been formally derived by structural modification of the enyne and acetate functions of the parent compound **1**. In field tests, trifluoroacetate ester **16** and the analog, **11**, with fluorine substitution at the olefin site, decreased the pheromone action, whereas epoxy derivative, **10**, from epoxidation of the olefin moiety in **1**, and propionate ester **15** gave synergistic activity. The formate **14** had a variable effect according to the composition of the lure. Formal reduction of the enyne to give the acetylene **2** was found to retain activity. Alcohols **12** and **13**, resulting from hydrolysis of the enyne **1** and acetylene **2**, respectively, inhibited the action of their parent compounds.

Key Words—Sex pheromone, inhibition, synergism, processionary moth, *Thaumetopoea pityocampa*, Lepidoptera, Thaumetopoeidae.

INTRODUCTION

It is generally accepted that in the perception process of insect sex pheromones, there are very strict structural requirements for successful interaction between

[1] Lepidoptera, Thaumetopoeidae.

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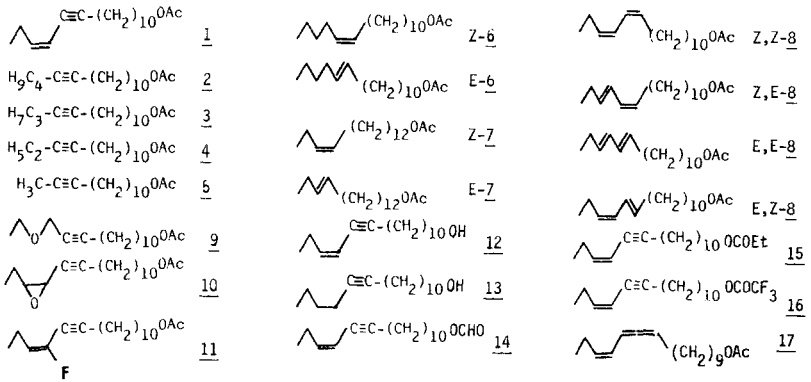
substrate and the receptor cells in the dendritic membrane of the antenna. This interaction may involve electrostatic, hydrophilic, hydrophobic, van der Waals forces or a hydrogen bonding mechanism and can be modulated by flexible receptor proteins, which can adopt the required conformational changes to achieve a successful recognition of the substrate molecule.

In this context, the knowledge of the sex pheromone critical molecular parts may be essential for a better understanding of the overall attractant perception process, and for the establishment of structure–activity relationships (Priesner, 1979; Liljefors et al., 1984) to help in the design of new synergists and inhibitors, capable of either increasing or decreasing the biological activity of the natural pheromone (Roelofs and Comeau, 1971).

In this paper, we report on the synthesis and biological activity of a variety of compounds structurally related to (*Z*)-13-hexadecen-11-ynyl acetate **1** (pityolure), the major component of the sex pheromone of the processionary moth *Thaumetopoea pityocampa* (Denis and Schiff.) (Guerrero et al., 1981; Camps et al., 1981a,b, 1983, 1987a; Cuevas et al., 1983; Michelot et al., 1982; Shani et al., 1983). These compounds, **2–17** (Scheme 1) proceed from modifications on the three putative key molecular parts of the parent molecule **1**, the double and the triple bonds and the acetate group, which may be directly involved in the interaction process with the receptor.



SCHEME 1.

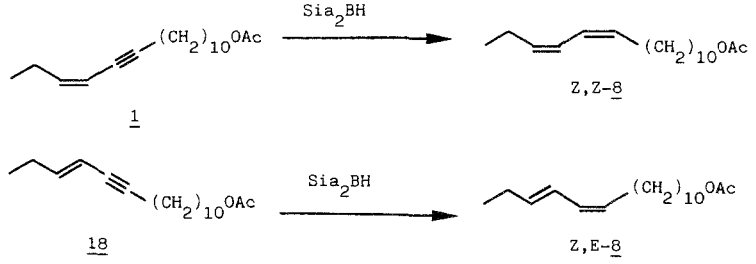
Thus, acetylenic derivatives **2–5** would result from saturation of the double bond and shortening of the chain length of the nonfunctionalized part of the sex pheromone component **1**. Likewise, partial reduction of the enyne moiety would lead to dienic compounds **8**, from which olefins **6** and **7** could be derived. On the other hand, whereas in oxa-analog **9** one of the olefinic carbons has been replaced by an oxygen atom, in the epoxy derivative **10** the double bond has been transformed into an oxirane ring. In fluorinated analog **11**, a fluorine atom

has been substituted for a vinyl hydrogen, whereas alcohols **12** and **13** would, obviously, derive from the corresponding esters **1** and **2**. Finally, in esters **14**, **15**, and **16**, the acetate moiety has been replaced by the formate, propionate, and trifluoroacetate groups, respectively.

METHODS AND MATERIALS

Boiling points are uncorrected. IR spectra were recorded in CCl_4 solution on Perkin Elmer 257 and 399B grating spectrometers [^{1}H]NMR spectra were determined in $CDCl_3$ solution on a Bruker WP80SY or on a Varian XL200 spectrometer, operating at 80 and 200 MHz, respectively, and absorptions are expressed in δ scale relative to TMS. [^{13}C]NMR spectra were recorded on a Bruker WP80SY instrument at 20.15 MHz in $CDCl_3$ solution, and the values are expressed in δ scale relative to TMS. GLC analyses were performed on Carlo Erba models 2350 and 4130 equipped with a FID detector, using 3% OV-101 glass column 2 m × 3 mm ID on Chromosorb W (nitrogen as carrier gas), or a fused silica capillary column SE-54 50 m × 0.32 mm ID (hydrogen as carrier gas).

Reactions requiring anhydrous and oxygen-free conditions were performed under dried inert atmosphere (N_2 or Ar). Anhydrous solvents were prepared as follows: tetrahydrofuran (THF) by distillation from Na/benzophenone, diethyl ether from lithium aluminium hydride (LAH), CCl_4 from P_2O_5, pyridine from KOH, and hexamethylphosphoric triamide (HMPT) from CaH_2.



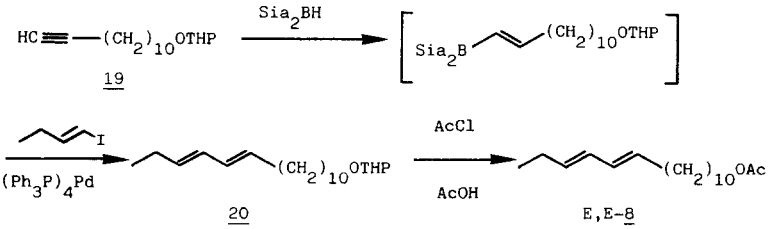
SCHEME 2.

Chemicals. Acetylenic acetates **2–5** were prepared by alkylation of the corresponding tetrahydropyranyloxy acetylides in HMPT (Brattesani and Heathcock, 1973) followed by hydrolysis and acetylation. Monounsaturated derivatives *Z*-**6**, *Z*-**7**, *E*-**6**, and *E*-**7** have been prepared by stereoselective reduction of the parent acetylenic compounds to the *Z* (H_2/Pd-C) or to the *E* isomer (Na/NH_3). The acetylenic precursor for preparation of compounds **7** was obtained by triple bond migration of tetradec-11-yn-1-ol with potassium

3-aminopropylamide (KAPA) in 1,3-propylenediamine (Brown and Yamashita, 1975), followed by hydroxyl protection as the tetrahydropyranyl derivative, alkylation of the resulting terminal acetylene with ethyl bromide, hydrolysis, and acetylation. The epoxyacetate **10**, which was prepared by epoxidation of the natural compound **1**, was subsequently transformed into fluorinated analog **11**, with a 2:1 *E*:*Z* isomer ratio, by a route previously described by us (Camps et al., 1986). Formate **14**, propionate **15**, and trifluoroacetate **16** have been prepared by esterification of the parent alcohol **12**. On the other hand, eneallene **17** was kindly supplied by Dr. Jahyo Kang (Sogang University, Seoul, South Korea).

(Z,Z)-*11,13-Hexadecadienyl Acetate* (Z,Z)-*8* (Scheme 2). In a two-neck 25-ml round-bottomed flask was placed 0.065 ml (0.5 mmol) of a 8 M BH_3 · THF complex. To this solution, previously cooled to 0°C, was added, under N_2, 0.070 g (1 mmol) of 2-methyl-2-butene. The resulting mixture was stirred for 2 hr at room temperature and cooled again to 0°C. Then, 0.115 g (0.42 mmol) of (*Z*)-13-hexadecen-11-ynyl acetate **1** in 2 ml of anh. ether was added. Stirring was kept at room temperature for 4 hr, then 0.3 ml of glacial acetic acid was added and the resulting solution further stirred for 12 hr at 30–35°C. Oxidation was carried out by stirring for 30 min in the presence of 1 ml of 6 N NaOH and 0.25 ml of 30% hydrogen peroxide, keeping the temperature below 40°C. The reaction mixture was poured into ice and extracted with hexane (4 × 8 ml). The combined organic layers were washed with brine and dried ($MgSO_4$) to yield, after evaporation of the solvent, 0.116 g of an oil, which was purified by column chromatography on silica gel, eluting with hexane–ethyl acetate 95:5, to afford pure (*Z*,*Z*)-**8** in 76% yield (isomeric purity higher than 99% by GLC analysis on capillary column).

Anal.: Calcd. for $C_{18}H_{32}O_2$: C, 77.14; H, 11.42. Found: C, 77.15; H, 11.72. IR: ν 2960, 2930, 1860, 1745, 1240 cm^{-1}. [^{1}H]NMR: δ 1.00 (t, J = 7.5 Hz, 3H, $C\underline{H}_3CH_2$), 1.28 (b, 14H, $CH_2C\underline{H}_2CH_2$), 1.62 (c, 2H, $C\underline{H}_2CH_2O$), 2.05 (s, 3H, $C\underline{H}_3CO$), 2.16 (c, 4H, $C\underline{H}_2C{=}C$), 4.05 (t, J = 6.7 Hz, 2H, $C\underline{H}_2O$), 5.44 (c, 2H, $CH_2C\underline{H}{=}$), 6.24 (c, 2H, ${=}C\underline{H}\text{-}C\underline{H}{=}$). [^{13}C]NMR: δ 64.7 (C-1), 28.7–29.7 (C-2 and C-4 to C-9), 26.0 (C-3), 27.5 (C-10), 133.6 (C-11), 123.2, 123.6 (C-12 and C-13), 132.1 (C-14), 20.9 (C-15 and C-1′), 14.2 (C-16), 171.0 (CO).



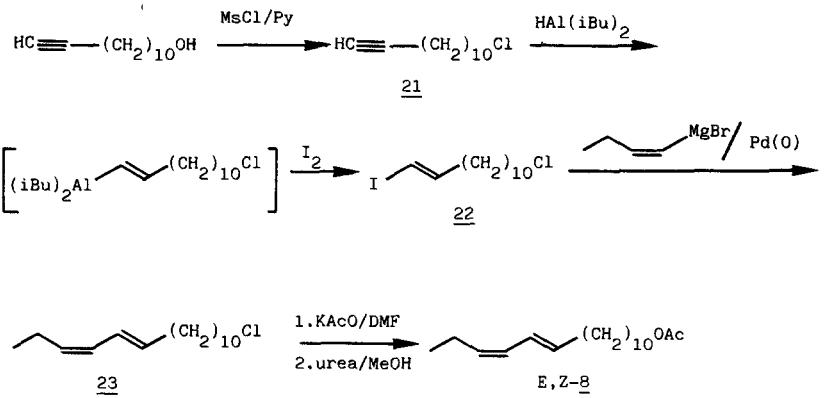
SCHEME 3.

(Z,E)-*11,13-Hexadecadienyl Acetate* (Z,E)-***8*** (Scheme 2). The same procedure described above for compound ***Z,Z*-8** was applied, using compound **18,** the *E* isomer of **1**, previously prepared by coupling of (*E*)-1-iodo-1-butene with 11-dodecynyl acetate in the presence of tetrakis(triphenylphosphine)palladium (Michelot et al., 1982). Thus, starting from 88 mg (0.31 mmol) of **18**, 72 mg (82%) of dienic acetate (*Z,E*)-**8** was obtained, after purification by column chromatography on silica gel, eluting with hexane–ethyl acetate 95:5. Compound (*Z,E*)-**8** had a 98:2 *Z,E*:*E,E* isomeric purity by GLC analysis on capillary column.

Anal.: Calcd. for $C_{18}H_{32}O_2$: C, 77.14; H, 11.42. Found: C, 77.17; H, 11.63. IR: ν 2960, 2930, 1745, 1240, 980 cm^{-1}. [^{1}H]NMR: δ 1.01 (t, J = 7.4 Hz, 3H, $C\underline{H}_3CH_2$), 1.29 (b, 14H, $CH_2C\underline{H}_2C\underline{H}_2$), 1.61 (c, 2H, $C\underline{H}_2CH_2O$), 2.05 (s, 3H, $C\underline{H}_3CO$), 2.16 (c, 4H, $C\underline{H}_2C{=}$), 4.05 (t, J = 6.7 Hz, 2H, $C\underline{H}_2O$), 5.31 (dt, J = 10.8 and 7.3 Hz, 1H, H-14), 5.70 (dt, J = 15 and 6.7 Hz, 1H, H-11), 5.95 (t, J = 10.8 Hz, 1H, H-13), 6.31 (dd, J = 15.1 and 10.9 Hz, 1H, H-12). [^{13}C]NMR: δ 64.5 (C-1), 28.6–29.7 (C-2 and C-4 to C-9), 25.8 (C-3), 27.6 (C-10), 130.0 (C-11), 128.6 (C-12), 124.7 (C-13), 136.0 (C-14), 25.8 (C-15), 13.6 (C-16), 20.9 (C-1′), 170.8 (CO).

(E,E)-*11,12-Hexadecadienyl Acetate* (E,E)-***8*** (Scheme 3). In a three-neck round-bottomed flask was placed, at 0°C under N_2, a mixture of 0.178 ml (1.52 mmol) of a 8 M $BH_3 \cdot S(CH_3)_2$ solution and 0.210 g (3 mmol) of 2-methyl-2-butene. The resulting solution was stirred at room temperature for 2 hr and then a solution of 0.353 g (1.27 mmol) of 2-(11-dodecynyloxy)tetrahydropyran **19** in 2 ml of anh. THF was added. The mixture was stirred for 2 hr to complete the formation of the intermediate vinylborane. After this time, a solution of 8 mg (1% molar) of tetrakis(triphenylphosphine)palladium in 1 ml of anh. THF, previously stirred at room temperature for 30 min on the dark and under N_2, was added along with 0.231 g (1.27 mmol) of (*E*)-1-iodo-1-butene (Michelot et al., 1982). The mixture was stirred for 2 hr more at room temperature and treated with a solution of 0.1 g of NaOH in 1.2 ml of deoxygenated water for 14 hr at 70°C. Oxidation was carried out by addition at 0°C of 1.2 ml of 6.5 N NaOH and 0.4 ml of 30% H_2O_2, followed by stirring for one additional hour at room temperature. The reaction mixture was poured into ice and extracted with hexane (5× 10 ml). The combined organic layers were washed with brine and dried ($MgSO_4$) to afford, after evaporation of the solvent, 0.36 g of the expected THP-protected dienic alcohol **20**, which was directly acetylated by treatment with 0.25 ml of acetyl chloride and 1 ml of acetic acid in 2 ml of CCl_4 under reflux for 7 hr. Then, the reaction mixture was cooled, and after addition of solid $NaHCO_3$, poured into ice and extracted with hexane (5× 10 ml). The organic layers were washed with brine and dried ($MgSO_4$). Removal of the solvent and purification by column chromatography on silica gel, eluting with hexane–ethyl acetate 95:5, afforded 0.155 g (43%) of compound (*E,E*)-**8**, isomerically pure (99%) by GLC analysis.

Anal.: Calcd. for $C_{18}H_{32}O_2$: C, 77.14; H, 11.42. Found: C, 77.04; H, 11.30. IR: ν 2960, 2930, 2860, 1745, 1240, 990 cm^{-1}. [^{1}H]NMR: δ 0.99 (t, J = 7.4 Hz, 3H, $C\underline{H}_3CH_2$), 1.29 (b, 14H, $CH_2C\underline{H}_2CH_2$), 1.62 (c, 2H, $C\underline{H}_2CH_2O$), 2.04 (s, 3H, $C\underline{H}_3CO$), 2.06 (c, 4H, $C\underline{H}_2C{=}$), 4.05 (t, J = 6.7 Hz, 2H, $C\underline{H}_2O$), 5.58 (c, 2H, $CH_2C\underline{H}{=}$), 6.00 (c, 2H, $=C\underline{H}\text{-}C\underline{H}{=}$). [^{13}C]NMR: δ 64.5 (C-1), 28.5–29.3 (C-2 and C-4 to C-9), 25.8 (C-3), 32.5 (C-10), 132.2 (C-11), 130.5 (C-12), 130.3 (C-13), 132.0 (C-14), 25.4 (C-15), 13.5 (C-16), 21.0 (C-1′), 170.9 (CO).



SCHEME 4.

12-Chloro-1-dodecyne ***21***. In a three-neck round-bottomed flask provided with a magnetic stirrer, N_2 inlet, septum, reflux condenser, and calcium chloride tube were placed 1.13 g (6.2 mmol) of 11-dodecyn-1-ol and 0.54 g (6.8 mmol) of anh. pyridine in 3 ml of anh. dimethylformamide (DMF). To this solution was added 0.52 ml (6.76 mmol) of methanesulfonyl chloride, and the reaction mixture was heated at 75°C for 16 hr. The resulting solution was poured into ice-water and extracted with hexane (5× 20 ml). The combined organic layers were washed with brine and dried ($MgSO_4$). Removal of the solvent left a residue which was distilled (bp 60–65°C/0.3 torr) to yield 1.0 g (88%) of pure chloroalkyne **21**.

Anal.: Calcd. for $C_{12}H_{21}Cl$: C, 72.07; H, 10.58. Found: C, 72.15; H, 11.00. [^{1}H]NMR: δ 1.32 (b, 14H, $CH_2C\underline{H}_2CH_2$), 1.79 (c, 2H, $C\underline{H}_2CH_2Cl$), 1.94 (t, J = 2.5 Hz, 1H, $\underline{H}C{\equiv}$), 2.16 (c, 2H, $C\underline{H}_2C{\equiv}$), 3.55 (t, J = 6.6 Hz, 2H, $C\underline{H}_2Cl$). [^{13}C]NMR: δ 68.0 (C-1), 84.4 (C-2), 18.2 (C-3), 28.4–29.2 (C-4 to C-9), 26.8 (C-10), 32.5 (C-11), 44.8 (C-12).

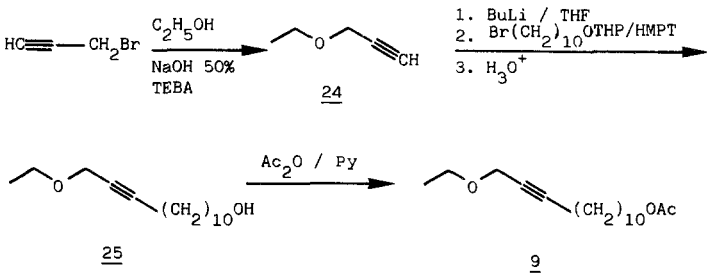
(E)-*12-Chloro-1-iodo-1-dodecene* ***22***. To a solution of 1.4 g (7 mmol) of alkyne **21** in 7 ml of anh. hexane was added dropwise, under N_2, 7 ml (7 mmol)

of a 1 M diisobutylaluminiumhydride (DIBAH) soln. in hexane. The reaction mixture was stirred at 50°C for 4 hr, cooled to −40°C, and subsequently treated with 1.77 g (7 mmol) of iodine in 7 ml of anh. THF. Stirring was continued for 1 hr at room temperature. The mixture was cooled to −10°C and 5 ml of 20% H_2SO_4 was slowly added. After pouring into ice and extraction with hexane (5× 20 ml), the combined organic layers were washed with 5% $NaHSO_3$ and brine and dried ($MgSO_4$). Evaporation of the solvent yielded a crude, which was chromatographically purified (silica gel) to afford 1.42 g (62%) of compound **22**, isomerically pure (>99%) by GLC analysis on a capillary column.

Anal.: Calcd. for $C_{12}H_{22}ClI$: C, 43.94; H, 6.76, Cl, 10,81; I, 38.69. Found: C, 43.77; H, 7.09; Cl, 10.79; I, 38.66. IR: ν 2930, 2860, 1605, 950 cm^{-1}. [^{1}H]NMR: δ 1.31 (b, 14H, $CH_2\underline{CH_2}\underline{CH_2}$), 1.82 (c, 2H, $\underline{CH_2}CH_2Cl$), 2.05 (c, 2H, $\underline{CH_2}C=$), 3.55 (t, J = 6.6 Hz, 2H, $\underline{CH_2}Cl$), 5.97 (dt, J = 14.4 and 1.6 Hz, 1H, $=\underline{CH}I$), 6.52 (dt, J = 14.5 and 7.4 Hz, 1H, $=\underline{CH}CH_2$). [^{13}C]NMR: δ 74.2 (C-1), 146.7 (C-2), 36.0 (C-3), 28.3–29.4 (C-4 to C-9), 26.9 (C-10), 32.6 (C-11), 45.0 (C-12).

(E,Z)-*11,13-Hexadecadienyl Acetate* (E,Z)-**8** (Scheme 4). A THF solution of the Grignard reagent of (*Z*)-bromo-1-butene, obtained as previously described (Norris, 1959), was prepared from 0.4 g (3 mmol) of the alkene and 86 mg (3.6 mmol) of magnesium. The organomagnesium reagent was slowly added, via syringe, to a solution of 0.329 g (1 mmol) of compound **22** and 0.173 g (0.15 mmol) of tetrakis(triphenylphosphine)palladium in 7 ml of anh. THF. The resulting mixture was stirred at room temperature for 2 hr, poured into ice-water and extracted with hexane (5× 10 ml). After conventional work-up, 0.170 g of crude (*E,Z*)-11,13-1-chlorohexadecadiene **23** was obtained, which was directly treated with 0.490 g (15 mmol) of KOAc in 10 ml of DMF at 120°C for 90 min. The reaction mixture was quenched and worked up as usual to provide 0.162 g of a crude, which was purified by column chromatography on silica gel, eluting with hexane–ethyl acetate 94:6, to furnish 0.146 g (52% from **22**) of diene acetate (*E,Z*)-**8**, of isomeric purity *E,Z*:*E,E* 82:18 on GLC analysis. Removal of the contaminating *E,E* isomer was achieved by treatment with a saturated solution of urea in methanol to afford compound (*E,Z*)-**8** in 97% stereomeric purity.

Anal.: Calcd. for $C_{18}H_{32}O_2$: C, 77.14; H, 11.42. Found: C, 77.42; H, 11.36. IR: ν 2980, 2930, 1745, 1240 cm^{-1}. [^{1}H]NMR: δ 0.99 (t, J = 7.5 Hz, 3H, $\underline{CH_3}CH_2$), 1.28 (b, 14H, $CH_2\underline{CH_2}\underline{CH_2}$), 1.6 (c, 2H, $\underline{CH_2}CH_2O$), 2.05 (s, 3H, $\underline{CH_3}CO$), 2.12 (c, 4H, $\underline{CH_2}C=$), 4.05 (t, J = 6.7 Hz, 2H, $\underline{CH_2}O$), 5.30 (dt, J = 10.7 and 7.3 Hz, 1H, H-14), 5.6 (dt, J = 14.9 and 7.2 Hz, 1H, H-11), 5.92 (t, J = 10.7 Hz, 1H, H-13), 6.33 (dd, J = 14.9 and 10.7 Hz, 1H, H-12). [^{13}C]NMR: δ 64.5 (C-1), 28.6–29.4 (C-2 and C-4 to C-9), 25.8 (C-3), 32.8 (C-10), 134.6 (C-11), 125.5 (C-12), 128.0 (C-13), 131.5 (C-14), 20.9 (C-15), 14.2 (C-16), 20.9 (C-1′), 170.8 (CO).



SCHEME 5.

Ethyl 2-propynyl Ether ***24***. To a mixture of 5.6 ml (0.1 mol) of ethanol, 40 ml (0.5 mol) of a 50% solution of NaOH and 1.14 g (5 mmol) of benzyltriethylammonium chloride, previously cooled to 0°C, was added 13 g (0.11 mol) of freshly distilled propargyl bromide. Stirring was continued for 2 hr at room temperature, the organic layer was decanted and washed with brine, dried ($MgSO_4$), and distilled to yield 6.0 g (71%) of pure acetylene **24** (bp 58–60°C/170 torr). IR: ν 3300, 2970, 2860, 1100 cm^{-1}. [^{1}H]NMR: δ 1.19 (t, J = 7 Hz, 3H, $C\underline{H}_3$), 2.37 (t, J = 2.4 Hz, 1H, $\underline{H}C\equiv$), 3.50 (q, J = 6.9 Hz, 2H, $CH_3C\underline{H}_2O$), 4.10 (d, J = 2.4 Hz, 2H, $OC\underline{H}_2C\equiv$). [^{13}C]NMR: δ 62.6 (C-1), 95.6 (C-2), 57.6 (C-3), 65.4 (C-4), 14.7 (C-5).

14-Oxa-hexadec-11-ynol ***25***. To a solution of 0.504 g (16 mmol) of ether **24** in 10 ml of anh. THF at −10°C under Ar, was added 5 ml (5mmol) of a 1 M *n*-BuLi soln. in hexane. The resulting solution was stirred for 15 min and then 1.44 g (4.5 mmol) of 2-(10-bromodecyloxy)tetrahydropyran in 5 ml of anh. THF was added dropwise. The stirring was continued for 4 hr and the reaction mixture poured into ice and extracted with hexane (5× 20 ml). The combined organic layers were washed with brine and dried ($MgSO_4$) to give, after evaporation of the solvent, an oily residue which was treated with pyridinium tosylate (60 mg) in 20 ml of ethanol for 6 hr under reflux. The reaction mixture was worked up in the usual manner to yield 1.08 g (70%) of alcohol **25**, pure enough to be used in the next step without further purification. IR: ν 3600, 3300, 2920, 2850, 1090, 860 cm^{-1}. [^{1}H]NMR: δ 1.22 (t, J = 7 Hz, 3H, $C\underline{H}_3$), 1.40 (b, 16H, $CH_2C\underline{H}_2CH_2$), 2.20 (c, 2H, $CC\underline{H}_2C\equiv$), 3.55 (q, J = 7 Hz, 2H, $CH_3C\underline{H}_2O$), 3.65 (t, J = 6.7 Hz, 2H, $C\underline{H}O_2OH$), 4.12 (t, J = 2.2 Hz, 2H, $OC\underline{H}_2C\equiv$).

14-Oxa-hexadec-11-ynyl Acetate ***9*** (Scheme 5). Acetylation of alcohol **25** was carried out under conventional conditions (acetic anhydride/pyridine). Thus, starting from 0.856 g (3.2 mmol) of alcohol **25**, acetic anhydride (6 ml) and anh. pyridine (6 ml), pure acetate **9** (0.733 g, 83%) was obtained after purification on silica gel, eluting with hexane–ethyl acetate 90:10. Anal.: Calcd. for $C_{17}H_{30}O_3$:

C, 72.28; H, 10.60. Found: C, 72.20; H, 10.65. IR: ν 2930, 2850, 1740, 1240, 1090, 910 cm^{-1}. [^{1}H]NMR: δ 1.20 (t, J = 7.2 Hz, 3H, $C\underline{H}_3CH_2$), 1.38 (b, 16H, $CH_2C\underline{H}_2CH_2$), 2.05 (s, 3H, $C\underline{H}_3CO$), 2.20 (c, 2H, $CC\underline{H}_2C\equiv$), 3.55 (q, J = 7.2 Hz, 2H, $CH_3C\underline{H}_2O$), 4.05 (t, J = 7 Hz, 2H, $C\underline{H}_2OAc$), 4.11 (t, J = 2.1 Hz, 2H, $OC\underline{H}_2C\equiv$). [^{13}C]NMR: δ 64.4 (C-1), 28.5–29.3 (C-2 and C-4 to C-9), 25.8 (C-3), 18.6 (C-10), 76.2 (C-11), 86.5 (C-12), 58.2 (C-13), 65.0 (C-15), 14.8 (C-16), 20.7 (C-1′), 170.8 (C-0).

EAG Bioassays. The electroantennogram activity of the test compounds was determined on an EAG set-up with a new non-operator-dependent sample injection system (Guerrero et al., 1986).

Field Tests. The required amount of test compound in each bait was mixed with 2.5 mg of paraffin wax to slow down the release rate in the field (Cuevas et al., 1983), and dissolved in 1 ml of nanograde hexane. The solutions were transferred into closed polyethylene vials (3 × 1.1 cm ID) which were used as dispensers. Field trials were carried out in Mora de Rubielos (Teruel) and Cieza (Murcia) from 1984 to 1986. Traps used throughout the test seasons were "dry" (no glue added) and specially designed for processionary moth catches (Montoya, 1984). Traps were hung on trees at a height of 1.7–2.0 m above ground and those located in the same parcel were spaced 50 m apart. Minimum distance between parcels was 150 m apart. They were set out in statistically randomized blocks and revised and rotated every day. Ten traps were generally used for each formulation. In each parcel, the number of catches of the different formulations was compared with that of pityolure. Trap catches were subjected to a square-root transformation followed by analysis of variance, and the data were analyzed statistically for significance according to Duncan's multiple-range test. When appropriate, Student's t test was used to assess the significance of differences between mean numbers of catches within the same parcel.

RESULTS AND DISCUSSION

As shown in Table 1, the biological activity of fluorinated analog **11** was studied in 1984 and 1985 field trials. While traps baited with compound **11** alone were only slightly active (parcel I, test 1), lures with mixtures of **11** and the natural pheromone **1** caught variable number of males, depending on their composition. Remarkably, when a 3:1 ratio of **11**:**1** was used, an approximately 50% inhibitory effect on the activity of the pheromone was observed (parcel III, tests 1 and 2), whereas the results were not statistically significant when the 1:1 ratio was utilized.

The effects exhibited by propargylic ether **9**, epoxide **10**, formate **14**, propionate **15**, and allene **17** were studied in other experiments carried out in 1984 and 1985 (Table 2). Significant synergistic effects appeared to be displayed by

TABLE 1. CAPTURE OF *Thaumetopoea pityocampa* MALES WITH BLENDS OF FLUORINATED COMPOUND **11** AND PITYOLURE **1** IN COMPARISON WITH NATURAL COMPOUND **1**

		Composition of the lure (mg)		
Test[a]	Parcel	Fluorinated analog **11**	Pityolure **1**	Mean catch/trap/week[b]
1	I	1.0	—	12.5a
			1.0	58.4a
	II	0.9	0.1	4.5b
			1.0	17.6b
	III	0.75	0.25	15.2c
			1.0	40.2c
	IV	0.5	0.5	11.9d
			1.0	24.2d
	V	0.25	0.75	17.1NS
			1.0	23.2NS
	VI	0.1	0.9	23.8NS
			1.0	24.9NS
2	I	0.1	1.0	24.2NS
			1.0	23.2NS
	II	1.0	1.0	10.9NS
			1.0	11.7NS
	III	3.0	1.0	12.4a
			1.0	24.3a

[a] Tests 1 and 2 were conducted in Mora de Rubielos (Teruel) in 1984 and 1985, respectively.
[b] Means within parcel followed by the same letter are significant at $P < 0.05$, Student's t test. NS = nonsignificant. Ten replicates per treatment.

ether **9** and epoxide **10** when mixed with pityolure **1** in 1:10, 1:1 and 2.3:1 ratios (parcel I, test 1, and parcels I and II, test 2 for compound **9**; and parcel III, test 1, and parcels VII and VIII, test 2, for compound **10**). On the other hand, formate **14** behaved in a strikingly different way, depending upon the composition of the bait. Thus, when this compound was mixed with pityolure in a 1:10 ratio, it showed a significant synergistic effect (parcel IV, test 2), whereas at higher ratios (2.3:1 parcel II, test 1, and 10:1 parcel VI, test 2) a marked decrease in catches was observed. Almost no effect was shown in baits charged with 1 mg of both compounds (parcel V, test 2).

Previous authors have also reported on the synergistic effect exhibited by ethers obtained by replacing an olefinic carbon by oxygen in the pheromone structure of a different species, such as the red-banded leaf roller moth *Argyrotaenia velutinana* (Roelofs and Comeau, 1971). In this case, both oxygen analogs exhibited similar activities. Unfortunately, in the present study, we

TABLE 2. EFFICIENCY OF LURES BAITED WITH COMPOUNDS **9**, **10**, **14**, **15**, AND **17** IN COMBINATION WITH PITYOLURE **1**

		Composition of bait (mg)						
Test[a]	Parcel	Ether **9**	Epoxide **10**	Formate **14**	Propionate **15**	Allene **17**	Pityolure **1**	Mean/catch/ trap/week[b]
1	I	0.7					0.3	19.32a
							0.3	10.92a
	II			0.7			0.3	8.80b
							0.3	22.12b
	III		0.7				0.3	15.12c
							0.3	6.80c
2	I	0.1					1.0	36.86a
							1.0	29.36a
	II	1.0					1.0	13.11b
							1.0	9.82b
	III[c]					1.0	1.0	14.2NS
							1.0	18.63NS
	IV			0.1			1.0	38.46c
							1.0	27.03c
	V			1.0			1.0	27.5NS
							1.0	27.3NS
	VI			10			1.0	14.4d
							1.0	29.4d
	VII		0.1				1.0	41.06e
							1.0	29.3e
	VIII		1.0				1.0	36.3f
							1.0	28.4f
	IX				0.1		1.0	40.3g
							1.0	29.3g
	X				1.0		1.0	29.8NS
							1.0	32.6NS

[a] Test 1 was carried out in Cieza (Murcia, 1984) and test 2 in Mora de Rubielos (Teruel, 1985).
[b] Ten traps per treatment. Means within parcel followed by the same letter are significant at $P < 0.05$, Student's t test. NS = nonsignificant.
[c] Only two traps per formulation.

could not prepare the alternative positional isomer of ether **9**, 13-oxahexadec-11-ynyl acetate, due to its inherent chemical instability.

With regard to the variable effect of the formate **14**, it should be noted that, in other cases, blends of natural pheromones with other synthetic compounds can considerably increase or decrease trap catch, depending on the relative ratios used in the bait (Kamm and McDonough, 1980).

When propionate **15** was mixed with pityolure **1** in a 1:10 ratio (parcel

IX, test 2), an increase in the number of catches was observed, whereas no significant effect was found when both compounds were mixed in a 1 : 1 ratio (parcel X, test 2). This nonsignificant effect differs from the inhibitory action elicited by a mixture of the sex pheromone of the red-banded leaf roller moth and the corresponding propionate analog with the same relative ratio. Therefore, it might be assumed that effects shown by the same type of analogs are species-specific and that no general validity may be extrapolated from the results obtained in one particular case. On the other hand, when the allene **17** was tested in baits with pityolure **1** (1 : 1 ratio, parcel III, test 2), no appreciable variation in trap catches was observed.

A wide range of EAG activities was exhibited by compounds depicted in Scheme 1. While compounds **4**, **5**, *E*-**6**, *Z*-**7**, and *E*-**7** with very low EAG responses in comparison with that of pityolure **1** (<20%) were not further investigated, the intrinsic activity of the remaining analogs was studied in a new set of field experiments (Table 3). In comparison with the sex pheromone component, some of these analogs, such as epoxide **10**, ether **9**, (*E*,*Z*)-**8**, and acetylene **3**, had slight attractant activity. However, we found that acetylene **2** had a fairly good activity amounting up to 65% of the efficiency of natural compound **1** (Camps et al., 1987b). Likewise, propionate **15** was also a good pheromone mimic, with a 40% relative trapping efficiency.

Figure 1 shows the trap catch vs. time for the sex pheromone component **1**, propionate **15**, and acetylene **2**. Although the activity pattern of the propionate vs. pheromone is similar, the activity of acetylene **2** seems to be better than

TABLE 3. RELATIVE INTRINSIC ATTRACTANT ACTIVITY OF ANALOGS **2**, **3**, *Z*-**6**, **8**, **9**, **10**, **14**, AND **15** COMPARED WITH PITYOLURE **1** (Mora de Rubielos, 1985)

Attractant	Total catch[a]	Relative efficiency (%) vs. pityolure
9	123d	9
3	79d	6
2	858b	65
Z-**6**	9e	0.6
15	523c	40
14	8e	0.6
10	182d	14
1	1322a	100
Z,*Z*-**8**	5e	0.4
Z,*E*-**8**	18e	1.4
E,*Z*-**8**	106d	8
E,*E*-**8**	18e	1.4

[a] Ten replicates per trap. Values followed by the same letter are not significantly different at $P = 0.05$ (Duncan's multiple-range test).

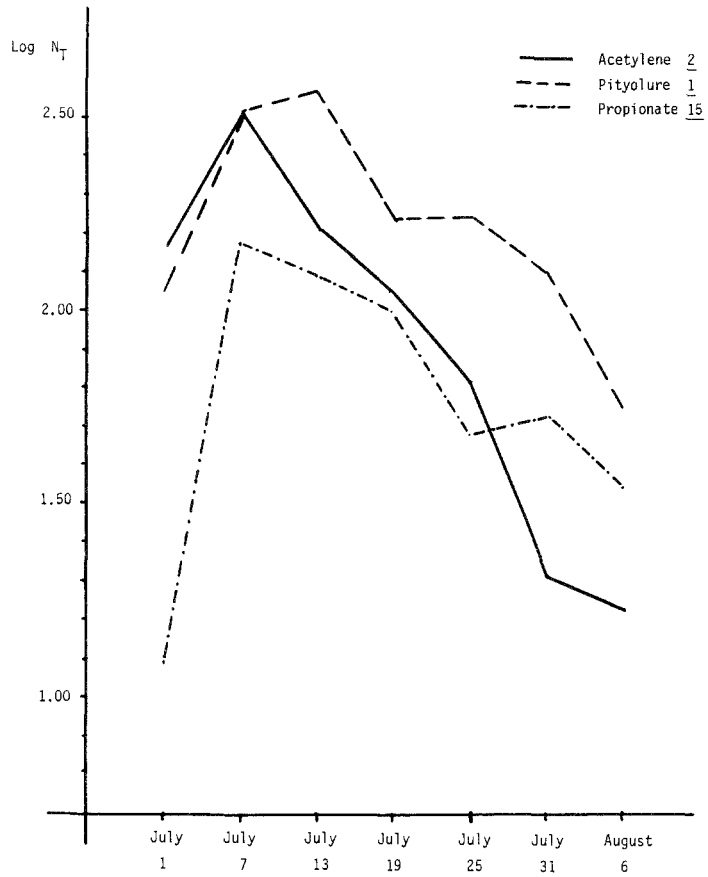


FIG. 1. Evolution of total number of catches of *Thaumetopoea pityocampa* males by lures baited with 1 mg of pityolure **1**, acetylene **2**, and propionate **15** (Mora de Rubielos, 1985).

propionate **15** except for the last part of the experiment. This was initially rationalized in terms of the possibly higher inhibitory effect of alcohol **13**, resulting from partial hydrolysis of the parent acetylene **2**, when compared with that of alcohol **12**, similarly originated from the natural pheromone **1** and propionate **15** under environmental conditions. However, analysis of the corresponding carriers after field trials showed a very small amount of alcohols **12** and **13**.

Furthermore, as shown in Table 4, in field tests designed to investigate the above hypothesis, it was found that the inhibitory effect shown by alcohol **12** on pityolure **1** was apparently higher than that promoted by alcohol **13** on its

TABLE 4. INHIBITORY EFFECTS OF ALCOHOLS **12** AND **13** AND TRIFLUOROACETATE **16** ON PITYOLURE **1** AND ACETYLENE **2** (Mora de Rubielos, 1986)

	Bait formulation (mg)					Total No.
Parcel[a]	Alcohol **12**	Alcohol **13**	Acetylene **2**	Trifluoroacetate **16**	Pityolure **1**	of catches[b]
I	0.1				1	72b
	1				1	82b
	10				1	64b
					1	540a
	1					10b
II		0.1			1	350abcd
		1			1	389abc
		10			1	414ab
					1	449a
		1				6e
III		0.1	1			220b
		1	1			138bc
		10	1			105bcd
			1			343a
		1				19d
IV				0.1	1	140b
				1	1	44c
				10	1	38c
					1	228a
				1		4c

[a] Several km of distance between parcels.
[b] Five replicates per trap. Means followed by the same letter are not significantly different at $P = 0.05$ Duncan's multiple-range test.

parent compound **2**. These results were further confirmed in EAG bioassays (see below). Other alternative possibilities to explain these differences in the activity pattern of these compounds are being considered in planning further studies. On the other hand, when alcohol **13** was mixed with pityolure **1**, no significant decrease in catches was observed. This fact combined with the clear inhibitory effects shown by alcohols **12** and **13** on pityolure **1** and the acetylene **2**, respectively, may suggest the presence of two different specific receptor cells, with high affinity for compounds **1** and **2**. However, this assumption has not been appropriately substantiated by cross-effect experiments or single-cell recording studies.

Remarkably, the trifluoroacetate **16**, although intrinsically inactive, inhibited significantly the activity of the sex pheromone component when mixed with **1** in 0.1 : 1, 1 : 1, and 10 : 1 ratios (Table 4). Both effects were also confirmed in EAG bioassays by presaturation of the antennal receptors for 4 hr with dif-

ferent amounts (0.1, 1, 10, 100, and 1000 μg) of trifluoroacetate **16** and further exposure to pheromone (10 μg) "puffs," whereby a very small response was recorded. In addition, we have found that, under field conditions, trifluoroacetate **16** is not substantially hydrolyzed to alcohol **12**, which might be held responsible for this inhibitory effect. In this context, Albans et al. (1984) observed an antipheromone action in field trials with the trifluoroacetate analog of the sex pheromone of a different species (*Heliothis virescens*).

Conclusions. Selected structural modifications of the three putative critical molecular parts of pityolure **1** have led to the synthesis of analogs **2–17**, which have exhibited diverse effects, mimicking, enhancing, or decreasing the activity of the sex pheromone component in field and laboratory bioassays. In general, preservation of the acetylene group at C-11 of the original structure is shown to be essential for high activity. On the other hand, fluorine substitution for vinyl hydrogen at C-13 or replacement of the acetate moiety by a trifluoroacetate group induced antipheromone activity. Likewise, alcohols **12** and **13** exhibited inhibitory effects on the corresponding acetates. In addition, synergistic effects have arisen from the transformation of the double bond into the corresponding epoxide or into an oxymethylene group, as well as when the acetate group was replaced by propionate or formate functions. However, surprisingly, in this latter analog, the synergistic action could be reversed into inhibition, according to the relative ratios used in the bait, when mixed with the sex pheromone component.

It is noteworthy to point out that the conjugated enyne moiety of the major component of the female sex pheromone of *Thaumetopoea pityocampa* has not been found in any other insect pheromone structure. From our results, it appears that, in this unique moiety, the triple bond is essential to elicit pheromonal activity.

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RUBBER SUBSTRATES AND THEIR INFLUENCE ON ISOMERIZATION OF CONJUGATED DIENES IN PHEROMONE DISPENSERS

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Abstract—Release rate and degree of isomerization of pheromones with conjugated double bonds were studied in dispensers prepared from several rubber substrates. The substrates compared were made of rubber, cured with elemental sulfur or accelerators based on organic sulfur compounds or organic peroxides. Isomerization of the double bonds occurs immediately after impregnation of the substrate, and the degree of isomerization increases during field use and/or storage. The propensity of the isomers to isomerize corresponds to their proportion in the equilibrium mixture. An *E,Z* isomer is isomerized faster than the *E,E* isomer, and finally a near-equilibrium mixture of the four isomers is present. Minimal isomerization was found in non-sulfur-cured substrates which are the material of choice.

Key Words—Insect pheromones, conjugated dienes, pheromone formulations, rubber pheromone dispensers, isomerization, dispensers.

INTRODUCTION

The use of sex pheromones in traps for insect monitoring ideally requires a uniform and prolonged release of the effective dose of the active compound during the whole period of the pest flight. Many controlled-release pheromone systems have been described (see review by Zeoli et al., 1982). However, these systems hardly meet all the requirements for a good pheromone dispenser. Currently, rubber septa are the most common substrates for pheromone dispensers

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in monitoring traps (Daterman, 1982, and references therein). Important information on their preparation, particularly from multicomponent mixtures, is given by Kydonieus (1977), Butler and McDonough (1979, 1981), and McDonough and Butler (1983). In recent years, a growing number of papers point out that not all rubber substrates are suitable for use as pheromone releasers, since some of them contain components with adverse effects on pheromones (Steck et al., 1979; Daterman, 1982; Minks, 1984).

Besides controlling the evaporation rate, an effective pheromone dispenser must protect the biologically active compounds against atmospheric oxidation, light, and other adverse environmental factors. Pheromones with conjugated double bonds are less stable, undergoing photooxidation (Shani and Klug, 1980; Ideses et al., 1982) as well as rubber-induced isomerization (Guerin et al., 1983; Davis et al., 1984; Brown and McDonough, 1986; Hrdý et al., 1986). In spite of the fact that the function of dispensers depends critically on the release rate and purity of the active compounds, the dispensers are usually characterized only by the amount and ratio of the active compounds applied. In order to develop standardized commercial lures so that all field data could be comparable, it is necessary to know the effect of various substrates on the purity and release rate of the active compounds.

Many sex pheromones of economic pests contain conjugated diene systems (Reed and Chisholm, 1985; Arn et al., 1986). Not all the species, however, are equally tolerant to the presence of other geometric isomers; thus, according to electroantennograph measurements, (8*E*,10*E*)-8,10-dodecadien-1-ol acetate is the most active stimulant for the pea moth, *Cydia nigricana* (F.), and has also been found in the extracts of abdominal tips of females (Greenway, 1984). On the other hand, when formulated into the dispensers, this compound shows only a limited activity in field tests (see, e.g., Greenway and Wall, 1981). However, the same dispensers are effective for the lucerne moth, *Cydia medicaginis* (Kuzn.) (see Bournoville, 1979; Horák et al., 1980; Hrdý et al., 1983).

Thus, high-purity compounds can be degraded to give low or even no activity when formulated in substrates containing additives that may react with the pheromones. Brown and McDonough (1986) investigated the effect of sunlight on the isomerization of (7*E*,9*Z*)-7,9-dodecadien-1-ol acetate, (9*Z*,11*E*)-9,11-tetradecadien-1-ol acetate, and (8*E*,10*E*)-8,10-dodecadien-1-ol and its acetate in sulfur-cured natural rubber septa and phenolic resin-cured halobutyl elastomer septa. The two most important factors for minimizing the isomerization noted in these studies are the absence in the rubber of isomerization catalysts such as sulfur, and minimum exposure to sunlight.

In this paper, we report the results of studies on the effect of various rubber substrates on the isomerization rates of pheromones with conjugated double bonds evaporated from the dispensers.

METHODS AND MATERIALS

Pheromone Chemicals. (8*E*,10*E*)-8,10-Dodecadien-1-ol (*E*8,*E*10–12:OH), purity 99.5%, with less than 0.1% of the other isomers; (8*E*,10*E*)-8,10-dodecadien-1-ol acetate (*E*8,*E*10–12:Ac), purity 99.0%, with less than 0.1% of the other isomers; and (7*E*,9*Z*)-7,9-dodecadien-1-ol acetate (*E*7,*Z*9–12:Ac), purity 93.1%, with 6.3% of the *E*,*E* isomer were prepared in this laboratory. (7*E*,9*Z*)-7,9-Dodecadien-1-ol acetate, purity 86.7%, containing 10.7% of the *E*,*E* isomer, was purchased from the Wolfson Unit of Chemical Entomology, Department of Chemistry, University of Southampton, Southampton, Great Britain.

Formulation Substrates. These were supplied by the following manufacturers: (A) Gumárne SNP n.p. (Dolné Vestenice, Czechoslovakia): (1) Aa—205 IR (ČSN 62 2625.14), synthetic isoprene rubber, sulfur-cured, 15 × 15 × 2-mm sheets, dispenser weight 0.6 g; Ab—159 (ČSN 62 2616.07), natural styrene–butadiene rubber, thiuram-cured, 15 × 15 × 2-mm sheets, dispenser weight 0.55 g; Ac—321 (ČSN 62 2035.03), sulfur-cured blend of isoprene, chloroprene, and styrene–butadiene rubber, 15 × 15 × 2-mm sheets, dispenser weight 0.6 g; Ad—3263 (ČSN 62 2245.14), sulfur-cured natural rubber, tubes, external diameter 7 mm, length 20 mm, wall thickness 1 mm, weight 0.55 g; Ae—162 (ČSN 62 2446.07), styrene–butadiene rubber, sulfur-cured, 15 × 15 × 2-mm sheets, dispenser weight 0.7 g; Af—rubber septa CM-4408 (ČSN 62 0011), sulfur-cured, thiuram-accelerated blend of synthetic isoprene and styrene–butadiene rubber, diameter 18 mm, dispenser weight 1.5 g.

All these substrates are commercially available and contain various amounts of other components such as fillers, antioxidants, plasticizers, and pigments.

(B) Rubena n.p., (Náchod, Czechoslovakia): Ba—3303, natural + isoprene rubber, cured with a mixture of zinc diethyldithiocarbamate, tetramethylthiuram disulfide and orthotolylbiguanide; septa, diameter 12 mm, thickness 2 mm, dispenser weight 0.45 g.

(C) JZD Květen-Nehvizdy (Czechoslovakia): custom-made substrates from rubber mix of simple composition, slightly conical cups of 10 mm diameter, height 10 mm, wall thickness 1.7 mm. They had the following compositions: Ca—natural rubber SMR 20, cured with dicumyl peroxide (2% wt), dispenser weight 0.55 g; Cb—natural rubber SMR 20, (82% wt), carbon black HAF (16% wt), cured with dicumyl peroxide (2% wt), dispenser weight 0.6 g; Cc—natural rubber SMR 20 (80% wt), carbon black HAF (16% wt), cured with dicumyl peroxide (4% wt), dispenser weight 0.6 g; Cd—natural rubber SMR 20 (90% wt), zinc oxide (5% wt), stearic acid (2% wt), phenyl-β-naphthylamine (1% wt), cured with tetramethylthiuram disulfide (2% wt), dispenser weight 0.6 g.

Impregnation of Substrates with Pheromone: General Procedure. A solu-

tion of the active compound (0.5, 1.0, or 2.0 mg per dispenser) in toluene (usually 0.5 ml per dispenser) was agitated in a rotary evaporator under atmospheric pressure and room temperature. The solutions soaked completely within 1–2 h, depending on the substrate type. The dispensers were then aged for 12 h in a closed vessel, and the solvent was evaporated on exposure to air.

Pheromone Analysis. All the analyses were performed with dispensers exposed in the laboratory at room temperature. The pheromone, released from individual dispensers, was quantitatively analyzed using the relative static method according to Baker et al. (1980). The dispenser was hung in a 250-ml Erlenmeyer flask, stoppered with a ground joint stopper (29/32) with a hanger, and the flask was kept at 25° ± 0.2°C for 2 h. After taking out the dispenser, the pheromone adsorbed on the flask walls was washed out with hexane (5 ml; for residue analysis, Merck, Darmstadt, F.R.G.), and a defined amount of tetradecanol was added as internal standard. The solution was concentrated to 10 μl, and the whole residue was injected into a gas chromatograph.

The gas–liquid chromatography was carried out on an HP 5880 Hewlett-Packard (Mountain View, California) chromatograph equipped with a FID, on a 25-m × 0.3-mm-ID fused silica column with cross-linked 5% phenyl methyl silicone (HP-5, Hewlett-Packard); splitless injection. The oven program for analysis was as follows: (A) 50°C, 30 sec; (B) 30°C/min; (C) 190°C, 20 min; a 10-min purge at 250°C. Quantitative analyses were done using the ISTD method. Each analysis was repeated three to five times. For all the substrates analyzed, blank analyses without pheromones were run. No substances of retention times similar to those of the analyzed pheromone isomers evaporated from the substrates. The identity of all four isomers, formed from the individual pure pheromones, was proved by GC-MS analysis (Hewlett-Packard, model 5985A).

RESULTS

Since isomerization of aliphatic pheromones containing a conjugated diene system results in equilibrium mixtures of about 65–69% *E,E* isomer, 12–23% *E,Z* isomer, 9–11% of *Z,E* isomer, and 3% *Z,Z* isomer, the substrates were preliminarily selected using the isomerization of the more sensitive *E*7,*Z*9–12:Ac. The above-mentioned composition is not the same for all conjugated dienes; our values agree with those published by Ideses et al. (1982) and Davis et al. (1984). First, the degree of isomerization of *E*7, *Z*9–12:Ac with 10.7% of the *E,E*-isomer in substrates Aa—Af was estimated at 24 hr and then 22 days after impregnation (Table 1). In spite of the good results in the laboratory test, polyethylene tubes were excluded since these corresponding dispensers do not protect the pheromones from sunlight and gave the worst results in the field tests. Substrates with lowest degree of isomerization were used in the second

TABLE 1. EFFECT OF RUBBER SUBSTRATES ON ISOMERIZATION OF *E*7,*Z*9–12 : Ac[a]

Substrate	After 24 hr (%)		After 22 days (%)	
	E,Z	*E,E*	*E,Z*	*E,E*
Aa	79	21	68	32
Ab	90	10	86	14
Ac	78	22	67	33
Ad	85	15	90	10
Ae	81	19	69	31
Af	74	26	53	47
PE[b]	89	11	86	14

[a] Percentage of *E,Z* and *E,E* isomer, $N = 5$, median. Content of the *E,E* isomer: 10.7%; 2 mg per dispenser.
[b] Polyethylene tube.

experiment in which the evaporation rate and degree of isomerization were determined during the first, 13th, and 29th day after impregnation (Table 2).

According to the laboratory experiments, the substrate Ab is suited best for manufacturing pheromone dispensers. In the field tests during the 1984 season, these dispensers proved to be better for monitoring the codling moth, *Cydia pomonella* (L.), than the commercial dispensers CM (Zoecon, U.S.A.) and CP (Chemica, Czechoslovakia) (Hrdý et al., 1986). Dispensers, made from a different batch of the same substrate, were again field-tested in 1985. Since the biological effectiveness of these dispensers in the field test with *Cydia pomonella* was substantially lower than in 1984, a new batch of the substrate was prepared by the manufacturer. With this material the same results were obtained as in 1984. Therefore, we concluded that the difference in quality was due to either manufacturing and/or quality control problems. We therefore decided to prepare our own substrates of as simple composition as possible, in which the presence of sulfur would be excluded or at least minimized (Vrkoč et al., 1986) because we regarded sulfur as one of the possible isomerizing factors.

The degree of isomerization of *E*7,*Z*9–12 : Ac with 6.3% of the *E,E* isomer in substrates Cb–Cd is given in Table 3. Table 4 contains the isomerization data for *E*8, *E*10–12 : Ac. The isomerization data for *E*8,*E*10–12 : OH (0.5 mg) also demonstrated better stability on the same substrates as compared with the sulfur-cured substrate Af.

The amounts of the individual isomers, evaporated during 2 hr at 25 ± 0.2°C from selected substrates up to 47 days after their preparation and exposure in the laboratory, are compared in Figures 1 and 2. Figure 1 compares the release rate and degree of isomerization of *E*7,*Z*9–12 : Ac in sulfur-cured sub-

TABLE 2. EFFECT OF RUBBER SUBSTRATES ON RELEASE RATE AND ISOMERIZATION OF *E*7,*Z*9–12 : Ac[a]

	Day 1				Day 13				Day 29			
	Amount (ng)		%		Amount (ng)		%		Amount (ng)		%	
Substrate	*E*,*Z*	*E*,*E*	*E*,*Z*	*E*,*E*	*E*,*Z*	*E*,*E*	*E*,*Z*	*E*,*E*	*E*,*Z*	*E*,*E*	*E*,*Z*	*E*,*E*
Aa	40	10	79	21	36	12	75	25	22	10	68	32
Ab	95	10	90	10	65	8	89	11	46	6	88	12
Ad	76	14	84	16	27	10	72	28	17	10	64	36
Ae	64	13	83	17	25	8	75	25	16	14	53	47

[a] Amount in nanograms and percentage of *E*,*Z* and *E*,*E* isomer, $N = 5$, median. Content of the *E*,*E* isomer: 10.7%; 2 mg per dispenser.

TABLE 3. EFFECT OF RUBBER SUBSTRATES ON ISOMERIZATION OF *E*7,*Z*9–12 : Ac[a]

	Day 1		Day 16		Day 30		Day 44	
Substrate	*E,Z*	*E,E*	*E,Z*	*E,E*	*E,Z*	*E,E*	*E,Z*	*E,E*
Cb	94	6	87	13	90	10	86	14
Cc	90	10	88	12	86	14	83	17
Cd	89	11	85	15	84	16	76	24

[a] Percentage of *E,Z* and *E,E* isomer, $N = 5$, median. Content of the *E,E* isomer: 6.3%; 2 mg per dispenser.

strate Af and in substrate Cd cured with tetramethylthiuram disulfide, Figure 2 compares the same parameters for *E*8,*E*10–12 : Ac in sulfur-cured substrate Af and in substrate Cc, cured with dicumyl peroxide.

DISCUSSION

The isomerization of conjugated dienes in the dispensers during storage and application can be influenced mainly by the substrate used and by variable weather conditions, primarily the sunlight intensity (Shani and Klug, 1980; Nesterova et al., 1984; Brown and McDonough, 1986). The substrate itself affects weather-induced isomerizations, thus influencing the biological utilizability of the dispenser.

Rubber substrates, suitable for the preparation of insect monitoring systems, should be selected not only according to their physicochemical properties, but also according to the components used in their manufacturing. From the data presented above, rubbers cured with elemental sulfur should be avoided; better results were obtained with rubbers cured with organic sulfur accelerators, but rubbers cured with organic peroxides are best. Although organic peroxides readily isomerize double bonds, they are thermally decomposed during the curing and, moreover, their residues can be removed by an additional thermal treatment of the final products. Our conclusions agree with those of Brown and McDonough (1986), who report that the absence of sulfur is the most important factor for minimizing isomerization of conjugated dienes in rubber septa and suggest substrates based on phenolic resin-cured halobutyl elastomer.

Since rubber septa for the monitoring systems are used only for a short time, no stabilization is necessary. The substrates, however, must have an appropriate degree of cross-linking to enable a relatively uniform release during a sufficiently long time period. Because of possible unfavorable effects of additives, their number as well as amount should be minimized. The possible sta-

TABLE 4. EFFECT OF RUBBER SUBSTRATES ON ISOMERIZATION OF *E*8,*E*10–12 : Ac[a]

	Day 3			Day 17			Day 33			Day 46		
Substrate	*Z,E*	*E,E*	*E,Z* + *Z,Z*	*Z,E*	*E,E*	*E,Z* + *Z,Z*	*Z,E*	*E,E*	*E,Z* + *Z,Z*	*Z,E*	*E,E*	*E,Z* + *Z,Z*
Af	10	78	12	12	72	16	15	68	17	14	70	16
Cb	2	95	3	3	91	6	2	93	5	2	93	5
Cc	2	94	4	2	94	4	2	93	5	0	94	6
Cd	4	91	5	3	92	5	3	91	6	4	90	6

[a]Percentage of *Z,E*, *E,E*, and *E,Z* + *Z,Z* isomers, $N = 5$, median; 1 mg per dispenser.

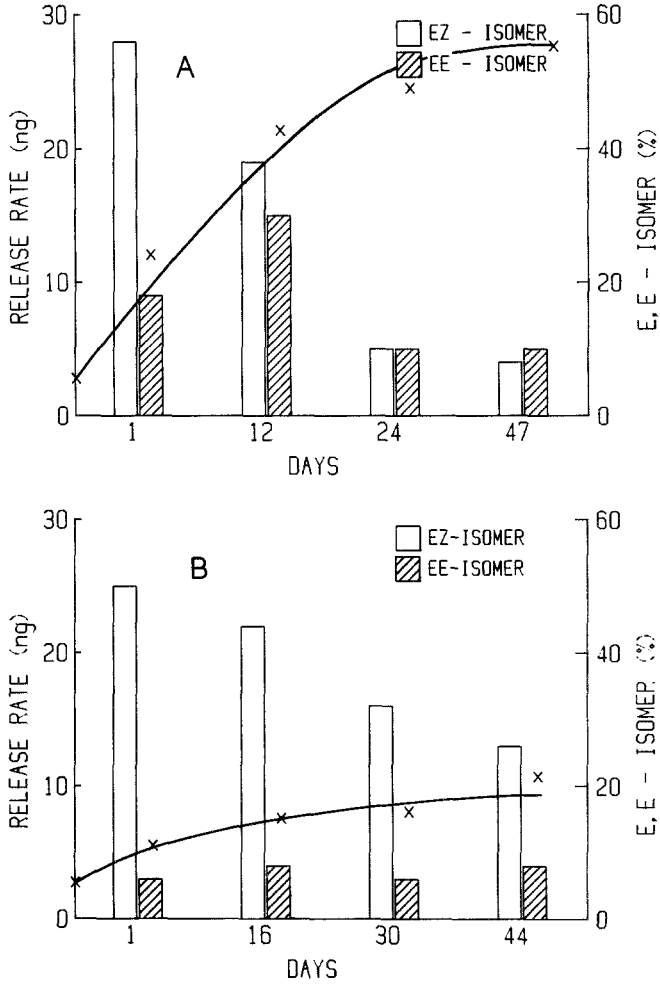

FIG. 1. Isomerization and release rate (ng) of *E*7,*Z*9–12 : Ac (containing 6.3% of *E*,*E* isomer; 2 mg per dispenser) in pheromone dispensers made from substrate Af (A) and substrate Cd (B) and dynamics of pheromone release during 47 and 44 days, respectively, at room temperature. x——x: % unwanted isomer formed.

bilization of the pheromones by some antioxidants (Greenway and Wall, 1981; Reed and Chisholm, 1985) can be achieved by their application together with the pheromones.

It follows unequivocally from the data presented that the isomerization of conjugated dienes takes place in freshly prepared dispensers. The degree of isomerization depends on the type of the substrate, increases during storage and

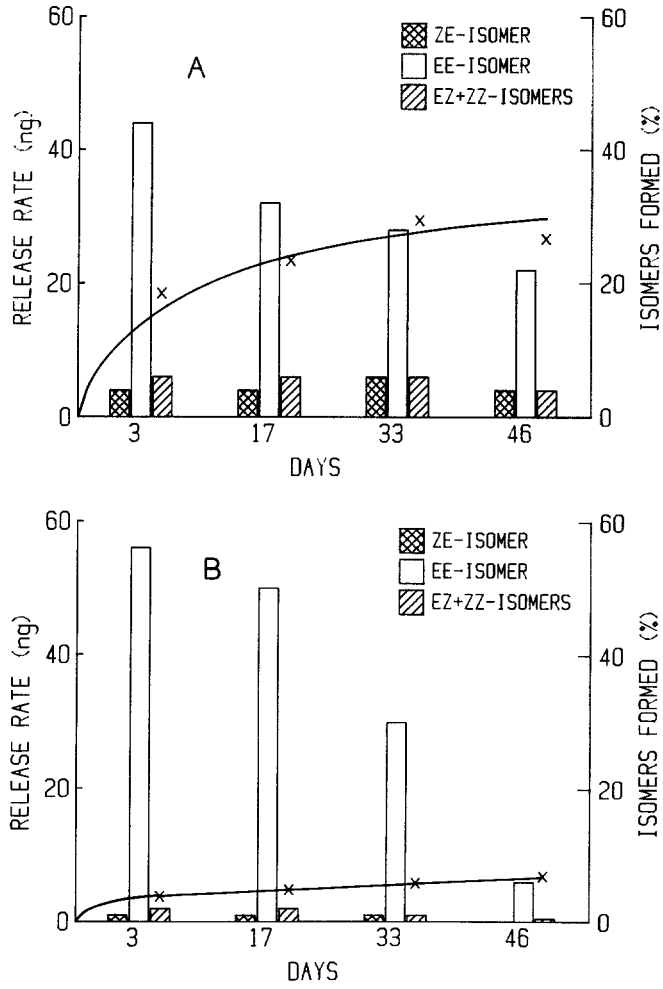

FIG. 2. Isomerization and release rate (ng) of *E*8,*E*10–12:Ac in pheromone dispensers (1 mg per dispenser) made from substrate Af (A) and substrate Cc (B) and dynamics of pheromone release during 46 days at room temperature. x——x: % unwanted isomers formed.

application, and can substantially affect the biological activity of the dispensers. Individual insect species obviously differ in sensitivity towards the presence of other isomers. Further data about the isomerization of *E*8, *E*10–12:OH and its effect on the catch of the codling moth (*Cydia pomonella*), and isomerization of *E*8,*E*10–12:Ac and the effect on the pea moth (*Cydia nigricana*), lucerne moth (*Cydia medicaginis*), and European grapevine moth (*Lobesia botrana*)

catch will be reported elsewhere. The pea moth males are very sensitive to the presence of isomers with different configurations of the conjugated double bonds and only substrates with the lowest degree of isomerization can give sufficiently active formulations. Also the preparation of active formulations for the European grapevine moth is difficult because the *E,Z* isomer is very susceptible to isomerization.

Steck et al. (1982) have recently reviewed the field test screening and recommend a careful interpretation of the results. According to these authors, the great majority of the chemical lure components used are of a purity less than 99.9% even before they are deteriorated by weather conditions or by the substrate. Many pheromone blends of noctuids have optional minor component contents of 1% or less; contents of 0.1% or even 0.01% are quite sufficient for biological activity. Thus, the good results with impure (or at least undefined) lures may result from the main component as well as from one of the impurities or even from a fortuitous effect of the multicomponent mixture. Further complications arise from degradation of pure compounds by weather conditions and/or various carriers. Consequently, interpretation of such results may be misleading.

The preparation of standard dispensers, allowing objective and qualified interpretation of the biological activity, requires (in addition to the usually published amount and ratio of the active compounds): (1) data on purity of the active compounds used, (2) identification of the possible impurities, (3) chemical characterization of the formulation substrates and, particularly, for pheromones containing conjugated double bonds, (4) an isomerization test in the formulation substrate.

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SEX PHEROMONE OF EUROPEAN CORN BORER, *Ostrinia nubilalis:* Polymorphism in Various Laboratory and Field Strains

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Abstract—Sex gland extracts of *Ostrinia nubilalis* females collected in the wild or laboratory-reared from Switzerland, Italy, and Hungary were analyzed. Individuals collected in the north of Switzerland contained (*Z*)- and (*E*)-11-tetradecenyl acetate at the approximate ratio of 97:3 (*Z* type), in accordance with field responses of males and previous findings. On the other hand, females from a laboratory culture derived from field collections made in the same area and reared for four to five generations contained the *Z* and *E* isomers at ratios of ca. 3:97 and 35:65, respectively. In the south, one of the eight wild females analyzed was of the *Z* type and the rest intermediate, whereas males were predominantly trapped with blends of the two isomers containing 60 to 97 *E*. In a laboratory culture reared for one to two generations from corn borers collected in Hungary, three of nine females were of the intermediate type and the rest *Z*. Small amounts of (*Z*)-11-hexadecenyl acetate were detected in female glands of the *E* strain; however, no effect of this compound could be observed in the field.

Key Words—Sex pheromone, European corn borer, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, (*Z*)-11-hexadecenyl acetate.

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INTRODUCTION

The European corn borer, *Ostrinia nubilalis*, represents a classical case of pheromone polymorphism. Klun and Brindley (1970) identified (*Z*)-11-tetradecenyl acetate (*Z*11–14:Ac) as the main pheromone component. Kochansky et al. (1975) verified the existence of two strains in North America, one in New York (*E* strain) with a ratio of 4:96 *Z*11–14Ac and (*E*)-11-tetradecenyl acetate (*E*11-14Ac), and one in Ontario (*Z* strain) at a 97:3 ratio; the latter resembled the population in Iowa (Klun and Robinson, 1971). Hybridization of the two was accomplished in the laboratory (Liebherr and Roelofs, 1975); males responding to blends of the two isomers were trapped in Pennsylvania (Cardé et al., 1975) and elsewhere.

In Europe, a survey by Klun and cooperators (1975) revealed the prevalence of the *Z* type; catches with 3:97 and 1:1 blends of *Z*:*E* were made in the north of Italy. Analyses and field tests by Maini et al. (1978), Büchi et al. (1982), Anglade et al. (1984), and Barbattini et al. (1985) confirmed that the *E* strain is present south of the Alps and occasionally hybridizes with the *Z* strain. However, there have been reports of occurrence of the *E* strain as far north as the Ruhr region in northern Germany (Langenbruch et al., 1985).

Additional components have been identified in the sex gland of *O. nubilalis*. Tetradecyl acetate (14:Ac) occurs in both *Z* and *E* strains (Kochansky et al., 1975) and was found synergistic at high levels (Stockel, 1980). (*E*)-9-Tetradecenyl acetate (*E*9–14:Ac), a compound detected in small amounts in the *Z* strain (Klun and Junk, 1977), inhibits male response (Klun et al., 1979). Attygalle et al. (1987) found (*Z*)-11-tetradecen-1-ol (*Z*11–14:OH) in the *Z* strain using solid sample injection of female gland material; this compound had no effect on male catch in North America (McLeod and Starrat, 1978).

For the European corn borer, a reliable monitoring technique would be desirable in integrated control programs, e.g., when using the parasite *Trichogramma maidis* (Bigler, 1986). In Europe, pheromone traps are not routinely used for that purpose, possibly due to general uncertainty about the suitable composition of the attractant. We therefore decided to reinvestigate the sex pheromone of *O. nubilalis* in Europe, by chemical analysis and field tests.

METHODS AND MATERIALS

Insects. Collections were made as diapausing larvae, unless noted. In laboratory cultures adults from the same origin were allowed to mate freely. The following sources were investigated: (1) Fricktal wild: collection made in the autumn of 1985 at Elfingen near Basel (north of Switzerland); (2) Fricktal culture: a collection made in the same location reared on an artificial diet (Bathon, 1978; diet 2), analyses were made from the fourth and fifth generations; (3) Ticino wild: nondiapausing larvae of the first generation collected at Giubiasco

(south of Switzerland) at the end of July 1986; (4) Bologna wild: a field collection made in the fall of 1985 in northern Italy; (5) Hungary culture: field collection made at Hódmezövásárhely in the southeast of Hungary in September 1985 and reared on an artificial diet (Nagy, 1970). Analyses were made on females of the first and second generations.

Insects were sexed as pupae and held at 80% relative humidity in a 18:6 hr photoperiod with 3000–6000 lux, 26°C in the photophase and 18°C in the scotophase (Loughner and Bringley, 1971; Maini et al., 1978).

Extraction and Cleanup. Extracts were made by severing the everted ovipositor tips of 2- to 3-day-old calling females and collecting them in hexane (Merck, for residue analysis) for 5–10 min. Batches of 20–25 females were extracted using 2–3 μl/female. Individual gland extracts required between 7 and 10 μl. The crude preparations were sealed in 50-μl micropipets and kept at −18°C.

To prevent interference from cuticular hydrocarbons, the pooled extracts used to detect minor components were chromatographed through a 1.2-mm × 4-cm silica column (Merck, 70–230 mesh). After removing nonpolar components with 3× 35 μl hexane, the sample eluting with 4× 35 μl methylene chloride (Merck, for residue analysis) was concentrated with a gentle stream of N_2 to ca. 2 μl and injected. Analysis for alcohols, which could be retained by silica, was done on a crude extract.

Gas Chromatography–Mass Spectrometry (GC-MS). Analyses were carried out on a SP-2340 fused silica capillary column (30 m, 0.32 mm ID) coupled via fused silica capillary interface to a Finnigan 4000 quadrupole instrument (EI, 70 eV, 240°C, *m/z* (35–750). Samples were introduced by splitless injection and the carrier gas (He) set at 1.5 bar.

Extracts of individual females were analyzed without previous cleanup by selected ion monitoring of the *m/z* values 61 ($CH_3COOH_2^+$, for acetates), 194, and 222 (M^+-CH_3COOH and M^+-H_2O for both monounsaturated acetates and alcohols of 14 and 16 carbons, respectively). A typical temperature program was: 50°C for 2 min, 20°C/min to 100°C, and 5°C/min to 170°C, heating out to 240°C. Elution temperatures ranged from 110 to 170°C for dodecenyl to octadecenyl acetates.

About 50% of the Fricktal culture and Hungary individuals were analyzed with GC-flame ionization detection, under the same conditions as above. The electroantennographic detector (EAD) with a male antenna of *Mythimna unipuncta* Hw. was used for detection of (*Z*)-11-hexadecenyl acetate (Z11–16:Ac) (Guerin et al., 1985).

Field Tests. Tests were carried out to determine the occurrence of males responding to different blends of the two isomers and to establish the importance of the minor components at proportions similar to those found in the female glands (Table 1). Unsaturated compounds were purchased from Farchan Chem-

TABLE 1. CATCHES OF *O. nubilalis* MALES WITH VARIOUS BLENDS[a]

Composition of lure[b]					Trapping locations and dates		
Z11-14:Ac	E11-14:Ac	14:Ac	Z11-16:Ac	E11-16:Ac	Trasadingen July 1-Aug 18	Hornussen July 4-Aug 15	Giubiasco July 22-Aug 29
97	3				25a[c]	180a	14c
80	20				0b	8b	35c
60	40				0b	1b	66b
40	60				0b	1b	102ab
20	80				0b	1b	92ab
3	97				0b	0b	123a
97	3				51a		
97	3		0.2		40a		
97	3		1		44a		
97	3		5		53a		
97	3		20		34a		
97	3	5	5		36a		
3	97						80a
3	97		1				79a
3	97		5				68ab
3	97		20				71ab
3	97		5	1			69ab
3	97	5	5	1			42b

[a] Total of 10 replicates; numbers followed by the same letter are not significantly different at $P = 0.05$ as indicated by log $(x + 1)$ transformation, two-way analysis of variance, and Duncan's multiple-range test.
[b] Amounts in micrograms per rubber cap.
[c] One replicate only, placed July 4.

icals, Willoughby, Ohio, and purified by HPLC on silver nitrate–silica gel (Heath et al., 1975) to an isomeric purity of better than 99.9%; 14:Ac was purchased from NuChekPrep, Elysian, Minnesota. Overall purity of chemicals was >99.0% by GC. Compounds were applied to rubber caps which were suspended above the sticky surface of tetra traps; treatments were tested in 10 replicates in an experimental design used by Arn et al. (1986).

Traps were placed in maize fields at Trasadingen and Hornussen (north of Switzerland), the latter being located ca. 5 km from the "Fricktal wild" collection site and, during the second flight only, at Giubiasco (south). Treatments were placed 2.8 m, replicates 25–50 m apart; trap height was adjusted to plant growth, starting at the upper level of the crop up to a maximum of ca. 2 m.

RESULTS

Analysis of Z11-14:Ac and E11-14:Ac. Results of female extract analyses are given in Figure 1. Individuals can be classified into three types: one containing between 1 and 20 ng *E* and ca. 2% *Z* (*E* type), another with 1–10 ng *Z* and ca. 3% *E* (*Z* type), and one with a more or less constant blend of 65 *E* and 35 *Z* (hybrid).

The 10 females collected in the wild in Fricktal were all of the *Z* strain. However, individuals of a culture derived from material collected at the same location and reared for five generations were, in six cases, of the *E* type and in the two remaining hybrids. Batch analyses of 20 and 25 females of the fourth generation gave similar results (60 and 73% *E*).

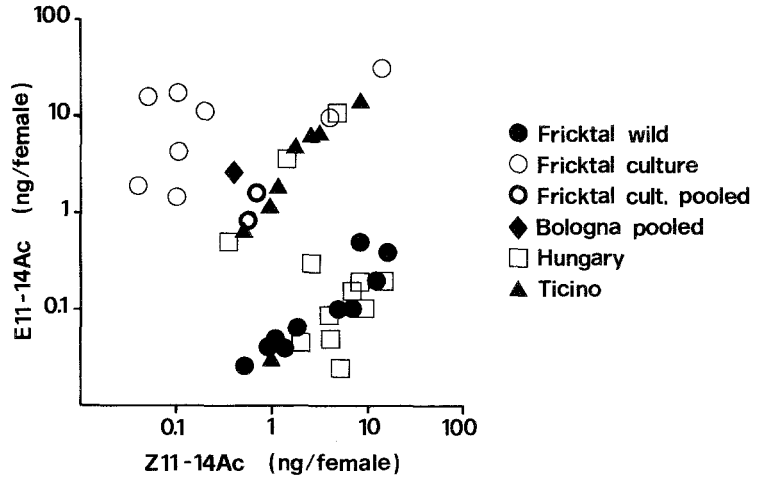

FIG. 1. (*Z*)- and (*E*)-11-tetradecenyl acetate content of *Ostrinia nubilalis* female glands of various sources.

In Giubiasco where we expected to find some *E* types, seven of eight were hybrids and one was *Z*. A batch of 22 wild females from Bologna contained ca. 87% *E*. Of the Hungarian culture, eight of 10 females from the first generation and one of two from the second were *Z*, while the remaining three were hybrids.

Other Components. Tetradecyl acetate was found in all samples, confirming earlier reports (Kochansky et al., 1975). It was generally associated with *Z*11–14:Ac. Samples with more than 50% of this isomer contained between 5 and 15% saturated acetate, the others 2–5%.

A hexadecenyl acetate was detected in all samples with a high proportion of *E*11–14:Ac, at 5–10% of this compound. Based on retention time and fragmentation patterns of standards, it was identified as *Z*11–16:Ac. The compound gave a response on the *Mythimna unipuncta* EAD equivalent to that elicited by synthetic *Z*11–16:Ac. None of the Δ5 to Δ13 hexadecenyl acetates, tested at a similar dose, gave a response with the male antenna. In most samples containing *Z*11–16:Ac, an amount 10 times lower of a second compound was observed which, on the basis of retention time and the typical fragments *m/z* 61 and 222, was tentatively identified as (*E*)-11-hexadecenyl acetate (*E*11–16:Ac).

No tetradecenyl alcohol could be detected in the female material; detection limit for *Z*11–14:OH was 50 pg per female. Similarly, we were unable to find *E*9–14:Ac (limit of detection ca. 30 pg per female), as reported by Klun and Junk (1977). No dodecenyl acetates (detection limit 10–20 pg) or additional saturated acetates were present.

Field Tests. Results of trapping tests confirmed previous reports from Switzerland (Büchi et al., 1982). At Hornussen and Trasadingen, near the northern border of the country, the population is entirely *Z*, and only sporadic catches were made with mixtures containing 20% or more *E* (Table 1). South of the Alps, on the other hand, highest catches were obtained with mixtures containing 60–97% *E*, with no significant differences between the three blends. Even lures with a lower percentage of *E* were still quite attractive; the trap baited with 97% *Z* made 3% of all catches.

The presence of additional compounds, at levels similar to those found in the females, had no effect on male attraction. *Z*11–16:Ac, which was associated with the *E* strain in female analysis, did not significantly enhance or suppress catches on either strain. The same holds true for 14:Ac which has been found synergistic at high doses (Stockel, 1980).

DISCUSSION

The results of this study generally confirm previous reports of the simultaneous presence of *E*, *Z* and hybrid corn borers south of the Alps and of pure *Z* in the north. However, some divergences were observed.

In Ticino, most of the males were trapped with a high proportion of *E* isomer, while females collected just 1 km away were hybrids or, in one case, *Z*. Similar differences were observed by Maini et al. (1978) in Bologna and Cardé et al. (1975) in Pennsylvania. Since our traps were placed in the middle of a large maize-growing area, it is unlikely that the males responding to *E* came from another host.

In the north, all females collected from the wild (Fricktal) were of the *Z* type, and male catches, with almost no exceptions, were made with 97% *Z*. More surprising was the presence of only *E* and hybrid types in the Fricktal culture. This raises the question of whether interbreeding occurred with Ticino types reared in the same laboratory, or if both strains are inherently present in most field populations. The results obtained in Hungary might be an indication for the latter: while previous records indicated presence of only *Z* corn borers (Anglade et al., 1984), a few hybrid females were found in a population kept for one to two generations in the laboratory. Pheromone types of *O. nubilalis* have been correlated with voltinism (Roelofs et al., 1985), and laboratory conditions are known to select out the nondiapausing part of the population (Stengel and Schubert, 1982). Furthermore, the *E* strain, as the more polyphagous (Langenbruch et al., 1985; Straub et al., 1986) might be better suited to survive on the artificial diet.

With the heterogeneity of the corn borer in Europe, we would recommend that at least three types of attractants be used throughout: blends of *E*11–14 : Ac and *Z*11–14 : Ac in the approximate ratios of 97 : 3, 60 : 40, and 3 : 97.

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VOLATILE COMPONENTS OF SCENT MATERIAL FROM COTTON-TOP TAMARIN (*Saguinus o. oedipus*): A Chemical and Behavioral Study

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Abstract—The first chemical analysis of the volatile components of scent material from the cotton-top tamarin, *Saguinus o. oedipus* is presented. In contrast to our previous findings in saddle-back tamarins (*Saguinus fuscicollis*), the chemical composition of the scent marks is quite variable. Analysis of secretion expressed manually from different areas of the scent pads of sedated animals shows individual and regional variability. Only three components (squalene, cholesterol, and *p*-methoxybenzaldehyde) were found in all scent mark samples analyzed. However, suprapubic secretions obtained from one sedated female contained, in addition, 12 butyrate esters as well as five acetate esters which were homologous to the butyrates. The butyrate esters have been previously identified in scent material from *Saguinus fuscicollis*. The variability of the composition of the scent material is discussed in relation to the scent-marking behavior of the cotton-top tamarins. Behavioral studies tested the ability of the cotton-top tamarins to discriminate between scent marks from conspecifics and scent marks from saddle-back tamarins. The group of 12 subjects discriminated between the scent marks from both species in choice tests during which they could freely contact the scent samples. However, when scent marks were presented under a screen, so that the subjects could smell but not contact the stimuli, no discrimination was shown. Four individuals displayed more interest in the screened scent stimuli than all other subjects. When these animals were tested in a second experiment for their ability to discriminate between material from the two species on the basis of only volatile cues, it became evident that they were able to do so. These results indicate that volatile cues alone enable the tamarins to recognize scent marks from conspecifics, but that additional cues per-

ceived during contact with the scent are important for its full attractiveness and/or informational content.

Key Words—Primate, callitrichids, *Saguinus o. oedipus*, chemical communication, scent marking, chemical analysis, GC-MS, volatile composition.

INTRODUCTION

Communication by means of chemical signals is known to be important to the New World callitrichid monkeys. These small marmosets and tamarins possess specialized skin glands located in the circumgenital region and on the midchest. The glands secrete material which, together with urine and possibly genital discharge, is applied to substrates in the environment using specialized scent marking behavior patterns (Epple et al., 1986).

Much of our research over the years has been concerned with the behavioral functions of scent marking and with the chemical composition of the scent marks in the saddle-back tamarin (*Saguinus fuscicollis*). Recently our studies have been extended to include another *Saguinus* species, the highly endangered cotton-top tamarin, *Saguinus o. oedipus*. Cotton-top and saddle-back tamarins have large scent glands in the circumgenital–suprapubic region, which are similar in morphological appearance and in histological structure (Perkins, 1966, 1969).

The scent glands of both species consists of apocrine and holocrine components and are sexually dimorphic, being larger in the female (Perkins, 1966, 1969; Zeller et al., 1988). The sexual dimorphism is particularly pronounced in the cotton-top tamarin. Females possess much larger scent glands than males and exhibit scent marking behavior to a much greater degree (French and Snowdon, 1981; Perkins, 1969; Wolters, 1978). Two distinct patterns of scent marking have been described for *Saguinus o. oedipus* by French and Snowdon (1981). Anogenital marking is performed in a sitting position and results in the application of glandular secretion, urine, and perhaps genital discharge and fecal residues. Suprapubic marking results in the application of skin secretions and other material adhering to the suprapubic part of the glands as the animals, assuming a sprawling position, rub this portion of the gland across the substrate.

Behavioral studies have shown that scent marks produced by the saddle-back tamarin contain a number of communicatory messages, among them the identity of gender, species, subspecies, and individual (Belcher et al., 1986; Epple et al., 1979). At least some of this information appears to be transmitted via volatile cues (Epple, 1978). Chemical analysis by means of gas chromatography–mass spectrometry (GC-MS) has demonstrated that the major volatile components (>90% by weight) of the scent marks of saddle-back tamarins consist of squalene and 15 esters of butyric acid (Yarger et al., 1977). Recent

studies on cotton-tops suggest that the scent marks of this species also convey information to conspecifics which is similar to that of saddle-back tamarins (Epple et al., 1988a).

In this study we present the results of the first chemical analysis of the volatile components of scent material from *Saguinus o. oedipus*. Concurrent behavioral studies, evaluating some of the signal content of volatile cues from scent marks of this species, are also presented. These studies have been undertaken to compare the behavioral and chemical nature of scent communication in these two closely related primate species.

METHODS AND MATERIALS

Maintenance of Experimental Animals and Performance Sites

The cotton-top tamarin is a highly endangered species (Mittermeier et al., 1986), and its availability for laboratory studies is quite limited. For this study six permanently cohabiting male–female pairs were available. The pairs were housed in small colony rooms which they shared with one or two groups of saddle-back tamarins. The rooms were maintained at a temperature of 26°C and 50% relative humidity and were lighted by means of fluorescent light for 12 hr a day. All tamarins were housed in stainless-steel cages consisting of several compartments, each measuring 50 × 80 × 130 cm. The cages were equipped with natural branches, resting shelves, and sleeping boxes. All compartments were available to the pairs most of the time. By inserting a sliding door it was possible to divide each cage into subunits and temporarily separate an animal from its mate for testing or scent collection without causing alarm or stress responses.

The monkeys received a mixed diet consisting of fruits, vegetables, small amounts of cooked meat, dairy products, monkey chow, and several types of cereal, supplemented with vitamins and minerals. The animals were maintained at the German Primate Center, and all behavioral testing was performed there. Chemical analyses were performed at both the Primate Center and the Monell Chemical Senses Center.

Behavioral Studies

Collection of Scent Material. Behavioral studies were performed to determine whether the tamarins could discriminate between natural scent marks from conspecifics and from saddle-back tamarins under two conditions. Under one condition the scent material could be freely contacted, under the second condition it could only be smelled, but not contacted.

The stimulus object on which scent was presented consisted of a glass rod

and a cover tube (Figure 1). The glass rods were closed on one end to form a smooth top. The cover was made from a perforated plastic centrifuge tube which could be placed over the rod, covering it completely. The rounded top of the glass rod was located just underneath the holes in the centrifuge tube. The entire assembly or the centrifuge tube or glass rod alone could be inserted into a hole drilled into a wooden sample carrier which was part of the permanent cage equipment. This made it possible to present scent marks directly on the centrifuge tube, in such a way that the subjects could contact them. Alternatively, scent material could be presented on the glass rod covered by a clean centrifuge tube, a condition under which the animals could sniff but not contact the scent material. Marks were collected on both types of objects by inserting the objects

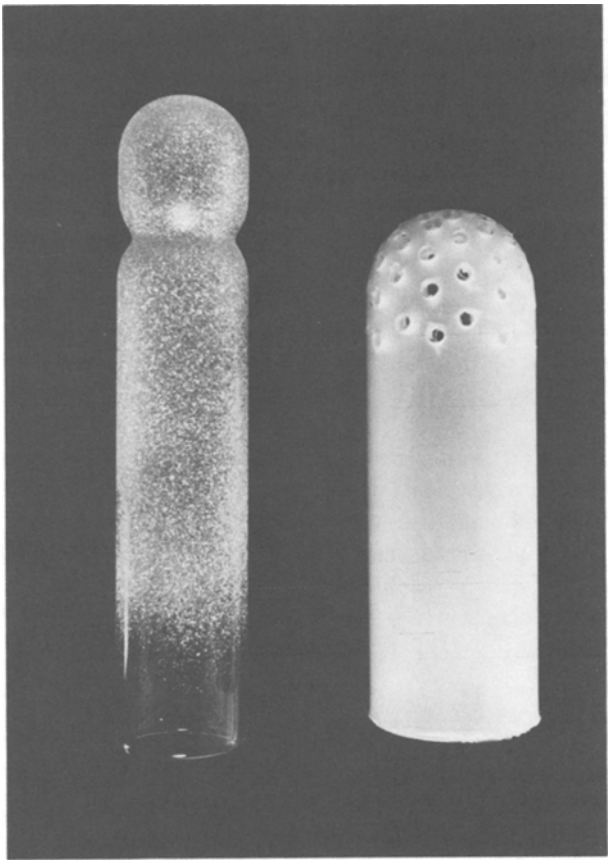

FIG. 1. Glass rod and plastic cover used as the stimulus assembly for behavioral testing of scent material.

into holes in the sample carriers and allowing the donor monkeys to scent mark them. Before use, the plastic tubes were cleaned by soaking them in a soap solution for a few hours or overnight. They were then scrubbed thoroughly with a hand brush to remove all contaminants and rinsed with water, distilled water, and methanol and then air dried. Before using the glass rods, they were thoroughly cleaned using a chromic–sulfuric acid solution, followed by rinses of distilled water, nanograde methanol, and finally, nanograde methylene chloride.

Scent Discrimination Tests. The ability of the tamarins to discriminate between scent marks from conspecifics and scent marks from saddle-back tamarins under the conditions described below was determined by means of choice tests. During each choice test, the subjects were presented with two stimulus objects, each carrying natural scent marks from one of the two donor species. Statistically significant differences in the responses of the tamarins to the two scent types were interpreted as proof that the animals could discriminate between them. All subjects were housed and tested in cages consisting of five compartments. Three of the compartments served as a testing area. In the right and left compartments of this testing area, level wooden shelves ($7 \times 7 \times 80$ cm), which served as sample carriers, were suspended between the back and front of the cages at a height of 65 cm above the cage floor. Each shelf was equipped with a hole into which the glass rod covered by the centrifuge tube could be inserted. The center compartment did not contain a carrier. The wooden sample carriers were part of the permanent cage equipment which also included natural branches. The cages and their equipment were washed in a cage washer at least once a month using hot water but no detergent.

The tamarins were allowed to use all five compartments when no tests were performed. During each test, the subject was confined to the three testing compartments while its mate remained in the other part of the home cage. The duration of each test was 10 min. The total testing time was divided into intervals of 5 sec, indicated by an audible signal. For each 5-sec interval the subject received a score of 1 per stimulus object for each of three behavioral responses if this response was shown.

The following behavioral responses (described in more detail by Epple et al., 1988a) were recorded: (1) contacting the wooden sample carriers; (2) sniffing the stimulus objects containing the samples; and (3) scent marking the stimulus objects. (Anogenital marking was the only marking response recorded. Suprapubic marking was very rare and is not included in this category.)

All animals were tested repeatedly in the course of each experiment (see below). The right–left position of the scent stimuli was counterbalanced across tests and across subjects. The order of testing of the subjects was determined at random. The observer recorded the behavioral responses while sitting approximately 2 m from the cage.

Experiment 1. This experiment tested the ability of the cotton-top tamarins

to discriminate between scent marks from conspecifics and saddle-back tamarins when the animals had full access to the stimulus material and when the animals could only smell but not contact the stimulus material. Six male and six female subjects were given choices between scent marks from conspecifics and from saddle-back tamarins under two conditions. Under the first condition, the plastic centrifuge tubes carried the scent marks from both donor species. The glass rods underneath the centrifuge tubes were clean. Under this condition the tamarins could contact, sniff, lick, and handle the stimulus material.

Under the second condition, the perforated centrifuge tubes were clean while the glass rods located underneath these screens had been scent marked by the donor monkeys. Under this condition the animals could bring their noses within 2–3 mm of the stimulus material but could not contact it directly.

Two cotton-top and two saddle-back females served as donors for each subject. However, due to the limited number of animals available, not all subjects were tested with the scent of the same donor females. All scent marks obtained from the cotton-top donors were anogenital marks. Saddle-back tamarins do not show two distinct marking patterns involving different parts of the circumgenital gland. Donors of this species use labial and suprapubic parts of the glandular pad to varying degrees without an obvious functional distinction.

Each subject received two choice tests under each condition, offering scent marks from the same donors. The succession of conditions was randomized. All subjects were tested only once a day. Mean behavior scores were computed for each subject under each condition. Scores were analyzed by means of a two-tailed paired t test for each condition. A significance level of $P \leq 0.05$ was accepted.

Experiment 2. This experiment tested the ability of four individual cotton-top tamarins to discriminate between scent marks from conspecifics and scent marks from saddle-backs solely on the basis of volatile cues. The four subjects also participated in experiment 1, which preceded experiment 2. Each of the four subjects received six tests offering a choice between scent marks from a female cotton-top and a female saddle-back. Scent marks were presented on glass rods covered by clean, perforated centrifuge tubes as described for experiment 1. Two females from each species served as scent donors. The response scores of each subject were analyzed individually by means of a two-tailed paired t test, accepting a significance level of $P \leq 0.05$.

Chemical Studies

Collection of Scent Material. The chemical studies are based on scent material collected from three adult females (Ri, Ma, and Em) using two different methods of collection. All glass used for collection was acid-cleaned as described for the glass rods in the behavioral methods.

Natural anogenital scent marks were collected by allowing the donor females to mark frosted glass rods. These were inserted into holes drilled into the wooden sample carriers making up the cage equipment. Marks collected in this way were applied by the donors almost exclusively with the labial part of the scent gland and consisted of gland secretions, urine, and perhaps genital discharge.

The scent-marked glass rods were rinsed in 30 ml nanograde methanol–methylene chloride (1 : 3, v/v). Scent-mark collections contained an average of 16 ± 3 SEM individual scent marks. Six collections of pooled scent marks were used for analysis. One individual (Ri) donated material for three collections (50, 20, and 11 marks); a second individual (Ma) donated material for two collections (4 and 6 marks), and the third individual (Em) donated material for one collection (5 marks).

Clean glandular secretion was collected from the suprapubic part of the scent glands of sedated females. For this purpose, the gland was washed thoroughly under running warm water, dried, cleaned with 70% ethanol, and secretion gently expressed several times by hand. Each time, the expressed material was taken up by rolling a frosted glass stopper across the gland, using a clean stopper for each wipe. Clean secretion from the labial part of the scent gland was obtained in a similar manner. The secretion was taken up in 30 ml nanograde methanol–methylene chloride (1 : 3, v/v) by immersing the glass stoppers in the solvent. Each secretion collection contained material from two to three manual expressions of the gland. Gland secretion was collected from two of the three females who donated scent marks (Ri and Em). One secretion sample was collected from female Em. Six samples, including one from the labia, were collected from female Ri. In addition, a control sample was obtained by rinsing a clean glass rod in methanol–methylene chloride using the same procedure as in scent material collection. Samples of natural scent marks and secretion were stored at −80°C until used for analysis.

Preparation of Scent Material for Analysis. Prior to analysis, each solution was carefully reduced in volume to about 0.5 ml using a rotary evaporator under reduced pressure, followed by final evaporation to 30 μl under a stream of nitrogen. The samples were then sealed in small glass vials for air transport from the German Primate Center (Göttingen) to the Monell Chemical Senses Center (Philadelphia). Analysis by gas chromatography at the Primate Center and after air shipment to the Monell Center using conditions identical to those employed for subsequent GC-MS analysis (see below) gave the same results.

Chemical Analysis. Gas chromatography–mass spectrometry (GC-MS) analysis was performed using a Finnigan 4510 GC-MS. The chromatography utilized a Chrompack Sil 8 fused silica column with helium carrier gas in a temperature programmed mode (100°C for 2 min, 4°C/min to 300°C). Mass spectrometry was performed using electron impact (EI) with a 1-sec scan rate.

Data Processing. The data were analyzed using the Nova 3 computer system for all runs in the following manner. The 25–30 largest peaks in each run were compiled into a scan list. Each scan list was processed to yield raw and enhanced spectra and area integration of each peak. Relative retention times of each peak were calculated, using a known component peak, squalene, as the reference. From these data, individual peaks could be compared among all sample runs. Using this procedure, a total of 144 different peaks were found among the 13 different samples analyzed. Those compounds which were identified as silicones or phthalates were assumed to be contaminants and were eliminated from consideration. Additional peaks were disregarded if they appeared in the control sample in comparable amounts. Finally, peaks were disregarded if they did not appear in at least half the samples of natural scent marks or $\frac{3}{5}$ of the samples of gland secretion. A final total of 36 peaks was compiled for analysis (see Table 1). Each mass chromatogram was then searched carefully using selected mass ions to determine if trace amounts of any of these compounds could be detected in any of the other samples.

Two aids to chemical identification were used: a computerized library search using the National Bureau of Standards (NBS) library, and comparison of spectra obtained from scent samples from cotton-top tamarins with spectra of previously identified components of the scent marks of saddle-back tamarins taken under comparable conditions. Positive identifications were made by comparing mass spectra and relative retention times. Identification of previously unknown compounds was confirmed by comparison with mass spectra and retention times of authentic samples. These samples were obtained from the following sources: Sigma Chemical Company, St. Louis, Missouri; Aldrich Chemical Co., and synthesis in our laboratory (Golob et al., 1979).

RESULTS

Behavioral Studies

Figure 2 illustrates the results of experiment 1. When the 12 subjects were presented with a choice between scent marks from cotton-top and saddle-back tamarins under conditions which allowed them to contact the scent marked object directly, they investigated scent from conspecifics significantly more frequently than scent from saddle-backs. Contacting of the sample carriers ($P \leq 0.003$) and sniffing of the samples ($P \leq 0.001$) were significantly more frequent in response to cotton-top scent. No discrimination was made in terms of scent marking. However, when both scent samples were presented under the condition in which the subjects could only sniff but not contact the stimulus material, no discrimination between the scent material was made. Moreover, the mean

TABLE 1. VOLATILE COMPONENTS FOUND IN SCENT MARKS AND SUPRAPUBIC SECRETION FROM COTTON-TOP TAMARIN

Peak	RRT[a]	Compound identification	Average % of total ± SEM	
			Marks	Secretion
1	0.19	*p*-methoxybenzaldehyde	11.0 ± 1.4	
2	0.21		10.2 ± 1.8	
3	0.35			0.4 ± 0.1
4	0.36	*p*-methoxyphenylacetic acid	15.1 ± 2.2	
5	0.40			2.4 ± 0.9
6	0.44	1-tetradecanol (C14:0)		0.3 ± 0.1
7	0.49	tetradecanoic acid (C14:0)		0.3 ± 0.0
8	0.54		1.7 ± 0.4	0.6 ± 0.2
9	0.56	1-hexadecanol (C16:0)		1.7 ± 0.2
10	0.61	hexadecyl (C16:0) acetate[b]	[c]	[c]
11	0.61	hexadecanoic acid (C16:0)	9.0 ± 2.2	1.0 ± 0.1
12	0.65		5.8 ± 0.5	
13	0.68	1-octadecanol (C18:0)		0.8 ± 0.2
14	0.72	hexadecyl (C16:0) butyrate		***
15	0.73	octadecyl (C18:0) acetate		1.1 ± 0.2
16	0.75		9.1 ± 2.9	
17	0.80		6.9 ± 2.1	
18	0.81	octadecenyl (C18:1, Δ9) butyrate		2.6 ± 0.3
19	0.81	octadecenyl (C18:1, Δ11) butyrate		6.5 ± 0.7
20	0.82	docosenyl (C20:1) acetate[b]		6.1 ± 0.5
21	0.82	octadecyl (C18:0) butyrate		[c]
22	0.82	docosenyl (C20:1) acetate[b]		6.3 ± 0.1
23	0.83		5.6 ± 1.2	
24	0.90	docosdienyl (C20:2, Δ11, 14) butyrate		[c]
25	0.90	docosenyl (C20:1, Δ11) butyrate		10.4 ± 0.8
26	0.90	docosenyl (C20:1, Δ13) butyrate		6.2 ± 0.5
27	0.91	eicosenyl (C22:1) acetate[b]		1.5 ± 0.3
28	0.92			0.8 ± 0.1
29	0.98	eicosdienyl (C22:2, Δ13, 16) butyrate		[c]
30	0.98	eicosenyl (C22:1, Δ13) butyrate		1.2 ± 0.2
31	0.98	eicosenyl (C22:1, Δ15) butyrate		0.8 ± 0.1
32	1.00	squalene	14.1 ± 2.3	30.6 ± 4.6
33	1.07	tetracosenyl (C24:1, Δ15) butyrate		0.2 ± 0.1
34	1.07	tetracosenyl (C24:1, Δ17) butyrate		0.2 ± 0.0
35	1.12	cholest-5-en-3β-ol	9.8 ± 1.4	17.0 ± 2.3
36	1.14	cholest-7-en-3β-ol	0.9 ± 0.4	1.0 ± 0.4

[a] Relative retention times; squalene = 1.00;
[b] Not present in scent marks of the saddle-back tamarin.
[c] Peak too small for area integration.

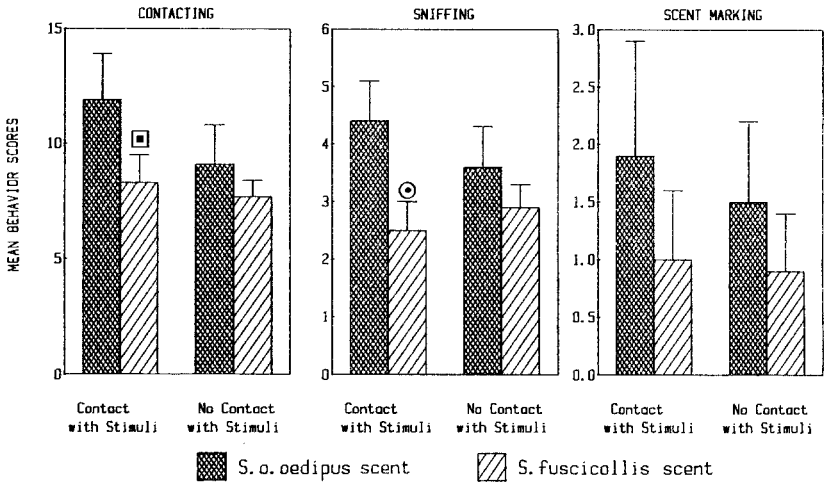

FIG. 2. Mean scores ± SEM for contacting, sniffing, and scent marking in response to scent marks from *Saguinus oedipus oedipus* and *Saguinus fuscicollis*. Scores for tests during which the subjects could contact the stimuli and for tests during which they could not contact the stimuli are shown separately. Significant difference, *t* test. P = ⊙, 0.0002; ▣, 0.003.

scores of contacting, sniffing, and scent marking were lower when the animals could not contact the material.

The results of experiment 1 suggest that the tamarins require direct contact with the stimulus material in order to discriminate between scent from conspecifics and from a related species. It was noted however, that two of the six subject pairs consistently showed more interest in the testing situation than the other pairs and had done so in previous choice tests. Because of this high motivation to investigate scent from conspecifics, it was decided to determine whether any of these four individuals would consistently discriminate between scent samples from the two species on the basis of volatiles alone.

Figure 3 illustrates the results of experiment 2. It shows the behavioral responses of each of the four individuals when presented repeatedly with choices between scent marks from cotton-tops and saddle-backs under conditions where they could smell but not contact the material. All four individuals sniffed scent from cotton-tops significantly more frequently than scent from saddle-backs (Em, $P \leq 0.004$; Ri, $P \leq 0.05$; Hu, $P \leq 0.02$; Rt, $P \leq 0.03$). Three of the subjects also contacted the shelf carrying cotton-top scent significantly more frequently than the shelf carrying saddle-back scent (Em, $P \leq 0.004$; Hu, $P \leq 0.03$; Rt, $P \leq 0.02$), and one female scent marked more on top of samples from conspecifics (Ri, $P \leq 0.01$).

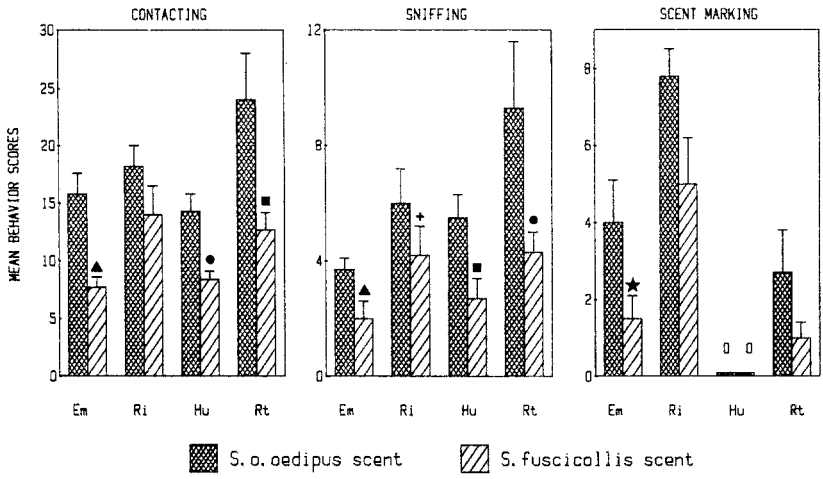

FIG. 3. Mean individual scores ± SEM for contacting, sniffing, and scent marking obtained by two females (Em, Ri) and two males (Hu, Rt) in response to volatiles from scent marks from *Saguinus o. oedipus* and *Saguinus fuscicollis*. Significant difference, *t* test. P = ▲, 0.004; ★, 0.014; ■, 0.02; ●, 0.03; +, 0.05.

Chemical Studies

Scent Marks. Thirteen different peaks were found in the scent-mark samples after processing the data as described above. Of these 13 peaks, only three appeared in all six samples. These three components have been identified as *p*-methoxybenzaldehyde, squalene, and cholest-5-en-3β-ol. These three compounds account for approximately 36% of the total volatile material in the scent marks. Figure 4 shows a mass chromatogram of a typical scent mark analysis from female Ri.

The 10 other peaks were found in very low concentrations. They were found in three to five of the six collections analyzed. Identification has been made for four of these compounds. They are *p*-methoxyphenylacetic acid, hexadecyl acetate, hexadecanoic acid, and cholest-7-en-3β-ol. These four compounds together account for approximately 25% of the total volatile material. Hexadecyl acetate was detected only in trace amounts mixed with hexadecanoic acid. The remaining six components have not been positively identified, although all but one appear to be aromatic compounds as evidenced by characteristic ions at *m/z* 91 and 77. See Table 1 for a summary of the data.

Difficulties were encountered in the identification of many of the components due to the low concentration of volatile material contained in the scent marks. Even large collections of scent mark material (up to 50 marks per collection) did not produce high peak intensities on analysis by GC-MS. Many of

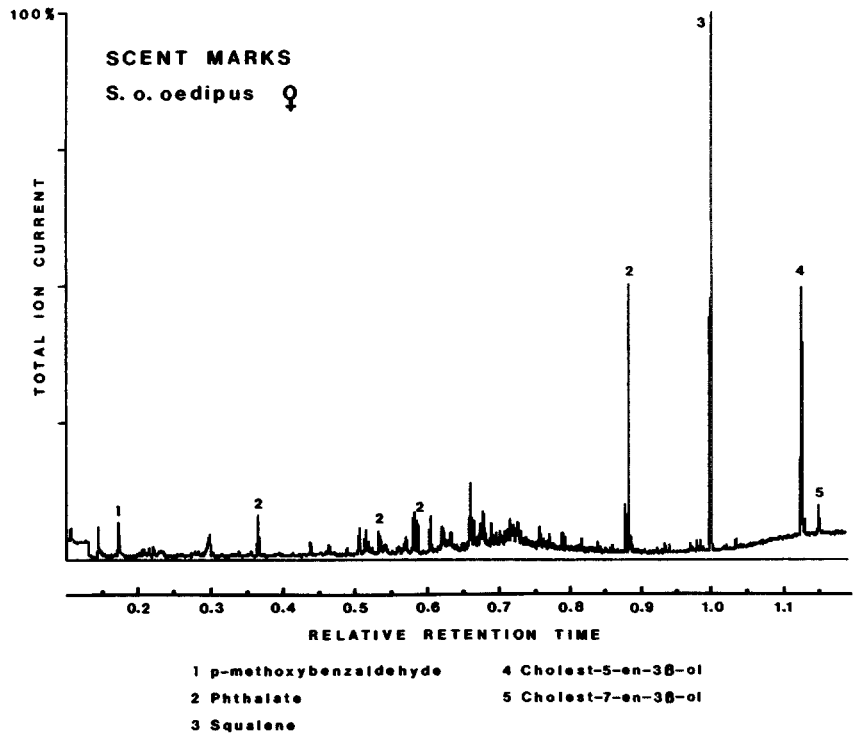

FIG. 4. A mass chromatogram of the volatile components of scent marks from a cotton-top tamarin female (Ri). Numbers refer to specifically identified components. Temperature program: 100°C for 2 min, 4°/min to 300°C.

the peaks are therefore at a very low signal-to-noise ratio and are often mixtures of components. In contrast, the intensity level of peaks in analyses of scent material from the saddle-back tamarins is much larger. It is estimated that one scent mark from the cotton-top tamarin contains approximately 400 ng of volatile material and that squalene and cholesterol account for approximately 150–200 ng of that material. One scent mark from the saddle-back tamarin, on the other hand, is estimated to contain approximately 1000–1500 ng of volatile material, i.e., three to four times as much, and only 15% of that is accounted for by squalene and cholesterol.

Gland Secretion. Chemical analysis of the volatile components of gland secretion manually expressed from the suprapubic glands of two sedated cotton-top females (Ri and Em) gave rather surprising results. Although both had well-developed scent glands, the secretion obtained from these two animals produced extremely different analytical results. The scent profile of suprapubic secretion

from female Em was similar to profiles obtained from the scent-mark samples. The composition of secretion from the labia of female Ri was also similar to that of scent marks; however, the composition of secretion obtained from the suprapubic area of her gland was not.

Analysis by GC-MS of the five suprapubic gland secretion samples from female Ri showed a much different profile than that obtained for any of the other samples. Figure 5 shows a mass chromatogram of a suprapubic gland secretion analysis from female Ri. Thirty different peaks were found in these five samples after processing the sample runs as described previously. Eighteen of these peaks appeared in all five of the secretion samples. Most of these components have now been identified and include, in addition to squalene and cholest-5-en-3β-ol, nine butyrate esters. Three additional butyrate esters were detected in smaller amounts in three or four of these samples. Squalene and cholesterol (cholest-5-en-3β-ol) account for approximately 48% of the total volatile material. The butyrate esters account for an additional 20%. *p*-Methoxybenzaldehyde, one of the three compounds found in all scent mark samples,

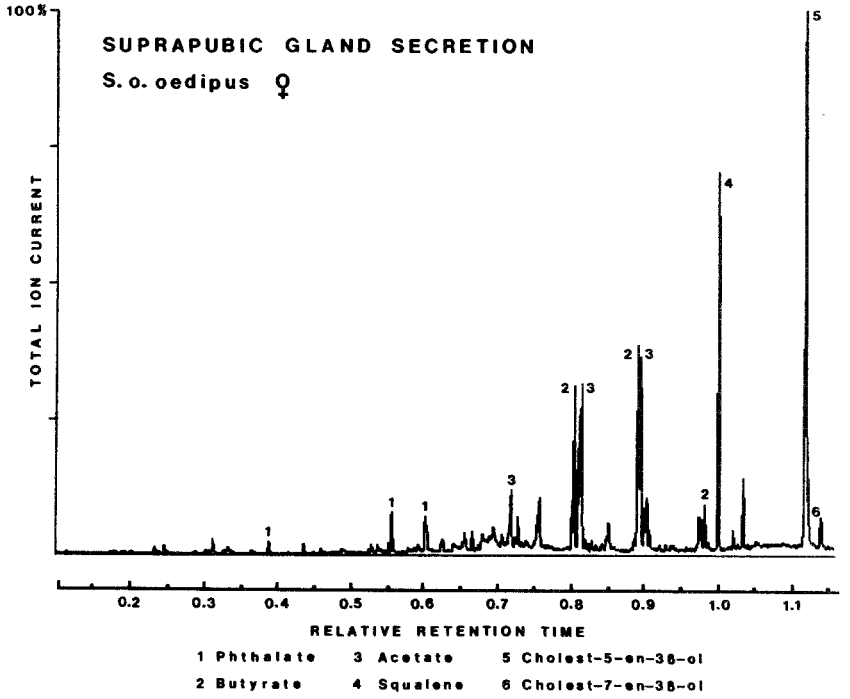

FIG. 5. A mass chromatogram of the volatile components of suprapubic gland secretion from a cotton-top tamarin female (Ri). Numbers refer to specifically identified components. Temperature program: 100°C for 2 min, 4°/min to 300°C.

was detected in only three of five secretion samples and appeared in these in low concentration (<0.5%).

Several additional components have been identified. Five of these compounds are acetate esters, including hexadecyl and octadecyl acetate as well as three monounsaturated esters. These esters, characterized by *m/z* ions of [61] and [M^+-60], account for approximately 15% of the total volatile material. Two of these unsaturated acetates have a 20-carbon alcohol moiety (C20:1), the other has a 22 (C22:1) carbon alcohol unit. Although the position and stereochemistry of the double bond has not yet been established, tentative identification of these unsaturated acetates suggest that they are analogous to three of the butyrates (i.e., C20:1Δ11, C20:1Δ13, and C22:1Δ13 or Δ15).

DISCUSSION

Our present behavioral studies as well as some of our earlier work (Epple et al., 1988a) show that cotton-top tamarins can discriminate between scent marks from conspecifics and from a related tamarin species. This discrimination is shown when the animals can contact the scent material directly. When they can only smell but not contact the stimulus material, their response is decreased and scent from conspecifics is no longer investigated preferentially. These results indicate that the full stimulus qualities of scent marks are available only when the animals can contact the material. The fact that the group of 12 subjects did not discriminate between the marks of the two species on the basis of volatiles alone suggests that the volatile components do not convey the full attractiveness and/or signal content of the mark. However, volatile cues alone appear to provide sufficient information to allow species discrimination by animals who are highly motivated to investigate the material. It is currently unknown which sensory systems are involved in perception of these scents. Olfaction, however, is clearly implicated by the results of this study. In addition the vomeronasal system, which appears to be well developed in callitrichids (Epple, 1986), and other chemosensory systems may be involved.

Similar to cotton-tops, saddle-back tamarins are able to discriminate between scent marks from conspecifics and other callitrichids (Epple et al., 1979, 1988b). Taken together, these results indicate that within the family Callitrichidae different species produce species-specific scents. Under natural conditions such signals may be important in controlling a wide variety of interactions among conspecifics as well as among different species. It is important to document the structural basis for species specificity in any communicatory signal. In the case of chemical communication, comparative chemical analytical studies on scent in related species are indicated.

Previous studies on *Saguinus fuscicollis* have shown that the major volatile

constituents of the scent marks of this species are squalene and a number of butyrate esters. Figure 6 shows, for comparative purposes, a mass chromatogram of scent marks from a female saddle-back tamarin. These compounds are present in relatively consistent concentrations in scent material from males and females of two subspecies. In fact, the sex and subspecies identity of the donor can be accurately predicted solely from these scent profiles (Yarger et al., 1977; Smith et al., 1985; Belcher et al., 1986). In addition, we have documented that methanol–methylene chloride fractions of the scent material from saddle-back tamarins contain sufficient information for the identity of both gender and subspecies. Squalene and the butyrate esters are also present in the same relative concentrations in secretions obtained from both the suprapubic glandular pads and the anogenital glands of sedated saddle-back males and females under conditions identical to those described in the present paper (Belcher, unpublished results).

Based on the studies on the saddle-back tamarin, it was expected that scent material from cotton-tops would be similar. In particular one might expect a

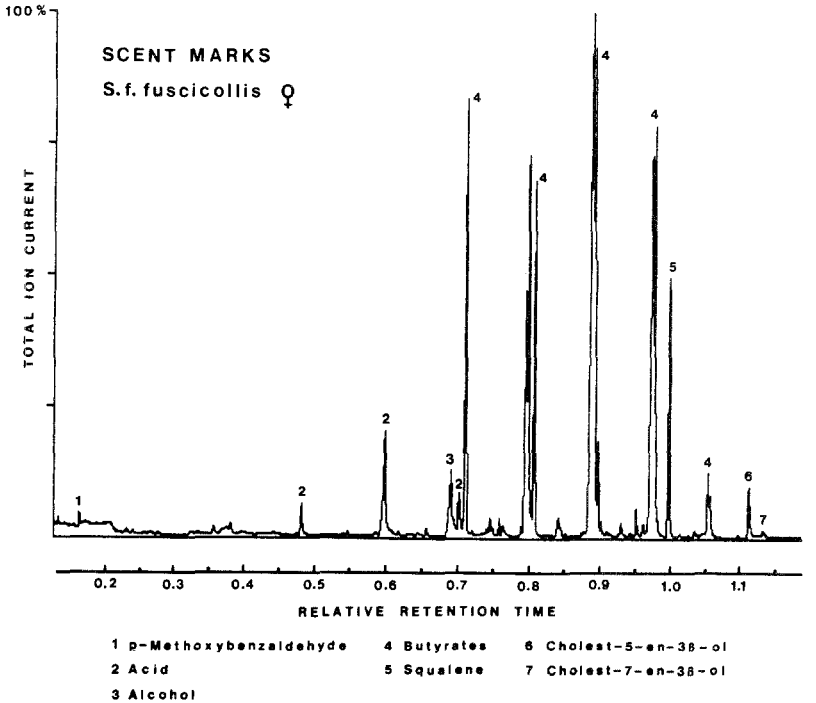

FIG. 6. A mass chromatogram of the volatile components of scent marks from a saddle-back tamarin female. Numbers refer to specifically identified components. Temperature program: 100°C for 2 min, 4°/min to 300°C.

homologous series of compounds, such as the butyrate esters, to be present, perhaps in concentrations different from those seen in the saddle-back species. Chemical analysis of the volatile components of the scent marks of cotton-top tamarins, however, reveals a much different, albeit also complex, profile from that found in saddle-backs. The scent marks of the saddle-back tamarin contain a relatively large number of compounds (16) which appear consistently in all samples. In contrast, the relative concentrations of the major volatile components in the scent marks of cotton-top tamarins do not show a consistent pattern. Only three components (squalene, cholesterol, and *p*-methoxybenzaldehyde) were found in all scent mark samples from cotton-top tamarins. All three are also present in the scent marks of the saddle-back, although only one, squalene, has been previously reported (Yarger et al.; 1977, Belcher, unpublished results). In contrast to our findings in saddle-back tamarins, the chemical composition of glandular secretion from cotton-tops differed from that of their scent marks. In addition, individual and regional variability were apparent. The copious amount of suprapubic secretion obtained from one female (Ri), contained 12 butyrate esters as well as five acetates which were analogous to the butyrates. These acetates were the only components not found in scent material of the saddle-back tamarin.

The chemical composition of secretion obtained from the labial part of the gland of this female (Ri), however, did not differ from that of the scent marks. Furthermore, no evidence of butyrates could be found in the suprapubic secretion of the other female (Em) or in any of the scent-mark samples. Nevertheless, the detection of the butyrates in the suprapubic secretion from one individual demonstrates that these compounds can be produced by this species as well.

The detection of acetates, as well as butyrates, in the suprapubic gland secretion from female Ri is of interest. However, only one of these, hexadecyl acetate, was detected in any of the scent-mark samples, and it was found there only in trace amounts. These acetates have been tentatively identified as analogous to several of the previously identified butyrate esters, including three of the monounsaturated types. Although positive identification has not been made for the unsaturated acetates, we tentatively assume them to be *cis* isomers, as has been previously determined for all of the unsaturated butyrates found in scent material from the saddle-back tamarin. Collection of large amounts of scent material will be necessary for complete structural identification.

It is difficult to explain the considerable variability in the chemical composition of the different scent materials from cotton-top tamarins. It is conceivable that this species can control the release of glandular secretions during scent marking and by doing so produces scent marks which are different in composition from material obtained by manual expression of the gland. Moreover, it is also possible that the suprapubic area of the glandular pad produces secretions different from those produced in the labial part. Cotton-top females appear to use the suprapubic and labial gland areas in two separate and distinct scent

marking patterns (French and Snowdon, 1981). While anogenital marking may be involved in sexual communication, suprapubic marking is used primarily in situations of aggressive arousal. Indeed, French and Snowdon (1981) have suggested that the two signals may differ in chemical composition.

In contrast to cotton-top tamarins, saddle-backs show much less pronounced sex differences in the size of the scent glands and in scent-marking frequency. Moreover, they do not appear to show a functional distinction between anogenital and suprapubic marking. Thus it appears that the two species have developed somewhat different mechanisms for the communication of information via chemical cues and that some of the cues may play different roles in behavior.

Clearly, more work is needed to evaluate the significance of the differences between the two species and the factors that may cause the variability in the chemical composition of the scent samples in *Saguinus o. oedipus*. Unfortunately a more detailed comparison of the composition of scent secretions with that of the scent marks is difficult. Large amounts of both scent marks and expressed secretion must be collected for chemical analysis. The frequent handling and sedation necessary for collecting these secretions, however, is strongly contraindicated in these endangered primates, since it often causes females to lose their pregnancies.

At this stage we cannot determine which of the volatile components of the scent of the two species are involved in encoding species specificity. Indeed, the behavioral studies reported here, as well as some unpublished work in *Saguinus fuscicollis* (Epple, unpublished results) suggest that, in addition to the known constituents, other components which do not lend themselves to chemical analysis by gas chromatography, such as high-molecular-weight, nonvolatile compounds, are necessary to complete the chemical messages. Our ongoing studies of chemical communication in both species are currently addressing this question.

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COMPUTATION OF RESPONSE FACTORS FOR QUANTITATIVE ANALYSIS OF MONOTERPENES BY GAS–LIQUID CHROMATOGRAPHY

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Abstract—Response factors for quantitative analysis by gas–liquid chromatography were computed for 10 monoterpenes using *p*-cymene as an internal standard. The results show that there is no significant variation in our estimates due to sample concentration, and very low variation due to the compound. The most significant deviation from $RF_c = 1.0$ occurred with myrcene. We conclude that the underlying assumptions of quantitative analysis are met using *p*-cymene as an internal standard for most plant or animal monoterpenes, and for total monoterpene content.

Key Words—Monoterpenes, gas chromatography, response factors, internal standards.

INTRODUCTION

Monoterpenes and their related compounds comprise one of the largest groups of secondary compounds in plants (Goodwin and Mercer, 1983). They also exhibit an extremely broad range of biological activities, including insect deterrents, attractants, pheromone precursors, and sequestered defensive agents, microbial inhibitors, allelopathic agents, and mammalian toxins, behavior modifers, and hormone precursors (e.g., Harborne, 1982; Brattsten, 1983). They are also widely used for chemotaxonomic classification. (e.g., von Rudloff, 1975).

In many systems, particularly in plant–animal interactions, qualitative descriptions of monoterpene composition are not sufficient to characterize behavioral or ecological effects. There can be critical differences in the ability

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of an herbivore to utilize a host plant due to quantitative variation (e.g., Raffa and Berryman, 1982; Cates et al., 1983; and others).

The most reliable way of analyzing monoterpenes is by gas–liquid chromatography (GLC) (Harborne, 1984). Quantification is typically achieved by the internal standard technique, in which the peak area is normalized to the response of a known quantity of a chemical that does not occur in the sample of interest. This method assumes that the detector responds uniformly to all components, and where this assumption is not met corrections must be made using the appropriate response factor (Debbrecht, 1977). Although there have been numerous reports dealing with quantitative variation in monoterpene content, we are not aware of any studies in which this assumption was critically tested. Response factors for other groups can sometimes show high variation (Edwards, 1978; Scanlon and Willis, 1985).

We describe in this paper an analytical system in which the internal standard method can be applied to the quantitative estimation of monoterpene content.

METHODS AND MATERIALS

Instrumentation. GLC was performed using a Shimadzu GC-9A with FID and a CR3A digital integrator. The column was 25 m × 0.25 mm bonded fused silica open tubular polyethylene glycol Superox® (= Carbowax 20 M) (Alltech Assoc., Deerfield, Illinois). Previous experiments demonstrated optimal separation of monoterpenes by this column.

Conditions. The injector and detector temperatures were at 220°C. The oven temperature was at 60°C for 10 min and rose at 10°C/min to 160°C. The carrier gas was helium at 30 cm/sec, with a methane retention time of 83.8 sec.

Monoterpenes were obtained from Aldrich Chemical Co. (Milwaukee Wisconsin). Beta-phellandrene was obtained from Duane Zinkel, USDA Forest Products Laboratory, Madison, Wisconsin. They were dissolved in analytical grade pentane before injection.

Calculation of Response Factor. The response factor is defined as the area ratio to weight ratio of the two constituents (Dietz, 1967): $RF_c = (A_c/A_a)/(W_c/W_a)$; where RF_c is the response factor for C; A_c and A_a are the peak areas of component C and internal standard A, respectively; and W_c and W_a are the corresponding weights.

p-Cymene was used as the internal standard because it is structurally similar to the other monoterpenes of interest, and does not occur at detectable levels in many conifer samples, in which we are particularly interested. *p*-Cymene is frequently used as an internal standard for studies on conifer–herbivore interactions (e.g., Wright et al., 1979; and others).

Each monoterpene was dissolved in pentane at a series of concentrations

to span the proportions we typically find in conifer oleoresin. These included 0.01, 0.1, 1.0, and 5.0%, with 15μl *p*-cymene. The weights of the monoterpene of interest and *p*-cymene in each sample were determined. The sample was injected, the resulting peak areas observed, and RF_c values were computed.

Statistical Analysis. The variation within monoterpenes due to concentra-

TABLE 1. GAS–LIQUID CHROMATOGRAPHIC RESPONSE FACTORS OF STANDARD MONOTERPENES RELATIVE TO INTERNAL STANDARD *p*-CYMENE[a]

Monoterpene	Retention time (min)	Concentration (%)	RF_c (± SEM, N)
Alpha-pinene	2.707	0.1	0.936 (±0.078, 2)
		1.0	0.942 (±0.072, 2)
		5.0	1.025 (±0.008, 2)
Camphene	3.200	0.01	0.945
		0.1	0.971
		1.0	0.948
		5.0	0.937
Beta-pinene	3.858	0.1	1.020
		1.0	0.944
		5.0	1.038
Sabinene	4.072	0.01	0.921
		0.1	0.839 (±0.300, 2)
		1.0	0.999 (±0.021, 2)
Myrcene	5.067	0.01	0.712
		0.1	0.681
		1.0	0.813
(−)-Limonene	6.023	0.1	0.966
		1.0	1.030
		5.0	0.984
(+)-Limonene	6.023	0.01	0.938
		0.1	1.266 (±0.133, 2)
		1.0	1.034 (±0.061, 3)
		5.0	1.015
Beta-phellandrene	6.22	0.1	1.043
		1.0	1.025
		5.0	0.943
Gamma-terpinene	7.717	0.01	1.086 (±0.016, 2)
		0.1	0.841 (±0.107, 2)
		1.0	0.936 (±0.033, 2)
		5.0	0.881 (±0.049, 3)
p-Cymene	8.423	—	—
Delta-3-carene	14.675	0.01	0.930
		0.1	1.055
		1.0	0.844

[a] RF_c = Response Factor; SEM = standard error of mean; N = number of trials where not 1.

tion was estimated by one-way analysis of variance (Steel and Torrie, 1980). The mean response factors for each monoterpene were compared by Duncan's multiple-range test at $P = 0.05$ using SAS (1982).

RESULTS AND DISCUSSION

The retention times and response factors of the various monoterpenes are shown in Table 1. None of the monoterpenes showed any significant variation in response factor due to concentration. Likewise, when all of the monoterpenes were combined, as would be the basis for computing total monoterpenes for example, there was no relationship between concentration and RF_c (Table 2). Therefore, we conclude that *p*-cymene is a suitable internal standard for the quantitative analysis of monoterpenes, at least within the concentration range shown in Table 1. For means-comparison analyses, therefore, we pooled all concentrations for each monoterpene.

The means-comparison analysis is shown in Table 3. There are no significant differences in response factors, except for myrcene vs. all other monoterpenes, and for the (+)-limonene vs. sabinene comparison. Underestimates of myrcene concentrations may be due to its tendency to polymerize at detector temperatures above 200°C. Therefore corrections must be made for estimates of myrcene concentrations in biological samples. For the other compounds tested, the assumption that the response factor for monoterpenes, relative to the

TABLE 2. ONE-WAY ANALYSIS OF VARIANCE OF CONCENTRATION EFFECTS ON MONOTERPENE RESPONSE FACTORS[a]

Monoterpene	Source	*DF*	SS	*F*	*P*
Alpha-pinene	Concentration	2	0.010	0.67	0.57
	Error	3	0.022		
	Total	5	0.032		
Sabinene	Concentration	2	0.031	7.36	0.07
	Error	3	0.006		
	Total	5	0.037		
(+)-Limonene	Concentration	3	0.099	1.73	0.33
	Error	3	0.057		
	Total	6	0.157		
Gamma-terpenene	Concentration	3	0.072	2.99	0.134
	Error	5	0.040		
	Total	8	0.111		
All	Concentration	3	0.002	0.040	0.99
	Error	43	0.682		
	Total	46	0.683		

[a]DF = degrees of freedom; SS = sum of square; F = variance ratio; P = probability level.

TABLE 3. MEANS COMPARIONS OF MONOTERPENE RESPONSE FACTORS

Monoterpene[a]	Response factor ± SEM
(+)-Limonene (a)	1.084 ± 0.061
Beta-phellandrene (ab)	1.004 ± 0.030
Beta-pinene (ab)	1.001 ± 0.029
(−)-Limonene (ab)	0.993 ± 0.019
Alpha-pinene (ab)	0.967 ± 0.033
Camphene (ab)	0.950 ± 0.007
Delta-3-carene (ab)	0.943 ± 0.061
Gamma-terpinene (ab)	0.930 ± 0.039
Sabinene (b)	0.906 ± 0.035
Myrcene (c)	0.735 ± 0.039

[a]Compounds followed by the same letter are not significantly different at $P < 0.05$.

internal standard *p*-cymene, is 1.0 appears to be acceptable within normal physiological ranges.

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EFFECTS OF DIETARY PROTEIN AND LUPINE ALKALOIDS ON GROWTH AND SURVIVORSHIP OF *Spodoptera eridania*

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Abstract—Toxic chemicals and nutrients are often positively correlated within and among plants. We studied how such correlations affect the suitability of plants as food for herbivores by assessing the growth and survivorship of *Spodoptera eridania* (army worm) on artificial diets containing lupine alkaloids and casein. We found that (1) the effects of casein were determined by other dietary components: increased dietary casein led to increased larval growth only when the diet was also high in wheat germ. (2) Dietary alkaloids were effective at very low concentrations, reducing both growth and survivorship. The alkaloids lupanine and sparteine were not synergistic in their effects, and the interaction between alkaloids and casein was significant only in the low-wheat-germ diets. (3) The effects of casein and alkaloids were generally apparent only in the first instar, and the growth of fifth-instar larvae was unaffected by diet. (4) Using these artificial diet experiments, we can make simple predictions about the food quality of plants grown under various nutrient regimes. These predictions are consistant with recent ideas about optimal plant defenses.

Key Words—*Lupinus*, Fabaceae, lupines, *Spodoptera eridania*, Lepidoptera, Noctuidae, southern army worm, sparteine, lupanine, alkaloids.

INTRODUCTION

Herbivores can use plants for food, water, and protection from enemies, but only after overcoming barriers such as poisonous hairs, toxic chemicals, and dry, slippery leaves (Scriber, 1977; Tingey and Laubengayer, 1981; Edwards, 1982; Lawton, 1983). Thus, the suitability of a particular plant as food for

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herbivores depends on the balance between factors with positive and negative effects. In this paper we focus on two such factors: nitrogen and the quinolizidine alkaloids. Nitrogen is one of the most important nutrients for herbivorous insects (Slansky and Feeny, 1977; White, 1978; Mattson, 1980), while the quinolizidine alkaloids are toxic chemicals in the large genus *Lupinus* (Fabaceae) (Smolenski et al., 1981).

Because the food value of plants is controlled by the interaction between variable plant nutrients and variable antiherbivore agents, predicting the food value of a plant can be a complex task. This task is especially complicated for alkaloid-producing plants, which often have positive correlations between toxic alkaloids and protein (Rhoades and Cates, 1976; Waller and Nowacki, 1978; Johnson et al., 1987). In these cases the relative food value of high-protein and low-protein plants depends on whether increased protein offsets the increases in alkaloid levels. Consequently, experiments with controlled diets are usually required to understand how protein and alkaloids affect the food quality of plants for herbivores (Duffey et al., 1986).

Although the biochemistry of nutrient–toxic chemical interactions has been studied (Reese, 1979), few experiments have used controlled diets to test how nutrient levels affect the toxicity of plant secondary chemicals, and these suggest that more research on nutrient and toxic chemical interaction is needed. Studies of a lepidopteran herbivore (Lincoln et al., 1982) and of rats (Morcos, 1970) found that high protein levels can reduce the negative effects of toxic chemicals. A third study found that the chrysomelid *Trirhabda diducta* had constant growth regardless of the levels of protein and leaf resin (Johnson et al., 1985). Finally, a fourth study found that rutin and tomatine were most effective when incorporated into high protein diets at reducing the growth rates of *Spodoptera exigua* and *Heliothis zea* (Duffey et al., 1986).

In this study we report the effects of dietary protein and the quinolizidine alkaloids sparteine and lupanine on the polyphagous herbivore, *Spodoptera eridania* (Soo Hoo and Fraenkel, 1966). In particular, we ask: (1) Do alkaloid and protein levels interact to affect larval growth? (2) Do different alkaloids interact (e.g., are alkaloids synergistic)? (3) What can we predict about the effects of natural levels of protein and alkaloids on larval growth and consumption? (4) At what developmental stage are larvae most vulnerable to the effects of dietary composition?

The two alkaloids that we have chosen for our study, lupanine and sparteine, are common in the genus *Lupinus* (Kinghorn et al., 1980). They have previously been shown to have antiherbivore activity against generalists, but not against lupine specialists (Smith, 1966; Wink et al., 1982; Wink, 1984). We therefore chose the generalist *Spodoptera eridania* as a convenient model for investigating the toxicity of these alkaloids.

METHODS AND MATERIALS

Larvae and Diets. All *Spodoptera eridania* were obtained as eggs from a laboratory colony maintained for several years by Dr. T. Anderson, Boyce Thompson Institute, Cornell University. Protein sources for diets were casein (ICN Biochemicals) and raw wheat germ. Sparteine was obtained from Sigma Chemical Co. (St. Louis, Missouri), and lupanine was extracted from pods of locally collected *Lupinus perennis*.

Protein and alkaloid concentrations were varied within two basic diets designated diet C (high casein) and diet Wg (high wheat germ). In diet C, each diet contained 6% wheat germ (dry weight) with an additional 16.7% and 33% casein added to the low- and high-protein diets, respectively. In the Wg diets, wheat germ was added to 33% of each diet with the high-protein diets having an additional 16.7% casein. The weight of casein + cellulose (alphacel nonnutritive bulk) was constant within each set of diets, totaling 40% in the C diets and 20% in the Wg diets. All other constituents are as in the artificial diet of Lincoln et al. (1982).

We refer to larval diets in the discussion below using a short-hand code with the first part of the code describing the diet type, the second part denoting the protein level, and the subscript giving the alkaloid concentration. For example, diet $WgL_{.02}$ is a high-wheat-germ diet with low protein levels and 0.02% dry weight alkaloids. This code is varied slightly to designate the two diets containing lupanine, with $CL\text{-}L_{.60}$ containing only lupanine and $CL\text{-}LS_{.60}$ containing lupanine + sparteine. The protein and alkaloid contents of our experimental diets are shown in Table 1, with the protein content including both wheat germ protein and casein using the conversion: wheat germ = 50% protein.

Experimental Procedures. Larvae were reared in 30-ml plastic cups containing small squares of diet and moist paper towels, and diets were changed 3× each week. Cups with larvae were placed in plastic containers lined with damp towels and kept in a growth chamber with a 16-hr photoperiod, 25°C day : 16°C night, and 70% relative humidity. Experiments with the two basic diets (i.e., diets C and Wg) used slightly different protocols. For experiments with diet C, newly hatched larvae were fed an alkaloid-free C diet for 24 hr and then 120 larvae were placed on each of the C diets in Table 1 except diets $CL_{.60}$ and $CH_{.60}$, which began with only 60 larvae. Larvae were initially stocked at 12/cup and then subcultured into new cups at two per cup after five days, and larvae were weighed on day 14. For diet Wg, 40 newly hatched larvae were reared on each of the 18 Wg diets shown in Table 1 at a stocking density of four larvae per cup. Larval instar and survivorship were censused at five 2- to 3-day intervals over the first 11 days, and all larvae were weighed on the 11th day after hatching. Larvae began molting into the fifth instar 14 days after

TABLE 1. ARTIFICIAL DIETS

Diet C									
20% protein	$CL_{.01}$		$CL_{.06}$	$CL_{.60}$[a]	$CL\text{-}L_{.60}$[a]		$CL\text{-}LS_{.60}$[a]		
Sparteine	0.01		0.06	0.60	0		0.30		
(% dry wt)									
Lupanine	0		0	0	0.63		0.32		
36% protein	$CH_{.01}$		$CH_{.06}$	$CH_{.60}$					
Sparteine	0.01		0.06	0.60					
Diet Wg									
16.7% protein	WgL_0	$WgL_{.01}$	$WgL_{.02}$	$WgL_{.03}$	$WgL_{.05}$	$WgL_{.075}$	$WgL_{.10}$	$WgL_{.60}$	$WgL_{1.20}$
Sparteine	0	0.01	0.02	0.03	0.05	0.075	0.10	0.60	1.20
33% protein	WgH_0	$WgH_{.01}$	$WgH_{.02}$	$WgH_{.03}$	$WgH_{.05}$	$WgH_{.075}$	$WgH_{.10}$	$WgH_{.60}$	$WgH_{1.20}$
Sparteine	0	0.01	0.02	0.03	0.05	0.075	0.10	0.60	1.20

[a] Diets $CL_{.60}$, $CL\text{-}L_{.60}$, and $CL\text{-}LS_{.60}$ are equimolar in alkaloids.

hatching, and newly molted fifth-instar larvae were kept without food overnight and then used for short-term feeding studies (described below). Because we expected no survivors on the 1.2% sparteine diets and low survivorship on the 0.6% sparteine diets, we kept an additional stock of 50 larvae on the $WgL_{.60}$ and $WgH_{.60}$ diets for use in the short-term feeding studies.

For our short-term feeding studies, larvae were allowed to feed on their original diets during one 16-hr photoperiod. Measurements were performed as in Waldbauer (1968). Briefly, we measured initial food and larval wet weights, both of which were converted to initial dry weights (larva = W_0, food = F_0) using measurements of larval and food percent dry weight; and we measured final food (F_f), larva (W_f), and frass dry weight (E).

Calculations and Statistical Analyses. We assessed the effects of diet on larval performance by calculating growth rates and survivorship over the first 11 (Wg diets) or 14 days (C diets), by estimating the time between instars, and by calculating short-term feeding indices. Because larval performance differed among cups within the same diet, the mean squares among cups was used as the error term in analyses of variance rather than the mean squares among larvae. All analyses of variance were done using type III sums of squares of the General Linear Models procedure of SAS (SAS Institute Inc, Cary, North Carolina).

Exponential growth rates were calculated as $R = \ln (A_T/0.074)/T$, where 0.074 mg = average larval weight at hatching, and A_T = the average weight of larvae in a cup at day T (T = 14 for C diets, T = 11 for Wg diets). We did not use data for 1.2% sparteine, because the $WgL_{1.2}$ estimate was based on only one surviving larva, compared to 4–40 larvae surviving on the other diets. The variates for testing the effects of diet on survivorships were the mean survivorship of each group of four larvae.

The overall effects of protein and sparteine on growth rates and survivorship were tested in a two-way analysis of variance (ANOVA) of protein (discrete) and sparteine (continuous) effects. Individual diets were contrasted using Sidak's correction for multiple comparisons (Sidak, 1967) and the error mean squares from the appropriate ANOVA. Thus, the effects of protein on larval survivorship were tested with high-to-low protein comparisons for each alkaloid concentration (nine comparisons) and the error mean squares of a two-way ANOVA (protein and sparteine, both discrete). The effects of alkaloids on 11-day survivorship and on 11-day larval weights were tested by comparing diets lacking alkaloids to diets with alkaloids (eight comparisons) for each protein concentration, using the error mean squares of an ANOVA of sparteine concentration (discrete).

The duration of the first-instar period was estimated using linear interpolation from our measurements of instar number. The total time spent as second to fourth instars was estimated by subtracting the duration of the first-instar

period from the time from hatch to fifth instar. The effects of protein and sparteine on first and second to fourth instars were tested in a two-way ANOVA of protein and sparteine (continuous).

Short-term feeding indices were calculated as in Waldbauer (1968) and Gordon (1968). Thus, for $t = 1$ day, we calculated relative growth rate: $RGR = \ln(W_f/W_0)$; average weight: $W_{av} = (W_f - W_0)/RGR$; relative consumption rate: $RCR = (F_0 - F_f)/W_{av}$; approximate digestibility: $AD = (F_0 - F_f - E)/(F_0 - F_f)$; efficiency of conversion of ingested food into biomass: $ECI = RGR/RCR$; and efficiency of conversion of digested food into biomass: $ECD = RGR/(RCR \times AD)$.

Feeding indices and fifth-instar weight were analyzed using a three-way ANOVA, with the eight-day period during which larvae reached the fifth instar as a random blocking factor. The main effects for the ANOVA were thus sparteine level (continuous), protein (discrete), and date of reaching fifth instar (random discrete).

RESULTS AND DISCUSSION

Effects of Protein on Larval Growth and Survivorship. The effects of added protein on larval growth were strikingly different in the C diets and the Wg diets. Larvae fed C diets grew more slowly on high-casein than on low-casein diets (Table 2), whereas added casein increased both the growth and survivorship of larvae fed the Wg diets (Figure 1).

We presume that our Wg diets mimic leaves more accurately than do our C diets, because plants with high protein levels are generally more nutritious than those with low protein, all other things being equal (White, 1978; Mattson, 1980). The protein levels in the C and Wg diets are not unusual for the leaves of herbaceous plants, but a heterogeneous protein source, such as leaves or the

TABLE 2. EFFECTS OF DIETARY SPARTEINE AND CASEIN: DIET C[a]

Alkaloid content	Mean larval wt at 15 days, mg (SE, N)	
	20% Protein	36% Protein
0.01% sparteine	439a (10.8, 60)	182b (5.4, 59)
0.06% sparteine	265a (9.8, 58)	146b (5.1, 55)
0.60% sparteine	64a (8.9, 8)	58a (7.4, 11)
0.63% lupanine	162 (6.6, 28)	—
0.32% lupanine/0.30% sparteine	118 (7.8, 37)	—

[a] N = number of cups, each of which contains one to three larvae. Values within each row followed by the same letter do not differ significantly ($P > 0.05$).

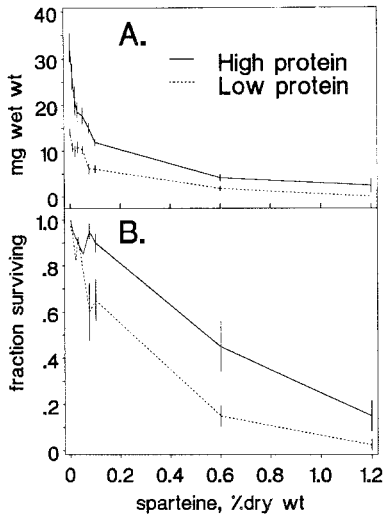

FIG. 1. Larval weight and survivorship of 11-day-old larvae fed Wg diets. (A) Mean weight of larvae; error bars indicate standard errors. High vs. low casein: $F_{1,154} = 42.8$, $P < 0.0001$. $N = 10$ cups/diet, except as follows: $WgL_{.05} = 9$; $WgL_{.60} = 3$; $WgL_{1.20} = 1$. (B) Mean survivorship with standard errors. High- and low-protein diets differ significantly at the following sparteine levels: 0.075%, $t_s = 3.6$, $df = 162$, $P < 0.005$; 0.1%, $t_s = 2.6$, $df = 162$, $P < 0.1$; and 0.6%, $t_s = 3.0$, $df = 162$, $P < 0.05$. $N = 10$. Error bars are omitted at low sparteine levels for clarity.

Wg diets, may be less toxic or may provide a more appropriate amino acid mixture than a homogeneous source such as casein. Alternatively, the differences between the C and Wg diets may be due to important nutrients found in wheat germ that are limiting in the low-wheat germ C diets. Because larvae feeding on low-protein diets have higher relative consumption rates (see below), they may consume adequate amounts of some dietary nutrients from wheat germ in the low-protein C diets that would otherwise be growth-limiting.

Effects of Alkaloids on Larval Growth and Survivorship. Dietary alkaloids decreased both larval weight and survivorship (Table 2, Figure 1). Larval size at 11-days was significantly reduced by as little as 0.02% sparteine in both high- and low-protein diets ($P < 0.05$ and $P < 0.005$, respectively). Larval survivorship, on the other hand, did not decrease until dietary sparteine was >0.05% at low protein and >0.1% at high protein (WgL_0 vs. $WgL_{.10}$, $t = 3.5$, $df = 81$, $P < 0.01$; WgH_0 vs. $WgH_{.60}$, $t = 7.6$, $df = 81$, $P < 0.001$).

Because alkaloids and protein are both quite variable in lupine plants (Johnson et al., 1987), it is important to determine whether the toxicity of alkaloids is affected by protein content. One effect of low protein content is simply

to cause increased alkaloid consumption, since larvae have greater consumption rates on low-protein diets (see below). Thus, we might expect to see a significant interaction between protein and alkaloid content, with alkaloids being more toxic in low-protein diets. However, our two diets gave conflicting results: the Wg diets showed no significant interaction between sparteine and protein, but sparteine was more active at lower protein concentrations in the C diets ($F_{1,247} = 27.2$, $P < 0.0001$). This difference between the C and Wg diets may be due to dietary constituents other than protein and sparteine, since larvae feeding on low-protein diets will have higher consumption rates of all diet components (see below). Neither of our two diets gave results similar to those of Duffey et al. (1986), however. These workers found that rutin and tomatine depressed *Spodoptera exigua* and *Heliothis zea* growth rates more strongly at higher protein concentrations.

We assessed the toxicities of lupanine and sparteine both alone and in combination because both lupanine and sparteine are often produced in a single lupine plant (Kinghorn et al., 1980). When tested individually in diets with 0.6% alkaloid, sparteine retarded larval growth significantly more than lupanine (Table 2, $P < 0.001$). This difference in the toxicities of lupanine and sparteine can be shown to be about twofold using linear interpolation: 0.63% lupanine was equivalent to about 33% sparteine. Wink (1984), using mollusks, also found lupanine to be less toxic than sparteine.

When tested in combination, lupanine and sparteine were not synergistic, since the toxicity of the 0.30% sparteine–0.32% lupanine diet was intermediate between those of the 0.60% sparteine and 0.63% lupanine diets. This lack of synergism can also be shown by using linear interpolation: the 0.32% lupanine–0.30% sparteine diet is equivalent to about 0.45% sparteine, and therefore 0.32% lupanine = 0.15% sparteine. Thus, lupanine seems to be about half as toxic as sparteine, as noted above. Because the plot of larval weight vs. sparteine is concave (Figure 1A), linear interpolation yields a maximum estimate of lupanine toxicity.

Effects of Protein and Alkaloids on Short-Term Feeding Indices and Fifth-Instar Weight. Dietary sparteine had no significant effects on any of the short-term feeding indices (Figure 2A–E), and protein levels did not affect growth or digestion efficiency (Figure 2A,E). In addition, neither sparteine nor protein significantly affected the weight of larvae upon reaching the fifth instar (protein: $F_{1,7} = 0.25$, $P = 0.64$; sparteine: $F_{1,7} = 0.26$, $P = 0.63$).

Thus, *S. eridania* larvae either died before reaching the fifth instar or adjusted to utilize their diets effectively. Larvae adjusted to diets with low protein by consuming more food with lower digestibility and efficiency (Figure 2C–E, all $P < 0.001$), similar to the results of others (Slansky and Feeny, 1977; Johnson et al., 1985).

Suceptibility of Young Larvae to High Sparteine and Low Protein Levels.

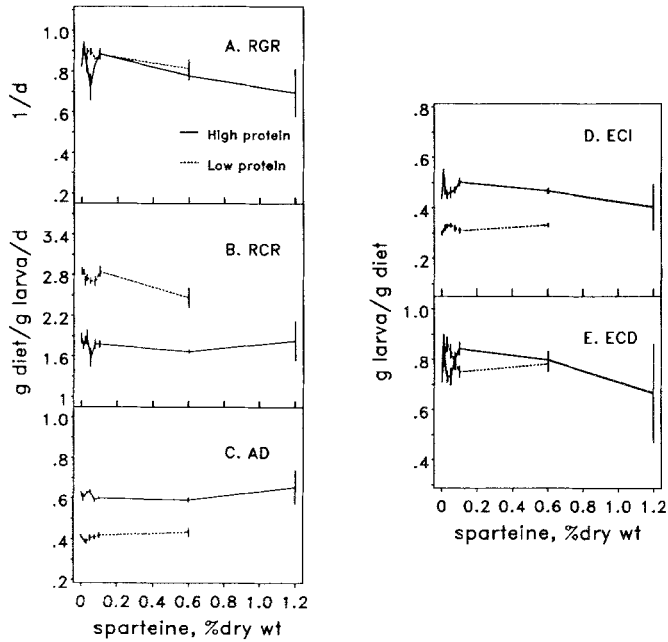

FIG. 2. The effects of dietary protein and alkaloid content on short-term feeding indices. Error bars have been omitted for clarity from some plotted points in A and E. Sample sizes (cups): $WgL_0 = 9$, $WgL_{.01} = 10$, $WgL_{.02} = 9$, $WgL_{.03} = 9$, $WgL_{.05} = 9$, $WgL_{.075} = 7$, $WgL_{.10} = 8$, $WgL_{.60} = 2$, $WgH_0 = 5$, $WgH_{.01} = 5$, $WgH_{.02} = 7$, $WgH_{.03} = 5$, $WgH_{.05} = 7$, $WgH_{.075} = 9$, $WgH_{.10} = 8$, $WgH_{.60} = 6$, $WgH_{1.20} = 3$.

Although surviving larvae had acquired resistance to high sparteine and low protein levels by the fifth instar (Figure 2), sparteine and low protein clearly affected growth at an earlier stage (Figure 1A). To determine when larvae were most affected by diet, we estimated the duration of the first-instar period and the total time that larvae spent as second to fourth instars. The durations of first and second to fourth instars were then tested in a two-way ANOVA of sparteine level and protein (Figure 3, Table 3).

Dietary sparteine had a strong effect on larval development rate only for first-instar larvae, while protein affected the durations of both first and subsequent instars (Figure 3, Table 3). However, the effects of protein on the duration of later stages were primarily evident in the high-sparteine diets, and protein had no significant effect if diets with $>0.6\%$ sparteine were excluded from the analysis.

Larval Growth on Diets with Natural Levels of Sparteine and Protein. Artificial diets can be used to predict the relative performance of herbivores on

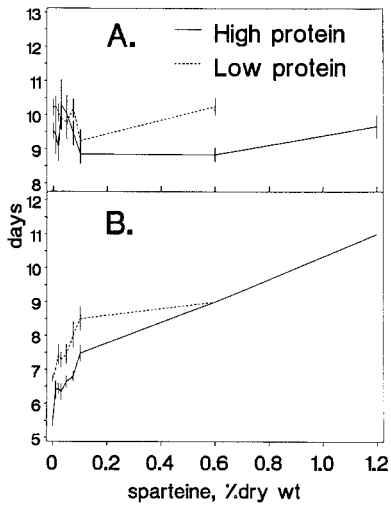

FIG. 3. The duration of larval developmental stages. (A) Duration of 2nd + 3rd + 4th instars. (B) Duration of 1st instar. Error bars indicate standard errors. Sample sizes as in Figure 2.

leaves with various protein and toxic chemical compositions (Berenbaum, 1981; Lincoln et al., 1982). We therefore designed our diets so that our results could be directly applied to recent experiments with *Lupinus succulentus* (Johnson et al., 1987).

The protein and alkaloid levels of *Lupinus succulentus* are strongly affected by available soil nitrogen, and thus soil nitrogen may also affect herbivores of

TABLE 3. EFFECTS OF ALKALOIDS AND PROTEIN ON 1st AND 2nd–4th INSTARS[a]

	df	*SS*	$F_{1,111}$	*P*
1st instar				
Protein	1	17.6	32.8	<0.0001
Sparteine	1	36.6	68.1	<0.0001
Protein × sparteine	1	0.2	0.4	0.56
Error	111	59.7		
2nd–4th instars				
Protein	1	4.3	4.2	0.04
Sparteine	1	1.1	1.0	0.31
Protein × sparteine	1	0.5	0.5	0.50
Error	111	113.2		

[a] Data from diet $WgH_{1.2}$ were not used because no larvae from $WgL_{1.2}$ survived to the 5th instar.

L. succulentus. We can use our artificial diet data to examine this idea because the different soil nitrogen treatments of Johnson et al. (1987) yielded *L. succulentus* foliage that resembled two of our diets. The mature foliage from low-nitrogen soils had about 17% protein and 0.01% alkaloids (similar to $WgL_{.01}$), while foliage from plants in high-nitrogen soils had about 30% protein and 0.03% alkaloids (similar to $WgH_{.03}$). Since larvae grew over 80% faster on $WgH_{.03}$ than on $WgL_{.01}$ (Figure 1A), despite the threefold difference in alkaloids, we suggest that the foliage from high-nitrogen soils will be a better food for *Spodoptera eridania* than the foliage from low-nitrogen soils (Figure 1). These results are consistant with preliminary experiments using foliage from lupines grown in high- and low-nitrogen soils (N. Johnson, unpublished).

Plants from high-nitrogen soils generally have high growth rates, and it has recently been suggested that plants with high growth rates should have lower levels of defenses (Coley et al., 1985). Our data are consistant with this idea, since we predict that lupines from high-nitrogen soils will be more suitable as food for herbivores than lupines from low-nitrogen soils. Thus high-nitrogen lupines may produce more alkaloids than low-nitrogen lupines, but these defenses are not enough to offset the benefits to herbivores from higher protein levels.

Conclusions. We chose a nutrient and toxic chemicals that are positively correlated in plants and used controlled diets to study the effects of variability in both nutrients and toxins on larval growth and feeding. In particular we conclude that:

1. Protein and sparteine had relatively simple effects on the growth of *Spodoptera eridania* larvae, each altering the growth rate by a constant amount regardless of the content of the other. This lack of interaction between protein and toxins has practical value because it simplifies the assessment of the food quality of plant tissues. However, this result is complicated by the effects of diet composition, since growth on our C diets was affected by sparteine more strongly at low protein levels. Although we presume that the effects of dietary protein in our high-wheat-germ diets mimicked those of plant proteins, the appropriateness of various protein sources and diet compositions clearly deserves more study.

2. We found no evidence of synergism between sparteine and lupanine, two lupine alkaloids that often occur together (Kinghorn et al., 1980). Many lupine species make more than one kind of alkaloid, but our data suggest that such an alkaloid mixture may not be any more toxic than would be expected from the sum of the toxicities of its components.

3. Larvae responded to poor diets by either dying or prolonging the time spent as first instars. However, larval performance beyond the first instar was unaffected by dietary sparteine. These results are similar to those of Larsson et al. (1986), who concluded that *Neodiprion sertifer* larvae were only vulnerable as early instars to the resin acids of Scots pine.

4. We predict that larvae will grow more quickly on *Lupinus succulentus* plants growing in nitrogen-rich soils than on plants in low-nitrogen soils, despite the higher alkaloid levels of plants from nitrogen-rich soils. This prediction is consistant with the idea that the suitability of plants as food for herbivores will be positively correlated with plant growth rate (Coley et al., 1985).

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ALGAL PHAGOSTIMULANTS FOR MARINE HERBIVOROUS GASTROPODS[1]

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Abstract—Methanol extracts of the green alga *Ulva pertusa* contain four kinds of glycerolipids that are active as feeding-stimulants for marine herbivorous gastropods. These compounds are digalactosyldiacylglycerol (DGDG), 1,2-diacylglycerly-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (DGTH), 1-monoacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (MGTH), and 6-sulfoquinovosyldiacylglycerol (SQDG). The various gastropods exhibit marked specificity, however, as young abalone *Haliotis discus* respond to DGDG and DGTH at minute dosages of 20–30 μg/sample zone, but do not respond to 300 μg of SQDG, which is a phagostimulant for two other kinds of gastropods, *Turbo cornutus* and *Omphalius pfeifferi*.

Key Words—Phagostimulants, feeding stimulants, herbivorous gastropods, abalone, *Haliotis discus, Aplysia juliana, Turbo cornutus*, digalactosyldiacylglycerol, phosphatidylcholine, 6-sulfoquinovosyldiacylglycerol, 1,2-diacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine, 1-monoacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-monoserine, *Ulva pertusa*, alga, glycerolipids.

INTRODUCTION

Gastropods are know to have olfactory and gustatory receptors and are considered to find their foods by chemoreception (Kohn, 1961; Croll, 1983). Such behavior provides a chemical basis to help understand the feeding preferences

[1] Chemical Studies on Phagostimulants for Marine Gastropods. Part VI. For Part V, see Sakata et al. (1986b).

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of marine herbivorous gastropods for particular species of seaweeds (Frings and Frings, 1965; Uki et al., 1986). Few chemical studies, however, have included the feeding behavior of such important gastropods as the abalone species of *Haliotis* that is now expected to provide a substantial harvest of about 18,000,000 seedlings for liberation a year in Japan (Uki, 1979). There is accordingly a clear need to establish the identity of the feeding attractants[4] for juvenile abalone *H. discus* (Harada et al., 1986) and the phagostimulatory responses of sea hares *Aplysia* spp. (Carefoot, 1986; Jahan-Parwar, 1972). Recently developed artificial diets for juvenile abalone could be improved by the addition of these chemicals, as current practice is to add powdered seaweed as a feeding stimulant.

Observation of the feeding behavior of juvenile abalone *H. discus* in aquaria led to the development of a simple and reliable bioassay for chemical study of their feeding behavior (Sakata et al., 1984). Recently this unique bioassay was found to be equally useful for studies of feeding behavior of other marine herbivorous gastropods including the turban shell *Turbo cornutus*, and the top shell *Omphalius pfeifferi* (Sakata et al., 1986a), both of which are also important seafoods in Japan.

We also established a similar bioassay using filter paper for isolation of the active principles in an ether extract of *Ulva* sp., which contains a feeding stimulant for a Japanese sea hare *A. juliana* (Sakata et al., 1986b). Presumably, a comparable feeding stimulant is involved in the feeding behavior of the Hawaiian sea hare *Aplysia* spp., which respond to a water extract of *Ulva* sp. (Frings and Frings, 1965).

Thus the isolation and purification of the active substances in preferred seaweeds can be monitored at each step for the presence or absence of activity against four kinds of gastropods described above. Following these preliminary trials, methanol extracts of several kinds of seaweeds were subjected to this bioassay, but while most extracts showed feeding-stimulatory activity to varying degrees, the extracts of the green alga *Ulva pertusa* were the most active for all gastropods tested. Accordingly, *U. pertusa* is the one source of algal material used in the present investigation.

METHODS AND MATERIALS

Bioassay Procedure for Phagostimulants for Gastropods. For abalone, *Haliotis discus*, the bioassay procedure was previously reported in detail together with collection and maintenance of the test animals (Sakata et al., 1984). Typical bioassay results are shown in Figure 1.

[4]The various activators of feeding behavior are defined as feeding attractants, arrestant, stimulants, and so on (Mackie, 1982). This paper follows his definition. "Phagostimulants" means feeding stimulants in this paper.

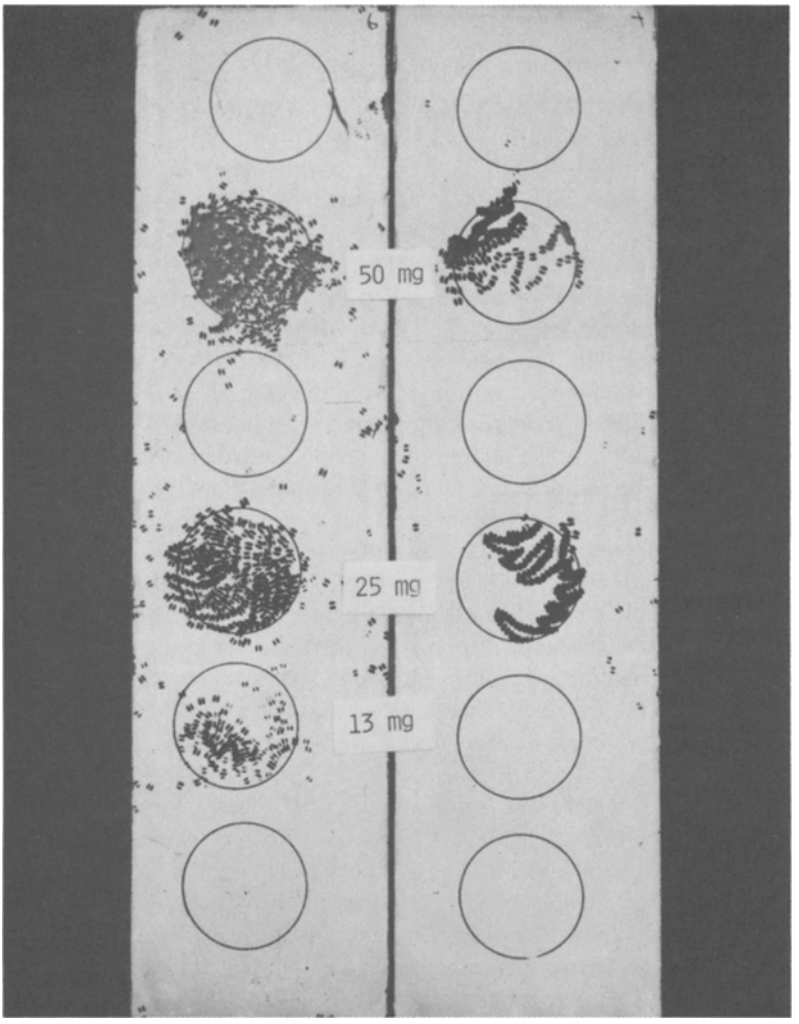

FIG. 1. Typical assay results obtained by using young abalone *H. discus*. The MeOH extracts equivalent to 13, 25, and 50 mg of air-dried brown alga *Eisenia bicyclis* were absorbed in each sample zone (2.5 cm in diameter) on the Avicel plate (0.25 mm thick, 5 × 10 cm). After evaporation of the solvent, each test plate was set, after sunset, at the bottom of each test aquarium which contains 10–20 young abalones (2–2.5 cm in shell length). Next morning, the plates were taken out of the aquarium and the activity was judged as ++, +, and −, according to the ratio of the feeding traces (a pair of characteristic feeding traces) inside the sample zone to those outside. Details of the assay procedure have been reported (Sakata et al., 1984).

For sea hare, *Aplysia juliana*, the assay was performed following the procedure reported previously (Sakata et al., 1986b).

For turban shell, *Turbo cornutus* (Sakata et al., 1986a), the test animals (average diameter of their operculam, 20–25 mm) were bought at a fish market and kept in a plastic aquarium (32 × 61 × 18 cm) into which the seawater filtered through sand was introduced, keeping the depth of the water about 5 cm with a drain. They were fed on dried brown alga *Eisenia bicyclis* as a maintenance food. They were starved for at least a day before the assay. Test sample solutions (50–100 μl) were applied on the sample zone (35–40 mm in diameter) made on an Avicel plate by a compass in the same manner as reported in the assay for the abalone (Sakata et al., 1984). The assay duration was determined for 3–4 hr after sunset. The assay was repeated at least twice. Judgment of the activity of each sample was performed as in the case of the abalone (Sakata et al., 1984): ++, the characteristic biting traces similar to those of abalones are observed almost all over the sample zone, and the activity can be judged clearly; +, many more biting traces are found in the sample zone than those outside in a unit area, but the judgment is not very clear; ±, a few more biting traces are observed in the sample zone than outside; and −, none or nearly the same number of the biting traces are left inside of the sample zone as those outside.

For top shell, *Omphalius pfeifferi*, the test animals (10–15 mm diameter of the opercula) were obtained from a fish market, kept in the same test aquarium as the one used for the abalone bioassay. The assay was carried out exactly the same way as in the case of the abalone (Sakata et al., 1984).

Isolation of Phagostimulants from Green Alga. The isolation procedure is outlined in Figure 2. Fresh *U. pertusa* (900 g), harvested in March 1984, was washed with tap water. The damp-dry alga was finely cut and soaked in a mixture of chloroform–methanol (1 : 3, 2.4 liters) to give the crude extract. Repetition of the extraction yielded ca. 12 liters of the extract. After evaporation of the solvent, the aqueous phase was taken up to ca. 1.5 liters, extracted with hexane (400 ml ×3) and then ethyl acetate (400 ml ×4). Half the combined organic extracts was fractionated by silica gel column chromatography [Wako gel C-300, 4 × 30 cm; 400 ml each of $CHCl_3$–MeOH (2, 5, 20, 30, 50, and 100% MeOH), 5 and 10% aq. MeOH] into six fractions as shown in Figure 2 as suggested by preliminary thin-layer chromatographic analyses (silica gel GF_{254}; BuOH–AcOH–H_2O = 4 : 1 : 2 and $CHCl_3$–EtOAc–MeOH–H_2O = 50 : 25 : 25 : 2) and bioassay of the eluates.

The sample amount of each fraction subjected to the bioassay was determined depending on each assay procedure as follows: 0.5 g equivalent amount of fresh *Ulva* for *Aplysia*, 0.05–0.1 g equivalent for *Haliotis* and *Turbo*, and 0.05 g equivalent for *Omphalius*. The crude algal extracts equivalent to those shown above always showed ++ activity in each bioassay. As the separation

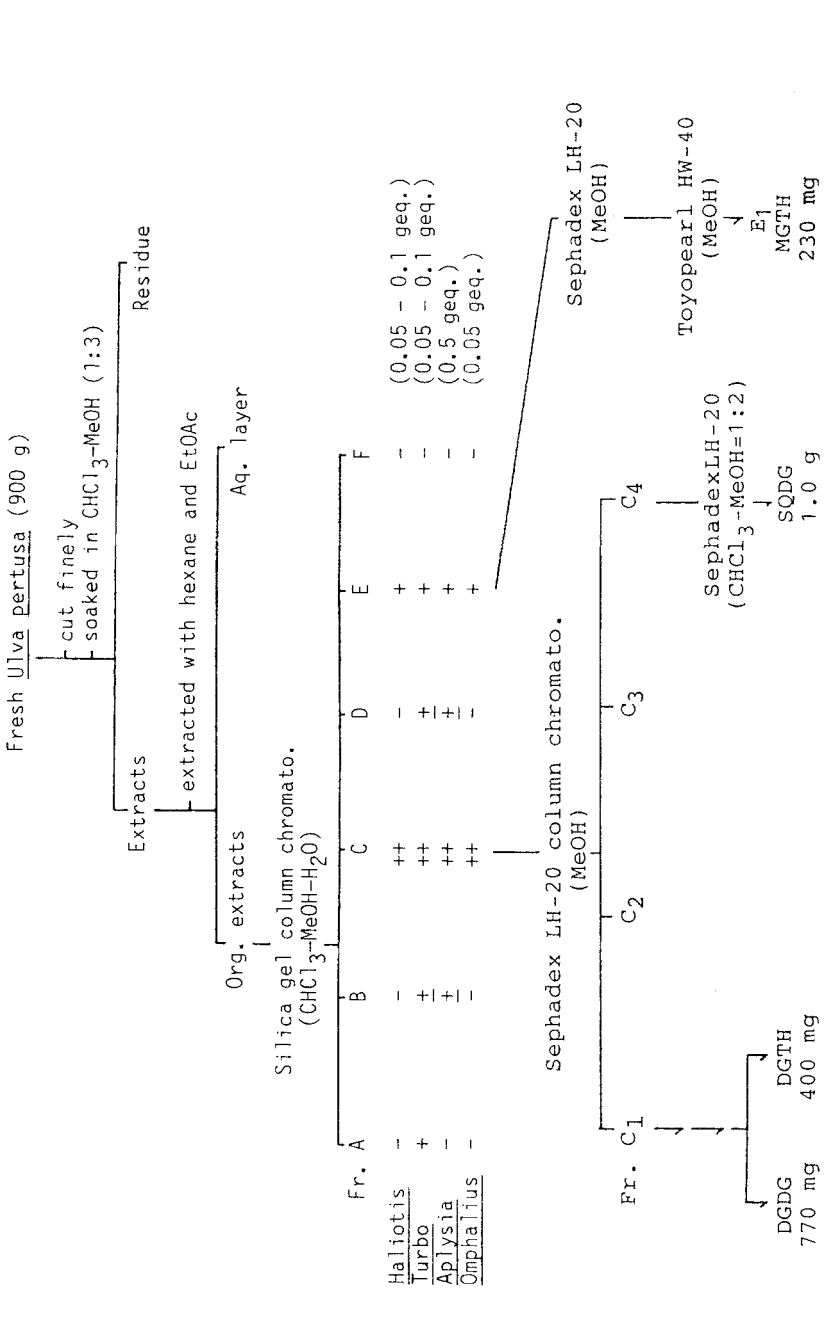

FIG. 2. Outline of the isolation procedure of phagostimulants for the herbivorous gastropods from the green alga *Ulva pertusa*.

of active principles proceeds, the sample amount applied was increased twice or three times more because the activity of the crude extract proved to be based on a few stimulants in it.

The most active fraction (Fr. C) was further fractionated by Sephadex LH-20 (MeOH) column chromatography. Fr. C_1, active against all test animals, was further purified by column chromatography using silica gel (Wako gel C-300, $CHCl_3$–MeOH), Toyopearl HW-40 (MeOH), Wako gel C-300 ($CHCl_3$–EtOH), and Sephadex LH-20 (MeOH) to finally give two active components: the earlier eluting one (770 mg) and the later eluting one (400 mg). Spectroscopic studies established that they were digalactosyldiacylglycerol (DGDG) (Sakata and Ina, 1985) and 1,2-diacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (DGTH), (Sakata et al., 1985), respectively.

The activity of Fr. C_4 was restricted to *Turbo* and *Omphalius*. Further purification by Sephadex LH-20 ($CHCl_3$–MeOH = 1:2) column chromatography yielded an active compound S (1.0 g): [^{13}C]NMR (22.5 MHz, $CDCl_3$–MeOH-d_4 = 4:1) δ 174.4, 173.5, 131.0, 130.3, 100.1, 74.9, 71.7, 69.7, 64.2, 54.3, 35.6, 35.3, 30.9, 30.5, 30.4, 28.4, 26.1, 23.9, 15.1. This was identified as 3-*O*-(6′-deoxy-6′-sulfo-α-D-quinovopyanosyl)-1,2-di-*O*-acyl-glycerol (6-sulfoquinovosyldiacylglycerol, SQDG) as shown below

Fraction E was purified by Sephadex LH-20 (MeOH) and then Toyopearl HW-40 (MeOH) column chromatography, yielding an active component E_1 (230 mg): [^{1}H]NMR (400 MHz, $CDCl_3$) (Figure 3); relative R_f to R_f of PC, 1.17, silica gel GF_{254}, $CHCl_3$–MeOH–AcOH–H_2O = 100:25:12:5. This was identified as 1-monoacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (MGTH) as shown below.

Methanolysis of Compound S. Compound S (62 mg) in methanol (10 ml) was treated with 0.1 ml of N methanolic KOH at 35°C for 1 hr. After neutralization with N HCl, 2 ml of water was added to the reaction mixture, and extracted with hexane (10 ml ×3). The hexane extract was analyzed by capillary GLC.

The residual aqueous solution was concentrated and chromatographed on Sephadex G10 (10% methanol). The fractions showing a single spot (R_f 0.51) on TLC (silica gel G, BuOH–AcOH–H_2O = 2:1:1) were combined, concentrated, and lyophilized to yield 1-glyceryl-α-D-6′-sulfoquinovoside: $[\alpha]_D^{23}$ + 80.4° (c = 1.96, H_2O); [^{1}H]NMR (400 MHz, D_2O) δ 2.89 (H-6′a, dd, 14.3, 9.8), 3.08 (H-4′, dd, 9.8, 9.0), 3.20 (H-6′b, dd, 14.3, 1.3), 3.26 (H-1a, dd, 10.0, 7.1), 3.40 (H-2′, dd, 10.0, 3.7), 3.42 (H-3a, dd, 11.5, 6.1), 3.51 (H-3b, dd, 11.5, 3.7), 3.55 (H-3′, dd, 10.0, 9.0), 3.76 (H-1b, dd, 10.0, 3.7), 3.80 (H-2, m), 3.87 (H-5′, dt, 9.8, 1.3), 4.71 (H-1′, d, 3.7); [^{13}C]NMR (22.5 MHz, H_2O) δ 99.2, 73.9, 73.4, 72.3, 71.7, 69.9, 69.0, 63.6, 53.1). The [^{13}C]NMR spectrum was identical with that previously reported (Johns et al., 1978).

Methanolysis of Compound E_1. Methanol solution of E_1 (38 mg/25 ml)

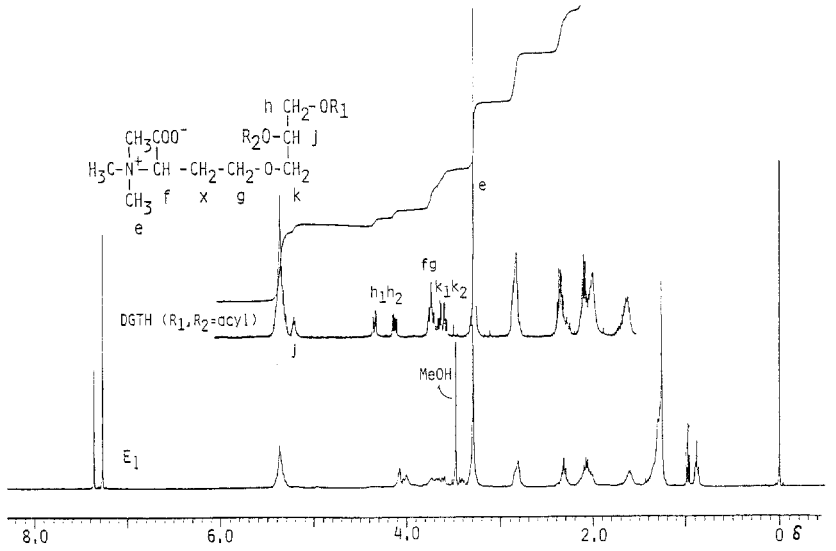

FIG. 3. [^{1}H]NMR spectra of the active component E_1 (MGTH: R_1 = acyl, R_2 = H) and DGTH (R_1, R_2 = acyl) ($CDCl_3$, 400 MHz).

containing about 50 μg of BHT was treated with 10 drops of methanolic N KOH at 40°C for 30 min and neutralized with ^{d}HCl. After addition of 1 ml of water, the reaction mixture was extracted with hexane (5 ml ×3). The hexane extract was washed with NaCl saturated water, dried with anhydrous Na_2SO_4, and subjected to capillary GLC analysis.

The aqueous layer was subjected to carbon column chromatography (Wako activated charcoal, ϕ1.8 × 5 cm). Elution was made by gradient elution between water and methanol (100 ml each). Fractions showing a single spot (R_f 0.25) on TLC (silica gel G, BuOH–AcOH–H_2O = 2:1:2) were combined, concentrated, and lyophilized to yield l-glycerly-4′-O-(*N*,*N*,*N*-trimethyl)-homoserine: [^{13}C]NMR (22.5 MHz, MeOH-d_4) δ 29.04 (t), 52.50 (q), 64.31 (t), 68.48 (t), 72.28 (d), 73.58 (t), 78.06 (d), 171.8 (s). The spectrum was identical with that obtained from DGTH (Sakata et al., 1985).

Capillary GLC Analysis of Fatty Acid Methyl Esters Obtained from Complex Lipids from U. pertusa. Analyses were carried out on a Hitachi gas chromatograph model 163 (FID) under the following conditions: column, PEG-HT G-SCOTT fused silica capillary column, ID 0.25 mm × 50 m; oven temperature 210°C; carrier gas, N_2, 1.5 ml/min; injection and detection temperature 240°C. Analytical data are shown in Table 1.

TABLE 1. FATTY ACID COMPOSITION OF GLYCEROLIPIDS ISOLATED AS FEEDING STIMULANTS FOR MARINE HERBIVOROUS GASTROPODS

Fatty acids	DGDG	DGTH	MGTH	SQDG
$C_{14:0}$		0.9[b]		
:1	1.6			
$C_{16:0}$	27.9	28.1	28.9	58.3
:1(9)	1.0	1.6	1.8	
:2(9,12)[a]	3.8			
:3(7,10,13)[a]	2.1			
:3(8,11,14)	8.0	2.0		
:4(4,8,11,14)	6.6			
$C_{18:0}$				
:1(9)	4.6	7.5	15.7	11.8
:2(7,10)	9.2	4.9	5.9	2.0
:3(9,12,15)	30.8	10.9	12.3	25.9
:4(6,9,12,15)	3.6	19.4	11.0	
$C_{20:3(11,14,17)}$		2.3		
:4(8,11,14,17)			1.7	
:5(5,8,11,14,17)		6.7	2.4	
$C_{22:5(7,10,13,16,19)}$[a]		6.2	11.4	
Unknown	0.8	9.2	8.9	2.0

[a] Supposed to be by comparison with the data reported (Jamieson and Reid, 1972).
[b] Given as area % of the peak of each fatty acid methyl ester on GLC.

RESULTS AND DISCUSSION

We have already established a simple and reliable bioassay procedure for phagostimulants for abalone, *H. discus* (Sakata et al., 1984). Figure 1 illustrates how the pattern of feeding traces provides a good semiquantitative bioassay of their feeding activity. Recently we applied this unique bioassay method to the other marine herbivorous gastropods including the turban shell *Turbo cornutus*, and the top shell *Omphalius pfeifferi*. On the test plate, they left pairs of typical feeding traces made by their radula. The traces were very similar to those left by the abalone. Judgment of the activity of each sample was performed exactly as in the case of the abalone (Sakata et al., 1984).

In the case of the turban shell, their feeding is often so active, especially in summer, that the Avicel on the test plate has been totally eaten not only inside of the sample zone but also outside, when the test plate was left in a test aquarium overnight as in the case of abalone. In such a case, judgment of the activity was impossible. We found, however, that good results could be obtained when the assay duration was for 3–4 hr after sunset. As we have already estab-

lished an assay method for *Aplysia* (Sakata et al., 1986b), bioassays for feeding stimulants for four kinds of marine herbivorous gastropods, therefore, are available now.

Isolation of phagostimulants for the gastropods from fresh *U. pertusa* was guided by the results of the four kinds of bioassays we established and is outlined in Figure 2. The combined organic extracts were separated by silica gel column chromatography using a mixture of $CHCl_3$–MeOH and aqueous MeOH into six fractions.

All four of the test animals, responsed to Fr. C (strong) and Fr. E (moderate) but not at all or weakly to Fr. A, Fr. B, Fr. D, and Fr. F. Fr. C was further fractionated by Sephadex LH-20 (MeOH) column chromatography. All test animals responded strongly to Fr. C_1. Further chromatographic purification finally gave two active components, which were identified as digalactosyldiacylglycerol (DGDG) and 1,2-diacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (DGTH), respectively.

These results thus confirm the activity of DGDG (extracted from the brown alga *Undarai pinnatifida*) as a feeding stimulant together with phosphatidylcholine (PC) for the young abalone *H. discus* (Sakata and Ina, 1985) and for the sea hare *A. juliana* (from the green alga *U. pertusa*) (Sakata et al., 1985). The activity of DGTH (from the green alga *U. pertusa*) (Sakata et al., 1985) as a phagostimulant was confirmed for the sea hare *A. juliana* (Sakata et al. 1985).

The activity of Fr. C_4 was restricted to *Turbo* and *Omphalius*. Further purification by Sephadex LH-20 ($CHCl_3$–MeOH = 1:2) column chromatography yielded an active compound S (1.0 g). Its [^{13}C]NMR spectrum suggests that it is 6-sulfoquinovosyldiacylglycerol (SQDG) (Johns et al., 1978). This identification was confirmed by comparison of the [^{13}C]NMR spectrum of its methanolysis product with that previously reported (Johns et al., 1978). The fatty acid composition of SQDG is rather simple ($C_{16:0}$, 58.3%; $C_{18:1}$, 11.8%; $C_{18:3}$, 25.9%) compared with that of DGTH (Sakata et al., 1985), DGDG, and PC (Sakata and Ina, 1985) (Table 1).

From the other active fraction, Fr. E, was isolated an active component E_1 (230 mg). Thin-layer chromatographic mobility of compound E_1 is very similar to that of PC, but it did not react to the Dittmer-Lester reagent. The similarity of the [^{1}H]NMR spectrum of compound E_1 to that of DGTH (Sakata et al., 1985) suggests that E_1 is a lyso form of DGTH (Figure 3).

The polar part of the methanolysis product of E_1 was shown to be identical with that obtained from DGTH (Sakata et al., 1985) by direct comparison of their [^{1}H]- and [^{13}C]NMR spectra. In the 400 MHz [^{1}H]NMR spectrum of E_1 (Figure 3), the H-2′ signal, which was observed at δ 5.21 (m) in the spectrum of DGTH, shifted up field ($\delta < 4.1$), implying the C-2′ hydroxyl of the glycerol moiety is not acylated. Accordingly, the structure of E_1 was determined to be 1-monoacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (MGTH) having a

fatty acid composition very similar to that of DGTH (Table 1). The phagostimulatory activity of the organic extracts of this green alga can, therefore, be attributed to the glycerolipids DGDG, DGTH, MGTH, and SQDG (Figure 4).

One very interesting point is that while DGDG, DGTH, MGTH, and PC were found to be effective for all the gastropods being tested, SQDG was active only for *T. cornutus* and *O. pfeifferi*. The abalone *H. discus* did not respond to SQDG even at dosages as high as 300 μg (Table 2). The young abalone was, however, very responsive to amounts of DGDG and DGTH as low as 20–30 μg (Table 2).

Another interesting point is that these glycerolipids are the first examples of lipophilic feeding stimulants for marine organisms. All the feeding attractants and stimulants for fish so far elucidated are water-soluble compounds like amino acids, nucleosides, etc. (Mackie, 1982; Carr and Derby, 1986). The feeding stimulants thus appear to be of great potential use for the culture of marine herbivorous gastropods that could feed on artificial diets, provided that a suitable feeding stimulant was added.

The wide occurrence of DGDG and PC in the plant kingdom strongly suggests that marine herbivorous gastropods could feed on not only any algae but also on terrestrial plants. The abalone *H. discus hannai* is, however, reported to show quite different feeding preferences, and some algal species are not eaten by them (Uki et al., 1986). Some feeding deterrants may be present in these particular species of algae. Our new bioassay methods for feeding stimulants for herbivorous gastropods are now being applied to study this interesting phenomenon.

The sea urchin is another marine herbivore that is an important seafood in Japan. Indeed, the sea urchin *Strongylocentrotus intermedius* is now easily cul-

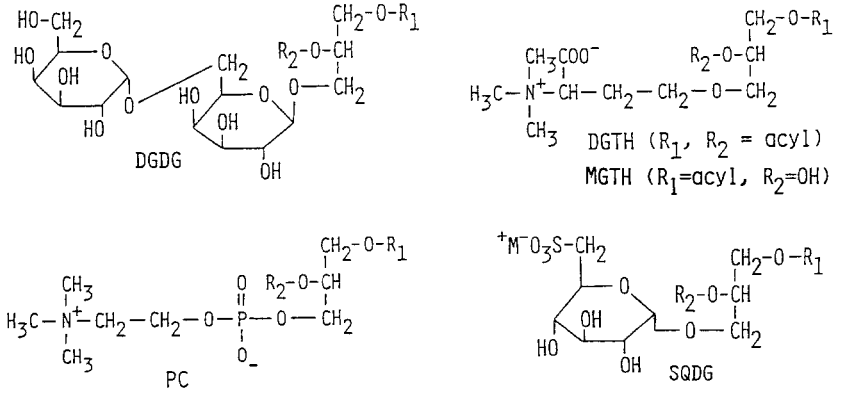

FIG. 4. Glycerolipid structures.

TABLE 2. FEEDING STIMULATION ACTIVITY OF GLYCEROLIPIDS ISOLATED FROM THE ALGA *Ulva pertusa*

	Minimum effective dose (μg)[a]			
	Haliotis discus	*Turbo cornutus*	*Omphalius pfeifferi*	*Aplysia juliana*
DGDG	14–28	15–25	18–23	800[b]
DGTH	10–20	24	<10	100
MGTH	23–50	25–50	<23	
SGDG	>300	20–40	<20	>1000
PC[c]	<10	15	<15	>50

[a] Minimum effective dose value expresses minimum sample amount/sample zone necessary to show + activity. These values are preliminary.
[b] In the case of *Aplysia* minimum sample amount/filter paper (Toyo filter paper No. 2, 5.5 cm in diameter).
[c] Isolated from the brown alga *Undaria pinnatifida*.

tured on a diet of the terrestrial plant, *Reynoutria sachalinensis* ("ooitadori" in Japanese). This technique has been extended to seedling production of the sea urchin in the Hokkaido district in Japan (Department of Mariculture, 1984).

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SEX PHEROMONE OF PURPLESTRIPED SHOOTWORM, *Zeiraphera unfortunana*[1] POWELL

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Abstract—The analyses of virgin female sex pheromone gland extracts and gland volatiles by GC, GC-EAD and GC-MS, followed by field trapping experiments, have identified (*E*)-9-dodecenyl acetate (*E*9-12 : Ac) as the primary sex pheromone component of the purplestriped shootworm, *Zeiraphera unfortunana*. Dosages of 1.0–10.0 μg of *E*9-12 : Ac impregnated in rubber septa provide an effective trap bait and can be used for monitoring purposes.

Key Words—*Zeiraphera unfortunana*, purplestriped shootworm, *Zeiraphera destitutana*, sex pheromone, (*E*)-9-dodecenyl acetate, electroantennograms, Lepidoptera, Tortricidae.

INTRODUCTION

There are many species of Lepidoptera in North America in the budmoth group found on spruce foliage (Martineau, 1984). Of particular economic importance in New Brunswick are two sympatric species: the spruce budmoth *Zeiraphera canadensis* Mutuura and Freeman and the purplestriped shootworm *Zeiraphera unfortunana* Powell [= *Z. destitutana* (Wlk.)]. These insects are considered important pests because their feeding activity deforms and/or kills new shoots and leaders, thus stunting tree growth (Magasi, 1983, 1984).

[1] Lepidoptera: Tortricidae.

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We report the identification of the primary sex pheromone component for *Z. unfortunana*. Identification was by GC-EAD and GC-MS analyses of female sex pheromone gland extracts and gland volatiles, and effects on males were verified in field trapping experiments.

METHODS AND MATERIALS

Insects. Zeiraphera unfortunana larvae were collected in 1984 and 1986 from a J.D. Irving, Limited, plantation in St. Leonard, New Brunswick. Branch tips (45 cm) were collected from white spruce [*Picea glauca* (Moench) Voss.] that had a high proportion of budcaps secured in place, apparently by feeding *Zeiraphera* larvae. In the laboratory, these branches were placed on 0.64-cm mesh hardware cloth trays in constant-temperature rooms at 25°C ± 2°C and high relative humidity (>80%). Subsequently, larvae dropped from the trays onto moist vermiculite where pupation occurred. Pupae were separated from the vermiculite by washing in a bleach solution (ca. 1% sodium hypochlorite in demineralized water) for ca. 10 min. They were rinsed in cold tap water for 30 min and then air-dried on paper towels. Pupae were separated by sex and maintained at ca. 25°C, 70% relative humidity on a 16:8 light–dark cycle until emergence. Adults were collected daily and held in cylindrical window-screen cages, misted with water twice daily, and allowed to mature under the same temperature, humidity, and photoperiod regime as were pupae.

Instrumental Analysis. GC analyses were performed using either an SPB-5 (30 m × 0.32 mm) or an SP2330 (30 m × 0.32 mm) fused-silica capillary column (Supelco) on a Varian 3700 gas chromatograph equipped with a flame ionization detector (FID); hydrogen was used as the carrier gas at an inlet pressue of 0.5 kg/cm^2. Injections were made in the splitless mode. The splitter was opened after 30 sec, and the column temperature programmed either at 8°C/min from 50°C to 210°C or 15°C/min from 50°C to 200°C as noted. GC-MS analysis was performed on a Finnigan 4021 EI-CI-INCOS system using the SPB-5 capillary column. Helium was used as the carrier gas with injections made in the splitless mode, with the column at ambient temperature, and then programmed as above.

EI mass spectra were generated at 70 eV, such that 1–5 ng of the synthetics produced excellent full-scan mass spectra with adequate resolution. We examined the respective *E*- and *Z*-monounsaturated isomers of Δ9- and Δ11-dodecenyl acetates (Δ9–12:Ac, Δ11–12:Ac), dodecenols (Δ9–12:OH, Δ11–12:OH), dodecenals (Δ9–12:Ald, Δ11–12:Ald), as well as Δ9- and Δ11-tetradecenyl acetates, alcohols, and aldehydes (Δ9–14:Ac, Δ11–14:Ac, Δ9–14:OH, Δ11–14:OH, Δ9–14:Ald, Δ11–14:Ald) and the saturated compounds dodecyl acetate (12:Ac) and tetradecyl acetate (14:Ac).

Chemicals. Chemicals were obtained from Chemical Samples Company, Columbus, Ohio; Koken Fine and Aromatic Chemicals, Tokyo, Japan; or were synthesized and purified in our laboratory. All chemicals were >99% pure as determined by capillary GC analysis and were used without further purification.

Preparation of Pheromone Gland Extracts and Volatiles. Sex pheromone glands from 2- to 3-day-old females were manually everted and excised during the first 3 hr of scotophase, placed in hexane (Spectrograde) and held at −10°C prior to analyses. Subsequently, glands were sonicated for ca. 1 hr, filtered through precleaned glass wool, and the extract was used for GC, GC-EAD, and GC-MS analyses. Volatiles from extruded virgin female sex pheromone glands of *Z. unfortunana* (2–4 days old; 3–4 hr into scotophase) were collected on glass wool by the method of Baker et al. (1981). Gland volatiles (240 female minutes) were eluted from the glass wool with hexane and were analyzed by GC-EAD using a male (2–4 days old) *Z. unfortunana* antenna.

GC-EAD Analysis. Sex pheromone gland extracts were analyzed by a capillary GC-EAD system (modified after Struble and Arn, 1984). Briefly, effluent from the GC column in a Varian 3700 GC was split 50/50 with a Varian glass-lined splitter. Half of the effluent (ca. 1 ml/min) was delivered to a conventional FID detector while the other half was delivered to the antennal detector. A male *Z. unfortunana* antenna was severed near its base, then the last few segments were excised, and the antenna was placed between the tips of two glass capillaries filled with a saline solution (Roelofs, 1977). Chloridized silver wire electrodes were placed in the solution in each capillary and connected to a 10× EAG amplifier. GC effluent was flushed over the antennal preparation by a water-condenser-cooled air stream passing at right angles over the splitter outlet at a flow rate of ca. 100 ml/min and ca. 100% relative humidity. EAD and GC responses were simultaneous [calibrated with male *Choristoneura fumiferana* (Clem.) antennae for EAD and the primary sex pheromone components 95:5 (*E/Z*)-11-tetradecenal (Silk et al., 1980) as the stimulus]. EAD responses of male *Z. unfortunana* were recorded to GC-separated extracts (sex pheromone glands and gland volatiles) and synthetic standards on both SPB-5 and SP2330 capillary columns.

Field Tests. Field trapping studies were conducted in 1984 and 1986 in the same J.D. Irving, Limited, property where larvae were collected (as above) near St. Leonard, New Brunswick. In 1984, two sites were used; each was comprised of white spruce planted in 1970 (plot 1) and in 1976 (plot 2). In 1986, plot 2 was used again. Red-rubber septa (Arthur H. Thomas Co., Ltd.) impregnated with 10 μl hexane solutions of synthetic chemicals were used as trap baits. Traps (Pherocon 1C) were placed out in a randomized complete block design (8 treatments × 7 blocks × 2 sites, 1984; 6 treatments × 9 blocks, 1986) with 25-m intertrap spacings within and between blocks. All traps were suspended from white spruce foliage at ca. 1.5 m from the ground. In 1984,

traps were placed out for six nights, then brought back to the laboratory for counting. Specimens were randomly selected from traps and sent to the Biosystematics Research Institute (P.T. Dang) in Ottawa for confirmation of species identification. In 1986, trap bottoms were changed daily, or as necessary (>5 moths captured), and the entire array was rerandomized after the third night. All moths were removed from trap bottoms and genitalia examined to confirm species identification. Traps were placed in the field between July 17 and July 23, 1984, and from July 19 to July 25, 1986. Trap capture data were analyzed by two-way analysis of variance and means separated using Tukey's multiple comparison test.

RESULTS

GC and GC-EAD Analysis. A total of 80 sex pheromone glands were available for analysis. Approximately five female gland equivalents (5 FE) in hexane were injected onto the SPB-5 capillary column for GC-EAD analysis (Figure 1; $N = 2$). Although several small EAD responses were noted, only one FID

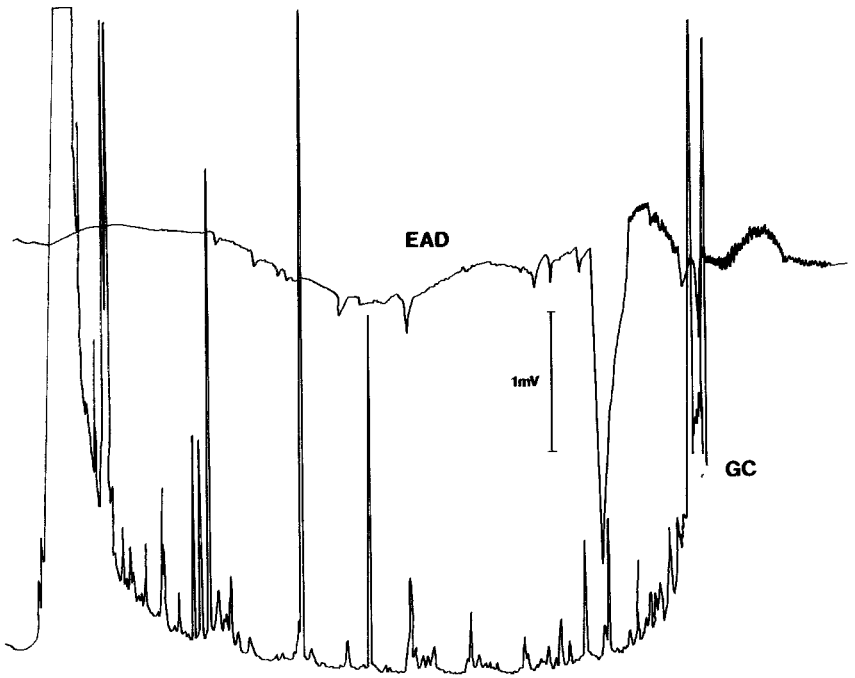

FIG. 1. GC-FID [30 m SPB-5 (0.32 mm) fused silica capillary column; see Methods and Materials] response (lower line) and EAD response (upper line) of a male *Z. unfortunana* antenna to five female pheromone-gland equivalents (equal time scales).

peak corresponded to a large EAD response. The synthetic Δ9–12 : Ac's, Δ9–14 : Ac's, and their saturated analogs (12 : Ac and 14 : Ac) gave noticeable EAD responses (Table 1). The GC retention time and large EAD response of the gland-derived material closely matched that of Δ9–12 : Ac with the retention time, suggesting the *E*-configuration of the double bond (Table 1). GC-EAD analysis of gland extracts (30 FE) on the SP2330 capillary column, which baseline-resolved *E*- and *Z*9–12 : Ac, confirmed the *E* configuration of the double bond (retention times of synthetic *E*9–12 : Ac, 9.90 min; *Z*9–12 : Ac, 10.10 min; gland-derived compound, 9.85 min; 50°C to 200°C at 10°/min). Under these conditions, *Z*9–12 : Ac (< 1% *E*9–12 : Ac) could not be detected in gland extracts. GC-MS analysis was then carried out with the remaining gland extract (40 FE). Although phthalate and other interferences were present, the GC peak of high EAD response had mass spectral (EI) and retention time characteristics of Δ9–12 : Ac [*m/e* 166 (M^+-60) and 61 ($CH_3COOH_2^+$) and fragments at *m/e* 68 and 82] which matched those of synthetic *E*9–12 : Ac. Insufficient material was available to confirm the double-bond position by ozonolysis or other derivatization techniques.

TABLE 1. MEAN EAD RESPONSES OF MALE *Z. unfortunana* ANTENNAE TO SOME SYNTHETICS AND PHEROMONE GLAND EXTRACT

Component	EAD response (mV)[a]
*E*11–14 : Ac	0[b]
*E*9–14 : Ac	1.6 ± 1.3 (N = 6)
*E*7–14 : Ac	0
*E*7–12 : Ac	0
*E*9–12 : Ac	4.2 ± 1.5 (N = 5)
*E*9–14 : OH	0
*Z*11–14 : Ac	0
*Z*9–14 : Ac	0
*Z*7–14 : Ac	0
*Z*7–12 : Ac	0
*Z*9–12 : Ac	2.8 ± 0.7 (N = 3)
12 : Ac	2.1 ± 0.9 (N = 3)
14 : Ac	0.6
12 : OH	0.4
14 : OH	0
Major component[c,d]	2.3 (5 FE)

[a] 1 ng stimulus, i.e., 2 ng injected on GC.
[b] Not distinguishable from background response (ca. 0.05 mV).
[c] See Figure 1; five female equivalents.
[d] On SPB-5 capillary column, 50°C–210°C at 8°C/min; synthetic *E*9–12 : Ac retention time = 16.3 min, gland component 16.25 min.

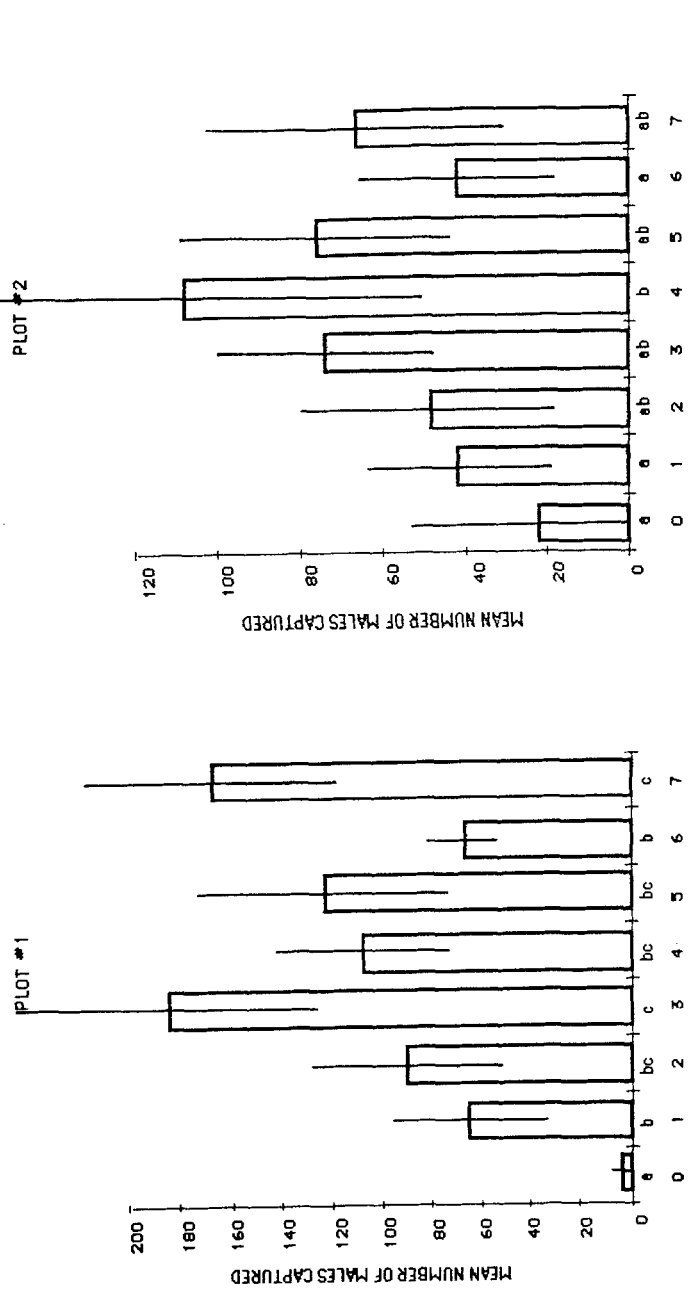

FIG. 2. Mean trap capture of male *Z. unfortunana* in white spruce stands, 1984. Plot 1 (planted in 1970); plot 2 (planted in 1976). Traps were baited with rubber septa impregnated with the following treatments: 0, unbaited trap; 1, 0.1 μg *E*9–12:Ac; 2, 0.3 μg *E*9–12:Ac; 3, 1.0 μg *E*9–12:Ac; 4, 3.0 μg *E*9–12:Ac; 5, 10.0 μg *E*9–12:Ac; 6, 100 μg *E*9–12:Ac; 7, 3.0 μg *E*9–12:Ac + 0.3 μg *Z*9–12:Ac. Means ($\pm$1 SD) followed by the same letter are not significantly different ($P < 0.05$).

GC-EAD analysis of sex pheromone gland volatiles indicated only one EAD-active region with the retention time (SPB-5, 50°C to 200°C at 15°/min; EAD-active peak, 10.10 min, synthetic *E*9–12:Ac, 10.15 min) corresponding to *E*9–12:Ac, confirming the emission of this compound from the sex pheromone gland.

The evidence from GC, GC-EAD, and GC-MS analyses, therefore, supported the assignment of *E*9–12:Ac as the major EAD-active gland component and as the EAD-active component volatilized from the sex pheromone gland surface. An approximate pheromone-gland titer of 100–200 pg/female was computed from the comparison of the magnitude of EAD responses to gland extract vs. synthetic *E*9–12:Ac.

Field Tests. In 1984 in plot 1, all baited traps captured significantly more moths than unbaited controls (Figure 2; $P < 0.05$). In both field plots, a dosage–response relationship was evident, with the 1- to 3-μg dosage apparently producing the highest trap capture rates. The admixture of 10% *Z*9–12:Ac to the 3-μg dosage of *E*9–12:Ac appears to have no effect on mean trap capture. In 1986 (Figure 3), all treatments were significantly different than the control (unbaited trap) but not significantly different from each other ($P < 0.05$). The addition of 10% of either *Z*9–12:Ac or 12:Ac did not appear to affect mean

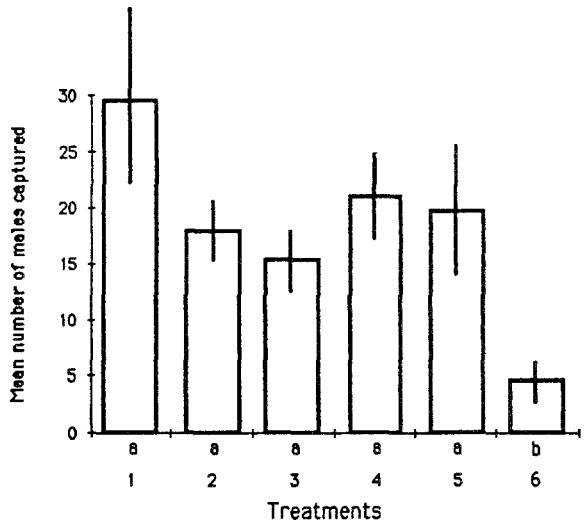

FIG. 3. Mean trap capture of male *Z. unfortunana* in a white spruce stand, 1986: plot 2 (as in 1984). Traps were baited with rubber septa impregnated with the following treatments: 1, 1 μg *E*9–12:Ac; 2, 10 μg *E*9–12:Ac; 3, 100 μg *E*9–12:Ac; 4, 1 μg *E*9–12:Ac; + 0.1 μg *Z*9–12:Ac; 5, 1 μg *E*9–12:Ac + 0.1 μg 12:Ac; 6, unbaited trap. Means ($\pm$1 SD) followed by the same letter are not significantly different ($P < 0.05$).

trap capture at the 1-μg dosage of *E*9–12 : Ac; both these compounds produced noticeable EAD activity but were not detected in the sex pheromone gland extracts of *Z. unfortunana*.

In plot 2 (1984), only the 3-μg dosage was significantly higher than the blank traps. However, one blank trap contained 101 moths and, if eliminated from the analysis, then all baited traps were significantly different from the control traps ($N = 7$; mean $= 22 \pm 35.6$; $N = 6$; mean $= 8.3 \pm 7.9$).

DISCUSSION

GC, GC-MS and GC-EAD analyses have identified *E*9–12 : Ac as the primary sex pheromone component of *Z. unfortunana*. In addition, field trapping studies indicate that *E*9–12 : Ac, impregnated in rubber septa in the 1- to 10-μg range, effectively traps *Z. unfortunana* males. This represents the first chemical identification of a sex pheromone for a North American *Zeiraphera* sp. The *Z* isomer and the saturated analog, at the dosage tested, do not appear to affect mean trap capture rates. Wind-tunnel studies are underway to determine whether *E*9–12 : Ac can elicit upwind flight responses from *Z. unfortunana* males.

At present, few additional data are available on the sex pheromones of North American *Zeiraphera* spp.; however, several European *Zeiraphera* spp. pheromones have been identified. In particular, some Swiss populations of *Zeiraphera diniana* (larch form) utilize *E*11–14 : Ac as their primary sex pheromone component (Roelofs et al., 1971; Guerin et al., 1984), while other *Z. diniana* populations (cembran pine form) utilize *E*9–12 : Ac as their primary component (Baltensweiler et al., 1978; Guerin et al., 1984).

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FLUORINATED ANALOGS OF ALDEHYDE COMPONENTS OF BOLL WEEVIL PHEROMONE: Synthesis and Biological Activity

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Abstract—Analogs of the two geometrical isomers of the dimethylcyclohexylidene acetaldehyde component of the pheromone of the boll weevil *Anthonomus grandis* were synthesized in which the α-vinylic proton or the aldehydic proton were replaced by fluorine. These isosteric substitutions substantially alter charge distribution and reactivity of the enal system, as documented by spectroscopic changes and changes in reactivity. The electrophysiological activity of the (*E*)- and (*Z*)-acyl fluorides is two orders of magnitude lower than that of the natural aldehyde. In contrast, the EAG response of female antennae to the (*E*)- and (*Z*)-α-fluoro compounds show that the thresholds are quite similar to (and in one isomer lower than) those of the natural aldehyde isomers.

Key Words—*Anthonomus grandis*, boll weevil, Coleoptera, Curculionidae, pheromone analog, fluorinated analogs, acyl fluoride, olfaction, electrophysiology, isosteric replacement.

INTRODUCTION

The shape and charge distribution of odorant molecules are important in their interaction with odorant binding sites in receptive tissues. In our efforts to describe the molecular properties of insect pheromone reception systems, we have focused on the design of pheromone analogs with altered chemical reactivity and charge distribution. These analogs can serve as potential affinity labels

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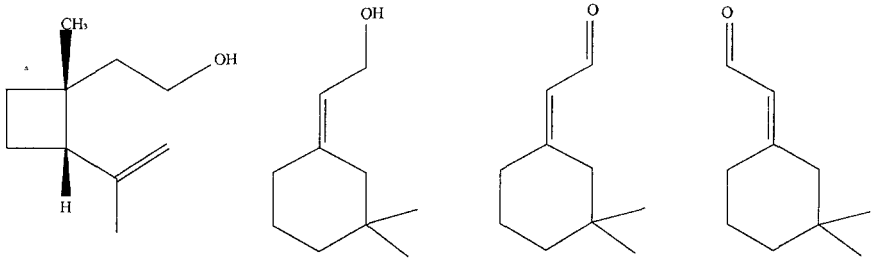
for pheromone binding proteins (Vogt *et al.*, 1988) or as inhibitors of the catabolic proteins which degrade pheromones (Prestwich, 1987). Current efforts have focused on the bioorganic chemistry of the catabolism and reception of moth pheromones (Prestwich et al., 1987), including the use of fluorinated analogs of pheromones (Prestwich, 1986, 1987; Prestwich and Streinz, 1988; Ding, 1987; Camps et al., 1984a,b). In this paper, we extend our studies to include fluorinated analogs of an aggregation pheromone component from a coleopteran, the boll weevil *Anthonomus grandis*, for which detailed electrophysiological results have been recently reported for the four components I–IV of "grandlure" (Figure 1) (Dickens, 1984). We describe the synthesis of fluorinated analogs of the (*E*)- and (*Z*)-isomers of the dimethylcyclohexylidene acetaldehyde component of the pheromone, and electroantennogram (EAG) studies elucidating their biological activity.

METHODS AND MATERIALS

General Synthesis. 3-Methyl-2-cyclohexene-1-one (V) was purchased from Aldrich and ethyl bromofluoroacetate was purchased from SCM. Solvents were distilled before use. Anhydrous tetrahydrofuran (THF) and diethyl ether were distilled from sodium benzophenone ketyl prior to use. Flash column chromatography and medium-pressure liquid chromatography (MPLC) were carried out using Woelm silica gel (32–63 μm). Thin-layer chromatography (TLC) was performed using MN Polygram Sil G/UV_{254} silica gel plates (4 cm × 8 cm × 0.25 mm). The developed TLC plates were visualized by staining with 3% vanillin (w/v) in ethanol or with UV light. Proton nuclear magnetic resonance ([^{1}H]NMR) spectra were obtained on a Varian FT-80 spectrometer using 0.03% tetramethylsilane (TMS) as an internal standard in chloroform-d_3. Chemical shifts (δ) are expressed as parts per million downfield from TMS. [^{19}F]NMR spectra were obtained on a Nicolet NT-300 spectrometer using trichlorofluoromethane ($CFCl_3$) as an internal standard in acetone-d_6. Chemical shifts (ϕ) are



I II III IV

FIG. 1 Structures of four boll weevil pheromone components.

expressed as parts per million upfield from $CFCl_3$ (ϕ = 0 ppm). Mass spectra (MS), as well as high-resolution mass spectra (HR-MS), (70 eV, electron impact) were obtained using a Spectros MS 30 spectrometer with a DS 50 data system. Gas chromatography was carried out on a Varian 3700 equipped with a fused silica capillary column (DB-5, 30 m × 0.263 mm). All glassware, syringes, and needles were dried in an oven at 110°C before use. The glassware was assembled hot and cooled under a flow of dry nitrogen. All of the reactions were carried out under a small positive pressure of dry nitrogen, using a mercury bubbler as a pressure relief valve.

(E)- *and* (Z)- *Methyl 3,3-dimethylcyclohexane-*$\Delta^{1,\alpha}$*-acetate (VII).* Sodium hydride (490 mg, 60% dispersion in mineral oil, 12.3 mmol) was washed with dry hexane (2× 4 ml) and suspended in 5 ml of anhydrous THF. A solution of trimethyl phosphonoacetate (2.17 g, 11.9 mmol) in 15 ml of anhydrous THF was added dropwise to this suspension at room temperature over 0.5 hr. The mixture was stirred for an additional 0.5 hr, then placed in an ice-water bath. A solution of 3,3-dimethylcyclohexanone VI (495 mg, 3.9 mmol), prepared from enone V as described by Pelletier and Mody (1976), was dissolved in 12 ml of anhydrous THF and added dropwise over 0.5 hr to the above mixture. The cooling bath was removed, and the reaction mixture was then refluxed for 4 hr. After cooling the reaction mixture down to room temperature, 250 ml of water was added to decompose the excess hydride. The aqueous phase was extracted with ether (3× 50 ml) and the combined ether extracts were washed with brine (1× 10 ml), dried ($MgSO_4$), and concentrated in vacuo. The crude product was purified by flash column chromatography (hexane–ethyl acetate, 10:1) to yield 680 mg (94%) of methyl 3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetate VII as a mixture of *E* and *Z* isomers (*E*:*Z* = 3:2). The isomers could be only poorly resolved by MPLC. [^{1}H]NMR (*E*)-(VII), δ 5.57 (1H, s), 3.68 (3H, s), 2.75 (2H, t), 1.97 (2H, s), 1.0–1.6 (4H, m), 0.90 (6H, s); (*Z*)-(VII), δ 5.68 (1H, s), 3.67 (3H, s), 2.63 (2H, s), 2.13 (2H, t), 1.0–1.6 (4H, m), 0.92 (6H, s).

(E)- *and* (Z)-*3,3-Dimethylcyclohexane-*$\Delta^{1,\alpha}$*-acetic acid (VIII).* The unresolved methyl ester isomers VII (374 mg, 2.1 mmol) were dissolved in 24 ml methanolic KOH (0.25 N, methanol–water, 1:1). The solution was gently refluxed for 3 hr. After cooling, the solution was diluted with 120 ml of water, washed with ether (1× 50 ml), and acidified with 2 N HCl to litmus red. The aqueous phase was extracted with ether (3× 10 ml), and the combined ether extracts were washed with brine (1× 10 ml), dried ($MgSO_4$) and concentrated in vacuo to yield 320 mg (92%) of the acid VIII as a white needle crystals. The *E* and *Z* isomers were separated by MPLC using two consecutive silica gel columns (2.2 cm ID × 30 cm each) eluting with hexane–ethyl acetate (20:1). [^{1}H]NMR (*E*)-VIII, δ 5.58 (1H, s), 2.77 (2H, t), 1.99 (2H, s), 1.2–1.6 (4H, m), 0.90 (6H, s); (*Z*)-VIII, δ 5.70 (1H, s), 2.65 (2H, s), 2.15 (2H, t), 1.2–1.6 (4H, m), 0.92 (6H, s).

(E)-*3,3-Dimethylcyclohexane-*$\Delta^{1,\alpha}$*-acetyl fluoride* [(E)-*IX*]. To a solution of the (*E*)-acid (*E*)-VIII (20 mg, 0.12 mmol) in 1 ml of anhydrous ether at 0°C was added dimethylaminosulfur trifluoride (DAST) (19 mg, 0.12 mmol). The mixture was warmed to room temperature and stirred for 20 min. The solvent was removed in vacuo, and the crude product was purified by flash column chromatography (hexane–ethyl acetate, 20:1) to yield 9 mg (48%) of the (*E*)-acyl fluoride (*E*)-IX. [^{1}H]NMR δ 5.54 (1H, s), 2.76 (2H, t), 2.04 (2H, s), 1.0–1.6 (4H, m), 0.92 (6H, s).

(Z)-*3,3-Dimethylcyclohexane-*$\Delta^{1,\alpha}$*-acetyl fluoride* [(Z)-*IX*]. The (*Z*)-acetyl fluoride (*Z*)-IX was prepared according to the procedure described above starting with the (*Z*)-acid (*Z*)-VIII. [^{1}H]NMR δ 5.69 (1H, s), 2.63 (2H, s), 2.18 (2H, t), 1.0–1.6 (4H, m), 0.92 (6H, s).

Ethyl 3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$*-*α*-fluoroacetate (X).* Sodium hydride (165 mg, 60% dispersion in mineral oil, 4.1 mmol) was washed with dry hexane (2× 4 ml) and suspended in 2 ml of anhydrous ether. A solution of triethyl 2-fluorophosphonoacetate (998 mg, 4.1 mmol), prepared from ethyl bromofluoroacetate (Machleidt and Wessendorf, 1964), was dissolved in 5 ml of anhydrous THF and added dropwise to this suspension at room temperature over 0.5 hr. The mixture was stirred for an additional 30 min and then placed in an ice-water bath. A solution of ketone VI (189 mg, 1.5 mmol) in 3 ml of anhydrous ether was added dropwise to the above mixture over 0.5 hr. The cooling bath was removed and the reaction mixture was refluxed for 5 hr. After cooling down to room temperature, 15 ml of water was slowly added to decompose the excess hydride. The aqueous phase was extracted with ether (3× 10 ml) and the combined ether extracts were washed with brine (1× 10 ml), dried ($MgSO_4$), and concentrated in vacuo. The crude product was purified by flash column chromatography (hexane–ethyl acetate, 20:1) to yield 275 mg (86%) of ethyl 3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-α-fluoroacetate X as a mixture of *E* and *Z* isomers (*E*:*Z* = 2:3). The *E* and *Z* isomers could not be separated by MPLC. [^{1}H]NMR, δ 4.26 (2H, q), 2.52 (CH_2-2, d, J_{HF} = 0.9 Hz), 2.14 (CH_2-2, d, J_{HF} = 2.6 Hz), 1.33 (3H, t), 0.92 (6H, s). [^{19}F]NMR, ϕ 131.56(s), 133.26(s).

3,3-Dimethylcyclohexane-$\Delta^{1,\beta}$*-*β*-fluoroethanol (XI).* The unresolved fluoroacetate isomers X (260 mg, 1.2 mmol) and lithium aluminum (51 mg, 1.3 mmol) were dissolved in 5 ml of anhydrous THF and refluxed for 6 hr. Upon cooling to room temperature, the excess hydride was slowly decomposed with 1 ml of ethanol, followed by 10 ml of water. The aqueous phase was extracted with ether (3× 10 ml) and the combined ether extracts were washed with brine (1× 10 ml), dried ($MgSO_4$), and concentrated in vacuo. The crude product was purified by flash column chromatography (hexane–ethyl acetate gradient, 25:1 to 6:1) to yield 180 mg (86%) of the incompletely separated *E* and *Z* isomers XI. [^{1}H]NMR *E* isomer δ 4.22 (2H, dd, J_{HF} = 22.7 Hz, J_{HH} = 6.0 Hz), 1.88 (2H, s), 0.89 (6H, s); *Z* isomer δ 4.26 (2H, dd, J_{HF} = 22.5 Hz, J_{HH} = 6.0

Hz), 2.1–1.9 (4H, m), 0.90 (6H, s). [^{19}F]NMR *E* isomer ϕ 122.09 (t, J_{HF} = 23.7 Hz); *Z* isomer, ϕ 123.70 (t, J_{HF} = 22.0 Hz).

3,3-Dimethylcyclohexane-$\Delta^{1,\alpha}$-α-fluoroacetaldehyde (XII). The unresolved β-fluoro ethanol isomers XI (52 mg, 0.3 mmol) were combined with manganese dioxide (300 mg, 3.5 mmol) in 3 ml of anhydrous CH_2Cl_2 and stirred for 18 hr at room temperature. TLC showed a considerable amount of the starting β-fluoroethanol still present. Additional manganese dioxide (200 mg, 2.3 mmol) was added, and the stirring was continued for another 12 hr. The reaction was quenched with 15 ml of ether, and the dark brown solution was filtered through Florisil. The precipitate was washed with ether (2× 10 ml), and the combined either filtrates were concentrated in vacuo. The crude product was purified by flash column chromatography (hexane–ethyl acetate, 20:1) to yield 32 mg (62%) of the incompletely separated (*E*)- and (*Z*)-α-fluoroacetaldehyde. [^{1}H]NMR *E* isomer of XII δ 9.74 (1H, d, J_{HF} = 18.4 Hz), 2.3–2.5 (4H, m), 0.97 (6H, s); [^{1}H]NMR *Z* isomer of XII δ 9.80 (1H, d, J_{HF} = 18.4 Hz), 2.56 (2H, t), 2.21 (2H, s), 0.96 (6H, s). [^{19}F]NMR *E*-isomer ϕ 135.95 (d, J_{HF} = 19.4 Hz); *Z* isomer ϕ 137.71 (d, J_{HF} = 19.2 Hz).

Electrophysiology. Adult female boll weevils used in this study emerged from a small laboratory colony annually infused with feral insects. Upon emergence, groups of five females were held in separate Petri dishes on moist filter paper (Whatman No. 1) at ca. 26°C on a photoregimen of 16 hr of light (ca. 600 lux) and 8 hr of darkness. Each group of five females was fed daily two fresh cotton squares. Only virgin females four to six days postemergence were used for recordings.

Methods used in recording electroantennograms (EAGs) were modified after previous methodologies (Schneider, 1957; Payne, 1970; Dickens, 1979; Dickens and Payne, 1977) and are detailed elsewhere (Dickens, 1984). Briefly stated, following insertion of Ag–AgCl capillary electrodes in the distal tip of the antennal club (recording electrode) and scape (indifferent electrode), electrical activity was amplified 10 times by a Grass P-16 DC preamplifier. EAG waveforms were displayed on a storage oscilloscope and their amplitudes were registered with a strip-chart recorder.

Dosage–response curves were constructed from serial dilutions (0.01–1.0 μg) of the *E* and *Z* isomers of both the α-fluoro analogs and acyl fluorides. Stimulus compounds diluted in hexane were delivered as 1-μl aliquots placed on filter paper (8 × 18 mm) inserted into glass odor cartridges (80 mm × 5 mm ID). A 10-μl aliquot was used to obtain the 10-μg dosage. Once these cartridges were oriented toward the antennal preparation at a distance of 1 cm, hydrocarbon-free air (filtered and dried) carried molecules evaporating from the filter paper over the preparation.

As in several previous EAG studies of the boll weevil (Dickens, 1984) and other insects (Dickens and Boldt, 1985; Dickens 1986; Light et al., 1988), 1-

hexanol at the 100-μg dosage was used as a standard for the normalization of all responses so that responses from different preparations could be compared. Odorous stimuli were presented from the lowest to the highest dosage; all compounds at one dosage were presented, followed by all compounds at the next higher dosage. Stimulation with the standard preceded and followed response to the series of compounds at each dosage. Responses to the compounds at each dosage were represented as a percent of the mean of the two nearest responses to the standard (Dickens, 1978, 1979, 1981). Three replicates were recorded for each dosage of each compound.

The magnitude of the negative transient of the EAG following stimulation was considered to be a measure of the relative number of responding acceptors (Payne, 1975; Dickens and Payne, 1977). The threshold of response was defined as the dosage at which the lower standard error of the mean was greater than zero. Saturation level was defined as that dosage at which the standard error of the response elicited overlapped with the standard error of the response at the highest dosage. Responses were compared for significant differences using the *t* test for two means (Ostle, 1969).

RESULTS AND DISCUSSION

The four sex pheromone components produced by male boll weevils, *Anthonomus grandis* Boheman, were isolated, identified, and first synthesized by Tumlinson et al. (1969). The four components are oxygenated monoterpenoids: (+)-*cis*-2-isopropenyl-1-methyl-cyclobutaneethanol (I), (*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\beta}$-ethanol (II), (*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde (III), and (*E*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetaldehyde (IV) (Figure 1). Several efficient syntheses of these compounds have been reported (Babler and Mortell, 1972; Pelletier and Mody, 1976; Wolinsky and Bedoukian, 1975), including synthesis of optically active grandisol I (Hobbs and Magnus, 1976; Mori et al., 1978; Webster and Silverstein, 1986). Laboratory bioassays and field tests (Cross, 1973; Hedin et al., 1973; Dickens, 1984, 1986) of the synthetic and chemically related compounds have also been reported.

Our effort in the boll weevil pheromone receptor system was to synthesize fluorinated isosteric analogs of II, III, and IV. Our target molecules were *E* and *Z* isomers of 3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetyl fluoride, (*E*)-IX and (*Z*)-IX, and the *Z* and *E* isomers of 3,3-dimethyl-cyclohexane-$\Delta^{1,\alpha}$-α-fluoroacetate, (*Z*)-XI and (*E*)-XII. Note that the change in nomenclature priorities (H < C < F) means that the α-fluoro analog with the *E* geometry is in reality an analog of the *Z* aldehyde.

We have chosen components III and IV as our parent compounds, because EAG studies have shown that both male and female weevils were significantly

more responsive to II and a mixture of III + IV (1 : 1) than to I, but the difference between II and III + IV was not significant (Dickens, 1984). Furthermore, geometrically pure III and IV are difficult to obtain, and the EAGs had not been described in earlier work.

Starting from commercially available 3-methyl-2-cyclohexen-1-one V, the (*E*) and (*Z*)-acyl fluorides (*E*)-IX and (*Z*)-IX could be synthesized in four steps as outlined in Figure 2, in 37% overall yield. A conjugate addition of lithium dimethylcuprate to the starting material V gave 3,3-dimethylcyclohexanone VI in 90% yield (Pelletier and Mody, 1976). Condensation of the resultant ketone with trimethyl phosphonoacetate in THF afforded, methyl 3,3-dimethyl-cyclohexane-$\Delta^{1,\alpha}$-acetate VII, in 94% yield as a mixture of *E* and *Z* isomers (*E*:*Z* = 3:2, GC). Subsequent hydrolysis of the esters in methanolic KOH solution gave the corresponding acids VIII in 92% yield. Separation of the *E* and *Z* isomers was then carried out by using medium-pressure liquid chromatography (MPLC) with two consecutive silica gel columns (2.2 ID × 30.0 cm, each), eluting with hexane–ethyl acetate (20 : 1). Selected fractions were analyzed by gas chromatography (DB-5, fused silica capillary column, 30 m × 0.263 mm). Fractions containing a single component and like retention times were then combined. The assignment of double-bond geometry to these acids was made

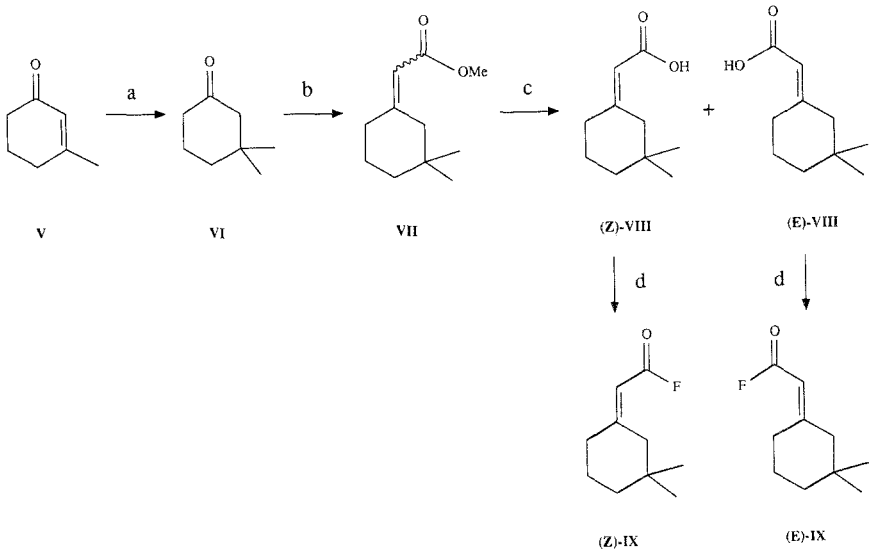
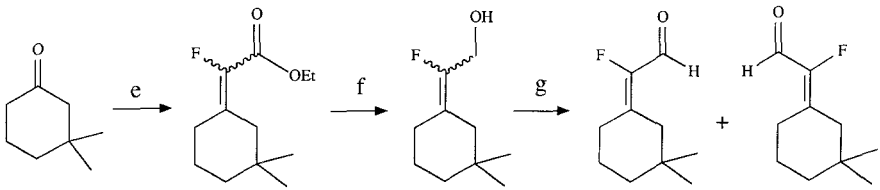


FIG. 2. Syntheses of acyl fluorides. Reagents: (a) CH_3Li, CuI, ether, 20°C, 20 min (90%); (b) NaH, $(CH_3O)_2P(O)CH_2CO_2CH_3$, THF, reflux, 4 hr (94%); (c) KOH, CH_3OH–H_2O (1 : 1), reflux, 3 hr (92%), and then MPLC separation; (d) DAST, ether, 20°C, 20 min (48%).

by converting the acids to the corresponding methyl esters with diazomethane and comparing spectra data with literature values (Tumlinson et al., 1971). The isomerically pure acids thus obtained were each reacted with diethylaminosulfur trifluoride (DAST) to give the acyl fluorides (*E*)-IX and (*Z*)-IX in 48% yield. These α,β-unsaturated acyl fluorides were stable and could be purified by flash chromatography without decomposition back to the starting acids. Acyl fluorides lacking the conjugated olefin (e.g., Z9–14:Acf) are readily hydrolyzed in air and do not survive chromatography (Prestwich et al., 1986; Ding, 1987).

The (*E*)- and (*Z*)-α-fluoroacetates (*E*)-XII and (*Z*)-XII could be synthesized in four steps also starting from V, as outlined in Figure 3, in 28% overall yield. To generate the α-fluoroacetate, triethyl 2-fluorophosphonoacetate was first obtained by reacting triethyl phosphite with ethyl bromofluoroacetate (Machleidt and Wessendorf, 1964). The triethyl 2-fluorophosphonoacetate was then added to ketone VI, giving the ethyl 3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-α-fluoroacetate X in 86% yield, as a mixture of *E* and *Z* isomers (*E*:*Z* = 2:3, GC). Reduction of the resulting acetate with lithium aluminum hydride (LAH) gave 3,3-dimethylcyclohexane-$\Delta^{1,\beta}$-β-fluoroethanol XI. Unlike the usual ester reductions with LAH in refluxing ether solution, the presence of the α-fluoro group reduced the rate of reaction significantly. In order to overcome this problem, a higher boiling solvent (THF) and longer reaction time (6 hr) was used. The yield of this reaction was 86%. The same problem was also encountered upon oxidation of the β-fluoroethanol with active manganese dioxide to the target molecules, 3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-α-fluoroacetaldehydes XII. The reaction time was 30 hr at room temperature and the yield was 62%. Surprisingly, these α-fluoroacetaldehydes, (*E*)-XII and (*Z*)-XII, have quite distinct R_f values: they showed up as two spots on TLC. They could be separated by flash column chromatography easily, as could the β-fluoroethanol compounds



VI X XI (E)-XII (Z)-XII

FIG. 3 Syntheses of α-fluoroacetates. Reagents: (e) NaH, $(C_2H_5O)_2P(O)CHFCO_2C_2H_5$, ether, reflux, 5 hr (86%); (f)$LiAlH_4$, THF, reflux, 6 hr (86%); (g) MnO_2, CH_2Cl_2, 20°C, 30 hr (62%), and then flash column chromatography separation. NOTE: The F atom changes the nomenclature priorities; that is, the α-fluoro compound (*Z*)-XII is a structural analog of the (*E*)-aldehyde IV.

(*E*)-XI and (*Z*)-XI. In contrast, separation of III and IV by flash column chromatography was not possible; even MPLC gave only low yields of pure III and IV, with the bulk remaining as III + IV.

Dosage–response curves constructed from EAGs of female *A. grandis* to the acyl fluorides and the α-fluoro analogs of III and IV revealed striking differences in the reactivity of these compounds with antennal olfactory acceptors (Figure 4). The EAG threshold for the α-fluoro analogs of both III and IV was at least 100-fold to 1000-fold lower for these compounds when compared to their acyl fluoride counterparts. Furthermore, the threshold for α-fluoro-IV [i.e., compound (*E*)-XII] was similar to that obtained for the natural pheromone IV. Antennal acceptors were somewhat more sensitive to the α-fluoro analog of III [i.e., compound (*Z*)-XII] than to III itself at lower concentrations.

Replacement of hydrogen by fluorine is a common strategy in producing biologically active compounds with modified binding properties and having different metabolic stabilities. Fluorinated pheromone analogs are able to effect behavioral responses and electrophysiological responses of insects by several modes of action. They can function as antagonists, blocking access of the pheromone to the receptor and thus inhibiting responses, or as agonists, mimicking the pheromone and producing a modified response. By judicious placement of the fluorine in a chemically reactive position, one can obtain an irreversible antagonist or agonist effect. Finally, a fluorinated analog may be active in disrupting the catabolism of a pheromone, thereby producing aberrant responses due to sensory adaptation to unremoved pheromone.

Camps and his co-workers (Camps et al., 1984a,b) have indicated that vinylic fluorines in olefinic pheromones act as agonists and do not cause major changes in responses of moths to their own pheromone. Our efforts to produce 2-fluorovinyl analogs of the aldehydes of two moth species have revealed a poor mimicry of the aldehyde by this functionality (Prestwich, 1987; Ding, 1987). Similarly, Mason et al. (1987) found that 2-fluoro-1-heptene was a poor analog of heptanal in conditioning experiments with tiger salamanders. When the fluorine is in the terminal ω position, we have observed trail-following activity by *Reticulitermes* to 12-fluoro analogs of dodecadienols similar to that of the unfluorinated compounds (cf. Prestwich, 1986; 1987). In addition, these ω-fluorofatty acid derivatives are potent toxicants by virtue of their in vivo conversion to fluoroacetate and then to fluorocitrate (cf. Prestwich, 1986, 1987).

Fluorination adjacent to carbonyl groups has not been extensively explored in pheromone biochemistry. Ding (1987) has prepared 2-fluoro- and 2,2-difluoroaldehydes analogous to Z11–16:Al, a pheromone component of several moths including *Heliothis virescens*. These aldehydes are so strongly hydrated that they appear unable to stimulate antennal responses; moreover, they are only moderately effective as inhibitors of the antennal aldehyde dehydrogenase of *H. virescens* (Ding, 1987; Prestwich, 1987; Prestwich et al., 1987). The boll

weevil system offered an opportunity to make α-fluoroaldehydes that would not be strongly hydrated, thus allowing us to observe the effects of a *less* electrophilic carbonyl carbon on pheromone response.

The effects of trifluoromethyl ketones as good mimics of acetates for binding or for enzymatic hydrolysis have also been explored in *Trichoplusia ni* (B.D.

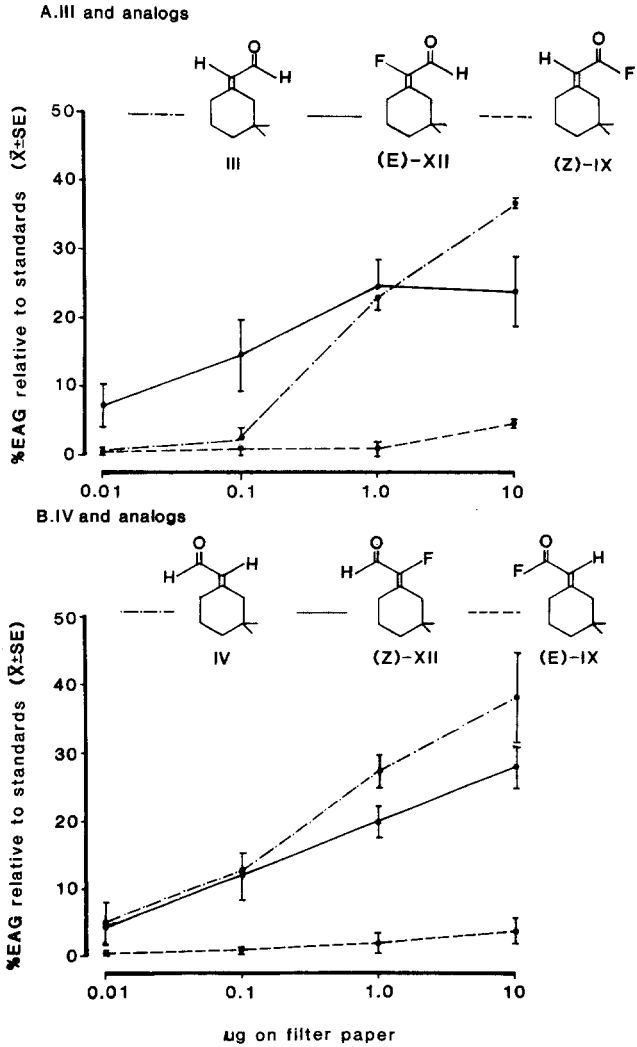


FIG. 4 EAG response ($\bar{X} \pm$ SE) of female antennae relative to 1-hexanol standard. (A) Responses to III (-·-·-·) and to analogs of III: acyl fluoride analog (*Z*)-IX (------) and α-fluoro analog (*E*)-XII (———). (B) Responses to IV (-·-·-·) and to analogs of IV: acyl fluoride analog (*E*)-IX (-----) and α-fluoro analog (*Z*)-XII (———).

Hammock, unpublished, cited in Prestwich, 1987) and in *Plutella xylostella* (Prestwich and Streinz, 1988). Finally, the sequential replacement of acetate hydrogens by halogens in Z11–16:Ac (Prestwich and Streinz, 1988) clearly showed that for *P. xylostella*, mono-, di-, and trifluoroacetates were moderately good stimulants (ca. 50% of acetate in EAG). Other halogens, while not changing the polarity, greatly affected the steric bulk of the acetate and resulted in haloacetate analogs with lower potency in both EAG and enzyme competition assays.

To date, the most striking example of a reactive pheromone mimic has been the Z9–14:Acf and Z11–16:Acf acyl fluoride analogs of the *H. virescens* aldehydes (Prestwich et al., 1986). Irreversible agonist activity was observed in treated males; disorientation and irreversible extrusion of the genitalia and hair-pencils resulted from in vivo exposure to 5 mg of the mixed acyl fluorides. Unfortunately, at low levels this acyl fluoride is quite labile to hydrolysis in the air. EAG assays and flight-tunnel experiments have been uniformly disappointing, because at low levels, hydrolysis, attachment to the substrate (e.g., paper), and release, all occur at competitive rates. Thus, the opportunity to evaluate a more stable α,β-unsaturated acyl fluoride in the boll weevil system offered a route to observe the low-level, longer-term effects of this isosteric replacement to give a *more* electrophilic carbonyl carbon.

The results of the EAG experiments clearly show that enhancing the reactivity of the carbonyl group as the acyl fluoride dramatically reduces its ability to stimulate the aldehyde receptor. In contrast, decreasing the electrophilicity with α fluoro substitution of the vinylic hydrogen produces a potent agonist. Further studies with the α-fluoro compounds will be reported in due course.

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CHEMICALLY MEDIATED HABITAT RECOGNITION IN SHORE INSECTS (Coleoptera: Carabidae; Hemiptera: Saldidae)

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Abstract—Adults of 14 species of Carabidae and mixed nymphs and adults of two species of Saldidae, collected in habitats associated with a saline lakeshore, sedge fen, vernal pond, stream mud flat, salt spring, mud and sand river bank, pebble river bank, and a marine sand beach, aggregated in choice chambers above volatiles (allelochemics) collected from their habitats. In other tests adults of some carabid species responded to volatiles from habitats other than their own, and to arbitrarily selected individual components and mixtures of components of saline lakeshore volatiles. These results suggest that shore insects recognize habitat allelochemics and aggregate in areas where these compounds are emitted by resident microflora. It is proposed that some habitat allelochemics are short-range signals that indicate locations of microhabitats used by shore insects for behaviors such as feeding, mating, and resting; in contrast, long-range allelochemics allow these insects to select their habitats from a distance. This hypothesis provides a mechanism for explaining how different shore habitats are partitioned among different species of shore insects.

Key Words—Habitat selection, habitat allelochemics, chemical cues, volatiles, shore habitats, arrestants, short-range cues, long-range cues, Coleoptera, Carabidae, Hemiptera, Saldidae.

INTRODUCTION

Even though they straddle the land–water interphase, shore environments are often named after the aquatic component of these ecotones because they are strongly influenced by the nature of the nearby water. Currents and wave action can erode or build up shorelines, and water temperature, pH, salinity, and other

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such factors determine the composition and structure of the biota of shore as well as aquatic communities (Úlehlová and Přibil, 1978). Yet, except for the intensively studied intertidal zone, little attention has been given to athalassic shore habitats such as those found adjacent to ponds, streams, lakes, springs, and rivers. When these places are examined, a distinct insect fauna is evident (Obrtel, 1972, 1978; Stehr and Branson, 1938; Holeski and Graves, 1978; Shelford, 1978; Richardson, 1921; Andersen, 1968, 1970, 1978, 1985; Lindroth, 1963). The most conspicuous are beetles of the families Carabidae (particularly bembidiines, agonines, and elaphrines), Staphylinidae, and Heteroceridae. Shore bugs (Saldidae) are also common as are scavenging, predatory, or algae-feeding flies (Ephydridae, Chironomidae, Canaceidae, Anthomyiidae, and Dolichopodidae). Collembolans and mites are abundant in some shore habitats, and hunting spiders (Lycosidae, Linyphiidae), normally associated with terrestrial vegetation, frequently forage in the same general areas as ground beetles and staphylinids. Similar insect communities live on some marine beaches and rocky shores (Evans, 1980). In western Canada the shore biota consists invariably of shore insects and an algal flora growing in wet substrates close to the water or in damp depressions on beaches.

Shore habitats vary in extent from narrow zones, scarcely more than 20 cm wide on the edges of meadow pools to vast beaches on gradually sloping shores of large lakes; because they are neither terrestrial nor aquatic, both flooding and desiccation are major hazards. Periodically, shore habitats are inundated by floodwaters or higher than normal tides and, after these subside, they leave deposits that completely blanket the habitats of the resident organisms. Wind-driven algal blooms are often deposited on beaches of large eutrophic lakes and these masses of decaying algae radically alter trophic pathways of the shore communities. Other shore habitats are endangered when lake and river levels subside, and small bodies of water dry out during droughts. However, shore organisms are adapted to these cyclic (intertidal) or intermittent (streams, rivers, ponds, springs, and lakes) fluctuations of water levels (Andersen, 1968; Thiele, 1977). The biota is soon reestablished in suitable sites due to rapid recolonization by pioneering microorganisms, particularly blue-green algae and diatoms, and the subsequent influx of shore insects and other animals. At some sites the successional transition from aquatic to terrestrial communities involves shore communities as early seral stages (Hefley, 1937).

Many shore insects overwinter in more sheltered places than exposed shorelines. Although short distances are involved in some localities, such as from a river bank to a nearby wood, for small insects these movements constitute migrations. Also, individuals of some species, particularly those from river banks prone to flooding or from ponds that dry out in late spring, have to migrate at other times as well. Movements of these insects between habitats require them to locate the general habitat area, the habitat itself, and the microhabitat;

or, in a broad sense (Prokopy, 1986), the habitat, patch, and resource, respectively (Hassell and Southwood, 1978).

Obviously, habitat recognition is an essential component of these kinds of activities. Recognition occurs when perception of certain signals or cues that are congruent with neural cognitive processes invokes specific behavioral responses (Dethier, 1982). The kind of response depends on the nature of the signal: Long-range signals may elicit anemotaxes that enable insects to find general habitat areas from a distance by flying upwind towards the signal source (Evans, 1983); intermediate-range signals may invoke some form of searching behavior; short-range signals induce orthokinetic and klinokinetic movements in relation to the boundaries of the microhabitat, resulting in increased time spent in the area (arrestment) (Jones, 1986).

During migrations, shore insects must distinguish their own habitats from a diverse array of similar habitats by recognizing specific habitat cues. These signals are token stimuli since they represent an integration of the physical, chemical, and biological nature of the habitat (Evans, 1983). Presumably, the species composition of communities differs because members of a particular habitat collectively recognize similar cues such as specific chemical compounds (allelochemics) or specific proportions of chemicals in a multicomponent allelochemic mixture. Components of volatile algal or other microbial metabolites have been implicated as sources of habitat recognition signals for some shore insects (Evans, 1982). These results prompted this investigation on whether other shore insects (from a broad range of shore habitats) also respond to habitat volatiles, the extent of recognition of other habitat volatiles, and whether components of allelochemic mixtures elicit responses.

METHODS AND MATERIALS

Ground beetles (Carabidae) and shore bugs (Saldidae) were chosen as experimental shore insects because they are common, conspicuous, and relatively easy to collect in most shore environments. These two groups of insects are primarily predaceous, their members feeding on smaller insects and mites, and, according to Silvey (1935), on freshly stranded zooplankton. A wide range of habitat types (8) in western Canada, from Saskatchewan to British Columbia, was selected for study. These sites were associated with a pond, salt spring, floating fen, stream, two kinds of river banks, a saline lake, and a marine sand beach. From among these sites adults of 14 carabid species and mixed nymphs and adults of two saldid species were collected, then kept in a laboratory incubator at 10°C until needed for tests. Groups of 10 individuals were placed in plastic boxes provided with damp filter paper and cotton-stoppered vials of water. All insects were fed weekly on vestigial-winged adults of *Drosophila*

melanogaster. Most of the carabids were *Bembidion* spp. along with *Elaphrus* (two species) and *Diplous aterrimus* Dejean and *Opisthius richardsoni* Kirby. The shore bugs consisted of *Saldula comatula* Parshley and *S. pallipes* (Fabr.). However, *S. pallipes* is actually a group of species that has not yet been differentiated taxonomically (Polhemus, personal communication); hence, their designation here as *S. pallipes* complex.

To test responses of the insects to habitat volatiles, they were allowed to choose, in still air, between half filter paper disks (Whatman No. 2, 5.5 cm diameter) treated with volatiles (from their own habitats or, in some experiments, from other habitats) or untreated filter papers, in choice chambers. The papers were positioned 2 mm below circular fine-mesh stainless-steel screens holding the insects, thus exposing them only to volatiles (see Evans, 1982, for details). Aggregation of insects on the sides containing the treated papers was considered evidence that the volatiles act as arrestants. All filter papers were initially rinsed in a mixture of 10% methanol and 90% acetone in a Soxhlet apparatus to remove residual chemicals. In each microhabitat, 50–100 filter papers were treated by pressing them onto the surface of damp soil or exposed algae, or by placing them under logs or stones in the habitats (see next section), and leaving them there for seven days or more. This allowed enough time for adsorption of volatile or contact chemical substances directly from the surface of substrates upon which insects walked. Papers collected in the field were transported to the laboratory in a cooler, then stored in a freezer until needed, and air dried before use. The papers from some habitats were eluted with carbon tetrachloride (four 5-ml aliquots per 20 papers), and the combined eluants were concentrated to about 4 ml with an N_2 gas stream. The concentrates were then applied with a syringe to fresh papers. Other papers from the habitats were used directly in the choice chambers. Groups of up to 10 insects were tested in each choice chamber, with each replicate consisting of between 5 and 10 counts of the insects on each side of the choice chambers at a count rate of one every 2 min.

Insects collected from one of the habitats (saline lakeshore) were tested for responses to individual components of habitat volatiles as well as to mixtures. These components were extracted from filter papers left in the habitat for 14 days and identified using gas chromatography and mass spectrometry. About 100 papers that had been left in the habitat for 14 days were crumpled with forceps and put in two glass tubes 20 cm long × 5.5 cm ID. A male 55/50 ground glass joint on the end of one tube connected with a female joint on the other tube, forming a sealed tube. The other ends of these tubes tapered to 8 mm OD × 7 cm tubing. Using Swagelok fittings, one end of the sample holder was connected to an N_2 source and the other end to a 9 cm long × 5 mm ID stainless tube containing 0.3 g of Tenax-GC stoppered with glass wool. The volatiles were flushed from the sample holder with N_2 at 50 ml/min for 2 hr

and adsorbed on the Tenax-GC, which was then eluted with three 0.5-ml aliquots of acetone.

Prior to injection into a gas chromatograph (Varian Vista 6000 connected to a VG Analytical 70E mass spectrometer), the acetone was concentrated with an N_2 stream to about 10 μl in a 15 mm long $\times$ 10 mm diameter screw-capped vial tapered at the bottom to a 10 mm long $\times$ 1 mm ID capillary tube. Separation of the components in extracted volatiles was performed on 1-μl injections with the use of two columns: a polar OV 351 programmed for 2 min at 35°C then 7°C/min to 200°C; and a nonpolar DB-1 which also started at 35°C for 2 min but continued to 290°C at 7°C/min. The resultant chromatograms and mass spectra showed that sulfur compounds, alkanes, alkenes, and saturated and unsaturated methyl esters of C_{16} to C_{18} acids were common. Therefore, acetone solutions of some of these chemicals (synthetic) were applied to half filter paper disks to serve as the treated side of the choice chambers, with acetone alone applied to the papers on the untreated sides. Three saline lakeshore species (*B. obtusidens* Fall, *B. scudderi* Lec., *B. versicolor* Lec.) and a river species (*B. interventor* Lindr.) were tested with these solutions.

Data were analyzed using replicated goodness of fit tests for observed ratios to expected ratios of 1:1 (G test; Sokal and Rohlf, 1969). The statistic "G pooled" considers all replicates as though they were a single large replicate and tests it for goodness of fit, whereas G heterogeneity indicates whether the trend in any direction is uniform; however, the magnitudes of G pooled and G heterogeneity are proportional to the number of replicates (*N* in tables), other factors being equal. Preliminary control tests using either solvent-treated or untreated papers on both sides of the choice chambers showed no preference by the insects for any side.

Habitats

Vernal Pond. Victoria, British Columbia. This habitat consists of mud flats left by receding water of a pond surrounded by couch grass *Agropyron repens* (L.) Beauv. The surface of the mud, particularly near and among the grass shoots, is covered with a layer of algae consisting mainly of chlorophytes *Oedogonium* spp., *Closterium* spp., *Protoderma* spp.; xanthophytes *Tribonema* spp.; euglenophytes *Trachelomonas* spp.; and cyanophytes *Anabaena* spp. Of the insect predators active in this habitat the carabids *Elaphrus americanus sylvanus* Goulet, *Bembidion coloradense* Hayward, and *B. versicolor* LeConte are the most abundant. Usually, by midsummer the water in the pond has dried out. Filter papers were pressed onto the algal layer.

Sedge Fen. Saltspring Island, British Columbia. This floating fen habitat differs from the vernal pond habitat in being a permanent body of water, although levels fluctuate seasonally, as do the kind of vascular plants present. The insect

predators are similar to those in the vernal pond habitat even though the algal complex overlaps only slightly. Algal aggregations are found on decomposing vascular plants and among stands of bulrush *Scirpus lacustris*. Dominant algae include the chlorophytes *Closterium* spp., *Klebsormidium* spp., and *Gloeocystis* spp.; species of the diatom genera *Pinnularia*, *Rhoicosphenia*, *Nitzschia*, *Eunotia*, *Fragilaria*, and *Hannea*, and the xanthophyte *Heterothrix* (*Tribonema*). Insects collected for testing were the same as those collected in the vernal pond habitat with the addition of *Saldula comatula*. Filter papers were placed on the algal mats.

Stream; Mud Flats. Amisk Creek, Tofield, Alberta. The algal mats on mud surfaces exposed by receding waters of this habitat consist mainly of members of the diatom genera *Nitzschia*, *Bacillaria*, *Navicula*, and *Pinnularia* along with the euglenophyte *Euglena* spp. and the cyanophyte *Chroococcus* spp. The carabids *Bembidion versicolor*, *B. graphicum* Casey, and *Elaphrus californicus* Mannerheim, and the saldid *Saldula pallipes* complex are common. Filter papers were pressed onto the algal layers.

Riverbank; Shingle. Goldstream River, Victoria, British Columbia. This clear, fast-flowing small river flows over a cobbled bottom forming numerous riffles and eddies. The pebbly substrate directly next to the water is the habitat of the patrobine carabid beetle, *Diplous aterrimus*. Both larvae and adults live in the spaces between pebbles, preying and scavenging on aquatic animals, including fingerling salmon stranded on the shore. Surfaces of pebbles in this habitat were coated with diatoms, *Eunotia* probably *lunaris* (Ehrenberg) Grunow. Filter papers were placed close to the water under stones large enough to cover them.

Riverbank; Mud and Sand. Pembina River, Barrhead, Alberta. This microhabitat is located up to 2 m away from the water and debris such as stones, twigs, and leaves serve as shelters for numerous shore insects, with *Bembidion interventor* Lindroth, *B. inequale* Say, *B. timidum* LeConte, and *Opisthius richardsoni* being dominant during the tenure of this study. A faint greenish color on the surface of the caked muds and sands is due to diatoms (*Eunotia*, *Nitzschia*, and *Pinnularia* spp.), so filter papers were placed directly on this substrate.

Saline Lakeshore. Wells Lake, Marsden, Saskatchewan. This sulfate-dominated, 5 × 1 km, saline lake is connected by a stream to Manito Lake [salinity = 19.3 g/liter; (Hammer, 1978)] and is typical of many such lakes in western Saskatchewan and eastern Alberta in having salt-encrusted sandy-loam beaches that provide habitats for many shore insects. One kind of habitat consists of shallow depressions containing 0.5- to 1.5-cm-thick mats of salt crystals, sand, and dense growths of blue-green algae (*Oscillatoria animalis* Agardh and *O. subbrevis* Schmidle). Diatoms, brineflies (*Ephydra* sp.), springtails, mites, and other organisms occupy this habitat as well as the carabids *Bembidion obtusi-*

dens, *B. scudderi*, *B. diligens* Casey, *B. versicolor*, and *B. henshawi* Hayward. On the open areas of the beach *Elaphrus lecontei* Crotch is occasionally present, whereas *Saldula pallipes* complex is very abundant. The wetter parts of the beach, especially near the water, are usually covered by sheets of *Cladophora*, which attract many brineflies but few carabids or saldids. *Salicornia rubra* (A. Nels) is common on the upper shore. Filter papers were placed directly on the salt-encrusted *Oscillatoria* mats.

Marine Sand Beach. This high intertidal habitat is situated on the southwest side of a protected sand spit (Sidney Spit) facing Sidney channel on Sidney Island, British Columbia. Spaces under cast-up logs at the base of sand dunes provide daytime resting sites for adults of the carabid beetle *Bembidion indistinctum* (Dej.) along with amphipods (*Orchestia* spp.), marine weevils (*Elassoptes marinus* Horn), and grapsid crabs [*Hemigrapsus oregonensis* (Dana)]. During nocturnal low tides, these animals forage over the surface in company with other sand-burrowing intertidal insects such as *Thinopinus pictus* LeConte (Staphylinidae), *Phycocoetes testaceous* LeConte (Curculionidae), *Endeodes collaris* (LeConte) (Melyridae); and *Dyschirius obesus* LeConte (Carabidae). The filamentous marine green alga *Percursaria percursa* (Agardh) Rosenvinge grows under those logs that are stranded near the high-tide mark. Filter papers were treated by placing them under logs for seven days.

Salt Spring. Saltspring Island, British Columbia. Sodium chloride is the major salt in this hypersaline spring (salinity = 73.1 g/liter) which oozes from the side of a low hill over an area of about 6 m^2. Collembolans (*Anurida* spp.), brineflies (*Ephydra* spp.), saldids (*Saldula comatula*), intertidal chironomids [*Thalassosmittia marinus* (Saunders)], (Saunders, 1928), and carabid beetles (*Bembidion indistinctum*) are fairly common here. *Salicornia rubra* and *S. virginica* L. dominate the areas downhill of the spring. Dense growths of *Percursaria percursa* give the general area of the spring a bright green color. Thus this marine alga and *B. indistinctum* occur together in two very different kinds of habitats. Filter papers were pressed onto the surface of *P. percursa* mats.

RESULTS

Dominant Microflora of Habitats. The eight shore habitats are readily segregated into four groups by algal composition: (1) The marine sand beach and salt spring are distinct because of the green alga *P. percusa*, which is dependent on the NaCl-dominated waters of these habitats. (2) Blue-green algae, *O. animalis* and *O. subbrevis*, associated with saline waters high in sulfates, characterizes the saline lakeshore habitat. (3) Diatoms encrusted on stones or growing in sand are characteristic of the microflora of riverbanks. (4) Fens, ponds, and

mudflats next to lentic waters, including quiet backwaters of streams, showed the greatest variety of algae such as greens, blue-greens, euglenophytes, xanthophytes, and diatoms growing on surfaces of wet substrates near water.

Responses to Habitat Volatiles. In all tests the total numbers on the treated side of the choice chambers exceeded those on the untreated and the pooled G tests show these differences to be real (Table 1). The treated–untreated ratios varied from 1.4 : 1 for *B. inequale* (river bank) to 4.3 : 1 for *S. pallipes* complex (saline lakeshore), indicating variability in magnitude of responses of species from different habitats. These results suggest that shore carabids and saldids recognize microhabitat signals and tend to aggregate in places that emit them. In more than two thirds of the tests, G heterogeneity was significant; the reasons for this are not known, but habituation of the insects to repeated disturbances when counts were taken cannot be discounted.

The volatiles are produced by the floral constituents of the habitats, as suggested by Evans (1982, 1986) and by a consideration of the *B. indistinctum*/*P. percursa* relationship. The hypersaline environment of the salt spring must have been conducive to *P. percursa* development (after colonization by windblown spores), even though the soils, slope, aspect, and general appearance of the surroundings are very different from marine shores. The common denominator of the salt spring and the sand beach habitats is the alga, indicating that chemical cues emitted by this plant have a significant role in guiding adults of *B. indistinctum* to both habitats.

Responses to Volatiles from Other Habitats. The results (Table 2) show that the insects that were tested can be grouped into those species (*B. coloradense*, *B. obtusidens*, *B. versicolor*) that tolerate a wide range of environmental conditions (eurytopic) and those species (*B. indistinctum*, *B. inequale*, *E. a. sylvanus*, *O. richardsoni*) that tolerate narrower ranges (stenotopic). Although *B. indistinctum* also responded to saline lakeshore volatiles, when given a choice between those and salt spring volatiles individuals of this species chose the latter. *E. a. sylvanus* appears to be restricted to nonsaline environments. Curiously, *B. coloradense*, a nonhalophile from the sedge fen responded to salt spring volatiles, although not to those from the sea shore habitat.

Because of the great contrast between river bank and lake shore environments, as well as the differences in salinity of the waters, river bank insects (*B. inequale* and *O. richardsoni*) did not aggregate above saline lakeshore volatiles. An exception was *B. versicolor*. This is a well-documented eurytopic species (Lindroth, 1963); in this study, individuals were collected at the sedge fen, river bank (sand and mud), and the saline lakeshore (Table 3) habitats. Thus responses of these beetles collected from the sedge fen to volatiles from the vernal pond and from the stream mud flats were not unexpected.

Individuals of *B. obtusidens* are usually found in highly saline environments where salts are precipitated on soil surfaces, but there are collection rec-

TABLE 1. OLFACTORY RESPONSES OF SHORE INSECTS IN CHOICE CHAMBERS TO FILTER PAPERS EXPOSED TO HABITAT VOLATILES (hv) OR TREATED WITH CCl_4 EXTRACTS OF HABITAT VOLATILES (ex) VERSUS UNTREATED PAPERS[a]

Species	Papers	N	Totals		G test	
			Treated	Untreated	Pooled	Heterogeneity
Vernal pond						
Bembidion coloradense	ex	15	1003	357	319.6 $P < 0.005$	80.9 $P < 0.005$
Elaphrus a. sylvanus	ex	9	490	290	51.9 $P < 0.005$	5.8 ns
Sedge fen						
Bembidion coloradense	ex	18	555	348	47.9 $P < 0.005$	35.7 $P < 0.005$
Bembidion versicolor	ex	18	613	376	48.8 $P < 0.005$	57.3 $P < 0.005$
Saldula comatula	ex	10	246	59	123.2 $P < 0.005$	25.3 $P < 0.005$
Stream; mud flat						
Bembidion graphicum	hv	12	559	181	202.5 $P < 0.005$	44.3 $P < 0.005$
Elaphrus californicus	ex	21	306	204	20.5 $P < 0.005$	22.1 ns
Saldula pallipes compl.	ex	6	198	72	61.1 $P < 0.005$	9.7 ns
Riverbank; shingle						
Diplous aterrimus	ex	12	336	162	62.1 $P < 0.005$	36.3 $P < 0.005$
Riverbank; sand and mud						
Bembidion inequale	hv	11	263	187	17.7 $P < 0.005$	29.5 $P < 0.005$
Bembidion interventor	hv	26	598	292	107.4 $P < 0.005$	18.9 ns
Bembidion versicolor	hv	12	383	199	59.2 $P < 0.005$	31.6 $P < 0.005$
Opisthius richardsoni	hv	6	93	57	8.7 $P < 0.005$	1.9 ns
Saline lake						
Bembidion diligens	hv	9	193	77	51.5 $P < 0.005$	8.5 ns
Bembidion henshawi	hv	9	289	161	36.9 $P < 0.005$	28.7 $P < 0.005$
Bembidion obtusidens	hv	12	340	90	154.9 $P < 0.005$	32.5 $P < 0.005$
Bembidion scudderi	hv	9	196	89	41.7 $P < 0.005$	2.3 ns
Saldula pallipes compl.	hv	25	855	195	515.9 $P < 0.005$	57.1 $P < 0.005$
Sand beach; marine						
Bembidion indistinctum	ex	15	871	581	58.3 $P < 0.005$	46.7 $P < 0.005$
Salt spring						
Bembidion indistinctum	hv	17	637	285	137.9 $P < 0.005$	64.6 $P < 0.005$
Saldula pallipes compl.	hv	21	497	252	81.6 $P < 0.005$	54.0 $P < 0.005$

[a] N = number of replicates; ns = not significant; G test explained in text.

TABLE 2. OLFACTORY RESPONSES OF SHORE INSECTS IN CHOICE CHAMBERS TO VOLATILES FROM HABITATS OTHER THAN THOSE FROM WHICH THEY WERE COLLECTED (Treated) VERSUS UNTREATED PAPERS (Other Details as in Table 1)

Species (habitat)	Source of volatiles	Totals			G test	
		N	Treated	Untreated	Pooled	Heterogeneity
B. coloradense (sedge fen)	vernal pond	9	320	85	145.3 $P < 0.005$	9.6 ns
	seashore	6	132	168	4.3 ns	12.0 $P < 0.005$
B. coloradense (vernal pond)	salt spring	3	117	33	49.9 $P < 0.005$	0.2 ns
B. obtusidens (saline lakeshore)	salt spring	3	81	14	52.2 $P < 0.005$	2.2 ns
	stream, mud flat	6	125	175	8.4 $P < 0.005$	66.1 $P < 0.005$
	vernal pond	12	482	113	246.4 $P < 0.005$	30.2 $P < 0.005$
B. versicolor (sedge fen)	vernal pond	15	692	270	189.3 $P < 0.005$	98.3 $P < 0.005$
	stream, mud flat	6	209	61	85.8 $P < 0.005$	4.7 ns
B. indistinctum (salt spring)	saline lakeshore[a]	6	233	57	105.0 $P < 0.005$	1.0 ns
		6	189	92	34.2 $P < 0.005$	30.8 $P < 0.005$
B. inequale (riverbank)	saline lakeshore	10	284	276	0.1 ns	41.0 $P < 0.005$
E. a. sylvanus (sedge fen)	saline lakeshore	4	57	43	1.9 ns	2.1 ns
	stream mud flat	10	223	97	51.0 $P < 0.005$	10.1 ns
O. richardsoni (riverbank)	saline lakeshore	6	254	258	0.03 ns	14.1 $P < 0.05$

[a] Treated = salt spring volatiles; untreated = saline lakeshore volatiles.

TABLE 3. OLFACTORY RESPONSES OF SALINE LAKESHORE INSECTS IN CHOICE CHAMBERS TO 0.02 ml ACETONE SOLUTIONS/cm^2 OF COMPONENTS OF HABITAT ALLELOCHEMICS (Treated) APPLIED TO FILTER PAPER HALVES VERSUS ACETONE-TREATED PAPERS (Untreated) (Other Details as in Table 1)

Volatile component(s)	Species	N	Treated	Untreated	G test	
					Pooled	Heterogeneity
Ester[a]	*B. obtusidens*	12	323	277	3.5 ns	29.5 $P < 0.005$
Ester[b]	*B. obtusidens*	6	173	127	7.1 $P < 0.01$	37.7 $P < 0.005$
Esters[c]	*B. scudderi*	9	374	77	212.9 $P < 0.005$	39.6 $P < 0.005$
	B. obtusidens	10	604	296	107.5 $P < 0.005$	64.7 $P < 0.005$
	B. versicolor	12	348	239	39.8 $P < 0.005$	101.6 $P < 0.005$
Sulfide[d]	*B. obtusidens*	3	178	122	10.5 $P < 0.01$	10.1 $P < 0.005$
Sulfide[e]	*B. scudderi*	3	197	63	72.5 $P < 0.005$	2.0 ns
Alkene[f]	*B. scudderi*	12	640	308	118.8 $P < 0.005$	43.3 $P < 0.005$
Alkene + sulfide[g]	*B. scudderi*	3	188	112	19.5 $P < 0.005$	7.7 $P < 0.05$
	B. obtusidens	3	197	103	29.9 $P < 0.005$	18.6 $P < 0.005$
Alka(e)nes[h]	*B. scudderi*	6	363	237	26.3 $P < 0.005$	39.6 $P < 0.005$
	B. interventor	6	243	177	10.4 $P < 0.005$	38.3 $P < 0.005$
	B. obtusidens	9	275	175	22.4 $P < 0.005$	11.9 $P < 0.005$

[a] 17.0 μg/cm^2 methyl ester of hexadecanoic acid.
[b] 19.0 μg/cm^2 methyl ester of 9-octadecenoic acid.
[c] 25.0 μg/cm^2 methyl ester of hexadecanoic acid + 9.0 μg/cm^2 methyl ester of 9-octadecenoic acid.
[d] 3.3 μg/cm^2 dimethyl disulfide.
[e] 2.0 μg/cm^2 dimethyl sulfide.
[f] 17.0 μg/cm^2 1-undecene.
[g] 17.0 μg/cm^2 1-undecene + 8.0 μg/cm^2 dimethyldisulfide.
[h] 1.0 μg/cm^2 1-pentadecene + 8.0 μg/cm^2 pentadecane + 4.0 μg/cm^2 1-heptadecene + 1.2 μg/cm^2 heptadecane.

ords of this species from hyposaline areas near Edmonton (Lindroth, 1963). Nevertheless, although responses of these insects to salt spring volatiles are expected (even if this habitat is outside their geographical range), it is not clear why they responded to the vernal pond volatiles but not to those from the stream mud flat.

Responses to Components of Saline Lakeshore Volatiles. Individuals of three species (*B. obtusidens*, *B. scudderi*, *B. versicolor*) aggregated above the filter papers treated with components alone or mixtures of these chemicals (Table 3). The ester mixture was shown previously to invoke responses of *B. obtusidens* adults in choice chambers (Evans, 1982) as well as anemotactic responses in a wind tunnel (Evans, 1984). This ester mixture is much more effective in eliciting aggregation of the insects than either of the individual components alone. The results of the methyl 9-octadecenoate tests were barely significant (G pooled), whereas the distribution in response to methyl hexadecanoate did not differ from that occurring by chance.

Although the mass spectra did not positively pinpoint the sulfur compounds, tests using both dimethyl sulfide and dimethyl disulfide [volatiles associated with anaerobic decomposition of organic sulfur (McLaren, 1963)] also induced responses as did the combination of 1-undecene with dimethyl disulfide. The C15:0, C15:1, C17:0, and C17:1 hydrocarbon mixture invoked responses in saline lakeshore species (*B. obtusidens*, *B. scudderi*) and in a river bank species (*B. interventor*). The responses of shore insects to arbitrarily selected concentrations and mixtures (Table 3) indicate that specific blends of volatiles are not required as habitat recognition cues.

DISCUSSION

The behavior of shore insects in choice chambers (Table 1) in response to habitat allelochemics demonstrates that they recognize these chemical signals. Recognition of these cues provides them with a means of monitoring varying degrees of habitat quality in relation to spatial aspects (although it does not explain how the habitat is selected initially by flying insects). The integration of this information with that provided by visual and tactile cues can then enable them to recognize microsites within the habitat for feeding, mating, resting, ovipositing, and other resource-related activities. This mechanism of habitat recognition can help to explain why shore insects appear to be restricted to specific areas of shore environments. *D. aterrimus*, for instance, is found only in a very narrow zone of moist pebbles next to the water. Chemical cues associated with diatoms growing on these stones may be more important than other cues in this process. Adults of *Elaphrus a. sylvanus* lurk in spaces between grass stems bordering ponds and dash out to catch prey on exposed areas next to the water. In one locality, the foraging distance from grass to water was only

about 40 cm. Although visual stimuli probably have a large role in the orientation behavior of these predatory insects, allelochemics also must have been involved (Table 1). Evans (1986) also records the role of these chemicals in habitat recognition in an intertidal carabid beetle inhabiting rock crevices.

These examples represent responses of insects to chemical cues that are short-range signals, i.e., arrestants. Specific concentrations or blends of these chemicals appear to be unnecessary to obtain arrestant responses in the laboratory (Table 3), explaining the responses of shore insects to volatiles from other habitats (Table 2). Perhaps a wide range of habitats produces some chemicals that are common to all of them. Preliminary unpublished results suggest that this may be so, at least in the case of volatiles of river bank soils and saline lakeshore sands. Data on the attraction of a river bank species (*Bembidion interventor*) to mixtures of saline lakeshore hydrocarbons (Table 3) support this idea.

Some shore insects fly readily when disturbed. Even if they are displaced a short distance away from the habitat, intermediate-range cues could keep them informed of their spatial relationship with the habitat, enabling them to fly back to the area. However, the localized effectiveness of short and intermediate range cues precludes their use in habitat selection by migrating insects at considerable distances from the habitat (several kilometers). Therefore, it is postulated that long-distance chemical cues must serve to bring migrants into the general area of the appropriate habitat and that partitioning of shore insect species among the many kinds of shore habitats is thus a function of recognition by these insects of long-range habitat cues. This proposed mechanism of habitat selection further supports the suggestion that components of short-range (microhabitat) signals may occur in more than one kind of habitat, resulting in bioassays that show responses to other habitat volatiles (Table 2) and apparent lack of specificity of responses to particular blends, mixtures, or concentrations of these allelochemics (Table 3).

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ANTINUTRITIONAL EFFECTS AND ECOLOGICAL SIGNIFICANCE OF DIETARY CONDENSED TANNINS MAY NOT BE DUE TO BINDING AND INHIBITING DIGESTIVE ENZYMES

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Abstract—The digestive enzymes alkaline phosphatase and 5′-nucleotide phosphodiesterase, solubilized from bovine intestinal mucosa and purified to homogeneity, were found to be strongly inhibited in vitro by condensed tannins (proanthocyanidins) purified from sorghum seeds and from quebracho. Tannin inhibition was prevented and reversed by the detergent Triton X-100 (protein-binding agent), by soluble polyvinylpyrrolidone (tannin-binding agent), or by phosphatidylcholine (membrane component). When tested as a crude particulate membrane fraction more characteristic of their in vivo condition, both enzymes were inhibited much less than either purified enzyme at the same tannin concentration. Because the enzymes appear to be relatively insensitive to inhibition by tannin in conditions which mimic in vivo conditions, and because the proportion of the dietary tannin which is available to interact with these enzymes in the digestive tract is likely to be rather small, we suggest that the antinutritional effects and ecological significance of dietary tannins are not due to tannin inhibition of these or other digestive enzymes by direct binding to them.

Key Words—Dietary tannins, digestive enzymes, phosphatase, 5′-nucleotide phosphodiesterase, enzyme inhibition.

INTRODUCTION

Tannins are plant polyphenols capable of forming strong complexes with proteins. Tannins are generally beneficial to the plants that produce them, func-

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tioning as part of the plants' chemical defense system against herbivores and/or microorganisms (Fellows, 1980). Tannins are effective antifeedants, but the mechanism of this effect has not been established (Mole and Waterman, 1987a). In animal diets tannins generally show antinutritional effects such as diminished weight gains and poor feed efficiency (Blair and Mitaru, 1983).

The growth-depressing effect of dietary tannins has been attributed, in part, to a decrease in protein digestibility (Vohra et al., 1966), possibly due to formation of a complex between the tannin and endogenous proteins such as digestive enzymes (Glick and Joslyn, 1970). Digestive enzymes such as trypsin and α-amylase have been reported to be inhibited in vitro by tannins (Griffith and Moseley, 1980). The digestive enzyme enterokinase, which plays a crucial role in the activation of digestive proteases (Maroux et al., 1971), has also been reported to be inhibited in vitro by (dietary) tannins (Oh and Hoff, 1986). These investigators also reported that tannin may either inhibit or stimulate in vitro digestion by intestinal proteases, depending upon the reaction conditions. Mole and Waterman (1985, 1987b) have also questioned the assumption that the effects of dietary tannins are due to their inhibition of digestive enzymes, and several observations inconsistent with inhibition of digestion as the sole or primary mechanism of the antinutritional effects of dietary tannin in mammals have been summarized (Butler et al., 1986). It has recently been concluded that dietary tannins do not reduce the nutritional value of insect foods by inhibiting digestion (Martin et al., 1987).

Condensed tannins (proanthocyanidins) have been shown to be highly selective in their binding; large, conformationally loose, proline-rich proteins are bound several orders of magnitude more strongly than small, compact proteins (Hagerman and Butler, 1981). Dietary tannin has opportunities for complexing with a wide variety of dietary proteins and other proteins of the digestive tract which have a very high affinity for tannin (Mehansho et al., 1983) before it is exposed to the major digestive enzymes. Tannin may therefore not be free to bind digestive enzymes when it encounters them. Moreover, some digestive enzymes occur in membrane-bound forms, which may be less susceptible to inhibition by tannin than the soluble forms purified and tested in vitro. In an attempt to assess the nutritional significance of the inhibition of digestive enzymes in vitro, we have investigated the inhibition, by condensed tannin purified from sorghum seeds and from quebracho, of two digestive enzymes as they occur in a particulate, membrane-bound form as well as the soluble form which has been purified and characterized in vitro. Because the effects of tannin on proteolytic enzymes are complicated by tannin binding to both the protease and the protein substrate, we have chosen to study phosphohydrolases, for which tannin binds only to the enzymes and not to the substrates. These enzymes, alkaline phosphatase (APase) and 5′-nucleotide phosphodiesterase (PDase), are from mucosal cells of the bovine intestinal brush border.

METHODS AND MATERIALS

Materials. The soluble forms of APase (Landt et al., 1978) and PDase (Landt and Butler, 1978) were purified from bovine intestine as previously described. Purified soluble enzymes were used in all experiments in which particulate enzyme is not specified. To prepare particulate enzymes (all steps carried out at 0–5°C), 2 g frozen bovine intestinal mucosa was suspended in 18 ml of buffer A (100 mM Tris HCl, 12 μM $ZnSO_4$, pH 8.0) using a ground glass homogenizer. The homogenate was centrifuged 20 min at 35,000*g* and the supernatant discarded. The pellet was suspended in 18 ml of buffer, and centrifuged again. The pellet from the second centrifugation was resuspended in buffer A and homogenized on a Brinkmann Polytron equipped with a PT-10 head, at full power for five bursts of 12 sec each, with 2-min intervals for chilling to 0°C. In order to show that Polytron treatment did not convert the enzyme to a soluble form, a sample of the homogenate was centrifuged 90 min at 200,000*g*. Most of the enzyme activity sedimented; only 3.5% of the APase activity and 4.5% of the PDase activity remained in the supernatant.

Substrates (4-nitrophenyl phosphate and 4-nitrophenyl phenylphosphonate), bovine liver phosphatidylcholine, soluble polyvinylpyrrolidone (PVP), average mol wt 10,000, and Triton X-100, average mol wt 625, were from Sigma (St. Louis, Missouri). Sorghum tannin was purified from the seed of the bird-resistant sorghum hybrid, BR 64, by the method of Hagerman and Butler (1980a). Quebracho tannin was purified from quebracho extract (Trask Chemical Corp., Marietta, Georgia) as previously described (Asquith and Butler, 1985).

Enzyme Assays. APase and PDase activities were determined as previously described (Landt and Butler, 1978) using 1 mM 4-nitrophenyl phosphate and 4-nitrophenyl phenylphosphonate, respectively, as substrates at pH 8.0.

RESULTS

Soluble highly purified PDase is strongly inhibited by tannin (Figure 1). Under the conditions used in these assays, no precipitation of the enzymes by tannin is observed; both PDase and APase are glycoproteins, which may account for the solubility of their complexes with tannin (Asquith et al., 1987). Inhibition is not instantaneous, but it is essentially complete in 15 min. Tannins purified from sorghum seeds or quebracho are equally effective as inhibitors, with 50% inhibition at about 20 μg/ml in the subsequent assay. The experiment shown in Figure 1 was carried out at pH 8.0. Similar effects were observed when the enzyme was incubated with tannin at pH 6.0 and assayed at pH 8.0; physiological pH in the small intestine should be between pH 6 and pH 8.

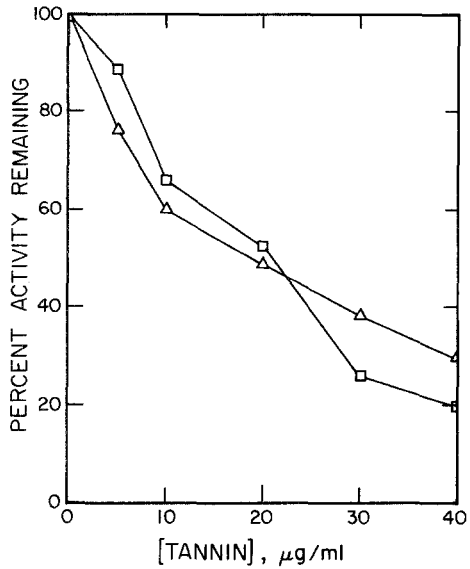

FIG. 1. PDase inhibition by sorghum and quebracho tannin. PDase was incubated for 15 min at 30°C in 0.1 M Tris (HCl), 10 μM $ZnSO_4$, pH 8.0 (buffer A) containing the indicated concentration of sorghum (squares) or quebracho (triangles) tannin. The mixture was then diluted 3.3-fold into assay mix and the activity measured as described in Methods and Materials.

Purified APase is somewhat less sensitive to inhibition by tannin (Figure 2). In contrast to PDase, the two sources of tannin are not equivalent for APase; up to eight times more quebracho tannin is required to obtain the same degree of inhibition observed with sorghum tannin. Sorghum tannin is likewise more effective than quebracho tannin in protein binding and precipitation assays (Asquith and Butler, 1985).

Effect of Triton X-100, Phospholipid, and PVP. Figure 3 shows the pronounced effect of 10 mM Triton X-100 on inhibition of PDase by tannin. Tannin concentrations, which otherwise produce 80% inhibition of PDase, are not inhibitory in the presence of Triton X-100. Tannin even slightly stimulates PDase when 10 mM Triton X-100 is present. Tannin likewise does not inhibit APase in the presence of 10 mM Triton X-100 (Butler et al., 1984). Similarly, in the presence of phosphatidylethanolamine, a prominent membrane component, tannin inhibition of PDase (Figure 4) and APase (not shown) is greatly reduced.

When Triton X-100 is added to tannin-inhibited APase, inhibition is completely reversed (Figure 5). The inhibitory effect of tannin, therefore, does not

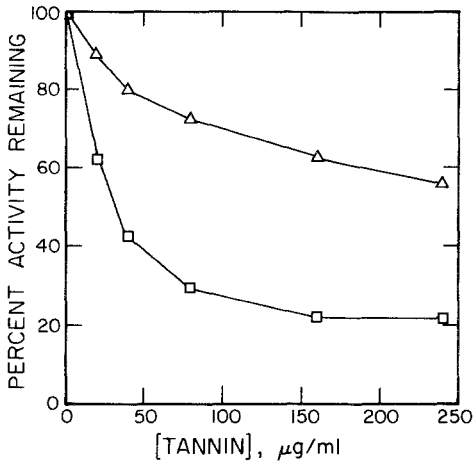

FIG. 2. APase inhibition by sorghum and quebracho tannin. APase was incubated for 15 min at 30°C in Buffer A containing the indicated concentration of sorghum (squares) or quebracho (triangles) tannin and assayed as in Figure 1.

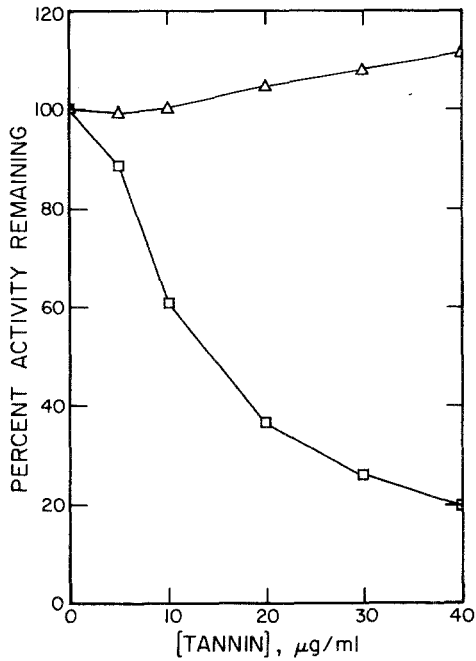

FIG. 3. Effect of Triton X-100 on tannin inhibition of PDase. PDase was incubated for 15 min in buffer A (squares) or in buffer A containing 10 mM Triton X-100 (triangles) in the presence of the indicated concentrations of sorghum tannin.

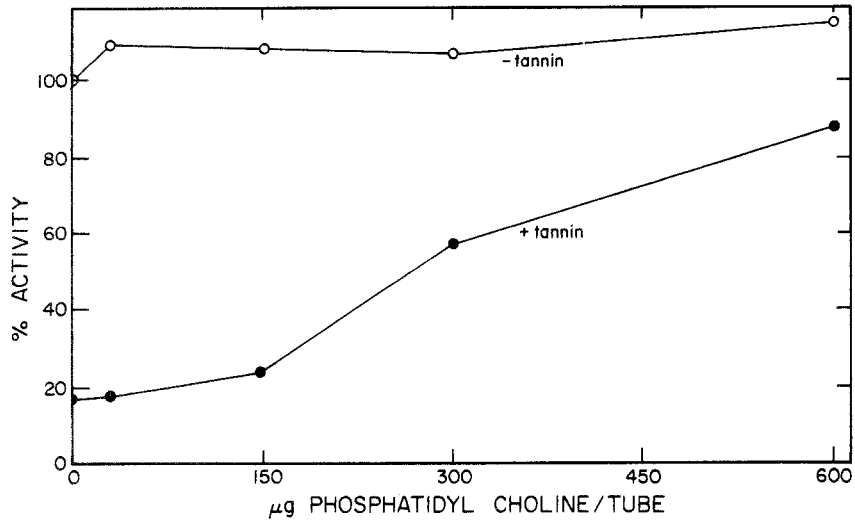

FIG. 4. Effect of phosphatidylcholine on tannin inhibition of PDase. PDase, 1.1 μg protein, was incubated for 30 min at 30°C with the indicated amount of phosphatidylcholine in buffer A after which 30 μg of sorghum tannin was added for a final volume of 0.5 ml another 30 min before assay.

depend on irreversible denaturation or modification of the enzyme. Triton X-100 also reactivated PDase after tannin inhibition (data not shown).

Addition of soluble PVP to tannin-inhibited PDase leads to complete reactivation (Figure 6). Tannin-inhibited APase was also reactivated by PVP, but the recovery of activity after PVP addition was not quite as complete as with PDase. Thus, the inhibition of these enzymes by tannin can be reversed by addition of agents that bind either protein (Triton X-100) or tannin (PVP). On the other hand, 10% (v/v) methanol, which enhances protein precipitation by tannin (Hagerman and Butler, 1980b), did not have a significant effect on tannin inhibition of PDase (data not shown).

When the enzyme sample is a particulate fraction instead of the purified soluble enzyme, the effect of tannin is much smaller. As shown in Figure 7, 50 μg/ml tannin only inhibits the particulate PDase by 11%, whereas the soluble purified PDase is inhibited 45% at this tannin concentration. The particulate enzyme preparation contained about the same level of enzyme activity as the soluble purified PDase, but it had approximately 200 times as much protein. In a previous study (Butler et al., 1984) a higher concentration of the crude particulate enzyme was used, and 50 μg/ml tannin caused only 3% inhibition of the PDase activity. APase activity of the particulate fraction is likewise less

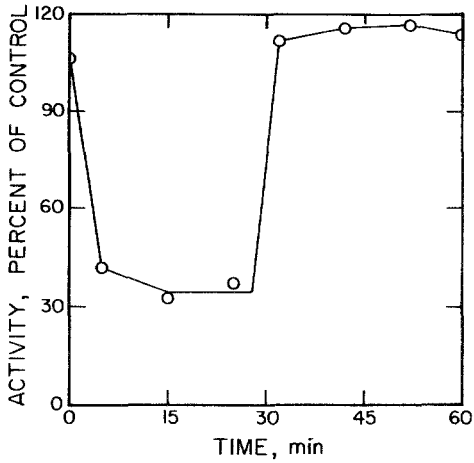

FIG. 5. Reversal of tannin inhibition of APase by Triton X-100. APase was incubated for the time shown at 30°C in buffer A containing 200 μg/ml sorghum tannin and 8 mg/ml methanol. A control sample contained methanol without tannin. At 28 min, both samples were made 12 mM in Triton X-100.

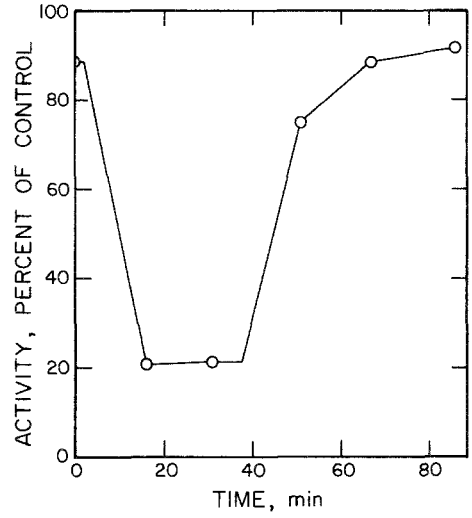

FIG. 6. Reversal of tannin inhibition of PDase by PVP. PDase was incubated for the time shown at 30°C in buffer A. At 2 min, the experimental sample, but not the control, was made 31.2 μg/ml in sorghum tannin. At 38 min, both samples were made 3.85 mg/ml in soluble PVP.

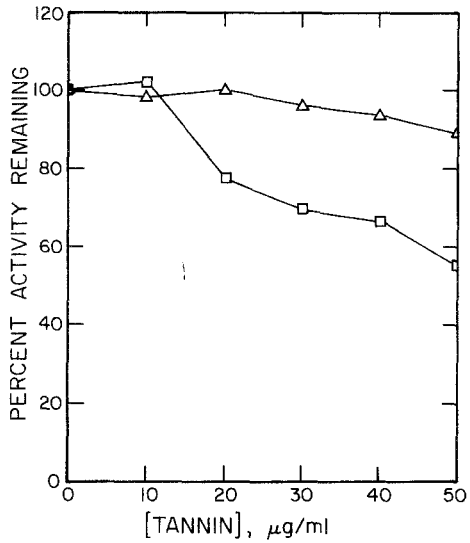

FIG. 7. Comparison of the tannin inhibition of soluble versus particulate PDase. Soluble purified PDase (squares) or the washed particulate fraction from bovine intestinal mucosa (triangles) was incubated for 30 min at 30°C in buffer A containing the indicated concentrations of sorghum tannin.

inhibited by 250 μg/ml tannin (44% inhibition) than is the purified soluble enzyme (79% inhibition).

DISCUSSION

Our results demonstrate, as expected, that in their purified soluble form these digestive enzymes are strongly inhibited by purified condensed tannins. This in vitro inhibition of digestive enzymes may not, however, account for the reported lower digestibility in ruminants (McBrayer et al., 1983; Kumar and Singh, 1984) and nonruminants (Featherston and Rogler, 1975; Martin-Tanguy et al., 1976) of diets containing tannin.

Both APase and PDase occur predominantly in a particulate, membrane-bound form is freshly prepared homogenates of intestinal mucosa; in the rat intestine, brush border membrane the binding of APase has been shown to be hydrophobic in nature (Seetharam et al., 1985). These enzymes in the washed particulate fraction proved to be less susceptible than the soluble form to inhibition by tannins at comparable tannin concentrations. This diminished susceptibility is likely due to the presence in the particulate form of other proteins

which compete for binding the tannin; the specific activity (units of activity per milligram of protein) of the particulate enzymes was approximately 0.5% that of the corresponding soluble purified enzymes. The membrane-bound enzymes may also be protected by bound phospholipids which, like Triton X-100, diminish tannin inhibition of the soluble form of the enzymes. In our early attempts to solubilize and purify PDase (Kelly et al., 1975), phospholipids were found to be strongly associated with this enzyme.

In addition to the diminished susceptibility to tannin of the enzymes in their predominant in vivo form, under physiological conditions dietary tannin is probably not accessible to digestive enzymes. Dietary proteins (Butler et al., 1984) or specialized tannin-binding proteins of the saliva (Mehansho et al., 1983) are available to form complexes with dietary tannin before it is exposed to digestive enzymes. Even if digestive enzymes are subject to inhibition by dietary tannins, the effect is likely to be reversed by detergents (Figure 5) (Oh and Hoff, 1986) such as bile acids or by various tannin-binding materials (Figure 6) encountered in the digestive tract. Our observations are consistent with those of Armstrong (1983), who found other digestive enzymes to exhibit full activity in the intestines of rats fed diets containing tannins from sorghum and other sources.

We conclude that the antinutritional effects (and ecological significance) of dietary tannin are probably not due to binding and inhibition of digestive enzymes by tannin, especially those which predominantly occur in a membrane-bound form. The relative insensitivity to tannin of membrane-bound enzymes suggests that other membrane-associated processes such as absorption may be little affected by dietary tannin. The antinutritional effects of dietary tannin that result in reduced protein digestibility may be due to formation of less-digestible complexes of dietary proteins with tannins (Butler et al., 1984). Other antinutritional effects are unrelated to digestibility (Rogler et al., 1985; Mehansho et al., 1985).

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CASTANOSPERMINE—A PLANT GROWTH REGULATOR

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Abstract—Castanospermine, 1,6,7,8-tetrahydroxyoctahydroindolizine, has been shown to be a potent dicot phytotoxin. The effective concentration to inhibit root length elongation of dicot roots by 50% is 300 ppb, while the effective concentration against monocot roots is 200 ppm, i.e., 10^3 times less effective. In contrast swainsonine, 1,2,8-trihydroxyoctahydroindolizidine, is ineffective as an inhibitor of root length elongation.

Key Words—Castanospermine, phytotoxin, glucosidase inhibitor, swainsonine.

INTRODUCTION

The indolizidine alkaloid, castanospermine (Scheme 1, **1**), 1,6,7,8-tetrahydroxyoctahydroindolizine, isolated from the seeds of the Australian legume *Castanospermum australe* (Hohenschutz et al., 1981), is a potent inhibitor of fibroblast α- and β-glucosidases and almond emulsion β-glucosidase (Saul et al., 1983). In addition, it inhibits the processing of the oligosaccharide portion of the influenza viral hemagglutinin (Pan et al., 1983) as well as the processing of plant *N*-linked oligosaccharides in soybean cells (Hori et al., 1984). Nojirimycin (**2**) (Inouye et al., 1968), an antibiotic structurally related to castanospermine, is a potent inhibitor of α- and β-glucosidases and amylases (Reese and Parrish, 1971) and of plant auxin-induced cell extension, while castanospermine itself is an effective inhibitor of cell-wall associated β-glucosidase from corn roots (Nagahashi et al., 1986). In view of these results, we felt that it would be of interest to determine whether castanospermine was phytotoxic by a test system previously found successful to delineate phytotoxicity (Stevens,

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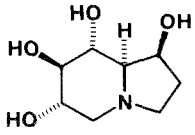

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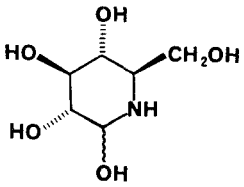

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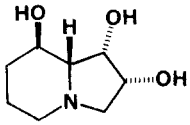

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SCHEME 1.

1986). The involvement of glucosidases in cell-wall degradation and synthesis (Inouhe, 1984; Koyama et al., 1983; Nevins, 1970, 1975; Nishitani and Masuda, 1983; Sakurai and Masuda, 1977) further suggests the potential utilization of castanospermine as a probe into the biochemical mechanism of cell growth.

METHODS AND MATERIALS

Isolation of Castanospermine. Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine), mp 218–220°C, was obtained (0.3% yield) from mature seeds of *Castanospermum australe* by water extraction and purified by ion-exchange chromatography on Dowex 50W-X8 (Hohenschutz et al., 1981).

Isolation of Swainsonine. Swainsonine (1,2,8-trihydroxyoctahydroindolizidine, mp 144–146, was obtained from *Astragalus lentiginosus* as previously described (Molyneux and James, 1982).

Bioassay. The effect of castanospermine on root length elongation of lettuce (*Lactuca sativa*, black-seeded Simpson), alfalfa (*Medicago sativa*), barnyard grass (*Echinochloa crusgalli*), and red millet (*Panicum miliaceum*) was tested at 0, 10, 20, 40, 80, 100, 200, 400, 800, and 1000 ppb (or ppm in the case of monocots) in 40 ml of 0.5% agar (Bacto-Agar, Difco Laboratories, Detroit, Michigan) in 9-cm Petri dishes. All test seeds were first germinated in Petri dishes on 0.5% agar in a growth chamber (58°F, 8-hr nights; 68°F, 16-hr days). Twelve germinated seedlings were transferred to Petri dishes containing castanospermine and incubated in the dark (21–23°C, 45–72 hr). The lengths of the roots of the 12 seedlings were measured to the nearest millimeter and the

highest and lowest values were discarded. The remaining 10 root length measurements were subjected to statistical analyses. Two replicates were run for each test species. A parallel series of experiments was run using swainsonine (**3**) rather than castanospermine (**1**).

Data Analysis. Seedling growth assay results were statistically analyzed separately at the Washington, D.C., Computing Center facilities and Statistical Analysis System (SAS Institute, Inc., Cary, North Carolina, 1982). The transformed data were subjected to Cochran's test for homogeneity of variances of all treatments and the least significant difference (LSD) test for differences between all treatment means.

RESULTS

The effect of castanospermine (**1**) on lettuce root (*Lactuca sativa*) elongation is shown in Figure 1. At 300 ppb (1.6×10^{-6} M), castanospermine inhibits root elongation by approximately 50%. Addition of 10^{-10} M indole acetic acid (IAA), a concentration determined to promote root elongation, did not nullify the inhibiting effects of castanospermine. Although many plant growth inhibitors exhibit "auxin-like" characteristics, i.e., they display a growth-promoting activity at lower concentrations, castanospermine showed no such activity down to 10^{-10} M. The effects of castanospermine on alfalfa (*Medicago sativa*) was similar to that of lettuce, i.e., a 50% inhibition of root length elongation was observed at 250 ppb. Again the addition of IAA at a concentra-

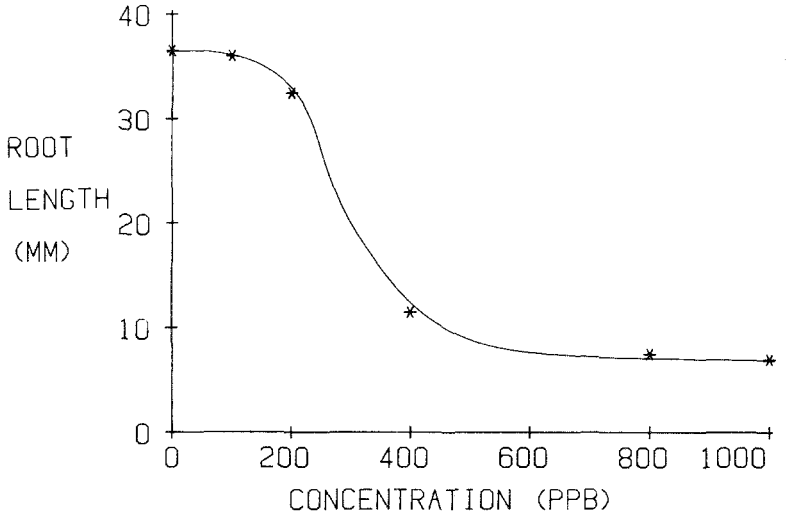

FIG. 1. Effect of castanospermine (**1**) on root growth of lettuce.

TABLE 1. EFFECT OF CASTANOSPERMINE ON ROOT LENGTH OF RED MILLET

Conc. (ppm)	Mean root length (mm)[a]	N^b
control	30.2 a	14
1	27.9 a	15
2	31.9 b	14
4	33.3 b	15
10	29.6 b	15
20	26.7 b	15
40	20.8 c	15
100	14.9 d	15
200	10.0 e	15
400	8.7 e	15
1000	7.2 f	15

[a] Means associated with a given test seedling with different letters are significantly different at the $\alpha = 0.05$ level according to Duncan's multiple-range test performed on transformed values.
[b] Number of roots measured.

tion that promotes elongation did not nullify the inhibitory effects of castanospermine.

Monocotyledons, such as red millet (*Panicum miliaceum*) and barnyard grass (*Echinochloa crusgalli*), were also inhibited by castanospermine (Table 1) but at a much higher concentration than for the dicots. For instance, red millet root elongation was inhibited 50% at 200 ppm (8×10^{-4} M) castanospermine. Barnyard grass gave similar results (not shown). Again, the addition of IAA had no apparent effect on nullifying the effects of castanospermine on these grasses.

DISCUSSION

The differential effect of castanospermine on the root length elongation (or inhibition) of dicots and monocots may well reflect the preferential inhibition of enzymes present in living plant tissue needed to degrade primary cell-wall structures. Evidence is substantial (Inouhe et al., 1984; Koyama et al., 1983; Nevins, 1970, 1975; Nishitani and Masuda, 1983; Sakurai and Masuda, 1977) that the auxin-induced elongation of cells is correlated with the degradation or depolymerization of cell-wall xyloglucans which lead to cell wall loosening and therefore cell extension growth. Studies with *Vigna angularis* (azuki bean) (Nishitani and Masuda, 1983) offer evidence that IAA-induced changes in the xyloglucans are related to the degree of cell-wall loosening rather than cell extension growth.

Substantial structural differences exist in the xyloglucans of monocots and dicots (Kato and Matsuda, 1976; Kato et al., 1981; Masuda, 1980), which may account for the differences in the inhibition of the root elongation of these plants by castanospermine. In addition, monocots have β-1,3- and 1,4-linked glucans, hemicelluloses not generally found in dicots. Furthermore, monocots have substantial amounts of glucuronoarabinoxylan (Burke et al., 1974), another hemicellulosic component. These differences could readily account for the observed differences in the effectiveness of castanospermine since it is an effective inhibitor of glucosidases (Saul et al., 1983), enzymes necessary for the degradation of dicot hemicelluloses. Since the structurally related compound, nojirimycin, is a much weaker inhibitor (by a factor of 1000) of glucanases vs. glucosidases (Reese and Parrish, 1971), enzymes necessary for the degradation of monocot hemicelluloses, castanospermine would be expected to have similar inhibitory activity against glucanases. Therefore, dicots would be expected to be inhibited more effectively than monocots by castanospermine. The specific enzyme inhibitory activity of castanospermine thus would interfere with primary cell-wall degradation, a step necessary to cell-wall elongation. Castanospermine does inhibit glucanases; however, a much higher concentration is required than for glucosidases. The effective concentration of castanospermine to inhibit root length elongation of monocots is about 900 times higher than that for dicots, approximately the difference in effectiveness of nojirimycin toward glucanases and glucosidases (Reese and Parrish, 1971).

Analogous experiments with swainsonine (**3**), a known inhibitor of α-mannosidase (Colegate et al., 1979; Kang and Elbein, 1983) but having little effect on glucosidases, showed no effect on the root length elongation or inhibition of either monocots or dicots. These data are consistent with the fact that the cell wall contains little mannose, hence α-mannosidase is not involved in the degradation of cell walls leading to cell elongation.

It has been observed that mannitol inhibits auxin-induced cell elongation by osomosis (Nevins, 1975). Since castanospermine may be considered to have many structural features analogous to carbohydrates, the possibility exists that its effect on monocots is a result of the same phenomenom as mannitol. Mannitol is effective at about 250 mM which is about 10^3 less active than castanospermine. Hence, it seems unlikely that castanospermine is decreasing the turgor of the cells by osmosis, thus interferring with growth.

A closer look at the specific enzymes inhibited by castanospermine and the relative amounts necessary to induce inhibition will lead to a better understanding of cell growth of both monocots and dicots. Castanospermine thus may be used as a probe to precisely define and understand the various polymers that make up the primary cell wall and the mechanisms of depolymerization and/or polymerization leading to cell growth.

We have observed that in order for *C. australe* seeds to germinate they

must be irrigated with water for a considerable period of time. It appears possible that the castanospermine present may therefore be self-inhibitory to germination and growth of the seeds, perhaps until rainfall is sufficient to support further growth. In addition to its effect upon root elongation, castanospermine is a powerful antifeedant to the pea aphid (Dreyer et al., 1985) and has been shown to differentially inhibit disaccharide-hydrolyzing enzymes in a broad taxonomic spectrum of insects (Campbell et al., 1987). Castanospermine may therefore serve a multiple ecological role in conferring an advantage to *C. australe* in competition with other plants and insect predators.

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HOST EGG KAIROMONES ESSENTIAL FOR EGG-LARVAL PARASITOID, *Ascogaster reticulatus* WATANABE (HYMENOPTERA: BRACONIDAE) I. Internal and External Kairomones

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Abstract—An artificial egg with a Parafilm membrane was devised for the oviposition of *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid of the smaller tea tortrix, *Adoxophyes* sp. (Lepidoptera: Tortricidae). Both external and internal kairomones were essential. The external kairomone, needed for host location and acceptance, was extracted with 70% ethanol, and the internal kairomone, needed for oviposition, was extracted with water. Female parasitoids responded to the external kairomone and oviposited through the membrane into the artificial egg when the supernatant of host egg-mass homogenate was inside, whereas they did not when water or saline solutions were inside. Thus an internal kairomone is responsible for the oviposition in the host egg. The internal kairomone apparently was not specific for the host egg mass because oviposition activity was found not only in egg, larval, and pupal stages of the host, but also in larvae of other species of Lepidoptera and Coleoptera.

Key Words—Kairomone, oviposition, parasitoid, *Ascogaster reticulatus*, Hymenoptera, Braconidae, smaller tea tortrix, *Adoxophyes* sp., Lepidoptera, Tortricidae.

INTRODUCTION

Ascogaster reticulatus, Watanabe (Hymenoptera, Braconidae) is an egg-larval endoparasitoid of the smaller tea tortrix, *Adoxophyes* sp. (Lepidoptera: Tortri-

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cidae). The female parasitoid responded to a filter paper disk treated with 70% ethanol extract of host egg masses (Kainoh et al., 1982). They performed antennal host searching and ovipositor host searching in response to the extract; however, they did not deposit eggs on the disks and left them in less than a minute. They were present on the egg mass for ca. 1 hr, during which time they laid eggs (Kainoh and Tamaki, 1982). This indicates that other stimuli inside the host egg are responsible for oviposition. Parafilm tubes containing host hemolymph were used to bioassay and identify amino acids and other chemicals as ovipositional stimulants for the ichneumonid parasitoid, *Itoplectis conquisitor* (Say) (Arthur et al., 1969, 1972; Hegdekar and Arthur, 1973). Yamaguchi (1987) used a Parafilm-based artificial egg for the bioassay of marking pheromone which is injected inside the host eggs at oviposition by female *A. reticulatus*.

In this study, an improved artificial egg was used to establish the bioassay of the internal kairomone as the oviposition stimulant. Additionally, the internal kairomone was examined for host specificity with various insects.

METHODS AND MATERIALS

Insects. All insects used were from our stock culture. Rearing was modified from Kainoh and Tamaki (1982). Females (10–20) were kept in a clear plastic container (15 cm diam., 12 cm high) with wet cotton on the bottom and honey on the wall. Virgin females were used for oviposition experiments. The conditions for the experiments were 25 ± 1°C, 60 ± 20% relative humidity, and a 16:8 light–dark photoperiod. Oviposition experiments were conducted in the latter half of a light period, when the activity of females is high (Kainoh, 1986).

Bioassay. An artificial egg was devised with a Petri dish and two sheets of Parafilm (Figure 1). The lid of a Petri dish (5.2 cm diam.) was covered with a sheet of Parafilm and either 30 μl of test solution for the internal kairomone activity or the supernatant of the host egg-mass homogenate was placed at the center. A polyethylene ring (1.9 cm OD, 1.5 cm ID, 1 mm thick) was placed on the Parafilm with the sample in the center. It was then covered with a Parafilm sheet to hold the sample. Host egg-mass extract (20 μl) containing the external kairomone (Kainoh et al., 1982) was placed on the outer Parafilm sheet and was air-dried at room temperature. Another dish (4.6 cm diam.) was used as a cover, and a virgin female parasitoid was introduced to the arena. She was allowed to remain for 30 min (Figure 2), and the number of eggs deposited into the solution was counted using a dark-field stereomicroscope.

Extraction of External Kairomone. The egg masses (15,360; ca. 68g) of

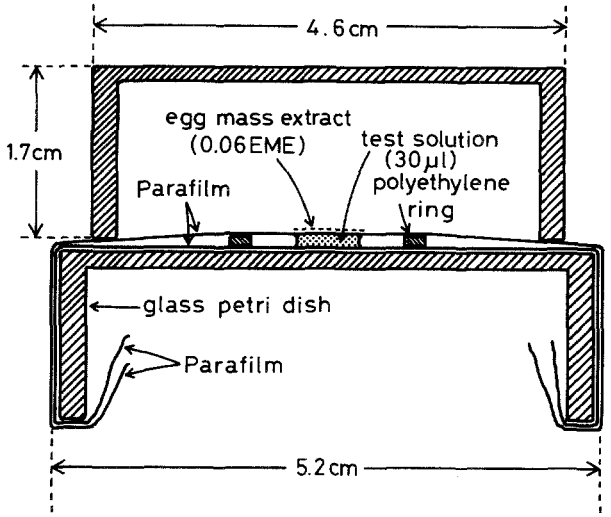

FIG. 1. An artificial egg for the bioassay of internal kairomone. A female *A. reticulatus* was introduced into the arena covered with a Petridish (4.6 cm diam.).

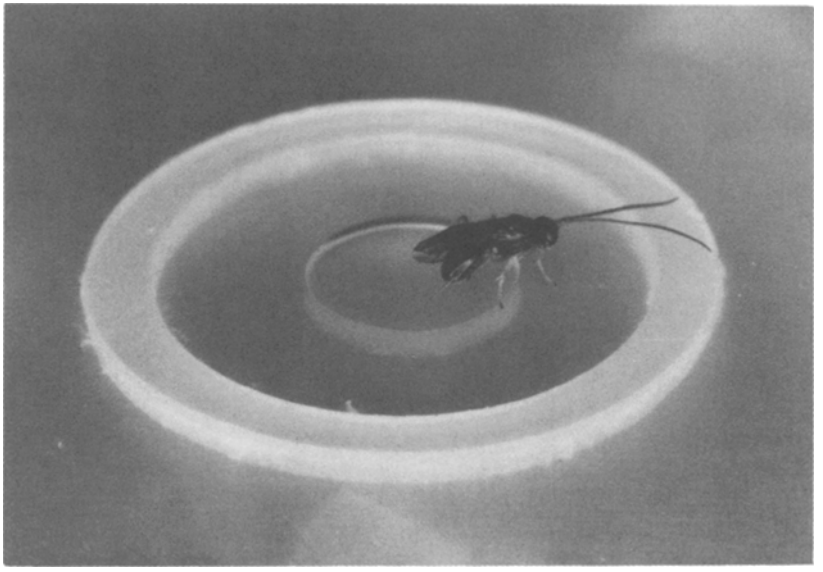

FIG. 2. *A. reticulatus* ovipositing on an artificial egg.

Adoxophyes sp. deposited on paraffin paper were collected according to Tamaki (1966) and soaked in 70% aqueous ethanol (3800 ml) for 1–9 months. The extract, filtered and concentrated to dryness under reduced pressure, was dissolved with 50 ml 70% ethanol. It was then filtered and refrigerated (5°C) until needed. The standard extract concentration for all experiments was set at 6.1×10^{-2} EME (egg mass equivalent)/20 μl, since higher concentrations with oily substances, applied to the Parafilm, affected the antennal response of female parasitoids when they palpated the treated part with their antennae.

Preparation of Physiological Salines. Table 1 shows the chemical composition of various physiological salines used for the homogenization of *Adoxophyes* sp. egg masses. Fifty egg masses on paraffin paper were homogenized with 1 ml of each saline or distilled water (Table 1) in a glass mortar with a pestle. The supernatant resulting from centrifuging the homogenate at 7000*g* for 10 min was used as the test solution. The control was either distilled water or one of the saline solutions without egg components.

Dilution of External Kairomone. Host egg-mass homogenate (50 egg masses in 1 ml distilled water) was centrifuged (7000*g*, 10 min) and 30 μl of the supernatant was used for the artificial egg as an internal kairomone. The egg-mass extract including external kairomone was serially diluted with 70% ethanol (6.1×10^{-7}–10^{-2} EME/20 μl) and 20 μl of each solution was placed on the outer Parafilm membrane. Artificial eggs treated with 70% ethanol (20 μl) were prepared as controls. A virgin female was allowed to remain with each artificial egg for 30 min after the extract or solvent was air-dried at room temperature.

Host Specificity Test. Whole bodies of various insects were homogenized in distilled water (ca. 0.4 g in 1 ml distilled water except the silkworm, *Bombyx mori* L.) with a glass mortar and a pestle, and centrifuged (7000*g*, 10 min): (1) egg masses of *Adoxophyes* sp.,; (2) final instar larvae of Adoxophyes sp.,; (3)

TABLE 1. CHEMICAL COMPOSITION OF PHYSIOLOGICAL SALINES USED FOR EGG-MASS HOMOGENATE (g/liter)

Chemicals	For *Drosophila*[a]	Neisenheimer's saline[b]	NaCl
NaCl	7.5	7.5	8.5
KCl	0.35	0.1	—
$CaCl_2$	0.21	0.2	—
$NaHCO_3$	—	0.2	—
pH	5.5	7.7	5.7

[a] Ephrussi and Beadle (1936).
[b] Rajendram and Hagen (1974).

female and male pupae of Adoxophyes sp.,; (4) final instar larvae of the small white butterfly, *Pieris rapae crucivora* Boisduval; (5) final instar larvae of *B. mori* (4 g in 10-ml distilled water); and (6) final instar larvae of the mealworm, *Tenebrio molitor* L. The artificial egg was prepared with 30 μl of each supernatant inside. A parasitoid was allowed to remain with each artificial egg for 30 min.

Probing Behavior and Oviposition. Two types of inner contents were used to prepare artificial eggs. One was the supernatant of the host egg-mass homogenate (50 egg masses in 1 ml distilled water) and the other was distilled water. A female parasitoid was allowed to oviposit in each artificial egg, and the duration and number of probes with an ovipositor were counted for 10 min. One probe was defined as one bout of continuous ovipositor host searching on the kairomone-treated part.

RESULTS

Bioassay. When a female parasitoid was introduced into the arena of an artificial egg, she groomed or walked inside the arena. If she encountered the area containing the external kairomone, she performed antennal host searching and ovipositor host searching (Kainoh et al., 1982) on the treated area. Most of the females released into the arena encountered and responded to the external kairomone within a few minutes. When the inner test solution was active, she began to oviposit, and ovipositor host searching lasted continuously throughout the bioassay. When the solution was inactive, ovipositor host searching was intermittent, and the female soon became indifferent to the treated part (cf. Table 4).

Effect of Physiological Salines. The rate of oviposition and the mean number of parasitoid eggs deposited in the supernatant of egg-mass homogenates were significantly higher when the solvent was distilled water (Table 2). Ovipositional activity was lowest in the case of the NaCl solution ($\bar{X} = 10.3$ eggs). There was no significant difference in ovipositional activity between the *Drosophila* and Neisenheimer's salines used as solvents. There was no ovipositional activity ($N = 10$) in the physiological salines devoid of homogenized egg masses.

Effect of External Kairomone. As the concentration of the egg-mass extract increased, ovipositional activity increased (Figure 3). More than 60 parasitoid eggs were deposited into artificial eggs at 6.1×10^{-4}–10^{-2} EME. No oviposition was detected in the artificial egg in the absence of the external kairomone ($N = 10$).

Host Specificity. Ovipositional activity was found not only in host insects but also in various nonhost insects (Table 3). Among the stages of *Adoxophyes*

TABLE 2. EFFECT OF PHYSIOLOGICAL SALINES ON OVIPOSITION OF *A. reticulatus* INTO SUPERNATANT OF EGG-MASS HOMOGENATE[a]

Salines or distilled water	Rate of oviposition[b]	No. of parasitoid eggs ($\bar{X} \pm$ SE)[c]
NaCl	9/25	10.3 ± 3.8 c
For *Drosophila*	12/20	22.0 ± 5.7 bc
Neisenheimer's saline	16/20	33.5 ± 5.7 b
Distilled water	35/35	56.9 ± 4.7 a

[a] Fifty egg masses (ca. 0.2 g) with each saline (1 ml) were homogenized.
[b] No. of artificial eggs in which the parasitoid oviposited/total number of artificial eggs used.
[c] Means followed by the same letters are not significantly different (5% level) as determined by Duncan's multiple-range test.

sp., there was no significant difference in ovipositional activity. Other lepidopteran larvae, *P. rapae* and *B. mori*, were also active, but they were not significantly different from host materials. Of all the materials tested in this experiment, the final instar larvae of *T. molitor* were most active ($\bar{X}$ = 105.9 eggs).

Probing Behavior and Oviposition. Total probing time on the artificial egg with an egg-mass homogenate was 2.7 times longer than that with only distilled water (Table 4). The number of probes on artificial eggs with egg-mass homog-

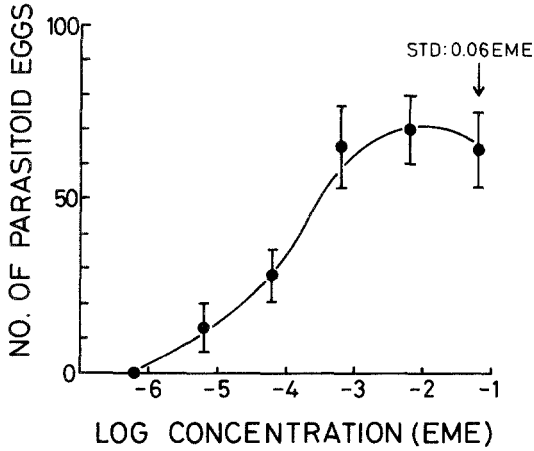

FIG. 3. Effect of concentration of the external kairomone on the ovipositional activity of internal kairomone. Vertical bars indicate the standard errors of the means ($\bar{X} \pm$ SE, N = 10).

TABLE 3. COMPARISON OF OVIPOSITIONAL ACTIVITY OF INTERNAL KAIROMONE IN HOMOGENATES OF VARIOUS STAGES OF *Adoxophyes* sp. AND OTHER NONHOST INSECTS

Materials (No.)	Rate of oviposition[a]	No. of parasitoid eggs ($\bar{X} \pm$ SE)[a]
Adoxophyes sp.		
Egg masses (100)	19/20	66.4 ± 7.2 b
Final instar larvae (9)	10/10	71.4 ± 8.4 b
Female pupae (10)	9/10	42.8 ± 10.7 b
Male pupae (18)	9/10	42.3 ± 9.2 b
Pieris rapae crucivora		
Final instar larvae (2)	7/10	61.6 ± 14.7 b
Bombyx mori		
Final instar larvae (1)	9/10	49.5 ± 9.0 b
Tenebrio molitor		
Final instar larvae (3)	10/10	105.9 ± 8.2 a

[a] See Table 2 for details.

enate was significantly less than for the control ($P < 0.001$). The mean time for one probe of the artificial eggs with the egg-mass homogenate was 16 times longer than that with distilled water. Average probing time required for the deposition of one parasitoid egg was 45.6 sec. Female parasitoids probed the artificial egg with distilled water but did not oviposit.

TABLE 4. PROBING BEHAVIOR AND NUMBER OF EGGS DEPOSITED IN ARTIFICIAL EGGS[a]

Items	Internal content of artificial egg		t test
	Supernatant of egg-mass homogenate[b]	Distilled water	
Total probing time (sec)[c]	528.5 ± 28.2	198.7 ± 30.2	$t = 8.0$ $P < 0.001$
No. of probes[c]	3.0 ± 0.8	10.0 ± 1.1	$t = 5.3$ $P < 0.001$
Mean time/probe (sec)[c]	325.7 ± 76.7	20.0 ± 2.7	$t = 4.0$ $P < 0.005$
No. of eggs deposited[c]	11.6 ± 1.8	0	

[a] A female parasitoid on the artificial egg was observed for 10 min.
[b] Fifty egg masses with 1 ml distilled water were homogenized.
[c] Values are $\bar{X} \pm$ SE ($N = 10$)

DISCUSSION

The bioassay of internal kairomone of *A. reticulatus* was established. The simple structure with two sheets of Parafilm membrane makes it easier to evaluate the activity of samples. The external kairomone, in amounts ranging from 6×10^{-4} to 6×10^{-2} EME is necessary to elicit appropriate ovipositional response.

The physiological salines used in this experiment adversely affected the ovipositional activity of the egg-mass homogenate (Table 2), which indicates that these salines are not suitable for the homogenizing solvent. Instead, distilled water is enough for the homogenization. Neisenheimer's saline was active by itself for the oviposition of *Trichogramma californicum* Nagaraja & Nagarkatti (Rajendram and Hagen, 1974) but was not active at all for the *A. reticulatus*. In *T. pretiosum* and *T. minutum* dilute solution of a mixture of KCl and $MgSO_4$ was an active stimulant for oviposition in the artificial egg (Nettles et al., 1982). This mixture was not active for *A. reticulatus* (Kainoh, unpublished).

The concentration of external kaironome critically affected the oviposition into the artificial egg (Figure 3). Although both the external and internal kairomone(s) were necessary for *A. reticulatus* to oviposit into the host, this differs from the case in *I. conquisitor* (Arthur et al., 1969) and *T. californicum* (Rajendram and Hagen, 1974). External kairomone may not be necessary, but physical properties of host may be more important for oviposition in these two species.

The activity of the internal kairomone for *A. reticulatus* was not specific for the host egg mass, since ovipositional activity was found not only in larval and pupal stages of *Adoxophyes* sp., but also in larval stages of other Lepidoptera and Coleoptera (Table 3). Also, in *I. conquisitor*, oviposition occurred in the serum of species of Lepidoptera, Hymenoptera, Coleoptera, and Trichoptera (Hegdekar and Arthur, 1973). The internal kairomone must not necessarily be specific, since the external kairomone for *A. reticulatus* was specific for some tortricids (Kawakami and Kainoh, 1986).

There was no activity as external kairomone for *A. reticulatus* in the larvae and just a little in the pupae of *Adoxophyes* sp. by the filter paper disk bioassay (Kainoh et al., 1982); however, they were both active in the Parafilm bioassay (Table 3). Furthermore, the diluted water solution of external kairomone (70% ethanol extract of host egg masses) was not active as internal kairomone by the Parafilm bioassay (Kainoh, unpublished). These results indicate that the external and internal kairomones were different chemicals.

Ovipositor host searching was important for detecting the internal contents of the host, since *A. reticulatus* females responded to the external kairomone and probed with their ovipositors even in the artificial egg with inactive internal

contents (Table 4). There may be some chemoreceptors on the tip of the ovipositor to detect the internal kairomone, as can be seen in other parasitoids (Gutierrez, 1970; Ganesalingam, 1972; Hawke et al., 1973; Greany et al., 1977) Dethier (1947) proved the response of an ovipositor to salt and acid solutions by the use of isolated abdomen of *Nemeritis canescens* (Grav.), and suggested the common mechanism of action for contact chemoreceptors. Morphological and electrophysiological studies will be necessary in *A. reticulatus* to understand the physiological aspects of oviposition.

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IDENTIFICATION OF TOBACCO HORNWORM ANTIBIOSIS FACTOR FROM CUTICULAE OF REPANDAE SECTION OF *Nicotiana* SPECIES

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Abstract—Cuticular components of the green leaves of the Repandae section of the *Nicotiana* species contain compounds that have been shown to be toxic to larvae of the tobacco hornworm larvae, *Manduca sexta*. The surface constituents of leaves of greenhouse-grown *N. repanda*, *N. stocktonii*, and *N. nesophila* were extracted with methylene chloride in order to isolate the active compounds. Solvent partitioning and gel chromatography was used to isolate a series of hydroxyacylnornicotines (HOAcylNN). The major component was identified as *N'*-(3-hydroxy-12-methyltridecanoyl)nornicotine. A number of minor 3-hydroxyacylnornicotines, with the acyl group containing C_{13}–C_{15}, were also identified. The HOAcylNN mixture in ethanol was topically applied to first-instar tobacco hornworm larvae at rates of 10, 50, 100, and 200 μg. Mortalities after 48 hr were 33, 44, 78, and 100% respectively.

Key Words—*Nicotiana repanda, Nicotiana stocktonii, Nicotiana nesophila*, nicotine alkaloids, hydroxyacylnornicotines, *Manduca sexta*, tobacco hornworm, Lepidoptera, Sphingidae, antibiosis.

INTRODUCTION

Several studies on the alkaloid contents of the species of the genus *Nicotiana*, particularly *Nicotiana tabacum*, have been performed (Jeffrey, 1959; Smith and

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Abashian, 1963). These reports generally examined the commonly occurring nicotine-related alkaloids, nicotine, nornicotine, anabasine, and anatabine, which generally comprise about 95% of the leaf alkaloids (Saitoh et al., 1985). Little work has been done, however, on the so-called minor or secondary alkaloid constituents and that which has been done was generally qualitative and lacking in biological activity data (Matsushita et al., 1979; Bolt, 1972; Miyano et al., 1979). Some investigators have found that the wild species of *Nicotiana*, in the section Repandae, contain "minor" nicotine alkaloids in the leaf exudates. These materials, in fact, impart a high level of resistance against certain phytophagous insects (Huesing and Jones, 1984). Therefore, it was the purpose of this study to identify the biologically active component(s) in the leaf exudate of the wild species, *N. nesophila*, *N. repanda*, and *N. stocktonii*, of the section Repandae.

METHODS AND MATERIALS

Extraction of Cuticular Leaf Components. Young leaves (30–60 g fresh wt.) were removed from greenhouse-grown plants at full-flower. Each leaf was dipped eight times into 170 ml of methylene chloride, in an 8-oz wide-mouth bottle, as described for the removal of cuticular components from *Nicotiana tabacum* (Severson et al., 1984). Leaf surface areas of the washed leaves were measured by a Licor model LI-3000[4] portable area meter.

Glass Capillary Gas Chromatography (GC-2). A portion of the extract, equivalent to about 20 cm^2 of surface area, was transferred to a test tube, and the solution volume was reduced under a stream of nitrogen. The residual solution was transferred to a micro-auto analyzer vial, the solvent was removed under nitrogen, and a 50-μl portion of 1:1 *N,O*-bis(trimethylsilyl)trifluoroacetamide–dimethylformamide (BSTFA/DMF, Pierce Chemical Company) was added. The vial was capped and heated for 30 min at 76°C. After cooling, a 50-μl portion of 1:1 BSA–pyridine was added and the sample was analyzed with a HP 5840 gas chromatograph, modified for GC-2 as described by Severson et al. (1982) and equipped with an HP 7672 autosampler and a 0.3 mm ID × 25 m thin-film SE-54 fused silica capillary column. The temperature program was 150–280°C at 4°/min with a 20-min hold at 280°C, hydrogen flow was 35 cm/sec, and flame ionization detection was used. Fraction isolates were analyzed in a similar manner.

Isolation of N'*-(3-Hydroxyacyl)nornicotines (HOAcylNN).* The isolation scheme for the hydroxyacylnornicotines is given in Figure 1. About 43 mg of combined cuticular extracts from *N. nesophila*, *N. repanda*, and *N. stocktonii*

[4] Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by USDA.

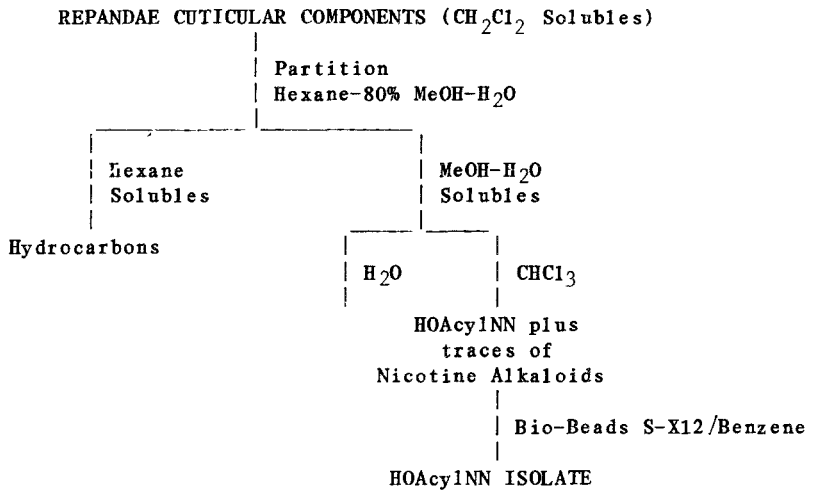

FIG. 1. Chromatographic isolation procedure for the cuticular hydroxyacylnornicotines (HOAcyNN).

were partitioned between hexane (5 ml) and 80% methanol–water (3 ml). The hexane and methanol–water fractions were then back-extracted with methanol–water (1× 2 ml) and hexane (3× 2 ml), respectively. After the addition of water (1 ml) and saturated KCl (1 ml) to the methanol–water-soluble fraction, it was extracted with chloroform (3× 2 ml), which yielded about 16 mg of the HOAcylNN mixture (95+% by GC-2). GC-2 analyses indicated the presence of the unknown compounds as well as a small amount of normal nicotine alkaloids and hydrocarbons. This chloroform extract was then separated by gel filtration chromatography on S-X12 Bio-Beads. The gel system consisted of four 1.25 × 109-cm Chromatronix LC columns, connected in series. The columns were packed with S-X12 Bio-Beads in benzene (Severson et al., 1976) and were eluted with benzene at a rate of 2 ml/min; 8-ml fractions were collected. The HOAcylNN eluted in fractions 28–33. Fractions 29–32 were combined to yield 10.3 mg of the HOAcylNN mixture (99+% by GC-2, average of two determinations).

A portion of the HOAcylNN gel isolate was subjected to preparative low-pressure, reverse-phase liquid chromatography on a 1.25 × 60-cm Chromatronix LC column packed with a 50-cm bed of Waters C_{18} reverse-phase packing. The 1:1 acetonitrile–H_2O eluant was pumped at 2 ml/min. A 94% pure (by GC) isolate of the iso-C_{14} HOAcylNN isomer was obtained.

Characterization of HOAcylNN Mixture. About 500 μg of the gel isolate were hydrolyzed in 2 N HCl for 2 hr at 100°C. After cooling, the sample was

extracted with methylene chloride. The methylene chloride extract was concentrated and treated with BSTFA/DMF. GC-2 analysis indicated the presence of fatty acids and unhydrolyzed hydroxyacylnornicotines. The aqueous portion was neutralized with KOH and was analyzed according to the GC-2 conditions for normal nicotine alkaloids (Severson et al., 1981). This analysis showed the presence of nornicotine. The HOAcylNN mixture was subjected to direct insertion probe mass spectrometry (MS) and also, after silylation, to GC-2-MS on a HP 5985B GC-MS system modified for GC-MS analysis, as described by Arrendale et al. (1984).

The iso-C_{14} isolate was subjected to NMR analysis: [^{1}H]NMR ($CDCl_3$)δ 0.93 [d, $J2$ = 6.5, 6H, $CH(C\underline{H}_3)_2$, 1.16–1.30 (m, 15H), 1.30–1.45 (m, 2H, $CHOHC\underline{H}_{2(n)}$, 1.85–2.13 (m, 4H, H-3 and 4 pyrrolidyl), 2.37–2.64 (m, 2H, $COC\underline{H}_2$), 3.64–3.85 (m, 2H, H-5 pyrrolidyl), 4.00–4.15 (m, 1H, $C\underline{H}OH$), 4.32 (s, 1H, OH), 5.02–5.29 (m, 1H, H-2 pyrrolidyl), 7.35 (m, 1H, H-5 pyridyl), 7.52 (m, 1H, H-4 pyridyl), 8.52–8.66 ppm (m, 2H, H-2 and 6 pyridyl).

Bioassay. The HOAcylNN isolate was bioassayed by topical application on first-instar *Manduca sexta* (tobacco hornworm) larvae at application rates of 10, 50, 100, and 200 μg of HOAcylNN in 1 μl of absolute ethanol. Additionally, 500 μg of nicotine (Sigma Corporation) was bioassayed in 1 μl ethanol as a control.

RESULTS

Isolation and Characterization of HOAacylNN. All three of the Repandae section species, *N. nesophila*, *N. repanda*, and *N. stocktonii*, yielded similar gas chromatographic profiles for their cuticular extracts. GC-MS analyses of this mixture indicated the presence of the typical *Nicotiana* aliphatic hydrocarbons and a homologous series of unknown components with one major homolog eluting near *n*-tricontane. These unknown components could be separated from the cuticular hydrocarbons by a simple hexane/80% methanol–water solvent partitioning step (Figure 1). GC-2 analysis of the resulting methanol–water solubles, after extraction with chloroform, showed the presence of a series of components, with one major component, and the presence of small amounts of the normal nicotine alkaloids, nicotine, nornicotine, anabasine, and anatabine. Subsequent chromatography of the above chloroform extract on Bio-Beads S-X12 gels resulted in the isolation of a series of components (Figure 2), free of the nicotine alkaloids.

Acid hydrolysis of the gel isolate yielded nornicotine and a series of hydroxy aliphatic acids, indicating that the unknown components were a series of *N'*-(hydroxyacyl)nornicotines, which are acid amides of nornicotine and β-hydroxy aliphatic acids. As shown in Table 1, probe mass spectral analyses

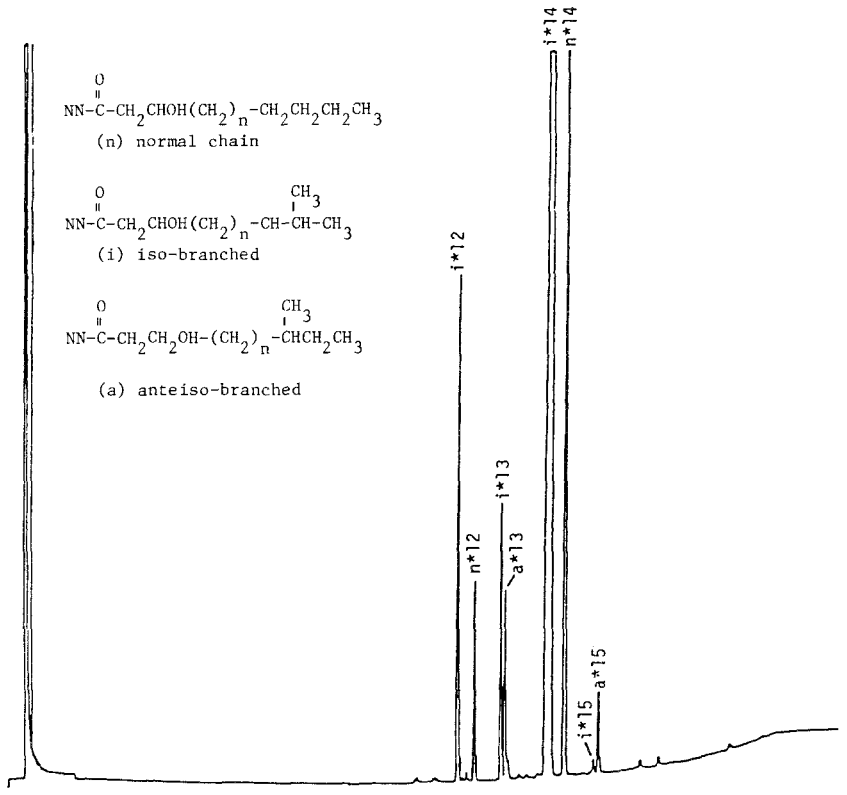

FIG. 2. The gas chromatographic profile of the hydroxyacylnornicotine isolate.

TABLE 1. HIGH MASS IONS OF HYDROXYACYLNORNICOTINES

m/e					
Neat		TMS Derivatives			Hydroxyacyl chain length
M+	M-H_2O	M+	M-CH_3	M-HOTMS	
346	328	418[b]	403	328	C_{12}
360	342	432[b]	417	342	C_{13}
374[a]	356	446[a,b]	431	356	C_{14}
388	370	460[a]	445	370	C_{15}

[a] Major component.
[b] Two or more isomers by GC-2.

of the underivatized mixture (neat) indicated a series of components differing by 14 amu, with a major homolog having a mass of 374, which readily lost water to yield an ion at 356. GC-2-MS of the TMS derivatives yielded ions with masses 72 amu higher than the neat sample. In agreement with the hydrolysis data, the mass spectral data showed the presence of HOAcylNN, with carbon chains in the acid groups ranging from C_{12} to C_{15}. The major acid was identified to be a C_{14}-hydroxy-acid, 3-hydroxy-12-methyltridecanoic acid. Previously, acylnornicotines ranging from formylnornicotine to octanoylnornicotine were isolated from *Nicotiana tabacum* leaves and cigarette smoke, and these compounds have been extensively studied by mass spectrometry (Bolt, 1972; Snook et al., 1984).

When the aliphatic chain has three or more carbons, major fragmentation ions are present at *m/e* 147, 175, 189, 190, and 203. The mass spectrum of the major C_{14} isomer, as its TMS derivative, is given in Figure 3, and its proposed fragmentation is given in Figure 4. All components, neat or as TMS derivatives, produced ions at *m/e* 147, 175, 189, and 190. In the neat sample, major fragment ions were at *m/e* 219 and 233. Due to the hydroxyl group on the β-carbon of the acid moiety, the intensive β-cleavage ion, normally observed in the spectra of compounds with an aliphatic chain (*m/e* 203), was now found 16 amu higher at *m/e* 219 (291 for TMS). Except for minor differences in the mass spectral data, due to methyl end-branching on the acid chains, and differences in molecular weight, all components yielded mass spectra with ion intensities similar to that shown in Figure 3.

It is well documented that *Nicotiana* species produce hydrocarbons and fatty acids with normal chains, isomethyl-branched chains, and anteisomethyl-branched chains (Severson et al., 1984; Snook et al., 1984). The mass spectral data from the TMS derivatives indicated that the numerous isomers observed in the isolate were also due to differences in the methyl-substitution on the acid chain. As shown in Figure 3, the iso-branched isomers lose the isopropyl group to give a weak M-43 ion and also a fairly intense 43 ion. The anteiso-branched chains yield weak M-15 and M-29 ions due to the loss of methyl and ethyl groups and the M-43 ion is essentially absent in the spectra of these compounds. Evaluation of the GC-MS data from the TMS derivatives of the isolate indicated that the C_{12} acids consisted of the isomethyl-branched acid, *N'*-(3-hydroxy-10-methylundecanoyl)nornicotine, and the normal-chained acid, from *N'*-(3-hydroxydodecanoyl)nornicotine. The C_{13} acids resulted from the iso-branched *N'*-(3-hydroxy-11-methyldodecanoyl)nornicotine, and from the anteiso-branched *N'*-(3-hydroxy-10-methyldodecanoyl)nornicotine. The C_{14} acids were derived from the iso-branched *N'*-(3-hydroxy-12-methyltridecanoyl)nornicotine and the normal *N'*-(3-hydroxytetradecanoyl)nornicotine. The C_{15} acids were iso-branched, namely *N'*-(3-hydroxy-13-methyltetradeca-

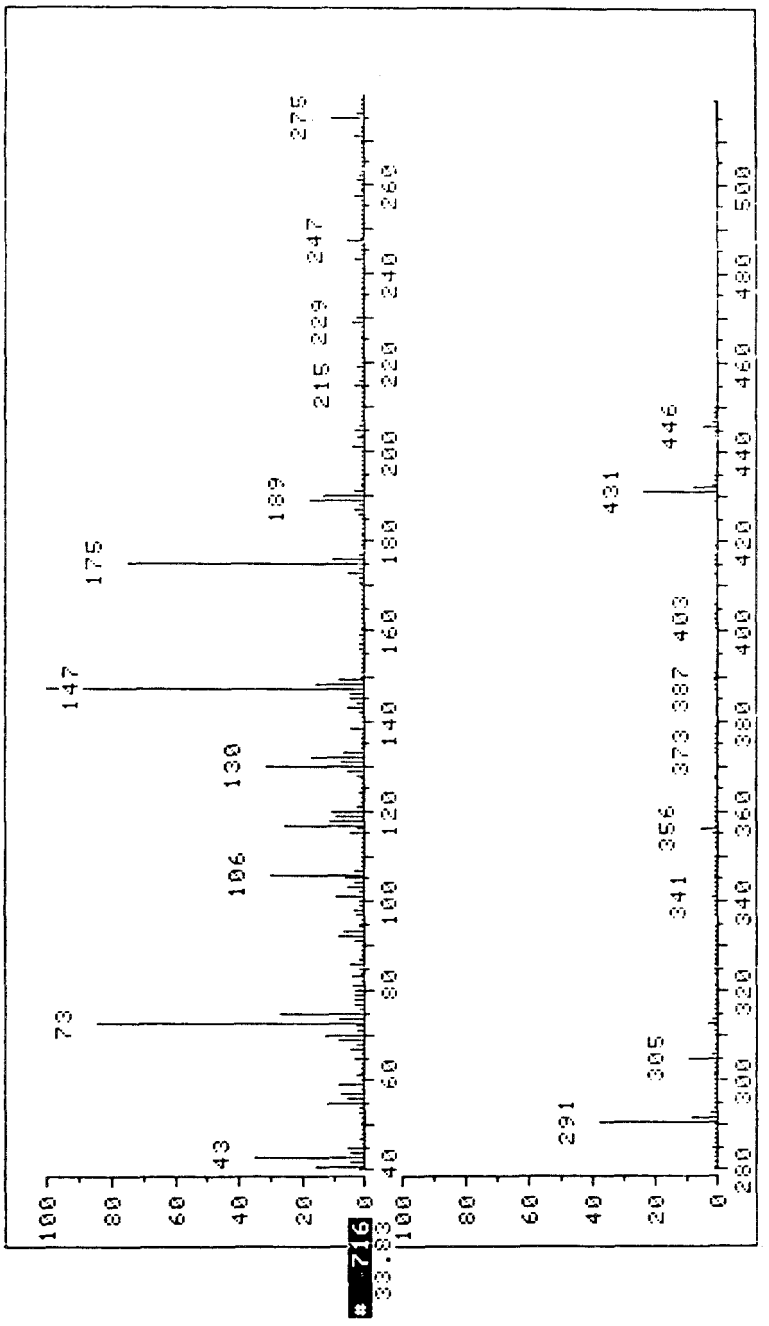

FIG. 3. The mass spectrum of the trimethylsilyl ether of *N'*-(3-hydroxy-12-methyltridecanoyl)normicotine.

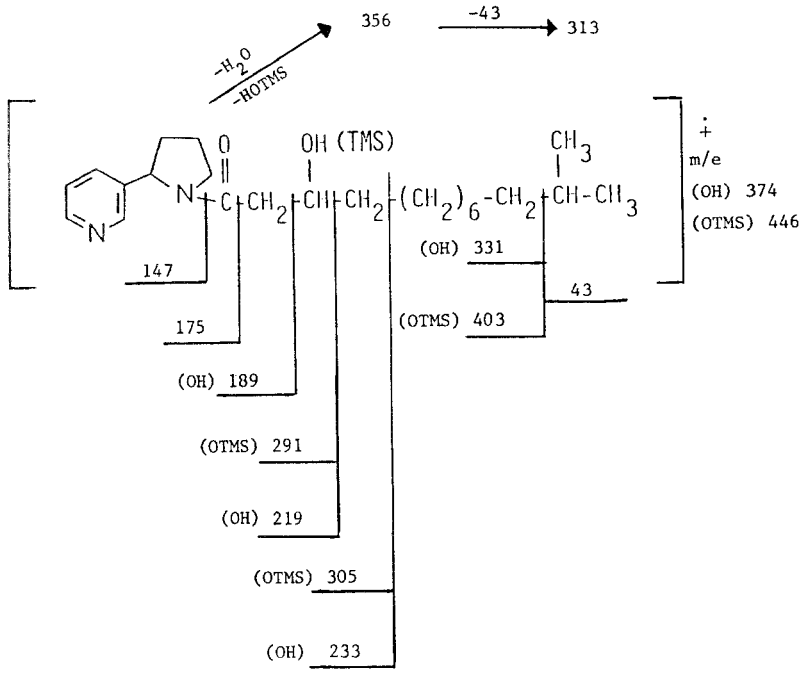

FIG. 4. Proposed MS fragmentation pattern for hydroxyacylnornicotines as shown in the C_{14} acid isomer.

noyl)nornicotine, and anteiso-branched, namely *N'*-(3-hydroxy-12-methyltetradecanoyl)nornicotine.

The NMR spectrum of *N'*-(3-hydroxy-10-methylundecanoyl)nornicotine was consistent with the mass spectral interpretation. The resonances for the pyridyl and pyrrolidyl ring hydrogens were consistent with those reported for acetylnornicotine (Warfield et al., 1972). The chemical shifts for the α-methylene and β-carbon protons were similar to those obtained from other β-hydroxy acid derivatives (Pouchert, 1983) and the presence of the isopropyl group was confirmed by a doublet at 0.93 ppm.

Bioassay. Topical applications of 200 μg of HOAcylNN produced 100% mortality to first-instar *Manduca sexta* within 48 hr (Table 2). Applications at the 50- to 100-μg level produced 80% or greater mortality within 96 hr. Even at the 10-μg level, 40% mortality was observed after 96 hr. This is in marked contrast to the 7% mortality observed when 500 μg of commercial nicotine, the most toxic nicotine alkaloid (Yamamoto et al., 1962), was applied.

TABLE 2. CUMULATIVE PERCENT MORTALITY OF *M. sexta* LARVAE[a] FOLLOWING APPLICATIONS OF HOAcylNN FROM REPANDAE

Treatment	*N*	Cumulative % Mortality[b]			
		24	48	72	96
10 μg	15	0	33	33	40
50 μg	25	8	44	64	80
100 μg	40	60	78	85	93
200 μg	30	90	100		
Control	15	0	0	0	0
EtOH (1 μg)	30	0	0	0	0
Nicotine (500 μg)[c]	15	0	7	7	7

[a] Larvae were dosed late during the first stadium.
[b] Includes moribund larvae. Moribund larvae are larvae which are extensively paralyzed but not yet dead.
[c] Commercial nicotine in 1 μl EtOH.

DISCUSSION

Several polyunsaturated straight-chain acyl derivatives of isobutylamine, tyramine, piperidine, and dihydropiperidine have been isolated from tribus Anthemideae (Bohlman et al., 1974; Burden and Crombie, 1969; LaLonde et al., 1980). Some of these amides, in particular the *N*-isobutylamides of 2,4-decadienoic acid, have demonstrated insecticidal action. However, this study has shown for the first time a class of acylated *Nicotiana* alkaloids produced by plant tissue which, unlike normal nicotine alkaloids, are biologically active against *M. sexta* larva (Bolt, 1972; Matsushita et al., 1979; Miyano et al., 1981). Our results indicate that this class of alkaloids is a major component of the leaf exudates of the Repandae species. It should be noted that these alkaloids have not been investigated from a human health perspective. The possible use of these alkaloids as antibiotic factors in conventional tobacco will make health-related studies mandatory.

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MATING STIMULANT OF THE PINE WEEVIL *Hylobius abietis* (L.)

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Abstract—Factors eliciting copulatory behavior in mature male *Hylobius abietis* were studied in the laboratory. Dead female weevils were sexually attractive, while dead mature males and pentane-extracted female weevils were not. The sexual attractiveness of dead females declined with time after death. Pentane extracts of whole female weevils or of the anterior or posterior parts of their bodies elicited a copulatory response when applied to decoys. In contrast, extracts of hindgut or frass were inactive. Juvenile males were sexually attractive for about four weeks, after which their attractiveness gradually declined. The results indicate that the mating stimulant is present on the body surface of female and juvenile male weevils, and it can be extracted with pentane.

Key Words—*Hylobius abietis*, Coleoptera, Curculionidae, sex attraction.

INTRODUCTION

The pine weevil *Hylobius abietis* (L.) is a common and widespread Palearctic forest insect. It feeds on conifer bark and causes damage in reforestation areas by killing seedlings. In regions where clear-cutting and reforestation by planting are practiced, *H. abietis* is one of the most harmful insects.

We have been studying the ecology, dispersal, and orientation of the species, with emphasis on host orientation and intraspecific communication. Pine weevils meet and copulate often, and oviposition extends over a long period (May to August, e.g., Lekander et al., 1985). Weevils are strongly attracted by host odors and find food as well as breeding substrate using olfactory ori-

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entation (e.g., Eidmann, 1974; Nordlander et al., 1986). They may aggregate at the host, but our studies suggest that *H. abietis* adults do not use pheromones to recognize each other over distances greater than a few centimeters (Tilles et al., 1986). On the other hand, at short distances sex-specific recognition does occur.

Selander and Jansson (1977) and Selander (1978) have described courtship and mating behavior in *H. abietis*. Their observations indicated that copulatory attempts by males were induced by a female-produced odor. After freeze-killing weevils, Selander (1978) reported that males attempted to copulate with dead juvenile and mature females but not with dead mature males. The dead weevils elicited this behavior for several hours following their return to room temperature. He also reported that juvenile males were sexually attractive to mature males. According to Selander (1978) the sex pheromone was present in female frass; however, his data do not support this conclusion.

Our goals have been to characterize more thoroughly the mating stimulant and determine some of its properties. In this paper we report on efforts to determine what parts of the body contain the mating stimulant and on the changes over time occurring in the sexual attractiveness of juvenile males.

METHODS AND MATERIALS

General. Weevils were collected during their flight period at a sawmill about 40 km northwest of Uppsala, Sweden. They were stored in total darkness at 10°C, and food was renewed at regular intervals. Weevils were sexed, and the sexes were transferred separately to a 20°C climate chamber with a 20:4 light–dark photoperiod ca. 7–10 days prior to their use. Males were used for six to eight weeks as responders, after which they were replaced with fresh males. Unless otherwise specified, all weevils used in the tests were sexually mature.

All bioassays were conducted under red light at 20°C and ca. 30% relative humidity. In the various tests, counts were made of male copulatory attempts (CA) with either living weevils, freeze-killed weevils, or decoys, i.e., dead weevils extracted in solvent and allowed to dry for 12–24 hr. A copulatory attempt was defined as occurring when a male mounted a living weevil or decoy, using its forelegs to stroke the latter's head while simultaneously attempting to initiate genital contact. Dead weevils and decoys used in the tests were pinned through their prothorax and placed in a 24-cm-diam. circular plastic arena with walls 6 cm high; the floor was covered with a 50-mm layer of moistened sand. When placed in the arena, male weevils tended to walk in circles around its periphery. To exploit this behavior, we placed the treated decoys around the circumference of the arena, 1 cm from the wall. When multiple treatments were

used, they were alternately placed around the arena to ensure that responding weevils had equal chances of contacting each treatment.

Fifty or 100 male weevils were placed in the arena and allowed about 5 min to acclimatize to the experimental setting before initiating a test. Each male attempting to copulate with a pinned specimen was immediately removed from the arena. Another male was then placed in the center of the arena so that the same number of males was present at all times during the tests.

A different method (see below) was used to test the attractiveness of living weevils.

Response to Dead Weevils. The attractiveness of dead female and male weevils was tested according to the procedures described above.

To determine how long dead females remain sexually attractive to males, 10 freeze-killed female weevils were tested in the arena at intervals for up to 97 hr after removal from the freezer.

Effect of Solvent Extraction on Female Attractiveness. Freeze-killed females were screen-tested in the arena with males for 30 min, and, based on these tests, five pairs of female weevils with similar sexual attractiveness were chosen. One weevil in each pair was then extracted in pentane for 60 min while the other was left untreated. The sexual attractiveness of the extracted and unextracted females was then tested.

Response to Extracts of Female and Male Weevils. Extracts of male and female weevils were prepared by extracting freeze-killed weevils in pentane. Approximately 5 ml solvent was used for every 100 weevils extracted, and the extracts were concentrated to about a fifth of the original volume in a water bath at 30°C. A test was then performed to determine whether the male and female extracts would stimulate male copulatory behavior. A 10-μl amount, equivalent to the extract from one weevil, of each pentane extract and a 10-μl pentane control were applied to each of 10 decoys and tested.

Localization of Mating Stimulant. Female weevils were divided into anterior (head and prothorax) and posterior sections, and the hindgut was removed. Each of these parts was then extracted separately. Extract preparation and bioassay methods were similar to those described above. A 10-μl amount, equivalent to the extract from one weevil, of each pentane extract and a 10-μl pentane control were applied to each of 10 decoys.

Freeze-killed females were screen-tested in an arena with males for 30 min, and, based on these tests, five pairs of female weevils with similar sexual attractiveness were chosen. The anterior section of each weevil was then separated from the rest of the body and half of them (one from each pair of weevils) were extracted in pentane for 30 min. These sections were then reconnected to the remainder of the body. The other weevils were also reassembled; however, their anterior sections had not been extracted. The sexual attractiveness of these five pairs of weevils was then estimated as described above. Parallel tests were

also conducted in which the posterior sections were extracted instead of the anterior ones. The tests were repeated twice. The results of the two repetitions were combined for statistical analysis using the Wilcoxon signed rank test.

To test frass, female weevils were placed in 16-cm-diam. glass Petri dishes and provided with small pine branches on which to feed. Their frass was collected daily and stored at −20°C. The frass (10.5 g) was then extracted in 23.5 ml pentane for 30 min. A 2-ml amount of this extract was then concentrated to 130 μl in a water bath at 35°C. Simultaneous comparisons were then made between five decoys treated with 20 μl frass extract, 20 μl concentrated frass extract, or 20 μl pentane. In addition, five unextracted females were included as controls in the test.

Attractiveness of Juvenile Males and Mature Females Compared over Time. The sexual attractiveness of each of 10 juvenile male and 10 mature female weevils (used as controls) was estimated periodically. The juvenile males were removed from their pupal chambers in the field and kept with fresh food at 5°C in total darkness for 36 days until the experiment was begun.

Throughout the experimental period, both juvenile males and adult females were stored at 20°C under a 20:4 light–dark cycle. Each weevil was uniquely color-marked on its elytra. The attractiveness of the weevils was measured during 50-min tests, three times weekly for about two months. During these tests, one juvenile male or mature female was placed in each of 10 open Petri dishes containing five mature males. The sides of the dishes were coated with polytetrafluoroethylene to prevent weevils from escaping, and a thin layer of moist sand was provided for the weevils to walk upon. A notation was made each time a mature male attempted to copulate with a marked animal. The responding male was then removed and replaced with another mature male held in reserve. Every 5 min the marked test animals were systematically moved to adjoining dishes with five males each. Thus, each test animal met at least 50 males per 50-min test.

RESULTS

The main results of the experiment are summarized in Table 1. Dead mature male weevils did not elicit copulatory behavior in the male responders, while all dead females stimulated males to mate (Table 1). The sexual attractiveness of dead females decreased slowly, reaching very low levels three days after death (Table 2).

Females extracted thoroughly in pentane no longer stimulated mating in male responders. When such extracted females (decoys) were treated with concentrated pentane extract of mature females, they became sexually attractive to males. The extract of mature males was inactive.

TABLE 1. SEXUAL ATTRACTIVENESS OF FREEZE-KILLED *Hylobius abietis*, MEASURED AS COPULATORY ATTEMPTS (CA) IN ARENA EXPERIMENTS

Exp.	No. weevils or decoys in exp.	No. male responders	Duration of exp. (min)	Weevils or decoys in treatment			No. weevils or decoys eliciting CA	No. CA	
				No.	Sex	Treated		Total	Range
A	20	50	60	10	♀	fresh	10	60	1–12
				10	♂	fresh	0	0	0
B	10	100	60	5	♀	fresh	5	35	4–10
				5	♀	pentane extracted	1	1	0–1
C	30	50	60	10	♀	extracted + extract of ♀ ♀	8	32	0–8
				10	♀	extracted + extract of ♂ ♂	1	1	0–1
				10	♀	extracted + pentane	0	0	0
D–F,total of 3 exp.				10^a	♀	extracted + extract of anterior ♀ ♀	8	20	0–6
	12–16	100	30	10^a	♀	extracted + extract of posterior ♀ ♀	10	54	2–11
				10^a	♀	extracted + extract of hindgut	0	0	0
				10^a	♀	extracted + pentane	0	0	0
G, H, total of 2 exp.				10^b	♀	anterior body extracted	9	27	0–7
	10	100	60	10^b	♀	fresh	10	77	4–17
I–K, total of 2 exp.				10^b	♀	posterior body extracted	6	11	0–3
	10	100	60	10^b	♀	fresh	10	50	2–9
L	20	50	60	5	♀	extracted + extract of frass	0	0	0
				5	♀	extracted + conc. extract of frass	0	0	0
				5	♀	extracted + pentane	0	0	0
				5	♀	fresh	5	23	1–10

[a] $N = 3$, 3, and 4, in 3 experiments.
[b] $N = 5$ and 5, in 2 experiments.

TABLE 2. SEXUAL ATTRACTIVENESS OF 10 FREEZE-KILLED FEMALE *Hylobius abietis* AT VARIOUS INTERVALS AFTER REMOVAL FROM FREEZER[a]

Time after removal from freezer (hr)	No. females eliciting CA	No. of CA	
		Total	Range
0	9	39	0–8
1	9	34	0–7
2	9	41	0–17
4	9	22	0–4
8	8	20	0–5
16	9	24	0–4
24	6	11	0–3
48	8	13	0–3
73	2	3	0–2
97	2	2	0–1

[a]The duration of each test was 30 min; 100 male responders. Each of the 10 females elicited copulatory attempts (CA) in one or more tests.

Extraction of either the anterior or the posterior parts of female weevils decreased sexual attractiveness to levels below those of unextracted females. The decrease was statistically significant ($P < 0.05$ for extraction of the anterior part, $P < 0.01$ for extraction of the posterior part). The decrease of attractiveness appeared to be more pronounced in the females with an extracted posterior body.

Pentane extracts of the anterior or posterior part of the body of mature females applied to decoys also elicited copulatory responses, while extracts of hindgut and pentane controls were inactive. The data suggest that the extract of the posterior body was more stimulatory than that of the anterior body.

Extracted females treated with frass extracts or pentane did not elicit mating behavior, while unextracted dead females, serving as controls, elicited numerous copulatory responses.

In the experiment monitoring the sexual attractiveness of juvenile males during their first months of adult life (Figure 1), the male weevils were only slightly attractive on day 1. They subsequently increased in attractiveness to levels comparable to those of the female control weevils and maintained these levels for about four weeks. During the following two weeks, they declined steadily in attractiveness, and by week seven they were unattractive. The female control weevils remained sexually attractive during the entire experimental period. Attractiveness varied between individual males as well as between indi-

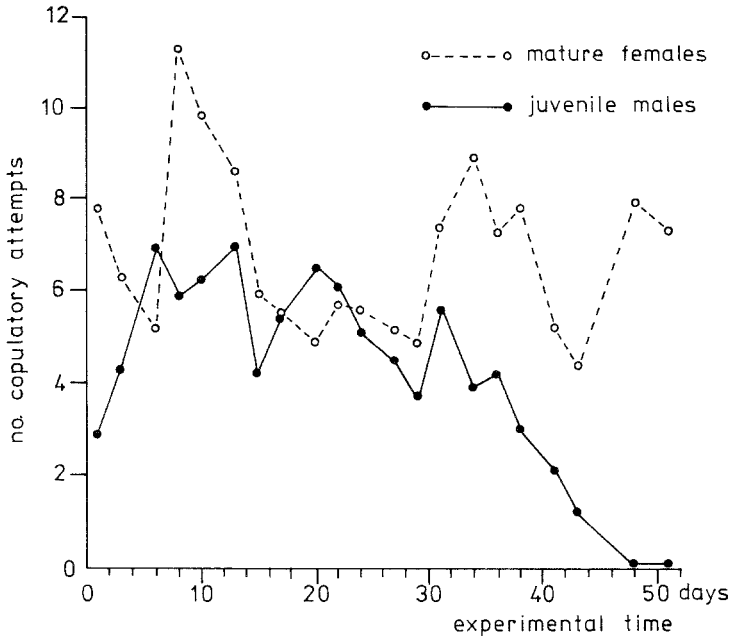

FIG. 1. Sexual attractiveness of 10 juvenile male and 10 mature female pine weevils during a long-term experiment. Attractiveness expressed as number of copulatory attempts per individual during a 50-min test period.

vidual females. The attractiveness of each individual also varied from test to test.

DISCUSSION

Our study substantiates Selander's (1978) observations that *H. abietis* females produce a substance releasing copulatory behavior in male pine weevils. However, we found frass and hindgut extracts lacking in activity. Instead, whole body extracts of female weevils rinsed in solvent elicited strong copulatory responses. These results indicate that the mating stimulant is present on the surface of the weevils.

Kalo and Nederström (1986) reported the existence of sex-specific compounds in the ovaries of female *H. abietis*. However, they were unable to show biological activity in these compounds, nor did we detect activity in hindgut extracts. It is also probable that ovarian tissues contain a variety of compounds—of no special biological interest—not found in other tissues.

The indications that the posterior part of the female body is more attractive than the anterior part may be explained by the fact that the former has a larger surface area than the latter.

Copulation release pheromones have been recovered from whole-body extracts of other Coleoptera, including *Leptinotarsa decemlineata* (Say) (Chrysomelidae) (Levinson et al., 1979), *Aleochara curtula* Goeze (Staphylinidae) (Peschke, 1978), *Cylas formicarius elegantulus* (Summers) (Curculionidae) (Coffelt et al., 1978), and *Callobruchus chinensis* L. (Bruchidae) (Tanaka et al., 1981). Peschke and Metzler (1987) reported that pheromone components of female *A. curtula* include C_{21} and C_{23} alkenes.

Selander and Jansson (1977) observed differences in the ability of female pine weevils to release copulatory behavior in males. We also have shown that the mating response of male weevils to an identical group of females fluctuated irregularly over a period of several weeks. Moreover, the number of mating attempts elicited by each female varied greatly between test periods. We interpret these observations as fluctuations in female sexual attractiveness. These fluctuations could indicate variability in the production or release of mating stimulant, and they could also, to some degree, be due to differences in male responsiveness.

As reported by Selander (1978), and as confirmed in our study, juvenile male weevils are sexually attractive to mature males. Peschke (1985, 1986) reported that juvenile males of *A. curtula* produce the female sex pheromone, which helps juveniles avoid aggressive encounters with adult males. We have not observed aggressive behavior in *H. abietis* males in the laboratory or in the field, nor have we come up with an explanation as to why production of a mating stimulant should be advantageous for juvenile males.

The decline that we observed in the sexual attractiveness of juvenile males after about four weeks could have been due to a cessation of pheromone production or to the production of a male recognition substance. We are addressing these alternatives in our present work.

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CAMPHOR FROM JUVENILE WHITE SPRUCE AS AN ANTIFEEDANT FOR SNOWSHOE HARES

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Abstract—One theory in plant antiherbivore defense predicts that slow growing late succession plants like white spruce (*Picea glauca*) make large investments in antiherbivore defenses. Juvenile stages of white spruce in the Yukon, Canada, are rarely browsed by snowshoe hares (*Lepus americanus*), an abundant herbivore, but mature spruce is a highly preferred food. The hexane-soluble fractions of the methanol extracts from juvenile and mature white spruce contain camphor and bornyl acetate. There is four times as much camphor in juvenile spruce as in mature spruce from GC analysis. Plant extracts were added to rabbit chow. Pairs of extracts were offered to hares in choice tests. These tests demonstrated that camphor in the juvenile spruce extracts deterred feeding. Bornyl acetate did not have a clear antifeeding effect.

Key Words—Camphor, bornyl acetate, antifeedant, *Picea glauca*, snowshoe hare, *Lepus americanus*, herbivore, plant defense.

INTRODUCTION

The boreal forest of North America has a generalist mammal herbivore, the snowshoe hare (*Lepus americanus*) which, when in high numbers, imposes a severe browsing impact on trees and shrubs. One theory on plant antiherbivore defense, synthesized by Coley et al. (1985) from earlier statements of Bryant and Kuropat (1980) and Bryant et al. (1983a), suggests that plants which are slow growing make large investments of their resources in antiherbivore de-

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fenses compared to the investments made by fast-growing plants. There has been much work on the antifeeding deterrents to snowshoe hares found in the fast-growing earlier succession shrubs such as birches, willows, and alders (Bryant, 1981; Bryant et al., 1983b, 1985; Reichardt et al., 1984, 1987; Palo, 1984; Palo et al., 1985; Tahvanainen et al., 1985; Clausen et al., 1986). However, there has been little attention paid to the slow-growing later successional plants such as white spruce (*Picea glauca*). In this species, Coley's hypothesis would predict high amounts of antifeedant chemicals because it is slow growing, it retains its needles, and it cannot resprout from the roots if it is ring-barked, as can the fast-growing angiosperms. Another theory (Bryant and Kuropat, 1980; Bryant et al., 1983a) proposes that the juvenile growth phase of boreal trees and shrubs is more heavily defended than the adult phase. This has been demonstrated by these authors for birches, willows, and shrubs but not yet for conifers.

In the Kluane region of southwest Yukon, white spruce is the dominant tree. It is obvious even to the casual observer that juvenile white spruce in that region are hardly touched by snowshoe hares although the trees are easily accessible. (For operational reasons we define "juvenile" here as plants below an arbitrary height of 2 m. Whether size or age are the relevant variables is the subject of other work. Similarly "mature" trees are those over 5 m.) Occasionally one sees a single sapling that has been almost completely browsed, but most juveniles are rarely browsed. On the other hand, mature trees that have fallen during windstorms in winter are clearly favorite food sources, with hares lining up along the fallen tree to feed side by side. Normally these trees, when upright, are out of reach of hares.

Feeding experiments at Kluane in winter (Sinclair and Smith, 1984) showed that mature white spruce was preferred over mature twigs of birches and willows when they were presented together. However, in similar experiments using mature birches and willows but with juvenile spruce, the latter was the least preferred plant type. In experiments where side branches of mature spruce and tops of juvenile spruce were offered together, there was a 10-fold greater amount of mature spruce eaten compared to the juvenile spruce. Despite these clear preference differences shown by hares for the two stages of white spruce, crude chemical analysis showed little difference: crude protein, total phenols, and protein-complexing phenols showed no significant difference, while total resins (ether extractable product) were higher in juvenile spruce but only by a small amount (Sinclair and Smith, 1984). It appeared, therefore, that a specific compound rather than the total resin or phenol content was affecting hare feeding.

This paper describes the analysis of organic extracts from juvenile and mature white spruce. We show that a major constituent of juvenile spruce is camphor, that this is in much lower quantity in mature spruce, and that in feeding trials it is the camphor that deters feeding by snowshoe hares.

METHODS AND MATERIALS

Sample Collection. The distal 30 cm of mature spruce side branches or the tops and side branches of juvenile spruce were clipped while frozen during winter at Kluane. The samples were shipped by air to the University of British Columbia and kept frozen until analyzed.

Extraction and Purification of Secondary Metabolites. The foliage of juvenile white spruce (660 g wet weight) was soaked in methanol (3 liters) at room temperature. After 24 hr, the methanol was decanted and concentrated in vacuo to near dryness. The resulting suspension was partitioned between water (100 ml) and hexane (3 × 300 ml) in a separatory funnel. The hexane layers were combined, dried over magnesium sulfate, and evaporated in vacuo to give a gum (3.0 g) referred to as the "juvenile hexane extract." Foliage of mature white spruce (660 g wet weight) was treated in an identical manner to generate the "mature hexane extract" (3.2 g).

[^{1}H]NMR spectra of the juvenile hexane extract (Figure 1) and the mature hexane extract (Figure 2) were run in $CDCl_3$, with TMS as an internal standard, on a Varian XL300 spectrometer.

Fractionation of the juvenile hexane extract was achieved by flash chromatography (Still et al., 1978) on silica gel 60 (230–400 mesh) using a step-gradient elution scheme involving mixtures of petroleum ether (40–60°C) and diethyl ether (200 ml each of 0, 10, 20, 50, 70 and 100% of petroleum ether in diethyl ether; 50-ml fractions were collected). The separation was monitored via silica gel thin-layer chromatography (TLC) of the collected fractions.

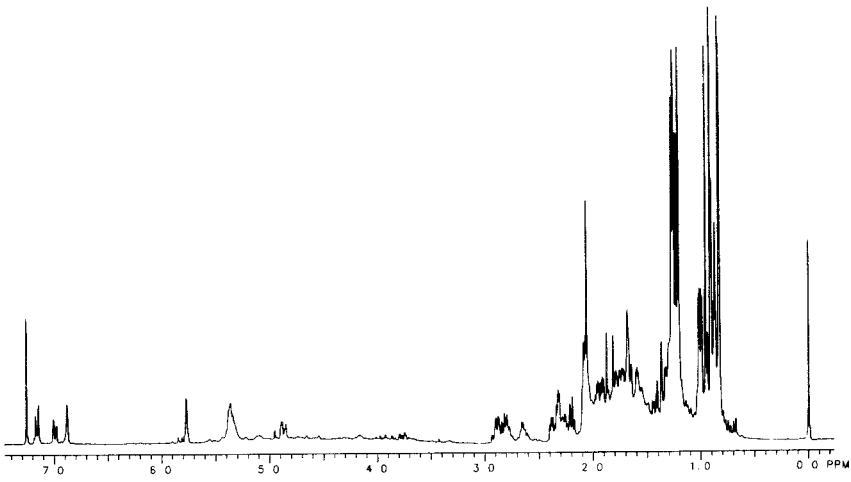

FIG. 1. [^{1}H]NMR (300 MHz) of juvenile hexane extract.

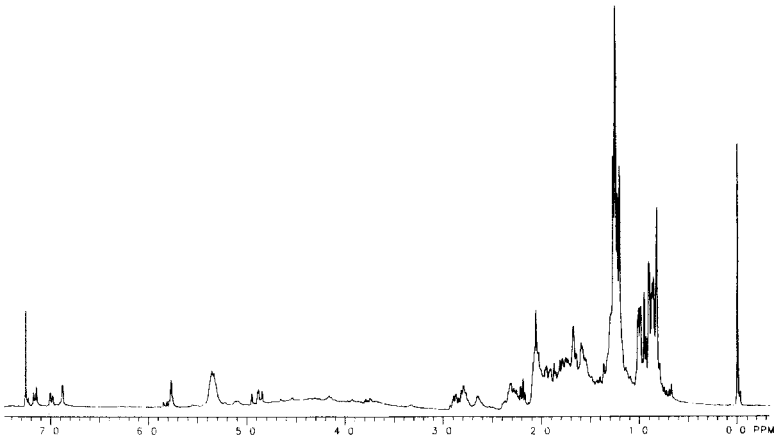

FIG. 2. [^{1}H]NMR (300 MHz) of mature hexane extract.

Preparation of Bioassay Samples. A "juvenile hexane extract with camphor" sample was prepared first by extracting the foliage of juvenile white spruce (660 g wet weight) as described above to get a juvenile hexane extract. This extract was then fractionated via flash chromatography as described above. All the fractions devoid of camphor by TLC analysis were recombined and concentrated in vacuo to give the "juvenile hexane extract without camphor" sample. A second batch of juvenile white spruce (660 g wet weight) was extracted and chromatographed in an identical fashion. All of the fractions from the second batch, including the ones containing camphor, were combined and concentrated in vacuo to give a "juvenile hexane extract with camphor control" sample. Similar procedures were used to prepare "juvenile hexane extract without bornyl acetate" and "juvenile hexane extract with bornyl acetate control" samples.

Samples for bioassay were dissolved in diethyl ether (300 ml) in a 1-liter round bottom flask. Rabbit chow (330 g dry weight) was added to the ether solution, and the solvent was removed in vacuo on a Buchi rotary evaporator to give "treated" chow. White spruce is 50% water, so the amount of rabbit chow used was equivalent to the dry weight of spruce from which the extracts were obtained.

Quantitative Analysis of Camphor and Bornyl Acetate. Quantitative analysis of camphor and bornyl acetate was carried out via analytical gas–liquid chromatography on a Hewlett Packard 5830A gas chromatograph using a 2 m $\times$ 2 mm (ID) stainless-steel column packed with 3% Carbowax 20 M on Chromosorb W. Helium, delivered at a flow rate of 10 ml/min, was used as a carrier

gas. Individual runs were temperature programmed from 100–180°C at a rate of 5°C/min. A known quantity of borneol was added to the methanol extracts of white spruce as an internal standard. The methanol extracts were then treated as described above to get juvenile and mature hexane extracts for GC analysis. Standard calibration curves were prepared for camphor, bornyl acetate, and borneol using weighed samples of authentic material. A Hewlett Packard 18850a computing integrator was used to quantify peak areas. The identity of peaks in the juvenile hexane extract and in the mature hexane extract was confirmed by GC-MS on a Kratos MS80 instrument.

Bioassay of White Spruce Extracts. The general procedure used approximately 80-g amounts of rabbit chow treated with extract. These were offered in bowls to the hares in separate cages overnight. The extracts were offered in pairs so that the animal could make a choice in its feeding. Weights of each chow type were recorded before and after feeding. This procedure was repeated for a minimum of four nights with the location of the two bowls in each cage switched each night to avoid location biases by the hares.

All statistical tests on the amount eaten used the nonparametric Wilcoxon matched-pairs signed ranks test (Siegel, 1956).

RESULTS

Isolation of Secondary Metabolites. The [^{1}H]NMR spectra of juvenile hexane extract (Figure 1) and mature hexane extract (Figure 2) showed significant differences. In particular, there were a series of intense high field methyl resonances present in the juvenile extract spectrum that were not present in the mature extract spectrum. We suspected that the secondary metabolite(s) responsible for the antifeedant activity of the juvenile hexane extract were also responsible for the additional high-field methyl resonances in the [^{1}H]NMR spectrum of the juvenile hexane extract.

The flash chromatography carried out on the juvenile hexane extract gave, in the order of elution, pure bornyl acetate, pure camphor, and a complex mixture of diterpenoic acids, that were only partially characterized. Camphor and bornyl acetate were shown to be identical to authentic samples by [^{1}H]NMR, [^{13}C]NMR, MS, TLC, and GC comparisons. It was possible to attribute all the additional high-field methyl resonances in the [^{1}H]NMR spectrum of juvenile hexane extract (Figure 1) to protons in either camphor or bornyl acetate.

Quantitative Determinations of Camphor and Bornyl Acetate. GC analysis showed that in juvenile white spruce the concentrations of camphor and bornyl acetate were 2.52 and 1.26 g/kg wet weight of foliage, respectively. In mature white spruce, the concentrations of camphor and bornyl acetate were 0.65 and

0.42 g/kg wet weight of foliage, respectively. Thus, we found that there was roughly four times as much camphor in juvenile white spruce foliage as there was in mature foliage.

Bioassay of Secondary Metabolites: Experiment 1. Camphor was added to chow at a concentration of 0.5 g/330 g of chow. This was approximately one fifth the concentration in an equivalent dry weight of juvenile white spruce. The control was ether-treated chow. The results for the amounts eaten are given in Table 1. The animals significantly avoided the camphor-treated chow ($P < 0.001$), and they ate approximately five times as much of the control chow compared with the camphor chow.

Experiment 2. The mature hexane extract was added to chow at the same concentration as that of the camphor chow (0.5 g extract/330 g chow). This concentration of mature hexane extract represented a sixth of that found in the plant. The dilute mature hexane extract was offered to hares with the camphor chow (Table 1). Figure 3A shows that the hares preferred the mature hexane

TABLE 1. AMOUNTS OF RABBIT CHOW TREATED WITH WHITE SPRUCE EXTRACTS EATEN BY SNOWSHOE HARES IN CHOICE TESTS[a]

Extract in chow	Amount eaten	
	Mean weight ± SE/day (g)	% of total
Experiment 1		
Ether solvent	51.5 ± 5.7	84.3
Camphor	9.6 ± 3.5	15.7
Experiment 2		
Dilute mature hexane extract	64.1 ± 4.3	86.3
Camphor	10.2 ± 3.2	13.7
Experiment 3		
Normal mature hexane extract	33.4 ± 5.6	46.2
Camphor	39.0 ± 5.7	53.8
Experiment 4		
Normal mature hexane extract	44.2 ± 5.4	82.8
Normal juvenile hexane extract	9.2 ± 3.8	17.2
Experiment 5		
Juvenile hexane extract without camphor	43.8 ± 4.9	75.1
Juvenile hexane extract with camphor control	14.5 ± 3.5	24.9
Experiment 6		
Juvenile hexane extract without bornyl acetate	35.1 ± 4.7	59.8
Juvenile hexane extract with bornyl acetate control	23.6 ± 3.1	40.2

[a] Four replicate runs were made for each experiment.

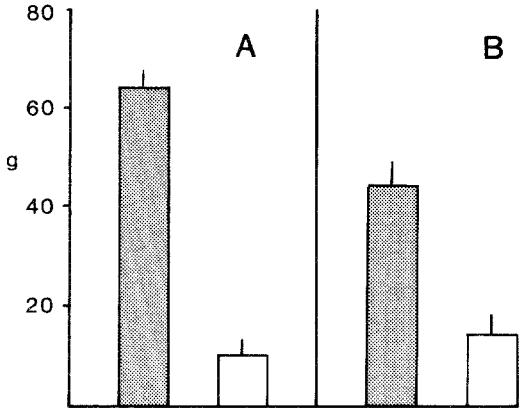

FIG. 3. The mean amounts of rabbit chow eaten per day by snowshoe hares. (A) Chow treated with mature hexane extract (shaded) and camphor (open) both at 0.5 extract/330 g chow. (B) Chow treated with juvenile hexane extract without camphor (shaded) and with camphor (open). Verticle lines are one standard error.

extract over the camphor chow ($P < 0.001$), eating approximately six times as much of the former.

Experiment 3. The mature hexane extract was added to chow at the concentrations found in the natural plant (3.2 g extract/330 g chow). This was offered to hares with the camphor chow. Table 1 shows that the hares did not show a preference between the two, eating similar amounts of both. The mature spruce extract contained 0.43 g camphor in 330 g of chow (from the quantitative analysis above), slightly less than that in the camphor-treated chow. The result of no significant choice is, therefore, to be expected since the camphor concentrations in the two choices were nearly the same.

Experiment 4. In experiment 3 the concentrations of the mature hexane extract and the camphor were markedly different, and the former was noticeably stronger smelling. To balance the secondary metabolites on offer, juvenile hexane extract was offered to hares at the same concentration as that of the mature hexane extract (3.2 g extract/330 g chow). The two hexane extracts were offered together. Table 1 shows that in this situation preference of the mature hexane extract was reestablished ($P < 0.005$).

Experiment 5. To test whether camphor was the major antifeedant in juvenile spruce, camphor was separated from the remainder of the juvenile hexane extract by flash chromatography as described above. In a portion of the remainder, camphor was replaced; in the other portion, camphor was excluded. This procedure controlled for all extraction processes. Juvenile hexane extract with camphor was tested against the same extract without camphor (Table 1). Figure

3B shows that hares preferred the juvenile hexane extract that did not contain the camphor ($P < 0.005$), eating three times as much of this than the extract with camphor.

Experiment 6. The other substance found largely in juvenile spruce was bornyl acetate. The procedure for treating chow with this compound was the same as that for camphor in experiment 5. Thus juvenile hexane extract with bornyl acetate in chow was offered to hares together with chow containing similar extract without bornyl acetate. Although the hares ate more of the extract without bornyl acetate, the result was not significant ($P > 0.05$).

DISCUSSION

The first feeding experiment showed that hares avoided camphor when it was added to chow. But it is possible that hares were simply avoiding any odoriferous substance, so that ether chow was not a proper control. Experiment 2 addresses this aspect by using a concentration of mature spruce extract similar to that of camphor. The avoidance of camphor in this case is more convincing; it appears to be the camphor itself that is being avoided.

The lack of preference shown by hares in experiment 3 could be due to either the similar quantities of camphor present in the two choices or to the higher concentration of other secondary metabolites in mature spruce (2.77 g extract/330 g chow). These other secondary metabolites could counteract the avoidance of camphor (0.5 g camphor/330 g chow). Experiment 4, therefore, was designed to balance the other secondary metabolites in mature and juvenile spruce, leaving only the camphor as the difference between them. Again in this case the animals avoided the juvenile hexane extract containing the camphor. Finally, experiment 5 was designed to show that camphor was the active antifeedant compound by comparing juvenile hexane extract with and without camphor. Extraction procedures were kept identical to avoid hidden biases. The hare preferences confirmed that it was the presence of camphor in the juvenile hexane extract that caused them to avoid it.

Bornyl acetate did not produce a significant antifeeding response in the hares (experiment 6). However, the results suggested there might be some avoidance and further testing is needed. In any case, avoidance of bornyl acetate was much less than avoidance of camphor.

In conclusion, we show that camphor, a specific compound in white spruce, acts as a major antifeedant for snowshoe hares. Camphor occurs at four times the concentration in juvenile spruce (<2 m high) compared to that in mature spruce (>5m high). Another compound, bornyl acetate, did not show significant antifeedant properties. Our results support the hypothesis of Coley et al. (1985) that late-succession slow-growing plants should be strongly defended

against herbivores. The results also support the growth-stage hypothesis of Bryant and Kuropat (1980) that early growth stages are more heavily defended than mature stages.

For these conifers, one evolutionary explanation could be that defense is against smaller mammals such as hares because the presence of camphor is associated with plant height. In addition, Sinclair and Smith (1984) found that preference for spruce tops at 3 m height was intermediate between preferences for spruce at <2 m and >5 m high. Defense against either insects or large mammals (browsing mastodons and giant ground sloths were present in these ecosystems less than 12,000 years ago; Dreimanis, 1968) would not be tied to height.

However, we note that the angiosperms, such as alder, also show a diminution in chemical defense in mature stages, but these stages are usually available to browsing hares. This suggests an alternative explanation that the lower defense of mature twigs is due to fundamental physiological changes in the plant rather than a relaxation of predation. What now remains is to see how camphor content is related to size and age of the spruce plant.

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ARTIFICIAL DEFLORATION AND FURANOCOUMARIN INDUCTION IN *Pastinaca sativa* (UMBELLIFERAE)

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Abstract—Damage simulating herbivory was tested as an inducer of furanocoumarins in the floral parts of the wild parsnip, *Pastinaca sativa* (Umbelliferae). In one experiment, primary umbels of *P. sativa* were partially deflorated over the course of nine days, and higher-order umbels as well as the remaining primary umbel floral parts were sampled. Total furanocoumarin concentration was not significantly affected by defloration, but one furanocoumarin, isopimpinellin, increased in one floral stage of the secondary umbel in damaged plants. In a second experiment, primary umbels were completely deflorated and the higher-order umbels allowed to set seed. No significant effect of defloration on furanocoumarin content was found in the seeds of the higher-order umbels.

Key Words—Wild parsnip, *Pastinaca sativa*, furanocoumarins, induction, defloration, optimal defense, Umbelliferae, allelochemicals, herbivore-plant interactions.

INTRODUCTION

Damage from herbivory can result in the induction of higher levels of plant allelochemicals (reviewed in Kogan and Paxton, 1983; Rhoades, 1983). Because resources are allocated for defense only when required, this flexibility in phytochemical response presumably represents the efficient use of limited materials and energy by the plant (Rhoades, 1979). In theory, induction should be especially important in the reproductive parts of monocarpic plants. For a plant

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reproducing only once during its lifetime, herbivore damage to any flowers or fruits increases the proportional contribution of the surviving floral units to its overall reproductive success; in other words, the more flowers are eaten, the more important the remaining flowers become to the plant. If levels of defensive chemistry are closely matched with floral value, the defense of surviving floral units should increase accordingly.

Simulated herbivory was tested as an inducer of one class of allelochemicals, the furanocoumarins, in the floral parts of the wild parsnip, *Pastinaca sativa* (Umbelliferae). *P. sativa* is a monocarpic perennial, flowering in its second year only if sufficient root mass has been accumulated (Thompson, 1978; Baskin and Baskin, 1979). Many of the furanocoumarins found in the flowers and seeds of *P. sativa* are either deterrent or toxic to herbivores (Yajima et al., 1977; Berenbaum, 1978; Muckensturm et al., 1981; Berenbaum and Neal, 1985; Ashkenazy et al., 1985), and several are involved in determining resistance of wild parsnip to two specialist insect species (Berenbaum and Feeny, 1981; Berenbaum et al., 1986). The production of furanocoumarins in response to other forms of stress makes it likely that these compounds can be induced by herbivore damage. Xanthotoxin and bergapten are phytoalexins in celery (Wu et al., 1972). Psoralen, bergapten, isopimpinellin, and xanthotoxin are induced in celery by exposure to cold temperatures, ultraviolet light, and sodium hypochlorite (Beier and Oertli, 1983). Xanthotoxin is a phytoalexin in parsnip root in vitro (Johnson et al., 1973), while angelicin, bergapten, psoralen, and xanthotoxin, collectively, can be 25 times higher in concentration in diseased parsnip roots compared to unspoiled roots (Ceska et al., 1986). There have been no studies, however, investigating the inducibility of furanocoumarins by herbivory or injury simulating herbivory.

METHODS AND MATERIALS

Flowers of *P. sativa* are borne on umbels, and initiation of floral development proceeds according to umbel order. The primary umbel terminates the main flowering stalk and develops first. Secondary umbels branch off the main stalk and develop next, while the tertiary umbels arise from the peduncles of the secondary umbels, and so on. The seeds of the primary umbel are therefore the first to mature and those of the highest umbel order the last to mature. This flowering pattern enabled me investigation of furanocoumarin content changes over two different time courses. In one experiment, I tested for chemical induction in developing flowers shortly after defloration of the primary umbel (short-term response). In a second experiment, seeds that had matured several weeks after primary umbel defloration were sampled from the higher order umbels (long-term response). In both experiments, herbivory was simulated by artificial

defloration to assure equal levels of damage among plants. Artificial defloration simulates insect herbivory with respect to compensatory floral growth in *P. sativa* (Hendrix and Trapp, 1981), so that plants may in general respond to artificial primary umbel damage as they would with natural herbivory.

Compensating floral growth in the higher-order umbels can occur after primary umbel damage in *P. sativa*, and the degree of compensatory growth is dependent on umbel order (Hendrix, 1979; Hendrix and Trapp, 1981). Because of this differential umbel order response, the different orders were sampled and analyzed separately for each plant so as to detect any similar differential response in furanocoumarin chemistry.

Test for Short-Term Response. In May 1984, one hundred *P. sativa* plants at the University of Illinois Phillips Tract natural area, Champaign County, were sprayed with acephate insecticide once a week for three weeks to prevent insect damage. Fifty of these plants were randomly assigned to the artificial defloration treatment and the other 50 to the undamaged control treatment. Five umbellets were clipped from the primary umbel of plants in the defloration group every other day from May 27 to June 6 for a total of four removals. This schedule of removal simulates damage as might occur from a flower-feeding insect such as the parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Oecophoridae), a species commonly found on wild parsnips. *P. sativa* is andromonoecious and protandrous, with each hermaphroditic flower passing through bud, male, and female stages. Primary umbel flowers were buds at the time of the first removal and were mostly males and females at the time of the last removal.

Two days after the last defloration, the following floral parts were sampled from each plant for chemical analysis: one umbellet from the periphery of the primary umbel, one umbellet from the periphery of one of the topmost secondary umbels, and an entire tertiary umbel branching off the secondary umbel that was sampled. An unidentified disease manifested itself in some of the plants in the population as flowering progressed. Only plants appearing healthy and showing no signs of stress were sampled, leaving 28 control plants and 37 artificially damaged plants. Plant samples were dried at 60°C, weighed, and extracted in ethyl ether; furanocoumarin content was quantified using high-performance liquid chromatography (Berenbaum et al., 1984). Seven furanocoumarins were detected: angelicin, imperatorin, bergapten, isopimpinellin, xanthotoxin, sphondin, and psoralen.

Plants varied in their degree of development at the time of sampling. Because furanocoumarin content varies with floral development (Nitao and Zangerl, 1987), each umbel order sampled was categorized into one of four floral developmental classes depending on the status of its flowers (bud, male, female, or fruit stage). Statistical comparisons were made within umbel order/floral stage classes to adjust for this developmental variation. Sufficient sample sizes existed

for comparisons between treatments for primary umbel male flowers, primary umbel female flowers, secondary umbel buds, secondary umbel male flowers, and tertiary umbel buds. The *t*-tests were used to test for a treatment effect on total furanocoumarin concentration and the relative amounts of each individual furanocoumarin. Data for proportions of compounds were arc-sine transformed prior to analysis.

Test for Long-Term Response. Nineteen overwintered *P. sativa* plants from Phillips Tract were transplanted as rosettes into pots in May 1985, kept in a greenhouse, and sprayed with acephate to prevent insect herbivore damage. Plants were randomly assigned to three treatment groups: control (no defloration); complete defloration of the primary umbel at the bud stage; and complete defloration of the primary umbel at the female flower stage. Earlier results showed that the timing of defloration affected growth in higher-order umbels (Nitao and Zangerl, 1987), suggesting that stage-dependent effects of defloration on furanocoumarin content in the resulting seeds might occur as well. All flowers in the umbel were clipped off when the primary umbel attained its preassigned stage to create as severe an impact as possible. All higher-order umbels were allowed to set seed.

Ten filled seeds were randomly chosen from the secondary and tertiary umbels of each plant and pooled for chemical analysis. Quaternary and pentanary umbel seeds were present in only a few of the plants and thus were not included in the analysis. Seeds were cut into halves to facilitate solvent penetration and extracted in ethyl acetate. Furanocoumarins were quantified by high-performance liquid chromatography as described earlier. One-way analysis of variance was used to test for a treatment effect on total furanocoumarin concentration and the relative proportions of individual compounds in the seeds. Data for proportions were arc-sine transformed prior to analysis (Sokal and Rohlf, 1981).

RESULTS

Test for Short-Term Response. An overall examination of the data revealed no consistent trends to suggest that total concentration of furanocoumarins increased in response to primary umbel damage; no significant treatment effect on total furanocoumarin concentration was found in any of the umbel orders and floral stages tested (Table 1).

No treatment effects were found on the relative proportions of the individual compounds except for a significant increase of isopimpinellin in the male flowers of secondary umbels; the mean percentage of isopimpinellin in the control group was 11.2 ± 2.68, whereas that of the deflorated group was $16.0 \pm$

TABLE 1. TOTAL FURANOCOUMARIN CONCENTRATION (μg FURANOCOUMARIN/mg DRY PLANT MASS) IN FLOWERS OF *Pastinaca sativa*[a]

Umbel order	Floral stage	Treatment[b]	
		Control	Deflorated
Primary	male	0.513 ± 0.1342 (6)	0.370 ± 0.0494 (6)
	female	0.938 ± 0.1175 (15)	0.949 ± 0.0751 (30)
Secondary	bud	0.420 ± 0.0526 (18)	0.410 ± 0.0578 (23)
	male	0.612 ± 0.0720 (8)	0.631 ± 0.0652 (14)
Tertiary	bud	0.611 ± 0.0720 (28)	0.592 ± 0.0772 (37)

[a] Primary umbels were either undamaged (control) or artificially deflorated. Mean ± SE. Sample size in parentheses.
[b] No significant treatment effects on total concentrations were found ($P > 0.05$, t tests).

1.13 ($P < 0.05$, t test, $t = -2.10$, $df = 20$). Because the mean total concentrations of furanocoumarins for the two treatment groups were not different, this change in relative amount signifies an absolute increase in isopimpinellin concentration as well. The 1.5-fold proportional increase in this compound was approximately equal to a 0.03 μg/mg increase in absolute concentration. Although several other compounds in turn tended to decrease in proportion, these changes were not large enough in any single compound to be significant. Across all umbel orders and floral stages, the range of mean relative proportions for angelicin was <0.01–0.1%, imperatorin 20.9–53.3%, bergapten 9.8–15.4%, isopimpinellin 8.1–21.2%, xanthotoxin 23.4–51.9%, sphondin 1.1–2.4%, and psoralen <0.1–5.3%.

Test for Long-Term Response. As with the experiment examining short-term effects, defloration of the primary umbel at either the bud or female stage failed to alter total furanocoumarin concentration in the seeds (Table 2). The mean total concentration of an umbel for control plants was intermediate to those of plants deflorated at either bud and female stages.

The relative proportions of the individual compounds were not significantly affected by primary umbel defloration. No consistent trend was found to suggest a change in the proportion of isopimpinellin similar to that observed in the secondary umbel/male flowers in the short-term response experiment. Across all umbel orders and treatment groups, the range of mean relative proportions

TABLE 2. TOTAL FURANOCOUMARIN CONCENTRATION (μg FURANOCOUMARIN/mg DRY PLANT MASS) IN SECONDARY AND TERTIARY UMBEL SEEDS OF *Pastinaca sativa*[a]

	Stage removed[b]		
Umbel order	Control ($N = 6$)	Bud ($N = 6$)	Female flower ($N = 7$)
Secondary	7.104 ± 0.8281	6.820 ± 1.1813	7.306 ± 0.9275
Tertiary	6.841 ± 1.1459	5.892 ± 0.9900	6.446 ± 0.9374

[a] Primary umbels were either undamaged (control) or artificially deflorated at bud or female flower stages. Mean ± SE.
[b] No significant treatment effects on total concentrations were found ($P > 0.05$, one-way ANOVA).

of angelicin was 1.0–1.7%, imperatorin 27.2–33.0%, bergapten 18.7–22.3%, isompimpinellin 10.7–19.8%, xanthotoxin 29.8–38.0%, and sphondin 1.0–1.8%. No psoralen was detected in these seeds.

DISCUSSION

No significant effect of artificial herbivory was found on total furanocoumarin concentration as either short- or long-term responses. Moreover, no consistent trends occurred to indicate that any true differences in total concentration might have been obscured by large variances. This result runs counter to what might be expected if plants defend tissues according to their value. Even though the value of the remaining floral units are, in theory, higher after defloration of the primary umbel, chemical defense as measured by overall furanocoumarin concentration did not correspondingly increase. Only one significant difference in the qualitative composition of the furanocoumarins was found in either experiment. The relative amount of isopimpinellin was higher in the male flowers of the secondary umbels in plants that had their primary umbel partially deflorated. This finding is in striking contrast to the dramatic 25-fold increase of four furanocoumarins in the presence of pathogens in parsnip roots (Ceska et al., 1986).

The experimental design may account for the absence of significant levels of induction. Insecticides can themselves be inducers of plant secondary compounds (Kogan and Paxton, 1983), so that the acephate used to prevent damage may have induced all plants regardless of defloration treatment. This appears not to have been the case, however, because the furanocoumarin values obtained in these experiments are comparable to those found in *P. sativa* plants of the same population not sprayed with acephate (Berenbaum and Zangerl, 1986; Nitao and Zangerl, 1987). Artificial herbivore damage does not always simulate

actual herbivore injury (Capinera and Roltsch, 1980); thus an unknown chemical stimulus that might occur in herbivore saliva may be required for the induction of furanocoumarins in damaged plants. Although this factor was not taken into account, Hendrix and Trapp (1981) have shown that, at least with respect to compensatory seed set, artificial defloration does simulate herbivory by the parsnip webworm.

Physical injury does not elicit increased production of secondary chemicals in all plant species (e.g., Chapin et al., 1985; Johnson and Brain, 1985), indicating that other ecological and physiological constraints can be important. Because reproductive parts directly determine fitness, there may be a high cost for even slight damage to these tissues; the resource cost for the plant to produce furanocoumarins (Zangerl and Berenbaum, 1987) may be outweighed by this low tolerance for damage. Plants may therefore allocate defenses according to the current value of the reproductive part instead of increasing allocation to defense only after flowers have already been lost.

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BEHAVIORAL RESPONSES OF WESTERN CORN ROOTWORM LARVAE TO VOLATILE SEMIOCHEMICALS FROM CORN SEEDLINGS

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Abstract—Corn seedling volatiles collected cryogenically are highly attractive to western corn rootworm larvae, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), in a laboratory bioassay. Carbon dioxide is known as an attractant for western corn rootworm larvae, and the amount of carbon dioxide in the cryogenic collections was measured with an infrared gas analyzer. In a choice test between a source containing carbon dioxide alone and a source containing corn seedling volatiles with an equal amount of carbon dioxide (verified by infrared gas analysis), western corn rootworm larvae chose the corn volatile source significantly more often than the side with carbon dioxide alone. This indicates that carbon dioxide is only one of the volatiles from corn seedlings that is behaviorally important and that other compounds of behavioral importance are present as well.

Key Words—*Diabrotica virgifera virgifera*, Coleoptera, Chrysomelidae, western corn rootworm, rootworm, corn, *Zea mays*, kairomone, volatile substances, attractants, carbon dioxide, semiochemical.

INTRODUCTION

Diabrotica virgifera virgifera LeConte, the western corn rootworm, is an annual threat to corn throughout much of the Midwest. Larval damage to corn roots results in reduced plant height, reduced yield, and lodging of corn, which interferes with harvesting (Branson et al., 1980). Members of the genus *Diabrotica* have been estimated to cause more than $1 billion in damage per year in the United States, mostly to corn (Metcalf, 1986).

Larvae emerge from overwintering eggs in the spring and can feed only

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on certain species of Gramineae (Branson and Ortman, 1967, 1970). Because the eggs are laid in soil the previous year, the burden of host plant location lies entirely with the newly hatched larvae. The larvae have been reported to crawl through as much as a meter through the soil to find the roots of a suitable host (Short and Luedtke, 1970), although estimates to date have been complicated by a number of experimental difficulties (Branson, 1986). In surface olfactometer choice tests between cut roots of corn and broad-leaved plants, Branson (1982) found that significantly more western corn rootworm larvae were attracted to the roots of corn than to the roots of soybean, squash, or sunflower (no difference was observed between roots of corn and tomato). Behavioral bioassays in a soil olfactometer did not reveal differences in attraction, perhaps due to the difficulty of establishing equal amounts of root tissue for the choice test (Branson, 1982). Roots of a number of grass species were as attractive as corn roots or more so to western corn rootworm larvae in surface olfactometer choice tests, including several grass species that were nonhosts (Branson, 1982).

The only chemical cue known to be involved in the orientation of western corn rootworm larvae to corn roots is carbon dioxide (Strnad et al., 1986, Strnad and Bergman, 1987), which is known to attract a number of other soil insects (Jones and Coaker, 1977; Doane et al., 1975; Pline and Dusenbery, 1987; and references therein). Carbon dioxide is produced by the roots of most plant species, and it does not appear to provide a basis for the ability of the larvae to distinguish the roots of host plants from those of many other plant species. We wished to determine if carbon dioxide was the only attractive compound produced by corn root tissue, or if other semiochemicals may be involved in host selection by western corn rootworm larvae as well. Compounds that affect larval behavior might be expected to include two principal categories: compounds that are volatile and disperse mainly by diffusion in the soil atmosphere, and those that are water-soluble and disperse mainly by diffusion in aqueous solution. Our initial studies have focused on the possible contribution of volatile constituents of corn plants.

Cryogenic collection was used to recover volatile compounds from corn seedlings in the present study. This technique has been used successfully for the recovery of other semiochemicals, such as insect pheromones (Browne et al., 1974; Golub and Weatherston, 1984). Unlike conventional cold trapping, cryogenic collection involves condensing the air itself, along with any volatile organic compounds that the air might contain. Low-temperature distillation allows nitrogen (bp $-196°C$) and oxygen (bp $-183°C$) to be removed after the collection is complete, while volatiles with boiling points higher than $-183°C$ are retained. The principal advantage of cryogenic collection is that compounds with a wide range of volatilities can be recovered. This is important in the present study because the very volatile carbon dioxide is already known as a semiochemical important in host location by western corn rootworm larvae

(Strnad et al., 1986; Strnad and Bergman, 1987), and we wished to evaluate the possible behavioral importance of additional compounds that may have an array of large and small molecular weights. In many other studies of volatile semiochemicals, compounds have been extracted with solid adsorbants such as Tenax or Porapak Q, but these techniques fail to recover compounds with very small molecular weights, such as carbon dioxide (Byrne et al., 1975).

Two behavioral bioassays were developed to determine the role of volatile semiochemicals from corn seedlings in host location by western corn rootworm larvae. A single-choice bioassay was developed to determine if the full complement of corn volatiles collected cryogenically was attractive to the larvae and to verify the conclusion of Strnad et al. (1986) that carbon dioxide alone is attractive to western corn rootworm larvae. A choice test bioassay was also developed, to allow behavioral comparison of carbon dioxide alone with a cryogenic collection of corn volatiles that contained an equal amount of carbon dioxide. Instead of using a choice test bioassay with carbon dioxide levels equal on both sides, the behavioral role of carbon dioxide can alternatively be evaluated by using KOH to remove carbon dioxide from a volatile blend, and using single-choice bioassays to compare the attractiveness of KOH-treated host plant volatiles with untreated host plant volatiles (Doane et al., 1975; Jones and Coaker, 1979). However, there are two problems with this approach: First, semiochemicals that are behaviorally relevant only in the presence of carbon dioxide would be detected in a choice test, but would not be detected in single-choice bioassays of a KOH-treated volatile blend. Second, KOH reacts with many compounds other than carbon dioxide and may remove or chemically alter other semiochemicals in addition to carbon dioxide. A choice test bioassay was adopted in the present study to obviate both these possible problems.

METHODS AND MATERIALS

Insect Colony. Eggs of a nondiapausing strain of *Diabrotica virgifera virgifera* were obtained in June and July of 1986 from Dr. Jan Jackson at the Northern Grain Insects Research Laboratory. The colony was maintained according to methods described by Jackson (1986), with some modifications. Modifications (Jackson, personal communication) included the use of soil in covered, small plastic containers (17 cm diam. $\times$ 10 cm high) in which 75 larvae were reared on corn seedlings for their first six days of growth. Two of these smaller containers (along with the larvae) were then transferred into a larger covered plastic container (18 $\times$ 32 $\times$ 8 cm), which contained sufficient seedling corn in soil for completion of larval development and pupation.

Corn Seedlings. Dried whole kernels of field corn, not treated with insecticide or fungicide, were purchased from a local supplier (variety unspecified).

Dried kernels were washed with detergent solution (Ivory liquid) for 5 min to inhibit fungal contamination, rinsed thoroughly, and soaked in water for 24 hr. Soaked seeds were washed again with detergent solution, rinsed thoroughly, placed on moist germination paper (Anchor Paper Co., St. Paul, Minnesota), covered with moist paper towels, and kept in a closed plastic container. Corn seedlings were removed three to four days later for bioassays.

Cryogenic Volatile Collection. A cryogenic collection technique similar to that described by Browne et al. (1974) was used, based on a simple pump in which a glass sample tube closed at one end (12 mm × 35 cm) was immersed in a liquid nitrogen bath. A boiling chip was placed in a clean sample tube, and a glass seed-holding tube (30 cm × 30 mm, tapering to 12 mm) containing 70 g of 3- to 4-day-old corn seedlings (seeds not treated with insecticide or fungicide) was connected to the sample tube with Teflon tubing. The sample tube was then immersed 20 cm into the liquid nitrogen bath. As air condensed in the sample tube, a vacuum was created that pulled air through the corn seedlings at 300 ml/min (measured with a bubble flowmeter). After 10 min, the sample tube was removed from the liquid nitrogen bath and disconnected from the seed-holding tube. The sample tube was immediately placed into a snugly fitting Styrofoam sheath that had been precooled in liquid nitrogen. The condensed air boiled away within 10 min, allowing liquid nitrogen (bp −196°C) and liquid oxygen (bp −183°C) to escape slowly and leaving compounds that were tested in behavioral bioassays for their importance in western corn rootworm larval orientation.

Single-Tube Bioassay. Plastic Petri dishes (10 cm diam.) were used as arenas for the bioassay. The top of the sample tube was connected with Teflon tubing to a 12-mm hole cut in the bottom of the Petri dish (see Figure 1). Ten larvae (5–6 days old and 4–6 mm long) were placed equidistant in a ring near the wall of the Petri dish, and the cover was replaced. We chose to use second-instar larvae (5–6 days old and 4–6 mm long) for our bioassays because initial bioassays indicated that they behaved similarly to first-instar larvae (the instar that must locate a host), and second-instar larvae were easier to handle for the large numbers of bioassays required. All bioassays were performed in dim light. The number of larvae that entered the tube was recorded at 5-min intervals for 30 min. To prevent possible effects from previous testing, larvae were not reused in the bioassays.

Two treatments were tested in larval behavioral bioassays as controls. Clean sample tubes were tested to verify that the sample tubes themselves were not inherently attractive. Sample tubes containing cryogenic collections of ambient air were tested to determine if the circumstances of the cryogenic collection procedure might influence the bioassay results.

Water was observed to condense in the sample tubes in cryogenic collec-

tions from corn seedlings. Measurement of the weight change in the sample tube before and after the cryogenic collection indicated that 75 ± 5 (SE) mg of water collected in a sample tube during the 10-min collection interval. Sample tubes containing the same amount of water were tested to determine if water alone was attractive to western corn rootworm larvae.

Because carbon dioxide has been reported as an attractant for western corn rootworm larvae (Strnad et al., 1986, Strnad and Bergman, 1987), we wished to verify that carbon dioxide was attractive in our bioassay as well. Carbon dioxide from a gas cylinder was introduced into clean sample tubes for 15 sec, and the tubes were inverted for 30 sec to reduce the concentration of carbon dioxide and disperse it evenly in the tube. An infrared gas analyzer interfaced with a Porapak N gas chromatograph column (GC-IRGA, details below) was used to determine the carbon dioxide concentration in the tubes. The number of larvae attracted into the sample tubes was counted every 5 min for 30 min.

Double-Tube Bioassay. Because carbon dioxide was highly attractive in our single-tube bioassays, the additional influence of other behaviorally important corn volatiles would have been difficult to detect using the single-tube bioassay. In order to test if compounds other than carbon dioxide were involved, a choice test bioassay was designed to give larvae a choice between a sample tube containing carbon dioxide alone and a sample tube containing an equal amount of carbon dioxide in association with other corn volatiles. A bioassay apparatus similar to that described by Branson (1982) was designed, consisting of three plastic Petri dishes (5 cm diam.) connected in series by Teflon tubing (10 mm diam.). Holes (12 mm) were cut into the end dishes to allow connection of sample tubes (see Figure 3). One choice was a sample tube containing 4 mmol/mol of carbon dioxide at bioassay initiation (see Figure 3). The sample tube was prepared by flushing with carbon dioxide from a gas cylinder and inverting it for 30 sec to reduce the concentration of carbon dioxide and disperse it evenly in the tube. The carbon dioxide concentration was verified with the GC-IRGA (details below). For the other choice, a sample tube containing a cryogenic collection of corn seedling volatiles was prepared, the liquid nitrogen and liquid oxygen were removed by low-temperature distillation, the sample tube was raised to room temperature, and the carbon dioxide concentration therein was adjusted to 4 mmol/mol at bioassay initiation (see Figure 3) (by flushing the sample tube with carbon dioxide, inverting it, and verifying the final carbon dioxide concentration with the GC-IRGA). Each sample tube was connected to one of the end dishes of the bioassay apparatus, and a 5-min delay was allowed for volatiles to begin diffusing before larvae were added. Ten larvae were placed in the center of a small Petri dish lid (40 mm diam. with a lip 5 mm high); this was placed in the center chamber of the bioassay apparatus, and the cover to the chamber was replaced. The number of larvae in each of

the three Petri dishes was recorded every 5 min for 30 min. Bioassays were performed in dim light. To prevent possible effects from previous testing, larvae were not reused in the bioassays.

Carbon Dioxide Levels in Bioassays. An infrared gas analyzer (IRGA, Beckman model 865) was used to measure carbon dioxide concentrations. An interface of our own design was constructed to allow the IRGA to be used as the detector for a Porapak N gas chromatograph column (3 mm × 2 m) operated isothermally at 25°C, and the output from the GC-IRGA was analyzed with a HP 3390A integrator-recorder. Gas samples (1 ml) were injected with a Pressure-Lok gas sampling syringe (Precision Sampling Co.) for analysis. A mixture of carbon dioxide in nitrogen equal to the atmospheric concentration was purchased (340 ppm, Union Carbide Corp., Linde Division) and used as a standard for quantitation of carbon dioxide by GC-IRGA analysis. In addition, a 99.9% carbon dioxide source (local welding supplier) was used to make dilutions to generate a calibration curve of GC-IRGA response with respect to carbon dioxide concentration. For the single-tube bioassays, gas samples were taken from the mouth of the sample tube (see Figure 1). For the double-tube bioassays, gas samples were taken from the edges of the middle dish (see Figure 3), because this is the location at which larvae must make a choice to leave the dish. Samples were taken every 5 min for 30 min for analysis with the GC-IRGA.

Statistical Analysis. The statistical package BMDP (BMDP Program Librarian, Department of Biomathematics, University of California, Los Angeles, California 90024) was used for data analysis. Behavioral bioassay data for 30 min were analyzed with one-way analysis of variance for larval attraction in the single-tube and double-tube bioassays, because larvae that entered the sample tubes were usually unable to come back out of the tubes and the bioassay data were therefore essentially cumulative with time. Duncan's multiple-range test (Duncan, 1955) was used to discern differences among treatment means. Data for carbon dioxide levels at all times sampled were compared in a repeated measures design (Anonymous, 1981), because sequential samples for GC-IRGA analysis were taken from each sample tube.

RESULTS

Significantly more ($P \leq 0.01$) western corn rootworm larvae were attracted to sample tubes containing cryogenically collected corn volatiles than to control treatments, which included sample tubes containing ambient air, cryogenic collections of ambient air, or water (Figure 1A). Samples tubes containing cryogenic collections of ambient air attracted more larvae ($P \leq 0.05$) than tubes with ambient air not cryogenically collected.

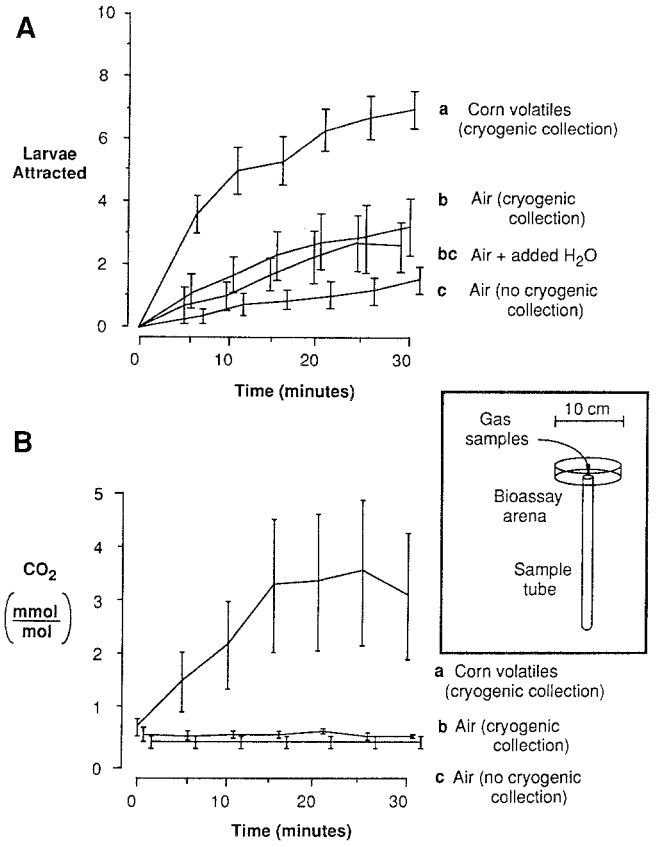

FIG. 1. (A) Single-tube bioassay to evaluate behavioral responses of western corn rootworm larvae to cryogenic collections of corn seedling volatiles and to control treatments. Treatment means at time 30 min were significantly different ($P \leq 0.01$) for curves followed by different letters according to Duncan's NMRT. Error bars represent 95% confidence intervals. (B) Carbon dioxide concentrations at the orifices of the sample tubes. Treatment means of overall carbon dioxide levels were significantly different ($P \leq 0.05$) for curves followed by different letters in a repeated measures analysis of all times according to Duncan's NMRT. Error bars represent 95% confidence intervals.

When a Petri dish with 10 larvae was added to sample tubes containing cryogenic collections of corn seedling volatiles, the first observable larval responses were head-waving, also observed by Strnad et al. (1986), and alignment of their body axes toward the center of the dish. Larvae crawled rapidly and more or less directly toward the source of corn volatiles, similar to the path that larvae took to carbon dioxide in Strnad et al. (1986). This behavioral sequence rarely occurred in response to control treatments (ambient air treat-

ment or water treatment). Larvae that lowered their heads into the sample tube usually crawled in and did not crawl back out.

The cryogenic apparatus for volatile collection provided an efficient vacuum. Air flow past the corn seedlings remained essentially constant throughout the 10-min collection period as indicated by measurement of the volume of liquid air that accumulated in the sample tube (0.35 ± 0.02 SE ml/min) and by measurement with a bubble flowmeter (300 ml/min). Collection rates remained relatively constant for collection periods as long as 30 min. Behavioral bioassay results indicated that it was effective in collecting compounds of interest to larvae, and after distillation of liquid oxygen and nitrogen, the odor of corn was apparent to the human nose as well.

Analysis of carbon dioxide standards with the GC-IRGA indicated a retention time for carbon dioxide of 1.8 min. No other peaks were observed in GC-IRGA analyses of air, cryogenic collections of air, or cryogenic collections of corn seedling volatiles. Calibration with known carbon dioxide concentrations showed the GC-IRGA to be linear in response ($r^2 = 0.995$) for the range of carbon dioxide found in the bioassays (0.34–30 mmol/mol). The use of the IRGA and GC in combination provided a sensitive, semiselective means of detecting carbon dioxide in conjunction with a characteristic retention time for this compound, allowing rigorous determination of carbon dioxide in the volatile samples even at low concentrations. The carbon dioxide concentration in ambient air was easily measurable (0.34 mmol/mol is atmospheric concentration).

The carbon dioxide concentration (Figure 1B) was significantly higher in sample tubes containing cryogenically collected corn seedling volatiles than in sample tubes containing cryogenically collected ambient air or sample tubes containing ambient air without cryogenic collection ($P \leq 0.01$). The concentration of carbon dioxide in sample tubes containing cryogenically collected ambient air (Figure 1B) was slightly higher than that found in ambient air (but significant, $P \leq 0.01$). The cryogenic collection evidently concentrated carbon dioxide from the surrounding air, and the greater carbon dioxide concentration may account for the significantly greater attraction of larvae to sample tubes containing cryogenically collected ambient air than to the sample tubes containing air alone.

Single-tube bioassays with carbon dioxide (Figure 2A) showed that this compound is highly attractive to western corn rootworm larvae ($P \leq 0.01$). The source of carbon dioxide in this experiment was a sample tube from which carbon dioxide emerged by diffusion. The dynamics of carbon dioxide release by the sample tube were determined by analysis with GC-IRGA (Figure 2B). For sample tubes prepared by filling them with carbon dioxide from a gas cylinder and inverting them for 30 sec to adjust the concentration to a low level,

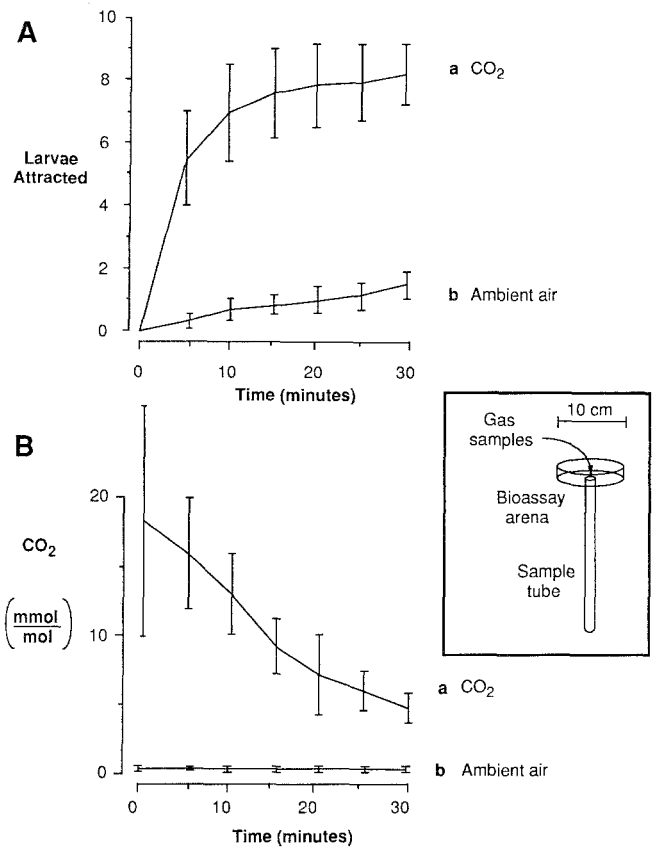

FIG. 2. (A) Single-tube bioassay to evaluate responses of western corn rootworm larvae to carbon dioxide and to ambient air. Treatment means at time 30 min were significantly different ($P \leq 0.01$) for curves followed by different letters according to Duncan's NMRT. Error bars represent 95% confidence intervals.(B) Carbon dioxide concentrations at the orifices of the sample tubes. Treatment means of overall carbon dioxide levels were significantly different ($P \leq 0.01$) for curves followed by different letters in a repeated measures analysis of all times according to Duncan's NMRT. Error bars represent 95% confidence intervals.

the average concentration of carbon dioxide at the orifice of the sample tube was initially 18 $\pm$ 8 (SE) mmol/mol and dropped to a third this value as the gas diffused from the sample tube during the 30 min bioassay (Figure 2B). This source of carbon dioxide was highly attractive to the larvae, in that 82% of the larvae tested entered the sample tube, and more than half of these entered the sample tube within the first 5 min of the bioassay (Figure 2A).

In a choice test between a sample tube containing carbon dioxide (4 mmol/mol at bioassay initiation; Figure 3B) in association with cryogenically collected corn seedling volatiles and a sample tube containing an equal amount of carbon dioxide alone (4 mmol/mol at bioassay initiation; Figure 3B), significantly more ($P \leq 0.01$) western corn rootworm larvae chose the sample tube

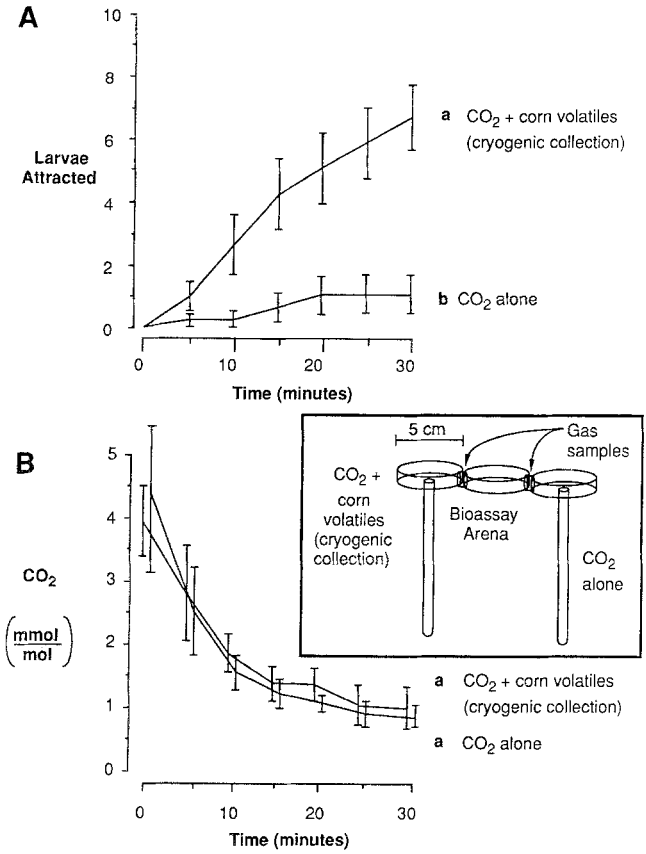

FIG. 3. (A) Double-tube bioassay to evaluate choice of western corn rootworm larvae between a sample tube containing carbon dioxide alone and a sample tube containing an equal concentration of carbon dioxide in conjunction with cryogenically collected corn seedling volatiles. Treatment means at time 30 min were significantly different ($P \leq 0.01$) for curves followed by different letters according to Duncan's NMRT. Error bars represent 95% confidence intervals. (B) Carbon dioxide concentrations at the orifices of the sample tubes. Treatment means of overall carbon dioxide levels were significantly different ($P \leq 0.05$) for curves followed by different letters in a repeated measures analysis of all times according to Duncan's NMRT. Error bars represent 95% confidence intervals.

with corn seedling volatiles (Figure 3A). It was essential that carbon dioxide concentrations be the same in both sample tubes, and GC-IRGA analysis verified that identical concentrations were effectively established by filling both types of sample tubes with carbon dioxide from a gas cylinder and inverting them for 30 sec (Figure 3B). The concentrations of carbon dioxide in each sample tube declined during the bioassay period as carbon dioxide diffused from the tubes, and the time course of carbon dioxide release from the two types of sample tubes was the same throughout the bioassay period (Figure 3B). The strong preference of the larvae for the sample tube containing corn seedling volatiles indicates that carbon dioxide is only one of the corn seedling volatiles attractive to western corn rootworm larvae and that other behaviorally important compounds are also produced by corn seedlings.

DISCUSSION

A central interest in studies in chemical ecology is to establish the complement of chemical compounds involved in a given ecological interaction and to determine how those compounds direct its course. We have shown that volatiles can be cryogenically collected from corn seedlings and that the isolated volatiles are attractive to western corn rootworm larvae. The only compound that has been implicated in host plant interactions with *Diabrotica* larvae is carbon dioxide (Strnad et al., 1986, Strnad and Bergman, 1987), a primary metabolite that is released by the roots of corn plants (Harris and van Bavel, 1957; Massimino et al., 1980). We found by GC-IRGA analysis that carbon dioxide is one of the compounds present in cryogenically collected corn seedling volatiles and verified that this compound was strongly attractive to larvae in our single-tube bioassay. The strong attraction of larvae to carbon dioxide in the single-tube bioassay (Figure 2A) would have made it difficult to determine any additional influence of other semiochemicals produced by corn seedlings. To make this distinction, we developed a choice test bioassay in which equal concentrations of carbon dioxide were present on both sides, and we found that the side containing corn root volatiles attracted significantly more western corn rootworm larvae. This indicates that a blend of volatile compounds from corn seedlings is involved in attraction of western corn rootworm larvae and that carbon dioxide is not the only compound in corn root volatiles of behavioral importance of *Diabrotica virgifera virgifera* larvae. Volatile secondary plant compounds have been shown to be important in location of host root systems by other species of insects that live in the soil but have not yet been implicated in host location by *Diabrotica* larvae (Jones and Coaker, 1978; Hsiao, 1985).

A number of volatiles from corn roots have been identified but have not been tested behaviorally with western corn rootworm larvae. Buttery and Ling

(1985) used the solid adsorbant Tenax to collect volatiles from the roots of young (60 cm high) and mature corn plants (2 m high). The principal volatiles recovered were sesquiterpene hydrocarbons that included beta-caryophyllene, logifolene, bazzanene (tentative), cyclosativene, alpha-ylangene, and a major hydrocarbon that was not identified. Volatile secondary compounds have also been identified from corn leaves (Buttery and Ling, 1984), buds (Thompson et al., 1974), silks (Flath et al., 1978; Cantelo and Jacobson, 1979), tassels (Buttery et al., 1980), and from husks and kernels (Buttery et al., 1978).

A review of the literature on volatile semiochemicals for soil insects indicates that two groups of compounds with very disparate vapor pressures have been considered. One group comprises gases such as carbon dioxide, nitrogen, oxygen, methane, and ethylene (see Jones and Coaker, 1977; Doane et al., 1975, and references therein). Carbon dioxide alone is attractive to a number of soil invertebrates, including insect larvae (Klingler, 1957, 1958, 1965, 1966; Doane et al., 1975; Städler, 1971, 1972; Paim and Beckel, 1963a, b; Meeking et al., 1974; Jones and Coaker, 1977), insect adults (Paim and Beckle, 1963a, b), mites (Moursi, 1962, 1970), chilopods (Moursi, 1970), nematodes (Pline and Dusenbery, 1987; Prot, 1980; Johnson and Viglierchio, 1961; Klingler, 1961, 1963, 1965), and bacteria (Scher et al., 1985). The other group of volatile semiochemicals for soil invertebrates comprises secondary compounds with considerably lower vapor pressures that can easily be collected by tissue extraction, steam distillation, or air extraction with solid adsorbants (Jones and Coaker, 1978; Hsiao, 1985).

Volatile secondary compounds important in underground attraction of larvae have been chemically identified for several insect species (all of them Diptera), including the carrot rust fly, *Psila rosae* (F)., the onion maggot, *Delia antiqua* (Meigen), the turnip maggot, *Delia floralis* (Fallen), and the related species *Delia brassicae* (Wiedemann). For the carrot rust fly *Psila rosae*, an insect that has a high degree of host specificity for certain species of Umbelliferae, carbon dioxide is one of several attractants that have been found for underground larvae (Jones and Coaker, 1977, 1979). Of 45 volatile secondary compounds that have been identified from fresh carrot root, five have been found to be most attractive to carrot rust fly larvae: bornyl acetate, 2,4-dimethyl styrene, alpha-ionone, beta-ionone, and biphenyl (Ryan and Guerin, 1982, Guerin and Ryan, 1984). Underground larvae of the onion maggot *Delia antiqua* are able to orient to onion volatiles (although host selection is primarily made by the ovipositing females). The larvae showed strong attraction to 27 synthetic sulfides, disulfides, and mercaptans in a laboratory bioassay (Matsumoto and Thorsteinson, 1968; Matsumoto, 1970, Soni and Finch, 1979), although only some of these compounds are known to be released from onions (Carson and Wong, 1961. Rotting onions are more attractive to larvae (and to ovipositing females) than are fresh onions, and it has been established that this is due to

the activity of *Klebsiella* bacteria, although it is not yet clear which volatiles of *Klebsiella*-infestated onions are responsible for the enhanced attraction (Ikeshoji et al., 1980, 1982, Yamada et al., 1981). Underground larvae of some *Delia* species feed only on certain species of Cruciferae. Larvae of the turnip maggot *Delia floralis* (Rygg and Somme, 1972) are attracted to allyl isothiocyanate, a characteristic volatile of many Cruciferae. A source of mustard oil glucosides is attractive (perhaps because of volatile degradation products) to the related species *Delia brassicae* (Finch and Skinner, 1974).

Volatiles have been shown to play a role in host location by underground larvae of several other insect species, but the compounds involved have not yet been chemically characterized. The false wireworm *Eleodes suturalis* (Say) was attracted to volatiles in an airstream passed over germinating wheat (Calkins et al., 1967). Sutherland (1972) found that subterranean larvae of the scarab beetle *Costelytra zealandica* (White) were attracted to fresh perennial ryegrass root in a laboratory bioassay. Neonate larvae of the clover root curculio *Sitona hispidulus* (F.) are attracted to roots of alfalfa *Medicago sativa* L. and red clover *Trifolium pratense* L. and are attracted in particular to *Rhizobium* nodules on the root systems (Wolfson, 1987).

Adults of some insect species have also been shown to orient underground to volatile secondary plant compounds, either for feeding or for oviposition. Underground adults of the scolytid *Hylastinus obscurus* Marsham, the clover root borer, feed on the diseased roots of red clover, *Trifolium pratense* (Leath and Byers, 1973), and behavioral bioassays for attraction have been performed with organic extracts of diseased roots and with organic extracts of a solid adsorbant (Tenax) used for air extraction of diseased roots (Kamm and Buttery, 1984). Of the compounds identified from diseased root tissue, the most attractive in a laboratory bioassay were ethyl laurate, ethyl benzoate, estragole, chavicol, pentadecanal, hexadecanal, and hexanoic acid. These compounds were not attractive in the field when presented alone or in combination (Kamm and Buttery, 1984). Adults of the pine weevil *Hylobius abietis* (L.) (Nordlander et al., 1986) and of the scolytid *Hylastes nigrinus* (Mann.) (Rudinsky and Zethner-Moller, 1967) locate conifer roots suitable for oviposition by using host volatiles diffusing through the soil.

The basis for host specificity in western corn rootworm larvae has not yet been determined. *Diabrotica virgifera virgifera* is an oligophagous species, feeding only on certain species of grasses. Branson and Ortman (1967, 1970) made a rigorous survey of the host plants suitable for larval development. Of 44 grass species tested, 18 supported rootworm larval growth for at least 10 days. No larvae survived 10 days on any broad-leaf species tested. In choice tests between cut roots of corn and of nonhost broad-leaved plants in a surface olfactometer, Branson (1982) found that significantly more western corn rootworm larvae were attracted to roots of corn than to roots of soybean, squash,

or sunflower (no difference was found for tomato roots vs. corn roots). In tests with a variety of grass species, many nonhost grass species were as attractive as corn or more so. Even the nonhost grass sorghum was found to be attractive (Branson, 1982) and to elicit feeding from western corn rootworm larvae (Branson et al., 1969), despite the fact that the roots contain cyanogenic glycosides that are lethal to the larvae. These results indicate that the olfactory cues used by western corn rootworm larvae in host location may be restricted to a range of grass species, limiting the amount of searching that must be done in the soil, but are not rigorously restricted to the grass species on which western corn rootworm larvae occur. We have shown that a blend of volatile compounds from corn seedlings is attractive to western corn rootworm larvae, and if these compounds are limited to some members of the Gramineae, this may provide a basis for olfactory discrimination by western corn rootworm larvae between grasses and broad-leaved plants.

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Book Review

Insect–Plant Interactions. J.R. Miller and T.A. Miller (eds.). New York, Springer Verlag, 1986. $67.00, DM 158.00, xii + 342 pp.

The title of this volume does not indicate that the book deals primarily with methods and techniques. A discussion of this subject is, however, most useful since everyone actively engaged in the field of insect–plant studies encounters experimental problems that arise from dealing with two completely different types of organisms. Insects and plants each have their own peculiar physiological features and their own typical degree of variation. Because of these essential differences, the proper design of experiments is often tremendously complicated.

This book, published within the Springer Series in Experimental Entomology, aims at presenting "approaches and methods at a level useful to nonexpert and expert researchers." Before reading the contents, I made a short list of topics that I would expect in a book of this format. It included: feeding and oviposition behavior under laboratory and field conditions, variability and adaptability of behavior (including learning), orientation, sensory responses (electrophysiological methods), artificial diets, nutritional parameters, plant resistance to insects, expression of plant responses, standardization of insects and plants (including genetic aspects), biological rhythms, insect–flower interactions, marking methods, data analysis and statistical tests, and terminology of insect and plant reactions. All of these subjects (with the notable exception of insect–flower interactions) have been discussed, in several instances in more than one place. Most of the authors selected by the editors have great experience in their fields of study, and all chapters reflect their authoritative position on the topics assigned to them. The format of the book is not that of a cookbook or a manual on techniques and methods. Rather, each chapter presents certain aspects of insect–plant relations, with an emphasis on methods, embedded in a discussion of the present state of understanding of that topic. The advantage of this approach is that the book is very readable, since it gives more than only techniques. Also, the presentation of views and hypotheses within a methodological framework often leads to a rearrangement of known facts, which makes stimulating reading. The disadvantage of this approach is that it often takes some effort by the reader to locate a certain method and the discussion of its ins and outs. However, this problem is to a large extent compensated for by a good index.

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The opening chapter by S.B. Opp and R. Prokopy is a well-balanced general introduction to the study of insect behavior, its pitfalls, and some of the technical aids available. Two chapters are devoted to behavioral studies in the field situation. The contribution by S. Fich contains a lucid discussion of experimental design and trapping devices, especially applicable to crop vegetations. M. Singer discusses behavioral studies in natural habitats and includes a highly informative case study of a butterfly. A.C. Lewis and H.F. van Emden present an incisive and critical review on feeding assays, historically the most important approach to the analysis of insect–plant relationships and still an extremely fruitful method. Three chapters are devoted to measuring food quality. M. Kogan elaborates on nutritional parameters and thus presents an appreciable extension to G.P. Waldbauer's famous founding paper (1968) on this subject. Postingestive effects of plant allelochemics on insects are authoritatively discussed in two chapters by M. Berenbaum and I. Ishaaya, respectively. Since plant chemicals play an overwhelming role in most insect–plant interactions, it seems appropriate to discuss problems associated with isolating and identifying chemical plant constituents, although this subject as a whole is clearly outside the scope of the present book. To treat this within the framework of one chapter is an unenviable task. I. Kubo and F.J. Hanke have chosen to discuss recent developments in analytical plant chemistry in this area, exemplified by one group of compounds of physiological importance, phytoecdysteroids. A special and very useful chapter (W.M. Tingey) is devoted to evaluating plant resistance to insects, the very heart of agricultural entomology. This is also the place where the effects that insects may induce in the plant receive some attention, thus justifying the term "interactions" in the title of the book. Last but not least comes the electrophysiological analysis of insect chemoreceptory capacities. J.L. Frazier and F.E. Hanson present a clear account of this approach, including its potential for the understanding of insect–plant relationships.

On one hand, the volume is a rich source of available methods and techniques although, as indicated before, one has occasionally to dig a bit to find the information desired. On the other hand, it is more than that. It also presents current lines of thinking in this rapidly expanding area of biology and makes one appreciate once more that scientific advancements are, to a large extent, determined by the techniques available. Clearly there is some dualism in the approach chosen, in which discussions of methods and useful techniques are combined with views on principles underlying insect–plant relationships. I would have preferred that the discussion was restricted to methods.

Of course, a book of this size cannot be expected to give a complete and exhaustive treatment of the subject. For instance Dethier's screen test is not mentioned, nor are Jermy's sandwich test and Saxena's grid test, all of them simple but rewarding methods. Some chapters (e.g., 7 and 9) hardly cite papers published after 1980, whereas others (e.g., 10) contain references up to 1984.

Addition of an author index would have increased its usefulness. The book is produced attractively.

In conclusion, this volume belongs in any library of experimental entomology, and it makes good reading for both advanced and beginning researchers in the field of experimental insect–plant studies. I suspect this volume will have a long half-life value.

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Book Review

Pheromone Biochemistry. G.D. Prestwich and G.J. Blomquist (eds.). New York, Academic Press, Inc. 1987. xix + 565 pp.

Pheromones are used throughout the animal kingdom for chemical communication among members of a species, particularly to ensure reproductive success. This book concentrates primarily on the biosynthesis, catabolism, and reception of sex pheromones in insects. Besides providing an excellent overview of this field, many of the chapters include methodological critiques and discuss new approaches for the study of these processes.

At the outset of the first section covering pheromone biosynthesis and its regulation, Tumlinson and Teal give a good overview of insect pheromone biochemistry as detailed in the remainder of the book. Then come technique-oriented chapters on sex pheromone gland ultrastructure and on pheromone biosynthesis in Lepidoptera. These chapters are crammed with specific methodology along with critical assessments, which should be extremely valuable to workers in these areas. Following are reviews of endocrine regulation of pheromone production in Lepidoptera, Coleoptera, Diptera, and in the ixodid ticks. For the Coleoptera and Diptera (primarily the house fly), these include a summary of the pheromones and their biosynthesis. Interestingly, the various groups use different endocrine signals to regulate pheromone production: ecdysteroids in the Diptera and ticks, juvenile hormone in the beetles, and neurosecretory hormones in the Lepidoptera. In beetles many of the pheromones are derived from plant host chemicals either by the beetle itself or by associated microorganisms. Plant-derived pheromones may also be important in sexual selection in Lepidoptera as portrayed in a series of short, thought-provoking vignettes by Eisner and Meinwald. The section ends with a detailed analysis of the terpenoid biosynthetic origin of cantharidin in blister beetles.

The second part of the book discusses reception and catabolism of sex pheromones, beginning with a nicely illustrated review of the functional morphology of the pheromone-sensitive sensilla. These sensillae are very selective, highly sensitive, and able to respond to rapid changes in pheromone concentration. Understanding these properties at both the neurophysiological and the biochemical levels is the basis of two reviews of recent studies that are providing new insights into the mechanisms involved. The study of the biochemical mechanisms of pheromone reception required new chemical approaches that Prestwich discusses in detail. The book closes with a short review of the molecular

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mechanisms of vertebrate olfaction that shows the many parallels with pheromone reception in insects.

In spite of the omission of the social insect pheromones, this book can be highly recommended to graduate students and postdoctoral and seasoned researchers in pheromone biochemistry. It provides an excellent basis for future research in this field.

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POTENTIAL FOR EVOLUTION OF RESISTANCE TO PHEROMONES
Worldwide and Local Variation in Chemical Communication System of Pink Bollworm Moth, *Pectinophora gossypiella*

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Abstract—Female *Pectinophora gossypiella* (Saunders) from most of the desert cotton-growing areas of southern California emitted significantly more pheromone in 1984 and 1985 than in preceding years (1982 and 1983). This increase amounted to almost 20% by 1985. It is unlikely that this small change would represent effective resistance to disruptant pheromones, but this increase could reflect the result of selection pressure imposed by the use of mating disruption for population control. A worldwide survey of emitted pheromone from this species found that there was much more variation in the emission rate than the blend ratio of the two pheromone components. The emitted blend ratio was remarkably consistent over time (in southern California) and throughout the worldwide range of the insect. Small differences in the blend ratio that were detected probably have no major biological significance because of the relatively broad response spectrum of males to changes in the blend of pheromonal components. Populations of males did not consist of several phenotypes, each with a different preference for specific blend ratios. Rather, the broad response spectrum to blend ratios in a population can be attributed to variation in the response of any individual. Therefore, selection for a response phenotype that is narrowly tuned to the blend emitted by females may be difficult.

Key Words—Resistance, mating disruption, sex pheromone, (*Z*,*Z*)-7,11-hexadecadienyl acetate (*Z*,*E*)-7,11-hexadecadienyl acetate, Lepidoptera, Gelechiidae, pink bollworm, *Pectinophora gossypiella*.

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INTRODUCTION

Selection imposed by the use of synthetic sex pheromones to disrupt mating of key agricultural insect pests could result in evolutionary changes in their chemical communication systems. The potential for rapid evolution of resistance to these mating disruptants will depend in part on heritable variation in the characteristics of chemical communication such as the emission rate, the emitted blend ratio of pheromone components, or phenotypic variation in the response of males to different blend ratios of pheromone components.

An ideal subject species for studying the potential for evolution of resistance to pheromones is the pink bollworm moth, *Pectinophora gossypiella* (Saunders), because its communication system has been examined extensively and certain populations have been exposed to mating disruptants for many generations. The sex pheromone of *P. gossypiella* consists of two components (*Z*,*Z*)- and (*Z*,*E*)-7,11-hexadecadienyl acetate (*ZZ*7,11-16:Ac and *ZE*7,11-16:Ac, respectively) (Hummel et al., 1973; Bierl et al., 1974). A blend of these components centered about a 1:1 *ZZ*/*ZE* ratio has been used by many cotton growers in the desert Southwest to disrupt mating of this species since the first commercially available mating disruptant was registered by the U.S. Environmental Protection Agency in 1978 (Doane and Brooks, 1981). Recently, Haynes et al. (1984) reported that these components were emitted from the sex pheromone gland in a blend ratio of 61:39 *ZZ*:*ZE*7,11-16:Ac, and Linn and Roelofs (1985) found that male *P. gossypiella* responded optimally to *ZZ*/*ZE* blend ratios between 60–65:40–35 at low emission rates of these components. Haynes et al. (1984) examined populations of *P. gossypiella* throughout southern California for evidence of resistance to sex pheromones used as mating disruptants. No differences in the emission rates or blend ratio of these pheromone components were documented in a comparison of females from fields with a long history of pheromone treatments to females from fields that received principally insecticide treatments. While these results were encouraging for continued effective use of mating disruption to control this species, it was important to expand this study in three critical ways. First, there was a need to continue this sampling for several years in case resistance to sex pheromones could not be detected in local populations because of migration among these populations. This procedure might establish a temporal record of changes in the communication system. Second, by sampling throughout the much broader geographical distribution of this species, in this case the worldwide distribution, one might detect differences between these populations that could correlate to the general pattern of use of mating disruptants in an area. In addition, this study of geographical variation in the emission rate and blend ratio might establish whether changes in the communication system of this species have occurred over the much longer period of time in which the species range has expanded. Third,

since heritable variation in the response of males to blend ratios could accelerate significantly the development of resistance, an analysis of phenotypic variation in the behavioral response to blend ratios was undertaken within a single field population.

METHODS AND MATERIALS

Collection and Handling of Insects from California. Cotton bolls were collected from selected cotton fields in the three major cotton-producing valleys in southern California (Coachella, Imperial, and Palo Verde valleys) during August through October. In each valley an attempt was made to select fields with different histories of use of mating disruptants. When possible, fields with no exposure to disruptant pheromones (called insecticide-treated fields) and fields with three to five years of treatment with disruptant pheromones (called pheromone-treated fields) were selected. Because of prevailing agricultural practices in some valleys (e.g., crop rotation) and changing patterns of disruptant pheromone use, it was not always possible to group fields into these two categories. For example, no pheromone-treated fields were identified in Coachella Valley in 1985, and every cotton field in Imperial Valley was treated with disruptant pheromone in 1982. Between 1000 and 3000 cotton bolls were collected in each field, and the bolls were transported to Riverside, California, where they were stored in screened cages in a lath house. Handling of cotton bolls and emerging insects was identical for all four years of the experiment and followed the protocol detailed by Haynes et al. (1984).

Laboratory *P. gossypiella* were reared on shredded wheat-germ diet in half-gallon cartons (Haynes et al., 1984). The procedure for handling the laboratory-reared pupae and adults was identical to that followed for field-collected insects. The laboratory population originated from insects collected before 1976 from fields in Coachella Valley.

Collection and Handling of Insects from Around the World. Collaborators in Argentina, Brazil, China, Egypt, Mexico, and Pakistan shipped field-collected last-instar larvae or pupae by the quickest practical method to the quarantine facility in the Division of Biological Control, Department of Entomology. At this facility pupae were separated according to sex. Adult females that had emerged within a 24-hr period were taken out of the quarantine facility in individual cages that were then housed in an environmental chamber with laboratory-reared females.

Collection and Quantification of Emitted Pheromone. Pheromone was collected from individual female *P. gossypiella* during their peak period of calling during the last half of the scotophase, following the procedures described by Haynes et al. (1984). The female's wings were folded back over her head and

she was inserted abdomen-first into a 2.0-mm-ID glass tube with a 0.5-mm (diam.) hole at the distal end. This hole was large enough to allow only the ovipositor and associated pheromone gland to emerge when light pressure was applied to the female's head with a pipe cleaner. The glass tube was then inserted through a Teflon-coated GLC septum into the collector. Volatiles emitted from the gland's surface were collected for 10 min (at ca. 25°C). An internal standard [3.0 ng of (*Z*)-7-hexadecenyl acetate (*Z*7–16:Ac) in 5 μl of CS_2] was added to the glass wool before the inside of the collector was rinsed with ca. 200 μl of CS_2. This volume of CS_2 was reduced under a nitrogen stream to ca. 6 μl before it was pulled up into a 10-μl syringe for injection onto the GLC column.

It was critical that the internal standard remained at a constant concentration throughout the four years of the experiment. We ensured this consistency by maintaining several reference standards of *Z*7–16:Ac that were not used as internal standards, but were compared by GC analysis to the internal standard on a daily basis. In this way a change in the concentration of the internal standard of a few percent was easily detected. An increase in concentration occurred during repeated opening of the vial containing the internal standard. An internal standard was discarded as soon as the concentration change was detected. All standards were stored at −20°C. Before each year's collections, a set of new standards were prepared from >98% pure *Z*7–16:Ac and were compared to the previous years'. No changes in concentration of the standards were noticed during storage between years.

Analyses were made on a Varian 3700 gas chromatograph equipped with a hydrogen flame detector, a Hewlett-Packard 3380A integrator, and a Silar 10C packed column (ca. 4 g of 10% Silar 10C on acid-washed 100–120 mesh Chromosorb W; glass column 3 m or 4 m; oven temperature ca. 175°C; N_2 flow rate of 30 ml/min). Over the four-year course of the experiment, several columns were used. Consistency in the ability of the columns to separate the pheromone isomers was checked by analyzing a standard that contained 2 ng *ZZ*7,11–16:Ac and 1.33 ng *ZE*7,11–16:Ac in 5 μl of CS_2 (60% *ZZ*7,11–16:Ac). The column was replaced or repacked if accuracy was lost in determining the blend ratio of this standard.

The amount of each isomer was calculated from a standard curve relating peak to mass. These values were corrected for recovery efficiency by standardizing the measurements relative to the internal standard. The lower analytical limit of our technique was ca. 0.1 ng (0.01 ng/min).

Variation in Males' Responses to Different Blend Ratios. Synthetic *ZZ*- and *ZE*7,11–16:Ac were purchased from Scentry Inc. (Buckeye, Arizona) and were determined to be greater than 93% free of other sex pheromone-like volatiles by GLC analysis. *ZZ*7,11–16:Ac contained as much as 4% *ZE*7,11–16:Ac. *ZE*7,11–16:Ac contained as much as 3% *ZZ*7,11–16:Ac. Hexane solutions of four blend ratios (38:62, 48:52, 58:42, and 68:32 *ZZ*:*ZE*) were

prepared taking into account the cross-contamination of the original materials. Using a pheromone collection device, it was determined that the emitted blend ratios from rubber septa treated with 5 mg of the two acetates were within 1% of the desired 40:60, 50:50, 60:40, and 70:30 blends of *ZZ*- and *ZE*7,11-16:Ac. Interestingly, the emitted blend ratio was always slightly biased towards the *ZZ* isomer relative to the blend loaded onto the rubber septum. The two pheromone components together were emitted at a rate of ca. 1.8 ng/min.

Sixty-four modified traps for marking males with fluorescent powders were deployed in a cotton field in Coachella Valley, California, during September 1984. These marking stations were evenly spaced in an 8 × 8 grid with 20 m between traps, and there was at least 40 m between every trap and the edge of the cotton field. The 64 marking stations consisted of 16 quartets arranged in a square. Each of the four blend ratios was used to bait a single marking station within each quartet.

The marking stations were cylindrical metal traps (height 13.5 cm, diam. 25 cm) with four 12.5-cm (width) by 3.5-cm (height) holes spaced evenly around the midline of the cylindrical surface. Each station contained a paper plate (25 cm diam.) that fit into the trap's bottom below the level of the holes. About 50 ml of fluorescent powder (Day-Glo Color Corp., Cleveland, Ohio) were spread evenly over the plate's surface. Four colors of fluorescent powder were used: Aurora Pink for the 40:60 blend, Horizon Blue for the 50:50 blend, Saturn Yellow for the 60:40 blend, and Blaze Orange for the 70:30 blend of the *ZZ* to *ZE* isomers. A pheromone-impregnated rubber septum was fixed with a color-coded straight pin to a cork that was in turn stapled to the paper plate.

Traps that were used to permanently capture males were similar to the marking stations, except paper plates coated with Stickem Special replaced the plates covered with fluorescent powder. Both marking stations and traps were placed at a height of about 1 m with three 1.2-m-long redwood stakes. Traps were placed at the center of each quartet of marking stations. Marking stations were only baited on the first, third, and sixth nights of the experiment, and traps were baited on the second, fourth, fifth, seventh, and eighth nights; thus marking stations and traps were not baited at the same time. Positions of traps were rerandomized before each trapping night of the experiment.

RESULTS

Comparison of Populations from California Valleys and Laboratory over Four Years. The emission rate of *ZZ*7,11-16:Ac from field-collected females was significantly higher during the last two years of the experiment (1984 and 1985) than during the first two years (1982 and 1983) (Table 1). On average a field-collected female emitted 19.3% more *ZZ*7,11-16:Ac during 1985 (0.117

TABLE 1. EMISSION RATE OF ZZ7,11–16:Ac (ng/min) FROM THREE VALLEYS IN CALIFORNIA AND LABORATORY COLONY FOR 1982–1985

	$\bar{X}$ Emission rate (ng/min) of ZZ7,11–16:Ac + SD (*N*)			
	Coachella	Imperial	Palo Verde	Laboratory
1982	0.086 ± 0.049 (47)cde[a]	0.099 ± 0.054 (145)bc	0.100 ± 0.056 (216)bc	0.094 ± 0.050 (52)cd
1983	0.082 ± 0.033 (76)cde	0.074 ± 0.031 (54)e	0.092 ± 0.042 (227)cde	0.080 ± 0.046 (80)de
1984	0.115 ± 0.041 (90)ab	0.117 ± 0.037 (100)a	0.131 ± 0.046 (77)a	0.085 ± 0.049 (76)cde
1985	0.120 ± 0.034 (35)a	0.119 ± 0.042 (62)a	0.114 ± 0.049 (101)ab	0.090 ± 0.054 (83)cde

[a] Any means followed by the same letter are not significantly different ($P > 0.05$, analysis of variance and Duncan's new multiple-range test).

ng/min) than during 1982 (0.098 ng/min). The difference between field-collected females and the laboratory-reared population was greatest in 1984 when the former (0.120 ng/min) emitted 41.2% more *ZZ*7,11–16:Ac than the latter (0.085 ng/min). Unlike the results of analyses of volatiles from field-collected females, the emission rate of pheromone did not change from year to year in the laboratory population.

The blend ratio of the two pheromonal components showed no significant trend towards increasing or decreasing over the course of our sampling in either the field populations or the laboratory population (Table 2). No valley consistently had females that emitted a higher or lower blend ratio than those from the other valleys or the laboratory. The significant differences that were documented between valleys were small—the range of mean percent *ZZ*7,11–16:Ac was only 60.1–62.9.

For the subsample of fields that could clearly be separated according to their history of pheromone use, there was no relationship between this history and the emission rate or blend ratio of components (Table 3).

Worldwide Variation in Chemical Communication. The highest emission rate of *ZZ*7,11–16:Ac was found in females from Californian populations (0.119 ng/min), followed by a population from Egypt (0.103 ng/min) (Figure 1). The average emission rate from females from a Chinese population was 0.057 ng/min, and was significantly lower than the other populations with the exception of that from Brazil, which was intermediate (0.082 ng/min).

The Chinese population was also unusual in terms of the emitted blend ratio of pheromonal components (Figure 2). Females from this population emitted 57.5% *ZZ*7,11–16:Ac, significantly lower than all other populations sam-

TABLE 2. EMITTED BLEND RATIO OF TWO PHEROMONE COMPONENTS EXPRESSED AS PERCENT OF *ZZ*7,11–16:AC ISOMER IN TWO-COMPONENT BLEND OF FEMALES FROM THREE VALLEYS IN CALIFORNIA AND LABORATORY COLONY FOR 1982–1985

	$\bar{X}$ % *ZZ*7,11–16:Ac ± SD (*N*)			
	Coachella	Imperial	Palo Verde	Laboratory
1982	60.4 ± 4.2 (45)e[a]	62.4 ± 5.4 (118)a	62.1 ± 4.0 (192)ab	61.0 ± 3.8 (51)bcde
1983	61.0 ± 2.9 (75)bcde	60.6 ± 3.2 (54)de	60.8 ± 3.3 (217)de	60.6 ± 3.5 (77)de
1984	61.8 ± 1.8 (90)abcd	61.8 ± 2.0 (100)abcd	62.2 ± 2.1 (77)abc	61.0 ± 2.8 (72)bcde
1985	60.1 ± 1.7 (35)e	60.1 ± 2.5 (62)e	62.9 ± 2.6 (98)a	60.9 ± 2.9 (76)cde

[a] Any means followed by the same letter are not significantly different ($P = 0.05$, analysis of variance of arc sin $\sqrt{\%\ ZZ}$ transformed data and Duncan's new multiple-range test).

TABLE 3. EMISSION RATE AND BLEND RATIO OF PHEROMONE COMPONENTS FROM FEMALES FROM PHEROMONE-TREATED AND INSECTICIDE-TREATED FIELDS

	Emission rate[a] ± SD (*N*)		Blend ratio[b] ± SD (*N*)	
	Pheromone-treated	Insecticide-treated	Pheromone-treated	Insecticide-treated
1982	0.095 ± 0.056 (252)	0.102 ± 0.053 (156)	61.7 ± 4.2 (218)	61.9 ± 5.0 (137)
1983	0.087 ± 0.040 (183)	0.087 ± 0.038 (174)	61.0 ± 3.2 (183)[c]	60.3 ± 3.2 (174)[c]
1984	0.127 ± 0.044 (109)	0.117 ± 0.036 (68)	61.6 ± 2.2 (109)	61.9 ± 2.0 (68)
1985	0.114 ± 0.044 (77)	0.118 ± 0.045 (121)	61.2 ± 3.2 (77)	61.7 ± 2.5 (118)

[a] Emission rate is ng of *ZZ*7,11-16:Ac emitted per minute.
[b] Blend ratio is % *ZZ*7,11-16:Ac in two-component blend.
[c] Significantly different pair of means ($P < 0.05$, Analysis of variance).

pled. The overall range in mean percent *ZZ*7,11-16:Ac emitted was small (57.5-63.1).

Variation in Males' Response to Different Blend Ratios. Over the course of this experiment we captured 13,303 male *P. gossypiella*, of which 331 (2.5%) were marked at least once. The 70:30 blend of *ZZ*- to *ZE*7,11-16:Ac captured significantly fewer moths than the 40:60 blend, and there were no other significant difference between blends. Because these traps were approaching their numerical capacity to capture and retain males, the absolute numbers captured are not useful. However, the proportion of males marked with fluorescent powders and subsequently captured should provide an accurate estimate of the proportion of males that actually visited the marking station.

Fluorescent marks were detected in the following proportions: 100 pink, 94 blue, 101 yellow, and 82 orange (pink = 40:60, blue = 50:50, yellow = 60:40, and orange = 70:30 *ZZ*:*ZE*7,11-16:Ac). A 4 × 4 chi-square analysis revealed that there was no significant relationship between marking and recapturing at specific blend ratios ($\chi^2 = 4.2$, 9 *df*). Figure 3 illustrates that some individuals that had responded to specific marking blends were subsequently recaptured in every capture blend. Fourty-two males (0.3% of total number of males captured) were marked with two or more colors. They were captured in the following numbers 10 P/B, 5 P/Y, 5 P/O, 6 B/Y, 7 B/O, 9 Y/O. Of these males, 20 were captured at a blend that was different from any to which they were marked, indicating that they had responded to at least three blends. Two males were marked at three different blend ratios, and one of these had visited every blend ratio that was available when the capture blend was considered.

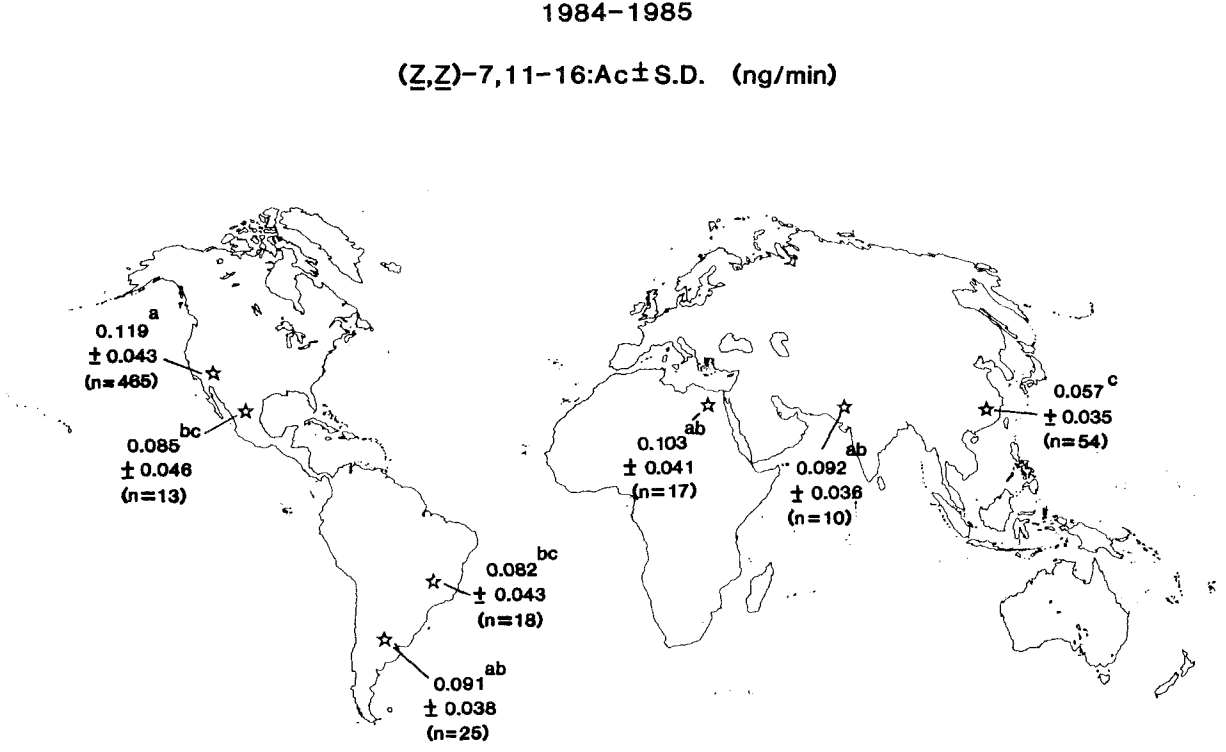

FIG. 1. Worldwide variation in the emission rate of *ZZ*7,11–16:Ac from the sex pheromone glands of female *P. gossypiella*. Means followed by the same letter are not significantly different ($P > 0.05$, analysis of variance and Duncan's new multiple-range test).

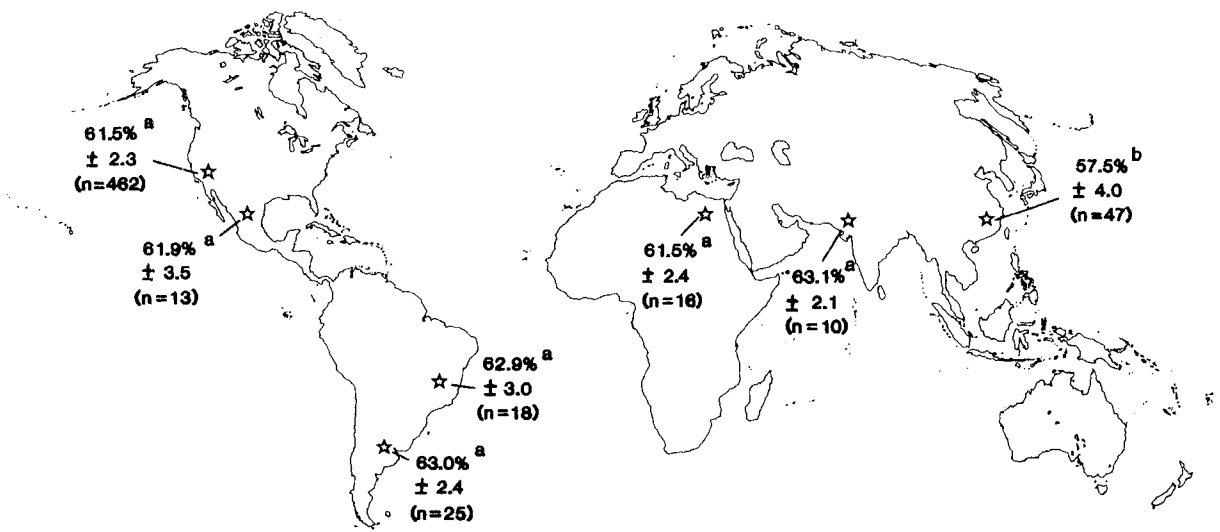

FIG. 2. Worldwide variation in the emitted blend ratio of ZZ7,11-16:Ac and ZE7,11-16:Ac expressed as % ZZ7,11-16:Ac, from female *P. gossypiella*. Means followed by the same letter are not significantly different ($P > 0.05$, analysis of variance of arc sin $\sqrt{\%\ ZZ}$ and Duncan's new multiple-range test).

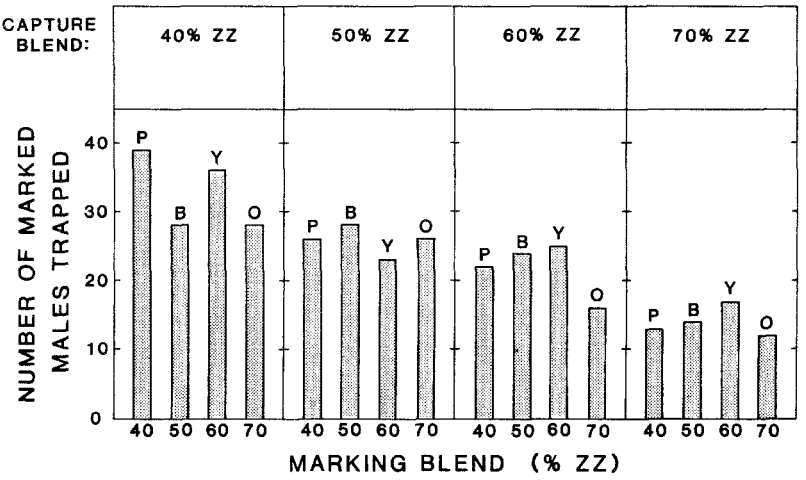

FIG. 3. The number of male *P. gossypiella* captured at indicated blend after being marked with fluorescent powders. Fluorescent marks established record of prior responses to specific blend ratios of pheromone components (P = pink = 40:60; B = blue = 50:50; Y = yellow = 60:40; O = orange = 70:30 *ZZ*- to *ZE*7,11-16:Ac).

DISCUSSION

The present study documents biological (phenotypic) variation in pink bollworm populations from southern California that has occurred over the four-year course of this experiment. Our data are not sufficient to unequivocally support the hypothesis that selection imposed by the use of mating disruption caused these changes. Rather this hypothesis should be considered as but one of a number of viable explanations, including undocumented changes in the environment with no underlying genetic change, selection imposed by some unknown factors that have resulted in the documented phenotypic change, or genetic drift with no underlying selection pressure. Regardless of the cause(s), however, the populations in 1984 and 1985 contained females emitting higher average amounts of pheromone and should have been, at least hypothetically, slightly less susceptible to the effects of synthetic disruptants than populations in previous years. We believe that any such advantage gained by females in these populations would have been too slight to result in significant loss of efficacy of the disruptant pheromone, and thus should not have had any major effect on overall population growth.

There is some indirect support for the idea that the increase in emission rate of pheromone in southern California in 1984 and 1985 could be related to the use of synthetic sex pheromone to disrupt mating. First, pheromones have been used there on a commercial basis for control of the pink bollworm moth

for over eight years. Second, field documentation of a possible selective advantage for a higher emission rate from point sources in disruptant-treated field was supplied by Doane and Brooks (1981), who showed that increasing emission rates of synthetic pheromone (*ZZ*- and *ZE*7,11-16:Ac) from traps lead to increasing captures of male moths in pheromone-treated fields. Third, in the laboratory, Collins and Cardé (1985) showed that the heritability of pheromone quantity was slightly greater than for blend quality, and they have been able to select for a line of *P. gossypiella* in which the females contain almost twice as much *ZZ*- and *ZE*7,11-16:Ac in their sex pheromone glands (personal communication). Thus the potential for a response to selection has been demonstrated in the laboratory.

One observation is not necessarily consistent with the hypothesis of selection for increased emission rate in field populations as a response to use of disruptant pheromone. In 1982 an areawide program was in effect in Imperial Valley in which all cotton growers utilized pheromone applications for control of *P. gossypiella* for part of the growing season. This program represented one of the most extensive uses of disruptant pheromones in agriculture to date, but it did not result in an immediate increase in pheromone emission rate from field-collected females. In fact, the emission rate from females collected in Imperial Valley in 1983 was 0.074 ng/min, the lowest average that was recorded.

Unlike the phenotypic variation in emission rate that was documented in field populations, the emission rate of *ZZ*7,11-16:Ac from laboratory females remained relatively constant throughout the four-year period. This consistency in the laboratory population was expected because of the constancy of environmental conditions and the lack of selection pressure for increased emission rate under laboratory conditions.

Based on our first year of sampling (Haynes et al., 1984), we anticipated that if any change occurred in the blend ratio, it would be an increase in the *ZZ* to *ZE* ratio, because a blend centered about a 50:50 ratio has been used as the standard commercial blend to disrupt mating, whereas females emit ca. a 60:40 blend. This could mean that directional selection might be imposed on the pheromone blend. However, in the present study the blend ratio of *ZZ*- to *ZE*7,11-16:Ac was relatively constant throughout the period of the study (Table 2). Selection on the emitted blend ratio may be more difficult because it would involve changes in both males and females, unless there was close genetic linkage between emission and response.

Our analyses of pheromone components emitted by females from around the world was consistent with greater interpopulational variation in the emission rate than in the blend ratio of components. While the blend ratios emitted by females from China were significantly lower that those from any other locality, the overall difference was small. In contrast, females from China emitted about half as much pheromone as those from southern California. It would be inter-

esting to obtain pink bollworm moths from Australia because *P. gossypiella* and *P. scutigera*, the pink-spotted bollworm, are sympatric in certain areas of this country. The two species respond to different blend ratios of *ZZ*- and *ZE*7,11–16:Ac (Rothschild, 1975), and thus interspecific selection pressure may have led to divergence of their blend ratios or to a narrowing of the broad response spectrum of males to blend ratios of the pheromone isomers. Our initial attempts to obtain sufficient numbers of moths from Australia were not successful because cotton production no longer occurs in areas where *P. gossypiella* occurs on wild plants (S.E. Learmonth and G.H.L. Rothschild, personal communication).

If selection is resulting in the changes in the pheromone communication system of *P. gossypiella*, then why are we unable to detect these differences between pheromone- and insecticide-treated fields in southern California? One possibility is that local differences between populations may be swamped by migration between fields within a valley and possibly between valleys. While the potential impact of migration on gene flow has not been evaluated in any quantitative way, the occurrence of such migration is well documented (Bariola et al., 1973; Flint and Merkle, 1981; Stern, 1979).

Since our marking–capture analysis of variation in the response of males to blend ratios from 40:60 to 70:30 indicated that there was no evidence of phenotypic variation between individuals, the potential for rapid selection for a preadapted phenotype that responds specifically to the blend released by the female and not to the blend used to disrupt mating seems unlikely. The major source of variation in the response of males to blends comes from variation within individuals rather than between individuals. It would seem that in order for resistance involving blend ratios to evolve rapidly, the population would need to begin with males having narrower blend ratio response spectrums than we measured here. Cardé et al. (1976), who first applied this marking–capture technique to the Oriental fruit moth, *Grapholita molesta*, also found no evidence of phenotypic variation in the response of males to different blends of the pheromone components of that species.

Considering the results of our experiments, it is possible that the observed increase in the females' emission rate of pheromone may be an early response to selection imposed by the use of disruptant pheromones, but other undocumented factors may play a role in this biological variation that has been followed for four years. Evolutionary changes in the blend ratio may be inherently more difficult because males have a broad response spectrum to variation in blend ratio (Flint et al., 1979; Linn and Roelofs, 1985) with no detectable phenotypic variation between individuals. Even so, shifts in the emitted blend ratio and the responses of males to blend ratios should continue to be considered as a possibility that may be expressed in individuals in field populations over evolutionary time.

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DEGRADATION OF JUGLONE BY SOIL BACTERIA

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Abstract—Bacteria that can degrade juglone (5-hydroxy-1,4-naphthoquinone) were isolated from soil beneath black walnut trees. Autecological studies with one of these bacteria (*Pseudomonas* J1), demonstrated that it could grow rapidly using juglone as its sole source of carbon and energy. Using nonlinear regression analysis and the Monod equation, it was determined that this bacterium had a high affinity for juglone (K_s = 0.95 μg/ml). *Pseudomonas* J1 can also utilize other aromatic compounds from plants as its sole source of carbon and energy. Compounds such as chlorogenic acid, ferulic acid, gallic acid, and 2-hydroxy-1,4-naphthoquinone (Lawson) were rapidly degraded by *Pseudomonas* J1. The rapid degradation of juglone and other suspected allelochemicals by soil bacteria make it unlikely that these compounds are important mediators of plant–plant interactions under natural conditions.

Key Words—Allelopathy, biodegradation, humic acids, *Juglans nigra*, juglone, nonlinear regression, *Pseudomonas putida* biovar A.

INTRODUCTION

Juglone is a toxic chemical produced by *Juglans nigra* L. and other members of the walnut family (Juglandaceae) (Thomson, 1971). It has long been known that juglone is toxic to certain plants, and this toxicity has been invoked to explain the antagonistic effect of black walnut trees on neighboring vegetation (Davis, 1928, Rietveld, 1983). Fisher (1978) presented evidence that the allelopathic potential of juglone was not expressed in well-drained soils, and he attributed this to the aerobic microbial mineralization of juglone in these soils. He went on to reason that, in wet soils, aerobic microorganisms capable of metabolizing juglone were not active, and therefore juglone was able to build up to concentrations high enough to inhibit the growth of red pines. However,

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no evidence was presented by Fisher (1978) or subsequent workers (Rietveld, 1983; Rietveld et al., 1983) that juglone was metabolized by soil microorganisms. Other hypotheses for the disappearance of juglone from well-drained soils, such as increased leaching, incorporation into soil organic matter (Mathur, 1972; Saiz-Jimenez et al., 1975), or sorption onto the mineral fraction of the soil also have not been explored (Fisher, 1987).

Many studies have demonstrated the microbial breakdown of simple aromatic compounds in soil (Alexander, 1977; Reber, 1975), but very little work has been done on the metabolism of polycyclic quinones such as juglone. Polycyclic quinones are toxic, very reactive, and therefore more likely to be incorporated into the humic fraction of soil than to be completely metabolized by soil microorganisms (Saiz-Jimenez et al., 1975). The present study was undertaken to determine if soil microorganisms are able to breakdown juglone at concentrations likely to occur under field conditions.

METHODS AND MATERIALS

Bacterial Isolate. Soil samples were collected from the top 10 cm of soil beneath a stand of black walnut trees in Boulder County, Colorado. Soil was also collected 20 m from the walnut trees at a site in which no black walnut or other trees were growing. These soils were clay-loams of the Niwot series and are characterized as being somewhat poorly drained and mildly alkaline (Moreland and Moreland, 1975). Five soil samples from each site were used in enrichment studies to determine if juglone-utilizing microorganisms could be isolated. Juglone (98%, Aldrich Chemical Co., Milwaukee, Wisconsin) was added to 3-g soil samples in sterile 20-ml vials at a concentration of 2 μg/g of soil (2 ppm). After seven days, an additional 6 μg of juglone in 0.2 ml of distilled water was added to the soil. This process was repeated twice more, and 0.5 g of soil was then transfered to 100 ml of distilled water containing inorganic salts and 5 μg of juglone per milliliter. The culture was incubated for seven days at 23°C. Serial dilutions were then plated on a medium containing inorganic salts, 5 μg of juglone and 15 mg of Difco Bacto-Agar per milliliter. Isolated colonies were selected and streaked on juglone plates several times successively until pure cultures were obtained.

Identification of the bacteria obtained from each soil was done using standard bacteriological techniques (Palleroni, 1984). Flagella were stained using the technique of Heimbrook et al. (1986), and the capacity of the organisms to metabolize allelopathic compounds other than juglone was tested using the auxanographic technique as described by Parke and Ornston (1984). The inorganic salts solution used in all enrichments and experiments contained 750 μg KH_2PO_4, 480 μg Na_2HPO_4, 40 μg NH_4Cl, 10 μg $MgSO_4 \cdot 7H_2O$, 10 μg $CaCl_2 \cdot 2H_2O$, and 0.02 μg $FeCl_3 \cdot 6H_2O$ per milliliter of distilled–deionized water.

Three of the enrichments from the walnut soil yielded bacteria capable of metabolizing juglone. One of these bacteria was chosen at random for further studies and is henceforth designated as *Pseudomonas* J1. Stock cultures of *Pseudomonas* J1 were grown in the inorganic salts solution containing 5 μg of juglone per milliliter. The inoculum of *Pseudomonas* J1 used in the experiments described below was grown in inorganic salts solution containing 10 μg of juglone per milliliter. Cells were grown to the early stationary phase and were then diluted with inorganic salts solution to obtain the initial cell densities used in the experiments. Experiments were conducted in glass-stoppered 250-ml Erlenmeyer flasks containing 100 ml of the inorganic salts solution and the indicated concentrations of juglone. The flasks were incubated at 23°C without shaking. At regular intervals, the number of cells was determined by using the spread-plate technique. Triplicate 0.1-ml portions of 10-fold dilutions were plated on a medium containing 15 mg of Difco Bacto-agar and 3 mg of Trypticase soy broth with glucose (BBL, Cockeysville, Maryland) per milliliter of deionized water, and colony counts were made after 72 hr of incubation at 23°C. The data represents means of triplicate plate counts from individual flasks.

Juglone concentrations were determined with a spectrophotometer. To obtain a standard curve, juglone was dissolved in the inorganic salts solution at concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 20, and 40 μg/ml. These solutions were acidified with concentrated sulfuric acid to a pH of approximately 0.8 and the absorbance was read at 420 nm. During the experiments, 2-ml samples were removed from the cultures and immediately acidified with one drop of concentrated sulfuric acid to a pH of approximately 0.8 to stop all microbial activity in the sample. The absorbance values for each sample were then determined and the juglone concentration was calculated using the standard curve.

Data Analysis. Nonlinear regression analyses were performed on the curve of juglone disappearance. For this purpose, the MARQFIT computer program was used (Simkins and Alexander, 1984). This program fits nonlinear data by minimizing the square of the differences between the data and the model curve using the Marquardt method (Bard, 1974). Nonlinear regression is a statistical tool that can be used to determine values for ecologically important parameters describing the growth and metabolism of microorganisms (Schmidt et al., 1985b). This information can then be used to help estimate the persistence of organic chemicals in the environment.

RESULTS

Juglone-metabolizing cultures were isolated only from soils collected under black walnut trees. Bacteria that could tolerate juglone were obtained from the other soil samples, but these bacteria could not metabolize juglone even after

three months of incubation in the presence of juglone. In addition, juglone-utilizing cultures did not develop in flasks containing juglone but no soil, when these were left open to the laboratory air for three months. Therefore, it is reasonable to conclude that the bacteria obtained from soils beneath walnut trees were indigenous to those soils.

Pseudomonas J1 is a short (1–2 μm) gram-negative rod with one to four polar flagella, requires no growth factors, reacts positively to the oxidase and catalase tests, and can hydrolyze arginine under anaerobic conditions. In addition, *Pseudomonas* J1 produces a yellow fluorescent pigment when grown under iron limitation. These characteristics warranted placing it in rRNA group I of the genus *Pseudomonas* (Palleroni, 1984). Of the species in rRNA group I, *Pseudomonas* J1 matches almost exactly the characteristics of *P. putida* biovar A (Palleroni, 1984).

Table 1 summarizes the results of tests conducted with a number or organic compounds to determine what compounds could serve as sole carbon and energy sources for *Pseudomonas* J1. Many of the aromatic compounds have been reported to be allelochemicals (Rice, 1984). In addition to these suspected allelochemicals, other compounds such as sugars and amino acids were also tested for their ability to support the growth of *Pseudomonas* J1. Of note in these results was the inability of this organism to utilize most of the simple sugars that were tested. Only glucose and fructose were degraded, and even these sugars were not used as rapidly by *Pseudomonas* J1 as were the aromatic compounds tested.

Pseudomonas J1 was able to metabolize juglone and grow rapidly using this compound as its sole source of carbon and energy (Figure 1). In the inorganic salts solution in absence of juglone, no growth of *Pseudomonas* J1 was detected (Figure 1), and in the inorganic salts solution in the absence of *Pseudomonas* J1, no degradation of juglone was observed (data not shown). The yield of *Pseudomonas* J1 cells was also proportional to the concentration of juglone (1.4×10^7 and 3.1×10^7 cells/ml for 10 and 20 μg of juglone per milliliter, respectively), as would be expected for an organism using a substrate as its sole source of carbon and energy. The cell yields obtained are also close to those for other soil *Pseudomonas* spp. that completely mineralize simple aromatic compounds such as phenol (Schmidt et al., 1985b).

The data presented in Figure 2 show the disappearance of juglone when *Pseudomonas* J1 utilized juglone as its only source of carbon and energy. A similar curve of juglone disappearance was obtained with an initial juglone concentration of 20 μg/ml (data not shown). The data for the mineralization of 10 μg of juglone per milliliter was fit by the Monod equation using nonlinear regression analysis (Simkins and Alexander, 1984). From this analysis, the half saturation constant (K_s) for the growth of this organism on juglone was estimated to be 0.95 ± 0.71 μg/ml ($P = 0.05$). The values for the other parameters

TABLE 1. ORGANIC COMPOUNDS THAT CAN (+) OR CANNOT (−) ACT AS SOLE SOURCE OF CARBON AND ENERGY FOR GROWTH OF *Pseudomonas* J1

Aromatic compounds		Sugars	
Anthranilic acid	−	Lactose	−
Benzoic acid	+	D-Mannitol	−
Caffeic acid	+	D-Mannose	−
Catechol	+	L-Rhamnose	−
Chlorogenic acid	+	D-Ribose	−
trans-Cinnamic acid	−	Starch (soluble)	−
Ferulic acid	+	Sucrose	−
Gallic acid	+	Trehalose	−
p-Hydroxybenzoic acid	+	D-Xylose	−
m-Hydroxybenzoic acid	−	Amino acids	
2-Hydroxy-1,4-naphthoquinone	+	Aspartic acid	+
Naphthalene	−	Arginine	+
1-Naphthol	−	Betaine	+
Naphthoresocrinol	+	Glutamic acid	+
Phenol	−	Glycine	+
Protocatechuic acid	+	L-Lysine	+
Quinic acid	+	L-Ornithine	+
Salicin	−	L-Phenylalanine	+
Salicyclic acid	+	Serine	+
Tannic acid	+	D-Tryptophan	−
p-Toluic acid	−	L-Tryptophan	−
Vanillic acid	+	Other compounds	
Vanillin	+	Acetic acid	+
Sugars		Citric acid	+
L-Arabinose	−	Ethanol	+
D-Fructose	+	Fumaric acid	+
Glucose	+	Glycerol	+
i-Inositol	−	Succinic acid	+
m-Inositol	−	L-Tartaric acid	+
Lactose	−	Testosterone	−

of the Monod equation were $X_0 = 0.53 \pm 0.18$ μg/ml, $\mu_{max} = 0.47 \pm 0.09$/hr, and $S_0 = 9.76 \pm 1.3$ μg/ml. X_0 is the initial population density expressed in terms of juglone concentration, μ_{max} is the maximum specific growth rate for *Pseudomonas* J1 on juglone, and S_0 is the program's estimate for the actual initial concentration of juglone. Independent estimates of these parameters also were determined from the growth data shown in Figure 1 and simply by knowing the concentration of juglone and cells added at the beginning of the experiment. Thus, $X_0 = 0.63$ μg/ml, $\mu_{max} = 0.56$/hr, and $S_0 = 10.0$ μg/ml. These independent parameter estimates all fall within the 95% confidence intervals of the computer-generated estimates from the data presented in Figure 2.

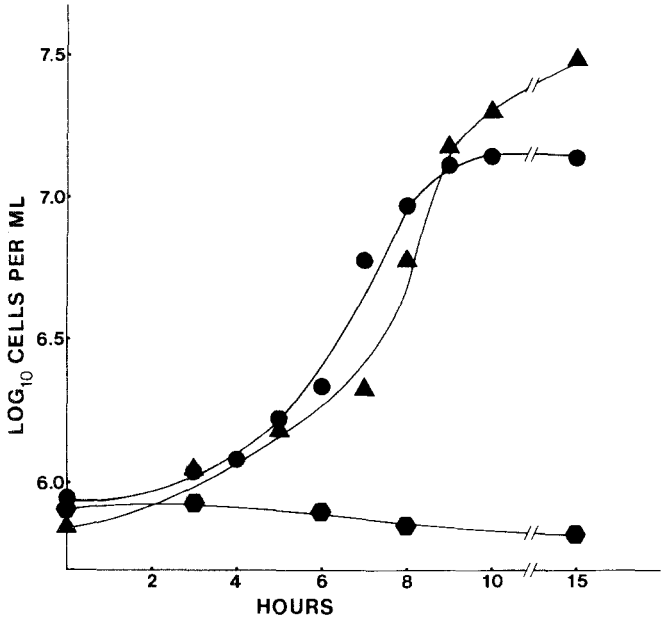

FIG. 1. Growth of *Pseudomonas* J1 on juglone at concentrations of 0 (⬣), 10 (●), and 20 (▲) μg/ml.

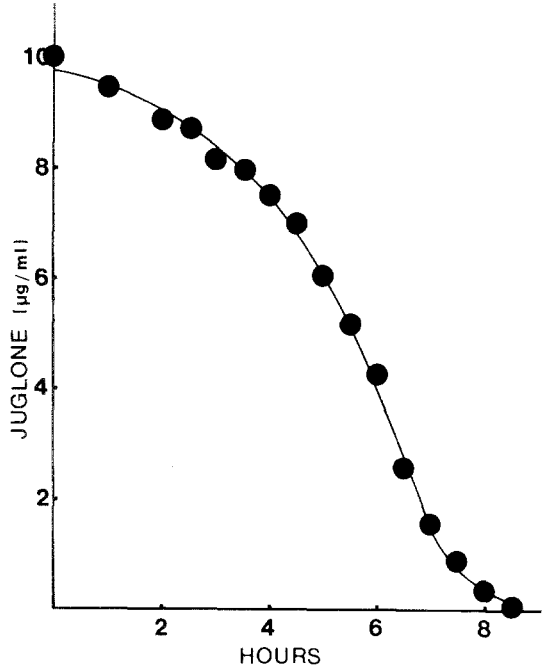

FIG. 2. Mineralization of 10 μg of juglone per milliliter by *Pseudomonas* J1. The curve is the best fit of the Monod equation to the experimental data (●). The curve was fit using nonlinear regression.

DISCUSSION

The presence of juglone-metabolizing bacteria in soils collected from under black walnut trees was confirmed in this study. This finding supports the hypothesis of Fisher (1978) that juglone is degraded and thus not allelopathically active in well-aerated soils. Juglone-mineralizing bacteria were not found in adjacent soils not exposed to black walnut litter or roots, indicating that juglone-utilizing bacteria are selected for in soils beneath black walnut trees. Juglone is structurally dissimilar to many aromatic compounds found in the soil solution (Alexander, 1977), and thus organisms adapted to metabolize simple compounds such as benzoate or phenol would not be expected to metabolize juglone. *Pseudomonas* J1, however, was able to grow on many simple aromatic compounds commonly reported to occur in soil. Compounds such as caffeic, chlorogenic, ferulic, and gallic acids have been reported to be allelochemicals (Rice, 1984), but most of these are readily degraded by a wide range of soil microorganisms (Alexander, 1977; Reber, 1975; Schmidt et al., 1987; Turner and Rice, 1975).

This is the first report of the microbial degradation of juglone. Previous work on the metabolism of polycyclic quinones has shown the fungus *Penicillum notatum* to be able to partially degrade lapachol (Rosazzo, 1982), an antitumor naphthoquinone from the wood of several species in the Bignoniaceae, Proteaceae, and Verbenaceae families (Thomson, 1971). *P. notatum* was able to carry out the partial degradation of lapachol using a monooxygenase enzyme under aerobic conditions (Rosazzo, 1982). Martin and Haider (1979), demonstrated that complexed anthroquinone mixtures were degraded by soil microorganisms, but they did not isolate the responsible organisms from soil.

Saiz-Jimenez et al. (1975) presented evidence for a different fate of polycyclic quinones in soil. In their work, the fungus *Eurotium echinulatum* catalyzed the oxidative linkage of anthroquinones and phenols to form dark-colored pigments similar to humic acids. Other workers have stressed the likelihood of an important role for polycyclic quinones in the formation of humic acids in soil (Mathur, 1972; Steelink and Tollin, 1967). Quinones also are very reactive with amino acids and proteins in the soil solution, forming complex molecules that are resistant to microbial degradation (Smith, 1982). Therefore, under aerobic conditions in soil, a multitude of fates is possible for polycylic quinones such as juglone. The fate of juglone in a particular soil may depend on several factors, such as aeration and the types of microorganisms present, but in any case it seems unlikely that juglone would persist in an unmodified form under aerobic conditions in soil in which black walnut trees are growing.

There is also reason to believe that juglone would not persist in an unmodified form under anaerobic conditions in soil. Recent work has demonstrated the bacterial breakdown of many aromatic compounds in anaerobic soil (Young, 1984). Soil bacteria can degrade aromatic compounds either by anaerobic (nitrate) respiration (Williams and Evans, 1975) or under methanogenic con-

ditions (Balba et al., 1979; Young, 1984). Even recalcitrant compounds such as halogenated benzoates are readily degraded by certain bacteria under anaerobic conditions (Suflita et al., 1982). In addition, 1,4-naphthoquinone and other naphthoquinones, which are structurally very similar to the K vitamins, have been shown to be utilized as growth factors by anaerobic bacteria such as *Succinivibrio dextrinosolvens* (Gomez-Alarcon et al., 1982). Thus, juglone may be subject to anaerobic attack, especially in soils that are repeatedly exposed to prolonged flooding. The persistence of juglone under wet moisture regimes in the lab (Fisher, 1978) may be due to the slower breakdown of juglone under anaerobic conditions, rather than a complete lack of degradation. It is also noteworthy that in his laboratory studies, Fisher (1978) used a soil that is not normally exposed to either anaerobic conditions or juglone. Thus, anaerobic bacteria adapted to metabolize juglone were probably absent from the soil used by Fisher (1978). Controlled studies are needed to determine if the die off of trees in certain soils is caused by juglone or if the effect is simply the result of anaerobiosis itself.

Autecological studies of microorganisms have been greatly enhanced by the use of nonlinear regression analysis and other computer-assisted techniques (Holder-Franklin and Tate, 1986; Simkins and Alexander, 1984). In the present study, the kinetic parameters obtained can be used to estimate the rate at which juglone will be degraded at different concentrations in the soil solution. Using the Monod equation and nonlinear regression techniques, an estimate for the half-saturation constant (K_s) for the utilization of juglone by *Pseudomonas* J1 was determined to be 0.95 μg/ml. K_s is inversely proportional to an organism's affinity for a given organic compound. A low K_s indicates a high affinity for the compound. At juglone concentrations greater than K_s, *Pseudomonas* J1 will be able to metabolize juglone at a rate approaching the maximum possible rate for this bacterium. The implications of this are that free juglone is unlikely to persist for long in the soil solution at concentrations around or above K_s. At concentrations below K_s, *Pseudomonas* J1 will metabolize juglone according to pseudo-first-order kinetics (Schmidt et al., 1985b). That is, juglone will be metabolized at a rate that is directly proportional to both the concentration of juglone and the size of the microbial population mineralizing juglone. The high affinity of *Pseudomonas* J1 for juglone is also evident from the rapid growth rate that this organism exhibited when growing on 10 μg of juglone per milliliter as its only source of carbon and energy.

At a juglone concentration approaching two orders of magnitude below K_s, *Pseudomonas* J1 may degrade juglone very slowly or not at all. Bacteria can exhibit threshold concentrations below which they are unable to mineralize organic compounds (Schmidt et al., 1985a). The threshold concentration for a *Pseudomonas* sp. that mineralizes *p*-nitrophenol (PNP) was approximately 100× lower than its K_s for growth on PNP (Schmidt et al., 1987). If *Pseudomonas*

J1 behaves similarly to this organism, it should be able to mineralize juglone at concentrations as low as 0.0095 μg/ml, a concentration below 10^{-7} molar. This is probably a conservative estimate of the lower limit for the degradation of juglone in soil. Scow et al. (1986) have demonstrated the rapid mineralization of many complex aromatic compounds at concentrations well below 1 ng/g of soil. In any case, *Pseudomonas* J1 is probably capable of mineralizing juglone at concentrations well below the lowest concentration that has elicited phytotoxicity, 10^{-6} molar (Rietveld, 1983), or significant toxicity to nitrogen fixing bacteria, 10^{-4} molar (Dawson and Seymour, 1983).

This study demonstrates the importance of considering the metabolic capabilities of soil microorganisms in studies of allelopathy (cf. Kaminsky, 1981). Soil microorganisms are carbon-limited most of the time (Lockwood and Filonow, 1981), and thus organisms such as *Pseudomonas* J1 are literally waiting to consume compounds such as juglone. The rapid degradation of potential allelochemicals by soil microorganisms should cause researchers to question claims of allelopathic activity if they are based solely on tests conducted in sterilized soil or laboratory media. Tests of the effects of a suspected allelochemical should be conducted using unsterilized soils from field sites where the alleged allelopathy is occurring. This simple procedure would give a more realistic assessment of the potential for a given chemical to cause allelopathic effects in nature.

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BIOLOGICAL ACTIVITY AND TENTATIVE IDENTIFICATION OF FLAVONOID COMPONENTS IN VELVETLEAF (*Abutilon theophrasti* Medik.) SEED COATS[1]

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Abstract—The biological activity of aqueous extracts of velvetleaf (*Abutilon theophrasti* Medik.) seed coats and their flavonoid components against three plant species and five soil fungi was investigated. Aqueous extracts slightly inhibited germination and significantly inhibited radicle growth of all plant species tested. Fungal growth was inhibited or not affected, depending on species, by aqueous extracts. The aqueous extracts were extracted into methanol and separated using paper chromatography for identification of compounds responsible for inhibition of seedling and fungal growth. Six flavonoid compounds were isolated and tentatively identified as delphinidin, cyanidin, quercetin, myricetin, (+)-catechin, and (−)-epicatechin. Bioassays revealed that the flavonoid compounds significantly inhibited germination and radicle growth of all test species at a concentration of 1.0 mM. Individual flavonoids had variable effects on fungi but appeared to inhibit growth and sporulation of potential seed-decomposing fungi rather than "beneficial" fungi. These results indicate that the flavonoid complement of velvetleaf seed coats may function in a dual defensive role against competing seedlings and seed-attacking fungi.

Key Words—*Abutilon theophrasti* Medik., allelopathy, antifungal activity, flavonoids, seed germination, soil fungi, weed biology, weed seeds.

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INTRODUCTION

Velvetleaf is a highly competitive annual dicotyledonous plant that is a major weed problem in row crops grown in the United States (Spencer, 1984). Velvetleaf produces large numbers of seeds followed by high dispersability near the parent plant for addition to the seedbank on and in the soil. The majority of the seeds arriving on the soil are dormant (possessing hard and impermeable seed coats) and remain viable in soil for several years during which germination gradually occurs (Egley and Chandler, 1983; Lueschen and Andersen, 1980). The hard-seeded attribute is considered a major factor for the occurrence of velvetleaf as a serious weed of row crops (Egley and Chandler, 1983).

Chemical compounds present in velvetleaf seeds have been reported to have various effects on plant seedlings and microorganisms. Velvetleaf seeds are sources of inhibitors to crop seed germination and seedling elongation (Gressel and Holm, 1964) and of growth regulatory compounds that cause abnormalities in cabbage (*Brassica oleracea* L.) root parenchyma cells (Retig et al., 1972). LaCroix and Staniforth (1964) qualitatively determined phenolic compounds in water extracts of velvetleaf seeds that inhibited velvetleaf seed germination. Elmore (1980) examined the effects of aqueous extracts of velvetleaf seeds on turnip (*Brassica rapa* L.) seed germination and concluded that plant phenolic fractions of the extract likely contributed the most to the observed inhibitory effects and urged further investigation of these compounds. Recent laboratory studies demonstrated considerable inhibition of microbial growth by intact, dormant seeds of velvetleaf placed on agar culture plates (Kremer, 1986). Chemical analyses of the seed diffusates within the inhibition zones around the seeds revealed the presence of phenolic compounds.

The occurrence of allelochemicals and the prolonged persistence in soil of velvetleaf seeds suggests that the seeds may influence the ecological balance of the soil microenvironment. The allelochemicals in the velvetleaf seeds inhabiting niches in the soil environment may be advantageous by reducing the germination and growth of competing plants (Friedman and Waller, 1983) and by inhibiting the growth of potential seed decomposers (Kremer, 1986). However, the simultaneous effects of allelochemicals of dormant seeds on adjacent germinating seeds and soil microorganisms are not well understood. Furthermore, the seed coat of dormant seeds is the only seed structure encountered by germinating seeds and microorganisms in the soil environment and, therefore, these organisms likely respond differently to the seed coats than to the seed contents. It is a common practice to assay alcoholic extracts of intact seeds (seed coat plus seed contents) in studies of allelochemicals of seeds. This may yield misleading information on the potential interaction of organisms with seed coats alone. Also, seed extracts obtained using organic solvents are likely not representative of natural conditions where biologically active chemicals are leached

out of seeds by water. In the present study, flavonoids in aqueous extracts of velvetleaf seed coats were investigated because of their widespread distribution in seeds (Bate-Smith and Ribereau-Gayon, 1958; Feenstra, 1960; Halloin, 1982; Young and Paterson, 1980) and their documented effects on seedling plants (Popovici and Reznik, 1976; Weston et al., 1987) and microorganisms (Byrde, 1963; Flood and Kirkham, 1960; Kraft, 1977; Rubia et al., 1987).

The overall objective of this study was to determine if aqueous extracts of velvetleaf seed coats exert allelochemical influences on other plant species and soil fungi. Specific objectives were to isolate and identify flavonoid compounds present in velvetleaf seed coats that may contribute to allelochemical activity and to assess the biological activity of seed coat extracts and identified compounds on both plant and microbial species.

METHODS AND MATERIALS

Collection and Processing of Velvetleaf Seed Coats. Mature velvetleaf seeds were collected from a plant accession grown at the University of Missouri Agronomy Research Center, Columbia, Missouri, in July 1984. The intact seeds were fractionated into seed coat and embryo components by grinding in a blender for 45 sec and then separating them on a seed blower for 1 min (Kremer et al., 1984). Seed coats were ground in a Wiley mill (mesh screen size 1 mm) and stored in sealed glass containers until used for extraction procedures.

Extraction of Velvetleaf Seed Coats. Seed coats (10 g) were extracted for 48 hr with 100 ml sterile distilled water on a shaker (60 rpm) at 27°C. Five seed lots were extracted to provide enough experimental material. Mixtures were filtered through Whatman No. 4 paper to remove particulate matter. Upon microscopic examination, the aqueous extracts appeared free of microorganisms, indicating that microbial by-products likely would not contribute to toxicity of the extracts detectable under bioassay conditions. Total phenolic content of the aqueous extract was estimated using the Folin-Denis technique (Horowitz, 1980). The aqueous extract was also tested with 10% lead acetate in 10% acetic acid and with 1% ferric chloride to detect the presence of tannins (Robinson, 1980). A 0.1% aqueous solution of tannic acid served as a control for these tests. The vanillin–HCl reaction (Broadhurst and Jones, 1978; Robinson, 1980) was used to detect catechins and leucoanthocyanidins in the aqueous extract. A solution containing 1 mg (+)-catechin per 1 ml methanol was used as a positive control.

Isolation and Characterization of Flavonoids from Extracts of Velvetleaf Seed Coats. The aqueous seed coat extract was evaporated to dryness by rotary evaporation at 45°C after which the dried residues were dissolved in 70% methanol (15 ml). The concentrated extracts were applied (20 μl) to Whatman No.

1 chromatography paper and developed two dimensionally in BAW (*n*-butanol–acetic acid–water, 4:1:5, upper phase) and in Forestal solvent (acetic acid–conc HCl–water, 30:3:10) (Harborne, 1984). After development and drying, the chromatograms were examined visually with and without longwave UV light and the position and color of each spot recorded. Chromatograms were also sprayed with 10% vanillin in conc HCl to detect the presence of catechins (Robinson, 1980). The flavonoid spots on the two-dimensional chromatograms were tentatively identified by direct comparison with authentic flavonoids through cochromatography. Cochromatography with authentic compounds is required to confirm that flavonoids occupying different positions on the chromatogram are different and those occupying similar positions are indeed the same compound (Harborne, 1984; Markham, 1982).

Seed Germination and Seedling Growth Bioassays. Concentrated aqueous extracts were diluted with sterile distilled water to concentrations representing 25, 50, and 100 mg seed coat per milliliter. Individual commercially available compounds, selected on the basis of chromatographic identifications, were initially dissolved in acetone followed by diluting with distilled water to a final concentration of 1 mM. Five milliliters of each test solution was added separately to sterile glass Petri dishes lined with Whatman No. 3 filter paper. A distilled water control was also included. Biological activity of the test solutions was assayed using seeds of cress [*Lepidium sativum* (L.) cv. Curlycress], radish (*Raphanus sativus* L. cv. Sparkler White Tip), and soybean [*Glycine max* (L.) Merr. cv. Williams 82]. Seeds were surface-sterilized by immersion in 70% ethanol for 4 min and then rinsed 10 times with sterile distilled water. Treatments consisted of 20 evenly spaced seeds per dish. Four replicates of each treatment were arranged in a completely randomized design and were repeated twice. Treated seeds were incubated in the dark at 27°C. Germination (radicle protrusion through the seed coat) and radicle lengths of all species were measured at 72 hr. Data were subjected to analyses of variance, and F values and 5% least significant differences were calculated to determine significance of treatment effects in each bioassay.

Fungal Growth Bioassays. Aqueous extracts of velvetleaf seed coats and test compounds at concentrations described previously were added to separate 125-ml Erlenmeyer flasks containing 50 ml sterile mineral salts medium. All test solutions were added through 0.2-μm filter membranes. The medium contained sodium nitrate (2.0 g/liter), dipotassium phosphate (1.0 g/liter), magnesium sulfate (0.5 g/liter), and potassium chloride (0.5 g/liter). Sucrose (15 g/liter) was added to control flasks. The fungi *Aspergillus niger*, *Gliocladium roseum*, *Penicillium diversum*, *Trichoderma viride*, and *Fusarium* sp. previously isolated from soil (Kremer et al., 1984) were used as indicator species. The fungi were cultured for five days on potato dextrose agar (PDA) after which a 5-mm plug of agar and mycelia from the actively growing edge of the fungus

was used as inoculum for each flask. Four replicate flasks were prepared for each test solution–fungus combination. The cultures were incubated in the dark at 27°C. After 14 days, mycelial mats were filtered onto preweighed filter papers and dried for 24 hr at 120°C prior to weighing. Data were analyzed as described previously.

RESULTS AND DISCUSSION

Aqueous extracts prepared from velvetleaf seed coats significantly decreased germination of cress seeds at extract concentrations of 50 and 100 mg seed coat extracted per milliliter of water (Table 1). Germination of radish and soybean seeds was significantly decreased only at 100 mg seed coat extracted per milliliter. Seed coat extracts at all concentrations inhibited radicle growth of all test species. Elmore (1980) reported similar inhibitory activity of aqueous extracts of whole velvetleaf seeds on both germination and radicle growth of turnip. Our results also agree with previous studies concerning bioassays of plant extracts in that seedling growth appears to be more sensitive to the effects of allelochemicals than does germination (Panasiuk et al., 1986; Rasmussen and Einhellig, 1977; Weston et al., 1987). In addition to effects on radicle growth, seedlings growing in seed coat extracts were generally less vigorous with greater occurrences of abnormal development, necroses, and chlorosis.

Seed coat extract concentrations at 25 mg seed coat extracted per milliliter of water had no significant effect on the growth of the test fungal species (Table 2). However, seed coat extract concentrations of 50 and 100 mg/ml inhibited growth of *A. niger*, *P. diversum*, and *Fusarium* sp., while the growth of *G. roseum* and *T. viride* were not affected. It appears that inhibitory activity of seed coat allelochemicals is dependent on both the nature of the fungal test

TABLE 1. EFFECTS OF AQUEOUS EXTRACTS OF VELVETLEAF SEED COATS ON GERMINATION AND SEEDLING GROWTH OF SELECTED PLANT SPECIES

	Cress		Radish		Soybean	
Extract concentration (mg seed coat/ml)	Germination (%)	Radicle length (mm)	Germination (%)	Radicle length (mm)	Germination (%)	Radicle length (mm)
0	94	14.0	100	16.2	90	24.4
25	94	9.6	98	11.2	82	22.0
50	85	9.2	90	9.2	82	14.2
100	80	2.4	88	7.8	70	10.1
LSD (0.05)	6	2.7	11	1.1	14	1.8

species and the concentration of the allelochemicals in the seed coat extract. Similar differential effects of allelochemical extracts have been observed for fungi associated with intact oat caryopses (Picman et al., 1984) and for soil fungi in contact with *Eupatorium riparium* residues (Rai and Tripathi, 1984). Extracts at concentrations of 50 and 100 mg seed coat per milliliter also inhibited sporulation of *A. niger*, *P. diversum*, and *Fusarium* sp. This is supported by previous reports on the inhibitory properties of seed coats on fungal sporulation (Picman et al., 1984; Kremer, 1986).

The aqueous seed coat extracts reacted positively to lead acetate and ferric chloride reagents and yielded a positive vanillin–HCl test. These reactions are consistent with the presence of phenolic compounds and, regarding the vanillin–HCl test, condensed tannins (Broadhurst and Jones, 1978; Robinson, 1980). The concentration of total phenolic compounds determined by the Folin-Denis method was 0.55 mg tannic acid equivalents per gram seed coats. Although this method does not measure any specific tannin or phenolic compound, a relative measure of phenolic content present can be used to assess the significance of these compounds in the total composition of the seed coat.

Paper chromatography of seed coat extracts revealed six distinct spots visualized under UV light or by the vanillin–HCl reagent (Table 3). The compounds, tentatively identified by cochromatography with authentic compounds, were members of the polyphenolic class of chemicals collectively known as flavonoids (Harborne, 1984). The intensities of the flavonoid spots on the chromatograms indicated that delphinidin was the major component, with catechin, epicatechin, and quercetin appearing as intermediate components. Cyanidin and myricetin were determined to be minor components. To our knowledge, the six flavonoid compounds detected are reported for velvetleaf seed coats for the first time. The classification of flavonoid compounds detected in this study is largely

TABLE 2. EFFECTS OF AQUEOUS EXTRACTS OF VELVETLEAF SEED COATS ON MYCELIAL GROWTH AND SPORULATION OF SOIL FUNGI

Extract concentration (mg seed coat/ml)	Mycelial dry weight (mg)				
	A. niger	*Fusarium* sp.	*G. roseum*	*P. diversum*	*T. viride*
0	103	102	75	80	30
25	91 (±)[a]	93 (±)	66 (++)	75 (+)	25 (++)
50	57 (−)	46 (−)	65 (++)	56 (−)	36 (+)
100	16 (−)	33 (−)	62 (+)	28 (−)	44 (+)
LSD (0.05)	13	24	14	22	9

[a] Visual sporulation ratings: (++), sporulation > control; (+), sporulation = control; (±), sporulation < control; (−), no sporulation.

TABLE 3. CHROMATOGRAPHIC DATA FOR FLAVONOIDS OF VELVETLEAF SEED COATS

Spot	R_f (×100) BAW[a]	R_f (×100) Forestal[b]	Color Vis/UV[c]	Compound
1	40	30	Purple/D	Delphinidin
2	43	28	Yellow/F	Myricetin
3	62	40	Yellow/F	Quercetin
4	66	49	Magenta/D	Cyanidin
5	65	55	Yelow/F	(−)-Epicatechin
6	75	63	Yellow/F	(+)-Catechin

[a] BAW: *n*-butanol–acetic acid–water, 4 : 1 : 5, upper phase.
[b] Forestal: acetic acid–conc HCl–water, 30 : 3 : 10.
[c] Reaction in UV light: D, dark absorbing; F, fluorescent.

preliminary and absolute identification using more detailed chemical and spectral techniques (Harborne, 1984; Markham, 1982) is necessary for specific analyses of the total chemical composition of the seed coats. Cyanidin and delphinidin have been shown to be common constituents in seed coats of several plant species (Bate-Smith and Ribereau-Gayon, 1958). However, species of Malvaceae, which include velvetleaf, were not included in previous analyses. Catechin, identified in cottonseed, was shown to be the major precursor of tannins in that malvaceous species (Halloin, 1982).

The flavonoids detected using chromatography were bioassayed to determine if these compounds might contribute to the inhibitory activity of velvetleaf seed coat extracts against plant and fungal species. The compounds were assayed at a concentration of 1.0 mM, a level approximating that occurring in the soil solution for other phenolic compounds (Williams and Hoagland, 1982) and a level that would likely occur in soil microsites adjacent to seeds. Bioassays revealed that flavonoid components had significant inhibitory effects on both germination and radicle growth of all three test species (Table 4). Visual observations included lack of root hair and/or lateral root formation and considerable necrosis of the developing root. Little information is available on the influence of flavonoids on seed germination and seedling growth. Quercetin and myricetin were shown to inhibit protoplasmic streaming in oat root hairs (Popovici and Reznik, 1976). Tricin, a flavonoid isolated from quackgrass [*Agropyron repens* (L.) Beauv.] shoots, inhibited seedling growth of several plant species (Weston et al., 1987).

The overall effects of individual flavonoids on the growth of soil fungi contrasted among fungal species (Table 5). For example, *T. viride* was considerably more tolerant of delphinidin, catechin, and epicatechin than was *Fusar-*

TABLE 4. EFFECTS OF FLAVONOID COMPOUNDS ON GERMINATION AND SEEDLING GROWTH OF SELECTED PLANT SPECIES

Compound	Cress		Radish		Soybean	
	Germination (%)	Radicle length (mm)	Germination (%)	Radicle length (mm)	Germination (%)	Radicle length (mm)
Delphinidin	19	3.4	56	3.0	62	9.5
Cyanidin	10	7.2	68	2.1	34	12.5
Myricetin	18	7.0	50	3.2	66	12.5
Quercetin	10	5.1	62	3.0	14	5.0
Catechin	14	5.8	45	2.2	14	7.5
Epicatechin	9	6.2	45	2.0	0	
Control	100	15.2	100	13.2	92	29.2
LSD (0.05)	4	3.0	6	3.3	7	4.4

ium sp. Quercetin significantly inhibited the growth of *A. niger* and *Fusarium* sp. but had no effect on the other fungi. All fungi were generally tolerant of or were stimulated by cyanidin. Although fungi were able to grow on several of the flavonoids, sporulation was commonly inhibited by these compounds. Previous studies have also shown that catechin and epicatechin may inhibit invading seed fungi (Byrde, 1963; Halloin, 1982). Delphinidin, a major flavonoid

TABLE 5. EFFECTS OF FLAVONOID COMPOUNDS ON MYCELIAL GROWTH AND SPORULATION OF SOIL FUNGI

Compound	Mycelial dry weight (mg)				
	A. niger	*Fusarium* sp.	*G. roseum*	*P. diversum*	*T. viride*
Delphinidin	20 (−)[a]	56 (−)	76 (±)	85 (+)	43 (−)
Cyanidin	53 (+)	186 (+)	116 (−)	106 (±)	70 (−)
Myricetin	6 (−)	80 (−)	70 (−)	96 (−)	40 (−)
Quercetin	26 (±)	66 (±)	76 (++)	96 (−)	40 (++)
Catechin	53 (+)	75 (+)	76 (++)	56 (−)	50 (++)
Epicatechin	50 (+)	43 (−)	53 (−)	66 (±)	36 (−)
Control	55	132	80	76	46
LSD (0.05)	8	16	9	14	6

[a] Visual sporulation ratings: see Table 2.

component in the testa of pea (*Pisum sativum* L.) accessions, is fungistatic and inhibits sporulation of the pathogen *Fusarium solani* f.sp. *pisi* (Kraft, 1977).

From the results of this study it may be seen that the flavonoid complement of velvetleaf seed coats shows biological activity in both seed coat extracts and as individual components against each type of test organism, thus suggesting a dual defensive role. This study supports the suggestion of Friedman and Waller (1983) that the allelopathic potential of seeds, produced in large numbers by the parent plant, i.e. velvetleaf, favors seedling establishment by reducing the germination and growth of competing adjacent vegetation. On a similar basis, allelochemicals likely protect weed seeds against microbial attack, as suggested by Halloin (1983). Indeed, these compounds may regulate the composition of organism populations in the seed–soil microenvironment. This is exemplified by the ability of certain seed coat flavonoids to inhibit the growth and/or sporulation of *Fusarium*, a potential seed pathogen, while not detrimentally affecting the activity of more beneficial (to the seed) antibiotic-producing fungi such as *Trichoderma* and *Gliocladium* spp. Elucidation of the presence and types of compounds present in seed coats and their effects on other organisms in vitro provides a basis for future studies aimed at demonstrating their ecological role under natural conditions. The impact of allelochemicals in seed coats of many hard-seeded plant species may be most pronounced in their interactions with other organisms when focused on the soil microsite perspective rather than on the broader soil system.

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BENEFICIAL ARTHROPOD BEHAVIOR MEDIATED BY AIRBORNE SEMIOCHEMICALS. III. Influence of Age and Experience on Flight Chamber Responses of *Microplitis demolitor* Wilkinson[1]

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Abstract—*Heliothis zea* (Boddie) larvae fed cowpea seedlings produced volatile semiochemicals to which *Microplitis demolitor* Wilkinson responded in a wind tunnel. However, most *M. demolitor* females reared from *H. zea* larvae fed an artificial diet were not responsive at emergence to the same volatile semiochemicals. A preflight contact with frass from *H. zea* fed cowpea was needed to stimulate a response of sustained flight in a wind tunnel. The most consistent flight response was 7–10 days postemergence. Response resulting from both antennal and ovipositor contact with host frass during preflight stimulation was no better than from antennal contact alone. Chilling the parasitoid pupae rendered most of the emerging females unresponsive to volatile semiochemicals.

Key Words—*Microplitis demolitor*, Hymenoptera, Braconidae, *Heliothis zea*, Lepidoptera, Noctuidae, Biological control, artificial diet, preflight behavior, wind tunnels, oviposition, age, chemosensory receptors, chilling pupae.

[1]Hymenoptera: Braconidae.

INTRODUCTION

Many authors, of whom we cite only a few, have shown that semiochemicals and other biological communication mediators serve vital roles in the foraging activities of entomophagous insects (Salt, 1935; Monteith, 1955, 1958; Arthur et al., 1964; Carton, 1971; Corbet, 1971; Hendry et al., 1973; Lewis et al., 1975; Lenteren, 1976; Weseloh, 1976, 1977; Prokopy and Webster, 1978; Nordlund et al., 1981a,b; Vet, 1983; Lecomte and Thibout, 1983; Vinson, 1976, 1984a,b; Noldus and Lenteren, 1985). An example is the report of Drost et al. (1986), who conducted wind-tunnel studies with the indigenous *Microplitis croceipes* (Cresson) and showed that responses of the females to volatile semiochemicals required appropriate preflight stimulation.

M. demolitor is an important part of the parasitoid community, which attacks *Heliothis* in several crops, especially soybeans in Australia. It was imported from Australia in 1981 through the Research Quarantine facility in Stoneville, Mississippi. Shepard et al. (1983) found that *M. demolitor* developed successfully in the cotton bollworm, *Heliothis zea* (Boddie); the tobacco budworm, *Heliothis virescens* (F.); the soybean looper, *Pseudoplusia includens* (Walker); and the cabbage looper, *Trichoplusia ni* (Hübner).

The studies reported here were designed to determine the response of the exotic *Microplitis demolitor* Wilkinson reared from *Heliothis zea* (Boddie) fed on artificial diet. Specific objectives were: to determine the effect of age and prior oviposition activity on flight response of *M. demolitor* females to odors emanating from the plant-host complex using a wind tunnel; to determine if a specified part of the plant–host complex was more particularly involved in preflight activation of females; to investigate the role of chemosensory receptors of antennae and ovipositor in integration of messages relative to presence of cues from the plant–host complex; and to test the effect of chilling *M. demolitor* pupae on the quality of females subsequently emerging.

METHODS AND MATERIALS

Culture of M. demolitor *in Hosts Reared on Artificial Diet.* All tests used *M. demolitor* that were reared from larvae of *H. zea* fed on artificial diet. Females prefer first- to third-instar larvae of *H. zea* for parasitization (Shepard et al., 1983). A detailed description of the rearing techniques for this parasitoid is provided by Hérard et al. (1988). Adult parasitoids were paired (1 male and 1 female) 1–15 hr after emergence. Each pair was placed in a 30-ml cup with honey and water until tested.

Preflight Experience. Response to olfaction can be affected by the parasitoid's previous experience (Thorpe and Jones, 1937; Narayanan and Subba Rao,

1955; Arthur, 1971). Drost et al. (1986) conducted wind-tunnel studies with the indigenous *Microplitis croceipes* (Cresson) and showed that responses of the females to volatile semiochemicals required appropriate preflight stimulation. Nordlund and Lewis (1985) observed initiation of host-seeking behavior when *M. demolitor* females contacted frass of *H. zea* and *T. ni* larvae fed pea cotyledons. They verified that 13-methylhentriacontane, a compound earlier identified from *H. zea* larval frass and shown to elicit antennation by *M. croceipes* (Jones et al., 1971), also stimulated the antennation behavior by females of *M. demolitor*.

Prior to these tests in the flight chamber, individual *M. demolitor* females were exposed to the components of plant–host complex in a 150-mm-diameter Petri dish for 2 min. The complete experience consisted of contact with greenhouse-grown, pink-eye, purple-hull cowpea seedlings freshly damaged by *H. zea* larvae; frass from the feeding host larvae; and one or two ovipositions.

Contact was limited to a specified part of the plant–host complex to determine the effect of various preflight stimuli. Twelve treatments were tested. Treatment 1 was the control, i.e., the female was tested without preflight exposures to plant–host-related materials. Treatment 2 was contact with an undamaged plant. Treatment 3 was contact with an artificially damaged cowpea seedling. Treatment 4 was contact with a plant that had been fed upon and then washed with water. Treatment 5 was contact with natural feeding damage and frass. Treatment 6 was contact with frass alone. In treatment 7, the parasitoid was exposed only to the host odor. The experience chamber contained second- and third-instar host larvae isolated in compartments. The female *M. demolitor* was placed above the host larvae in a chamber with a double bottom of organdy. In this way the parasitoid was able to detect the odors of larvae without contacting them. In treatment 8, the female was allowed to oviposit once. Treatment 9 was contact with the complete plant–host complex and one oviposition. Treatment 10 was contact with the same material as in treatment 9 and two ovipositions. Treatment 11 was contact with an artificially damaged seedling and frass. In treatment 12, the source of the odors was a 4-liter jar containing five cowpea seedlings and 25 actively feeding third-instar *H. zea* larvae producing damage to plants and frass. An *M. demolitor* female was placed in a 10-cm^3 cage with a double cover of organdy and the cage placed in the 4-liter jar so that the parasitoid could detect odors from the complete plant–host complex but could not contact the source.

Test in the Wind Tunnel. The wind tunnel provided laminar air flow at 16 cm/sec. The test section of the tunnel was 2 m long with a 75 × 75-cm cross-section. Overhead lighting was provided by four 80-W fluorescent light bulbs.

The source of airborne semiochemicals consisted of a damaged cowpea seedling bearing fresh frass and five second- and third-instar *H. zea* larvae, which were feeding actively at the time of each test. The source was prepared

by allowing the larvae to feed on the seedling, the stem of which was immersed in water, for 2 hr prior to testing. During most of the tests, the source was placed at the center of the upwind end of the flight tunnel.

Each naive female *M. demolitor* was transferred to the experience chamber (Petri dish) and then to the tunnel in clear 3.7-ml vials. The vial was placed 1.3 m downwind from the source with the open end up. When the females walked out of the vial and became exposed to the odor plume, they displayed two principle types of responses. One was a non-target-oriented flight that occurred either immediately or after a brief period of walking while drumming on the release vial with the antennae. The other was a sustained flight to the source of odors, which occurred after a period of upwind orientation on the vial. If a female conducted a non-target-oriented flight in the first trial, we placed her back on the vial and allowed a maximum of four other chances of flight to the source. We recorded which females displayed a sustained flight in the first through fifth trials.

We tested the possible role of vision when *M. demolitor* females approached the source in the flight chamber with the technique developed by Zanen et al. (1988). The source was placed outside the wind tunnel, and semiochemicals were introduced through a simple glass nozzle. Thus, the plant–host complex was not visible to the parasitoid as she flew. Females did not need visual cues because they oriented and flew to this nozzle.

Effect of Parasitoid Age. The level of response to volatile semiochemicals from a plant–host complex varied widely with the ages of *M. demolitor* females. We investigated the effect of age on flight response to plant–host odors by experienced females. Female *M. demolitor* 1–14 days old were separated into seven groups, each with an age range of two days.

Effect of Oviposition Activity. An *M. demolitor* female emerges from its cocoon with some mature eggs available for oviposition during the very first day. The females tested in the above-mentioned experiment were not allowed to oviposit prior to the test. In order to determine if the response to odors from the plant–host complex by 7-day-old females was due to their strong need to lay eggs, we allowed 20 females to deposit five eggs per day and another group of 20 to lay 20 eggs per day for seven days. Their flight responses were compared to 20 females that were not allowed to oviposit during the same period.

Role of Chemosensory Receptors of Antennae and Ovipositor in Integration of Messages Relative to Presence of Cues from Plant–Host Complex. While exploring an area containing some cues from the plant–host complex, a female frequently shows intense antennation activity followed by probing with the abdomen. Occurrence of a necessary information exchange process based on messages received by the chemosensory receptors of these two organs was suspected in the recognition of contact kairomones and sensitization to volatile semiochemicals.

An experiment was set up to observe the flight response of female *M. demolitor* to volatile semiochemicals following contact with host frass, either by the antennae only or by the antennae and ovipositor. These responses were compared with those of inexperienced females. To allow contact only by the antennae, a pellet of frass maintained on the tip of a pin was presented above the substrate to the female until she palpated it with her antennae. Normal behavior of the female was to catch the pellet with her fore tarsae, climb on the pin, and probe the pellet with her ovipositor. To avoid this contact, we moved the pellet back while the female moved forward still palpating the pellet with her antennae. We stopped the contact after 15–20 sec and then tested the female in the wind tunnel.

Effect of Chilling Pupae on Quality of Subsequently Emerging Females. Initially, it was observed from experiments designed for other purposes that *M. demolitor* and *M. croceipes* females emerging from chilled pupae were particularly unsuitable for behavioral bioassays. These females had a strong propensity to show only non-target-oriented flights. Since chilling of pupae is commonly practiced in many entomological laboratories, we suspected a general practical interest in this observation. We tested effect of chilling pupae at 13°C for 4, 7, and 13 days on behavioral and reproductive performance of *M. demolitor*.

Statistical Analysis. The numbers of females tested in each treatment was 20, except for 40 in the seven groups used to study the effect of age, and 30 in the study of the effect of chilling pupae. Incidences of sustained flight in the first trial were compared in the different treatments by using repeated G tests, or replicated goodness-of-fit tests, based on use of the log-likelihood ratio (Sokal and Rohlf, 1969; Zar, 1974). The 0.05 probability level was used for rejection of all null hypotheses.

RESULTS AND DISCUSSION

Description of Response to Contact and Airborne Semiochemicals. Females of *M. demolitor* that contacted material from the plant–host complex in an experience chamber exhibited intense antennation activity, drummed the plant material or frass with their antennae while walking or standing still, and probed at the material with the tips of their ovipositors.

Flight initiation and orientation sequences of *M. demolitor* females to odors from the plant–host complex in the wind tunnel were essentially the same as those described by Drost et al. (1986) for *M. croceipes*: a period of preflight orientation characterized by a sequence of walking while drumming, followed by standing still oriented upwind while holding the antennae in a vertical plane perpendicular to the wind direction, and followed by initiation of the flight pose.

During the flight pose, the female stood on her meta- and mesothoracic legs while making "walking" movements with her prothoracic legs. Takeoff usually was followed by a sequence of wide zigzagging, straight flight, recurrent zigzagging, another straight flight, hovering at about 10 cm downwind from the target, and quick darts toward the target, ending with landing on the target. This characterized sustained flight.

Effect of Age. Figure 1 shows that the response to semiochemicals by 1- to 6-day-old females was low and variable. In the youngest females, only a few responded in the first trial, and some did not fly in five trials. However, the percentage of females responding increased from 1 to 6 days of age.

Response to semiochemicals in 7- to 10-day-old females was much more consistent, with 55–75% of them conducting sustained flights in the first trial. In 11- to 14-day-old females, response to semiochemicals decreased and individual variability increased. Forty-five percent of females showed a sustained flight in the first trial, and 20% did not respond in five trials.

As no statistical difference was observed between the 7- to 8- and 9- to 10-day-old groups, we considered the 7- to 10-day-old group sufficiently homogeneous to be used in experiments where a high responsiveness to semiochem-

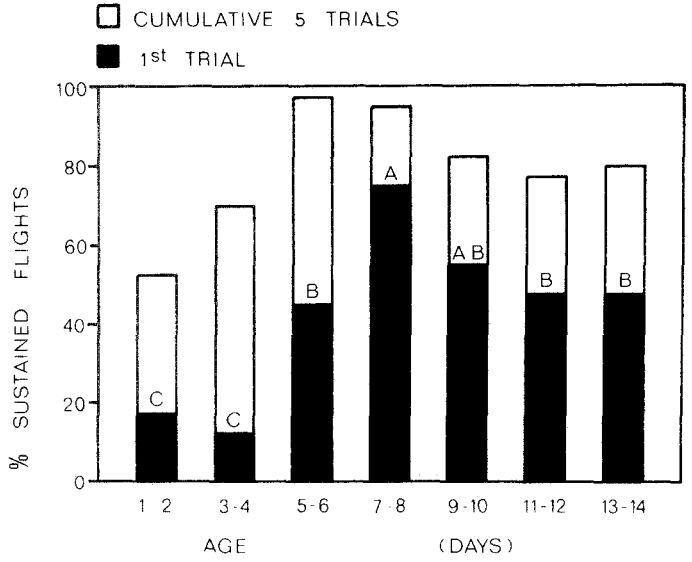

FIG. 1. Effect of age on flight response to odors from cowpea–*Heliothis zea* complex by experienced *Microplitis demolitor* females. The black portion of the bars represents the percentage of females flying in the first trial, and the white plus black bars those flying at least once in five trials. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

icals was needed. This also suggests that age of the female is an important consideration when releasing parasitoids in the field.

Effect of Oviposition Activity. There were no significant differences among the percentages of flight responses by the experienced females which had laid 0, 35, and 140 eggs, respectively, prior to the test (Figure 2).

These results show that previous egg depositions did not diminish the responsiveness of the females to volatile semiochemicals. On the other hand, the younger females did possess eggs and were capable of ovipositing. Therefore, it appears that the characteristics of possessing mature eggs and being held away from hosts is not sufficient of itself to induce the host-searching process by a female *M. demolitor*.

These results suggest that we cannot expect effective field performance by released *M. demolitor* merely because the females are inseminated and possess mature eggs.

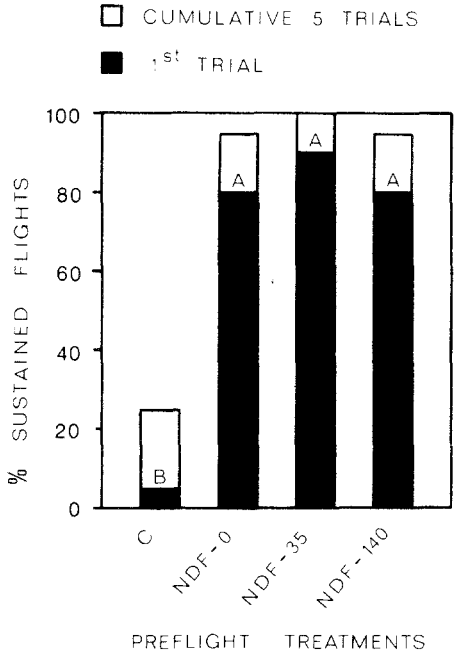

FIG. 2. Effect of preliminary egg deposition on flight response to odors from cowpea–*Heliothis zea* complex by 7- to 8-day-old experienced *Microplitis demolitor* females. C: no egg laid, no preflight experience; NDF-0: no egg laid, natural damage + frass; NDF-35: 35 eggs laid, natural damage + frass; NDF-140: 140 eggs laid, natural damage + frass. The black portion of the bars represents the percentage of females flying in the first trial, and the white plus black bars those flying at least once in five trials. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

Effect of Varying Preflight Stimuli. Data for the 12 preflight experience designs are shown in Figure 3. Treatments 2, 3, 4, 7, 8, 11, and 12 gave results not significantly different from the control. Contact with the complete plant-host complex (treatment 5) induced a sustained flight in the first trial in 75% of the females. When components of the plant-host complex were used separately in the experience chamber (treatments 2, 3, 4, 6, and 7), only frass (treatment 6) induced a stimulation (in 70% of the females) which was as good as the complete complex (treatment 5). Three other treatments containing the frass component induced a good sustained flight in the first trial: natural damage + frass (treatment 5), and complete plant-host complex with oviposition (treatments 9 and 10). The additional contact with natural feeding damage and the experience of one or two ovipositions did not significantly increase the degree

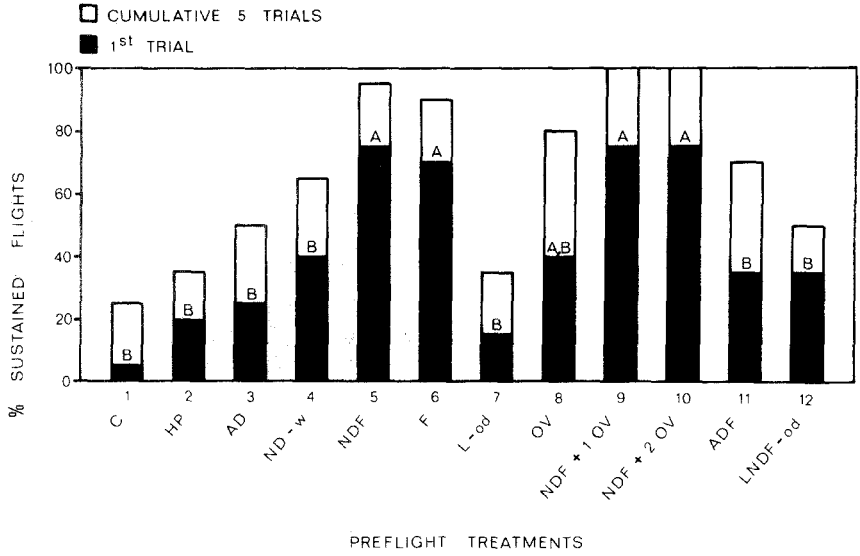

FIG. 3. Effect of varying preflight stimuli on flight response to odors from cowpea-*Heliothis zea* complex by 7- to 8-day-old experienced *Microplitis demolitor* females. Treatment 1 (C): no preflight experience; treatment 2 (HP): undamaged host plant; treatment 3 (AD): artificial damage; treatment 4 (ND-w): natural damage, water washed; treatment 5 (NDF): natural damage + frass; treatment 6 (F): frass; treatment 7 (L-od): larval odor; treatment 8 (OV): 1 oviposition; treatment 9 (NDF + 1 OV): natural damage + frass + 1 oviposition; treatment 10 (NDF + 2 OV): natural damage + frass + 2 ovipositions; treatment 11 (ADF): artificial damage + frass; treatment 12 (LNDF-od): odor of larvae, natural damage and frass. The black portion of the bars represents the percentage of females flying in the first trial, and the white plus black bars those flying at least once in five trials. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

of response observed with frass alone. Only the combination of preflight contact with frass and artificial damage (treatment 11) gave a response as poor as other treatments lacking frass. This suggests the possibility of a masking or repulsive effect of chemicals emitted by cowpea leaves damaged artificially.

A leaf from which the frass was removed was then washed (treatment 4) and did not induce host-seeking behavior in most of the females. Exposure to odors of larvae (treatment 7) or of the complete plant–host complex with no contact with the material (treatment 12) were not valuable preflight experiences. Even a brief contact with a host, limited to a fraction of a second, during which an egg was laid (treatment 8), did not render most of the females tested responsive to volatile semiochemicals. Contact with undamaged or artificially damaged plants (treatments 2 and 3, respectively) had no effect on responses to plant–host odors in most of the *M. demolitor* females. However, very few females (5%), with no preflight experience at all (treatment 1), showed a sustained flight in the first trial. This indicates that a few females have a sufficiently low inherent threshold of response to volatile semiochemicals such that no prior experience is required. This experiment showed that: (1) most of the *M. demolitor* females reared from hosts fed artificial diet needed a preflight experience to become responsive to volatile semiochemicals emitted by a target plant–host complex; (2) frass from the host was the most effective source for preflight stimulation and only a brief contact with the source was necessary; and (3) the act of oviposition alone did not increase responses nor did it enhance the effect of contact with the frass.

Role of Chemosensory Receptors of Antennae and Ovipositor in Integration of Messages Relative to Presence of Cues from Plant–Host Complex. Figure 4 indicates that there is no significant difference in percentages of females showing a sustained flight in the first trial after experience by contact with frass with only the antennae or with antennae and ovipositor. This indicates that the antennae are the necessary and sufficient olfactory organs involved in: (1) recognition of significant cues from the plant–host complex, (2) detecting host presence in the vicinity and continuation of active search for it, and (3) the threshold level necessary for flight response to volatile semiochemicals.

Contact with frass elicits responses of antennation, a short running, and subsequent probing with the abdomen by the female *M. demolitor*. As soon as the ovipositor touches the frass, running stops and antennation is resumed. Consequently, we assume that when the frass is touched with the antennae, it triggers a stereotypical behavior of oviposition, which ceases when the chemoreceptors of the ovipositor encounter a substance that is not a suitable host.

Very likely, an information exchange process occurs when the stimulation is received alternatively by antennae and ovipositor. Whereas the former would induce activation of search for the host by lowering the threshold of sensitivity

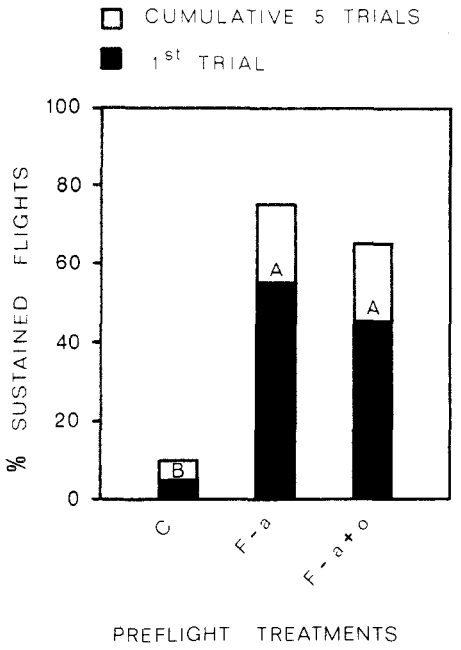

FIG. 4. Role of chemosensory receptors of antennae and ovipositor in establishing experience of frass, and effect on flight response to odors from cowpea-*Heliothis zea* complex by 7- to 8-day-old *Microplitis demolitor* females. C: no preflight experience; F-a: experience with frass by the antennae only; F-a + o: experience with frass with both the antennae and ovipositor. The black portion of the bars represents the percentage of females flying in the first trial, and the white plus black bars those flying at least once in five trials. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

to airborne semiochemicals and by initiating the reflex of oviposition, the latter would have a regulatory effect on this reflex and would act mainly in host recognition and host acceptance. Many parasitoids are guided to the host by volatile odors and are stimulated to accept it or reject it by chemotactile stimuli on the cuticle or in the body, perceived through receptors on the antennae, tarsi, or ovipositor (Herrebout, 1969; Vinson and Lewis, 1965; Weseloh and Bartlett, 1971; Corbet, 1971; Wilson et al., 1974; Weseloh, 1974; Prokopy and Webster, 1978).

Effect of Chilling Pupae on Quality of Subsequently Emerging Females. The percentage of experienced females that performed a sustained flight to the odor source in the first trial in each of the three chilled groups was significantly lower than in the nonchilled group (Figure 5). Even a four-day period of chilling led to a decrease in the response of subsequently emerging adults. Experienced

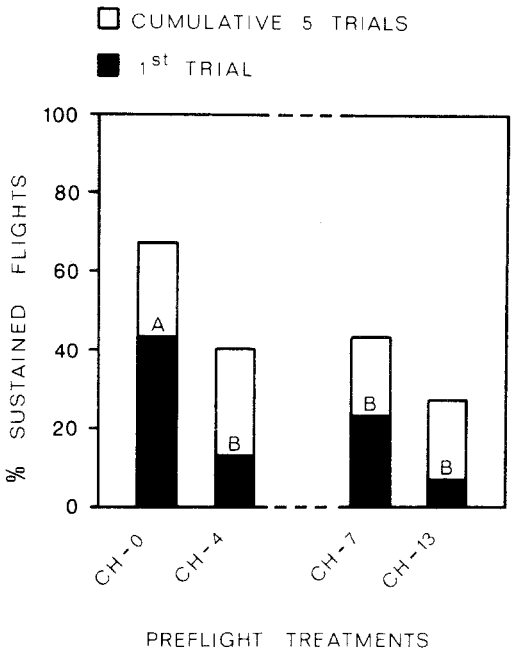

FIG. 5. Effect of chilling pupae on flight response to odors from cowpea–*Heliothis zea* complex by 7- to 8-day-old experienced *Microplitis demolitor* females, subsequently emerged from this pupae. CH-0: not chilled; CH-4: 4-day chilling; CH-7: 7-day chilling; CH-13: 13-day chilling. All the females were given a preflight exposure to natural damage + frass. The black portion of the bars represents the percentage of females flying in the first trial, and the white plus black bars those flying at least once in five trials. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

females did not respond to volatile semiochemicals in most cases. It may be that such parasites, released in the field, would be ineffective for biological control.

In addition, reproductive performance of the seven- and 13-day chilled individuals was so altered that 94% of their progeny were males. These data suggest that females were not inseminated because of a misfunctioning of the pheromonal system or because the sperm transfer was altered by the cold. In order to answer this question, we interbred females emerging from seven-day-chilled cocoons with normal males, and seven-day-chilled males with normal females. Observation of the males' response to the female pheromone suggested that chilled females produced the pheromone normally. Both chilled and unchilled males responded positively and chilled males were sensitive to the

female pheromone. Matings were observed in the two groups. The percentage of male progeny in both groups was higher than in typical rearing (60% instead of 40–50%). The results indicate that chilling of pupae altered the reproductive performance of both the females and males. The combination of these effects, when chilled males and females were interbred, led to production of a particularly high percentage of males among the progeny.

These studies demonstrated the importance of airborne semiochemicals in the host-searching behavior of *M. demolitor* and the importance of preflight handling on behavioral performance of the parasitoid. Understanding the process of preflight stimulation has a high practical value. It appears that conditioning of beneficial insects by contact with particular cues from the target plant–host complex before their release results in a much more effective host-searching behavior by them and their retention in the release area. Gross et al. (1975) demonstrated in greenhouse and field studies that stimulation with host-seeking stimuli just prior to release resulted in higher parasitization rates with *M. croceipes*, *Trichogramma pretiosum* (Riley), and *T. achaeae* Nagaraji. Lewis et al. (1975) showed that retention of *Trichogramma* spp. increased when a kairomone (Tricosane) was applied to plant foliage.

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BENEFICIAL ARTHROPOD BEHAVIOR MEDIATED BY AIRBORNE SEMIOCHEMICALS
IV. Influence of Host Diet on Host-Oriented Flight Chamber Responses of *Microplitis demolitor* Wilkinson[1]

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Abstract—*Microplitis demolitor* Wilkinson were reared from *Heliothis zea* larvae fed either an artificial diet or a diet of cowpea seedlings. Responses of females from these two sources to semiochemicals from the insect host–cowpea complex were compared in a flight tunnel. Very limited responses were obtained from females reared from hosts fed artificial diet unless they first had preflight contact with frass from plant-fed hosts. Female parasitc ls reared from plant-fed hosts were generally responsive without preflight contact. Contact with cocoons containing frass of the plant fed host at the time of emergence was an important source of stimulation. It increased their subsequent responsiveness to the volatile semiochemicals of the insect host–plant complex. The cocoons of females reared from artificial diet-fed hosts were apparently lacking plant chemicals that are vital to their subsequent responsiveness. Imprinting of the adults at time of emergence from the cocoon is strongly indicated.

Key Words—*Microplitis demolitor*, Hymenoptera, Braconidae, *Heliothis zea*, Lepidoptera, Noctuidae, biological control, rearing on plant, wind tunnels, flight response, preflight behavior, imprinting.

[1]Hymenoptera: Braconidae.

INTRODUCTION

Earlier work (Hérard et al., 1988) indicated that, at emergence, most *Microplitis demolitor* Wilkinson females reared from *Heliothis zea* (Boddie) larvae fed artificial diet could not orient and fly upwind to volatile semiochemicals emitted by the cowpea–*H. zea* complex. Optimally, they needed a preflight contact with frass from *H. zea* larvae fed cowpeas to show a sustained flight in a wind tunnel to odors from this plant–host complex. In addition, the most consistent flight responses were performed at 7–10 days of age following emergence (Hérard et al., 1988). Several authors have demonstrated that host frass can be a source of compounds that induce a behavioral change when parasitoids detect them (Kajita and Drake, 1969; Cardona and Oatman, 1971; Lewis and Jones, 1971; Greany and Oatman, 1972; Kennedy and Golford, 1972; Hendry et al., 1973). However, diet can affect the kairomonal content of host frass (Roth et al., 1978; Nordlund and Sauls, 1981). Sauls et al. (1979) and Nordlund and Sauls (1981) found that differences occur in the antennation response of *M. croceipes* (Cresson) females to frass from *H. zea* larvae fed different diets. Nordlund and Lewis (1985) demonstrated that host-selection response of *M. demolitor* to *H. zea* frass is also dependent on the diet on which the larvae fed. These authors observed that antennation behavior was elicited in *M. demolitor* females contacting the frass of *H. zea* and *Trichoplusia ni* (Hübner) larvae fed pea cotyledons, whereas it was not elicited in individuals contacting frass from larvae fed artificial diet. These findings prompted us to evaluate and compare flight responsiveness to volatile semiochemicals by *M. demolitor* females reared from hosts fed plant material and from hosts fed artificial diet.

METHODS AND MATERIALS

H. zea larvae, parasitized by *M. demolitor*, were reared individually on an artificial diet developed by Burton (1969). Following pupation, parasitoid cocoons were isolated in clean 30-ml cups with honey and water. Adults were paired (1 male and 1 female) a few hours after emergence. Each pair was isolated and provided with honey and water until the time behavioral testing. (See Hérard et al., 1987, for more detail on *M. demolitor* rearing techniques.)

The *M. demolitor* were also reared from *H. zea* fed on pink-eye purple-hull cowpea seedlings that had been grown in the greenhouse. Two hundred parasitized first-instar larvae were placed on two pans of cowpea seedlings in 70-liter cages until emergence of the parasitoid larvae from their hosts. The parasitoid cocoons, normally spun on the damaged leaves, were removed from the plant and placed individually in 30 ml cups. Thus, these adults did not have any contact with the damaged plant at emergence.

Those females provided with a preflight experience on the plant–host complex were placed for 2 min into a 150-mm-diameter Petri dish containing a cowpea leaf damaged by *H. zea* and frass from cowpea-fed *H. zea* larvae. The host itself was not presented since we knew from earlier studies (Hérard et al., 1988) that contact with and oviposition in hosts does not increase a parasitoid's stimulation. The wind tunnel used and test procedures were the same as described by Hérard et al. (1988). The odor source placed at the upwind end of the flight tunnel consisted of one cowpea seedling being fed upon by five third-instar *H. zea* larvae and fresh frass. The larvae were confirmed to be actively feeding at the time of each test.

Individual females were released from a vial 130 cm downwind from the odor source. A positive response consisted of sustained oriented flight from the release point to the odor source. A description of various responses by female *M. demolitor* are detailed in Hérard et al. (1988).

Characterization of Varying Response Thresholds to Volatile Semiochemicals. Results obtained in earlier studies (Hérard et al., 1988) showed that in female *M. demolitor* reared from *H. zea* fed artificial diet, 5% of individuals with no experience flew in the first trial to a source of volatile semiochemicals emitted by the cowpea–*H. zea* complex. This observation led us to ask two questions: (1) Do some individuals have an inherently lower response threshold; or (2) have they obtained some experience from inadvertent contaminations? One source of contamination could be from the surface of the cocoon. In the rearing containers that held the artificial diet, the parasite cocoons were spun on a layer of silk from the host and were generally contaminated with fragments of frass. They were removed from these containers before emergence of adults, but inevitably were contaminated with silk and frass from the host. Some contact by the parasitoid with this material could occur and serve as a preflight stimulation.

For this experiment, we used two groups of 20 parasitoid females reared from *H. zea* fed artificial diet. One group of females emerged normally from their cocoons. Females of the second group emerged from pupae that had been separated from their cocoons on the second day of pupal stage, by cutting the cocoon and the cast larval cuticle longitudinally all around in two halves, with a razor blade. The pupae were then removed and placed individually in 30-ml plastic cups on a layer of moist cotton. Proper humidity was crucial during imaginal molting to obtain adults with functional wings. In this manner we isolated 20 female *M. demolitor* that had not contacted a cocoon. We did not provide females of the two groups with any stimulation before testing them in the wind tunnel. Tests were with 7- and 8-day-old females.

Effect of Host Diet on Behavioral Performance of Parasitoid. At the end of the parasitoid larval development, the parasitized host on a cowpea leaf stops moving. The end of its abdomen seems paralyzed and fixed to the substrate.

The host does not feed but is still alive. The anterior part of its body remains free, and it spins a considerable amount of silk around itself. The parasitoid larva emerges from the host and begins to spin a cocoon attached to the leaf just beside the host on the layer of larval silk. At this time, the parasitoid larva and its cocoon are mixed with hemolymph, silk, frass, and larval cuticle which contain chemicals from the plant–host complex. We assumed that these host and plant products on cocoons could be a source of activation for the female at emergence. To test this hypothesis we reared two groups of *M. demolitor*: one from larvae fed on artificial diet and the other from hosts fed on cowpea seedlings at the same temperature (26 ± 1°C). They showed an equal period of development. Cocoons were removed from cups of artificial diet and from cowpea leaves and isolated until emergence of adults which were then fed and paired. Five- and 6-day-old females from the two cultures were tested in the wind tunnel on the same days. Half the females in each group were tested without preflight experience. The other half were exposed to frass and cowpea leaves from feeding *H. zea* larvae and frass.

Effect of Contact with Cocoon when Host Larvae were Reared on Plants. It was assumed that *M. demolitor* females emerging from cocoons spun on plants acquire the experience of the plant–host complex as soon as they palpate their cocoon with their antennae at the moment of emergence. To verify the role of contact with a cocoon, we tested in the wind tunnel three groups of parasitoids reared from hosts fed on cowpeas. In two of them, the females emerged from their cocoons normally. The first group received preflight experience with the natural plant damage and frass, and the second received no preflight experience before the test. In the third group, the cocoons were cut and the pupae isolated as described earlier. The adults emerged without contact with the cocoon. The females received no experience before the test.

RESULTS AND DISCUSSION

Characterization of Varying Response Thresholds to Volatile Semiochemicals. No female of the group removed from the cocoons flew in the first trial, but 15% showed a sustained flight to the source in 5 trials (Figure 1). This confirmed the natural ability of a few females to respond to semiochemicals without any prior stimulation. Several authors have given clear evidence that searching is partly genetically determined, because certain cues evoke an innate fixed searching pattern (Thorpe and Jones, 1937; Jones et al., 1971, 1973; Lewis et al., 1972; Hendry et al., 1973; Vinson et al., 1975; Sandlan, 1980). Among the females emerged from the cocoon of a host fed artificial diet, but given no other experience other than contacting the cocoon, 5% showed a sustained flight to the source in the first trial, and 25% flew in five trials (Figure

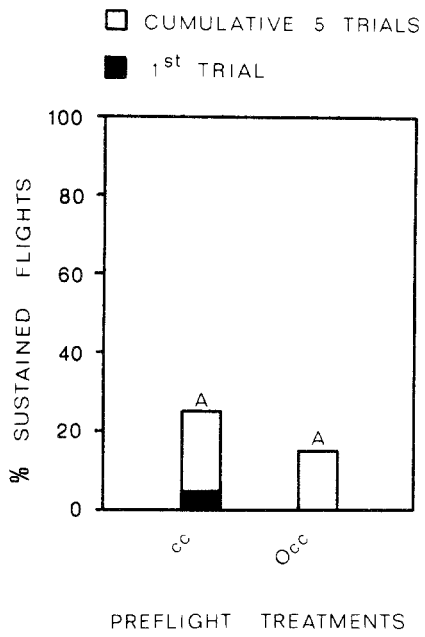

FIG. 1. Flight response to odors from cowpea–*Heliothis zea* complex by 7- to 8-day-old inexperienced *Microplitis demolitor* females reared from hosts fed artificial diet. cc: parasitoid emerged from cocoon. Occ: pupa developed and emerged outside cocoon. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

1, cc). These percentages are not significantly different from the results obtained with similar females emerging without contacting the cocoon (Figure 1, Occ). These results indicate that contact with the cocoon and associated materials from artificial diet was not a sufficient source of activation for *M. demolitor* females.

These data indicate that, from a practical standpoint, if *M. demolitor* is to have an effective host-selection behavior when reared in large numbers from hosts fed on artificial diet, it should have contact with appropriate semiochemicals from the target plant-host complex before release in the field. Most of them would disperse immediately and would be ineffective for biological control without this preflight stimulation. This may apply to other beneficial insects.

Effect of Host Diet on Behavioral Performance of Parasitoid. Percentages of females flying in the first trial to the source were significantly higher in the group obtained from hosts reared on plants, even when they did not receive preflight experience (Figure 2, p-0exp), than in the group obtained from hosts reared on artificial diet (Figure 2, a-0exp and a-NDF). Among the females

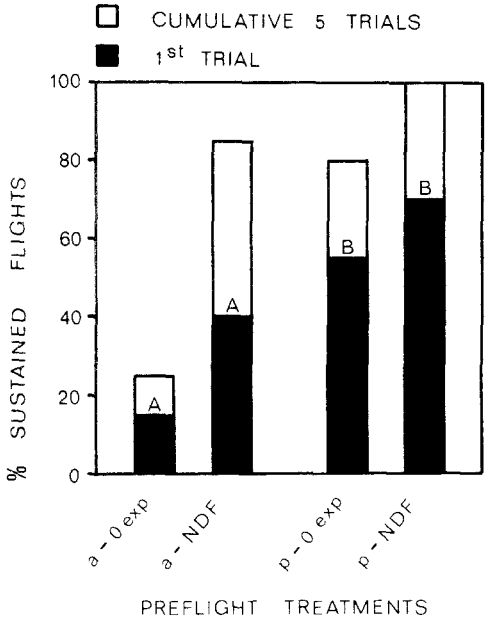

FIG. 2. Flight response to odors from cowpea-*Heliothis zea* complex by 5- to 6-day-old female *Microplitis demolitor* reared from hosts fed plant material or artificial diet. a-0exp: host reared on artificial diet, no preflight experience. a-NDF: host reared on artificial diet, preflight exposure to natural damage + frass. p-0exp: host reared on plant, no preflight experience. p-NDF: host reared on plant, preflight exposure to natural damage + frass. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

obtained from hosts reared on plants, the occurrence of an additional preflight experience (Figure 2, p-NDF) did not increase significantly the percentage of positive responses. Thus, parasitoids from hosts fed on the natural plant apparently receive valuable experience from the plant–host complex before or at time of emergence from the cocoon. Arthur (1966, 1971) gave evidence that the searching pattern can be influenced by previous experience in the ichneumonids *Itoplectis conquisitor* (Say) and *Venturia canescens* (Gravenhorst). Learning in insects has been shown to occur (Thorpe, 1956; Alloway, 1972, 1973) and takes different forms: habituation (Thorpe, 1938; Waage, 1979), preimaginal conditioning (Thorpe and Jones, 1937; Monteith, 1955; Waage, 1979; Kudon and Berisford, 1980), postimaginal latent learning (Thorpe and Jones, 1937; Thorpe, 1938), and associative learning (Monteith, 1963; Vinson et al., 1977). The ecological significance of the learning process was discussed by Vet (1983). The function of learning in search behavior would be to "optimize the discovery and utilization of resources which fluctuate in abundance and time" (Vet, 1983).

Our results confirmed that females obtained from hosts reared on artificial diet were not naturally experienced at emergence. Perhaps some essential compound existing in the plant is lacking in the artificial diet. Accordingly, the frass of hosts fed on this diet would not contain the kairomone(s) that contaminate the parasitoid's cocoons, activate the host-seeking behavior of emerging females, and lower the threshold response to volatile semiochemicals. Observations by Nordlund and Lewis (1985) showed that contact of *M. demolitor* females with frass of *H. zea* larvae fed artificial diet did not elicit host-seeking antennation behavior by the parasitoids.

Effect of Contact with Cocoon when Host Larvae were Reared on Plants. As shown in Figure 3 by the two first bars, a high percentage of experienced (cc-NDF) and inexperienced (cc-0exp) females that had emerged from their cocoons responded to odors from the plant–host complex in the first trial. Most

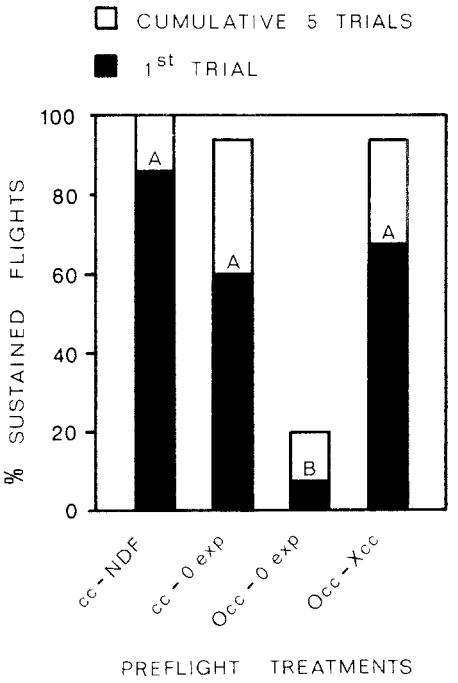

FIG. 3. Effect of contact with the cocoon on flight response to odors from cowpea–*Heliothis zea* complex by 7- to 8-day-old female *Microplitis demolitor* reared from hosts fed plant material. cc-NDF: parasitoid emerged from coccon, preflight exposure to natural damage + frass. cc-0exp: parasitoid emerged from cocoon, no preflight experience. Occ-0exp: parasitoid emerged outside cocoon, no preflight experience. Occ-Xcc: parasitoid emerged outside cocoon, preflight exposure to cut cocoons. Bars topped by the same letter do not differ significantly; $P = 0.05$; repeated G tests.

of the females that had emerged separated from the cocoons (Occ-0exp) did not fly to the target.

The females of this third group, which showed only negative responses in five trials, were later exposed for 2 min to the cut cocoons. They became very active at the surface of the cocoons, showing intense antennal activity and abdominal probing. They were tested again in the wind tunnel. A high percentage of them then flew to the odor source, as shown in Figure 3 (Occ-Xcc). Thus the cocoon of individuals reared from hosts fed on plants is a natural source of substances important for preparation of *M. demolitor* females for later host-finding performance.

In fact, cocoons spun in contact with damaged plants and dying hosts are likely to be contaminated by a complete set of cues from the plant–host complex (sap from the damaged plant, and silk, saliva, hemolymph, and frass from the host). These data show that the first preflight stimulation occurs naturally at the time of emergence through contact with the cocoon and surrounding substrates and that this contact is sufficient to obtain responsiveness by most females. It is a durable activation, since the females respond positively for at least seven days after the contact with a cocoon. Accordingly, true imprinting of emerging *M. demolitor* females from hosts fed plant material is strongly indicated.

CONCLUSIONS

These studies showed that when the parasitoids were reared from host fed artificial diet, the foraging behavior of female *M. demolitor* response to the insect–host plant contact was not activated by contact with its cocoon at emergence. When hosts were reared on plants, parasitoid cocoons were mixed with frass and semiochemicals from the plant–host complex. It was demonstrated in this case that simple contact of a *M. demolitor* female with its cocoon at emergence served as a necessary, valuable, and durable preflight stimulation in its subsequent search for a host.

These studies show that when parasitoids are reared from hosts fed artificial diet, they are missing vital experience necessary for their most effective performance. A few other questions arise at this point. Is imprinting on a host reared on a particular host plant specific to that plant–insect host association? What would be the responsiveness of a parasitoid to semiochemicals of the same host species reared on another host plant? From a practical standpoint, is it necessary to give the parasitoids a preconditioning on the target plant–insect host complex before release in the field in order to achieve the highest rate of parasitism? The validity of these ideas was not tested in the present study and requires experimental proof, as the results have significant implications concerning the effectiveness of the parasitoids in biocontrol programs. Thus, parasitoids reared from hosts fed cowpeas may not respond to semiochemical cues

from host feeding on cotton and vice versa. The specificity of the imprinting to semiochemicals from the plant–host complex is an important question that arises from this research. Studies designed to better understand the roles and mechanisms of these factors could greatly enhance biological control programs.

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BENEFICIAL ARTHROPOD BEHAVIOR MEDIATED BY AIRBORNE SEMIOCHEMICALS

V. Influence of Rearing Method, Host Plant, and Adult Experience on Host-Searching Behavior of *Microplitis croceipes* (Cresson), a Larval Parasitoid of *Heliothis*[1]

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Abstract—Rearing the parasitoid *M. croceipes* on hosts fed cowpea-seedling leaves instead of artificial diet increased the percentage of oriented flights to odors of a cowpea seedling–*H. zea* complex in a flight tunnel. However, the increase in response was much stronger after adult females had searched a fresh plant-host complex just prior to a test. The host plant appears to be of major importance in the parasitoid–host relationship: host-plant species, growth phase, and part of the host plant influence the parasitoid's response in the flight tunnel. The percentage of inexperienced females responding to infested leaves was higher for 4- to 5-day-old females than for 0- to 1-day-old females, while the response to uninfested flowers was equally high for both age groups. Olfactory experience with odors of an attractive plant–host complex increased the response to an unattractive plant–host complex. Possible implications of the results are discussed.

Key Words—*Microplitis croceipes*, Braconidae, Hymenoptera, *Heliothis zea*, Lepidoptera, Noctuidae, olfaction, flight, olfactory experience, rearing.

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INTRODUCTION

Host specialists may be more likely to have an innate response to stimuli that are directly related to the host or to the host habitat than are host generalists (Vet, 1983; Waage, 1979). Females of the host specialist *Microplitis croceipes* (Cresson), an important larval endoparasitoid of *Heliothis zea*, show an innate host-searching response when they make contact with feces of the host. They restrict the host-searching area by drumming the surrounding substratum with their antennae (Lewis and Jones, 1971). Yet, in a flight tunnel the females do not fly upwind to *H. zea* feeding on cowpea-seedling leaves unless they had previously contacted feces (Drost et al., 1986). Contact with feces may often be followed by oviposition once the host is found; however, oviposition was not required for obtaining increased flight responses. Thus, contact with feces induces a change in the behavior of the parasitoid. We will use the term "experienced" for adult females that had searched a plant–host complex and oviposited once and "inexperienced" for females that had no oviposition experience and no contact with host- or host-plant related cues since emergence.

Parasitoids used by Drost et al. (1986) were reared on *H. zea* larvae feeding on an artificial pinto-bean diet (Burton, 1969). Perhaps this rearing method has influenced the responsiveness of the adult females. Sauls et al. (1979) reported that feces from hosts fed artificial diet elicit less antennation in adult female *M. croceipes* than do feces from hosts fed cowpea-seedling leaves. During the development of the parasitoid in the host larvae, the parasitoid perhaps perceives chemical cues derived from the host's diet that are required as a later reference in the host-finding process.

Another factor that may cause low responsiveness of the parasitoids may relate to the quality of the source emitting the odors in the flight tunnel, which is determined by the host and the host plant. The host-plant species of *H. zea* influences the parasitization rates by *M. croceipes* in the field, as was reported by Danks et al. (1979). The likelihood of attack and the probability of successful parasitism is correlated with the host-plant species (Mueller, 1983). The influence of the host-plant species on host-searching behavior by inexperienced *M. croceipes* has not been determined previously. In addition to host-plant species, the growth phase and part of the plant might influence the response by *M. croceipes*. One might expect the parasitoid to fly more frequently to plant species, plant growth phases, and plant parts on which hosts are most likely to occur, such as fruits and young leaves on which third-instar *H. zea* larvae prefer to feed (Schmidt, 1985). For example, Elzen et al. (1987) found *M. croceipes* females responding to odors of uninfested cotton.

The age of the adult parasitoid may influence its response to host-related odors. Although Drost et al. (1986) found that the age of the adult female does not influence the flight response of experienced females, this might not be true

for inexperienced females. Influences on the responsiveness due to the age of the parasitoid female may have been overridden by the effect of experience.

We discovered that odors of a plant–host complex attractive to inexperienced females had an effect on the subsequent response to other plant–host complexes. This means that besides experience with cues perceived while searching a plant–host complex, olfactory experience might play a role in the host-searching process.

In the present paper, we show how the responsiveness of *M. croceipes* in a flight tunnel is influenced by the rearing method, host-plant species, host-plant growth phase, part of the host plant, presence of host larvae, age of the parasitoid, and experience of the adult with odors from a plant–host complex.

METHODS AND MATERIALS

Insects. Heliothis zea larvae were reared on artificial diet (Burton, 1969). *Microplitis croceipes* were reared using the method of Lewis and Burton (1970) (see also Drost et al., 1986). Oviposition experience was given just prior to a flight test with one host.

Flight Tunnel. For a complete description of the flight tunnel, see Drost et al. (1986), and for the odor supply system, see Zanen et al. (1988). Odors were injected into the flight tunnel (50 × 50 × 120 cm) through nozzles made of Pasteur pipets. Four separate flasks were connected to one nozzle outside the flight tunnel, which allowed individual separation of the four larvae that formed one odor source. This was necessary because the larvae are cannibalistic and odors emanating from cannibalism reduce the response by *M. croceipes*. The system allows shut-off of odor sources when they are not needed, which makes it possible to switch between odor sources without contaminating the flight tunnel. Wind speed was maintained at 50 cm/sec.

Odor Sources. Cowpea-seedling leaves (*Vigna unguiculata* L.) and mature leaves and squares (i.e., flower buds) of cotton (*Gossypium hirsitum*) were obtained from plants grown in a greenhouse. Mature leaves and flowers of cowpea and hyacinth bean (*Lablab purpureus* L.) were collected in the field. One day prior to an experiment, recently molted fourth-instar *H. zea* larvae were placed in 1-oz cups with limited food (1-cm^2 piece of leaf on the plant to be evaluated). On the test day, odor sources were prepared with one larva and 1–2 g of plant material per flask and four flasks per odor source. To ensure saturated adsorption of materials inside the glass tubing and nozzles and to remove volatiles emanating from the cutting wounds of the plant material (Zanen et al., 1988), we turned on the dispersing system for 60 min prior to testing.

Test Procedure. The parasitoid females were placed in the experiment room under an exhaust hood and supplied with fresh honey and water 30 min prior

to a test. A single female was introduced into the flight tunnel in a 1-dram vial while the wind speed was set on maximum (140 cm/sec) and the odor source shut off. Subsequently, the door of the tunnel was closed, the odor source opened, and the wind speed set on 50 cm/sec. By keeping the wind speed high until a test started, we could prevent pretest flight of the parasitoid. The occurrence of a sustained flight (i.e., a nonstop flight followed by a landing on the odor source; see Drost et al., 1986) was recorded for each female. All females were used once, and the percentage of sustained flights was calculated as the quotient of the number of females making a sustained flight and the total number of females tested, unless otherwise noted. All tests were single choice tests and were carried out on at least three different days. Homogeneity among days of number of sustained flights to a certain odor source was tested with a chi-square test with alpha = 0.05.

RESULTS

Experiment 1: Influence on Rearing Method on Responsiveness of Adult Female. To test the hypothesis that the rearing method influences the response to infested cowpea seedlings, we compared two groups of females: one group reared on hosts fed artificial diet and one group reared on hosts fed cowpea-seedling leaves. For parasitization, 20 third-instar *H. zea* larvae were confined with two 3-day-old *M. croceipes* females in a 16-oz cup for one night. Subsequently, the larvae were split into two groups and transferred to 1-oz cups, one larva/cup, with artificial diet or cowpea-seedling leaves. The larvae were kept at 28°C, 50–70% relative humidity, and a 16-hr photophase. The cowpea-seedling leaves were replaced every other day by fresh ones. We collected the parasitoid pupae and transferred them to clean 1-oz cups for emergence. Adults were collected from the cups before 10 AM each day and transferred to 16-oz cups, one female and one male per cup, until they were 4-5 days old. The females were released in acrylic cages (30 × 30 × 17 cm) the night prior to a test and provided with water and honey. We tested the responsiveness of the females in both groups (58 females reared on artificial-diet-fed hosts and 50 females reared on cowpea-seedlings-fed hosts) to odors of hosts feeding on cowpea-seedling leaves. All parasitoids were inexperienced when tests started. Females that made a sustained flight in either one of the first two trials were scored as responsive. Females that did not respond in the first two trials were given oviposition experience, which increases the probability of flight, and tested twice more to ensure that the alternative rearing methods had not diminished the flight capabilities of the parasitoids.

Females that were reared on hosts fed cowpea seedlings made more sustained flights to odors of hosts feeding on cowpea seedlings than females that

were reared on hosts fed artificial diet (Figure 1, open bars). For both groups, many of the nonresponding females would respond after oviposition experience and the total (inexperienced + experienced) number of responding females for both groups was not significantly different (Figure 1, solid bars). This indicates that the rearing method influences the olfactory response of adult females but does not change the flight capabilities of the females.

*Experiment 2: Influence of Odors from Different Plant–*H. zea *Complexes on Flight Response by Inexperienced Females.* In order to avoid possible conditioning to the host diet (Vet, 1983), we reared the parasitoids in this and all following experiments on hosts fed artificial diet. Responsiveness of 4- to 5-day-old inexperienced females was tested for three different odor sources in single-source experiments: (1) hosts feeding on mature cowpea leaves (N = 26), (2) hosts feeding on mature cotton leaves (N = 62), and (3) hosts feeding on mature hyacinth-bean leaves (N = 55). All plants were bearing flowers or flower buds when leaves were picked.

Odors from a hyacinth bean–*H. zea* complex elicited significantly more sustained flights in inexperienced females than did odor from cowpea leaves–*H. zea* or cotton–*H. zea* complexes (Figure 2). The three odor sources all elicited sustained flights in at least 40% of the females, which means that inexperienced females, even when reared on hosts feeding on artificial diet, are able to respond to volatile stimuli. Comparison of the results for cowpea with the results of experiment 1 for inexperienced females reared on hosts fed artificial diet suggested that the age of the host plant is another important factor determining the number of sustained flights. We verified this with the next experiment.

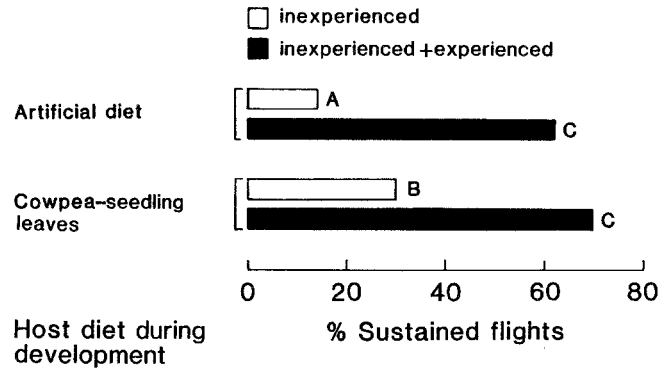

FIG. 1. Influence of rearing method on the flight response of adult females to odors of a cowpea seedling–*H. zea* complex. Similarly patterned bars with different letter indicate significant difference (G test, $P < 0.05$).

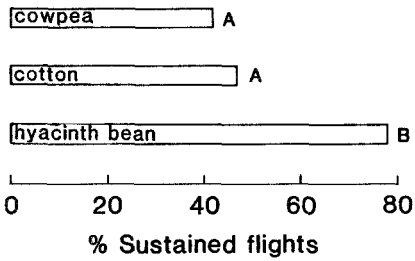

FIG. 2. Effect of host-plant species of *H. zea* on the flight response by *M. croceipes*. Bars with different letters indicate significant difference (G test, $P < 0.05$).

Experiment 3: Influence of Growth Phase of Host Plant on Flight Response. Inexperienced 4- to 5-day-old females were tested for their response toward plant–host complexes containing either cowpea-seedling leaves or mature cowpea leaves. The percentage females making a sustained flight toward a mature cowpea leaves–*H. zea* complex (42%, $N = 26$) was significantly (G test, $P < 0.05$) higher than that toward a cowpea seedling–*H. zea* complex (17%, $N = 34$).

Experiment 4: Flight Responses toward Different Parts of Plant in Relation to Presence of Hosts. We determined the responsiveness of inexperienced 4- to 5-day-old females toward infested leaves ($N = 55$), infested flowers ($N = 40$), uninfested leaves ($N = 44$), and uninfested flowers ($N = 50$) of hyacinth bean, all in single-source experiments. Both leaves and flowers were attractive without hosts present. Females did not respond differently to infested than to uninfested flowers although they did respond more frequently to infested leaves than uninfested leaves (Figure 3). Infestation significantly reversed the response lev-

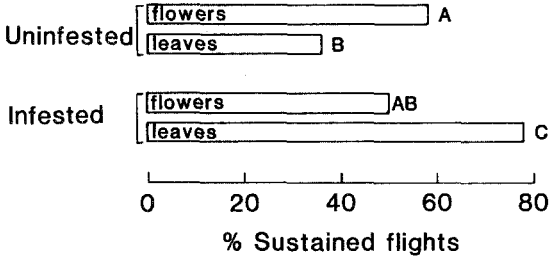

FIG. 3. Influence of the part of the host plant and *H. zea* infestation of the flight response. The host plant was hyacinth bean in all cases. Bars without same letters indicate significant difference (G test, $P < 0.05$).

els to flowers compared to leaves (G test, $P < 0.05$). We expected that infested flowers would be more attractive than uninfested flowers because they contain both hosts and food for the parasitoid. One explanation might be that the odor of the flowers masks the odor of the larvae.

Experiment 5: Influence of Age of Parasitoid on Flight Response. We tested 0- to 1-day-old females for their response toward infested hyacinth-bean leaves and uninfested flowers, again in single-source tests, and compared the responses with those of 4- to 5-day-old females from experiment 4. Comparison between experiments 4 and 5 was justified, because both experiments were carried out at the same days with parasitoids taken from the same populations. The age of the parasitoid is of significant (G test, $P < 0.05$) importance for the flight response when the parasitoid is inexperienced. While 78% ($N = 55$) of 4- to 5-day-old females respond to odor of a hyacinth bean leaves–*H. zea* complex, only 29% ($N = 41$) of 0- to 1-day-old females respond to the same odor source. In contrast, the response to host-free flowers was not significantly different for both age groups: 55% ($N = 20$) for the 0- to 1-day-old females and 58% ($N = 50$) for the 4- to 5-day-old females.

Experiment 6: Influence of Odors Encountered Previously on Flight Response. In the previous experiments, the percentage of females responding to a cowpea seedling–*H. zea* complex was consistently lower than to the hyacinth bean–*H. zea* complex. In this experiment, parasitoids were tested for their response to both these plant–host complexes in each of three sequences: cowpea/cowpea ($N = 19$), cowpea/hyacinth bean ($N = 25$), and hyacinth bean/cowpea ($N = 23$). One female was allowed to have no more than two trials and no more than one sustained flight to each odor source. This resulted in differences in the number of trials per female (minimum 2, maximum 4), but we thought it was more important to keep the flight experience to each source constant because flight experience may increase the level of response. However, when both odor sources contained infested cowpea seedlings, none of the females responded twice (Figure 4). Apparently, in females without preflight exposure to a plant–host complex, flight experience by itself does not increase the level of response as was the case for parasitoids with preflight exposure to a plant–host complex (Drost et al., 1986). When the first odor source contained infested cowpea seedlings and the second contained infested hyacinth bean leaves most of the females responded only to the odor of the hyacinth bean–*H. zea* complex. When the first odor source contained infested hyacinth bean and the second contained infested cowpea seedlings, most females responded to the hyacinth bean complex as well as to the cowpea seedling complex and less than 10% did not respond at all. Thus the encounter with the odors of an attractive odor source increases the response to odors of a less attractive odor source.

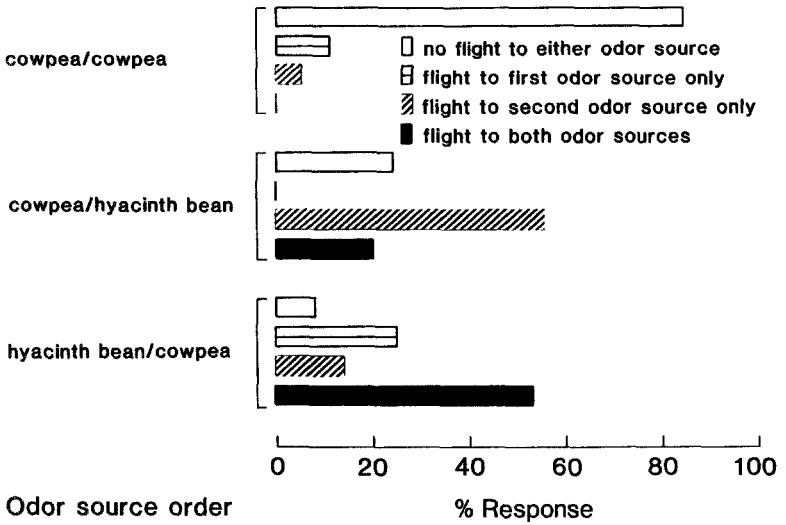

FIG. 4. Effect of previous encounter with odors on subsequent flight response. Odor sources were either a cowpea seedling–*H. zea* complex or a hyacinth bean–*H. zea* complex that were presented in different order to one female according to the treatment. The distributions of responses are significantly different between the three treatments with a repeated G test ($P < 0.05$).

DISCUSSION

The diet of the host larva during development of *M. croceipes* influences the responsiveness of the females toward odors of a plant–host complex in the flight tunnel. This may be caused by stimuli perceived either prior to eclosion or shortly after eclosion. During parallel studies in our lab on *M. demolitor*, Hérard et al. (1988) found that this parasitoid, which also parasitizes *H. zea* larvae, obtained the stimuli causing the increased responsiveness by contact with the cocoon. *M. demolitor* females reared on hosts fed cowpea seedlings and dissected from their cocoons had a low responsiveness. When these females were allowed to contact the cocoons, the responsiveness to a cowpea seedling–*H. zea* complex increased significantly compared to the initial response. A similar phenomenon may occur in *M. croceipes*. It is unclear whether stimuli perceived during development or upon eclosion condition the parasitoid, i.e., females reared on larvae fed artificial diet may be conditioned to respond to an artificial diet–*H. zea* complex, or decrease the response threshold to any plant–host complex. Whatever the mechanism, contact experience of the adult female with a fresh plant–host complex seems to be far more important for the respon-

siveness to host-related odors than the rearing method. Vet (1983) reported similar findings for the parasitoid *Leptopilina claviceps*, a larval parasitoid of fungivorous Drosophilidae.

The host plant of the *H. zea* larvae appears to be an important factor in the host-searching process of the parasitoid. The innate response of *M. croceipes* females differed with the host-plant species on which *H. zea* was feeding, and odors of an attractive plant–host complex increased the response to a less attractive plant–host complex. Hence, a less attractive host plant may benefit from being planted between more attractive host plants because the response threshold of the parasitoid is at least temporarily decreased by the more attractive host plants.

Flowers and extrafloral nectaries may influence the distribution of both pest and beneficial arthropod populations (Adjei-Maafo and Wilson, 1983; Maxwell et al., 1976). The presence of floral and extrafloral nectaries increases both the survival of the parasitoid and rates of parasitization in *Campoletis sonorensis*, another larval endoparasitoid of *H. zea* (Lingren and Lukefahr, 1977). Although we found only a small percentage of inexperienced recently emerged *M. croceipes* females responding to host-related odors, a much larger percentage was responding to host-free flowers. Upon visiting flowers, the prefered plant part by *H. zea*, a female might by chance encounter host feces or a host and thus become experienced, which increases her response level to subsequently perceived host-related odors.

The work reported here shows that different factors in the environment of the parasitoid as well as in the parasitoid itself affect the behavioral response in *M. croceipes*. It still remains to be investigated in what way the different factors interact with each other.

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VOLATILE METHYL KETONE SEED-GERMINATION INHIBITORS FROM *Amaranthus palmeri* S. WATS. RESIDUES[1]

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Abstract—The effects of nine methyl ketones previously identified in the mixture of volatiles released by *Amaranthus palmeri* (AMAPA) residues upon onion, carrot, AMAPA, and tomato seed germination were determined. Three-day exposures to these volatiles significantly inhibited germination of all assay seeds, and the degree of inhibition was dependent upon seed species, exposure time, and concentration. Based on the degree of inhibition observed in both time- and concentration-dependent assays, the following activity series was obtained: 2-octanone, 2-nonanone > 2-undecanone > 2-heptanone > 2-hexanone, 3-methyl-2-butanone, 2-pentanone, 3-hydroxy-2-butanone > 2-butanone. The activities of these compounds appear to be additive and dependent on relative volatility and hydrophilicity.

Key Words—Allelopathy, volatile allelochemicals, methyl ketones, germination inhibitors, onion, *Allium cepa*, carrot, *Daucus carota*, Palmer amaranth, *Amaranthus palmeri*, tomato, *Lycopersicon esculentum*.

INTRODUCTION

The presence of *Amaranthus palmeri* (AMAPA) residues in the soil reduces fresh weight accumulation in onions and carrots and markedly decreases seedling field establishment in carrots (Menges, 1985). Classic natural products iso-

[1] Name of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

lation techniques (Bradow, 1985, Fischer and Quijano, 1985) and solid-phase separation techniques applied to water extracts of soil containing AMAPA residues (Bradow and Connick, 1987) failed to isolate any inhibitory factors from either AMAPA residues or soil containing such residues.

However, volatiles emitted both by AMAPA residues and soil containing the residues were highly inhibitory of germination in onion, carrot, AMAPA, and tomato (Bradow and Connick, 1987). A number of volatile organic compounds associated with AMAPA seedhead, stem, and root residues were subsequently identified (Connick et al., 1987), and two of these volatiles, 2-heptanone and 2-heptanol, were shown to be potent inhibitors of seed germination. The high bioactivity of 2-heptanone and the detection of six other methyl ketones in the volatile mixtures associated with AMAPA residues led to the present examination of the seed germination effects produced by the identified methyl ketones (plus 2-hexanone and 2-octanone detected under anaerobic conditions), tested individually and in selected binary combinations. This paper describes the concentration and exposure-time dependence of these effects observed with nine C_4–C_{11} methyl ketones associated with AMAPA residues.

METHODS AND MATERIALS

All chemicals used in these assays were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). Assay seeds were either purchased from commercial sources or were gifts of Dr. R.M. Menges, USDA, Weslaco, Texas.

Time-Dependent Seed-Germination Bioassays. The desiccator seed-germination assay technique has been previously described (Bradow and Connick, 1987; Connick et al., 1987). Briefly, seeds of onion, carrot, AMAPA, or tomato were spread on double sheets of deionized water-saturated Whatman No. 1 filter paper placed on the porcelain plates of separate 2.5-liter (160-mm-ID) glass desiccators. Circles (22 mm diam.) had been removed from the filter paper sheets to facilitate diffusion of volatiles, and the filter paper circles were divided into eight equal segments (replicates) containing the same number of seeds (25 AMAPA, or 20 of the other species). Each desiccator well contained a 10-ml glass beaker resting on 50 g of pure sand in a crystallizing dish (100 × 50 mm). The sand was moistened with 10 ml of deionized water. The central beakers were left empty in the controls. In the time-dependence study, a volume equivalent to 34.4 μM of a volatile test compound was placed in the central beaker, and the separate seed species were incubated for 72 hr before germination evaluation (3-day data). Radicle protrusion was the germination criterion. All evaluations were made in dim light under an exhaust safety hood. The volatile

source beaker was then either removed (3 + 4-day data) or the test volatile was renewed by the addition of a second 34.4 μM equivalent (7-day data). All seeds were then incubated an additional four days before final evaluation. Controls (deionized water) was repeated at three-month intervals throughout the duration of the experiments.

Concentration-Dependent Seed-Germination Bioassays. The central beaker of the desiccator apparatus contained the appropriate volume of a single test compound to give a nominal volatile concentration of 3.4, 6.9, 17.2, or 34.4, μM, assuming total volatilization and negligible adsorption. The actual volumes of neat ketone placed by microsyringe in the central beakers were adjusted on the basis of compound density to produce equal initial volatile molarities, e.g., 0.8, 1.8, 4.5, and 8.0 μl 2-butanone compared to 1.8, 3.5, 8.9, and 17.8 μl 2-undecanone. The seeds were then incubated three days in the presence of the test volatile, germination was evaluated, the volatile source was removed, and the seeds were incubated an additional four days. In both assays humidity within the desiccators was maintained by the addition of 10 ml deionized water after germination evaluation at three days.

2-Heptanone × 3-Hydroxy-2-butanone Interaction Assays. The standard 3-day exposure seed-germination assay was repeated using total ketone concentrations of 34.4, 25.8, or 17.2 μM in ratios of 2-heptanone to 3-hydroxy-2-butanone of 4:0, 3:1, 2:2, 1:3, or 0:4.

Statistical Analysis. After normalization by the transformation $[(x + 0.5)^{0.5}]$, germination count data from the concentration-dependent bioassays and the 7-day and 3 + 4-day time-dependent data were compared separately for each seed species by two-way analyses of variance with 16 replicates. One-way analyses of variance were used to determine significant differences between the effects of the methyl ketones and appropriately incubated deionized water controls within the 3-day, 7-day, and 3 + 4-day data, using Tukey's Honestly Significant Difference procedure (significance level, $P = 0.01$); and concentration-dependent data were examined by regression analysis. Chi-square test and G-test goodness of fit procedures were used to test the hypothesized additive relationships between the activities of 2-heptanone and 3-hydroxy-2-butanone (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

All of the nine methyl ketones listed in the figures and tables were detected in the volatile mixtures above AMAPA residues (Connick et al., 1987 and unpublished data). 2-Hepatonone was a major component of these mixtures,

but 2-nonanone and 2-undecanone were observed only in trace amounts and principally in volatiles emitted by AMAPA root tissue. 2-Octanone and 2-hexanone were observed only when the residues were incubated anaerobically under nitrogen. Since plant residue decomposition frequently occurs under low oxygen conditions in the soil (Parr and Reuszer, 1959) and since volatiles from both AMAPA roots and seedheads inhibited germination in at least one test seed (Bradow and Connick, 1987), the homologous C_4–C_9 methyl ketone series plus 2-undecanone, 3-hydroxy-2-butanone, and 3-methyl-2-butanone were tested for effects on the germination of onion, carrot, AMAPA, and tomato seeds. Germination of seeds exposed only to deionized water vapor (control) showed no significant changes throughout the duration of these experiments.

Effects of Exposure to 34.4 μM Methyl Ketones upon Onion Seed Germination. After a 3-day exposure, all nine methyl ketones significantly inhibited onion seed germination in comparison to the control (Figure 1). 2-Butanone was the least inhibitory (39% reduction, compared to control), and 2-heptanone and 2-octanone were the most inhibitory (85% and 88% reductions, respectively). When the volatile source was removed after three days and the seeds were incubated for an additional four days, the germination of onion seeds exposed to 2-heptanone, 2-octanone, 3-methyl-3-butanone, 3-hydroxy-2-butanone was no longer significantly inhibited. The significant inhibitors induced by a 3-day exposure to 2-butanone and 2-pentanone persisted unchanged, regardless of whether the ketone source was removed or renewed before the additional 4-day incubation. Continuous 7-day exposure to 2-hexanone, 2-nonanone, and 3-methyl-2-butanone was necessary to maintain the degree of inhibition observed with these compounds at three days. Continuous exposure to 2-heptanone and 3-hydroxy-2-butanone retarded, but did not prevent, onion seed germination. 2-Undecanone produced the largest residual effect (67% reduction) after the ketone source was removed, and 2-octanone and 3-methyl-3-butanone produced the largest inhibition (76% reduction) after 7-day exposure. The significant differences observed among the activities of these methyl ketones correlate most closely with carbon chain length.

The effects of 2-butanone, the two substituted 2-butanones, and 2-pentanone were not significantly different in the 3-day or the 3 + 4-day results. 3-Methyl-2-butanone was more inhibitory than these compounds in the 7-day continuous exposure assays.

Effects of Exposure to 34.4 μM Methyl Ketones upon Carrot Seed Germination. Carrot seed germination was also significantly inhibited by 3-day exposure to all the methyl ketones tested (Figure 2). The level of inhibition ranged from the minimums observed with 3-hydroxy-2-butanone (29% reduction, compared to control) and 2-butanone (33% reduction) to elimination of

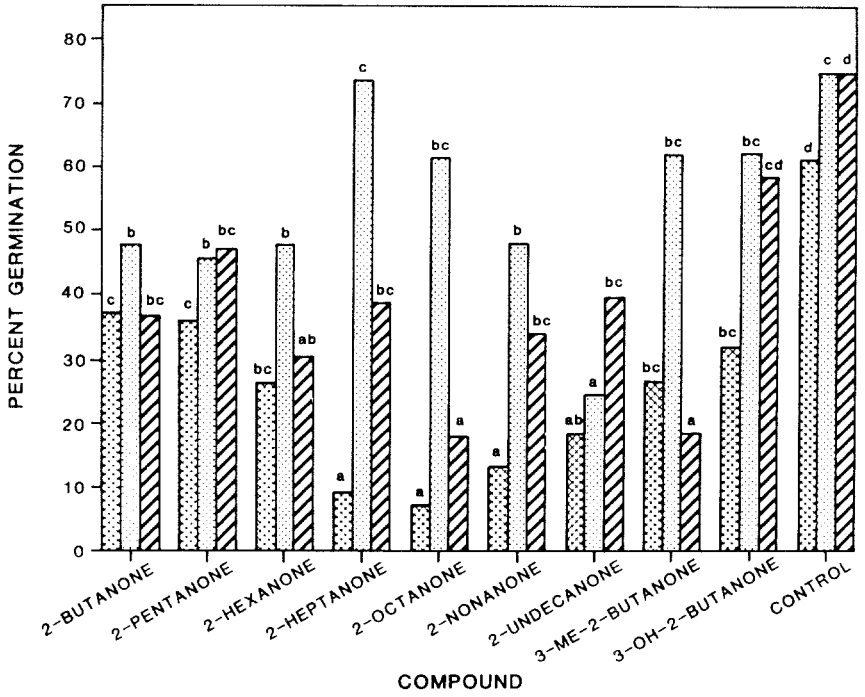

FIG. 1. Percent germination of onion (*Allium cepa* L.) seed exposed to 34.4 μM methyl ketones for three (first, crosshatched bar) and seven (third, diagonally striped bar) days or three days followed by four days' incubation after removal of ketone source (second, dotted bar). Percentages represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was $< 1.8\%$.

germination in the presence of 2-heptanone, 2-octanone, and 2-nonanone. The C_7-C_9 methyl ketones, which were the most inhibitory after three days, were the only test compounds to produce significant residual effects after removal of the ketone source. Continuous 7-day exposure to these same ketones also prevented germination, 2-undecanone decreased (22% reduction) germination under the same conditions. Although carrot seeds exposed for three days to 3-methyl-2-butanone and 3-hydroxy-2-butanone exceeded the germination percentages observed in the controls after seven days' total incubation, the increases in germination were not significant. In the carrot seed assays, activity differences were again correlated with carbon chain length. The activities of 2-butanone,

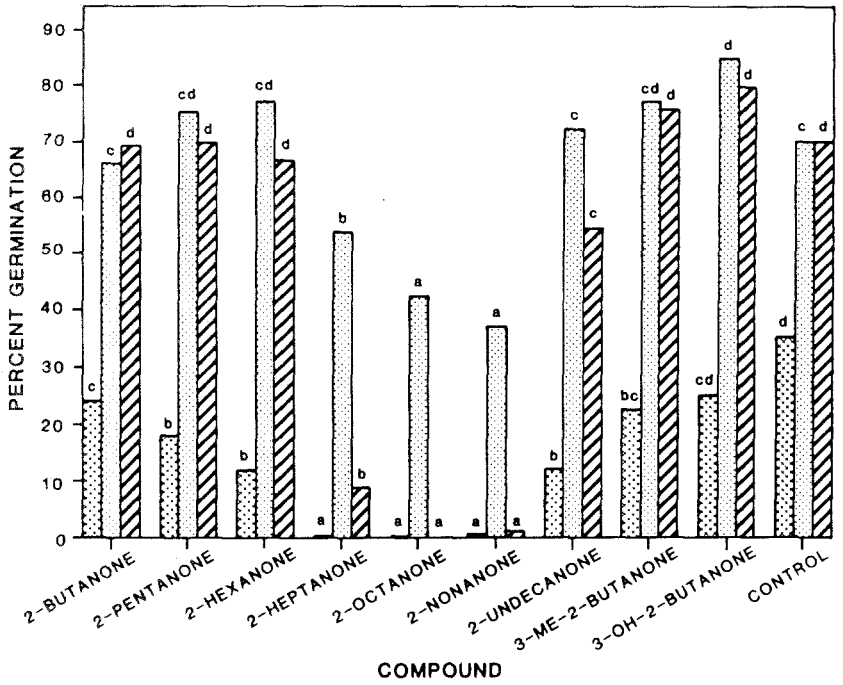

FIG. 2. Percent germination of carrot (*Daucus carota* L.) seed exposed to 34.4 μM methyl ketones for three (first, crosshatched bar) and seven (third, diagonally striped bar) days or three days followed by four days' incubation after removal of ketone source (second, dotted bar). Percentages represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was $< 1.5\%$.

3-methyl-2-butanone, 3-hydroxy-2-butanone, and 2-pentanone were not significantly different after either the 3-day or 7-day exposures.

Effects of Exposure to 34.4 μM Methyl Ketones upon AMAPA Seed Germination. All the methyl ketones profoundly inhibited AMAPA seed germination after a 3-day exposure (minimum inhibitory activity: 2-butanone, 86% reduction, compared to control) (Figure 3). When the ketone source was removed, the inhibitory effects of 2-butanone, 2-pentanone, and 2-heptanone disappeared by the end of the 7-day incubation. Residual inhibitory effects ranged from the minimum observed with 3-hydroxy-2-butanone (50% reduction) to the maximum induced by 2-nonanone (74% reduction). When exposure

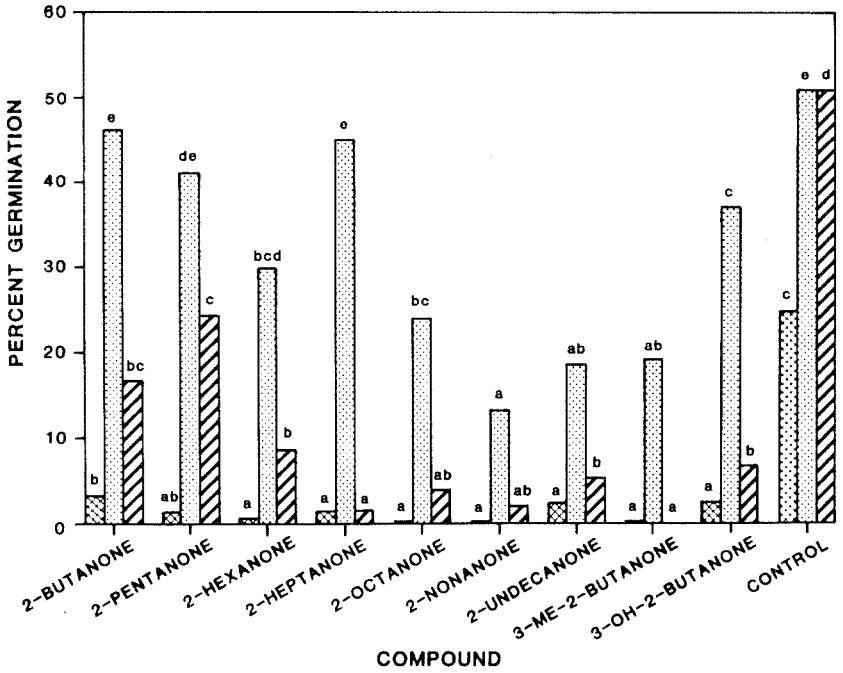

FIG. 3. Percent germination of AMAPA (*Amaranthus palmeri* S. Wats.) seed exposed to 34.4 μM methyl ketones for three (first, crosshatched bar) and seven (third, diagonally striped bar) days or three days followed by four days' incubation after removal of ketone source (second, dotted bar). Percentages represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was $<2.1\%$.

to the ketones was continuous throughout the incubation, the 3-day levels of inhibition were maintained in the presence of all but 2-butanone and 2-pentanone. 3-Methyl-2-butanone essentially prevented AMAPA germination as long as it remained present. Germination of AMAPA after a 3-day exposure to any of the tested methyl ketones was extremely low, but comparison of the effects of 2-butanone, 3-methyl-2-butanone, 3-hydroxy-2-butanone, and 2-pentanone after a 7-day exposure shows the following decreasing order of inhibition, 3-methyl-2-butanone > 3-hydroxy-2-butanone $\geq$ 2-butanone $\geq$ 2-pentanone. The correlation between carbon chain length and inhibitory activity observed in the other assay seeds was not apparent in the AMAPA data.

Effects of Exposure to 34.4 μM Methyl Ketones upon Tomato Seed Ger-

mination. A 3-day exposure to the C_5–C_9 methyl ketones, 2-undecanone, and 3-methyl-2-butanone prevented (<2% germination) tomato seed germination (Figure 4). 2-Butanone and 3-hydroxy-2-butanone were also highly inhibitory (70% and 75% reductions, respectively, compared to control). This length of exposure to 2-undecanone and 3-hydroxy-2-butanone, however, produced no significant residual inhibition in tomato seeds after the ketone source was removed. With the exception of 2-octanone (70% reduction), the residual inhibitory effects of the other methyl ketones were in the 26–33% reduction range. Even when the ketones were renewed before the second 4-day incubation period, the inhibitions initially caused by 2-butanone, 3-methyl-2-butanone, and 3-hydroxy-2-butanone did not persist, and the inhibitory effects of 2-pentanone, 2-undecanone, and 2-hexanone decreased. Only 2-heptanone, 2-octanone, and

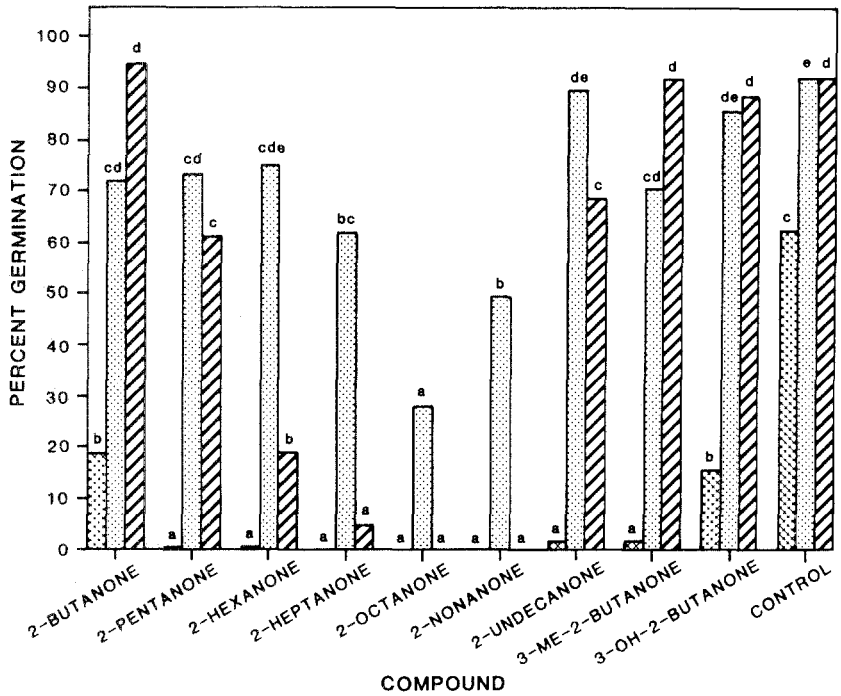

FIG. 4. Percent germination of tomato (*Lycopersicon esculentum* Mill.) seed exposed to 34.4 μM methyl ketones for three (first, crosshatched bar) and seven (third, diagonally striped bar) days or three days followed by four days' incubation after removal of ketone source (second, dotted bar). Percentage represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was <1.4%.

2-nonanone were profoundly inhibitory during the continuous 7-day exposure. The correlation between carbon chain length and inhibitory activity was apparent in both the 3-day and 7-day exposure data and, to a lesser degree, in the 3 + 4-day data for tomato seeds.

When the composite activities of these methyl ketones are considered across seed species and exposure time, the following approximate activity ranking is obtained: 2-octanone, 2-nonanone, 2-heptanone, 2-undecanone, 2-hexanone, 3-methyl-2-butanone, 2-pentanone, 2-butanone, 3-hydroxy-2-butanone. The two most active methyl ketones were observed as traces under aerobic conditions and were formed in increasing amounts when the AMAPA residues were incubated under nitrogen. 2-Heptanone, the third most active methyl ketone in these time-dependent experiments, was a major component in all AMAPA residue volatiles mixtures examined.

Although 34.4 μM of most of these methyl ketones inhibited all four assay seeds after the first 3-day exposure, the inhibition usually did not persist after the ketone source was removed. Residual inhibition was most often observed in seeds exposed to 2-undecanone, 2-nonanone, and 2-octanone, the latter two compounds having no significant residual effect on onion seeds. Continuous exposure to the methyl ketones for seven days was much more inhibitory, particularly in the case of the more inhibitory C_7–C_9 methyl ketones.

Concentration Effects of Exposure to Methyl Ketones. When the four species of assay seeds were exposed to 3.4, 6.9, 17.7, or 34.4 μM of each of the methyl ketones, the results shown in Table 1 were obtained. Two-way analyses of variance of the 3-day exposure data showed that concentration, compound, and the concentration $\times$ compound interaction were significant for all four species of seeds. When the appropriate control germination data were used as null concentration, direct plots of percent germination yielded hyperbolic curves, but transformation $[(x + 0.5)^{0.5}]$ of both x and y axes yield significant ($P > 0.10$) linear regression lines for all compounds and seeds. The slopes shown in Table 1 are for the transformed data.

Compared to the control, all concentration levels of the methyl ketones significantly inhibited onion seed germination, but not all these inhibitory effects were concentration dependent. 2-Octanone and 2-heptanone induced the most concentration-sensitive effects, while the inhibitory activity of 3-hydroxy-2-butanone did not change over the range of concentrations used. In comparison to the control, 2-butanone and 2-pentanone were inhibitory at all concentrations, but the degree of inhibition decreased as the concentration increased.

On the basis of analysis of variance ($P = 0.01$), carrot seed germination was insensitive to 3.4 μM levels of the methyl ketones. However, 3.4 μM 2-butanone and 2-undecanone induced inhibition that was significant ($P = 0.05$). All but 3-methyl-2-butanone and 3-hydroxy-2-butanone inhibited significantly

TABLE 1. GERMINATION EFFECTS AFTER THREE-DAY EXPOSURES TO VARIOUS CONCENTRATIONS OF METHYL KETONES

	Germination (%)[a]				
	3.4 μM Ketone	6.9 μM Ketone	17.7 μM Ketone	34.4 μM Ketone	Slope[b]
Onion					
2-Butanone	23.5 a	25.3 ab	32.3 bc	37.3 c	−0.0737 c
2-Pentanone	29.1 a	28.7 ab	26.4 bc	35.8 c	−0.1053 bc
2-Hexanone	43.8 b	29.1 ab	28.7 bc	26.4 bc	−0.0713 c
2-Heptanone	43.9 b	24.1 a	22.6 b	9.4 a	−0.3736 a
2-Octanone	23.9 a	27.8 ab	11.7 a	7.2 a	−0.3888 a
2-Nonanone	26.9 a	21.4 a	18.2 a	13.3 a	−0.2446 b
2-Undecanone	36.8 ab	35.0 b	28.9 bc	18.5 ab	−0.2146 b
3-Me-2-Butanone	43.2 b	42.2 b	29.5 bc	26.6 bc	−0.1354 bc
3-OH-2-Butanone	33.6 ab	31.8 b	35.3 c	31.9 bc	−0.0151 c
Control	61.1 c	61.1 c	61.1 d	61.1 d	
Carrot					
2-Butanone	23.9 a	24.3 ab	25.1 c	23.9 c	−0.0054 d
2-Pentanone	30.7 a	21.7 ab	28.3 cd	18.1 b	−0.1276 c
2-Hexanone	33.9 a	28.2 ab	18.1 b	12 b	−0.2196 bc
2-Heptanone	34.3 a	19.8 a	6.2 ab	0.3 a	−0.4039 a
2-Octanone	33.0 a	23.4 ab	0.7 a	0.3 a	−0.4772 a
2-Nonanone	30.2 a	20.8 a	2.1 a	0.7 a	−0.4418 a
2-Undecanone	21.9 a	21.9 ab	8.1 b	12.1 b	−0.2276 b
3-Me-Butanone	25.8 a	33.3 c	23.4 c	22.8 bc	−0.1008 cd
3-OH-Butanone	24.2 a	28.7 bc	27.2 cd	25.2 cd	−0.0062 d
Control	35.5 a	35.5 c	35.5 d	35.5 d	
AMAPA					
2-Butanone	5.7 b	5.6 b	4.0 b	3.5 b	−0.2247 a
2-Pentanone	2.5 ab	3.2 ab	2.2 ab	1.5 ab	−0.2512 a
2-Hexanone	1.2 a	4.4 ab	1.3 a	0.7 a	−0.2666 a
2-Heptanone	6.3 b	3.9 ab	0.9 a	1.5 a	−0.2896 a
2-Octanone	1.2 a	0.6 a	0.4 a	0.2 a	−0.2816 a
2-Nonanone	0.6 a	1.4 a	0.2 a	0.3 a	−0.2861 a
2-Undecanone	2.6 ab	1.2 a	1.3 a	2.5 a	−0.2312 a
3-Me-Butanone	1.5 a	1.5 a	0.9 a	0.2 a	−0.2707 a
3-OH-Butanone	3.5 ab	1.7 a	1.4 a	2.4 a	−0.2421 a
Control	24.7 c	24.7 c	24.7 c	24.7 c	
Tomato					
2-Butanone	72.1 d	66.1 c	74.0 c	18.9 b	−0.2649 b
2-Pentanone	63.6 d	30.8 b	22.5 b	0.5 a	−0.5602 a
2-Hexanone	9.4 a	5.5 a	1.2 a	0.5 a	−0.4766 a
2-Heptanone	7.0 a	2.8 a	1.2 a	0.0 a	−0.4713 a
2-Octanone	27.3 b	3.2 a	0.3 a	0.0 a	−0.5539 a
2-Nonanone	33.7 b	2.6 a	3.4 a	0.0 a	−0.5132 a
2-Undecanone	5.0 a	1.8 a	0.7 a	1.9 a	−0.4224 a
3-Me-Butanone	49.2 c	28.6 b	17.8 b	1.9 a	−0.4286 a
3-OH-Butanone	51.2 c	36.4 bc	20.9 b	15.5 b	−0.3511 ab
Control	62.5 cd	62.5 c	62.5 c	62.5 c	

[a] Percentages are the means of 16 replications. Values in columns followed by the same letter and associated with a given seed species are not significantly different ($P = 0.01$). Standard errors of the means were $< 2\%$.

[b] Slopes were obtained using transformed $(X + 0.5)^{0.5}$ germination count data.

at 6.9 μM. Higher levels of 2-heptanone, 2-octanone, and 2-nonanone essentially eliminated carrot seed germination. The relatively low inhibitory effects of 2-butanone, 2-pentanone, 3-methyl-2-butanone, and 3-hydroxy-2-butanone were not concentration dependent, while those of 2-heptanone, 2-octanone, and 2-nonanone increased significantly as the concentration increased.

All levels of the methyl ketones dramatically decreased AMAPA seed germination, and there was no significant difference in the concentration effects of the test compounds on these seeds. Tomato seed germination was not significantly affected by 3.4 μM 2-butanone, 2-pentanone, 3-methyl-2-butanone, and 3-hydroxy-2-butanone; however, 2-hexanone, 2-heptanone, and 2-undecanone were highly inhibitory at all concentrations tested. 2-Octanone and 2-nonanone inhibition was highly significant at concentrations above 3.4 μM. 2-Butanone inhibited tomato seed germination only at 34.4 μM.

A summary of the results based on the degree of inhibition at all assay concentrations and the relative concentration dependence as shown by the regression line slopes for each methyl ketone yields the following activity series: 2-octanone, 2-nonanone, 2-heptanone, 2-undecanone, 2-hexanone, 3-methyl-2-butanone, 2-pentanone, 3-hydroxy-2-butanone, 2-butanone. This series is essentially the same as that observed in the time-dependent studies.

Two-way analyses of variance of the 3 + 4-day data showed no significant ($P = 0.01$) concentration effects on carrot seed germination, and no methyl ketone induced a residual inhibition of 50% at 34.4 μM (Figure 2) or less (data not shown). There was no significant residual concentration effect on onion seed germination, and only 34.4 μM 2-undecanone inhibited more than 50% (Figure 1). There was also no significant difference between the residual effects of different concentrations of the test compounds on tomato seed germination, and no residual inhibition above 50% was observed, except after exposure to 34.4 μM 2-octanone (Figure 4).

Concentration, test compounds, and concentration $\times$ compound interaction effects were all significant in the 3 + 4-day AMAPA data. Exposure to 34.4 μM 2-octanone, 2-nonanone, 3-methyl-2-butanone, and 2-undecanone residually inhibited AMAPA 50% or more (Figure 3). In addition, under the 3 + 4-day exposure protocol, 6.9 and 17.7 μM 3-methyl-2-butanone reduced AMAPA seed germination, in comparison to the control at seven days, by 52 and 61% respectively.

2-Heptanone $\times$ 3-Hydroxy-2-butanone Interaction. All of the results discussed above were obtained by exposing the assay seeds to one methyl ketone at a time at differing concentrations or for varying lengths of time. Since the AMAPA residues give off mixtures of volatiles, assay seeds were exposed for three days to varying ratios of 2-heptanone and 3-hydroxy-2-butanone, the two major methyl ketone peaks in stem and root chromatograms (Connick et al., 1987). The effects of these mixtures and the pure compounds are shown in Table 2. Goodness-of-fit analysis permits the assumption of additivity in the

TABLE 2. OBSERVED GERMINATION EFFECTS AFTER THREE DAY EXPOSURES TO MIXTURES OF 2-HEPTANONE AND 3-HYDROXY-2-BUTANONE

Ratio heptanone-3-OH-2-butanone	Germination (%)[a] at total methyl ketone concentration		
	34.4 μM	25.8 μM	17.2 μM
Onion			
4:0	4.2 a	6.5 a	28.8 a
3:1	10.6 a	7.9 ab	35.9 a
2:2	11.3 b	16.5 ab	33.7 a
1:3	25.3 b	23.0 b	34.2 a
0:4	27.0 b	24.4 b	36.4 a
Carrot			
4:0	1.2 a	8.0 a	10.0 a
3:1	1.2 a	13.5 ab	16.1 ab
2:2	14.4 b	20.8 bc	22.8 ab
1:3	23.7 bc	23.5 bc	25.3 b
0:4	26.3 c	31.6 c	31.3 b
AMAPA			
4:0	0.4 a	0.6 a	1.6 a
3:1	1.0 a	1.0 a	2.2 a
2:2	0.6 a	1.9 a	2.8 a
1:3	1.2 a	0.2 a	1.2 a
0:4	0.8 a	0.2 a	0.2 a
Tomato			
4:0	0.0 a	0.0 a	5.1 a
3:1	0.2 a	0.0 a	12.8 b
2:2	6.2 a	12.5 b	15.5 b
1:3	10.3 b	13.0 c	17.7 b
0:4	12.6 b	18.9 c	20.9 b

[a] Observed percentages are means of 16 replications and values in a given column and associated with a particular seed species followed by the same letter are not significantly different ($P = 0.01$).

combined effects of 2-heptanone and 2-hydroxy-1-butanone at all combined concentration levels ($P = 0.01$). Since the concentration-dependence curves for these compounds are nonlinear, inhibitions observed at the three different total concentrations are not proportional. In all cases, the differences between observed and expected values were within experimental error for the germination assays.

The results of both the time- and concentration-dependent seed-germination studies show that the inhibitory activity of the methyl ketones is related to carbon chain length. The C_7–C_9 ketones were more inhibitory of all four seed species, the activity decreasing as the chain length increased or decreased out-

side this range. Branched-chain molecules (i.e., 3-methyl-2-butanone and 3-hydroxy-2-butanone) were no more inhibitory than the straight-chain ketones of similar molecular weight. The observed methyl ketone activity series may arise from differing, structure-related physiological activity, but the volatility of the individual compounds is probably also a significant factor. In all assays, the individual test compounds were allowed to vaporize from a dry glass beaker to equilibrate with the water-saturated interior of the desiccator. Thus, both the vapor pressures and the air–water partition coefficients of the test compounds would determine the effective concentrations within the desiccator. The vapor pressures of the methyl ketones show an approximately 100-fold decrease at the assay temperature over the C_4–C_{11} molecular weight range. At the micromolar levels assayed here, however, it can be assumed that the methyl ketones volatilize 100% after initial application to the beaker. The effective concentrations, with respect to the germinating seeds, would then be determined by the relative volatilities of the methyl ketones in dilute water solution. Under aqueous conditions, the higher-molecular-weight homologs in the methyl ketone series, up to C_9, are more volatile than the lower-molecular-weight homologs (Buttery et al., 1969). The air–water partition coefficient of 2-nonanone is approximately eight times greater than that of 2-butanone. However, this coefficient for 2-undecanone, which is less inhibitory than 2-nonanone, is 10 times that of 2-butanone, which is significantly less inhibitory.

The lower-molecular-weight compounds are more water soluble (Amoore and Hautala, 1983) and are also more likely to hydrogen bond with water, resulting in lower volatilization fluxes (Rathbun and Tai, 1984). Thus the air–water partitioning characteristics of the methyl ketones serve to increase the effective volatile concentrations of the compounds found most inhibitory in the bioassays. These same differences in hydrophilicity would also cause differential partitioning of the methyl ketones between membranes and cytoplasm within the germinating seeds. The various partitioning effects add weight to our current hypothesis which relates the physiological activity of these compounds to membrane disruption during seed rehydration and early stages of germination. This partitioning phenomenon may also explain the reciprocal relationship between molecular weight and seed-germination inhibition observed with millimolar aqueous solutions of acetone and the C_4–C_9 methyl ketones (Reynolds, 1977).

In most cases continual exposure to the methyl ketones was necessary for significant germination suppression. However, dried AMAPA residues, upon rehydration, emit inhibitory volatile mixtures for more than four months (Bradow and Connick, 1987). Thus, significant amounts of volatiles would be added to the soil atmosphere as long as these residues persist in the soil. Since the inhibitory activities of these methyl ketones appear to be additive, volatile mixtures, such as those arising from AMAPA residues, must be important, but relatively uninvestigated, factors in the soil atmosphere.

Volatile substances in the soil atmosphere, such as the methyl ketones and other compounds associated with AMAPA residues, probably have a decisive role in other cases of allelopathy besides the observed inhibitions of onion and carrot field establishment and growth which prompted this and earlier studies (Koller, 1972; Lill et al., 1979). In addition to decreasing or retarding seed germination, methyl ketones have been shown to stimulate the germination of competitive weeds (French and Leather, 1979; French et al., 1986) and of spores of pathogenic fungi (French, 1984, French et al., 1986). In the soil, other mixtures of volatile substances stimulate sclerotial (King and Coley-Smith, 1968; Linderman and Gilbert, 1969) and mycelial growth (Menzies and Gilbert, 1967). The components of these and other biologically active mixtures of volatiles (Afifi, 1975; Lill et al., 1979) have not been identified, but the inhibitory C_7–C_9 methyl ketones have been reported to be emitted by other plant residues (Buttery et al,, 1978, 1985; Flath et al., 1978). The broad distribution and biological activity of plant residue volatiles make them important factors, not only in allelopathy, but in phytopathology and agronomy, particularly in reduced tillage situations.

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SEED-GERMINATION INHIBITION BY VOLATILE ALCOHOLS AND OTHER COMPOUNDS ASSOCIATED WITH *Amaranthus palmeri* RESIDUES[1]

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Abstract—Effects of 3-pentanone and eight low molecular weight aliphatic alcohols and aldehydes identifed in the mixtures of volatiles released by *Amaranthus palmeri* S. Wats. (AMAPA) residues were determined on germination of onion, carrot, AMAPA, and tomato seeds. Three-day exposures to these volatiles significantly inhibited germination of these assay seeds, and the inhibition was dependent upon exposure time and concentration. Based on the degree of inhibition observed in both time- and concentration-dependent assays, the following activity series was obtained: 2-heptanol > 3-methyl-1-butanol, 1-hexanol > hexanal, 1-pentanol, 3-pentanone, acetaldehyde > ethanol, 2-methyl-1-propanol. The activities of this group of compounds with that of 2-heptanone appear to be additive and related to test compound volatility and hydrophilicity. 2-Heptanol and 2-heptanone also significantly inhibited the germination of other species, including shepherds-purse, soybean, lettuce, alfalfa, common purslane, oats, and lovegrass.

Key Words—Allelopathy, volatile allelochemicals, alcohols, aldehydes, germination inhibitors, onion, *Allium cepa*, carrots, *Daucus carota*, Palmer amaranth, *Amaranthus palmeri*, tomato, *Lycopersicon esculentum*.

INTRODUCTION

The presence of *Amaranthus palmeri* (AMAPA) residues in the soil reduces fresh weight accumulation in onions and carrots and markedly decreases seed-

[1] Name of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

ling field establishment in carrots (Menges, 1985). Classic natural products isolation techniques (Bradow, 1985; Fischer and Quijano, 1985) and solid-phase separation techniques applied to water extracts of soil containing AMAPA residues (Bradow and Connick, 1987) failed to isolate inhibitory factors from either AMAPA residues or soil containing such residues.

However, volatiles emitted by AMAPA residues and soil containing the residues were highly inhibitory of seed germination in onion, carrot, AMAPA, and tomato (Bradow and Connick, 1987). A number of the volatile allelochemicals associated with AMAPA seedhead, stem, and root residues were identifed (Connick et al., 1987) and have been tested individually and in selected binary ratios for seed germination effects. This paper describes the effects on seed germination induced by the vapors of 3-pentanone, six alcohols, and two aldehydes, all associated with AMAPA residues.

METHODS AND MATERIALS

All chemicals used in these assays were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). Assay seeds were either purchased from commercial sources or were gifts of Dr. R.M. Menges, USDA, Weslaco, Texas.

Time-Dependent Seed-Germination Bioassays. The desiccator seed-germination assay technique has been previously described (Bradow and Connick, 1987, 1988; Connick et al., 1987). Controls (deionized water) were repeated at three-month intervals throughout the duration of the experiments, and no significant changes were observed during the term of these experiments. In the time-dependence study, 34.4 μM of a volatile test compound were placed in the central beaker, and the separate seed species were incubated for 72 hr before germination evaluation (3-day data). The volatile source beaker was then either removed (3+4-day data) or the test volatile was renewed by the addition of a second 34.4 μM volume (7-day data). All seeds were then incubated an additional four days before final evaluation.

Concentration-Dependent Seed-Germination Bioassays. The concentration-dependent assays were performed as previously described (Connick et al., 1987, Bradow and Connick, 1988), using 3.4, 6.9, 17.2, or 34.4 μM of each test volatile. The seeds were incubated for three days in the presence of the test compound, germination was evaluated, the volatile source was removed, and the seeds were incubated an additional four days.

Assays of Binary Mixtures of 2-Heptanone × Alcohol. The standard 3-day exposure seed-germination assay was repeated using ratios of 2-heptanone × 2-heptanol or 2-heptanone × 3-methyl-1-butanol of 4:0, 3:1, 2:2, 1:3, or 0:4 to give total assay volatile concentrations of 34.4, 25.8, or 17.2 μM.

Multiseed Assays of 2-Heptanone and 2-Heptanol Activity. Under environmental conditions similar to those used in the desiccator studies, seeds of the species listed in Table 4 were germinated separately in an apparatus consisting of a sealed one-pint (0.473-liter) dome-lidded Mason jar in the mouth of which was suspended a perforated aluminum weighing dish (5.7 cm diam., Fisher Scientific). The bottom of the dish was perforated with a 1-cm central hole surrounded by six 4-mm holes, all made with a cork borer. A paper punch (6 mm diam.) was used to make six additional holes around the side of each dish to facilitate vapor phase movement. Each dish was lined with two sheets of 5.5-cm-diam. Whatman No. 1 filter paper, each sheet having a 1-cm central perforation. Depending on seed size, 30 or 40 seeds were placed on the filter paper sheets which had been moistened with 2 ml deionized water. To emulate the desiccator assays on the smaller scale of the Mason jar apparatus, all parameters were reduced by a factor of 0.19 based on the ratio of the relative volumes of the Mason jars and the desiccators. A Wheaton bottle (8 ml) that contained 9.5 g sand moinstened with 1.9 ml deionized water was placed in the bottom of each jar, and a microsyringe was used to place either 34.4 or 68.9 μM (2.3 μl or 4.6 μl) of 2-heptanol or 2 heptanone in the Wheaton bottle.

Statistical Analysis. After normalization by the transformation $[(x+0.5)^{0.5}]$, germination count data from the concentration-dependent bioassays and the 7-day and 3+4-day time dependent data were compared separately for each seed species by two-way analyses of variance with 16 replicates. One-way analyses of variance were used to determine significant differences between the effects of the test volatiles and appropriately incubated deionized water controls within the 3-day, 7-day, and 3+4-day data, using Tukey's Honestly Significant Difference procedure with a significance level of $P = 0.01$. Concentration dependent data were also examined by regression analysis, and the chi-square test and *G*-test goodness-of-fit procedures were used to test hypothesized additive relationships between the activities of selected pairs of volatile compounds (Sokal and Rohlf, 1981). To permit comparisons between these volatiles and the methyl ketones discussed in the preceding paper (Bardow and Connick, 1988), all analyses of variance were repeated on the combined data for the 18 volatile compounds.

RESULTS AND DISCUSSION

Prior work had shown that all the compounds listed in the figures and tables were detected in the volatile mixtures associated with AMAPA residues (Connick et al., 1987). 2-Pentanol and 1-hexanol were observed in atmospheres associated only with AMAPA roots. 3-Methyl-1-butanol was a major compo-

nent of the volatile mixtures, and 2-heptanol was always associated with 2-heptanone in the GC-MS analyses. Ethanol and traces of acetaldehyde were ubiquitous.

Effects of Exposure to 34.4 μM Test Volatiles upon Onion Seed Germination. After a 3-day exposure, all test compounds, except 3-pentanone, inhibited onion seed germination more than 50% with respect to the deionized water control (Figure 1). 2-Heptanol, 3-methyl-1-butanol, acetaldehyde, and hexanal were the most inhibitory. When the volatile source was removed after three days and the seeds were allowed to incubate an additional four days, seeds initially exposed to 3-methyl-1-butanol were still greatly inhibited (>80%), essentially the same degree of inhibition observed if the volatile concentration were maintained throughout the 7-day duration of the assay. Onion seeds exposed to 2-heptanol and hexanal were still significantly inhibited after seven days, regardless of whether the volatile sources were removed; but seeds inhib-

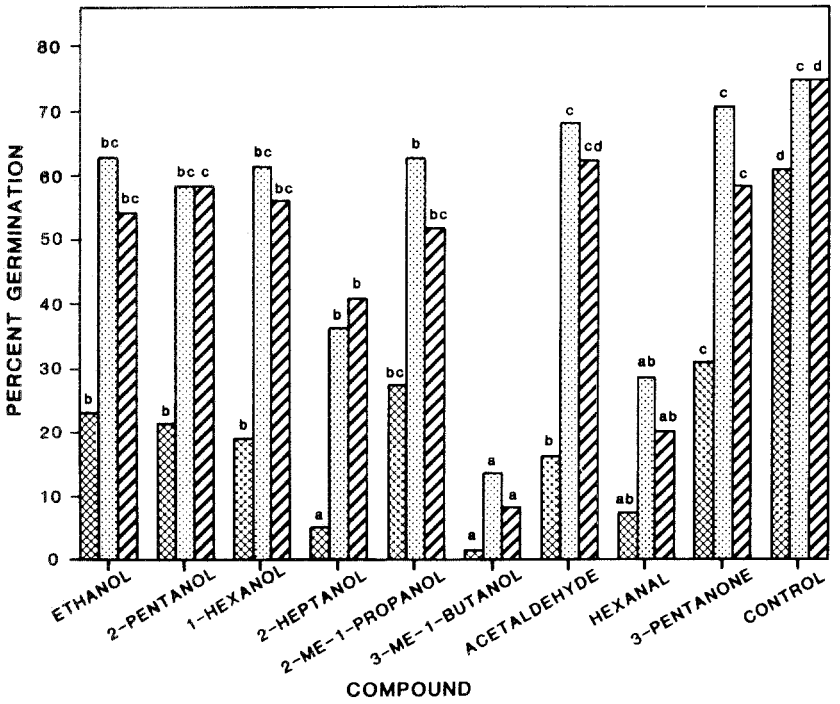

FIG. 1. Percent germination of onion (*Allium cepa* L.) seeds exposed to 34.4 μM test volatiles for three (first, crosshatched bar) and seven (third, striped bar) days or three days followed by a four-day (second, dotted bar) incubation after removal of ketone source. Percentages represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was <2.0%.

ited by a 3-day exposure to acetaldehyde germinated to the same extent as the control after seven days, both in the presence and the absence of this aldehyde. None of the other test compounds inhibited onion seed germination more than 30% after the initial 3-day exposure period.

On the basis of the analyses of variance combining data for this group of nine volatile compounds with data previously reported (Bradow and Connick, 1988) for nine methyl ketones, 3-methyl-1-butanol, hexanal, and 2-heptanol were more inhibitory of onion seed germination than the most active methyl ketones, i.e., 2-undecanone and 2-octanane.

Effects of Exposure to 34.4 μM Test Volatiles upon Carrot Seed Germination. 1-Hexanol, 2-heptanol, 3-methyl-1-propanol, 3-methyl-1-butanol, acetaldehyde, and hexanal all inhibited carrot seed germination more than 50% after the 3-day exposure (Figure 2). None of the test compounds had any significant inhibitory residual effect when the volatile source was removed after three days,

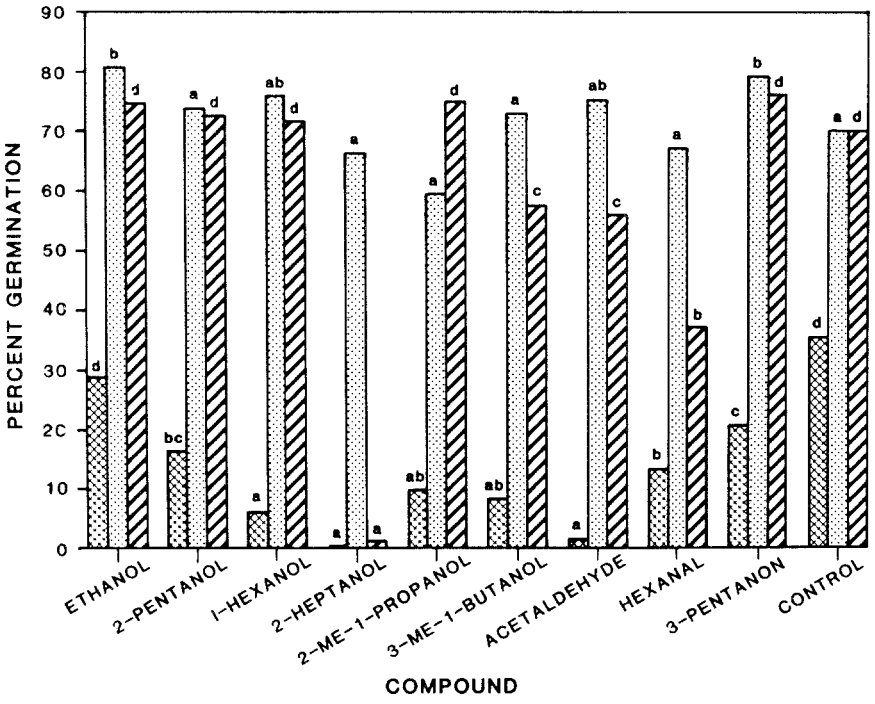

FIG. 2. Percent germination of carrot (*Daucus carota* L.) seeds exposed to 34.4 μM test volatiles for three (first, crosshatched bar) and seven (third, striped bar) days or three days followed by a four-day (second, dotted bar) incubation after removal of ketone source. Percentages represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was $< 1.4\%$.

and germination was slightly promoted in carrot seeds initially exposed to ethanol and 3-pentanone. A 7-day exposure to 2-heptanol eliminated carrot seed germination, and continuous exposure to 3-methyl-1-butanol, acetaldehyde, and hexanal also significantly inhibited germination.

The composite analysis of variance including the methyl ketone data shows that, in general, 2-heptanol was as inhibitory as the most potent methyl ketones, and the activities of hexanal, acetaldehyde, 1-hexanol, and 3-methyl-1-butanol were comparable to that of 2-undecanone in the carrot seed assays (Bradow and Connick, 1988).

Effects of Exposure to 34.4 μM Test Volatiles upon AMAPA Seed Germination. Again, a 3-day exposure to any of the test compounds inhibited AMAPA seed germination (Figure 3). 2-Methyl-1-propanol was the least inhibitory. Only low residual effects were observed when the sources of 1-pentanol, 2-heptanol, and 3-pentanone were removed. After a 7-day exposure, all test compounds

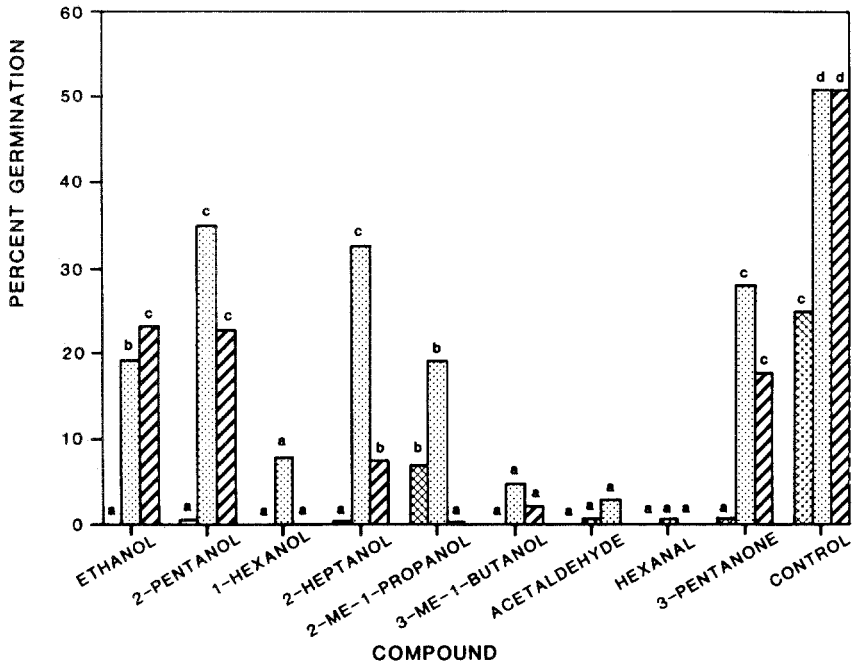

FIG. 3. Percent germination of AMAPA (*Amaranthus palmeri* S. Wats.) seeds exposed to 34.4 μM test volatiles for three (first, crosshatched bar) and seven (third, striped bar) days or three days followed by a four-day (second, dotted bar) incubation after removal of ketone source. Percentages represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was $<2.0\%$.

inhibited AMAPA seed germination more than 50%, in comparison to the appropriate control. Ethanol, 1-pentanol, and 3-pentanone were the least inhibitory under these conditions.

Acetaldehyde, hexanal, 3-methyl-1-butanol, and 1-hexanol had greater residual effects on AMAPA seed germination than all the methyl ketones (Bradow and Connick, 1988). Continuous exposure to 1-hexanol, hexanal, 2-methyl-1-propanol, 3-methyl-1-butanol, acetaldehyde, and 2-heptanol was as effective in suppressing AMAPA seed germination as similar exposure to the most inhibitory methyl ketones, 3-methyl-2-butanone, the C_6–C_9 methyl ketones, 2-undecanone, and 3-hydroxy-2-butanone.

Effects of Exposure to 34.4 μM Test Volatiles upon Tomato Seed Germination. Ethanol was the only test compound that did not inhibit tomato seed germination more than 70% after a 3-day exposure (Figure 4). Seeds exposed

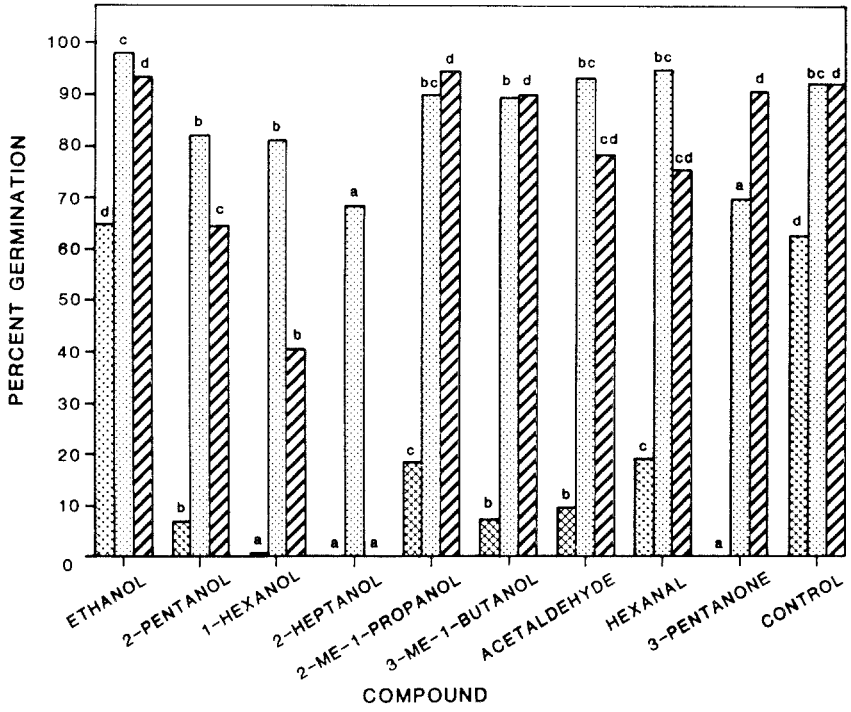

FIG. 4. Percent germination of tomato (*Lycopersicon esculentum* Mill.) seeds exposed to 34.4 μM test volatiles for three (first, crosshatched bar) and seven (third, striped bar) days or three days followed by a four-day (second, dotted bar) incubation after removal of ketone source. Percentages represented by bars with the same shading and lower case letter do not differ significantly ($P = 0.01$ with $N = 16$). Standard error of all means was $< 1.5\%$.

to all the test compounds, except 2-heptanol and 3-pentanone, germinated normally when the volatile sources were removed after the initial exposure period. During a 7-day exposure, 2-heptanol prevented tomato seed germination and 1-hexanol inhibited germination of tomato seeds more than 50%.

After the 3-day exposure, 2-heptanol, 3-pentanone, 2-pentanol, 3-methyl-1-butanol, and acetaldehyde were as inhibitory of tomato seed germination as the highly active C_5–C_9 methyl ketones and 2-undecanone (Bardow and Connick, 1988). Only 2-heptanol and 3-pentanone had residual effects equivalent to those observed with the more inhibitory methyl ketones, but continuous exposure to 2-heptanol was as effective in preventing tomato seed germination as exposure to the highly inhibitory C_6–C_9 methyl ketones. The effects of 2-heptanol were equivalent to those observed after exposure to 2-heptanone.

Overall, the most inhibitory compounds in these exposure-time-dependent studies were 3-methyl-1-butanol, hexanal, and 2-heptanol. The inhibitory activities of these nine compounds could be ranked 3-methyl-1-butanol, hexanal, 2-heptanol > 1-hexanol, acetaldehyde, 2-pentanol > 2-methyl-1-propanol, 3-pentanone >> ethanol. In comparsion to the activities of the methyl ketones discussed in the preceding paper (Bradow and Connick, 1988), the germination reductions caused by exposure to 3-methyl-1-butanol, hexanal, and 2-heptanol were equivalent to those produced by 2-heptanone. 1-Hexanol, 2-pentanol, and acetaldehyde inhibited to the same degree as 2-hexanone, 3-methyl-2-butanone, and undecanone; while the activities of 2-methyl-1-propanol and 3-pentanone were not significantly different from those of 2-pentanone and 2-butanone. Both 3-hydroxy-2-butanone and ethanol induced relatively little inhibition, compared to the other test compounds in the combined series. [Refer to Figures 1–4, in Bradow and Connick (1988) for corresponding methyl ketone results.]

Concentration Effects of Exposure to Volatile Test Compounds. When the four species of assay seeds were exposed for three days to 3.4, 6.9, 17.2, or 34.4 μM of each of the nine test compounds, the results shown in Table 1 were obtained. Two-way analysis of variance of the data showed that concentration, compound and the concentration × compound interaction were significant for all four species of seeds. When the appropriate control germination data were used as null concentration, direct plots of percent germination yielded hyperbolic curves, but transformation $[(x + 0.5)^{05}]$ of both x and y axes yields significant ($P < 0.01$) linear regression lines for all compounds and seeds. The slopes shown in Table 1 are for the transformed data and include null concentration data from the appropriate controls.

After a 3-day exposure, all of the test compounds significantly inhibited onion seed germination of all four assay concentrations. The most pronounced concentration-dependent effects were observed in onion seeds exposed to 3-methyl-1-butanol and 2-heptanol. The concentration-dependent effects of 2-heptanol and 3-methyl-1-butanol approximated to those observed with 2-octanone, and hexanal and acetaldehyde were as inhibitory as 2-nonanone. The

TABLE 1. SEED GERMINATION EFFECTS AFTER THREE-DAY EXPOSURE TO VARIOUS CONCENTRATIONS OF TEST VOLATILES

	Germination (%)[a] at test volatile concentration				
	3.4 μM	6.9 μM	17.7 μM	34.4 μM	Slope[b]
Onion					
Ethanol	33.9 ab	32.1 b	29.1 b	23 b	−0.108 d
2-Pentanol	32.6 ab	30.5 b	30.3 b	21.4 b	−0.161 c
1-Hexanol	40.5 ab	27.8 b	25.8 b	18.9 b	−0.247 bc
2-Heptanol	25.5 a	14.2 a	14.9 a	5.1 a	−0.321 b
3-Me-1-Butanol	37.4 ab	20.5 ab	7.1 a	1.5 a	−0.523 a
Acetaldehyde	24.7 a	25.3 b	20.0 ab	16.3 b	−0.125 cd
Hexanal	30.7 ab	26.3 b	21.1 ab	7.5 ab	−0.234 bc
3-Pentanone	44.4 b	43.3 c	3.8 c	30.9 c	−0.091 d
2-Me-1-Propanol	34.3 ab	32.1 b	27.1 b	27.4 bc	−0.199 c
Control	61.1 c	61.1 d	61.1 d	61.1 d	
Carrot					
Ethanol	34.9 b	36.4 cd	31.0 dc	29.0 d	−0.022 c
2-Pentanol	29.5 ab	36.3 c	31.7 d	16.7 bc	−0.046 c
1-Hexanol	22.5 ab	27.3 bc	24.9 c	6.4 a	−0.251 b
2-Heptanol	30.1 ab	14.9 a	6.7 a	0.3 a	−0.417 a
3-Me-1-Butanol	24.3 ab	24.8 ab	9.7 b	8.5 ab	−0.363 a
Acetaldehyde	42.4 b	35.0 c	25.0 c	1.5 a	−0.190 b
Hexanal	44.2 b	43.5 d	28.5 cd	13.4 b	−0.245 b
3-Pentanone	20.8 a	24.0 ab	24.7 cd	20.8 c	−0.043 c
2-Me-1-Propanol	24.2 ab	33.7 cd	30.2 cd	9.8 ab	−0.072 c
Control	35.5 b	35.5 cd	35.5 d	35.5 d	
AMAPA					
Ethanol	1.4 a	0.8 a	1.1 ab	0.1 a	−0.369 a
2-Pentanol	2.8 a	1.1 a	1.0 ab	0.6 a	−0.281 bc
1-Hexanol	2.6 a	2.5 a	0.0 a	0.0 a	−0.30 b
2-Heptanol	3.4 a	1.4 a	0.2 a	0.2 a	−0.31 b
3-Me-1-Butanol	5.0 a	0.4 a	0.0 a	0.0 a	−0.32 b
Acetaldehyde	1.2 a	1.2 a	0.0 a	0.0 a	−0.387 a
Hexanal	0.2 a	0.0 a	0.2 a	0.0 a	−0.343 a
3-Pentanone	2.1 a	2.7 a	2.5 b	0.9 a	−0.25 bc
2-Me-1-Propanol	29.2 b	27.1 b	19.3 c	7.2 b	−0.285 c
Control	24.7 b	24.7 b	24.7 c	24.7 c	
Tomato					
2-Ethanol	75.1 d	72.0 d	71.4 d	64.8 d	0.190 d
2-Pentanol	32.2 b	8.0 a	8.1 b	6.8 b	−0.405 bc
1-Hexanol	14.9 b	9.0 a	0.5 a	0.5 a	−0.513 a
2-Heptanol	9.5 a	2.3 a	0.3 a	0.2 a	−0.492 a
3-Me-1-Butanol	34.4 b	25.6 b	20.3 c	7.3 b	−0.374 c
Acetaldehyde	73.8 d	56.7 c	66.7 d	9.5 b	−0.056 d
Hexanal	70.5 d	72.8 d	62.7 d	19.0 c	−0.349 c
3-Pentanone	31.2 b	26.7 b	19.0 c	0.0 a	−0.435 ab
2-Me-1-Propanol	55.4 c	44.3 bc	24.5 c	18.4 c	−0.182 d
Control	62.5 cd	62.5 cd	62.5 d	62.5 d	

[a] Percentages are the means of sixteen replications. Values in column and associated with a given seed and followed by the same letter are not significantly different ($P = 0.01$). Standard errors of the means were <2%.

[b] Slopes were obtained using transformed $[(x + 0.5)^{0.5}]$ germination count data.

methyl ketone results are shown in Table 1 of the preceding paper (Bradow and Connick, 1988).

At the lowest assay concentration following a 3-day exposure, only 3-pentanone had a significant inhibitory effect on carrot seed germination. Significant inhibition was observed after exposure to 6.9 μM 2-heptanol, 3-methyl-1-butanol, and 3-pentanone and to 17.2 μM 1-hexanol and acetaldehyde. The effects produced by 3-methyl-1-butanol and 2-heptanol were the most concentration dependent in the carrot seed assays. The inhibitory effects of 2-heptanol approximated those of 2-nonanone, and those of 3-methyl-1-butanol approximated the inhibitory effects caused by exposure to 2-octanone and 2-heptanone.

All test compounds except 2-methyl-1-propanol essentially prevented AMAPA seed germination after a 3-day exposure. 2-Heptanol, 2-pentanol, 1-hexanol, 3-methyl-1-butanol, and 3-pentanone all significantly inhibited tomato seed germination after a 3-day exposure at 3.4 μM. When the concentrations of the test compounds were doubled, all test compounds but ethanol, 2-methyl-1-propanol, acetaldehyde and 2-methyl-1-propanol inhibited tomato seed germination more than 50%. Only ethanol was not inhibitory at 34.4 μM. The responses of tomato seeds to 1-hexanol and to 2-heptanol were most concentration dependent. Tomato seeds were insensitive to hexanal and acetaldehyde at concentrations below 34.4 μM.

A summary of the results based on the degree of inhibition at all assay concentrations and after an exposure of three days yields to the following activity series: 2-heptanol > 3-methyl-1-butanol, 1-hexanol > 2-pentanol, 3-pentanone, acetaldehyde > hexanal > ethanol, 2-methyl-1-propanol. Due to the insensitivity of seeds to hexanal and acetaldehyde at concentrations below 34.4 μM, this activity series differs from that obtained in the time-dependent studies. When the overall activities of these compounds are compared with those of the methyl ketones the series becomes: 2-nonanone, 2-heptanol, 2-octanone > 2-undecanone > 3-methyl-1-butanol, 1-hexanol > 2-heptanone > 2-hexanone > 3-methyl-2-butanone, hexanal, 2-pentanol, 3-pentanone, 2-pentanone, acetaldehyde, 3-hydroxy-2-butanone > ethanol, 2-butanone, 2-methyl-1-propanol.

Two-way analyses of variance of the 3+4-day data showed no significant ($P = 0.01$) residual germination effects in carrot and tomato seeds (data not shown). Overall, 3+4-day concentration effects were insignificant in onion seed germination, but 2-heptanol inhibited onion seed more than 50% at all but the lowest concentration. Concentration, compound, and concentration × compound interactions were significant in the AMAPA seed data. All concentrations of ethanol residually inhibited AMAPA germination more than 60%. At 17.2 and 34.4 μM, 1-hexanol inhibited AMAPA seeds more than 80%, 3-methyl-1-butanol more than 90%, and 2-methyl-1-propanol more than 70%. Even after the volatile source had been removed, acetaldehyde and hexanal were highly inhibitory at all concentrations, and an initial 3-day exposure to

34.4 μM of both aldehydes prevented AMAPA seed germination after the four-day recovery period.

2-Heptanone × 2-Heptanol Interaction. All of the results discussed above were obtained by exposing the assay seeds to a single test compound at differing concentrations or for varying lengths of time. The AMAPA residues, however, give off mixtures of volatiles, and 2-heptanone and 2-heptanol have been identified as the components of a major peak in the gas chromatograms of volatile mixtures from AMAPA residues (Connick et al., 1987). Therefore, assay seeds were exposed for three days to varying ratios of 2-heptanone and 2-heptanol at three concentrations (Table 2). When the observed germination percentages are compared with "expected" values calculated from results obtained for the pure

TABLE 2. OBSERVED SEED GERMINATION PERCENTAGES FOLLOWING THREE-DAY EXPOSURE TO MIXTURES OF 2-HEPTANONE AND 2-HEPTANOL

Ratio heptanone–heptanol	Germination (%)[a] at total test compound concentration		
	34.4 μM	25.8 μM	17.2 μM
Onion			
4:0	1.6 a	12.8 ab	28.7 a
3:1	2.7 a	16.5 b	41.9 a
2:2	3.3 a	11.3 ab	31.3 a
1:3	2.2 a	14.4 ab	28.5 a
0:4	2.2 a	5.8 a	25.1 a
Carrot			
4:0	0.9 a	2.2 a	12.8 a
3:1	0.2 a	2.1 a	19.1 a
2:2	0.2 a	0.5 a	12.6 a
1:3	1.0 a	3.1 a	15.7 a
0:4	1.6.a	1.9 a	13.0 a
AMAPA			
4:0	0.4 a	0.6 a	1.2 a
3:1	0.2 a	0.4 a	0.8 a
2:2	0.2 a	0.3 a	0.6 a
1:3	0.0 a	0.2 a	0.0 a
0:4	0.4 a	0.4 a	0.0 a
Tomato			
4:0	0.0 a	0.0 a	1.5 ab
3:1	0.2 a	0.0 a	0.0 a
2:2	0.2 a	0.0 a	0.5 ab
1:3	0.8 a	0.0 a	3.6 bc
0:4	0.2 a	0.0 a	8.0 c

[a] Observed percentages are means of 16 replications and value in a given column and associated with a particular seed followed by the same letter are not significantly different ($P = 0.01$).

compounds, goodness-of-fit testing permits the assumption of an additive relationship in the inhibition of all four species of assay seed exposed to 34.4 μM total 2-heptanone + 2-heptanol. Tomato seed germination was too low at 25.8 μM total concentration for the interaction to be characterized. Inhibition was lower than expected in onion seeds exposed to 25.8 and 17.2 μM mixed volatiles, and the null hypothesis of additive inhibition due to exposure to mixed 2-heptanol and 2-heptanone was rejected ($P = 0.01$). However, exposure to the mixtures did reduce onion seed germination significantly below that observed of onion seed controls (Table 1), and no antagonistic relationship was apparent.

2-Heptanone × 3-Methyl-1-Butanol Interaction. Two major chromatogram peaks observed in analyses of volatile mixtures from AMAPA residues were those representing 2-heptanone and 3-methyl-1-butanol (Connick et al., 1987). When the assay seeds were exposed to different ratios of these two compounds, the results shown in Table 3 were obtained. Germination of all four assay species was essentially eliminated by exposure to 34.4 μM volatiles, and AMAPA seeds did not germinate when exposed to the 25.8 or 17.2 μM binary volatile mixtures. Goodness of fit analysis permits the assumption of additivity in the combined effects of 2-heptanone and 3-methyl-1-butanol on onion, carrot, and tomato seeds exposed to the 25.8 μM mixtures. Again, onion and carrot seeds exposed to 17.2 μM mixtures of these two compounds were inhibited less than the activities of the individual compounds would indicate, but the mixtures significantly inhibited both species of seed, compared to the controls (Table 1). Germination was lower than expected in tomato seeds exposed to 17.2 μM mixtures of these two major components of the AMAPA residues volatiles mixtures.

Effects of 2-Heptanone and 2-Heptanol on Seed Germination of Weed and Crop Species. Using the Mason jar apparatus, seeds of 17 dicot and six monocot weed and crop species were exposed to 34.4 μM or 68.8 μM 2-heptanone or 2-heptanol in the dark under the same alternating temperature conditions used in the desiccator assays (Table 4). In comparison to the controls, 68.8 μM 2-heptanone or 2-heptanol significantly inhibited seed germination in several dicots, i.e., *Capsella bursa-pastoris*, *Daucus carota* cv. Imperator, *Glycine max*, light-sensitive and light-insensitive *Lactuca sativa*, *Portulaca oleracea*, *Rumex acetosella*, and *Sida spinosa*. The lower concentration of the two C_7 compounds inhibited *Capsella*, light-sensitive and light-insensitive *Lactuca*, *Medicago sativa*, *R. acetosella*, and *Sida* seed germination. Among the sensitive monocot species, *Avena sativa* and *Eragrostis curvula* were inhibited by both concentrations of the two compounds; and 68.8 μM of both compounds significantly inhibited *Lolium* seed germination. *Sorghum bicolor* seed germination was promoted by 68.8 μM of either 2-heptanol or 2-heptanone. The effects of the vapors of 2-heptanone and 2-heptanol upon the sensitive species of seeds did not differ significantly at either concentration. At five- and tenfold

TABLE 3. OBSERVED SEED GERMINATION PERCENTAGES FOLLOWING THREE-DAY EXPOSURE TO MIXTURES OF 2-HEPTANONE AND 3-METHYL-1-BUTANOL

Ratio heptanone–3-Me-1-butanol	Germination (%)[a] at total test compound concentration		
	34.4 μM	25.8 μM	17.2 μM
Onion			
4:0	0.0 a	4.4 a	13.5 a
3:1	0.0 a	7.5 a	15.9 a
2:2	0.0 a	5.6 a	10.1 a
1:3	0.0 a	2.5 a	11.1 a
0:4	0.0 a	3.1 a	6.3 a
Carrot			
4:0	0.0 a	0.0 a	7.4 a
3:1	0.0 a	5.6 a	8.9 a
2:2	0.0 a	8.7 a	14.2 a
1:3	0.0 a	5.0 a	12.1 a
0:4	0.0 a	11.9 a	7.6 a
AMAPA			
4:0	0.0 a	0.0 a	0.0 a
3:1	0.0 a	0.0 a	0.0 a
2:2	0.0 a	0.0 a	0.0 a
1:3	0.0 a	0.0 a	0.0 a
0:4	0.0 a	0.0 a	0.0 a
Tomato			
4:0	0.0 a	0.0 a	0.0 a
3:1	0.0 a	0.0 a	0.0 a
2:2	0.0 a	0.0 a	5.6 ab
1:3	0.0 a	19.4 c	14.4 bc
0.4	0.0 a	9.4 c	35.3 c

[a] Observed percentages are means of 16 replications and values in a given column and associated with a particular seed followed by the same letter are not significantly different ($P = 0.01$).

higher concentrations and under different germination conditions than used in these studies, 2-heptanone vapors have previously been reported to produce >50% inhibition of seed germination in *Amaranthus retroflexus* (light or dark germination) and to stimulate *R. acetosella* germination (French and Leather, 1979). In aqueous solution 0.57 mM 2-heptanol and 1.1 mM 2-heptanone, 50% germination was reported for Great Lakes lettuce seed (Reynolds, 1977).

There was no apparent correlation between carbon chain length and inhibitory activity. This is in contrast to the chain-length effect observed in the C_4–C_{11} methyl ketones (Bradow and Connick, in 1988). The branched-chain alcohol, 3-methyl-1-butanol, was more inhibitory than the straight-chain ketones and alcohol of the same molecular weight. The vapor pressures of the test com-

TABLE 4. EFFECTS OF THREE-DAY EXPOSURE TO 2-HEPTANONE AND 2-HEPTANOL ON SEED GERMINATION OF WEED AND CROP SPECIES[a]

Seed species	Germination (%)[b]				
	2-Heptanone		2-Heptanol		
	34.4 μM	68.8 μM	34.4 μM	68.9 μM	Control
Dicots					
Brassica napus L. Rape	30.1 b	21.7 ab	28.0 ab	13.2 a	28.9 b
Capsella bursa-pastoris Shepherdspurse	24.2 ab	17.2 a	36.9 b	29.7 ab	60.8 c
Daucus carota L. cv. Imperator Carrot	70.8 bc	63.0 ab	70.0 bc	56.4 a	77.2 c
Glycine max (L.) Merr cv. Wade Soybean	30.7 bc	15.5 ab	30.0 bc	4.6 a	45.6 c
Lactuca sativa L.					
Light insensitive	59.3 ab	51.4 ab	40.2 a	33.7 a	94.5 c
Light sensitive Lettuce	15.5 b	1.6 a	25.3 b	1.8 a	77.4 c
Lepidium sativum L. Garden cress	99.6 b	68.5 a	99.2 b	98.0 b	99.9 b
Medicago sativa L. Alfalfa	79.7 a	72.2 a	89.6 bc	81.2 ab	92.9 c
Portulaca oleracea L. Common purslane	79.7 a	72.2 a	89.6 bc	81.2 ab	92.9 c
Rumex acetosella L. Red sorrel	2.7 b	2.1 a	5.0 b	0.4 a	21.9 c
Sida spinosa L. Teaweed	36.2 a	24.8 a	22.6 a	17.1 a	65.3 b
MONOCOTS					
Avena sativa L. Oats	52.6 c	19.6 b	63.0 c	5.2 a	93.9 d
Eragrostis curvula (Shrad) Nees Lovegrass	14.8 ab	5.2 a	14.6 ab	4.3 a	22.1 b
Lolium spp. Ryegrass	69.8 b	47.2 a	53.7 ab	50.3 a	71.1 b
Sorghum bicolor (L.) Moench Sorghum	41.0 a	88.7 b	61.2 ab	85.7 b	32.6 a

[a] Species tested but not significantly affected: *Amaranthus retroflexus* L., redroot pigweed; *Cucumis melo* cv. Magnum 45, cantelope; *Cucumis sativus* L. cv. Marketmore, cucumber; *Cucumis sativus* L. cv. Ponseti, cucumber; *Linum usitatissimum* L., flax; *Phaseolus mungo aureus* cv. Golden Gram, mung bean; *Trifolium incarnatum* L., red clover; *Echinocloa crus-galli* (L.) Beauv., Barnyard grass; *Zea mays* L. P.S. 788, corn.

[b] Percentages are the means of eight replications. Values in the same rows and associated with a given seed and followed by the same letter are not significantly different ($P = 0.01$). Standard errors of the means were $< 3\%$.

pounds decrease as the inhibitory activity increases, ethanol and acetaldehyde having the highest vapor pressures and 2-heptanone the lowest. When attempting to compare the inhibitory effects produced by vapors of analogous methyl ketones, methyl alcohols, and aldehydes, the relative activities do not appear to be related to the air–water partition coefficients, since the less inhibitory but less water-soluble hexanal is approximately 12 times more concentrated in the vapor phase than 1-hexanol (Buttery et al., 1969). In the presence of water, the highly inhibitory alcohols are significantly less volatile than the analogous methyl ketones which, in turn, are less volatile than aldehydes. High water solubility could account for the low activities of acetaldehyde and ethanol vapors. There were also clear species differences in seed germination sensitivity to alcohols, ketones, and aldehydes. Further examination of the relative activities of analogous aldehydes, alcohols, and ketones is necessary before the modes of action and the relationships between molecular structure, functional group, volatility, and water solubility can be understood.

Both the C_4–C_{11} methyl ketones discussed in the preceding paper (Bradow and Connick, 1988) and the AMAPA residue volatiles discussed in this paper have been detected in volatiles emitted by a number of plant materials (Buttery et al., 1985; Flath et al., 1978; Johnson et al., 1971a,b; Lundgren et al., 1985; Ninnemann and Jüttner, 1981). The broad distribution of these volatile compounds suggests that plant residue volatiles may be active in selective regulation of seed germination and growth of annual weeds (Purvis, et al., 1985), as well as in observed cultivar and species specificity of insect attractants (Buttery et al., 1978, 1985; Flath et al., 1978). The species specificity of the volatile profiles from plant material has already been applied in the identification of somatic hybrids (Ninneman and Juttner, 1981) and chemotaxonomy (Lundgren et al., 1985), and the differing responses of plant species to the group of volatile compounds considered in this and the preceding paper indicate that the volatiles released from plant residues must be an important factor in the various detritus food webs encountered in both conventional and no-tillage ecosystems (Hendrix et al., 1986).

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FEEDING PREFERENCES OF EASTERN SPRUCE BUDWORM LARVAE IN TWO-CHOICE TESTS WITH EXTRACTS OF MATURE FOLIAGE AND WITH PURE AMINO ACIDS

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Abstract—The feeding preferences and feeding rates of eastern spruce budworm on extracts from mature foliage normally avoided by larvae indicate that the chemical composition of four major host plants does not account for this avoidance. Other factors such as the degree of lignification and/or moisture content of the older foliage may play a more important role. Of 14 amino acids tested, alanine, lysine, proline, and serine stimulated feeding. The interactions of amino acids and sucrose were studied, and the results are discussed in relation to feeding preferences and feeding rates.

Key Words—Eastern spruce budworm, *Choristoneura fumiferana*, Lepidoptera, Tortricidae, amino acids, proline, sucrose, extracts, host plants, feeding behavior.

INTRODUCTION

Eastern spruce budworm larvae, *Choristoneura fumiferana* Clem. (Lepidoptera: Tortricidae), normally feed on balsam fir [*Abies balsamea* (L.) Mill.], white spruce [*Picea glauca* (Moench) Voss.], red spruce (*Picea rubens* Sarg.), and black spruce [*Picea mariana* (Mill., B.S.P.)] in eastern North America. Sixth-instar larvae confine themselves to the newly developed shoots of these hosts; however, in cases of severe defoliation, larvae may be forced to feed on the older, mature foliage. In so doing, their pupal weight and adult fecundity are reduced (Blais, 1952; Miller, 1963).

In behavioral tests using extracts from new shoots of its four hosts, feeding

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was highly stimulated by sugars/glycosides, slightly stimulated by amino acids/bases, and organic acids had no effect or deterred feeding. The chloroform fractions (lipids, etc.) of white and red spruces stimulated feeding, while those of balsam fir and black spruce had no effect (Albert, 1982). Subsequent studies emphasized the importance of the sugars/glycosides (Albert and Parisella, 1985) and of sucrose in particular on the feeding behavior of the spruce budworm larva (Albert et al., 1982).

Since larvae normally avoid the older foliage from their host plants, we wished to determine whether this was due to their chemical composition. Does mature foliage have the ability to stimulate larval feeding, or is it actually deterrent?

The effects of amino acids on the feeding behavior of insects have been studied in Coleoptera (Wensler and Dudzinski, 1972; Mitchell, 1985), Lepidoptera (Schoonhoven, 1969; Dethier and Kuch, 1971; Ma, 1972; Ascher et al., 1976; Cobbinah et al., 1982), Diptera (Shiraishi and Kuwabara, 1970), Hemiptera (Hatfield et al., 1982), Homoptera (Srivastava and Auclair, 1974; Srivastava et al., 1983), and Orthoptera (Sugarman and Jakinovich, 1986), to cite a few. The amino acid compositions of balsam fir and of white spruce were described by Kimmins (1971). Heron (1965) showed that *l*-proline stimulated eastern spruce budworm larvae to feed. Results of experiments with pure amino acids are presented in the second part of this paper to illustrate the potential hazards in the interpretation of different types of feeding behavior data and to supplement our knowledge of the feeding behavior of the spruce budworm larva toward host-plant chemicals.

METHODS AND MATERIALS

Insects. Larvae were obtained from the Forest Pest Management Institute, Sault Ste Marie, Ontario. They were reared on the artificial diet developed by McMorran (1965) and supplied by the Maritimes Forest Research Centre, Fredericton, New Brunswick. Sixth-instar larvae, <1 day post molt and starved for 24 h, were used in feeding tests.

Plant Extracts and Amino Acids. Collections of foliage from all four host species coincided with the time of year (mid-June, 1982) when larvae in the field were actively feeding as sixth instars, at the Acadia Forest Experiment Station, New Brunswick. Plant material was freeze-dried and extracted with hot methanol, then separated by column chromatography on Amberlite resins as described previously (Albert and Jerrett, 1981). We obtained four fractions from each host species: sugars/glycosides, amino acids/bases, organic acids, and chloroform (lipids, esters, alcohols, aldehydes, waxes, etc.). All fractions were evaporated to dryness. Polar extracts were redissolved in distilled water to bring

their concentration to 1.0×, which was the equivalent of their concentration in the host plant at the time of its collection. Chloroform fractions were redissolved in hexane to bring their concentration to 1.0×.

Amino acids were obtained from Sigma Chemical Co. (St. Louis, Missouri). The concentrations of amino acids used in our experiments were based on those found in balsam fir and white spruce, as determined from the work of Kimmins (1971).

Two-Choice Feeding Tests. Two-choice feeding tests modified after Jermy et al. (1968) for spruce budworm larvae (Albert et al., 1982) were used. Results are presented as mean percent consumption (± SE) of test disks vs. control disks, and feeding rates: $FR = [(T + C)/H]$, where T = area eaten of test disks, C = area eaten of control disks, and H = time in hours. Multiplying the *FR* by 33.183 (the surface area of one disk) gives the total amount of feeding (mm^2) on the combined test and control disks per hour. Mean percent consumption data were analyzed using Wilcoxon's signed-ranks test (Sokal and Rohlf, 1969) and feeding rate data were analyzed with condescriptive statistics (Nie et al., 1975) to obtain means and 95% confidence limits.

RESULTS AND DISCUSSION

Extracts. The amounts of chemicals recovered in extracts from each of the four hosts are shown in Table 1. Feeding preferences for the extracts from 1-year-old foliage (Table 2) are similar to those described for new foliage (Albert, 1982). Sugars/glycosides are the preferred extracts. The most notable differences in the response are that the organic acids of balsam fir and red spruce are

TABLE 1. WEIGHTS OF CHEMICALS RECOVERED IN EACH FRACTION FROM FOUR HOST PLANTS' ONE-YEAR-OLD FOLIAGE

Chemical fraction	Weight (mg/g of freeze-dried needles)			
	Balsam fir	White spruce	Red spruce	Black spruce
SG[a]	103.9 (53.6)[b]	184.1 (73.8)	116.5 (37.1)	101.7 (48.5)
AA	3.8 (1.9)	8.2 (3.3)	3.3 (1.1)	4.7 (2.1)
OA	4.4 (2.3)	22.9 (9.2)	105.2 (33.5)	21.3 (10.2)
CH	81.8 (42.2)	34.0 (13.6)	88.5 (28.2)	81.9 (39.1)
Total	193.8 (100)	249.3 (100)	313.6 (100)	209.5 (100)

[a] SG, sugars/glycosides; AA, amino acids/bases; OA, organic acids; CH, chloroform fraction.
[b] Numbers in parentheses represent the percent of the total weight of chemicals recovered from each host species.

TABLE 2. PERCENT OF TOTAL CONSUMPTION FOR CONTROL DISKS AND TEST DISKS TREATED WITH EXTRACTS, AND FEEDING RATE OF ANIMALS IN EACH EXPERIMENT

Host extract[a]		Mean % consumption			Animals (N)	P[b]	Feeding rate (95% CL) [(T + C)/H]
		Control	Test	SE			
BF	SG	20.7	79.3	1.5	19	0	15.0 (12.7-17.4)
	AA	29.8	70.2	3.1	19	0	7.9 (6.2- 9.6)
	OA	40.5	59.5	3.7	17	0.031	7.2 (5.5- 8.9)
	CH	22.9	77.1	3.3	17	0	5.2 (3.0- 7.4)
WS	SG	12.7	87.3	1.9	19	0	12.3 (10.9-13.8)
	AA	34.5	65.5	3.3	18	0.001	7.8 (6.4- 9.2)
	OA	48.1	51.9	3.7	16	0.679 NS[c]	2.7 (2.1- 3.2)
	CH	15.9	84.1	4.4	18	0	7.4 (5.9- 8.9)
RS	SG	16.1	83.9	1.7	11	0.003	14.5 (12.8-16.3)
	AA	42.1	57.9	3.6	18	0.052	9.1 (7.6-10.7)
	OA	43.3	56.7	2.6	16	0.036	6.2 (5.2- 7.2)
	CH	33.4	66.6	4.6	15	0.008	5.2 (3.9- 6.4)
BS	SG	39.3	60.7	4.0	13	0.033	7.9 (4.6-11.2)
	AA	37.1	62.9	3.6	17	0.013	6.4 (5.1- 7.7)
	OA	43.0	57.0	4.7	16	0.140 NS	6.7 (4.7- 8.7)
	CH	48.6	51.4	4.8	15	0.807 NS	6.2 (4.6- 7.9)

[a] BF, balsam fir; WS, white spruce; RS, red spruce; BS, black spruce. SG, sugars and glycosides; AA, amino acids and bases; OA, organic acids; CH, chloroform fraction.
[b] *p*: Significance of differences in feeding between test and control.
[c] NS: not significant.

actually preferred to the water controls, whereas for white and black spruces there was no preference. Feeding rates on red and black spruce extracts from old foliage are more than double those using new foliage. Feeding rates on organic acids from white spruce, on the other hand, are 60% lower.

There is an increase in the preference for the balsam fir chloroform extract over the corresponding extract from new foliage, with no change in the feeding rate. Animals show the same preference for chloroform extracts from red and white spruce, but the feeding rates are reduced by one half compared to those on new foliage extracts. The only other notable difference is in the lowered preference for sugars from black spruce, with a 40% reduction in feeding rate, and a reduced preference compared to data from new foliage.

Feeding preference and feeding rate data suggest that the chemical composition of mature foliage is adequate to stimulate larval feeding. With the exceptions noted above, the responses to these extracts are remarkably similar to those previously reported for new foliage (Albert, 1982).

The actual chemical composition of the extracts from mature foliage is obviously expected to differ from that of new growth. If a feeding deterrent

such as pungenin is present in the mature foliage (Heron, 1965), it could account for the reduced preference and feeding rate for black spruce sugars/glycosides, although it does not explain the high preference for white spruce sugars/glycosides, which should also contain this same chemical since it has been identified in this host (Neisch, 1957). In a recent study, Strunz et al. (1986) found that pungenin was, at best, a modest feeding deterrent for spruce budworm larvae.

Larvae may avoid old foliage because it is tougher, as was suggested by Heron (1965). Another contributing factor could be the reduction in moisture content of old vs. new foliage. The moisture content of mature foliage in our collections was as follows: balsam fir, 51%; white spruce, 52%; red spruce, 42%; black spruce, 52%. These values are lower than those for new foliage, which are about 75–80%. Mattson and Haack (1987) discussed the effects of drought (moisture stress) on phytophagous insects. Since larvae fed readily on the extracts at the concentrations found in the host plants, we assume that leaf toughness is the most likely factor that discourages feeding on older foliage.

Amino Acids. Pure alanine, lysine, proline, and serine stimulate feeding (Table 3); valine deters feeding, and the remainder have no effect. The strongest

TABLE 3. PERCENT OF TOTAL CONSUMPTION FOR CONTROL DISKS AND TEST DISKS TREATED WITH PURE AMINO ACIDS, AND FEEDING RATE OF ANIMALS IN EACH EXPERIMENT

Acid	Conc. [mM]	Mean % consumption			N	P	Feeding rate (95% CL) $[(T + C)/H]$
		Control[a]	Test[b]	SE			
l-Alanine	100	38.8	61.2	3.9	20	0.002	9.8 (6.8–12.7)
l-Arginine	100	53.2	46.8	4.8	20	0.526 NS[d]	5.4 (4.4– 6.5)
Aspartic	20	46.3	53.8	3.4	20	0.126 NS	6.0 (4.5– 7.4)
Cystine	20[c]	52.9	47.1	3.7	20	0.296 NS	7.0 (5.5– 8.5)
l-Glutamic	2	45.2	54.8	4.8	20	0.218 NS	6.1 (4.5– 7.6)
l-Glycine	10	49.2	50.8	5.8	20	0.940 NS	4.8 (3.4– 6.1)
l-Histidine	30	51.4	48.6	4.6	20	0.455 NS	6.4 (4.9– 7.9)
l-Leucine	50	49.3	50.7	5.0	20	0.629 NS	7.2 (5.3– 9.1)
l-Lysine	50	43.7	56.3	3.9	20	0.048	6.4 (4.8– 8.1)
l-Proline	50	34.1	65.9	3.8	20	0.002	7.3 (4.8– 9.7)
l-Serine	50	37.9	62.1	5.6	20	0.044	6.2 (4.7– 7.7)
Threonine	50	55.1	44.9	3.3	20	0.198 NS	8.3 (6.5–10.1)
Tyrosine	20[c]	54.6	45.5	3.6	20	0.184 NS	7.7 (5.2–10.2)
l-Valine	20	60.1	39.9	3.1	20	0.006	7.9 (5.6–10.2)

[a] Distilled water.
[b] Amino acid dissolved in distilled water.
[c] Saturated solution.
[d] NS, not significant.

preference is for proline, which was described as a feeding stimulant by Heron (1965). Feeding rates are similar to those in experiments with amino acid extracts (Table 2).

To determine the possible effect of synergism between amino acids and sucrose, experiments were repeated using 25 mM sucrose on control disks, and the same amino acids dissolved in 25 mM sucrose on test disks. Under these conditions, alanine and lysine had no effect; glutamic acid and serine deterred feeding; and valine glycine, and proline stimulated feeding (Table 4). The most significant changes are seen in the feeding rates, which are increased by the addition of 25 mM sucrose. Serine, by itself, for example, is stimulating (Table 3), but when mixed with sucrose, it appears to deter feeding (Table 4, mean percent consumption data). However, the feeding rate of larvae in the latter experiment is 3.6× greater than in the comparable experiment without sucrose. The same is true for alanine, where the feeding rate is doubled, indicating a possible synergism. Similar effects were noted by Cobbinah et al. (1982) for the gum leaf skeletonizer *Uraba lugens*, where amino acids showed a low phagostimulatory activity that increased upon combination of the acids with sucrose.

TABLE 4. PERCENT OF TOTAL CONSUMPTION FOR CONTROL DISKS AND TEST DISKS TREATED WITH COMBINED AMINO ACID AND 25 Mm SUCROSE, AND FEEDING RATE OF ANIMALS IN EACH EXPERIMENT

Acid	Conc. [mM]	Mean % consumption			N	P	Feeding rate (95% CL) $[(T + C)/H]$
		Control[a]	Test[b]	SE			
l-Alanine	100	50.4	49.6	2.3	18	0.887 NS[d]	18.8 (15.3-22.1)
l-Arginine	100	47.3	52.7	3.7	17	0.680 NS	8.8 (6.2-11.3)
Aspartic	20	51.3	48.7	4.3	16	0.600 NS	8.8 (5.7-12.0)
Cystine	20[c]	42.7	57.3	3.8	16	0.980 NS	8.2 (5.8-10.5)
l-Glutamic	2	70.9	29.1	3.5	18	0	16.9 (13.1-20.7)
l-Glycine	100	37.4	62.6	3.0	16	0.003	8.5 (5.8-11.3)
l-Histidine	30	48.6	51.4	2.8	19	0.560 NS	11.9 (9.7-14.2)
l-Leucine	50	43.0	57.0	3.4	18	0.039	12.5 (10.5-14.5)
l-Lysine	50	53.0	47.0	4.1	19	0.571 NS	9.7 (7.5-11.9)
l-Proline	50	42.0	58.0	1.9	18	0.002	19.2 (16.3-22.2)
l-Serine	50	56.3	43.7	2.1	17	0.009	22.5 (19.9-25.1)
Threonine	50	50.0	50.0	2.1	19	0.965 NS	16.9 (14.1-19.6)
Tyrosine	20[c]	46.5	54.4	2.9	10	0.123 NS	9.1 (4.9-13.4)
l-Valine	20	44.5	55.5	2.6	20	0.044	13.4 (10.3-16.6)

[a] 25 mM sucrose.
[b] Amino acid dissolved in 25 mM sucrose.
[c] Saturated solution.
[d] NS, not significant.

A possible synergistic effect occurred with a combination of sucrose and arginine in their experiments.

Feeding Preference vs. Feeding Rate. An interesting phenomenon is observed in comparing the mean percent consumption data with the data on feeding rates in all experiments. Clearly, there is no direct correlation between the two measures of feeding behavior. The highest feeding rates were found in experiments with sugars/glycosides, as was also shown for new foliage (Albert, 1982). Feeding rates with pure amino acids are comparable to those for amino acid extracts. When combined with sucrose, however, feeding rates for eight of the 14 amino acids increased substantially. Glutamic acid and serine would be considered deterrents as judged from mean percent consumption data; yet, they obviously did not interfere with the actual ingestion of cellulose disks. Feeding rates for six of the amino acids increased only slightly, indicating that they suppressed the positive effect of sucrose. This information would not be gleaned from observing the mean percent consumption data alone; thus care must be taken in interpreting the results of feeding preference tests, as discussed by Dethier (1982). Evidence from electrophysiological experiments on the chemosensilla responsible for the reception of sucrose and amino acid stimuli may prove useful in explaining the above phenomena.

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CHEMICAL PROTECTION OF PHEROMONES CONTAINING AN INTERNAL CONJUGATED DIENE SYSTEM FROM ISOMERIZATION AND OXIDATION

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Abstract—Conjugated diene systems are common in natural products, including pheromones. The systems are sensitive to heat, light, and oxygen, among other things. They can be protected by antioxidants and UV absorbers, which slow down *cis–trans* isomerization and oxidation. Three sex pheromones (one as an analog) containing *Z,E*, *E,Z*, and *E,E* units were studied: (*Z,E*)-9,11-C_{14}OAc, (*E,Z*)-7,9-C_{12}OAc, and (*E,E*)-10,12-C_{16}OAc. The UV absorber 2-hydroxy-4-methoxybenzophenone and the antioxidants BHT and BHA were found to be effective in solution. The protective effect of the UV absorber against photoisomerization on paper carriers was not as good as that in solution. Preliminary studies on the utilization of formulations containing these compounds and (*Z,E*)-9,11-C_{14}OAc in the mass trapping of Egyptian cotton leafworm male in cotton fields showed the new combinations to be as good as a previously used formulation with UOP 688, a compound which is unpleasant to handle.

Key Words—*Lobesia botrana*, *Spodoptera littoralis*, *Earias insulana*, conjugated dienes, sex pheromone, photoisomerization, UV absorbers, antioxidants, protection of pheromones.

INTRODUCTION

As integrated pest management (IPM) becomes more sophisticated, the main role played by pheromones will be that of monitoring and disruption of communication (confusion) via controlled release of the active ingredients in the field. The longer the desired field life of the pheromones, the greater the quantity of pheromones that will be degraded by chemical and physical processes. Evaporation is known to be a major cause of the loss of material in short-lived

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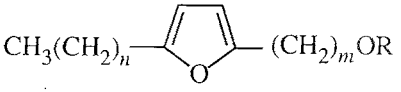
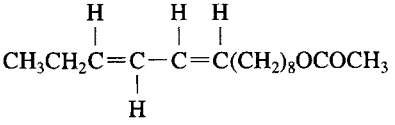
devices, but not much is known about chemical degradation under field and/or laboratory conditions. The few studies that have been performed have dealt mainly with the oxidation and isomerization of double bonds (Goto et al., 1974; Bruce and Lum, 1976, 1981; Fujiwara et al., 1977; Shani and Klug, 1980a, b; Ideses et al., 1982b, c; Shani et al., 1982; Vaintraub et al., 1983; Davis et al., 1984; Vylegzhanina et al. 1984; Guerin et al., 1984; Nesterova, 1985; Nesterova et al., 1985; Chisholm et al., 1985; Brown and McDonough, 1986; Sychev et al., 1987), with transformations of aldehydes (Weatherston et al., 1981; Shaver and Ivie, 1982; Dunkelblum et al., 1984), and with hydrolysis of acetates in the field (Shani and Klug, 1980b).

Our group (Shani and Klug, 1980a, b; Shani et al., 1982; Ideses et al., 1982b, c) has already shown that the conjugated diene systems in several sex pheromones of moths are isomerized into a mixture of the four possible geometric isomers and oxidized in the field (or under simulated field conditions in the laboratory) to a furan system (Scheme 1, structure I), via a peroxide.



I

Ia: $m = 6, 8, 9$; $n = 1, 2$; R = Ac
Ib: $m = 7$; $n = 0$; R = H



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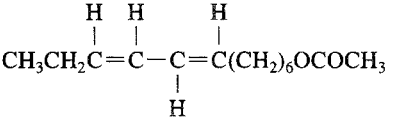

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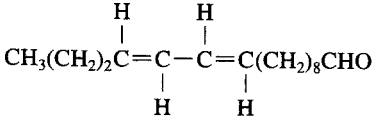
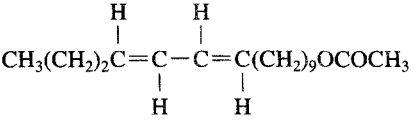


IV



V

Scheme 1.

With the aim of finding means of protecting these sensitive chemicals and prolonging their biologically active period in the field, we studied the effect of antioxidants and UV absorbers on pheromone stability both in the laboratory and under field conditions. Three internal conjugated diene systems known to be present in moth sex pheromones were studied: *Z,E* dienes as found in (*Z,E*)-9,11-tetradecadien-1-yl acetate (TDDA) (II), the main component of the sex pheromone of the female Egyptian cotton leafworm (*Spodoptera littoralis*) (Nesbitt et al., 1973; Tamaki et al., 1973); *E,Z* dienes as found in (*E,Z*)-7,9-dodecadien-1-yl acetate (DDA) (III), the sex pheromone of the female European grapevine moth (*Lobesia botrana*) (Roelofs et al., 1973; Buser et al., 1974); and *E,E* dienes, as found in (*E,E*)-*10,12*-hexadecadienal (HDAL) (IV) [studied as the corresponding ester (*E,E*)-10,12-hexadecadien-1-yl acetate (HDDA) (V)], the sex pheromone of the female spiny bollworm (*Earias insulana*) (Hall et al., 1980).

We report here the results of these studies and a preliminary study of the efficiency of protected pheromone carriers in cotton fields in Israel in mass trapping of Egyptian cotton leafworm males.

METHODS AND MATERIALS

Chemicals. The purity of the pheromones and the pheromone analog was determined by GLC: (*Z,E*)-9,11-C_{14}OAc 98%, 2% *E,E*-isomer; (*E,Z*)-7,9-C_{12}OAc 92%, 8% *E, E*-isomer; (*E,E*)-10,12-C_{16}OAc 100%. Antioxidants 3(2)-*tert*-butyl-4-hydroxyanoisole (BHA) and 2,6-di-*tert*-butyl-4-methylphenol [butylated hydroxytoluene (BHT)] were purchased from Sigma. UV absorbers 2-hydroxy-4-methoxybenzophenone [$\lambda_{max}^{C_6H_{12}}$ 288, 360 nm (ϵ = 14,770, 9,090); $\lambda_{max}^{t\text{-BuOH}}$ 286, 340 nm (12,860, 7,750)] (trade name Eusolex 4360), and a eutectic mixture of 4-isopropyldibenzoylmethane and 3-(4-methylbenzylidene)camphor [$\lambda_{max}^{C_6H_{12}}$ 312, 342 nm (ϵ = 15,790, 18,420); $\lambda_{max}^{t\text{-BuOH}}$ 299, 350 (22,470, 19,720)] (trade name Eusolex 8021) were purchased from Merck. UV spectra were determined with a Bausch & Lomb Spectronic 2000 in spectroscopic grade cyclohexane or in *tert*-butanol.

GLC analyses were performed on a Packard GLC model 417 fitted with a flame ionization detector and with either a fused silica capillary column of SP 2340 30 m × 0.25 mm, flow rate (He) 0.4 ml/min at 130–160°C or a WCOT Silar 9 25 m × 0.5 mm, flow rate (He) 2.7 ml/min at 130–160°C, depending on the pheromone studied.

All solutions contained 100 mg of a pheromone in 5 ml cyclohexane or *tert*-butanol in a corked Pyrex flask, and 50 μl were withdrawn and diluted to 0.5 ml with cyclohexane and then injected twice ($\pm$1%) into the GLC (1–2 μl).

Flasks were refilled to 5 ml every day to compensate for evaporation. Solid carriers (two replicates of each sample) were extracted with hexane (2 × 2 ml), evaporated and then diluted to 1 ml and injected into the GLC. All formulations (solutions or solids carriers) were subjected to diffused sunlight on the roof of the building for the greater part of the experiment, although for some of the time they were placed in direct sunlight. (Details of the light regime are given in the tables or in the text.) Samples were usually withdrawn every two to four days until no pheromone (or its isomers) could be detected by GLC; this period usually lasted five to six weeks. Thus, samples analyzed up to the third or fourth week contained enough material for detection (in general 20–40% of the starting material and isomers were present). In the absence of antioxidant, the pheromone decomposed much faster, and the last sample that contained measurable amounts is documented in the Tables.

RESULTS AND DISCUSSION

It has already been established that heat (50°C in the laboratory hood) causes thermal oxidation via $^3O_2$ and that the process can be slowed down by antioxidants such as BHA or BHT (Shani et al., 1982).

The effect of light seems to be more complicated. In the field, both direct and scattered sunlight may be present, a situation that may affect both pheromone carriers in traps and slow-release devices that are covered by leaves and other objects in the field. Moreover, the excitation of the diene system could be through direct absorption of light by its end absorption (Shani and Klug, 1980b; Shani et al., 1982) or via energy transfer from a sensitizer. In addition, in tropical or subtropical regions, exposure during the summer means that the pheromones are subjected to a combination of high-intensity sunlight and temperatures of 40°C or more. We have already found (Shani and Klug, 1980b) that the rate of direct photoisomerization in sunlight is two to three times faster in summer than in winter. These results are in keeping with the summer and winter figures for total solar energy in Be'er-Sheva (5500–6900 vs. 2300–3500 kcal/m^2/day)[1]. Therefore, in all the experiments described below, the pheromones were, in fact, exposed to a combination of heat, light, and oxygen. Thus, the protective effect of the antioxidant is crucial. The set of experiments performed by us was designed, therefore, to include all possible factors that also play a role in any study on the effect of light on isomerization of pheromones.

Effect of Antioxidants. When pheromones are subjected to heat and light, two competing chemical processes take place—thermal decomposition (oxida-

[1]We thank A. I. Kudish, Department of Chemical Engineering, Ben-Gurion University for the data.

tion) and photoisomerization. Heating alone (in the hood) caused decomposition of the pheromone within a few days (Shani et al., 1982), as was the situation in this experiment with pheromones exposed to sunlight and not protected by an antioxidant. In this case, very little isomerization took place (entries 1, 4, and 7 in Table 1). Pheromones protected by an antioxidant and hence exposed to longer periods of sunlight underwent more extensive isomerization, almost reaching the equilibrium composition, which was found to be 68–75% of the *E,E* isomer, 12–16% of each of the *Z,E* and *E,Z* isomers, and 1–3% of the *Z,Z* isomer (Shani et al., 1982; Ideses and Shaní, 1986). In an earlier study (Shani and Klug, 1980b), we observed that the rate of photoisomerization is somewhat slower in the presence of the antioxidant, as compared to the process in its absence. After 11 days of sunlight, 17% of (*E,E*)-9,11-TDDA was found when

TABLE 1. PHOTOISOMERIZATION OF (*Z,E*)-9,11-C_{14}OAc (TDDA), (*E,Z*)-7,9-C_{12}OAc (DDA) AND (*E,E*)-10,12-C_{16}OAc (HDDA) EXPOSED TO SUNLIGHT IN PRESENCE OF UV ABSORBERS[a]

		Without BHA						With BHA					
				Composition (%)						Composition (%)			
Entry	Pheromone	UV absorber	Period (days)[b]	*Z,E*	*E,Z*	*Z,Z*	*E,E*	UV absorber	Period (days)	*Z,E*	*E,Z*	*Z,Z*	*E,E*
1.	TDDA		7	75	5		20		6	80	8		12
									25[c]	25	15		60
2.	TDDA	E-4360	11	82	7		11	E-4360	11	90	5		5
								E-4360	23[c]	86	7		7
3.	TDDA	E-8021	11	58	20		22	E-8021	11	73	13		14
			14	50	24		26	E-8021	14	66	16		18
								E-8021	27[c]	47	23		30
4.	DDA		3		87		13		3		85		15
									30[c]	20	36	2	42
5.	DDA	E-4360	10		90		10	E-4360	10		89		11
								E-4360	30[c]		85		15
6.	DDA	E-8021	10		85		15	E-8021	10	5	74		21
								E-8021	32[c]	9	63	1	27
7.	HDDA		10	5	5		90		10	7	8		85
									25[c]	10	10		80
8.	HDDA	E-4360	10	2	2		96	E-4360	10	2	2		96
								E-4360	30[c]	3	3		94
9.	HDDA	E-8021	10	6	6		88	E-8021	10	6	6		88
								E-8021	20[c]	9	9		82

[a] Each sample contained 100 mg of pheromone, 100 mg of antioxidant, and 100 mg of UV absorber (when mentioned) in 5 ml cyclohexane.

[b] Due to fast decomposition, the last sample withdrawn which gave measurable results is presented. Without antioxidant, the oxidation product I was formed in some cases (Ideses et al., 1982c).

[c] Last sample withdrawn when some 20–40% of starting material and isomers were still present in solution.

BHT was added to the pheromone solution as opposed to 30% of the *E,E* isomer when it was not present. After 16 days of sunlight exposure, both solutions contained 35% of the *E,E* isomer. That study was performed during May 1979, when the total sunlight energy is very high (6000–6900 kcal/m^2/day), but temperatures in Be'er-Sheva are still moderate.[2] This might explain why we could still detect the isomers of TDDA after 16 days in sunlight while in the current study, which was performed in August 1985, decomposition was much faster, being complete within seven days (Table 1, entry 1). The first experiment with (*E,Z*)-7,9-C_{12}OAc (entry 4) was done in July 1981 and repeated in July–August 1985.

Effect of UV Absorbers. We studied the effect of two UV absorbers that have their main absorption bands in the region of 300 nm (see Methods and Materials). The photoisomerization of the three diene systems was followed in solution, and the results are summarized in Table 1.

The effect of the antioxidant is again demonstrated in that less pheromone is decomposed in the solution, and exposure period to sunlight is thus lengthened. The effect of the UV absorber is dramatic, as can be seen by comparing the composition in entries 2, 3, 5, 6, 8, and 9 with entries 1, 4, and 7 of Table 1. Exposure to sunlight for three to four weeks brings the isomeric mixture closer to equilibrium (see above), except in the presence of UV absorbers which slow down this process, allowing very little isomerization to take place (entries 2, 5, and 8 of Table 1). The difference between E-4360 and E-8021 (entries, 2, 5, 8 and 3, 6, 9 in Table 1) is clear. We attribute the protective effect to the intense absorption of E-4360 at 288 nm, which exactly covers the end absorption of the conjugated diene system (Shani and Klug, 1980a, b, Shani et al., 1982). The other UV absorbers (E-8021) is less effective, as its main absorption bands are at longer wavelengths, which do not mask the diene absorption. We may also assume that E-8021 does not act as a photosensitizer, since its components have essentially the same chromophor system as that of E-4360. As aromatic ketones, these chemicals probably possess a very high quantum yield for intersystem crossing in solution ($S_1 \rightarrow T_1$) (ΦISC 0.9–1.0) and, when excited, contain about the same triplet energy (ca. 70 kcal/mol) (Calvert and Pitts, 1966).

In order to learn more about the efficacy of both the antioxidant and the UV absorber on the photoisomerization process, we prepared solutions with different amounts of these two essential additives, as shown in Table 2. It is clear that a pheromone solution containing 10% of the antioxidant and 10% of the UV absorber is well protected from both thermal oxidation and photo-

[2] The average daily maximum temperatures (°C) in Be'er-Sheva during different periods of study were as follows: 1979, May 28.7, December 17.0; 1981, July 33.1; 1985, July 32.9, August 34.0. We thank Dov Mills of the Regional Meteorological Station in Be'er Sheva for the data.

TABLE 2. EFFECT OF DIFFERENT AMOUNTS OF UV ABSORBER AND ANTIOXIDANT ON PHOTOISOMERIZATION AND OXIDATION OF TDDA[a]

Entry	Pheromone (mg)	BHA (mg)	E-4360 (mg)	Period in sunlight (days)	Composition (%)				Oxidation product
					Z,E	*E,Z*	*Z,Z*	*E,E*	
1	100	100		16	55	15		30	none
2	100	100	100	16	94	3		3	none
3	100	100	10	17	90	6		4	none
4	100	10	10	17	92	2		6	none
5	100	10		17	47	16		37	none

[a] All samples were solutions in 5 ml cyclohexane.

isomerization of the internal conjugated diene system under sunlight (compare entries 2 and 4 in Table 2).

Effect of Photosensitizer. In the presence of a photosensitizer (in our case rose bengal) the photoisomerization is very fast, yielding an equilibrium mixture, and the process can be accomplished within 90 min (Shani et al., 1982) as compared with direct illumination for several weeks in sunlight without photosensitizer. The question whether the UV absorber could slow down this energy transfer and excitation was investigated, and the results are summarized in Table 3.

The effect of E-4360 in slowing down the sensitized photoisomerization is impressive, as compared with that of E-8021, the latter compound being completely ineffective (compare entries 3 and 4 in Table 3). These results are in keeping with the findings for these two UV absorbers in direct sunlight (Table 1). The photosensitizer (rose bengal) probably transfers its energy to E-4360,

TABLE 3. EFFECT OF UV ABSORBER ON PHOTOISOMERIZATION OF TDDA IN PRESENCE OF PHOTOSENSITIZER[a]

Entry	UV absorber	Exposure time (min)	Composition (%)			
			Z,E	*E,Z*	*Z,Z*	*E,E*
1		45	16	13	1	70
2		75	15	12	1	72
3	E-4360	60	65	10		25
4	E-8021	60	13	16	3	68

[a] Each sample contained 100 mg of (*Z,E*)-9,11-C_{14}OAc, 10 mg of rose bengal, and 100 mg of UV absorber in 5 ml *tert*-butanol.

which, having absorption bands at 288 and 360 nm, does not take part in energy transfer to the diene system. We thus may consider E-4360 as a good "filter."

It was interesting that the antioxidant (BHA) was also effective in slowing down the sensitized photoisomerization. As noted above (entries 1 and 2 in Table 3) and as has already been published (Shani et al., 1982), sensitized photoisomerization towards equilibration of the four isomers is fast, being accomplished in 30–100 min. However, when a solution of 100 mg of pheromone (TDDA or DDA) containing 10 mg of rose bengal and 100 mg of BHA was left in the sunlight, the rate of isomerization was much slower, and more than 50% of the starting isomer was detected in the tested solution after 4 hr. When the UV spectrum of an admixture of rose bengal and BHA was studied, two separate and unchanged spectra of the components were clearly observed. Still, energy transfer from rose bengal to BHA can take place since the absorption band of BHA is wide and intense at this concentration (100 mg in 5 ml solution) even beyond the 300 nm. Another explanation could be that the phenoxy radical reacts with the photosensitizer and thus decreased its efficacy as a photosensitizer. More experiments should be undertaken in order to determine the preferred mechanism.

The effect of radicals (I_2, C_6H_5SH) on the isomerization was also studied, and we found that the process with I_2, but not with the thiophenol, is slowed down by antioxidants as radical scavengers. The reaction is temperature dependent and can be considered as a catalytic process in 5–10% of iodine in solution (Ideses and Shani, in preparation).

Simulated and Real Field Conditions. The effect of the binary mixture of the antioxidants and the UV absorber was also checked in pheromone carriers, which are usually applied in the field in traps. We selected to study (*Z,E*)-9,11-$C_{14}OAc$, since this compound is used extensively in mass trapping of males of the Egyptian cotton leafworm in cotton fields in Israel. The pheromone is usually applied into the carrier as a solution of the active ingredients (the solvent evaporates in a short time and then the carriers are stored in a refrigerator until used). These carriers are installed into traps under a "cover" that protects them from direct sunlight. We thus investigated these carriers on the roof of our building in diffused sunlight. The results are shown in Table 4.

We found that the photoisomerization of pheromones in solution takes place at a slower rate than that in pheromones impregnated on a carrier. This phenomenon may be explained in terms of the observation that upon application of solution, "paper chromatography" took place and two or three zones appeared on the carrier. We cannot determine how much of overlapping of the components is effective, but it is interesting that the UV absorber is not as effective on the carrier as it is in solution, especially on the cigarette filter (entries 2 and 6 in Table 4). In fact, it seems that the antioxidant alone is almost as good as the binary mixture of antioxidant and UV absorber. This finding could be

TABLE 4. PHOTOISOMERIZATION OF (*Z*,*E*)-9,11-C_{14}OAc LOADED ON CARRIERS IN PRESENCE OF ANTIOXIDANT (BHA) AND UV ABSORBER (E-4360)[a]

Entry	Carrier	Relative amount (%)			Exposure time (days)[b]	Composition (%)			
		Pheromone[a]	BHA	E-4360		*Z*,*E*	*E*,*Z*	*Z*,*Z*	*E*,*E*
1	Solution	100	10		17[c]	47	16		37
2	Solution	100	10	10	17	92	2		6
					21	84	6		10
3	Cardboard	100	10		18	69	12		19
					25	60	16		24
4	Cardboard	100	10	10	17	81	8		11
					24	78	10		12
5	Cigarette filter	100	10		18	64	14		22
					21	55	20		25
6	Cigarette filter	100	10	10	18	61	19		20
					21	59	20		21

[a] Each carrier was loaded with cyclohexane solution containing 2 mg of pheromone, 0.2 mg of BHA, and 0.2 mg of E-4360 (when mentioned). The solution contained 100 mg of pheromone in 5 ml cyclohexane. The carriers (two of each sample) were kept in a Pyrex dish, covered with a watch glass. All samples were kept on the roof of the building in diffused sunlight.

[b] The amount of the starting isomer was 20–40% of its original quantity. For technical reasons, analyses of samples were performed on different days.

[c] The flask was broken.

explained by the protective effect of the solid support of the carrier, which may furnish better masking from sunlight than is available in a clear solution. It is also possible that some additives in the paper act as either UV absorbers or quenchers, as the antioxidant acts in the presence of a photosensitizer (see above). Such a phenomenon was found in carriers made of rubber septa (Fujiwara et al., 1977; Brown and McDonough, 1986; Teich and Shani, unpublished results). It is important to remember that without an antioxidant, the pheromone decomposed on the carriers in a few days and could not be detected on the GLC.

The real test of the biological activity of the pheromone carriers loaded with the binary protecting mixture was conducted in kibbutz cotton fields. The usual pheromone carrier on a cigarette filter was loaded with 2 mg of (*Z*,*E*)-9,11-C_{14}OAc and 8 mg of UOP 688 (*N*-phenyl-*N'*-(1-methylheptyl)-*p*-phenylenediamine). The latter compound is a dark, viscous, and unpleasant material that may act as a protecting "solvent" for the pheromone and also for slowing down evaporation. Preliminary results of catching efficacy of different preparations are summarized in Table 5. No significant difference ($P = 0.05$, Student's *t* test) was found between each of the five experimental preparations and the standard (UOP), or among the five compounds themselves, except for two

TABLE 5. MASS TRAPPING OF EGYPTIAN COTTON LEAFWORM MALES BY DIFFERENT PHEROMONE PREPARATIONS IN COTTON FIELDS IN 1986

		Average No. of males caught in a trap per night[b]			
		Kibbutz Mefalsim			
		1st experiment		2nd experiment	Kibbutz Reshafim
Entry	Pheromone formulation[a]	Jun 22–Jul 24	Jul 24–Aug 28	Jul 24–Aug 28	Jul 4–Aug 2
1	BHA + E-4360	0.3	6.4	12.2	13.8[c]
2	E-4360	0.4	10.4	11.2	10.8
3	BHA	0.7	7.2	8.7	10.7
4	BHT	1.1	10.3	11.9	14.7[c]
5	BHT + E-4360	3.1	10.9	12.5	12.7
6	UOP 688 (standard)	0.2	6.7	9.3	6.2

[a] Each carrier was loaded with a cyclohexane solution of 2 mg of (*Z*,*E*)-9,11-C_{14}OAc, 0.2 mg of BAH or BHT, and 0.2 mg of E-4360 (when mentioned). With UOP 688, 8 mg of the material was loaded on each carrier with 2 mg of pheromone.

[b] Dry traps with long sleeves were erected at random, 50 m apart, six replicates of each sample. Catches were monitored twice a week.

[c] Significantly different from UOP 688 at $P = 0.10$ (Student's t test).

formulations (entries 1 and 4 in Table 5) in the experiment carried out at Kibbutz Reshafim, which were significantly different from UOP 688 (entry 6) ($P = 0.10$, Student's t test). These results strengthen previous observations (Teich, Shani, and Klug, unpublished results) made during the 1981–1982 seasons, which showed not only that BHA and BHT were better protecting chemicals for (*Z*,*E*)-9,11-C_{14}OAc than UOP 688, but also that they increased catching efficacy. Therefore, it should be desirable to replace UOP 688 as an antioxidant in pheromone protections with BHA and BHT, at least for technical and handling reasons.

It is interesting that both the UV absorber and the antioxidant as a single additive prolonged the trapping efficacy of the pheromone. This fact may be in keeping with our earlier findings (see Table 4) that the behavior of the solid carriers was very much like that of one (antioxidant) or two additives and not to the positive effect observed for the UV absorber in solution (Tables 1, 2, and 4). The exact quantity of the pheromone and the relative composition of its geometrical isomers remaining on the solid carriers in the field can indicate the

correlation between the chemical processes and the biological activity (Shani and Klug, 1980b). At this stage we should remember that, when analyzing the results from the field study and comparing them with the chemical transformations of the pheromone, either in solution or on solid carriers, we are comparing two different processes and results. It could be, and we indeed found earlier in two cases, that not much of the real pheromone is needed for attraction, and the other geometric isomers do not interfere with trapping (Shani and Klug, 1980b; Ideses et al., 1982a). Therefore, the chemical results obtained in the laboratory do not necessarily reflect the biological findings in the field and vice versa. As long as enough active pheromone is present on the carrier and the other isomers are inactive and do not interfere, there may be high activity in the field despite the fact that isomerization and/or oxidation has destroyed much of the pheromone. In solutions studies, the pheromones and their isomers could be detected by GLC for up to six weeks afer the start of the experiment, while in the field the carriers attracted the Egyptian cotton leafworm for up to two months. The results in Table 5 show that, in the first experiment at Kibbutz Mefalsim, trapping in the second month (24.7–28.8) was almost as high as that in the first month of the second experiment.

We may summarize the results as follows:

Thermal Decomposition–Oxidation. Addition of an antioxidant prolonged the life-span of the diene system both in solution and in the carrier from several days to 2 weeks to six to eight weeks (Shani and Klug, 1980b; Ideses et al., 1982b; this work).

Isomerization. Addition of a UV absorber effectively slowed down isomerization in the diene system in solution and was less effective on the solid carrier. Both additives are crucial in solution, but the effect of the UV absorber becomes less important on the solid carriers.

Biological Activity. We have found that (*Z*,*E*)-9,11-C_{14}OAc attracts the Egyptian cotton leafworm in the field up to two months. The (*E*,*Z*)-7,9-C_{12}OAc isomer was found to attract the European grapevine moth in the field for up to seven weeks (Ideses et al., 1982a), which time is in the range of the chemical stability. The (*E*,*E*)-10,12-C_{16}Ald isomer is active in catching the spiny bollworm for four to five weeks (Kehat et al., 1981). The chemical behavior of this isomer was studied on the corresponding acetate, as the sensitive group is the aldehyde. The isomerization was very slow as the *E*,*E* isomer is the most stable one in the mixture.

Israeli entomologists and farmers usually change the carriers in traps for all three once in four to six weeks, although from scattered observations in the fields, some traps were found to be active for two months or more.

All these findings lead to the conclusion that results of laboratory studies and experimentation cannot necessarily be extrapolated to the real-life situation

in the field: the ultimate test of pheromone efficacy is the biological activity on the field, when all factors come into full expression and the weighted system is the governing power.

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Trirhabda canadensis (COLEOPTERA: CHRYSOMELIDAE) RESPONSES TO PLANT ODORS

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Abstract—The responses of the goldenrod leaf beetle *Trirhabda canadensis* to host and nonhost volatile odors were tested in a Y-tube olfactometer in the laboratory. Beetles preferred host to nonhost odors and were sensitive to concentrations of host odor. Beetles distinguished between host and nonhost volatiles of only one of the two nonhost *Solidago* species; host volatiles were preferred to all nonhost volatiles at the family and order levels. In other words, all nonhosts above the genus level had similar effects on beetle responses. Although the odors of most nonhosts were neutral (i.e., neither attractive nor repellent) to the beetles as tested against air, this neutrality disappeared if the odors of two or more nonhosts were added to the host odor and beetles were given a choice between this mixture and pure host odor. Given this choice, they strongly preferred pure host odor, which suggests that diversity of odors per se is unattractive to the beetles. Beetles walked rather than flew to locate their hosts in the field, and their movements suggest that they used olfactory cues to locate hosts.

Key Words—*Trirhabda canadensis*, Coleoptera, Chrysomelidae, *Solidago*, plant volatiles, host finding, olfaction.

INTRODUCTION

Host selection by insect herbivores comprises a number of behavioral responses to various stimuli associated with the host plant; stimuli may be olfactory, gustatory, visual, or mechanical (Visser, 1986). In particular, the orientation of phytophagous insects to chemical stimuli has been most extensively studied in agricultural systems (Finch, 1980; Visser, 1986). In these, it has been shown

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that volatile plant odors are important stimuli that aid specialist insects in short- and long-distance discovery of suitable host-plant patches and in selecting individual host plants. Since host-plant patches may vary in size, density, and the diversity of non-host-plant species associated with them, the volatile odors emanating from different patches may also vary. The concentration of host odor and the presence of nonhost odors may affect the ability of insects to find host plants in various ways (Root, 1973; Tahvanainen and Root, 1972; Kareiva, 1983; Stanton, 1983; Visser, 1986). Thiery and Visser (1986), for example, have shown experimentally that mixing nonhost odor with host odor neutralizes the normal response of the Colorado potato beetle, *Leptinotarsa decemlineata*, to host odor. They describe this effect as masking of the host odor by the nonhost odor.

The work described in this paper is part of a larger study that examined the responses of a specialized herbivorous beetle to patches of host plants in the field, and, in particular, how the density of the host and the presence of associated nonhost species influenced beetle responses. Here we present data from field and laboratory experiments on the responses of beetles to odors from host and nonhost species. These plant species are in the same genus or same family as the host or in different orders. All species are naturally cooccurring herbaceous perennials native to the tall grass prairie of North America (Table 1). We tested four hypotheses: (1) beetles prefer host volatiles to nonhost volatiles; (2) beetles detect differences in concentration of volatiles from the host plant; (3) volatiles from plants in the same genus as the host are more acceptable

TABLE 1. ARRAY OF PLANT SPECIES TESTED IN THREE SETS OF OLFACTOMETER EXPERIMENTS, AND THEIR TAXONOMIC AFFILIATIONS

Species	Order	Family	Tribe	Subtribe
Solidago altissima L. *Solidago nemoralis* Ait. *Solidago missouriensis* Nutt. *Solidago rigida* L.	Asterales	Asteraceae	Astereae	
S. altissima L.	Asterales	Asteraceae	Astereae	Solidaginae
Helianthus laetiflorus Pers.	Asterales	Asteraceae	Heliantheae	Helianthinae
Liatris aspersa (Michx.) Greene	Asterales	Asteraceae	Eupatorieae	Adenostylinae
Aster ericoides L.	Asterales	Asteraceae	Astereae	Asterinae
S. altissima L.	Asterales	Asteraceae	Astereae	Solidaginae
Asclepias tuberosa L.	Gentianales	Asclepiadaceae		
Lespedeza capitata Michx.	Fabales	Fabaceae		
Monarda fistulosa L.	Lamiales	Lamiaceae		

to beetles since they are closely related and are therefore expected to have some of the same volatile chemicals, while species that are more distantly related are less acceptable since these produce volatiles that are less similar; and (4) beetles prefer pure host volatiles to volatiles from host + non-host combinations.

METHODS AND MATERIALS

Goldenrod Leaf Beetle. Trirhabda canadensis Kirby (Coleoptera: Chrysomelidae) feeds on a narrow range of goldenrod *Solidago* species (Hogue, 1970). At Cedar Creek Natural History Area in Anoka and Isanti counties, Minnesota, the beetles feed on *S. altissima* L. and *S. missouriensis* Nutt. In May, larvae hatch from eggs that have overwintered in the soil at the base of goldenrod plants. They crawl up host plants to feed, complete three instars by mid-June, and drop to the ground to pupate in the soil. Adults 8–10 mm long emerge in early July and recolonize host plants, sometimes dispersing to new goldenrod clones. Beetles mate repeatedly and feed on the host until the end of the summer, usually early September.

Experimental Beetles and Plant Material. Adult beetles were obtained from Cedar Creek at intervals from July 21 to August 26, 1986. They were collected from *Solidago altissima* and *S. missouriensis* in the early morning and air-freighted to Boston the same day. They were maintained at Northeastern University on locally collected *S. canadensis* (which belongs to the *S. canadensis–S. altissima* polyploid complex) under controlled conditions (18°:15°C and 16:8 hr light–dark), and used for olfactometer tests within seven days of collection. Beetles were deprived of food for 2 hr before testing and were used only once in 75% of the tests. Because their number was limited, we used some beetles a second time. However, in those tests we ensured that: (1) the plant material in the second test was as different as possible from that used in the first (for example Air vs. *S. altissima* and then Air vs. the mint *Monarda fistulosa* L.); and (2) at least three days elapsed between the first and second tests. A preliminary trial showed that male and female responses did not differ, so beetles of both sexes were used for the tests.

Plant material was also obtained from Cedar Creek, where it was collected in the early morning, stored in a cooler with individual stems in Water-pics, and air-freighted to Boston the same day. Material was refrigerated and used for olfactometer tests within three days; preliminary trials showed that beetles did not respond in a reproducible manner to plant material after three days.

Experimental Procedures. The olfactometer was modeled after one described by Ascoli and Albert (1985), with minor modifications (Figure 1). A compressed-air cylinder provided the airstream, and a pipecleaner rather than copper wire was provided for beetles to climb. Plant material was placed in

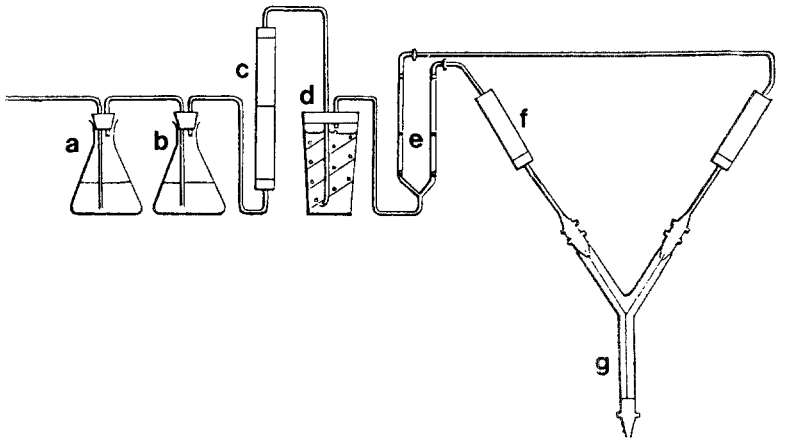

FIG. 1. Schematic diagram of the Y-tube olfactometer apparatus (after Ascoli and Albert, 1985): (a) drierite, (b) charcoal, (c) molecular sieve, (d) water, (e) flowmeters, (f) tube for plant material, and (g) Y tube.

polypropylene sample tubes, connected by polypropylene joints at either end to Tygon tubing, so that the airstream passed over the plant material in the sample tubes at a flow rate of 80 ml/min. We tested air flow in the olfactometer with smoke prior to using it, and determined that flow was smooth, with very little turbulence at the Y junction. The apparatus was positioned in a fume hood in which fluorescent lights provided illumination. Experiments were conducted at room temperature (25°C). The Y tube and pipecleaner were rinsed with acetone after each trial.

Plant material used in the tests was replaced every 30 min. Five to eight leaves were used at a time, except where indicated. In interspecific comparisons, the number of leaves was adjusted so that the amount of leaf material per species was the same.

Between 50 and 80 beetles were used for each test. For each trial, a beetle was placed on the pipecleaner 1 cm from the bottom of the Y tube and allowed to crawl up the pipecleaner. The trial ended when the beetle was within 1 cm of the top of either arm of the Y. A beetle was recorded as not having made a choice if it had not done so in 300 sec. Data were analyzed using chi-square tests for goodness of fit to an even distribution.

To establish whether beetles found a particular plant odor, or combination of odors, attractive, neutral, or repellent, the plant(s) in question were run against air. Then nonhost plants were individually tested against the host, *S. altissima*. Finally, we tested whether beetles distinguished between pure host odor and host odor plus odors from different taxonomic groupings of nonhost species. The nonhost groups were: (1) two species in the same genus, (2) three

species in different tribes or subtribes of the family Asteraceae, or (3) three species in different orders.

Individual Beetle Movements. We recorded movements of individually marked female beetles in the field to determine whether they typically move by walking and/or flying and to see whether their paths were directed or random with respect to host and nonhost plants. Eight observers recorded the movements of eight and seven female *T. canadensis* placed 4 m downwind of cut stems of *S. altissima* or *Helianthus* sp. (Asteraceae), respectively. A curved, open "fence" of stems, approximately 40 cm deep, 70 cm high, and 6 m long, was made from cut stems placed in buckets and jars that were dug into a lawn so that stems were at ground level. The *Helianthus* sp. was chosen because its leaves are similar in shape to *S. altissima*. Wind speed varied from 1.8 to 2.7 m/sec during the experiment. We released each beetle onto a 30-cm bamboo skewer and recorded her path across the lawn with numbered skewers.

RESULTS

Olfactometer Tests. In 85% of the olfactometer tests, beetles walked straight up the pipecleaner with their antennae moving as if to test the airstream, and they did not pause before choosing and walking up one of the arms. In 11% of the tests, beetles walked up one branch of the olfactometer for 10–15 mm before returning to the Y junction and walking up the other to make their final choice; these were included in the final sample as having made a choice. Beetles took 44.3 $\pm$ 9.3 sec ($\pm$SD) to complete a trial. Beetles made no choice in the remaining 4% of the olfactometer tests. These results were included in the calculation of χ^2 values (Siegel, 1956); they did not alter the significance of any of the tests.

Beetles were attracted (as shown by testing against air) to their host plants *S. altissima* and *S. missouriensis* in significant numbers (Figure 2a). They were not significantly attracted to or repelled by *S. nemoralis* Ait. and *S. rigida* L., two closely related nonhosts, although they showed a slight attraction to *S. nemoralis*. Beetles did not distinguish between their two hosts, *S. altissima* and *S. missouriensis*, when offered a choice (Figure 2b). However, when given a choice between *S. altissima* and the two nonhost goldenrods, the beetles did not distinguish significantly between *S. altissima* and *S. nemoralis*, although they did show a slight preference for *S. altissima*. They did significantly prefer *S. altissima* to *S. rigida* (Figure 2b). In short, *T. canadensis* did not distinguish among the *Solidago* species with the exception of *S. rigida*. *S. nemoralis* has been shown to contain a diterpene which is strongly antifeedant to *T. canadensis* (Cooper-Driver et al., 1986), but the volatile chemicals released by this species were not deterrent to the beetles.

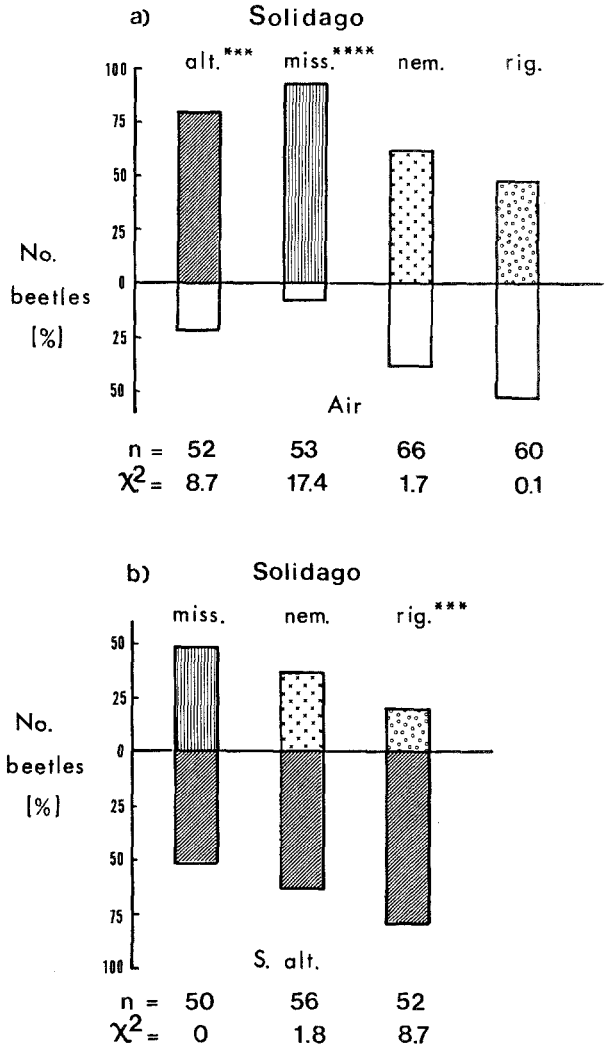

FIG. 2. The choices of adult *Trirhabda canadensis* of plant odors in a Y-tube olfactometer. Choices among plants of the genus *Solidago* were offered against (a) air and (b) the host, *S. altissima*. *S. altissima* = diagonal lines, *S. missouriensis* = vertical lines, *S. nemoralis* = crosses, *S. rigida* = open circles, air = white. ***$P < 0.01$, ****$P < 0.001$.

To test whether beetles responded to differences in odor concentration of the host, they were offered volatiles from one small leaf vs. two small leaves and five leaves vs. 20 leaves of *S. altissima* in two different tests. Beetles did not distinguish between volatiles from five leaves and 20 leaves, but showed a

TABLE 2. RESPONSES OF ADULT *Trirhabda canadensis* TO DIFFERENT CONCENTRATIONS OF HOST PLANT ODOR IN Y-TUBE OLFACTOMETER

Choice offered[a]		Choice made[b]			N	χ^2	P
1 Sa	2 Sa	14	38	0	52	4.00	<0.05
5 Sa	20 Sa	25	40	3	68	1.65	NS
5 Sm	20 Sm	25	27	1	53	0.04	NS

[a] Sa = *S. altissima*, Sm = *S. missouriensis*. Numbers preceding host species refer to the number of leaves offered.

[b] The first two columns indicate the number of beetles choosing each host odor, the third the number of trials in which beetles made no choice.

significant preference for two leaves over one leaf (Table 2). Thus, it appeared that a threshold concentration existed above which the odor was strong enough to elicit a positive response no matter what the concentration.

All three confamilial species tested [*Liatris aspersa* (Michx.) Greene, *Aster ericoides* L. and *Helianthus laetiflorus* Pers.] were neutral to *T. canadensis*, as shown by testing against air, although beetles appeared slightly to prefer air in all three tests (Figure 3a). However, they showed a significant preference for *S. altissima* to all three of these species (Figure 3b). In the orders group, *Monarda fistulosa* was highly repellent to *T. canadensis*, as tested against air, while the other two species were not (Figure 4a), and beetles significantly preferred the host plant to any of the three (Figure 4b). Thus, in the family and orders comparisons, one of the six species tested was repellent to the beetles; the other five appeared to be neutral, although there was a slight (but statistically nonsignificant) tendency for beetles to prefer air in all five tests. Beetles significantly preferred the host, *S. altissima*, to all six species.

Four of the six comparisons of beetle preference given *S. altissima* alone versus *S. altissima* plus one nonhost species showed that the nonhost species was neutral (Figure 5). Of the family group, *Helianthus* was the only species the odor of which caused the beetles to choose *S. altissima* alone over *S. altissima* + nonhost, in spite of being neutral to the beetles on its own; neither the odor of *Liatris* nor of *Aster* had this effect (Figure 5a). Of the three species in the orders group, *Monarda* was the only one that caused the beetles to prefer *S. altissima* on its own to *S. altissima* + nonhost (Figure 5b).

Equal numbers of beetles chose the odor from a "genus bouquet" (a mixture of *S. altissima*, *S. nemoralis*, *S. missouriensis*, and *S. rigida*) and odor from pure *S. altissima*, that is, beetles did not distinguish between the odor from pure *S. altissima* and that from the bouquet (Figure 6). When a larger amount of pure *S. altissima* (20 leaves) was offered equal to the total amount of leaf material in the genus bouquet, beetles only slightly preferred the odor

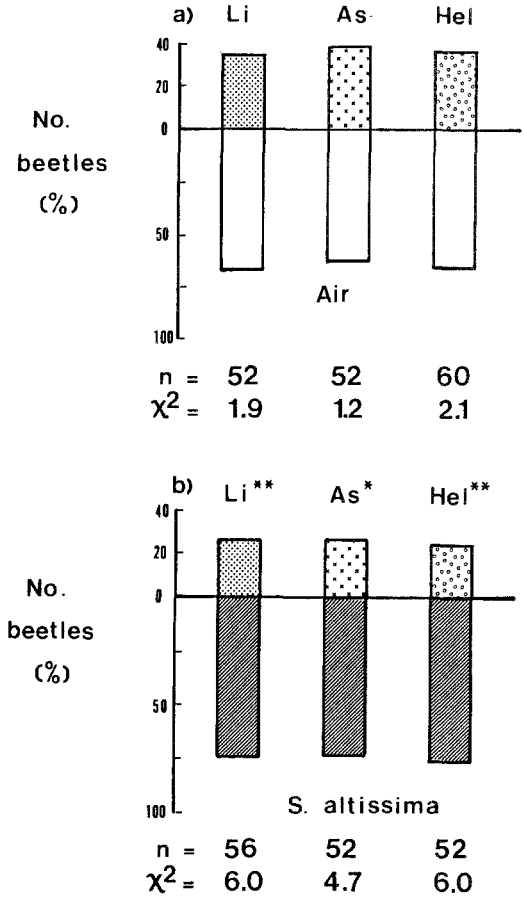

FIG. 3. The choices of adult *Trirhabda canadensis* of plant odors in a Y-tube olfactometer. Choices among different nonhost species in the family Asteraceae were offered against (a) air and (b) the host, *S. altissima*. *S. altissima* = diagonal lines, *Liatris aspersa* = dots, *Aster ericoides* = crosses, *Helianthus laetiflorus* = open circles, air = white. $*P < 0.05$, $**P < 0.02$.

of pure *S. altissima* (32 beetles vs. 19, $\chi^2 = 1.63$, 1 *df*, NS), but this result was not statistically significant.

S. altissima plus the three species from the family array was neutral to the beetles (as shown by testing against air) (Table 3). Interestingly, when *S. altissima* was removed from the family array, the number of beetles preferring air increased (Table 3), although the preference was not significant. However, beetles significantly preferred pure *S. altissima* to *S. altissima* plus the three non-

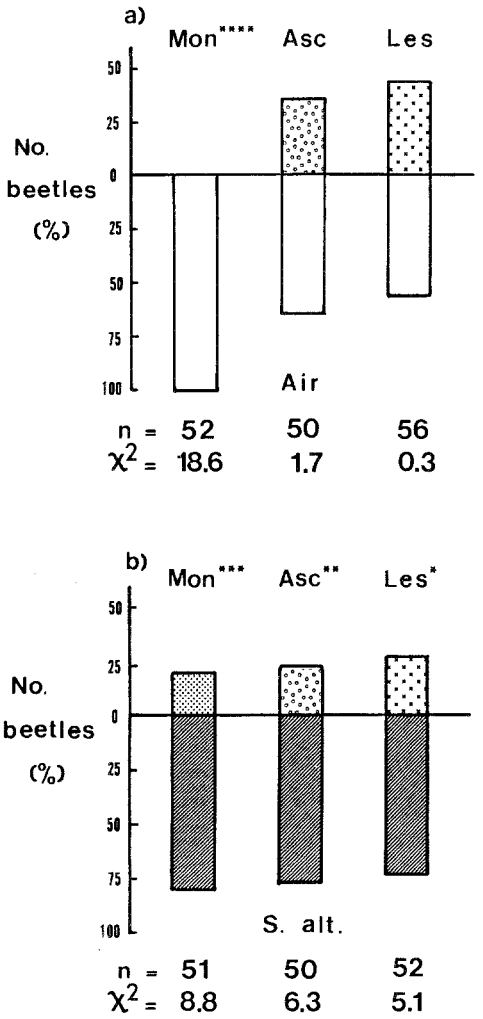

FIG. 4. The choices of adult *Trirhabda canadensis* of plant odors in a Y-tube olfactometer. Choices among different nonhost species in three different orders were offered against (a) air and (b) the host, *S. altissima*. *S. altissima* = diagonal lines, *Monarda fistulosa* = dots, *Asclepias tuberosa* = open circles, *Lespedeza capitata* = crosses, air = white. $*P < 0.05$, $**P < 0.02$, $***P < 0.01$, $****P < 0.001$.

host species in the family array (Figure 6). The "order bouquet" was repellent to the beetles when tested against air even though it had *S. altissima* present (Table 3), and this was most likely due to the presence of *Monarda*. Given the repellency of *Monarda* (Figure 4), it was not surprising that the beetles pre-

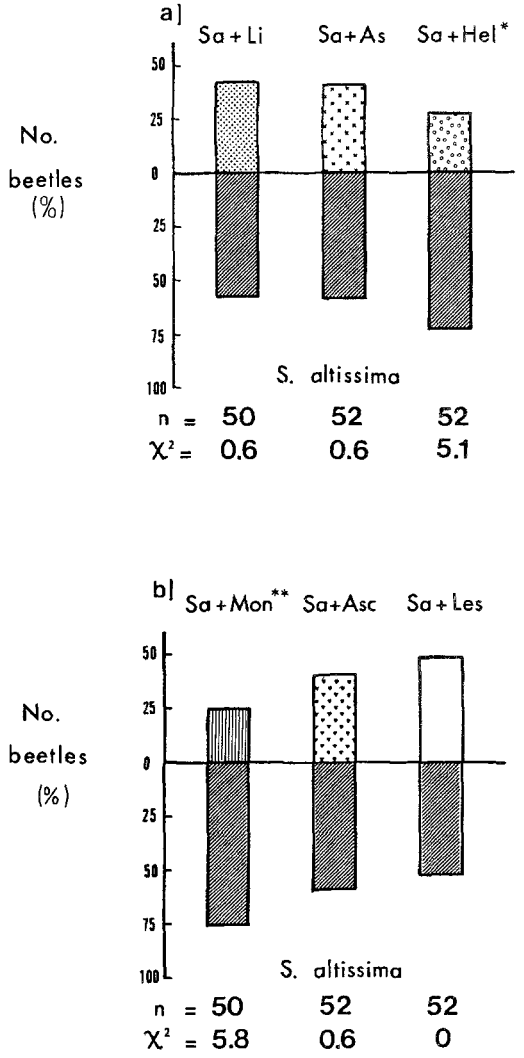

FIG. 5. The choices of adult *Trirhabda canadensis* of plant odors in a Y-tube olfactometer. Choices offered were (a) *S. altissima* (*Sa*) vs. *Sa* + different nonhost species in the family Asteraceae and (b) *Sa* vs. *Sa* + different nonhost species in three different orders. *S. altissima* = diagonal lines, *Sa* + *Liatris aspersa* = dots, *Sa* + *Aster ericoides* = crosses, *Sa* + *Helianthus laetiflorus* = open circles, *Sa* + *Monarda fistulosa* = vertical lines, *Sa* + *Asclepias tuberosa* = inverted triangles, *Sa* + *Lespedeza capitata* = white. $*P < 0.05$, $**P < 0.02$.

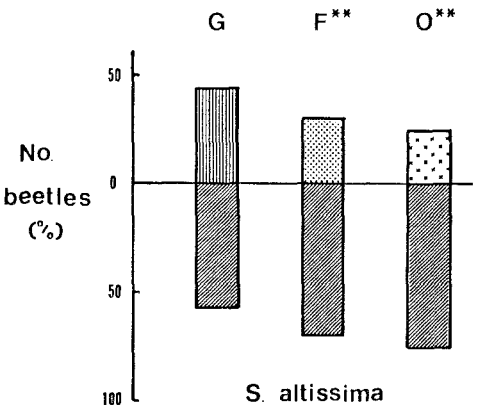

FIG. 6. The choices of adult *Trirhabda canadensis* between plant odors in a Y-tube olfactometer. Choices offered were between the host, *S. altissima* and a "bouquet" of plant species. G = genus bouquet comprising *S. altissima*, *S. missouriensis*, *S. nemoralis* and *S. rigida*. F = family bouquet comprising *S. altissima*, *Liatris aspersa*, *Aster ericoides* and *Helianthus laetiflorus*. O = orders bouquet comprising *S. altissima*, *Monarda fistulosa*, *Asclepias tuberosa* and *Lespedeza capitata*. $^{**}P < 0.02$.

ferred *S. altissima* to the order bouquet (Figure 6). However, even when *Monarda* was removed from the order bouquet, the remaining two species caused the beetles still to prefer *S. altissima* on its own to *S. altissima* plus these two species (44 beetles vs. 21, $\chi^2 = 4.01$, 1 *df*, $P < 0.05$).

Individual Beetle Movements. Beetles stayed on their release sticks for 5–120 min before walking across the lawn, and they climbed up grass blades frequently while walking. They moved their antennae as if to test the air in the

TABLE 3. ADULT *Trirhabda canadensis* CHOICES BETWEEN PLANT ODORS AND AIR IN Y-TUBE OLFACTOMETER, TESTING WHETHER ODORS ARE ATTRACTIVE, NEUTRAL, OR REPELLENT TO BEETLES

Choice offered[a]		Choice made[b]			N	χ^2	P
Air	F	36	30	2	68	0.26	NS
Air	F-Sa	42	20	6	68	3.56	NS
Air	0	47	20	2	69	5.28	<0.05

[a] F = "Family bouquet" comprising *Solidago altissima* (Sa) + *Liatris aspersa* + *Aster ericoides* + *Helianthus laetiflorus*; F-Sa = family bouquet minus Sa; 0 = "order bouquet" comprising *Solidago altissima* + *Monarda fistulosa* + *Asclepias tuberosa* + *Lespedeza capitata*.

[b] The first two columns indicate the number of beetles choosing between air and plant material, the third the number of trials in which beetles made no choice.

same way as they did in the olfactometer tests. One beetle flew after walking for 58 min. On average, beetles moved 5.5 ± 2.0 m/hr (±SD) and walked 4–12.9 m before finding a goldenrod stem (Figure 7). Of the eight beetles released downwind of *S. altissima*, seven moved upwind and six of these found a goldenrod stem. Of the seven beetles released downwind of *Helianthus*, two went downwind from their starting points and both eventually found *S. altissima* stems. The other five initially moved upwind: two reversed direction and one

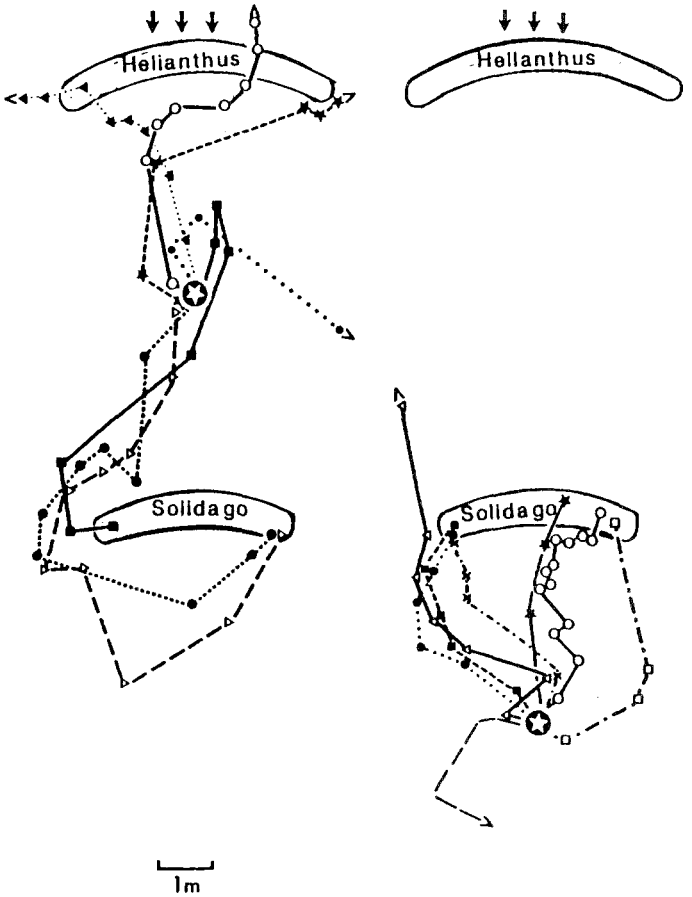

FIG. 7. Paths walked by 15 *Trirhabda canadensis* placed downwind of *Helianthus* sp. (left) or downwind of *S. altissima* (right). White stars indicate release points. Paths ending in v indicate beetles that did not find *S. altissima*. Both groups of beetles were followed simultaneously on the same grid but are shown separately for clarity. Arrows at top indicate wind direction.

of these located an *S. altissima* stem, while the other three passed around or through the *Helianthus*. The circuitous paths walked by the beetles suggested that they found goldenrod stems using olfactory rather than visual cues (Figure 7).

DISCUSSION

The behavior of goldenrod leaf beetles in the laboratory showed that beetles responded to olfactory cues from host and nonhost plants and preferred host to nonhost odors. In addition, beetles were sensitive to concentrations of host odor. Beetles distinguished between host and nonhost volatiles of only one of the two nonhost *Solidago* species; host volatiles were preferred to all nonhost volatiles at the family and order levels. In other words, all nonhosts above the genus level had similar effects on beetle responses. Although the odors of most nonhosts at the family or order levels were neutral (i.e., neither attractive nor repellent) to the beetles as tested against air, this neutrality disappeared if the odors of two or more nonhosts were added to the host odor and beetles were given a choice between this mixture and pure host odor. Given this choice, they strongly preferred pure host odor, which suggested that diversity of odors per se (or odors emanating from a diversity of species) was unattractive to the beetles. Beetles walked rather than flew to locate their hosts in the field, and their movements suggested that they used olfactory cues to locate hosts.

Beetle preference in these experiments largely supported the results of several experiments conducted in the field at Cedar Creek, testing the responses of goldenrod leaf beetles to the same set of host and nonhost species. In colonization experiments, beetles preferred monospecific plots to plots with *S. altissima* interplanted either with three species in the same genus or with three species in the same family or with three species in different orders (Morrow, Tonkyn, and Goldburg, unpublished data). It is likely that the final distribution of beetles in the colonization experiments was the result of beetle response to the different volatile "profiles" of the different plots.

The movements of individual beetles in the field experiment (Figure 7) also suggested that beetles were responding to host-plant odor. The likely mechanism was by means of odor-conditioned positive anemotaxis (Kennedy, 1977; Visser and Nielsen 1977; Visser, 1986). The general orientation of insects to chemical stimuli has been studied extensively (Shorey and McElvey, 1977; Visser, 1986, and references therein). Orientation may occur by means of chemotaxis, which comprises a response directed towards or away from a chemical stimulus and depends on a steep odor gradient. Therefore, chemotaxis can occur only at short distances from an odor source. The effective distance for chemotaxis in insect larvae ranges from 0.5 to 4 cm (Visser, 1986), while chemotaxis

occurs over slightly longer distances than this in adult insects since adults are more sensitive to odors than larvae (Baker, 1985). For longer distances, insects are thought to orient by means of odor-conditioned positive anemotaxis. This taxis comprises mechanical stimulation by wind which primes the insect; when it then perceives an attractive odor, it turns upwind (Visser, 1986). The concentration of the odor needs to be only just above the threshold of detection to bring this taxis into operation. Thus, it is thought to be effective over long distances, although the exact distance has not been determined experimentally (Baker, 1985).

The hypothesis that beetles would show a graded response to volatiles from plants that were closely to distantly related to the host (hypothesis 3 above), was only partly supported by our results, since we found that all nonhosts above the genus level had similar effects on beetle responses. This suggests that beetles recognize specific volatile components or profiles associated with *Solidago* species. Visser and Avé (1978) found that the specificity of potato plant odor in eliciting a positive response from Colorado potato beetles depended on the ratios of the individual volatiles present rather than the presence of any particular volatile. On the other hand, the importance of all non-*Solidago* volatiles appeared to depend on the total diversity present rather than on the recognition of specific volatiles.

Experimental methods for testing insect orientation to chemical stimuli have been reviewed (Kennedy, 1977). Although Y-tube olfactometers have many advantages, they have the disadvantage of not enabling the actual mechanism of attraction to be unequivocally assayed since chemoklinotaxis cannot be distinguished from odor-conditioned positive anemotaxis. This ambiguity in ability to attribute the behavioral mechanism responsible for beetle choices in this study was not a problem since we were interested in actual beetle responses to plant odors rather than in determining the mechanism by which beetles responded.

Of the plant volatiles so far isolated, over 40% are derived from either terpenes or glucosides (Finch, 1980). Visser and Avé (1978) found that leaf alcohols, aldehydes, and derivatives, which they called "green leaf" volatiles, induced positive anemotaxis by the Colorado potato beetle. We are currently isolating volatiles from the plant species used in the present study and testing these to determine which are responsible for observed beetle behavior.

It is possible that attractiveness, neutrality, or repellency of the volatiles of a given plant species or mixture of species is strictly a function of the chemical structure of these volatiles, as determined, for example, by the presence of a particular functional group, although Harborne (1982) cautions that the relationship between chemical structure and odor is not likely to be a straightforward one. Whatever the mechanism, the varied responses of beetles to host, nonhost and host + nonhost volatiles suggest that in natural systems beetles

may respond not only to host odors but may also use nonhost odors to gain additional information about their environment. For example, given the same concentration of host plant volatiles, the presence either of increasing concentrations of the volatiles from one nonhost species or of increasing numbers of volatiles from a larger number of nonhost species may signal a host plant patch with higher total plant density. In such a patch, the host might be less vigorous and therefore a poor-quality source of food.

This study is the first to our knowledge that has focused on insect odor perception in a natural system where plants and insects have been associated over long periods of time. We have shown the effects of plant diversity (as represented by volatile diversity) on the responses of a specialist phytophagous insect at a fairly close range in the laboratory. In future field and laboratory experiments, we plan to test the effects of plant diversity on long-distance attraction of these beetles. More experiments such as these are needed, since such effects have not been tested for any insect species to date (Thiery and Visser, 1986).

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CHEMOSENSORY RESPONSES IN TWO SPECIES OF ELEPHANTS TO CONSTITUENTS OF TEMPORAL GLAND SECRETION AND MUSTH URINE

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Abstract—This report discusses three areas of investigation: (1) The chemical components in the temporal gland secretion (TGS) of Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants were characterized by radioimmunoassay (RIA) for testosterone (T) and dihydrotestosterone (DHT) levels and by on-column capillary column gas chromatographic analysis of volatiles. An inverse relationship between TGS testosterone levels and (*E*)-farnesol levels was observed. (2). African elephants responded preferentially toward a particular constituent of African elephant TGS. (3) Urine from Asian bull elephants in musth was partially fractionated by high-performance liquid chromatography. Specific chromatographic regions elicited dramatic avoidance responses from female African elephants. These results support the suggestion that the TGS plays multiple chemocommunicative roles.

Key Words—Temporal gland secretion, *Elephas maximus*, *Loxodonta africana*, testosterone, urine, flehmen, palatal pits, (*E*)-farnesol.

INTRODUCTION

Elephants utilize a variety of sensory systems to communicate with each other: auditory (Heffner and Heffner, 1980; Berg, 1983; Payne et al., 1986; Buss, personal communication 1980), visual (Rensch, 1959; Kuhme, 1962; Markowitz et al., 1975), and tactile (Eisenberg, 1980; Gadgil and Nair, 1984). Especially important in communication are the olfactory (Eisenberg et al., 1971; McKay, 1973) and other chemosensory systems, including the vomeronasal organ (VNO) (Rasmussen et al., 1982, 1986). Chemicals from certain glands, body secretions, and/or excretions apparently convey information to other ele-

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phants on sex (Schmidt, 1978), sexual status (Hess et al., 1983; Rasmussen et al., 1982, 1984), individuality (Rasmussen et al., 1986), status within groupings (Schmidt, personal communication), and familial relationships (Rasmussen, unpublished).

Urine and secretions from the temporal gland (Rasmussen et al., 1982, 1984; Buss et al., 1976) are intimately involved in chemocommunication among elephants. The temporal gland is a modified sebaceous–apocrine skin gland, uniquely possessed by elephants (Perrault, 1734; Estes and Buss, 1976). The gland is located on the side of the head in the temporal fossa about midway between the eye and ear and opens to the surface by a duct near the center of its lower border. Various functions have been proposed for the gland in African elephants (Sikes, 1971, McKay, 1973; Buss et al., 1976; Wheeler et al., 1982) and in Asian elephants (Eisenberg et al., 1971; Rasmussen et al., 1984). Current thinking favors a multifunctionality for the gland.

Three probably chemosensory responses are distinguishable in both species of elephants (*Elephas maximus*, Asian elephant, and *Loxodonta africana*, African elephant). First, olfactory responses have been observed for centuries (Strabo, 63 BC–21 AD; Estes, 1972; McKay, 1973; Eisenberg et al., 1971; Buss et al., 1976). Systematic studies documenting specific olfactory responses such as sniffing (the trunk tip may be several centimeters to kilometers distant from the odor source) and checking (extreme tip of the trunk touches sampling area), their frequencies, and resulting behavioral responses are few (Eisenberg et al., 1971; Rasmussen et al., 1982; Rapaport et al., 1986). Second, flehmen responses have been documented for both sexes of Asian and African elephants (Rasmussen et al., 1982, 1984, 1986; Rapaport et al., 1986). The flehmen response by elephants apparently occurs directly after olfactory investigation, although it is possible that there may be involvement by as yet undescribed contact or tactile receptors in the trunk tip. Specifically, the flehmen response in elephants involves the contact of the trunk tip with a wet spot, e.g., urine or temporal gland secretion (TGS), followed by placement of the tip directly on the orifices of the paired ducts which connect with the VNO (Rasmussen and Hultgren, 1988). Highest flehmen frequencies exhibited by the Asian bull toward female urine correlate with ovulation and are inversely related to serum progesterone levels (Hess et al., 1983). Third, a response involving placement of the trunk tip on the palatal pit region of the inner upper lip has been observed among Asian elephants at the Washington Park Zoo (WPZ) (Rasmussen et al., 1986; Haight and Rasmussen, in preparation). These three behavioral responses by the elephants served as the basis of the response data recorded during the bioassay sessions.

The present study has (1) compared the chemical composition of temporal gland secretions (TGS) from two species of elephants (with careful attention to age, sex, diet, and physiological condition), (2) recorded the three described chemosensory responses in Asian and African elephants to both whole secre-

tions (TGS and urine) and subfractions derived from these secretions, and to specific compounds previously identified as present in temporal gland secretions, and (3) noted a behavioral response to yet unidentified molecules excreted in urine by male Asian elephants during musth.

METHODS AND MATERIALS

Bioassay Procedure

Bioassay procedures utilizing the three described responses have been developed for both African and Asian elephant groups in moderately sized enclosures involving free-roaming animals (Rasmussen et al., 1986).

Sites. Cooperative relationships have been established with three zoos—Washington Park Zoo (WPZ), Portland, Oregon; Wildlife Safari Park (WLSP), Winston, Oregon; and Point Defiance Zoo (PtDZ), Tacoma, Washington. Each has good facilities for bioassaying. Each zoo maintains small groups of free-roaming elephants in enclosures composed of sand and grass substrates varying in area from 0.25 to 5 hectares. The test enclosures contained several areas of concrete slabs. These slabs were rigorously cleaned with organic cleansers and hosed with hard streams of water prior to every bioassay session. The unique elephant facility, especially the elephant restraining crush, at the WPZ made possible the frequent collection of serum and TGS samples.

Test Animals. Twenty-two elephants in six separate groups of elephants (entirely Asian or African, and mixed species groups) were available for response monitoring (Table 1) either as groups or as individual animals.

TABLE 1. ELEPHANTS PARTICIPATING IN BIOASSAYS

Location	Sex	Status	Age (years)	Origin[a]
Elephas maximus				
Washington Park Zoo (WPZ)	3 bulls	mature and breeding	21–25	2 wc,1 cb
	7 cows	mature and cycling	22–38	5 wc,2 cb
	1 female	intermediate	3	1cb
Point Defiance Zoo (PtDZ)	1 male	intermediate	3	1cb
Loxodonta africana				
PtD2	3 females	maturing	9–12	3wc
Wildlife Safari Park (WLSP)	6 cows	mature	13–35	6wc
	1 male	maturing	12	1wc

[a]cb = captive born; wc = wild caught.

Sample Preparation and Placement. Intact urine samples or fractions thereof in 250 ml buffer were placed on the slabs. Physiological concentrations and their percentages were estimated for specific compounds using analytical data. Substances of limited water solubility were first dissolved in acetone or methanol and subsequently added to buffer. Controls included buffers, heterospecific urine, selected novel substances, and organic solvents in concentrations equivalent to levels used in separation procedures.

Samples of 250 ml volume were poured directly on level areas of concrete slabs. Three to nine samples (two to six test preparations and one to three controls) were placed at random positions prior to each bioassay session. Intervals between bioassay sessions, unless for special experiments described later, were at least 24 hr and usually 48 hr to five days. Samples were placed in the absence of the test animal(s). All bioassays were conducted with the observer uninformed of the test solution composition. Unlike the constant interactions observed between the elephants and their keepers, our experience demonstrated no interactions between the elephants and the observer during the bioassay sessions. The observer watched from a regular visitor viewing area, standing, visible to the test animal, about 30 feet from the samples with an unobstructed view of all the samples. Bioassay sessions lasted 1–4 hr.

Each sample was tested in duplicate during the first bioassay presentation and at least eight additional times during subsequent bioassay sessions, for a total of at least 10 tests. Depending on the purpose of the test, three to five discrete, successive bioassay test sessions were separated by 1–3 hr, at least 24 hr, or 6 months to 1 year. Habituation by the elephants to samples was a concern. The criterion for bioactivity (and lact of habituation) was a response of nondiminishing intensity elicited during five successive (intervals between 1 and 3 hr and/or 24 hr) tests on the same elephant. Selected compounds, especially single compounds testing positively, were also retested after periods of 6 months and 1 year. An earlier study in Asian bull elephants demonstrated that about one third of the chemical substances tested elicited responses upon initial presentation (Rasmussen et al., 1986). Subsequent retesting of these initial response-provoking substances demonstrated loss of response by the fourth bioassay except for selected natural substances from the elephant secretions (Rasmussen et al., 1986).

Responses. The responses monitored included three distinct responses: (1) olfactory responses [number of sniff (trunk tip not touching sample) responses per unit time, duration of sniff episodes, number of check (trunk tip touching sample) responses and duration of checks]; (2) the flehmen response; and (3) the palatal pit area contact response (PPAC). The latter response was described a "sideways tasting response (STR)" in Asian bull elephants (Rasmussen et al., 1986). Examination of recent videotapes clearly showed the trunk tip is actually placed on the area of the palatal pits. Histological examination has not

demonstrated any taste buds in this region (Haight, unpublished results). The response is identical to the palatal pit area contact respone (PPAC) described in female Asian elephants (Haight and Rasmussen, in preparation). The characteristics of these three responses are outlined in Table 2.

Data Collection

Scoring Procedures—Behaviors. We recorded the frequencies of these three responses (Table 2) during bioassay time periods of 1–4 hr (Hutt and Hutt, 1974, "event sampling"; Slater, 1978, "complete record").

Observers. Each person observed one or two elephants. During multiple animal bioassays, a focal animal was chosen at random. The number of elephants present during a bioassay session varied from 1 to 6 animals (always randomly selected). Only the principal investigator and three other trained personnel served as observers (see Acknowledgments).

Observers recorded the type of initial detection of test samples (a sniff or a check), its duration (in seconds), any repetitiveness or sequence, the number of flehmen responses and their duration, the number of palatal pit responses and their duration, and unusual responses, especially responses indicating agitation or avoidance. Records indicated whether scored responses were those of a single animal or if two or more animals were involved.

Recording Procedures. We utilized video taping as an accurate, supplemental recording technique. Video recordings were cross checked with both written and auditory observer records and with rapid sequence (3/sec) still photography substantiating both intraobserver and interobserver reliabilities. During multiple animal bioassays, the entire session was filmed by a preset automatically recording videocamera.

Sequential Scoring on Focal Animals. One set of experiments emphasized the recording of sequence of responses. The video recordings were an especially valuable check on accuracy of sequencing records (see Table 6).

Data Handling Procedures. The range of response durations was tabulated (Table 2), and average durations of responses were calculated. Frequencies (number per unit time) of flehmen and palatal pit responses were calculated, and these frequencies were compared. Interobserver reliability rate was determined both by the videotape methodology of Lehner (1979) and calculated from simultaneous data sheets by the Kappa method of Cohen (1960).

Procurement of Secretion Samples

Female Asian Elephant TGS. Ten samples of TGS from Asian females were collected during the 4-year period 1981–1985. Infrequent secretion occurred predominantly at stressful times such as changing of cow association groups and calving. Samples were collected by gently placing specially designed

TABLE 2. PROBABLE CHEMOSENSORY RESPONSES IN ELEPHANTS

Response	Description	Duration*		Occurrence	Behavior may be indicative of
		Range	Mean		
Olfactory		1 sec to 5 min.	15 sec		
Sniff	Trunk tip several cms to several meters distant from odor source				
Check	Extreme tip of trunk touches sampling area			solitary event or prior to flehmen	mating greeting offspring recognition
Flehmen	Contact of trunk tip with wet spot followed by placement of the tip directly on orifices of incisive ducts.	1–3 sec	1 sec	After prior olfactory investigation	male–female interactions: recognition of estrus/ nonestrus female–female interactions male–male interactions: musth/nonmusth female–maleinteractions maternal recognition
Palatal pit area contact response (PPAC)	Placement of trunk tip on palatal pit region of inner upper lip after sample contact	1 sec to 2+ min	?	pre- or post-olfactory or solitary or pre- or postflehmen and/or may be sequential from elephant to elephant	under investigation

widemouth collecting flasks to washed areas beneath the orifice of the temporal gland, or, for very viscous secretions, using special wide-bore Teflon needles connected to a glass syringe.

Male Asian Musth Secretion. Asian bull elephants experience an annual "musth" period characterized by radical behavioral changes including an increase in aggressiveness and dominance displays (Schmidt, 1978), a 10-fold increase in serum testosterone levels, and copious secretion from the temporal glands. Testosterone is extremely high in most TGS samples from musth bulls (Rasmussen et al., 1984). Between 2 and 101 TGS samples per bull were obtained from three Asian bull elephants in musth at intervals during entire musth periods (Rasmussen et al., in preparation) during the years 1981–1986. Twenty samples have been analyzed for this report. During light musth, some gland secretion and some aggressive behavior occur. During heavy musth, copious, odoriferous gland secretions, intermittent uncontrolled urine dribbling, lack of appetite, inability to lower the penis, and very aggressive, unpredictable behavior occur (Schmidt, 1978; Rasmussen et al., 1984). The procurement of the TGS was dangerous, but possible because of WPZ's restraining crush, which prevented bull movement during sample collection (Rasmussen et al., 1984). Samples were frozen in liquid nitrogen and stored at −20°C. Whenever possible, concurrent serum samples were obtained by the method described in Hess et al. (1983). Both the serum and TGS samples from both elephant species, in the wild and in zoos, were obtained consistently between 9 and 11 AM.

African Elephants. TGS samples were obtained from three young female elephants at PtDZ. Samples from wild culled African adult male and female elephants were collected and frozen by the method described in Rasmussen et al., (1984). Some TGS and serum samples were obtained from several African males observed to exhibit certain behaviors similar to those of Asian bulls in musth (Hall-Martin, personal communication, 1980; Poole and Moss, 1981; Hall-Martin and Van der Walt, 1986).

Collected samples were subdivided into six aliquots before being frozen; duplicate aliquots were used for radioimmunoassay. Three samples per group were analyzed by both capillary column gas chromatographic (gc) analyses and gas chromatographic–mass spectrometric (GC-MS) analyses. The GC analyses are based on 20 replicate GC runs of each of the three aliquots. Other aliquots were fractionated by HPLC, used for bioassay presentations, or assessed for freeze–thaw stability and stability during chemical manipulations.

Chemical Characterization of Components of TGS

Samples were analyzed for testosterone (T) and dihydrotestosterone (DHT) by the method of Resko et al. (1980) as described in Rasmussen et al. (1984).

Samples were prepared for gas chromatographic separation by three suc-

cessive extractions of 1 ml of TGS with dichloromethane using a secretion–solvent ratio of 1:3. Extractions were combined and dried by filtration through anhydrous sodium sulfate. An aliquot (0.5 μl) was chromatographed using a capillary column (J&W DB-5 fused silica column, 60 m, 0.32 μm, film thickness 0.25 mm), with helium as the carrier gas, and temperature programed from 35°C to 300°C at 6°/min using a Hewlett-Packard 5790 gas chromatograph. Flame ionization detection was used. Reference standards of the five previously identified compounds from African elephant temporal gland secretions—phenol (P), 4-methylphenol, (*E*)-farnesol, farnesol monohydrate (FMH), and farnesol dihydrate (FDH)—were used to identify those components in samples (Wheeler et al., 1982). These compounds were also used as internal standards. Identification was made on the basis of retention times relative to those of these standards, and by GC/MS (using a Hewlett-Packard 5790 GC coupled to a Finnigan mass spectrometer #4000 and/or a VG 7070 E-HF double-focusing mass spectrometer system with FD-EI and FAB ion source system and a 11/250 data system).

Identification of additional, new compounds (such as 4-ethylphenol, 2-propylphenol, 4-propylphenol, and 1-benzoylpyrrole) were tentatively made by GC/MS analyses and confirmed by additional on-column capillary column GC analyses in comparison to standards, both external and internal, and by analyses on a 90-MHZ NMR spectrometer. Other categories of compounds were only tentatively suggested, not postively identified, such as hydrocarbons and steroids.

Preparative high-performance liquid chromatography (HPLC) was employed to separate the organic-extracted, aqueous-soluble components of musth urine. Selective fractions, separated by HPLC on a semipreparative C-18 Bondpak reverse-phase column using a gradient elution system of 20:80, methanol–water to 90% methanol, were prepared for bioassay.

RESULTS

Chemical Analyses: Characterization of TGS Components

Testosterone. Testosterone (T) and dihydrotestosterone (DHT) levels were measured in 20 TGS samples from three Asian bull elephants in musth (Table 3). Bull #2 samples were taken serially during two different musth periods. Several concurrent serum samples were obtained (Table 3). TGS testosterone values were high, ranging from 17.5 to 2113.8 ng/ml. The wide range was apparent even when androgen levels were equated per TGS protein. Especially to be noted are the testosterone levels in the TGS extracts analyzed by GC (Figure 1). Testosterone values for serum and TGS are given for the samples analyzed (Figure 1, legend).

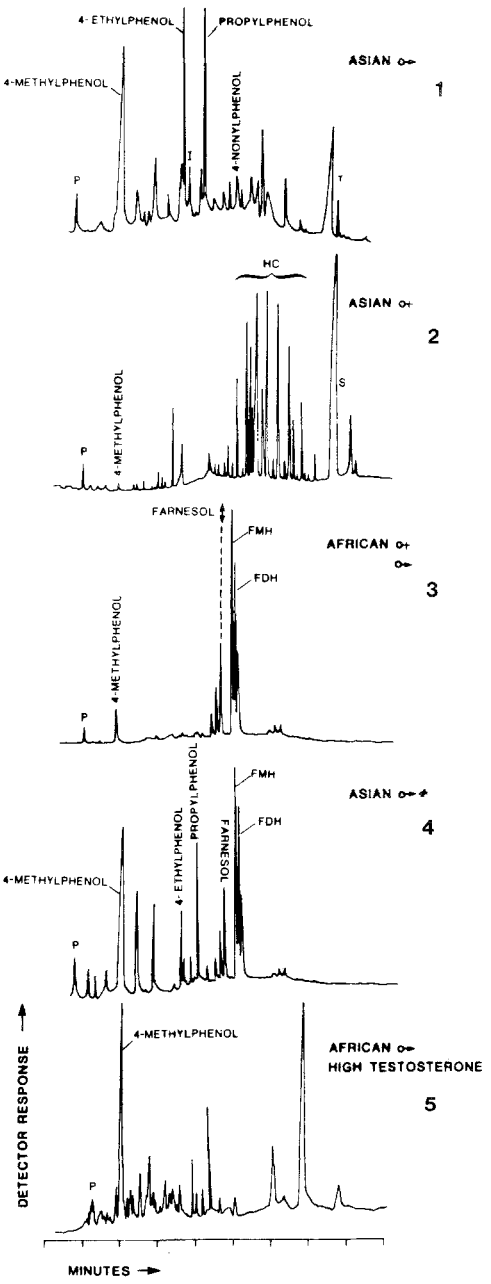
TABLE 3. TEMPORAL GLAND SECRETION TESTOSTERONE (T) AND DIDHYDROTESTOSTERONE (DHT) CONCENTRATIONS

2 (Serum T = 127.8)	Testosterone ng/ml	Dihydrotestosterone ng/ml
Bull #		
1	786.24	—
2 Musth period 1	385.50	201.10
2	810.00	560.50
2	196.90	—
2 (Serum T = 127.8)	921.61	1716.35
2 (Serum T = 40.1)	447.86	577.30
2 (Serum T = 73.8)	247.0	182.8
1	259.3	71.59
2 Musth period 2	151.61	62.74
2	195.88	43.81
2	232.16	39.25
2	236.64	50.64
2	511.04	557.04
2	2113.81	691.00
2	87.36	25.36
3	17.55	3.94
1	1363.3	890.01
3	512.98	600.06
3	126.67	98.69
3	57.98	27.22
Mean	483.07	355.52
SD	514.14	431.93
SE	=/± 8.9	9.8

Serum: range 0.08–127.8 ng/ml

Range distribution % of T levels in 20 TGS samples:	
5%	>2000 ng/ml
5%	1000–2000 ng/ml
25%	500–1000 ng/ml
50%	100–500 ng/ml
15%	<100 ng/ml

Gas Chromatographic Analyses. The volatiles extracted from TGS of various elephants and separated by the high resolution of on-column capillary column GC are depicted in Figure 1. Represented from top to bottom: #1, Asian bull elephant (musth); #2, Asian female elephant; #3, male/female African ele-


4-ETHYLPHENOL
PROPYLPHENOL
ASIAN
1
4-METHYLPHENOL
4-NONYLPHENOL
P
I
T
HC
ASIAN
2
4-METHYLPHENOL
P
S
FARNESOL
FMH
AFRICAN
3
FDH
4-METHYLPHENOL
P
FMH
ASIAN
4
FDH
4-METHYLPHENOL
4-ETHYLPHENOL
PROPYLPHENOL
FARNESOL
P
4-METHYLPHENOL
AFRICAN
HIGH TESTOSTERONE
5
P
DETECTOR RESPONSE
MINUTES

phant (dashed line indicates female captive African elephant); #4, Asian male elephant, estrone injected; and #5, African bull elephant with high serum and TGS testosterone. The TGS volatiles GC patterns from Asian male elephants in musth (#1) and African bulls with high serum testosterone (#5) are especially noteworthy to compare. The similarities between the TGS volatiles of an estrone-injected in-musth Asian bull (#4) and a African bull with normal serum T levels (#3) are equally remarkable.

Five volatiles [previously identified in the TGS of both sexes of culled African elephants (Wheeler et al., 1982)]—phenol (P), 4-methylphenol, (*E*)-farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-l-ol), farnesol monohydrate (FMH, 3,7,11-trimethyl-2,10-dodecadien-l, 7-diol), and farnesol dihydrate (FDH, 3,7,11-trimethyl-2-dodecen-l,7,11-triol)—were identified in TGS samples from several captive African females and one male African elephant (Figure 1, #3).

Variable concentrations of either or both phenol and 4-methylphenol were detected in all TGS samples from both elephant species, including Asian bulls in musth.

(*E*)-Farnesol was a component of the TGS from most African elephants of both sexes. (*E*)-Farnesol was present in low concentration in TGS of Asian female elephants (Table 4). In only five of 101 TGS samples from Asian bulls in musth were low levels (< 1%) of (*E*)-farnesol detected (Table 4) (Rasmussen et al., in preparation). However, (*E*)-farnesol was readily identified in the single TGS sample from an estrone-injected Asian bull in musth (Table 4; Figure 1, #4).

Other notable differences in volatiles from TGS included the presence in

FIG. 1. On -column capillary GC separation of volatiles in dichloromethane extracts of temporal gland secretions (TGS) from following indicated elephants: Chromatogram #1, captive Asian bull at WPZ, serum T = 73.8 ng/ml, TGS T = 247.00 ng/ml, F = 1%. Chromatogram #2 Asian female elephant sample obtained shortly after parturition, serum T = 0.05 ng/ml, TGS T = 0.05 ng/ml. Chromatogram #3, African female or male with low serum T 0.05–0.5 ng/ml, TGS -T = 1.45 ng/ml. Dashed lines indicate farnesol peak from captive female African elephant TGS, serum T = 0.02 ng/ml, TGS T = 0.75 ng/ml. Chromatogram 4, estrone treated Asian male elephant, TGS T = 2.50 ng/ml. Chromatogram #5, African male elephant (wild) serum T = 6.64 ng/ml, TGS T = 78.06 ng/ml. Compound codes: P = phenol, propylphenol = mixture of 2- and 4-propylphenol, T = testosterone, S = nontestosterone steroid, HC = hydrocarbons including triacontanes, pentatriacontanes; other compounds identified in this region included 2-benzothiazole, *n*-propylbenzamide and other benzoic acid derivatives. FMH = farnesol monohydrate, FDH = farnesol dihydrate, I = 1-benzoylpyrrole, 4-nonylphenol (contains other isomers).

TABLE 4. ON-COLUMN CAPILLARY COLUMN GC SEPARATION OF CONSTITUENTS OF TEMPORAL GLAND SECRETIONS

	Asian elephants			African elephants			
	Male			Female		Male	
Species	Captive	Captive estrone-treated	Female captive	Wild	Captive	Wild	Wild aggressive
T levels (ng/ml)							
Serum	127.8		0.05	2.39	0.02	2.90	6.64
TGS	511.04	2.50	0.01	6.94	0.99	8.90	78.06
	High	Low	Low	Moderately low	Low	Moderately low	High
Compounds							
Phenols	10%	30%	5%	2-42%	47%	24%	1-11%
Phenol							
4-methylphenol							
4-ethylphenol							
2- and 4-propylphenol							
Farnesols	0-1%[a]	65%	?	48-73%	53%	39%	2-9%
	7-13%[b]						
	9-52%[c]						
(*E*)-farnesol							
Farnesol monohydrate							
Farnesol dihydrate							
RT 24-26[d]	-50%	1%	60%	2-4%	1%	2%	18-44%
Steroids	84-92%[a]						
Testosterone	High	Low	Low	Low	Low	Low	High
	37-59%[b]						
	21-24%[c]						
Other			40% unidentified				

[a] 7 samples—early musth.
[b] 7 samples—late musth.
[c] 6 samples—late, late musth.
[d] Hydrocarbons.

female Asian elephant secretion of a number of high-molecular-weight hydrocarbons (tentative identifications include various substituted pentatriacontanes, tetratriacontanes, and tritetracontanes of undetermined substitutions), benzoic acid derivatives, and at least one nontestosterone steriod (S) (Figure 1, #2). A high level of (*E*)-farnesol was present in the TGS of a young zoo female African elephant as indicated by the dashed line in Figure 1, #3 and Table 4.

The GC pattern of volatiles from the male Asian elephant TGS "musth" was distinctive. The majority of identified peaks, including 1-benzoylpyrrole (I), 4-methylphenol, 4-ethylphenol, 2-propylphenol, and 4-propylphenol, eluted before the farnesols (Table 4, Figure 1). By GC/MS analysis on Finnigan #4000 under the particular GC conditions, 2-propylphenol and 4-propylphenol co-eluted (Figure 1, propylphenol). GC separation of extended times resulted in the elution of 2-propylphenol (bp 224–226°C) several minutes prior to 4-propylphenol (bp 232°C. Identifications were confirmed by high-resolution GC/MS (VG 7070), internal standards, and NMR. A similar protocol was followed for 1-benzoylpyrrole. GC-MS and internal standard data established the retention time of testosterone (T) as indicated in Figure 1. The testosterone peak, varying quantitatively, was detected in all Asian musth bull and several African bull TGS samples. The volatiles from the TGS of the male African elephant with high testosterone (Figure 1, #5) bear an interesting resemblance to the volatiles from the TGS of an Asian bull in musth (Figure 1, #1 and Table 4). Noteworthy similarities were the presence of various phenols, the reduced farnesol content, and the high levels of testosterone. Further identifications will be presented in Rasmussen et al., in preparation.

HPLC Analyses. The indicated eluting region on the HPLC chromatogram of fractionated Asian male musth urine (Figure 2) elicited a characteristic response from female African elephants (both at PtDZ and WLSP). Upon initial detection of the test sample, there was a high frequency of flehmen and PPAC responses, immediately followed by a dramatic avoidance alarm response. The ears were held in an erect position characteristic of alarm (Buss, personal communication). Trumpeting and roaring occurred. Both at PtDZ and WLSP, the elephants herded together, ran to the other end of yard, and thereafter avoided the sample. Further fractionation of this chromatographic region is currently in progress.

Bioassay Results: Natural Substances

The interobserver reliability was calculated to be 0.81. Three responses were recorded independently. Comparisons between them are expressed primarily as frequency. The *y* axis scale of response frequency differs in Figures 3 and 4. Data on response duration are contained in Tables 2 and 5.

Asian Elephant Responses. The bioassays were conducted several times a week at WPZ for a total of 1351 samples tested. Each sample was bioassayed by each bull 10 or more times and by each cow 5–10 times.

Among females, the highest flehmen frequencies were to male musth urine and male nonmusth urine with no significant differences between these two samples (Figure 3). All six samples, including male musth TGS and female conspecific TGS (Figure 3, top), elicited significantly higher responses than the controls.

Both self and sequential palatal pit responses were observed toward musth urine and less frequently toward female urine. Within our study group of samples, female Asian elephants performed more PPAC responses than male Asian elephants.

Several of the eight female Asian elephants tested at WPZ exhibited a less dramatic, less intense avoidance response than that demonstrated by African females to Asian musth urine. Cow behavioral responses observed anecdotally, after introduction into enclosures previously occupied by bulls in musth and

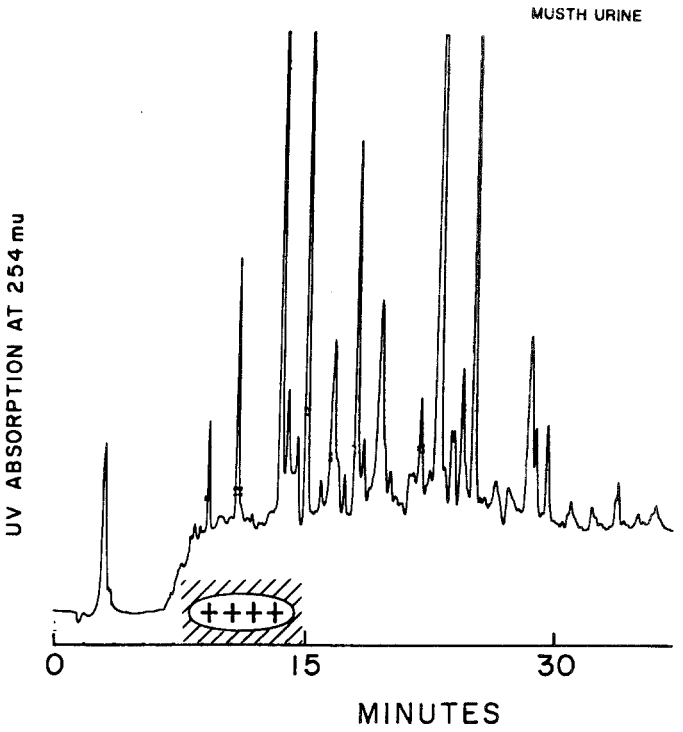


FIG. 2. HPLC chromatogram of "musth" urine fraction from male Asian elephant. Bioassay animals: African female elephants. Biologically active region: Sample: concentrated sonicated aqueous fraction from desiccated dichloromethane extract. Code: ++++ response, especially avoidance reaction, trumpeting. Separation conditions: reverse-phase C-18 bonded silica column, 30 min separation time, linear gradient 20 : 80 methanol–water to 90 : 10 methanol–water.

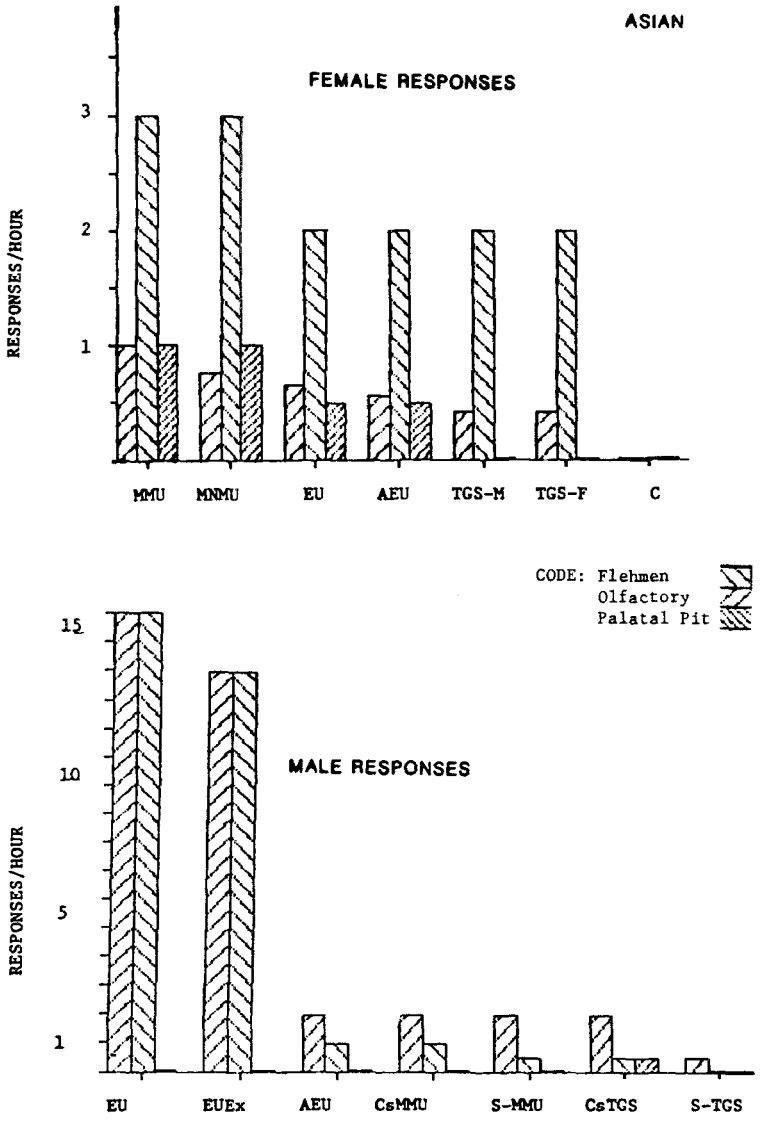


FIG. 3. Responses by Asian elephants to urine and temporal gland secretions. Sample size: TGS, 0.5 ml; urine, 500.0 ml. Each female tested 10 times; each male tested 25 times, at intervals of at least 24 hr MMU = male nonmusth urine, MNME = male nonmusth urine, EU = estrous urine, AEU = anestrous urine, TGS-M = conspecific temporal gland secretion (male), TGS-F = conspecific temporal gland secretion (female), EUEx = estrous urine extract, CSMMU = conspecific male musth urine, S-MMU = self male must urine, CsTGS = conspecific temporal gland secretion, S-TGS = self temporal gland secretions, C = controls all samples were from *Elephas maximus*. The calculated standard error (SE) for flehmen data: 0.125–0.175 [standard deviations (SD) 0.392–0.553]; for PPAC data, 0.115–0.145 (SD 0.284–0.601); and met the criteria for 0.05% confidence limit. The *t* test was used to test for significant differences, $P < 0.05$.

containing voided musth urine, included agitation. The familarity of the cows with the particular bulls, the normally maintained physical separation of bulls and cows, and the stage of musth at which the urine is obtained may be contributing factors affecting their behavioral responses. Studies employing musth urine from bulls whose serum T levels have been measured are currently in progress.

In regard to responses by males, previous studies demonstrated a high flehmen frequency by Asian bulls to estrous urine and estrous urine extract (Rasmussen et al., 1982). This high response was significantly different in frequency (10-fold) than flehmens to other urines (Rasmussen et al., 1984). The second-rank flehmen frequencies exhibited by Asian bull elephants were to conspecific male musth urine and to anestrous urine (Figure 3, bottom). Nonmusth urine, either self or conspecific, elicited no responses.

Among bulls, musth TGS also elicited detectable flehmen responses. The initial positive responses toward testosterone, a single and major component of Asian elephant TGS, were novel substance responses that diminished on successive bioassays (Rasmussen et al., 1986).

Olfactory response frequencies and flehmen frequencies appeared complementary. However, as the frequency of flehmen and olfactory checks increased with approaching ovulation, the duration of olfactory investigations did not lengthen.

Palatal pit area responses were only observed to the following samples: conspecific TGS, acidified artificial samples, and selected purified fractions from estrous urine.

African Elephant Responses. Bioassays were carried out on 10 occasions at PtDZ and five times at WLSP.

The frequencies of the three responses by female African elephants to selected urine and TGS samples are depicted in Figure 4 (top). The samples tested included male musth urine (MMU), temporal gland secretions (TGS) from conspecifics and Asian elephants, and four categories of Asian elephant urine, including one extract of female Asian elephant estrous urine. Data are from 10 tests at PtDZ on three young African female elephants and five tests at WLSP on six adult female elephants (see Table 6). Standard deviation between the two data sets at two locations were within the 0.05% confidence limit; the data were summarized together.

Among the African female elephants, the flehmen response frequencies were greatest toward male musth urine. After sample detection, high-intensity responses occurred, samples were avoided subsequently. The olfactory response frequencies were highest toward estrous urine. The frequencies of palatal pit responses were greatest to female urine, both estrous and nonestrous.

Female African elephants showed a low, but detectable, response to both male and female conspecific TGS. None of the TGS samples tested were from African bulls with high serum testosterone.

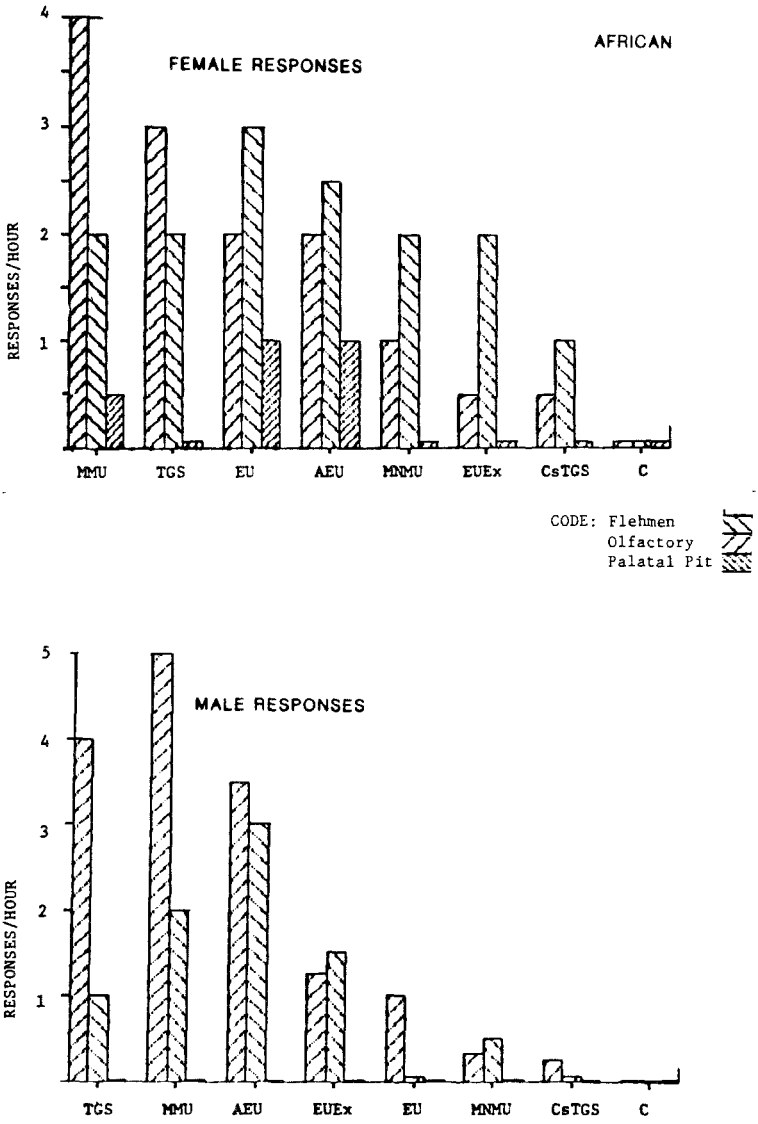


FIG. 4. Responses by African elephants to urine and temporal gland secretions Sample size: TGS, 0.5 ml; urine, 500.0 ml. Females were tested 10 times at 24-hr and 6-month intervals. Sites: PtDZ, 3 females; WLSP, 6 females. Avoidance reactions (trumpeting, herding of young elephant by older elephants, ears in laid back position, and agitated running) were observed. Males were tested 12 times at 24-hr and 6-month intervals; two successive tests per time; WLSP- 1 male MMU = male musth urine, TGS = temporal gland secretion, EU = estrous urine, AEU = anestrous urine, EUEx = estrous urine extract, MNMU = male non-musth urine, CsTGS = conspecific temporal gland secretion, C = controls. All samples were from *Elephas maximus*, except CsTGS which was from *Loxodonta africana*. The SE for flehmen and PPAC data was within the 0.05% confidence limit. Olfactory data was less rigorous. The *t* test was used as criteria of significant differences, $P < 0.05$.

The most striking responses exhibited by female African elephants to male musth urine were the high-frequency flehmen response (4/hr), trumpeting, roaring, erectile ears, and persistence of avoidance. The response by the African female elephants to male Asian musth urine was similar to the reactions to the fractions previously described. A response of four flehmen per hour is a high flehmen frequency for female elephants. Olfactory investigation times of musth urine were long (Table 5). TGS from heterospecifics also elicited interest (Figure 4, top).

In regard to the response by males, the only bull available for bioassay testing exhibited the highest flehmen frequency to Asian musth urine and the greatest frequency of olfactory responses to anestrous urine. Few palatal pit area responses were observed. Urine from African female elephants with hormonally well-characterized estrous cycles was not available.

Bioassay of Synthetic Mixtures of Identified Components of African TGS. Figure 5 and Table 6 show the responses of one maturing African bull and 10 African females (ages 9–35 years) at two locations to the three available (of five) volatile components identified in African TGS and of selected biological secretions. Each sample was tested at least 10 times. Three bioassays were done in immediate (1–3 hr) succession; three bioassays were separated by 24 hr; three bioassays were separated by 6 months to 1 year. The maximum response obtained from a variety of controls was a cursory olfactory check (Table 6). Responses were observed to several concentrations of (*E*)-farnesol and to mixtures of farnesol with 4-methylphenol and cholesterol. The greatest response was elicited by a low farnesol concentration (0.033%). The response to 0.033% (*E*)-farnesol was 50% the response level seen to musth urine. The response frequency to single components was not of the magnitude of the response by the Asian bull to estrous urine; rather it was the order of magnitude of the detectable response of the bull to anestrous urine. Its repeatability in successive bioassays was remarkable.

FMH and FDH were used as standards during GC analyses, but sufficient quantities were not available to allow the bioassay of these two components. No funds were available for their synthesis during this study.

Of the male Asian elephant responses, three Asian bull elephants demonstrated individual variability in response to components from African GS. One bull with former associations with African elephants responded to all three tested components [phenol, 4-methylphenol and (*E*)-farnesol] and their mixtures from African TGS, whereas the responses of the other two bulls were negative.

Proportional Use of Three Responses. The complete record of three responses to four investigator-placed samples in a single experiment are listed in Table 5. As listed in Table 5, the total number of olfactory responses and their duration was higher than the number or duration of flehmen responses. The flehmen responses were greater in number and total duration than palatal pit responses.

TABLE 5. RESPONSES BY PtDZ ELEPHANTS[a] TO INVESTIGATOR-PLACED SAMPLES[b]

	Controls				Musth urine extract[c]				Anestrous urine[d]				0.02% Farnesol and 0.01% cholesterol			
	F1	F2	F3	MI	F1	F2	F3	MI	F1	F2	F3	MI	F1	F2	F3	MI
Olfactory investigations																
Number	2	2	3	1	8	8	8	6	5	4	4	2	3	4	4	1
Duration (sec)	4	4	3	2	24	30	17	18	6	4	4	10	6	8	9	1
Flehmen responses																
Number					5	4	4	3	1	2	1	2	1	1	2	2
Duration (sec)					5	8	6	8	1	3	1	3	1	1	3	2
Palatal pit responses to self																
Number					1	—	1	—					1	0	1	0
Duration (sec)					5	—	3	—					3		3	
Sequential palatal pit responses to conspecifics																
Number (only single sequences)					1	1	5	5					1	0	1	0
					↓	↓	↓	↓					↓		↓	
					F2	F1	MI	F3					F2		MI	
Duration (sec)					15	10	5	4					5		6	
Other reactions																
Trumpeting					T	T	T	—								
Roaring					R											

[a]F1, F2, F3, African females; M1 Asian male, intermediate age.
[b]Bioassay time: 1 hr, mean of 2 sessions.
[c]From Asian mature male Asian in musth.
[d]From nonrelated animal.

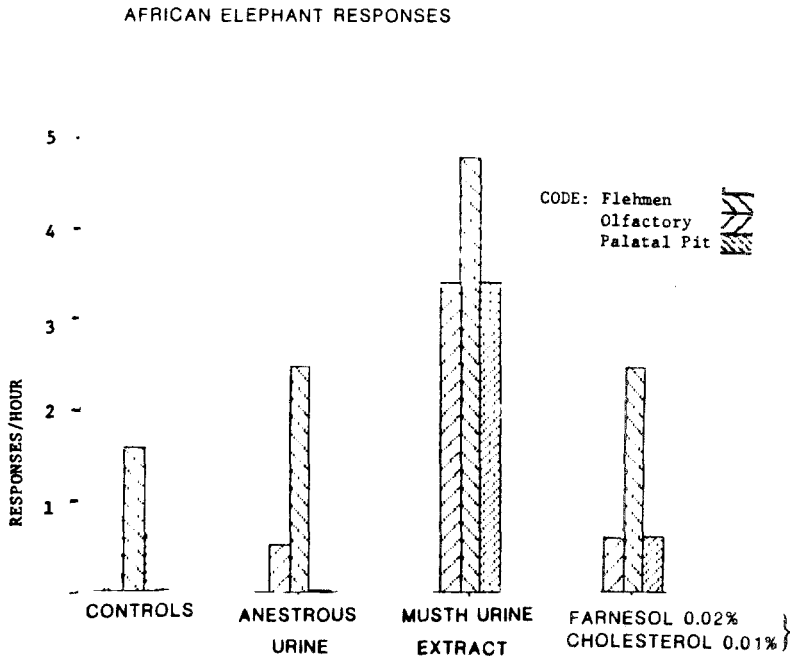


FIG. 5. Female African elephant responses to four biological samples. Sample size: TGS, 0.5 ml; anestrous urine, 500.0 ml; musth urine Extract, 500.0 ml equivalent. Controls: all solvents used in preparations various urines. TGS was from *Loxodonta africana*; the other samples were from *Elephas maximus*. The *t* test for significant differences was set at $P < 0.05$.

The data from one female are depicted in Figure 5. The high response of African elephants to Asian musth urine (Figure 4) is also seen in Figure 5. Olfactory responses tabulated were threefold higher toward musth urine samples than to controls and twofold higher toward musth urine than to 0.02% farnesol and 0.02% cholesterol mixture or to anestrous urine. Flehmen responses were fivefold higher to musth urine than to the other two samples. The number and duration of sequential palatal pit responses were greatest when musth urine was tested.

DISCUSSION

The response frequencies observed to whole secretions and urines, to unpurified fractions, and to single components were greater than expected from earlier investigations. Bioassay procedures, using aerosol spraying or air blowing for sample dispersion followed by subsequent monitoring of olfactory

TABLE 6. AFRICAN ELEPHANT RESPONSES TO COMPONENTS FROM AFRICAN ELEPHANT TGS

Substance tested	Concentration (%)	Responses per hour		
		Olfactory	Flehmen	Palatal Pit
Farnesol[a]	0.033	3	2	2
	0.066	2	1.5	nr[c]
	0.1	2	0.5	nr
	0.2	1	0.1	nr
Farnesol	0.02			
Cholesterol	0.01	1	1.0	nr
Farnesol	0.1			
Cholesterol	0.01			
4-Methylphenol	0.3	1	0.2	nr
Farnesol	0.2			
4-Methylphenol	0.3	1	0.1	nr
Controls				
Ethanol		—	—	—
Water		—	—	—
Acetone		—	—	—
Cinnamic acid		—	—	—
Hydrocinnamic acid		—	—	—
No response or nonrepetitive response				
0.3% 4-methylphenol				
2-Pentanone				
0.1% farnesol and cholesterol				
0.1% farnesol and 0.3% 4-methylphenol				
0.5% 4-methylphenol				
1-Benzoylpyrrole				
Testosterone, several concentrations				
FMH				
FDH				

[a] Response to 0.033% (*E*)-farnesol (3,7,11-trimethyl 2,6,10-dodecatrien-l- ol) was 50% the level seen to musth urine.
[b] FMH and FDH and mixtures were tested twice (nr) during first bioassays. The high cost of these compounds has prevented further testing.
[c] nr = no response.

responses, did not reveal any responses by African or Asian elephants to African TGS components (Buss, personal communication; Gorman, 1986a, b). However, Asian bull elephants were observed to flehmen both to their own

urine and TGS and that of conspecifics (Rasmussen et al., 1984). In the current study, presentations of natural secretions and appropriate synthetic compounds in liquid media resulted in measurable olfactory, palatal pit, and flehmen responses in a variety of contexts in both species.

The flehmen response in elephants is seen only after prior olfactory investigations. The trunk tip physically transports liquids to the openings of the vomeronasal passages during flehmen. The flehmen response in elephants, used with dramatic intensity by Asian bull elephants in response to estrous urine (Rasmussen et al., 1982), is seen in both sexes during a variety of situations, which include not only sex identification and sex state information but also the establishment and maintenance of mother–young recognition, in female–female interactions, and in the mediation of male–male interactions. In the wild, certain African bulls have been observed to flehmen to the penile area and urine of other more dominant, more aggressive bulls (Rasmussen et al., 1986; Hall-Martin and Rasmussen, in preparation). Current observations show that flehmen is used in sequence with prior olfactory responses and often with successive palatal pit responses. (The possibility that these three responses represent different intensities of a similar response is currently under investigation.) Such sequences involving the PPAC response were most evident in the premating sequences and alarm–avoidance reactions. Moderately high frequencies of palatal pit response and high frequencies of flehmen responses by female African elephants occurred during initial response to Asian male musth urine. After evidencing alarm by trumpeting, roaring, and positioning ears, the animals did not return to the test sample.

Positive responses to novel substances could be sorted out from real meaningful responses by successive, repetitive testing. Habituation was not evident during frequent testing of Asian bulls with biologically active compounds (Rasmussen et al., 1986). In the present study, the lack of habituation for selected compounds in successive tests may indicate that elephants recognize, innately or through learning, meaningful compounds. These molecules elicit responses, consistently and repetitively, if presented in appropriate concentrations and presentation media.

Factors such as age, sex, diet, physiological condition, and season might affect gc, GC/MS, and HPLC results. Some of these factors are not controllable, but with the WPZ bulls these factors remained relatively constant. The consistency of triplicate samples and samples from successive days (within groupings of animals), the reproducibility of bioassays in repetitive sample testing, and the demonstration of similar GC patterns of TGS from animals with similar testosterone levels support the validity of the GC analyses.

The dramatic reaction by African female elephants to male Asian musth urine (and specific fractions), in contrast to female Asian elephants' lower

response to Asian musth secretion and Asian TGS, raises several questions. Which chemicals or combinations are meaningful to each species? Is a spectrum necessary? Do the species' repertoires gradate into each other? Maybe a clue exists in the chemical similarity between Asian musth TGS and the TGS from African bulls exhibiting a behavioral musth (Hall-Martin and Rasmussen, in preparation) and having high serum and TGS testosterone.

Figure 1 and Table 3 show an inverse relationship between farnesol and testosterone levels in TGS. Especially noteworthy to compare are the estrone-injected Asian bull in musth with the normal African bull and the high testosterone African bull with an Asian bull in musth. The TGS sample from an African male elephant with high serum and TGS testosterone does not have the proportion of (*E*)-farnesol normally resolved from African male and female TGS.

Such observations as this inverse relationship between testosterone levels and farnesol levels in TGS, and an avoidance reaction by female African elephants to Asian male musth urine, and an earlier description of the triggering of TGS secretion release in Asian bulls (Rasmussen et al., 1984), suggest definitive chemocommunicative functions for this unique gland.

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Announcements

INTERNATIONAL SYMPOSIUM ON INSECT-PLANT RELATIONS

The 7th International Symposium on Insect-Plant Relationships will be held in Budapest, Hungary, July 3–8, 1989. Its organizers, Drs. T. Jermy, Chairman, and A. Szentesi, Secretary, have grouped sessions according to the special fields of physiology, behavior, ecology, phytochemistry, evolution, and applied aspects. Interested scientists may acquire more details by writing to A. Szentesi, 7th International Symposium on Insect-Plant Relationships, Plant Protection Institute, Department of Zoology, Budapest, Pf. 102, H-1525, Hungary.

CURRENT AND FUTURE TRENDS IN BIOLOGICAL CONTROL OF INSECTS

To honor the retirement and many contributions of Professor Harry C. Coppel to the field of biological control, the University of Wisconsin Entomology Department is sponsoring a symposium entitled Current and Future Trends in Biological Control of Insects, to be held on the university campus September 27–29, 1988. The agenda includes presentations by nationally recognized leaders in biological control and a poster session for contributed papers. For more information contact: D. L. Mahr, Department of Entomology, University of Wisconsin, Madison, Wisconsin 53706 (608–262–3228) or N. E. Beckage (608–263–7924).

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BEHAVIORAL RESPONSES OF FEMALE MEXICAN FRUIT FLIES, *Anastrepha ludens*,[1] TO COMPONENTS OF MALE-PRODUCED SEX PHEROMONE

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Abstract—The behavioral responses of virgin female Mexican fruit flies elicited by components and combinations of the components of male-produced pheromone were measured in a laboratory wind-tunnel bioassay where test chemicals were applied to the undersides of some leaves on a treated tree but to none of the leaves of a control tree. Only treatments containing at least (*Z*)-3-nonenol and/or (*Z,Z*)-3,6-nonadienol in combination with (*S,S*)-(−)-epianastrephin elicited strong behavioral responses. Responses included attraction to the vicinity of the pheromone but not to point sources, increased searching rate, changes in searching strategy, and agonism. The results support a model of pheromone-component function in which components act as a unit to stimulate all behaviors of the pheromone-mediated behavioral repertoire.

Key Words—Sex pheromones, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*.

INTRODUCTION

Extracts of male Mexican fruit flies (*Anastrepha ludens* Loew) elicit a repertoire of behaviors from conspecific virgin females (Robacker and Hart, 1986). Behaviors include attraction to pheromone, stimulation of searching activity, changes in searching strategy, and an increase in female–female agonism. Robacker and Hart (1985b) demonstrated biological activity for several chemicals previously identified from male extract (Esponda-Gaxiola, 1977; Battiste

[1] Diptera: Tephritidae.

et al., 1983; Stokes et al., 1983; Nation, 1983). Robacker and Hart (1985b) showed that (*Z*)-3-nonenol, (*Z*,*Z*)-3,6-nonadienol and (*S*,*S*)-(−)-epianastrephin each elicited weak but significant attraction and/or locomotor arrest in combinations containing the components (*R*,*R*)-(+)-epianastrephin and (*R*,*R*)-(+)-and (*S*,*S*)-(−)-anastrephin, and that combinations containing either alcohol with (*S*,*S*)-(−)-epianastrephin elicited synergistically enhanced activity. However, effects of chemicals on specific behaviors of the behavioral repertoire were not tested.

The purpose of this work was to test components of male extract, singly and in combinations, shown to be important by earlier research (Robacker and Hart, 1985b) for their effects on attraction, searching, agonism, and other behaviors from the behavioral repertoire of virgin female *A. ludens*. This research also addresses the broader question: do pheromone components in multicomponent systems function individually to elicit distinct behaviors or do all components of a blend act as a unit to elicit any and all behaviors of a pheromone-mediated behavioral repertoire?

METHODS AND MATERIALS

General. All flies were from a laboratory culture maintained for at least 50 generations with no wild-fly introductions. Experiments were conducted in the laboratory. Temperatures varied between 20 and 30°C and relative humidity between 40 and 70%. Photoperiod (14 : 10 light–dark) was shifted so that lights came on at 0230 hr and went off at 1630 hr.

Preparation of Chemical Treatments. Chemical treatments per 10 μl of hexane were: (*Z*)-3-nonenol (Z3N), 100 ng; (*Z*,*Z*)-3,6-nonadienol (ZZ36N), 40 ng; (*R*,*R*)-(+)-anastrephin (RRANA), 100 ng; (*S*,*S*)-(−)-anastrephin (SSANA), 100 ng;(*R*,*R*)-(+)-epianastrephin (RREPI), 350 ng; (*S*,*S*)-(−)-epianastrephin (SSEPI), 350 ng; Z3N/SSEPI combination, 100/350 ng; ZZ36N/SSEPI combination, 40/350 ng; the six-chemical combination, 100/40/100/100/350/350 ng; and extract of male *A. ludens* abdomens. These amounts equal 1.0 male equivalent (ME) per chemical (Robacker and Hart, 1985b).

Each chemical was at least 97% free of contamination from the other five chemicals (J.L. Nation, University of Florida, Gainesville; M. Jacobson, USDA-ARS, Beltsville, Maryland; and gas chromatography by author when possible). Male extract was prepared by grinding abdomens of sexually mature, virgin male *A. ludens* in hexane. Extract was filtered through glass wool, concentrated under nitrogen, and stored at −20°C.

Bioassay. The bioassay cage was a simple wind tunnel consisting of an aluminum-framed cage (2.0 m long × 0.7 m wide × 1.3 m high) with aluminum window screening covering the top and all sides. An airflow of about 1 m/

sec was directed through the length of the cage by fans on the upwind and downwind ends. The downwind fan exhausted the chemical-laden air to outdoors. A bank of four fluorescent lights on top of the downwind half of the cage attracted the test insects away from the upwind end when they were not responding to pheromone. A vertically placed aluminum sheet completely divided the upwind end of the cage into two compartments to prevent flies entering one compartment from detecting chemicals in the other. A 1-m-tall, potted, grapefruit tree (*Citrus paradisi* MacFad.) with about 30 leaves occupied the center of each compartment. Canopy diameters were ca. 0.4 m. Twenty filter-paper squares were attached to the undersides of leaves of both trees as in Robacker and Hart (1986). Thus, the treated (T) tree had 10 T leaves and 10 control (C) leaves and the C tree had 20 C leaves.

We conducted one test each day beginning at 1500 hr when flies were sexually active. One ME of one of the chemical treatments was applied to each T leaf and 10 μl of hexane were applied to each C leaf. Fifty sexually mature, virgin females of the same age were then released into the downwind end of the cage about 1.5 m from the tree canopies. Cohort females were used to test each of the 10 treatments within a replication, when possible. Thus female age increased during testing of the 10 treatments. Previous work (Robacker et al., 1985) showed the age-range (9–30 days posteclosion) had little effect on response to pheromone. Behavior observations and bioassay procedures were conducted as in Robacker and Hart (1986) with the following exceptions. Arrivals of flies to trees were monitored for 4 min per tree instead of 2 min during the last arrival-monitoring period of each half test. Also, we did not monitor arrivals to T and C leaves during this time. Instead, we monitored the numbers of within-leaf flights on T and C leaves of the T tree and on C leaves of the C tree for 4 min per tree. A within-leaf flight is a flight which originates and ends on the same leaf. Total observation time (all behaviors combined) following each of the two pheromone applications in each test was 21 min.

We investigated the following aspects of behavior: attraction to the vicinity of test chemicals, leaf visitation rate and searching strategy in the vicinity of the chemicals, attraction directly to chemical point sources, searching strategy after arrival to point sources, and agonistic behavior near and at chemical sources. The methods used to quantify these behaviors were described previously (Robacker and Hart, 1986), except that searching strategy after arrival to point sources was determined from within-leaf flights as well as from time spent by females on point sources.

After each test we cleaned the wind tunnel with acetone and the trees with soap and water. Ten trees were used to test the 10 chemical treatments, with the same tree always used for the same treatment. Further, the same tree served as the C tree in all tests and the same leaves served as T and C leaves. The experiment was conducted in this way to prevent problems from residual chem-

icals on leaves. We kept trees as identical in appearance as possible to minimize tree and leaf effects arising from factors other than those imposed by the treatments. Nine replications of each chemical treatment were conducted.

Statistical Analyses. Data for arrivals to trees (T and C), arrivals to leaves (T leaves, C leaves of T tree, C leaves of C tree), within-tree flights, within-leaf flights, agonistic behavior on trees, agonistic behavior on leaves, fly density on trees, and fly density on leaves were analyzed as separate randomized complete-block analyses of variance (ANOVA). All eight ANOVAs included the 10 chemical treatments and contained nine replications. Analyses of variance comparing trees had 20 treatments (2 tree types $\times$ 10 chemical treatments), and those comparing leaves had 30 treatments (3 leaf types $\times$ 10 chemical treatments). Operations performed on data such as converting to a per-fly basis were done before analyses. Within ANOVAs, means were compared by t tests (LSD). When results for a particular chemical departed from overall data trends, we conducted t tests or ANOVAs using only behavioral data from that chemical. Tendencies for behaviors to vary either with fly density or with other behaviors were measured by regression. Data for time spent by flies on trees and leaves fitted bimodal distributions and were analyzed by chi square (2×2 contingency tables) comparisons of the proportion of flies spending less than 2 min with the proportion that spent greater than 2 min on T and C treatments.

RESULTS AND DISCUSSION

Chemical combinations containing Z3N and/or ZZ36N with SSEPI elicited significantly greater responses ($P < 0.05$) to T than to C locations for the behaviors: arrivals to trees (Table 1); within-tree flights (Table 2); and agonism on trees and leaves (Table 3). These treatments also stimulated greater ($P < 0.05$) fly density on T than on C trees (Table 4). Fly density is a positive function of arrival rate and locomotor arrest. The ZZ36N/SSEPI combination elicted more attraction to trees ($P < 0.05$) (Table 1) and greater fly density on trees ($P < 0.05$) (Table 4) than the Z3N/SSEPI combination, but this pattern did not hold for all behaviors. Male extract and the six-chemical combination did not generally elicit greater behavioral responses than the alcohol/SSEPI combinations. However, extract and the six-chemical combination were the only treatments that consistently elicited significantly greater time spent on T trees than on C trees ($P < 0.01$) and on T leaves than on C leaves ($P < 0.05$) (Table 5). Futhermore, they both stimulated greater fly density on trees ($P < 0.05$) than either of the alcohol/SSEPI combinations (Table 4). Individual chemicals did not elicit behavioral responses greater than controls (blanks) with one exception. The number of arrivals to the ZZ36N T tree was significantly higher ($P < 0.05$) than to the C tree (Table 1) according to a paired t test of the data for

TABLE 1. NUMBERS OF ARRIVALS OF VIRGIN FEMALE MEXICAN FRUIT FLIES TO PHEROMONE-TREATED (T) TREE COMPARED TO UNTREATED (C) TREE AND TO TREATED (T) LEAVES OF T TREE COMPARED TO UNTREATED (C) LEAVES OF BOTH TREES[a]

Pheromone treatment[b]	Arrivals to trees[c]		Arrivals to leaves[c,d]		
			T tree		C tree
	T tree	C tree	T leaves	C leaves	C leaves
Z3N	7 x ab	8 x a	0.20 x a	0.14 x a	0.20 x a
ZZ36N	13 x bc	7 x a	0.14 x a	0.12 x a	0.14 x a
RRANA	3 x a	4 x a	0.18 x a	0.04 x a	0.10 x a
SSANA	4 x ab	5 x a	0.04 x a	0.14 x a	0.04 x a
RREPI	7 x ab	4 x a	0.46 x a	0.28 x a	0.18 x a
SSEPI	9 x ab	8 x a	0.22 x a	0.14 x a	0.28 x a
Z3N/SSEPI	22 x cd	6 y a	0.28 x a	0.16 x a	0.27 x a
ZZ36N/SSEPI	36 x e	5 y a	0.34 x a	0.28 x a	0.18 x a
6-Chem comb	29 x de	5 y a	0.28 x a	0.18 x a	0.37 x a
Male extract	37 x e	9 y a	0.22 x a	0.18 x a	0.28 x a

[a] Means in a horizontal row and in the same group (trees or leaves) followed by the same letter (x, y), and means in the same vertical column followed by the same letter (a, b, c, d, e), are not significantly different from each other at the 5% level by LSD.
[b] Z3N = (*Z*)-3-nonenol; ZZ36N = (*Z*,*Z*)-3,6-nonadienol; RRANA = (R,R)-(+)-anastrephin; SSANA = (*S*,*S*)-(−)-anastrephin; RREPI = (*R*,*R*)-(+)-epianastrephin; SSEPI = (*S*,*S*)-(−)-epianastrephin.
[b] Per 12 min for trees or 8 min for leaves (= per replication).
[d] Per T or C leaf on tree, per fly present on tree.

this individual chemical (conducted in addition to the ANOVA shown in the table). Conversely, the amount of agonistic behavior on the RREPI T tree was not significantly greater than on its C tree (Table 3), also according to an additional paired *t* test.

We conducted regressions of within-tree flights per female, within-leaf flights per female, and agonistic behavior per female on fly density and on other behaviors to determine if the tendencies of females to perform these behaviors were related to and perhaps caused by either fly density or to tendencies to perform other behaviors. All regression coefficients calculated were too small to account for the large differences in behavior between T and C locations. For example, the coefficient from the regression of agonistic interactions per female on the number of females on the male-extract T tree was 0.02 ($F = 0.7$).

These results generally agree with the data from the bioassay of all 64 possible combinations of the six chemicals (Robacker and Hart, 1985b). Both experiments showed that individual components had little effect on any of the behaviors. However, in both experiments ZZ36N elicited a weak behavioral

TABLE 2. NUMBER OF FLIGHTS BEGINNING AND ENDING WITHIN SAME TREE, AND NUMBERS OF FLIGHTS BEGINNING AND ENDING ON SAME LEAF, BY VIRGIN FEMALE MEXICAN FRUIT FLIES ON A PHEROMONE-TREATED (T) TREE CONTAINING TREATED (T) LEAVES AND UNTREATED (C) LEAVES COMPARED TO UNTREATED (C) TREE CONTAINING ONLY UNTREATED (C) LEAVES[a]

	Within-tree flights[c]		Within-leaf flights[c,d]		
			T tree		C tree
Pheromone treatment[b]	T tree	C tree	T leaves	C leaves	C leaves
Z3N	3.8 x ab	4.0 x a	0.1 x a	1.1 x a	5.1 x a
ZZ36N	4.3 x ab	3.8 x a	1.6 x a	0.1 x a	0.8 x a
RRANA	4.6 x ab	3.1 x a	1.3 x a	4.0 x ab	3.4 x a
SSANA	6.4 x abc	3.8 x a	0.1 x a	3.4 x a	1.7 x a
RREPI	7.6 x abcd	7.1 x a	1.0 x a	0.6 x a	2.2 x a
SSEPI	3.4 x a	2.4 x a	2.2 x a	1.4 x a	2.1 x a
Z3N/SSEPI	10.8 x bcde	2.4 y a	10.6 x b	2.9 y a	1.0 y a
ZZ36N/SSEPI	15.7 x e	7.8 y a	13.3 x b	10.6 x d	0.5 y a
6-Chem comb	14.0 x de	4.0 y a	12.6 x b	14.7 x cd	1.6 y a
Male extract	12.5 x cde	2.6 y a	12.4 x b	11.2 x bc	2.7 y a

[a] Means in a horizontal row and in the same group (trees or leaves) followed by the same letter (x, y), and means in the same vertical column followed by the same letter (a, b, c, d, e), are not significantly different from each other at the 5% level by LSD.
[b] Z3N = (*Z*)-3-nonenol; ZZ36N = (*Z*,*Z*)-3,6-nonadienol; RRANA = (R,R)-(+)-anastrephin; SSANA = (*S*,*S*)-(−)-anastrephin; RREPI = (*R*,*R*)-(+)-epianastrephin; SSEPI = (*S*,*S*)-(−)-epianastrephin.
[c] Per 4 min (= per replication), per fly present on tree or leaf.
[d] Per T or C leaf on tree.

response by itself. The present work shows that ZZ36N attracts virgin females to the vicinity of the chemical source. Combinations containing either Z3N or ZZ36N with SSEPI elicited much stronger responses in both experiments, and combinations of the six components elicited stronger responses than the minimal alcohol/SSEPI treatments. Also, combinations containing ZZ36N outperformed those with Z3N in both experiments.

Robacker and Hart (1986) studied the effects of *A. ludens* male extract on essentially the same behaviors of conspecific females as in the present work. The results of the two experiments were similar, but some differences occurred, possibly due to procedural differences. Three major modifications were: bioassays were conducted in a pheromone-free room; the bioassay cage was used as a wind tunnel; and flight activity ending on leaves was partitioned into arrivals and within-leaf flights.

TABLE 3. NUMBERS OF AGONISTIC INTERACTIONS BY VIRGIN FEMALE MEXICAN FRUIT FLIES ON PHEROMONE-TREATED (T) TREE COMPARED TO UNTREATED (C) TREE AND ON TREATED (T) LEAVES OF T TREE COMPARED TO UNTREATED (C) LEAVES OF BOTH TREES[a]

	Agonism on trees[c]		Agonism on leaves[c,d]		
			T tree		C tree
Pheromone treatment[b]	T tree	C tree	T leaves	C leaves	C leaves
Z3N	0.4 x a	0.8 x a	0.0 x a	1.0 x abc	0.8 x a
ZZ36N	0.4 x a	0.4 x a	0.5 x ab	1.0 x abc	0.5 x a
RRANA	0.6 x a	0.7 x a	0.1 x a	0.7 x ab	0.6 x a
SSANA	0.7 x ab	0.1 x a	0.0 x a	1.3 x abc	0.2 x a
RREPI	2.0 x bc	0.3 y a	2.2 x bc	0.8 xy ab	0.3 y a
SSEPI	0.3 x a	0.8 x a	0.1 x a	0.0 x a	0.4 x a
Z3N/SSEPI	2.4 x c	0.3 y a	3.2 x c	1.2 y abc	0.3 y a
ZZ36N/SSEPI	2.3 x c	0.6 y a	2.8 x c	2.2 xy bcd	0.6 y a
6-Chem comb	2.6 x c	1.3 x a	2.4 xy c	3.7 x d	1.0 y a
Male extract	2.6 x c	0.5 y a	2.5 x c	2.6 x cd	0.4 y a

[a] Means in a horizontal row and in the same group (trees or leaves) followed by the same letter (x, y), and means in the same vertical column followed by the same letter (a, b, c, d), are not significantly different from each other at the 5% level by LSD.
[b] Z3N = (*Z*)-3-nonenol; ZZ36N = (*Z*,*Z*)-3,6-nonadienol; RRANA = (R,R)-(+)-anastrephin; SSANA = (*S*,*S*)-(−)-anastrephin; RREPI = (*R*,*R*)-(+)-epianastrephin; SSEPI = (*S*,*S*)-(−)-epianastrephin.
[c] Per 4 min (= per replication), per fly present on tree or leaf.
[d] Per T or C leaf on tree.

Conducting the tests in a pheromone-free room apparently increased overall response to pheromone treatments. This is evident in many of the behaviors. For example, compare within-tree flights (per female) on the male-extract T tree in Table 2 (12.5) with the same from Table 1 in Robacker and Hart (1986) (5.6). On average most behavioral responses were two to three times higher in the present work. The increase in pheromone sensitivity may have triggered some undesirable effects not evident in Robacker and Hart (1986). Crowding caused by the greater attractiveness may have caused an overflow of flies from preferred T leaves to C leaves, where they performed behaviors normally associated with T leaves. Also, time spent on T leaves may have been reduced. Although we recorded time spent only for "undisturbed" flies, premature departures due to activity on nearby leaves may have occurred.

Converting the bioassay cage into a wind tunnel was done to better evaluate attraction from a distance. The change was apparently successful as can

TABLE 4. NUMBERS OF VIRGIN FEMALE MEXICAN FRUIT FLIES ON PHEROMONE-TREATED (T) TREE COMPARED TO UNTREATED (C) TREE AND ON TREATED (T) LEAVES OF T TREE COMPARED TO UNTREATED (C) LEAVES OF BOTH TREES[a]

	Number of trees[c]		Number of leaves[c,d]		
			T tree		C tree
Pheromone treatment[b]	T tree	C Tree	T leaves	C leaves	C leaves
Z3N	0.6 x a	0.8 x a	0.03 x ab	0.02 x a	0.02 x a
ZZ36N	1.0 x a	0.8 x a	0.02 x a	0.01 x a	0.02 x a
RRANA	0.6 x a	0.8 x a	0.03 x ab	0.03 x a	0.05 x a
SSANA	0.4 x a	1.0 x a	0.05 x abc	0.02 x a	0.03 x a
RREPI	0.6 x a	0.7 x a	0.07 x bc	0.05 x a	0.04 x a
SSEPI	0.6 x a	0.6 x a	0.03 x a	0.03 x a	0.02 x a
Z3N/SSEPI	3.6 x b	0.8 y a	0.06 x abc	0.03 x a	0.05 x a
ZZ36N/SSEPI	6.0 x c	1.0 y a	0.05 x abc	0.02 x a	0.02 x a
6-Chem comb	7.5 x d	0.5 y a	0.08 x c	0.02 y a	0.04 xy a
Male extract	7.6 x d	0.8 y a	0.07 x bc	0.02 y a	0.02 y a

[a] Means in a horizontal row and in the same group (trees or leaves) followed by the same letter (x, y), and means in the same vertical column followed by the same letter (a, b, c, d), are not significantly different from each other at the 5% level by LSD.
[b] Z3N = (Z)-3-nonenol; ZZ36N = (Z,Z)-3,6-nonadienol; RRANA = (R,R)-(+)-anastrephin; SSANA = (S,S)-(−)-anastrephin; RREPI = (R,R)-(+)-epianastrephin; SSEPI = (S,S)-(−)-epi-anastrephin.
[c] Per count, per tree or leaf.
[d] Per fly present on tree.

be seen by comparing the ratio of arrivals to the male-extract T and C trees in Table 1 (37/9) to the same in Table 1 of Robacker and Hart (1986) (6.2/3.8). The aluminum-sheet partition also may have affected behavior by preventing flies on C trees from perceiving pheromone on T trees. Thus, flies on C trees performed relatively less pheromone-mediated behavior than in Robacker and Hart (1986) in which no partition was placed between T and C trees: Compare the ratio of agonism (per female) on male-extract T and C trees in Table 3 (2.6/0.5) to the same in Table 1 of Robacker and Hart (1986) (0.28/0.088).

Partitioning behavior on leaves into arrivals and within-leaf flights resulted in a different interpretation of attraction behavior than was reported previously. Robacker and Hart (1986) reported that pheromone probably attracts females directly onto T leaves, based on a ratio of arrivals (per female) to T and C leaves of 0.25/0.098 ($P < 0.05$) (Table 1). This conclusion is challenged by the present work in which the ratio was 0.22/0.18 (not significantly different from a ratio of 1) for T and C leaves (Table 1). The discrepancy arose because

TABLE 5. PERCENTAGES OF VIRIGIN FEMALE MEXICAN FRUIT FLIES REMAINING MORE THAN TWO MINUTES ON PHEROMONE-TREATED (T) TREE COMPARED TO UNTREATED (C) TREE AND ON TREATED (T) LEAVES OF T TREE COMPARED TO UNTREATED (C) LEAVES OF BOTH TREES[a]

Pheromone treatment[b]	Percentages remaining >2 min on trees		Percentages remaining >2 min on leaves		
			T tree		C tree
	T tree	C tree	T leaves	C leaves	C leaves
Z3N	30(33) x	56(25) y	32(22) x	42(12) x	48(29) x
ZZ36N	35(52) x	45(29) x	21(19) x	18(11) x	18(28) x
RRANA	40(45) x	32(40) x	20(20) x	29(21) x	34(38) x
SSANA	56(27) x	55(31) x	30(20) x	27(11) x	38(21) x
RREPI	29(49) x	23(43) x	26(31) x	4(23) y	20(46) xy
SSEPI	39(38) x	35(23) x	38(21) x	25(16) x	21(24) x
Z3N/SSEPI	21(57) x	23(47) x	16(32) x	24(29) x	18(57) x
ZZ36N/SSEPI	39(49) x	41(32) x	24(25) x	24(25) x	32(34) x
6-Chem comb	55(31) x	9(23) y	44(25) x	28(25) xy	20(40) y
Male extract	44(68) x	20(56) y	47(62) x	15(40) y	21(73) y

[a] Percentages in a horizontal row in the same group (trees or leaves) followed by the same letter are not significantly different from each other at the 5% level by 2 × 2 chi-square contingency tests. Each value is the percentage summed over all observations in all replications. The number of observations is in parenthesis.

[b] Z3N = (*Z*)-3-nonenol; ZZ36N = (*Z*,*Z*)-3,6-nonadienol; RRANA = (R,R)-(+)-anastrephin; SSANA = (*S*,*S*)-(−)-anastrephin; RREPI = (*R*,*R*)-(+)-epianastrephin; SSEPI = (*S*,*S*)-(−)-epianastrephin.

in Robacker and Hart (1986) we recorded all flights ending on a leaf as arrivals to that leaf, whereas in the present work we counted as arrivals only those flights that originated at some place other than the observed leaf. We recorded flights that began and ended on the same leaf as within-leaf flights. Table 2 shows that the ratio of within-leaf flights (per female on leaf of type) on T vs. C leaves for the male-extract T tree was also not significantly different from 1 (12.4/11.2). However, multiplying these values by the number of females on each leaf type and dividing the results by the number of minutes of observation time shows the ratio of within-leaf flights (summed over females) on the T tree is ca. 12/3. The arrival's ratio after similar operations is 2.2/1.7. Adding numerators and denominators yields an arrival ratio, as defined in Robacker and Hart (1986) of 14.2/4.7 or about 3/1. This is similar to the ratio from Robacker and Hart (1986) of 0.25/0.098 or about 2.6/1. Thus, flies behaved similarly in both experiments but behavior partitioning showed that flies did not arrive at higher rates to T than to C leaves. The apparently higher rate in Robacker and Hart

(1986) was probably due to greater numbers of flies performing within-leaf flights on T leaves. This suggests that females are attracted to the vicinity of males by pheromone but are attracted to male-held leaves by other stimuli, possibly by visual and/or acoustical signals from males (Robacker and Hart, 1985a; Sivinski et al., 1984).

Do within-leaf fights really constitute different behavior from arrivals to leaves, or is it just a matter of the experimenter's point of view as to where a fly alights? Assuming the null hypothesis that they represent just two different outcomes of the same behavior, a fly taking off from a leaf should land on a different leaf more often than on the leaf of flight origin, since there are many different leaves available compared to only one that it left. From the above paragraph, the number of arrivals to T and C leaves on the male-extract T tree was ca. 3.9 per minute (2.2 + 1.7) compared to ca. 15 within-leaf flights per minute (12 + 3). Thus, flies performed about four times as many within-leaf flights as arrivals. This contradicts the null hypothesis and demonstrates that within-leaf flights and arrivals to leaves are distinct behaviors. Possibly, females perform arrivals when they are searching for pheromone sources (males), whereas they perform within-leaf flights at or near pheromone sources to increase their visibility to males.

Do females alter their searching strategy at or near pheromone sources by performing within-leaf flights, as suggested in the above scenario? Table 2 shows little difference in within-leaf flights per female on T and C leaves of T trees, with the exception of the Z3N/SSEPI treatment. Reanalysis of the Z3N/SSEPI data by a paired t test found this difference nonsignificant also. Table 2 also shows that more within-leaf flights per female took place on both T and C leaves of the T tree than on C leaves of the C tree ($P < 0.05$) for Z3N/SSEPI, ZZ36N/SSEPI, the six-chemical combination and male extract. These results show that females do alter their searching strategy in the vicinity of pheromone but not on individual leaves (pheromone point sources), at least with respect to within-leaf flights.

Data for time spent by females on various locations were bimodally distributed, suggesting that at least two different behaviors were involved. Most flies spent either less than 1 min or more than 3 min at any location. Flies spending less than 1 min were probably engaged in searching activity. This could include searching for food as well as mates and searching by visual and acoustical as well as by olfactory modes. Some flies spending greater amounts of time were preening and possibly resting. This was especially evident on C leaves of the C tree where females often exhibited these behaviors for a half hour or more. Finally, some females spent more time where they perceived pheromone, as reported earlier (male extract and six-chemical combination). We interpret this as an altered searching strategy in response to pheromone (Robacker and Hart, 1986), but not necessarily locomotor arrest since time spent

included time that females performed within-tree and/or within leaf flights. Unfortunately, data from behaviors other than response to pheromone diluted the searching strategy alteration and probably increased variability. This variability is evident as large differences in responses to C locations from one treatment to another (Table 5). Because of the different behaviors involved in "time-spent" data, differences within columns of Table 5 (among chemical treatments) could not be compared meaningfully. Thus, only comparisons between T and C locations within a chemical-treatment test were conducted.

Pheromone Component Functions. A major aim of this research was to evaluate two models of pheromone component function in multicomponent systems using the *A. ludens* pheromone as a test system. One model, proposed by Jacobson et al. (1970), is that each component functions to elicit a different behavior from the pheromone-mediated behavior repertoire. Cases that fit this model have been reported (Baker et al., 1976; Nakamura, 1980; Linn and Gaston, 1981). The alternate model is that pheromone components function as a unit to elicit each and all behaviors (Baker and Cardé, 1979).

Our results fit the unit-function model. For all behaviors, individual pheromone components stimulated little or no response. The only exception was that ZZ36N elicited weak attraction (Table 1, $P < 0.05$ by a paired t test). Because the effect was weak compared to that elicited by blends such as ZZ36N/SSEPI, this does not fit the model in which individual components elicit specific behaviors out of a repertoire of behaviors. We interpret the effect as partial stimulation of behavior, complete stimulation requiring all critical components.

Further evidence for the "unit" model is that pheromone blends elicited most behaviors either greatly or not at all. Also, adding more components to the blend did not generally elicit additional behaviors, just higher levels of the same behaviors. One exception was that the two alcohol/SSEPI combinations did not stimulate females to spend more time on T than C locations, whereas the six-chemical combination and male extract did (Table 5). Fly density data in Table 4 also reflect this behavior, since fly density depends in part on time spent. Since no individual components increased time spent, one possible explanation is that several, perhaps all, components are necessary to elicit locomotor arrest, which translates into greater time spent. This result is consistent with the "unit-function" model, although it suggests that some behaviors may require the unit to be more complete than others. These conclusions do not rule out the possibility that other as yet unknown pheromone-mediated behaviors of this species may be controlled by the individual components studied here or by other unknown components.

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HAGEN'S GLAND MORPHOLOGY AND CHEMICAL CONTENT ANALYSIS FOR THREE SPECIES OF PARASITIC WASPS (HYMENOPTERA: BRACONIDAE)[1]

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Abstract—The morphology and chemical content analysis of Hagen's glands of the braconid wasps *Biosteres longicaudatus*, *B. tryoni*, and *B. arisanus* were shown to be useful taxonomic markers. *B. longicaudatus* glands contain two components tentatively identified as (3aα,5β,6aα)-5-butyltetrahydrofuro[3,2-b]furan-2(3H)-one and a corresponding 5-hexyl derivative, while *B. tryoni* glands contained dodecan-4-olide and *B. arisanus* glands contained ethyl octanoate, ethyl Z-4-octenoate, and octan-4-olide.

Key Words—*Biosteres longicaudatus*, *Biosteries tryoni*, *Biosteres arisanus*, Hymenoptera, Braconidae, Hagen's gland, ethyl octanoate, ethyl octenoate, octan-4-olide, dodecan-4-olide, (3aα,5β,6aα)-5-butyltetrahydrofuro[3,2-b]furan-2(3H)-one, (3aα,5β,6aα)-5-hexyltetrahydrofuro[3,2-b]furan-2(3H)-one.

INTRODUCTION

Glands originating near the tip of the abdomen in males of various opiine braconid parasitoids have been implicated in the production of fragrant substances (Hagen, 1953; Buckingham, 1968, 1975). Both Hagen (1953) and Buckingham (1968) discussed these glands in connection with mating or courtship behavior but were unwilling to attribute a function to the gland because of insufficient data. Haramoto (1957) suggested a possible role in protection against natural

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enemies, but presented no quantitative data. Buckingham (1975) later presented evidence for the role of gland secretions in defense against predators in one opiine species, *Opius concolor siculus* Monastero, and one alysiine braconid species, *Alysia alticola* (Ashmead). He also noted that the glands did not originate on the true pygidium in some species and suggested that they be called Hagen's glands rather than pygidial glands. As far as we can determine, no other purpose for these glandular secretions has been brought forward.

In the work presented here, we describe the glands and identify volatile chemicals found in them for three species of opiine Braconidae: *Biosteres longicaudatus* Ashmead, *B. arisanus* (Sonan), and *B. tryoni* (Cameron). *B. arisanus* has recently been treated as a senior synonym of *B. oophilus* (Fullaway) (Wharton and Gilstrap, 1983).

METHODS AND MATERIALS

Parasitoid wasps examined during this study all belong to the braconid subfamily Opiinae. They include the three *Biosteres* species listed above, and two *Opius* species, *O. incisi* Silvestri and *O. fletcheri* Silvestri. All insects were laboratory reared in Honolulu, Hawaii, from fruit samples originally collected on the island of Maui. Adult parasitoids were shipped to College Station, Texas, soon after emergence from their tephritid hosts *Ceratitis capitata* (Wiedemann), *Dacus cucurbitae* (Coquillett), and *Dacus dorsalis* Hendel.

Glands were dissected from the males immediately after their arrival in Texas. Glands were removed in a small drop of distilled water, blotted, placed in vials containing a minimum amount of glass-distilled diethyl ether, and crushed prior to analysis. GC analysis was performed on a Tracor 550 FID instrument equipped with a 105–0 : 1 variable ratio all-glass splitter system and a Brownlee-Silverstein thermal gradient collector (Brownlee and Silverstein, 1968). A 1.83-m × 4-mm-ID glass column packed with 3% OV-101 on Chromosorb 750 100–125 mesh was used, with an N_2 flow rate of 60 ml/min, temperature programmed from 80 to 270°C at 10°/min. Fractions were collected in 1.3-mm-OD glass capillary tubes cooled by liquid nitrogen and were flame sealed until used. Comparisons with standards and analyses of purity were performed on a Varian 3700 GC equipped with a 25-m × 0.33-mm-ID vitreous silica BP1 capillary column with helium carrier gas at 15 psig head pressure, temperature programmed at 60°C for 1 min, then to 220°C at 10°/min, using flame ionization detection. Mass spectral data were obtained on Hewlett Packard 5993A and Finnigan 1020 OWA quadrupole instruments in electron impact mode. The former was equipped with a 1.83-m × 2-mm-ID OV-1 on Supelcoport 100–125 mesh column, He flow rate 30 ml/min, temperature programmed from 120 to 240°C at 10°C min, the latter with a 25-m × 0.33-mm-

ID vitreous silica BP1 capillary column, He head pressure 15 psig, operated at 60°C for 1 min, then temperature programmed to 220°C at 10°/min. NMR spectra were recorded using $CDCl_3$ solvent on a JEOL FX 90Q Fourier transform instrument or using acetone d_6 solvent on a Bruker 500 Fourier transform instrument. NMR shift and coupling assignments for component 6 were made using the fixed decimal PANIC® algorithm on the Bruker instrument. Known compounds for comparison were obtained as gifts from International Flavors and Fragrances or were purchased from Aldrich Chemical Company.

RESULTS AND DISCUSSION

Males of all three *Biosteres* species exuded strong, pleasant fragrances. As first noted by Hagen (1953), there were interspecific differences in the odors produced. Gland extracts exhibited these same aromas, indicating that the glands were their sources. Relative shapes and sizes of the male Hagen's glands for these species are shown in Figure 1. They compare favorably with illustrations of *B. arisanus* (oophilus) and *B. longicaudatus* glands given by Buckingham (1968, 1975). Glands of *O. incisi* and *O. fletcheri,* which were much smaller than those of *Biosteres*, did not produce a detectable aroma, and did not produce peaks on GC analysis.

The gland contents of *B. arisanus* (Table 1) were studied first due to their

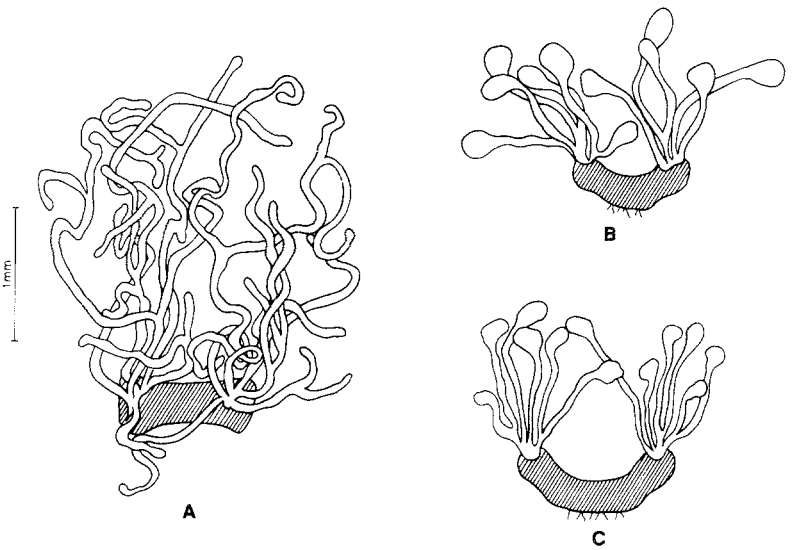

FIG. 1. Hagen's glands of *Biosteres* species showing placement on internal surface of 9th tergum. (A) *B. arisanus*, (B) *B. tryoni*, (C) *B. longicaudatus*.

TABLE 1. GC ANALYSIS OF GLAND VOLATILES[a]

Species	Component	RT	Relative percentage
B. arisanus	1	7.60	17.4
	2	7.73	8.7
	3	8.46	73.9
B. tryoni	4	14.23	100
B. longicaudatus	5	12.20	7.7
	6	15.10	92.3

[a]25-m × 0.3-mm-ID BP1 vitreous silica capillary, 15 psig He head pressure, 60°C for 1 min, then to 220°C at 10°/min.

fragrance, which was highly reminiscent of fresh coconut. GC analysis indicated the presence of two minor components (**1** and **2**) and one major component (**3**) in the gland. Component **3** had the characteristic coconut aroma. The mass spectrum of component **3** showed a base peak at *m/z* 85, characteristic of a γ-lactone (Honkanen et al., 1965). A parent peak at *m/z* 142 indicated a possible molecular formula of $C_8H_{14}O_2$. NMR analysis at 90 MHz showed a 3H distorted triplet at δ0.9, a complex broad 10H multiplet at δ1.2–2.4 and a 1H multiplet at δ4.36, characteristic of an oxygen substituted methine. The spectrum was identical to that of γ-octanoic lactone (octan-4-olide) purchased from Aldrich Chemical Co. GC and MS data also were identical. The structure is therefore as shown in Figure 2, component **3**. This compound is, in fact, a component of coconut oil and is used as artificial coconut flavoring.

The minor components **1** and **2** gave parent MS peaks at *m/z* 170 and 172, indicating possible molecular formulas of $C_{10}H_{18}O_2$ and $C_{10}H_{20}O_2$, respectively. Component **2** exhibited the characteristic *m/z* 88 and 101 fragments of a saturated ethyl ester. This assignment was confirmed by NMR analysis, which showed a 3H triplet at δ1.28 coupled to a 2H quartet at δ4.15. The single 3H distorted triplet at δ0.92 combined with the broad multiplet centered at δ1.3 indicated no branching. Component **2** was therefore ethyl octanoate (Figure 2).

The NMR spectrum of component **1** showed a δ1.28 3H triplet and a δ4.15 2H quartet, indicating that it too was an ethyl ester. The molecular formula indicated one unit of unsaturation in an eight-carbon acid unit, which was also indicated by the presence of two broad 1H doublets at δ5.32 and 5.42, with a 4Hz *cis* coupling constant. One of these was coupled to a broad δ2.05 2H triplet which in turn was coupled to a δ2.33 2H triplet, the methylene next to the carbonyl, indicating that the double bond is in the 4 position. A single methyl triplet at δ0.98 indicated that no branching occurred. The compound is therefore ethyl (*Z*)-4-octenoate (component **1**, Figure 2). NMR and mass spectra are in

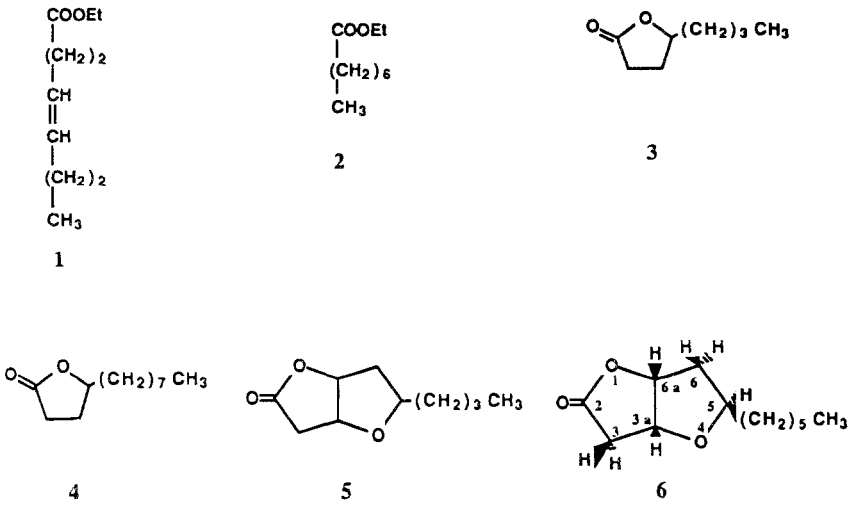

FIG. 2. Volatile chemicals identified from *Biosteres* spp. Hagen's gland extracts.

good agreement with those of Naf and Degen (1971). Naf-Muller and Willhalm (1971) identified this compound as a component of pineapple essential oil.

GC analysis of *B. tryoni* gland contents showed that it consisted of a single component of greater than 99% purity (Table 1). Its sweet, lingering fragrance is very similar to that of the blacktail deer lactone (*Z*)-6-dodecen-4-olide (Ravid et al., 1978). The mass spectrum again showed a base peak at *m/z* 85 characteristic of a γ-lactone (Honkanen et al., 1965), but the parent peak was at *m/z* 198, indicating a molecular formula of $C_{12}H_{22}O_2$. The NMR spectrum was almost identical to that of γ-decanolactone purchased from Aldrich Chemical Co., the only difference being that integration of the broad singlet at δ1.3 indicated the presence of two more methylenes in the side chain. The compound is therefore dodecan-4-olide (component **4**, Figure 2).

B. longicaudatus gland contents presented a more difficult analytical problem. The mass spectra of the two components present (Table 2) showed parent peaks at *m/z* 184 and 212 (major compound), respectively, indicating possible molecular formulas of $C_{10}H_{16}O_3$ and $C_{12}H_{20}O_3$. Base peaks in both instances were at *m/z* 127, and the spectra were virtually identical in the *m/z* 35–127 range, indicating the presence of common core fragments of possible molecular formula $C_6H_7O_3$. [^{1}H]NMR at 90 MHz ($CDCl_3$) showed complex patterns, the spectra of the two compounds differing only in the broad multiplet at δ1.0–1.6. Distorted triplets in the spectra of both compounds (**5**, δ0.91, **6**, δ0.90) with broad multiplets at δ1.0–1.6 indicated straight hydrocarbon side chains.

The spectrum of the major component was taken at 500 MHz (acetone d_6,

TABLE 2. MASS SPECTRA.

Mass	%	Mass	%	Mass	%
Component 5					
41	13.6	57	6.7	99	25.5
42	4.9	67	1.8	126	4.6
43	13.7	69	3.0	127	100.0
44	3.1	71	12.5	128	6.9
45	1.9	81	6.3	140	2.1
53	3.1	82	3.1	143	2.9
54	1.9	83	5.0	156	2.4
55	20.8	85	1.9	184	3.1
Component 6					
41	14.0	81	6.4	117	0.9
42	5.0	82	4.0	123	0.6
43	17.7	83	5.2	124	0.7
44	2.0	84	1.0	125	2.0
45	1.2	85	0.9	126	4.5
53	2.9	89	0.8	127	100.0
54	2.3	95	1.3	128	7.1
55	21.4	96	3.2	129	1.7
56	2.8	97	0.9	134	2.1
57	3.8	98	0.6	135	1.0
67	2.4	99	22.6	143	6.8
68	1.0	100	1.4	144	0.6
69	2.5	101	0.9	152	0.6
70	1.2	107	0.7	194	1.2
71	10.9	109	0.6	195	0.3
79	1.1	113	1.1		

Figure 3, Table 3). From this spectrum and the mass spectrum, structure **6** is proposed for the major component based on the following analysis. Three 1H multiplets at δ4.01 (complex), 4.80 (d of d), and 5.16 (apparent triplet) indicated the presence of three O—C—H fragments. Decoupling experiments showed that the two lower field signals were coupled to each other (4.7 Hz) but not to the signal at δ4.01. The peak at δ4.80 was also coupled to an AB system at δ2.42 and 2.82 with couplings of <1 Hz and 6.4 Hz, respectively. The strong coupling of the AB hydrogens to each other (18.4 Hz) indicated these were geminal methylene protons with a strong electronegative influence. The peak at δ4.80 was thus assigned to the methine at C-3a and the peaks at δ2.42 and 2.82 are assigned to C-3β and C-3α, respectively. The proton at δ5.16 shows a coupling (5.1 Hz) to a multiplet at δ1.71. This peak shows a geminal coupling (J = 13.7 Hz) to the signal at δ2.28. These peaks are therefore due

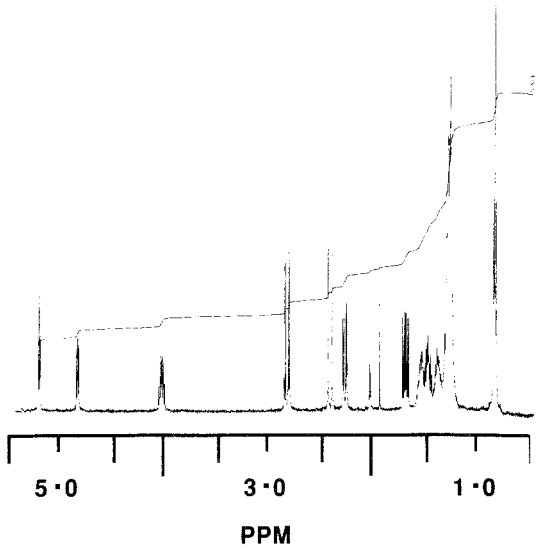

FIG. 3. 500-MHz [^{1}H]NMR spectrum of component 6 in acetone d_6.

TABLE 3. NMR DATA OF COMPONENT 6

^{1}H chemical shift		^{1}H coupling			^{13}C chemical shift ($CDCL_3$), ppm
Hydrogen	(Acetone d_6) ppm	Hydrogen 1	Hydrogen 2	J	
H3α	2.82	3α	3β	18.4	175.8
H3β	2.42	3α	3a	6.4	84.8
H3a	4.80	3β	3a	0.7	78.1
H5	4.01	3β	6a	0.6	77.2
H6α	1.71	3a	6a	4.7	38.7
H6β	2.28	3a	6β	0.3	36.5
H6a	5.16	5	6α	10.1	34.6
H7(1)	1.48	5	6β	4.9	31.6
H7(2)	1.56	5	7(1)	5.2	29.1
H8-(10)	1.37-1.24	5	7(2)	7.2	25.8
H11	0.87	6a	6α	5.1	22.4
		6a	6β	13.7	13.8
		C-11	C-12	7.0	

to the C-6 methylene protons (C-6β, δ2.28; C-6α, δ1.71). The methine proton at δ4.01 is coupled to both methylene protons on C-6 ($J_{C\text{-}6\alpha}$ = 10.1 Hz; $J_{C\text{-}6\beta}$ = 4.9 Hz) and to the first two side-chain methylene protons, broad multiplets at δ1.48 and δ1.57. Kojima and Kato (1979) studied 5-*t*-butyltetrahydrofuro[2,3-b]furan-2(3H)-one), a compound derived from an antifeedent for *Spodoptera litura* F., which is useful as an NMR model. Their measured geminal couplings of 17.3 and 13.0 Hz and *cis* ring juncture methine coupling of 4.9 Hz are in excellent agreement with those of component **6**.

The configuration of the hydrogen at C-5 was determined using the Karplus equation (Karplus, 1959) and dihedral angles were calculated using computer simulation. For H_5 in the β position, couplings to $H_{6\alpha}$ and $H_{6\beta}$ would be expected to be 6.0 and -0.2 Hz, while with H_5 in the α position, the couplings are calculated to be 7.9 and 6.3 Hz. The measured values of 10.1 and 4.9 Hz are more nearly those calculated for $H_{5\alpha}$. All other couplings calculated were within 3 Hz of the measured values. The [^{13}C]NMR spectrum showed a lactone carbonyl at 175.8 and three H—C—O linkage carbons at δ84.8, 78.1, and 77.2. Component **5** differs from component **6** only in having two fewer carbons in the side chain.

Chemically, the major gland component of *B. tryoni* appears to be more closely related to that of *B. arisanus* than to those of *B. longicaudatus*, but it must be noted that removal of the oxygen bridge in the *B. longicaudatus* furan would produce the *B. tryoni* lactone. *Biosteres tryoni* and *B. longicaudatus* are closely related and are members of a distinct Indo-Pacific species group (the tryoni complex) characterized by the apomorphic possession of a sinuate ovipositor (Fullaway, 1951; Wharton and Gilstrap, 1983). In addition, these two species share wing venation patterns, and propodeal, thoracic, frons, and vertex sculpture patterns that are distinctly different from *B. arisanus* and other members of the persulcatus complex to which *B. arisanus* belongs. Although both *B. arisanus* and *B. longicaudatus* have the second abdominal tergum longitudinally striate, the striations are different (Wharton and Gilstrap, 1983), and probably not homologous. Thus, our preliminary results suggest that gland chemistry may not be useful for studying relationships at higher taxonomic levels. Gland chemistry is sufficiently distinct to discern species, however, and may be useful in solving certain speciation problems. For example, further work on the chemistry of glands of *B. longicaudatus* collected from populations throughout its natural range should help to clarify the status of this species. A number of varieties and species have been described (Fullaway, 1953; Fischer, 1966, 1971), all of which have been treated as synonyms of *B. longicaudatus* (Wharton and Marsh, 1978; Wharton and Gilstrap, 1983).

As suggested by Buckingham (1975), gland morphology may also be useful for elucidating higher taxonomic relationships. *Opius incisi* and the closely related *O. fletcheri* have much smaller glands, with different morphological fea-

tures (Buckingham, 1975, Figure 38), than the glands discussed here. Similarly, the Indo-Pacific species of the tryoni and persulcatus complexes, all of which attack fruit-infesting tephritids, have distinctly different glands from *B. carbonarius* and related species (Buckingham, 1974), which attack leaf-mining Anthomyiidae. Host and gland morphology differences are supported by a number of external morphological differences (e.g., Fischer, 1977), suggesting that these two groups should probably be treated as different genera.

Buckingham (1975) concludes that the primary and possibly sole function of Hagen's glands is defensive. He based his work largely on experiments with *O. concolor siculus*, which has glands similar to *O. incisi* and *O. fletcheri*. We believe that differences in gland morphology and chemistry of species in the present study suggest other functions, which may either be in addition to, or to the exclusion of, defense. While gland secretions of *O. concolor* and related species may be primarily for defense, those of the species treated herein may function during mating or courtship.

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INDIVIDUAL VARIATION IN AGGREGATION PHEROMONE CONTENT OF THE BARK BEETLE, *Ips typographus*[1,2]

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Abstract—The total amounts of, and proportions among, components of the aggregation pheromone produced by *Ips typographus* were found to vary considerably among individuals excised from attacks on standing spruce trees. Chemical analyses of 392 individual male beetles were made by GC-MS. Both unmated and mated males had log-normal frequency distributions in their content of the pheromone components 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol (cV), since a large fraction of males had a low content. The amount of MB in male hindguts varied independently of cV and the other oxygenated monoterpenes, while the amount of cV covaried with the other pinene alcohols and showed a variation between beetles from different spruce trees. Mated males had, on average, lower amounts of MB than unmated, while the average content of cV in mated males varied with the resin content of their host trees. Ipsdienol and ipsenol were only found in mated males, but in less than 40% and 10%, respectively, of these mated males. Even-aged males exposed to α-pinene in the laboratory showed slightly less variation in the amounts of verbenols, and the variations in ratio between cV and tV were similar to those among males attacking the same spruce tree.

Key Words—*Ips typographus*, Coleoptera, Scolytidae, bark beetle, individual variation, 2-methyl-3-buten-2-ol, *cis*-verbenol, *trans*-verbenol, ipsdienol, ipsenol, α-pinene, biosynthesis, principal component analysis, aggregation pheromone.

[1] Coleoptera, Scolytidae.

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INTRODUCTION

Until recently bark beetle pheromone systems have been seen primarily from the point of view of the group or the species. However, after Alcock's seminal paper (1982), the evolutionary background of aggregation pheromones is largely seen in a perspective of individual selection (Birgersson et al., 1984; Borden et al., 1986; Schlyter and Birgersson, 1989). An understanding of the importance of individual selection has, together with developments of chemical analysis technology, opened the field of indvidual variation for ecological, physiological, and evolutionary lines of study.

The first study on individual variation in content of pheromone components in bark beetles was done on naturally attacking males of *Ips typographus* L. (Birgersson et al., 1984). Additional studies have been done in laboratory experiments with the North American species *Dendroctonus ponderosae* Hopkins and *Ips pini* (Say) (Slessor et al., 1985; Borden et al., 1986; Hunt et al., 1986). Typical for all these quantitative studies are the large variances among individuals; many are "low producers," while a few have large amounts of pheromone.

What is the reason for these very skewed distributions, and which factors influence it? Apart from the quantification methods, ecological and physiological factors in the attacking bark beetles and in the host trees can be important for the beetles' production of semiochemicals. In the study by Birgersson et al. (1984), the individually analyzed males were all unmated and collected from a spruce tree that had been characterized as having weak resistance to attack. This could have been an atypical tree, due to its low resin content. One question is whether qualitative and quantitative differences in host resistance have an influence on the individual variation between the attacking beetles. Moreover, will beetles from resistant trees include more "high producers" and thus show less skewed distribution in pheromone yield than did the males (Birgersson et al., 1984) from a nonresistant tree? In laboratory experiments with the North American species *Ips paraconfusus* (Lanier), Byers (1981) showed that the production of *cis*- and *trans*-verbenol, and myrtenol increased with the amount of the host precursor, α-pinene, to which they were exposed.

Part of the indvidual variation could also depend on the genotype, which is the only part of the variation subject to natural selection and hence to adaptive evolutionary change. Such genotypically based variation would be indicated by variation in homogeneous groups where variation in external factors (methods of analysis and ecological and physiological factors) are minimal. A large variation in such groups would confirm the view that beetles may differentially employ "strategies" as pioneers, hitchers, or sneakers (Borden et al., 1986; Schlyter and Birgersson, 1989) or that different population levels may give different, alternating selection pressures.

The phenotypic variation in amounts and proportions of the pheromone components is here studied from the following aspects: (1) in relation to tree resin production and composition among trees; (2) in relation to spatial variation of attack within a tree; and (3) in relation to temporal variation between the attack phases of the bark beetles.

We will focus on the pheromone components that are of known behavioral significance and thus are ecologically and evolutionarily relevant: 2-methyl-3--buten-2-ol and *cis*-verbenol, which are essential for attraction of *I. typographus* in the field (Schlyter et al., 1987a), and ipsdienol, ipsenol, and verbenone, which are known to modify the beetles' attraction to methylbutenol and *cis* verbenol (Bakke, 1981; Schlyter et al., 1987b)

To elucidate the nature and reasons for variation in pheromone components, we have quantified the content of volatiles in individually analyzed hindguts from unmated and mated males of the spruce bark beetle, *I. typographus*, in well-defined attack phases originating from six spruce trees with known composition of monoterpene hydrocarbons and different levels of resin production. The naturally attacking males are also compared with males exposed to α-pinene in the laboratory.

METHODS AND MATERIALS

Biological Material

Males from Naturally Infested Trees. Males of *I. typographus* were excised from the bark of four standing (baited) and two windthrown (unbaited) trees of Norway spruce [*Picea abies* (L.) Karst.], north of Torsby, province of Värmland, central Sweden, during the main swarming period June 1–15, 1982 (Table 1). In order to ensure adequate attack, the trees were baited with the commercial attractant for *I. typographus*, Ipslure (Borregaard a/s, Norway). Males from two attack phases (as defined by Birgersson et al., 1984) were collected: phase 3, unmated males with complete nuptial chambers; and phase 6, mated males joined by one or two females, in 2- to 4-cm-long egg galleries (see Figure 5). The beetles were immediately transferred to a cooling box and within 1 hr to a thermos with liquid nitrogen as described by Birgersson et al. (1984).

Fifteen to 20 samples (2 × 3 × 40 mm) of fresh phloem (attacked and brown-stained phloem excluded), evenly distributed over the part of the bole where beetles were collected, were taken from each tree. Samples from each spruce tree were pooled before extraction. The samples contained mainly the phloem and small amounts of adjacent cambium and cork layers, i.e., the layers of bark contacted by the beetles.

Laboratory-Exposed Males. Emerging male brood adults from the 11th generation of laboratory-reared bark beetles, originating from Lardal, southern

TABLE 1. CHARACTERISTICS OF SPRUCE TREES (*Picea abies*) AND SAMPLING REGIMES OF MALE *Ips typographus* FOR INDIVIDUAL ANALYSES (TORSBY, VÄRMLAND, SWEDEN, JUNE 1982)

Tree	State	Synthetic pheromone baited	Resin flow, response to attack	Date of first attack	Sampling period, June	Number of beetles analyzed		
						Phase 3	Phase 6	Total
I	Standing, fresh	Yes	Rich, resistant	May 30	1–6	37	32	69
II	Standing, fresh	Yes	Poor, taken	May 30	1–9	29	18	47
III	Windthrown, still green	No	Very poor, taken	May 17	1–4	3	42	45
IV	Standing, fresh	Yes	Rich, resistant	May 30	3–15	73	47	120
V	Standing, fresh	Yes	Poor, taken	May 30	3–8	21	40	61
VI	Windthrown, still green	No	Rich, resistant	June 3	4–8	48	2	50
Total number of male beetles analyzed						211	181	392

Norway (Anderbrant et al., 1985), were separated from the females by their lower bristle density on the pronotum (Schlyter and Cederholm, 1981). The males were initially divided into two groups: (1) one group with 50 males was kept at +4°C and 90–100% relative humidity in a refrigerator after emergence; and (2) the second group, with about 100 males, was given the possibility for flight exercise for 24 hr at +25°C, 95% relative humidity, and 10,000 lux (light : dark = 20 : 4), and then tested in a Y-tube olfactometer (Schlyter and Löfqvist, 1986) at +24°C and 70% relative humidity for 2 hr. Approximately 50% of the males were attracted to a synthetic source of pheromone (5 mg/day of 2-methyl-3-buten-2-ol and 0.1 mg/day of *cis*-verbenol released) as they walked up the Y-tube and were collected at the end of the Y-tube arms (Schlyter and Löfqvist, 1986). The Y-tube tested group of males was, accordingly, divided into responding and not responding groups (2A and 2B, respectively), which were kept dark and cool for 4 hr before α-pinene exposure.

The three groups (1, 2A, and 2B) were exposed simultaneously to α-pinene in separate Petri dishes (ID 9 cm) with moistened filter paper (100% relative humidity at +22°C and 6000 lux for 18 hr). The monoterpene hydrocarbons, (+)-(1*R*, 5*R*)-α-pinene and (−)-(1*S*, 5*S*)-α-pinene (>99%, Fluka, purum) were released from two separate 10-μl glass capillaries attached to the top inside of each Petri dish. Following exposure, release rate was estimated by the amount

of liquid remaining in each capillary. The exposed beetles were kept in liquid nitrogen until dissection and chemical analyses.

Chemical Analyses

The beetle hindguts were dissected (with penis and dorsal abdominal tergite fragments removed) and frozen to the wall of a sharply coned dissection vial (Reacti-Vial, Pierce) chilled on dry ice. Ten microliters of pentane, with an internal standard of 10 ng/μl of heptyl acetate (C_7Ac), was added, and the gut was crushed with a glass rod and left for 10 min at room temperature for extraction. The extract was concentrated, and about 3 μl was injected into a Finnigan 4021 gas chromatograph–mass spectrometer (GC-MS). A 25 m fused silica column, ID 0.20 mm, coated with OV-351 (Supelco, d_f = 0.38 μm, HETP = 0.26 mm, $k'_{C_8\text{-ol}}$ = 4.4) was used in the analyses of the field-collected beetles. The males exposed to α-pinene in the laboratory were analyzed with a 25 m fused silica column, ID 0.15 mm, coated with Superox FA (RSL, d_f = 0.26 μm, HETP = 0.17 mm, $k'_{C_8\text{-ol}}$ = 7.8). Both columns were made at the Department of Chemical Ecology, Göteborg, as described in Lanne et al. (1987), and the temperature program used was 60°C for 4 min, followed by an increase of 8°C per minute to 200°C, and finally isothermal for 7 min. Helium was used as carrier gas (Q = 29 cm/sec) for both columns.

Quantification was done using the extracted ion current profiles (EICP) on characteristic and prominent ions of each substance, relative to the 100 ng of C_7Ac added (Birgersson et al., 1984). To assure the accuracy and precision of the quantifications, each day of analysis was begun with a run of one of the concentrations in the dilution series: 0.1, 0.3, 1.0, 3.0, . . ., 3000, 10,000 ng/3.0 μl plus the internal standard, 100 ng of C_7Ac. The dilution series was made from a stock solution, containing all major compounds found in male hindgut extracts (Birgersson et al., 1984): 2-methyl-3-buten-2-ol (MB), ipsenol (Ie), *cis*-verbenol (cV), ipsdienol (Id), *trans*-verbenol (tV), myrtenol (Mt), *trans*-myrtanol (tM), and 2-phenylethanol (PE), plus verbenone (Vn), in equal amounts. The logarithmic standard curves obtained were essentially linear (r = 0.98–1.00) in the interval from 10^{-1} to 10^4 ng/injection, allowing a conservative estimate of the limit of detection (LOD) as <1 ng/individual gut and of the limit of quantification (LOQ) as <5 ng/individual (Anonymous, 1980). However, using EICP, LOD was better than 0.1 ng and LOQ was 1.0 ng/injection. The dissected beetles were frozen, and dry weight (60°C, 24 hr) and fat content (percent extractable by petroleum ether) were estimated, as described in Anderbrant et al. (1985).

The phloem samples were cut up into millimeter-sized pieces and extracted with 2 ml of pentane (Fluka, p.a.) for 30 min in an ultrasonic cleaning bath.

The extracts were analyzed for the contents of monoterpene hydrocarbons with a Hewlett-Packard 5830A GC. A 50-m glass capillary column, ID 0.32 mm, coated at the Department of Chemical Ecology, Göteborg, as described in Lanne et al. (1987), with Carbowax 40 M (Chrompack, $d_f = 0.66$ μm, HETP = 0.35, $k'_{C_8\text{-ol}} = 6.2$,) was used. The temperature program used was 60°C for 5 min, followed by an increase of 5°C/min for 20 min. Nitrogen was used as carrier gas ($Q = 20$ cm/s). The monoterpene content is expressed in the results as arbitrary integrator units per gram of dried phloem (80°C, 5 hr). For the enantiomeric analyses of α-pinene compare Lindström et al. (1988).

Statistics

The frequency distributions for most of he variables (amounts of hindgut volatiles and percent fat) showed significant skewness and kurtosis. Since ordinary statistics (such as ANOVA, MANOVA, regressions, and correlations) were insufficient to investigate the patterns of variation and covariation among the variables, principal components analysis (PCA) was used. The method employed was SIMCA (soft independent modelling of class analogies), as developed by Wold et al. (1984), which facilitates an analysis of a multivariate data matrix. PCA class models were developed by using 10 individuals from each host tree in attack phase 3. The males were randomly selected, and all or a subset of the variables were used. This method also enabled us to provide an independent check of the "host-tree model," by adding a sample of individuals that had not been used to build the model. The SIMCA method was also used to model phase-3 and phase-6 males from the same tree and to study the separation between the attack phases based on all variables.

The transformation log [amount + 1] was used in both ANOVA, were normality is an essential assumption (Sokal and Rohlf, 1981), and in SIMCA, where log transformation is recommended for GC data (E. Johansson, personal communication).

RESULTS

Naturally Attacking Males

Overall Variation Patterns. In total, 392 male bark beetles, collected during their attack on Norway spruce, were analyzed individually for their content of nine volatiles, using extracted ion current profiles (EICP). The males, all together or separated by attack phase or host tree, showed variation that ranged over three orders of magnitude for most of these compounds (Figures 1 and 2). Representative gas chromatograms (Figure 1) illustrate that the ratios between cV and tV appeared constant in males collected from the same spruce tree,

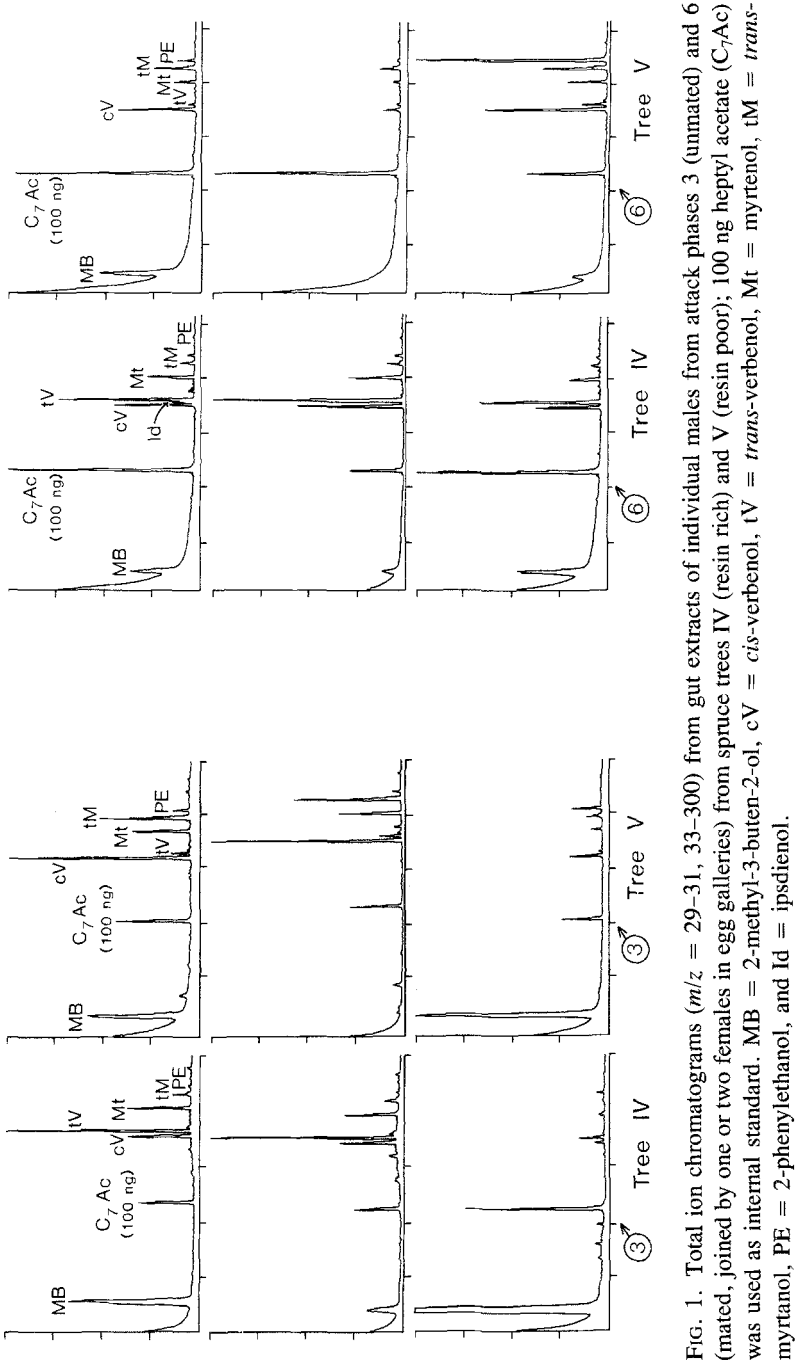

FIG. 1. Total ion chromatograms (m/z = 29–31, 33–300) from gut extracts of individual males from attack phases 3 (unmated) and 6 (mated, joined by one or two females in egg galleries) from spruce trees IV (resin rich) and V (resin poor); 100 ng heptyl acetate (C_7Ac) was used as internal standard. MB = 2-methyl-3-buten-2-ol, cV = *cis*-verbenol, tV = *trans*-verbenol, Mt = myrtenol, tM = *trans*-myrtanol, PE = 2-phenylethanol, and Id = ipsdienol.

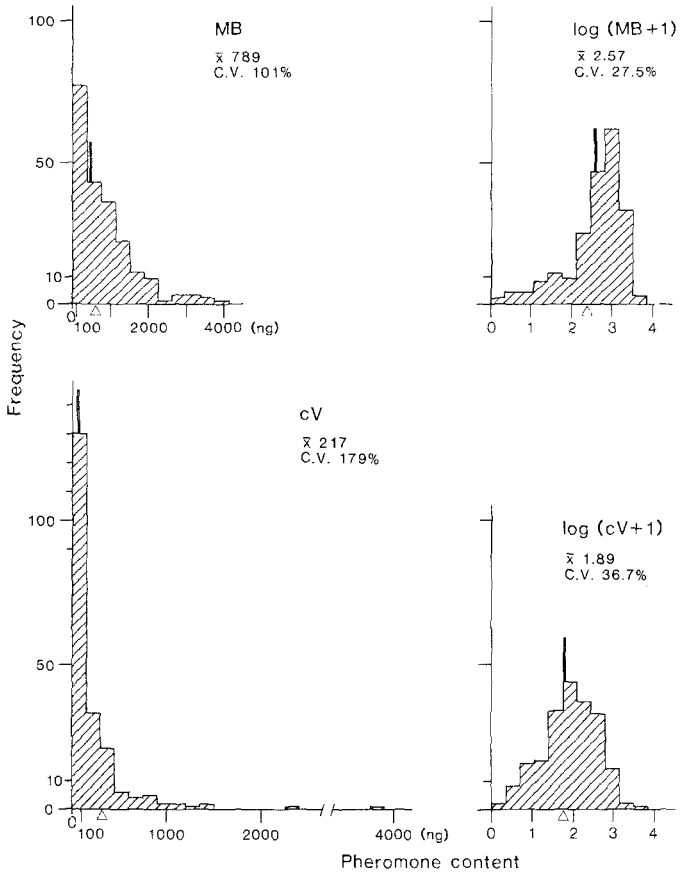

FIG. 2. Frequency histograms of hindgut content in males from attack phase 3 (unmated) from all trees (N = 211) of 2-methyl-3-buten-2-ol, and *cis*-verbenol, untransformed and transformed by log [absolute amount + 1]. Triangles indicate the arithmetic means and vertical lines the medians.

while MB seemed to vary independently of the monoterpene alcohols (cV, tV, Mt, tM, Id, and Ie) and PE.

Many indviduals had low amounts of some or all compounds. However, the sensitivity of the GC-MS method appeared to be sufficient as very few males (< 1% for MB and cV) had amounts below LOD, and less than 3% and 5% for MB and cV, respectively, had amounts too low for a reliable quantification (LOQ).

The large quantitative variation and the high number of individuals with low amounts resulted in very skewed and peaked distributions of both MB and cV, especially in phase-3 males (see Figure 2 and Table 2). This is also indicated by the means being much larger than the medians. A simple logarithmic

TABLE 2. FREQUENCY MEASURES OF VARIABLES QUANTIFIED FROM INDIVIDUAL *Ips typographus* MALES FROM NATURAL ATTACKS ON *Picea abies*; ATTACK PHASE 3

Variable	Frequency distribution measure				
	Mean	Median	CV (%)	Skewness	Kurtosis
Fat (%), all trees	8.3	7.3	48.2	1.43***[a]	2.34***
Dry weight (mg) all trees	3.59	3.56	15.6	0.38*	0.54
MB (ng)					
Tree I	754	467	125	1.95***	3.75***
II	504	302	106	1.42**	1.41
IV	960	783	91.1	1.80***	4.12***
V	964	861	82.5	0.80	−0.02
VI	681	569	90.9	1.25***	1.65**
All trees	831	581	101	1.75***	3.66***
Log (MB + 1)					
Tree I	2.40	2.67	35.5	−0.91**	0.11
II	2.34	2.48	32.7	−1.43**	2.36***
IV	2.74	2.89	21.9	−1.57***	2.76***
V	2.76	2.94	19.7	−1.09*	0.93
VI	2.52	2.76	28.2	−1.28***	0.92
All trees	2.59	2.76	27.4	−1.36***	1.78***
cV (ng)					
Tree I	395	100	188	3.38***	12.8***
II	99.4	36.4	137	2.67***	8.49***
IV	112	67.7	130	2.92*	10.6***
V	141	79.2	120	2.03	4.79***
VI	354	287	99.7	1.37	1.58**
All trees	179	76.6	166	6.31*	49.6***
Log (cV + 1)					
Tree I	2.40	2.67	32.0	0.02	0.01
II	2.34	2.48	36.5	−0.39	0.08
IV	1.77	1.85	30.1	−0.24	−0.34
V	1.79	1.90	38.5	−0.78	0.17
VI	2.20	2.46	33.2	−1.06**	0.22
All trees	1.83	1.89	33.9	−0.13	0.17
tV (ng), all trees	165	66.9	151	3.21***	13.6***
Mt (ng) all trees	70.9	34.8	119	4.49***	26.4***
tM (ng) all trees	31.0	13.2	111	3.32***	15.0***
PE (ng) all trees	14.6	7.3	165	4.38***	24.6***
%cis [cV/(cV + tV) × 100]					
Tree I	69.5	0.2	8.8	−1.24***	2.01**
II	45.1	44.2	16.3	0.25	−0.53*
IV	31.8	31.1	20.3	0.55	−0.24
V	90.2	90.9	1.4	−0.64	−0.83
VI	51.5	51.0	8.9	1.25***	2.55**
All trees	49.6	48.5	40.4	0.62**	−0.48

[a] Significant departure from normality: $*P < 5\%$, $**P < 1\%$, $***P < 0.1\%$.

transformation, log [amount + 1], gave almost normal distribution of MB and cV with medians very close to the means, but with a left-hand skewness remaining in MB (Figure 2), especially after partition of males for attack phases and host trees. The mean dry weight of unmated males (phase 3) was lower than that for mated males (phase 6) ($P < 0.05$, t test). However, the weight variation for individuals from different trees was small and its distribution was close to normal (Tables 2 and 3). The MB content was weakly correlated with weight ($r = 0.18$, $N = 200$, $P < 0.01$, Spearman rank correlation), but cV content was not ($P > 0.10$). The amounts of MB and cV showed no consistent difference among beetles from the same tree sampled in the morning and in the afternoon or in subsequent days ($P > 0.10$, ANOVA).

Verbenone was never found in amounts exceeding 1 ng in any hindgut extract and in most cases it was below LOD (<0.1 ng, EICP).

Variation among Males from Different Spruce Trees. The ratio between the pinene alcohols (i.e., cV, tV, Mt, tM) was almost constant in the beetles attacking the same spruce tree, independent of absolute amounts of the compounds and of whether the males were mated or not (Figure 3). When ANOVA was used on the total material, the differences among the males from different host trees seemed to be significant for the absolute amounts of MB and cV. However, as many of the amounts of volatiles, after separation of the material by host trees, had heterogeneous variances ($P < 0.05$, Cochrans C and Barlett box tests), we also used principal component analysis (SIMCA) to study the "between tree variation" among males in phase 3. Discrimination among males from different spruce trees based on all variables (i.e., the amounts of MB, cV, tV, Mt, tM, PE, Id, and Ie, and percent fat) was very poor for any pair of "host-tree models" (class models). However, when only the four pinene alcohols (cV, tV, Mt, and tM) were used as variables, a clear separation among males from different host trees was obtained (Figure 4A; cf. Figure 3). The close covariation and the modeling power of the pinene alcohols can be seen in the loading plot for all variables where cV, tV, Mt, and tM form a tight cluster (Figure 4B). MB, PE, and percent fat are widely separated from each other and from the pinene-derived components because they varied independently of each other and of the pinene alcohol group. Id and Ie had no modeling power, as they were never found in phase-3 males.

The amounts of cV, tV, Mt, and tM in males attacking the same spruce tree varied very little in their proportions (Figure 3) and had approximately normal distributions (Tables 2 and 3). However, the proportions of the pinene alcohols varied greatly among males collected from different trees. This "among-tree" difference could be due to the variation in the amounts of monoterpene hydrocarbons present in the phloem from different host trees. The percentage of *cis*-isomer of the verbenols [$\%cis = cV/(cV + tV) \times 100$] was correlated with the percentage of the (−)-enantiomer of host tree α-pinene ($r = 0.92$, $N = 6$, $P < 0.001$; cf. Lindström et al., 1988). However, there is a small difference in the $\%cis$ between males in attack phases 3 and 6, collected

TABLE 3. FREQUENCY MEASURES OF VARIABLES QUANTIFIED FROM INDIVIDUAL *Ips typographus* MALES FROM NATURAL ATTACKS ON *Picea abies*; ATTACK PHASE 6

Variable	Frequency distribution measure				
	Mean	Median	CV (%)	Skewness	Kurtosis
Fat (%), all trees	10.3	9.6	41.7	0.74***[a]	0.17
Dry weight (mg), all trees	3.77	3.76	17.5	0.43**	0.43
MB (ng)					
Tree I	127	6.7	263	4.34***	20.9***
II	6.2	3.8	123	2.16***	5.07***
III	94.7	22.0	168	2.20***	4.31***
IV	206	39.2	146	1.73***	2.69***
V	22.8	6.2	177	2.41***	4.86***
All trees	108	8.7	19.9	3.82***	18.1***
log (MB + 1)					
Tree I	1.18	0.89	77.7	0.65	−0.83
II	0.68	0.68	57.6	0.50	−0.56
III	1.48	1.36	44.6	0.46	−0.58
IV	1.69	1.60	49.9	0.12	−1.36
V	0.98	0.86	55.9	0.83*	−0.03
All trees	1.23	0.99	66.5	0.71**	−0.59
cV (ng)					
Tree I	354	209	101	1.68***	3.16***
II	43.7	19.6	109	1.55**	1.70
III	7.2	1.5	394	6.32***	40.5***
IV	164	129	86.1	2.61***	9.94***
V	40.2	16.2	124	1.28***	0.23
All trees	156	85.3	146	3.21***	13.3***
Log (cV + 1)					
Tree I	2.31	2.32	22.7	−0.56	−0.12
II	1.41	1.32	35.0	−0.16	0.30
III	1.48	1.36	44.6	0.46	−0.58
IV	2.09	2.11	17.0	−0.37	0.30
V	1.25	1.23	48.5	0.09	−1.15
All trees	1.81	1.94	37.0	−0.42*	−0.43
tV (ng), all trees	141	65.5	132	2.27***	7.26***
Mt (ng), all trees	63.0	38.3	129	2.59***	8.89***
tM (ng), all trees	23.3	16.9	99.6	1.79***	3.79***
PE (ng), all trees	6.67	1.90	29.2	8.69***	85.1***
%cis [cV/(cV + tV) × 100]					
Tree I	70.4	71.8	8.8	−0.89*	−0.08
II	40.1	36.4	32.2	0.20	1.32
III	35.3	34.4	14.1	1.50	3.34**
IV	36.0	36.1	14.6	0.80*	2.72***
V	87.7	86.2	3.8	0.11	−1.09*
All trees	52.2	43.4	39.6	0.36*	−1.36***

[a] Significant departure from normality: *$P < 5\%$, **$P < 1\%$, ***$P < 0.1\%$.

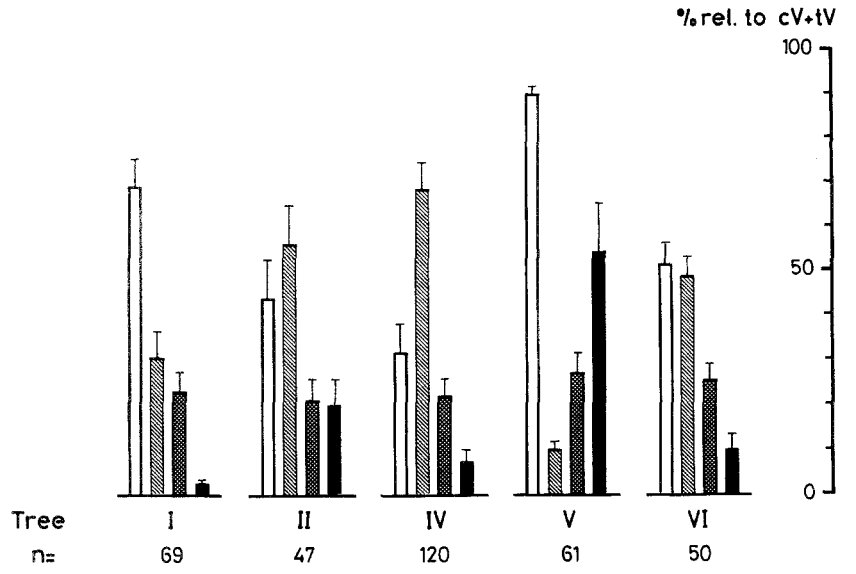

FIG. 3. Differences in relative amounts of pinene alcohols among males collected from different spruce trees. The amounts of *cis*-verbenol, □; *trans*-verbenol, ▨; myrtenol, ▩; and *trans*-myrtanol, ■; are related to the sum of *cis*- and *trans*-verbenol. n = number of beetles analyzed from each tree.

from spruce trees of different resistance. For the males collected from trees IV and V, the difference was significant ($P < 0.05$, t test) while it was not for the males in trees I and II. This shows a tendency for the percentage cV to decline in males from trees fully colonized by the beetles (trees II and V), while it is constant or increases in males attacking resinous and resistent trees (I and IV) (Tables 2 and 3).

To check the validity of the "host-tree separation," we included two randomly selected "test objects" from each of the five trees (Figure 4A). The validity was good, as eight of the 10 test objects were unambiguously assigned to the correct tree (Figure 4C), and none was incorrectly assigned. The two test objects belonging to tree I could not be assigned to tree I or any other tree.

Variation between Males in Different Attack Phases. The males from phase 3 and phase 6 showed the same relative magnitude of variation for all the volatiles, with standard deviations of the same magnitude as the means, which gave very large coefficients of variation [%CV = ($\bar{x}$/SD × 100] (Tables 2 and 3). The differences between attack phases 3 and 6 seemed highly significant for both MB and cV when all males were included in the ANOVA. The amount of MB was much lower in phase 6 than in phase 3 for males from all trees ($P < 0.001$, Wilcoxon signed rank test and Kruskal-Wallis one-way analysis by ranks; Figure 5). For cV and the other pinene alcohols, the picture was more compli-

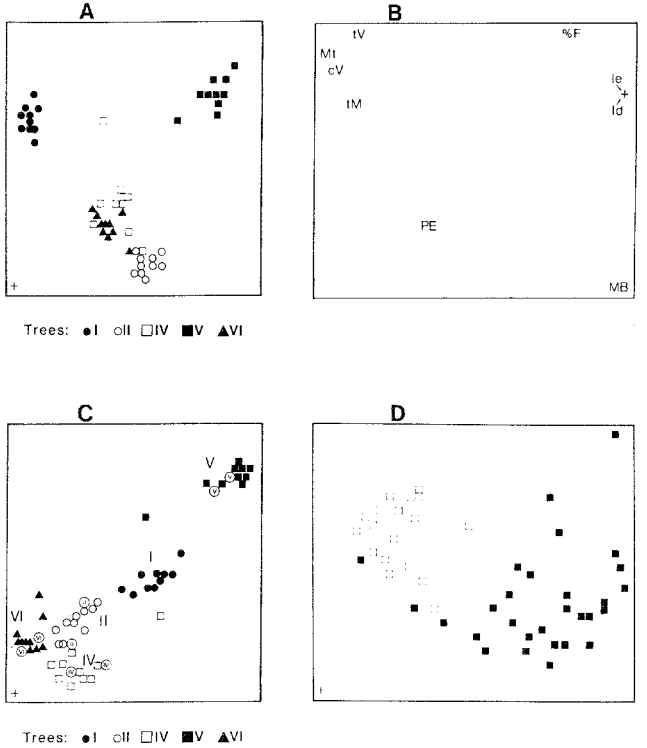

FIG. 4. (A) Plot of the residual standard deviations (SDD plot) from the "host-tree models" of trees I and II for 10 unmated males from each of five trees, where the models were based on the four pinene alcohols (cV, tV, Mt, and tM); + indicates origin. (B) A "loading plot" showing the modeling power of the nine variables orginally used in the classification of males from phase 3 from the five spruce trees. A position far from the origin indicates a high modeling power and a position close to another variable indicates a covariation. Id and Ie had no modeling power as they were never detected in any unmated male. (C) An SDD plot of the same type as in A, but based on the residuals from the class models of trees IV and VI, which gave the best overall resolution. From each tree, two "test objects," which were not used in building of the models and provides a test of the validity of the models, are plotted with the tree number inscribed in a circle. (D) A residual standard deviation plot of phase 3 (■, $N = 29$) against phase 6 (□, $N = 18$) for males from the same spruce tree (No. II) using all variables except Id and Ie and all males of each attack phase to build the "attack-phase model."

cated. The overall arithmetic mean in phase 6 was only slightly lower than in phase 3. Males from trees II and V followed this general pattern ($P < 0.001$, t tests). However, the medians for the pinene alcohols increased in trees I and IV between attack phases 3 and 6 (Figure 5).

Id and Ie were characteristic for phase 6, as none of them were detected

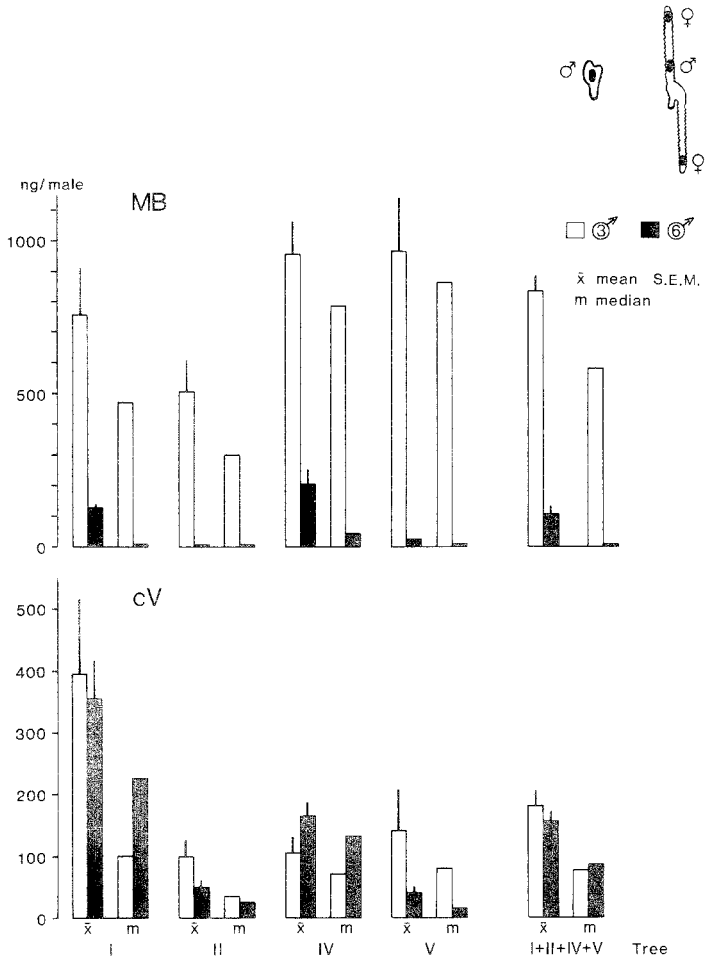

FIG. 5. Mean ($\bar{x}$ + SEM) and median (m) gut content of 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol (cV) in males from attack phases 3 (□) and 6 (■) in the four spruce trees where both attack phases had adequate sample sizes.

(<0.1 ng, EICP) in any individual from phase 3. Still, neither of the two compounds was found in more than 60% of the mated males (Table 4). Two thirds of the males in which Id was detected contained less than 1 ng. Ie was found in fewer than 10% of the phase-6 males in amounts from much less than 1 ng up to 157 ng, but almost all of these males had less than 10 ng.

To evaluate the difference between the attack phases, we class-modeled males from tree II in phase 3 and phase 6 by SIMCA, using all variables except Id and Ie simultaneously. The reason for excluding Id and Ie was that only two males had Id in amounts above LOD, and none had Ie. The classes showed a

TABLE 4. OCCURRENCE OF IPSDIENOL AND IPSENOL IN INDIVIDUAL MALES OF DIFFERENT ATTACK PHASES AND DISTRIBUTIONS IN DIFFERENT TREES IN ATTACK PHASE 6

Phase	Occurrence				
	Neither	Only Id	Only Ie	Both Id + Ie	Sum
Phase 3	211	0	0	0	211
	(100)[a]	(0)	(0)	(0)	(100)
Phase 6	113	51	0	17	181
	(62.4)	(28.2)	(0)	(9.4)	(100)

Variable	Frequency distribution measure						
	Mean	Median	CV (%)	Skewness[b]	Kurtosis[b]	Minimum	Maximum
Id (ng)							
Tree I	0.07	0.0	305	3.65	12.9	0.0	1.0
II	0.01	0.0				0.0	0.2
III	11	0.0	262	3.00	8.32	0.0	121
IV	1.0	0.002	271	2.94	7.47	0.0	10.5
V	2.1	0.002	603	6.31	39.9	0.0	79.2
All trees	3.3	0.0	448			0.0	121
Ie (ng)							
Tree I	0.0	0.0				0.0	<0.1
II	0.0	0.0				0.0	0.0
III	1.3	0.0	395	4.30	18.0	0.0	25.6
IV	0.60	0.0	317	3.29	39.9	0.0	7.9
V	3.9	0.0	613	6.22	39.9	0.0	157
All trees	1.3	0.0	900			0.0	157

[a] Values within parentheses are percent of total.
[b] All values of skewness and kurtosis indicate significant departure from normality, $P < 1\%$.

certain overlap, mainly due to some individuals in phase 3 having very low amounts of volatiles (Figure 4D).

Terpene-Exposed Unmated Males in the Laboratory.

In order to reduce the variability introduced by external factors such as variation in composition and amounts of monoterpene hydrocarbons (e.g., α-pinene), and phenotypic variance, males as homogeneous as possible (bred in laboratory and bioassayed for pheromone response) were exposed to α-pinene.

Beetles given flight exercise (group 2) had larger amounts of MB present in their hindguts than cold-stored ones (group 1), but still much less than those collected in the field (Figure 6). The differences in mean cV content between males from cold storage responding or not responding to a pheromone source in a Y tube after flight exercise were related only to the amount of (−)-α-pinene released during the exposure ($r = 0.998$, $N = 3$), but the deviation around the

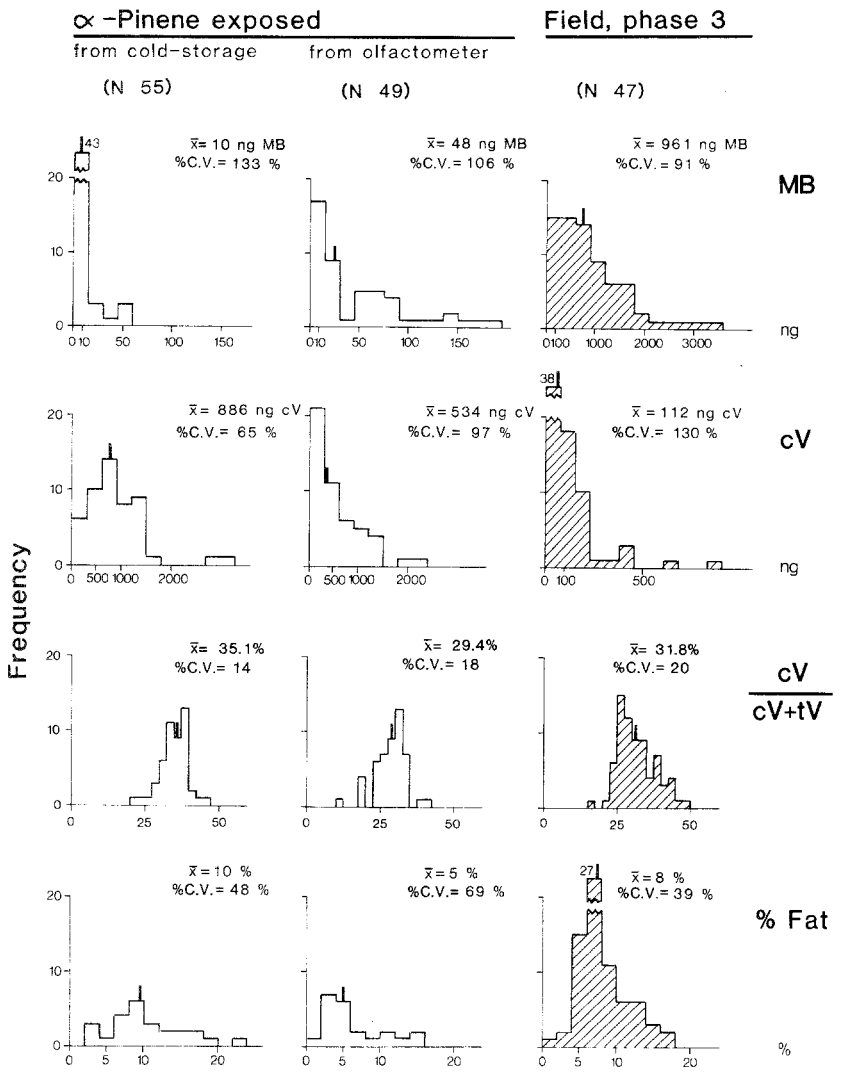

FIG. 6. Frequency histograms of hindgut content of MB, cV, *%cis* [= cV/(cV + tV) × 100], and % Fat in body of males exposed to α-pinene in the laboratory and males in attack phase 3 from spruce tree IV. The left column of diagrams shows beetles kept in refrigerator after emergence. The middle column shows beetles having responded to pheromone in olfactometer after flight exercise before α-pinene exposure. Right column shows males in attack phase 3 sampled from tree IV. Vertical lines indicate the medians, % CV = coefficient of variation [(SD/$\bar{x}$) × 100].

mean of each group was very large (Table 5). The untransformed values of MB and cV were skewed, leptokurtic, and significantly different from a normal distribution. Transformed values of MB and cV (log [amount + 1]) had, together with *%cis*, distributions that were normal or close to normal (Table 5), as was the case with the field-collected material (Tables 2 and 3). The coefficient of variation (%CV) of studied parameters from α-pinene-exposed beetles were generally similar to, or smaller than, those from unmated males collected from tree IV (Figure 6) and other trees (Tables 2 and 3). The fat content was lower

TABLE 5. DISTRIBUTION MEASURES OF VARIABLES QUANTIFIED FROM INDIVIDUAL *Ips typographus* MALE LABORATORY BEETLES EXPOSED TO α-PINENE IN THREE GROUPS[a]

	Frequency distribution measure				
Variable	Mean	Median	CV (%)	Skewness	Kurtosis
Fat (%)					
Cold storage	10.2	9.8	47.9	0.44	−0.44
Not responding					
Responding	5.2	4.7	69.5	1.31***[b]	1.34
Dry weight (mg)					
Cold storage	3.36	3.30	15.7	0.02	−0.38
Not responding					
Responding	3.06	3.07	18.6	0.00	−0.88
MB (ng)					
Cold storage	10.1	5.9	133.	2.51***	5.81***
Not responding	41.0	32.6	90.7	1.78***	3.89***
Responding	48.5	26.2	106.	1.13**	0.30
Log (MB + 1)					
Cold storage	0.83	0.84	51.1	0.12	0.19
Not responding	1.44	1.53	32.6	−0.94*	1.08
Responding	1.35	1.44	44.7	−0.44	−1.01
cV (ng)					
Cold storage	886	782	64.6	1.65***	4.41***
Not responding	704	560	81.6	1.55***	2.94***
Responding	534	425	97.4	1.41***	2.15***
Log (cV + 1)					
Cold storage	2.86	2.89	10.6	−0.62	0.45
Not responding	2.69	2.75	15.7	−0.75*	0.29
Responding	2.45	2.53	22.5	−0.46	−0.70
%cis [cV/(cV + tV) × 100]					
Cold storage	35.1	35.4	13.5	−0.69*	1.29
Not responding	28.4	28.5	11.5	−0.63	1.35
Responding	29.4	28.9	18.5	−0.69*	1.46*

[a] The three groups are: cold storage ($N = 55$), not responding ($N = 42$), and responding ($N = 49$) to pheromone in Y-tube olfactometer after flight exercise.
[b] Significant departure from normality: $*P < 5\%$, $**P < 1\%$, $***P < 0.1\%$.

in the two groups given flight exercise ($P < 0.001$, t test). Comparing only the most relevant laboratory group (2B; flight exercise and pheromone responding) and using only those variables that are at least close to normal distributed, we find that the laboratory group (2B) for log(MB + 1) had 45% CV, while the field groups ranged from 45 to 78% CV; for log(cV + 1) 2B had 22% CV, while the field groups had 17–49% CV; and for *%cis* 2B had 18% and the field groups had between 4 and 32% CV (Tables 2 and 5).

The transformed amounts of cV (log [amount + 1]) were weakly correlated to the dry weight and percent fat for the exposed males in Figure 6 (r = 0.27 and 0 .24, respectively, $P \leq 0.01$), but there were no correlations between the content of MB and the dry weight or the percent fat, for males from any exposure group or all groups combined. However, the amounts of the two pheromone components were significantly intercorrelated ($P \leq 0.001$), especially for the two groups given flight exercise (r = 0.62–0.86).

DISCUSSION

Quantities of the pheromone components in individual male bark beetles appear to be based on a multitude of factors, including external ones and the individual variation in a strict sense. The intrinsic individual variation, i.e., the deviation from the mean of a homogeneous subset of individuals defined by external factors, is in turn composed of phenotypic and genetic factors. Is there a pattern of the quantified compounds that can illuminate these factors?

Variation due to External Factors

Methodology. The sampling regime could possibly have introduced some additional variance in pheromone production among males in the same attack phase, as we sampled beetles throughout and during several days. However, the influence from the time of collection was small in phase 3 as we did not detect any differences among beetles collected in the morning and the afternoon or among different days. This means that males attacking the trees during the first days of swarming did not contain significantly larger amounts of aggregation pheromone components than later attacking ones. Our results do not support the hypothesis put forward by Borden et al. (1986) that the pioneers would have a larger capacity for pheromone production. Another possible factor contributing to the large variation in phase 6 is that most males had only one female, and these were not separated from those with two or more females. An earlier study with batch samples of males with one or two females in attack phases 4 and 5 showed virtually no difference in pheromone content (Birgersson et al., 1984). The individual variation in amounts of volatile in phase 6 is thus most probably not affected by the number of females present.

Monoterpene Composition in Host Trees. The highly significant differences among beetles from different trees are shown by both ANOVA and SIMCA, especially when the analyses were based on the pinene alcohols cV, tV, Mt, and tM (Figures 3 and 4A and B). This result was not unexpected as we earlier found consistent differences in proportions of pinene alcohols among batch samples of males from different spruce trees and were able to correlate the amount of some host monoterpenes and pheromone components in beetle hindguts (Birgersson et al., 1984). It has earlier been shown in laboratory experiments that the production of cV, tV, and Mt depends on the amount and enantiomeric composition of the precursor, α-pinene (Renwick et al., 1976; Klimetzek and Francke, 1980; Byers, 1981).

In our field-collected material, the agreement between the absolute amounts of monoterpene hydrocarbons (MT) in host-tree phloem and pinene alcohols in the beetle hindguts was weak. A possible reason for this is that our analyses of the bark samples measured only the content of MT in constitutive resin present in the phloem, while the beetles interact mostly with the secondary resin produced by the host in response to the bark beetle excavating activity (Wright et al., 1979). The absolute amounts of MT in secondary resin is not correlated with the amounts in primary resin. However, the relative amounts of MT in primary and secondary resin is constant throughout the attack (Leufvén and Birgersson, 1987).

In contrast to the absolute amounts, the relative amounts of cV and tV in the hindguts of both phase-3 and phase-6 males corresponded very closely to the proportions of (−)-(2*S*, 5*S*)- and (+)-(2*R*, 5*R*)-α-pinenes in the phloem, as was reported by Lindström et al. (1988). The relative amount of Mt was very constant among males attacking different spruce trees (Figure 3), while the relative amount of tM was correlated to the amount of β-pinene, relative to α-pinene, in the host-tree phloem (Birgersson, 1989). The enantiomeric composition of Mt, which was not investigated, is probably the same as in the precursor α-pinene, as Mt is produced at a constant percentage independent of the ratios between the produced cV and tV or between (+)- and (−)-α-pinene in the host tree or during the laboratory exposures. The production of tM from β-pinene seems to be of much lower efficiency than that of cV, tV, and Mt from α-pinene; this is probably because tM is not used as a pheromone component (Schlyter et al., 1987a), but is rather, together with Mt, a product of detoxification.

Resin Production in Host Trees. Averaged over all trees, the mated males in phase 6 differed from the unmated phase-3 males mainly in their low content of MB. However, rate of decrease of MB was clearly related to resin flow (Figure 5). While there is a drastic decrease in all medians, the means for males from trees I and IV, which were classified as "resinous," showed a smaller decrease. This means that some males still have a large content of MB, even

almost one week after initiating their attack. On the other hand, there is no clear decrease in cV content of mated males (Figure 5). As in the case of MB, the amount of cV was also greater in beetles from "resinous" trees (I and IV) than in those from "dry/resin poor" (II and V) trees. Additionally, males on "dry" trees (especially tree V) decreased in cV content as attack progressed, while the males in the "resinous" trees clearly increased in their average cV content between attack phases 3 and 6. For males in tree IV, both the mean and the median increased between attack phases 3 and 6. The means of cV content for phase-3 and phase-6 males from tree I is not significantly different, but the median is more than doubled for the mated males. This increased content of cV in males from these resistant trees is probably a result of the continued resin production. Not enough beetles were attracted to the baited standing trees to reach the threshold number of beetles needed to overcome the resin flow (Berryman, 1982) of the resistant trees I and IV. A larger production and release of the aggregative cV, and also MB, by bark beetles attacking a resistant host tree, will increase the number of attracted beetles, which is in agreement with the findings by Raffa and Berryman (1983), where trees with higher resin flow attracted more of the swarming beetles. This continued host resistance probably contributed to the large variation in phase 6, for both the attractants MB and cV and probably also for Id and Ie.

Id and Ie were only found in the mated males, but neither compound was detected in more then 60% of the males in phase 6 (Table 5). The hypothesis by Hughes (1974) and Fish et al. (1979), that Ie is produced from Id, is supported by the fact that Ie was only found together with Id. Both compounds have been shown to influence the attraction of *I. typographus* to synthetic MB and cV; Id slightly increases, while Ie decreases, the attractivity (Bakke, 1976, 1981; Schlyter et al., 1987b). The trace amounts of Id and Ie that were found in the hindguts suggest that their role as natural semiochemicals might be small.

In many bark beetle species, including *I. typographus* and *I. paraconfusus*, Vn is known as an antiaggregation compound (Byers and Wood, 1980; Bakke, 1981). For some *Dendroctonus* species it has been shown that Vn is produced by the beetles themselves (Borden, 1982), even without an apparent precursor (Byers, 1983). However, for the spruce bark beetle, we were only able to detect trace amounts in the hindgut extracts. The quantitative and qualitative variations among cV, tV, and Vn during the colonization part of the attack are discussed by Francke and Vité (1983). It was recently shown that microorganisms isolated from *I. typographus* are able to convert cV and tV to Vn (Leufvén et al., 1984). In a recent study, Leufvén and Birgersson (1987) showed that the amounts of Vn in the phloem surrounding the nuptial chamber and the galleries increased as the attack proceeds, and this compound is also released from the beetles' entrance holes (Birgersson and Bergström 1989).

Variation due to Factors Within the Beetle

A large variation in the natural material remains even in males from the same attack phase and host tree (Figure 6). Unmated males in phase 3 from a certain spruce tree could (except from genetic variation) vary based on two main factors: within-tree variation of resin flow, which was, in fact, observed during the sampling, and variation due to the history of the individual beetle. This intrinsic variation could be due to the fact that an emerging individual faced with severe larval competition will, on the average, have a lower weight, fat content, and pheromone-producing capacity (Anderbrant et al., 1985). Other factors affecting the adults, such as differences in hibernation conditions and flight exercise, might also influence the physiological quality and thereby the pheromone production systems of the beetle.

The different groups of beetles exposed to α-pinene vapors in the laboratory also showed a large variability in content of volatiles, despite virtually identical environmental factors in each Petri dish during the exposure. The reason for the difference in the content of MB between males given flight exercise or not could be that the higher temperature and light levels during the flight exercise prepare the males for MB production, but exposure to α-pinene is neither a sufficient stimulus for this production, nor is α-pinene a precursor (Hackstein and Vité, 1978; Birgersson et al., 1984). The production of MB may be under a stricter hormonal or nervous (CNS) control, stimulated by their excavating behavior, as male spruce bark beetles also produce MB in large amounts after "attacking" spruce logs without a resin flow in the laboratory (unpublished results).

Some swarming bark beetles have to fly until they acquire a low lipid content before they respond to pheromone sources (Atkins, 1969). In the exposure experiments, the males were found to produce cV independent of flight exercise. The average cV content within the different groups was, instead, strongly correlated to the amounts of (−)-α-pinene to which the beetles were exposed. Compared to the different groups of beetles exposed to α-pinene, the males in attack phase 3 collected from tree IV show a distribution of cV content that is more peaked and skewed towards low amounts (Figure 6, Tables 2 and 5). The distribution of the males in each group closely resemble the frequency distributions for tV in *D. ponderosae* females exposed to α-pinene, and Id in *I. pini* males boring in lodgepole pine logs or in *D. ponderosae* males exposed to myrcene (Borden et al., 1986; Hunt et al., 1986).

The variation in *%cis* was almost the same in laboratory exposed and naturally attacking males, as it depends on the enantiomeric composition of the precursor, α-pinene. However, there is still considerable variation (CV up to 15–20%) among the males from the same exposure or host tree. For the attack-

ing males, this variation might be due to spatial or temporal differences of the enantiomeric composition in the host-tree resin.

Is it possible for a single enzyme system to produce cV and tV with this variation, or are the verbenols produced by two separate biosynthetic pathways working in parallel? This latter hypothesis is supported by the fact that *I. typographus* females collected in spruce trees contain very little cV (Birgersson et al., 1984) or none at all after exposure to (−)-α-pinene, although they do produce tV (Anderbrant et al., 1985).

In this study we have measured the content of volatiles in hindguts of male bark beetles at the time of collection. A low amount of compounds can be the result of either a high or normal production followed by a high release, or a low production and release; a large content can be the sum of either a large production and a low release, a very high production and a medium release, or a production without release. If the beetles release their semiochemicals periodically in repetitive bouts of flatulence, the amounts of these compounds in their hindguts may vary according to the production rate and the time after each single release. However, aerations for 3-hr periods outside individual entrance holes (Birgersson and Bergström, 1989) did not show this kind of variation. It is likely that there is a steady state between the amounts of volatiles produced and released, and probably the rate of production is reflected by the content of the hindguts.

Evolutionary Implications

The three species of bark beetles analyzed individually so far (Birgersson et al., 1984; Borden et al., 1986; Hunt et al., 1986) do not only demonstrate a large variance in the content of their pheromone components, but the reason for these high variances is a large positive skewness, corresponding to a large fraction of the population samples from these species that contain very small amounts of pheromone (cf. Figure 2 and Figure 1 in Schlyter and Birgersson, 1989). Such low contents (only a few percent of the mean) would imply a poor signaling capacity and consequently low mating success and low fitness for these individuals.

Variation in pheromone components seems to be higher in bark beetles than in moths (Schlyter and Birgersson, 1989). The reason might be that, while female moths call singly, bark beetles—except for a few "pioneers"—call in groups in which some males mates despite low pheromone production. There is a difference between tree-killing species, with their cooperative component, and those species that breed in windbroken or otherwise severely weakened trees or logging debris. The bark beetle species studied individually so far kill trees in mass attacks either occasionally, at high population levels (*Ips* species), or more often (*D. ponderosae*). In such species, low-producing individuals might "hitch-hike" or "sneak in" on the signal of a large group and hope for

a mate to arrive, more or less by chance, at the gallery entrance where other steps in the mating behavior, such as stridulation or physical contact, may take over. Consequently, at this high population level, selection pressure for high pheromone production will be weaker. Conversely, there should be stronger selection pressure for high pheromone production at lower population levels, when a large proportion of the individuals will find themselves pioneers or members of very small groups. The average genetic structure in a bark beetle population might be the result of alternating periods of selection pressures of different strength operating at different population densities.

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Rumex obtusifolius L: Release of Allelochemical Agents and Their Influence on Small-Scale Spatial Distribution of Meadow Species

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Abstract—Decomposing *Rumex obtusifolius* L. leaves and their extracts were most toxic for germination and root growth of meadow species *Lolium perenne*, *Trifolium repens*, *Poa pratensis*, and *Dactylis glomerata* after seven days' decomposition, although the toxicity level was, in some cases, still high after 21 days. The hypothesis that *R. obtusifolius* exerts allelopathic control over meadow species is supported by small-scale distribution of meadow species in the neighborhood of *R. obtusifolius* plants. The area affected and the intensity of the effect both increase with the size of the individual *R. obtusifolius* plant.

Key Words—Allelopathy, *Rumex obtusifolius* L., meadow species, bioassays, spatial distribution.

INTRODUCTION

It has long been recognized that plants are not randomly distributed, but exhibit patterns with respect to both their physical environment and other plants (Fowler and Antonovics, 1981). Numerous techniques for sampling and analyzing plant communities have been developed to test distribution models and to investigate the causes of the patterns observed. However, many of these techniques are unsuitable for small-scale studies required for the elucidation of interactions among plants (Turkington et al., 1985). For these purposes, the most sensitive methods are based on point-quadrat sampling procedures (Turkington et al., 1985; Fowler and Antonovics, 1981; Nicolas et al., 1980; Stowe and Wade, 1979).

One of the mechanisms by which interspecies interaction may affect plant

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distribution in natural or agricultural ecosystems is allelopathy (Rice, 1984; Bhowmik and Doll, 1984; Numata, 1982; Moreira, 1979; Putnam and Duke, 1978). It is nevertheless extremely difficult to distinguish in the field between allelopathy and competition, making it necessary that distribution studies be supported by experimental data (Jong et al., 1983; Carballeira et al., 1988). Conversely, laboratory bioassays are not by themselves sufficient to determine whether allelopathy occurs under natural conditions; most work in this area has consisted of laboratory studies unsupported by small-scale distribution analysis (Stowe, 1979).

This research was designed to determine the extent to which the capacity of *R. obtusifolius* to invade artificial grassland is due to allelochemical mechanisms, and whether such mechanisms are responsible for the poor growth of the major meadow species in the neighborhood of *R. obtusifolius* (Figure 1). Similar repression of meadow species has been observed by Einhellig and Rasmussen (1973) in the neighborhood of *Rumex crispus*; Fowler and Antonovics (1981) found that *Poa pratensis* and *Trifolium dubium* were negatively associated with *Rumex acetosella* in the neighborhood of the latter. Turkington et al. (1985) reported similar negative affinities of *Poa pratensis*, *Trifolium dubium*, *Poa annua*, and *Poa trivialis* for *Rumex acetosa*.

METHODS AND MATERIALS

Site. The site studied was an artificial meadow located in Santiago de Compostela (42°50′N, 80°27′W) at an altitude of 250 m. The meadow faces 218°N with a slope of 26:100. The soil is a humic andosol (FAO) over colluvial deposits, although small variations in depth or density create localized areas of humic cambisol with andic properties. The soil profile is polycyclic. According to Papadakis' classification, the area has a temperate maritime climate with a warm maritime temperature and a humid regime (Carballeira et al., 1983). The mean temperature was 12.9°C, the mean minimum of the coldest month 5.2°C and the mean maximum of the warmest month 23.3°C. Mean summer rainfall was 137 mm and mean annual rainfall 1288 mm. The meadow was established five years ago with a mixture of *L. perenne*, *T. repens*, *P. pratensis*, and *Dactylis glomerata* and was periodically treated with slurry and mineral fertilizer rich in phosphorus and nitrogen.

Bioassays. Juste et al. (1985) found that the main route by which *R. obtusifolius* releases allelochemical agents into the environment is the decomposition of fallen leaves. Accordingly, leaves were picked from *R. obtusifolius* plants at the study site. A sample of these leaves was used to determine the ratio of fresh weight to dry weight at 80°C.

Fresh leaf material was divided into lots equivalent to 30 g dry weight, 30

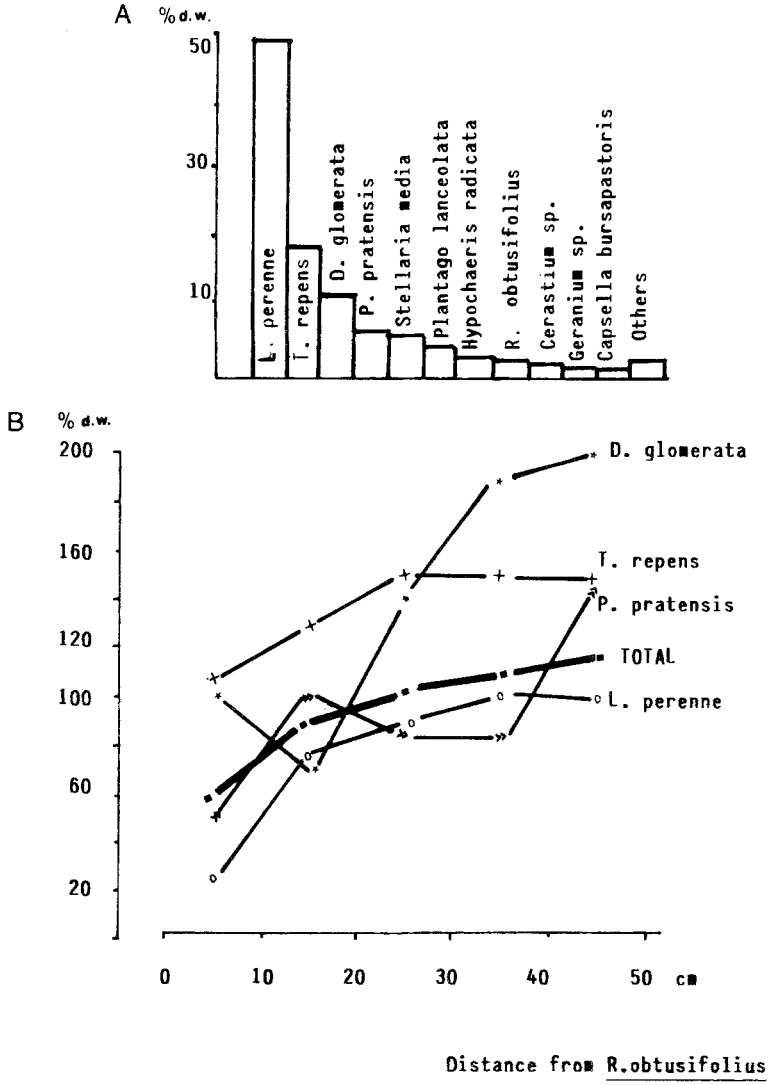

FIG. 1. (a) Composition of the biomass of the study site. (b) Variation of biomass in the neighborhood of *R. obtusifolius*.

of which were placed in 12-mm mesh nylon litter bags. The bags were placed in groups of six at five random points of the study site, and 7, 14, and 21 days later two bags were removed from each group and the contents of each pair pooled. A sample was taken from each pool for dry weight determination, and

the remainder of the material recovered was used for bioassays. Bioassays were also performed on freshly collected leaves (day 0).

Three bioassays were performed, two using leachates (one twice as strong as the other) and one using the leaves as picked or recovered from the litter bags (Woods and Raison, 1982; Kelman and Lang, 1982). For the stronger leachate (1 : 4), leaves saturated with distilled water were left in the dark for 24 hr at 20°C and then gently sprayed with distilled water until the collected drippings amounted to 4 ml/g dry weight. The weaker leachate (1 : 8) was obtained by diluting a portion of the 1 : 4 leachate to twice its volume. For each meadow species whose sensitivity was to be tested, both leachates were bioassayed by sowing 50 seeds on Whatman 3MM paper in a Petri dish and sprinkling them with 4 ml of leachate. For the third bioassay (H), 50 seeds were sown on Whatman 3MM over a layer of leaves on the bottom of a Petri dish and sprinkled with 4 ml of distilled water. In all cases, five replicate dishes were prepared, one from each group of litter bags. Dishes with 50 seeds sown on Whatman 3MM and sprinkled with 4 ml of distilled water were used as controls. All dishes were incubated in the dark at 28 ± 1°C and 80% humidity. Percentage germination and root growth were determined for *T. repens* and *L. perenne* after 144 hr, and for *P. pratensis* and *D. glomerata* after 216 hr.

The data were tested by two- and three-way analysis of variance (Vesereau, 1960).

Spatial Distribution. The spatial distribution of the meadow species considered with respect to *R. obtusifolius* was investigated using a modified form of Stowe and Wade's (1979) "species-region" method. Of the *R. obtusifolius* plants of 20 cm or more in diameter in the study site, 160 were chosen at random. Four 1-m-long transects were drawn radially from the outer edge of each plant's projection area, one in the direction of maximum upward slope and the others at 90° intervals about the plant. A 1-m frame holding a 60-cm sliding needle was placed over each transect. The needle was shifted centimeter by centimeter along the frame, and at each centimeter point the species, if any, in contact with the needle at ground level was recorded, regardless of the organ touched.

The species-region method compares the relative frequencies of occurrence of a secondary species B with respect to the average plant population density inside and outside the region of influence R_1 of a primary species A. Specifically, the tendency of B to associate with A is measured by an association index: $AI = (ad)/(bc)$, where a is the number of occurrences of B within R_1; b is the number of occurrences of species other than B or A in R_1; c is the number of occurrences of B in R_2, a region assumed to be free from A's influence; and d is the number of occurrences of species other than B or A in R_2. $AI < 1$ indicates negative affinity of B for A, $AI > 1$ positive affinity, and $AI \simeq 1$ indif-

ference. A χ^2 test may be used to establish the statistical significance of the observed value of *AI*.

In the present study, the dependence of association on distance was investigated in two ways. In the first (the "accumulative" method), R_1 was defined as a segment of the transect comprising the *r* cm nearest the *R. obtusifolius* plant. In the second (the "annular" method), R_1 was defined as a 10-cm segment centered *r* cm from the *R. obtusifolius* plant. In both cases, the 50 cm furthest from the *R. obtusifolius* plant were used as R_2 (the validity of this was checked in preliminary experiments), and *AI* was plotted against *r*.

RESULTS

Bioassays. The rate of decomposition during the time the picked leaves were exposed in the litter bags was limited only by temperature (Esser et al., 1982). Thermal efficiency was low, and the differences among the three sampling times were small (Figure 2). Heavy rains during the study probably caused leaching of the leaves, thus reducing the concentration of toxins in subsequent bioassays.

Figure 3a shows that whatever the meadow species, germination and root growth averaged over the four sampling times were most depressed in the decomposing leaf bioassay, H. The relative sensitivities of the species were not the same for both germination and root growth in this assay, the rankings of *L. perenne* and *D. glomerata* being interchanged. For all the species, the inhibition averaged over the three kinds of assay was greatest when material exposed for seven days was used (Figure 3b). The extracts from this material were those of lowest pH and highest electrical conductivity (Table 1). In the decomposing

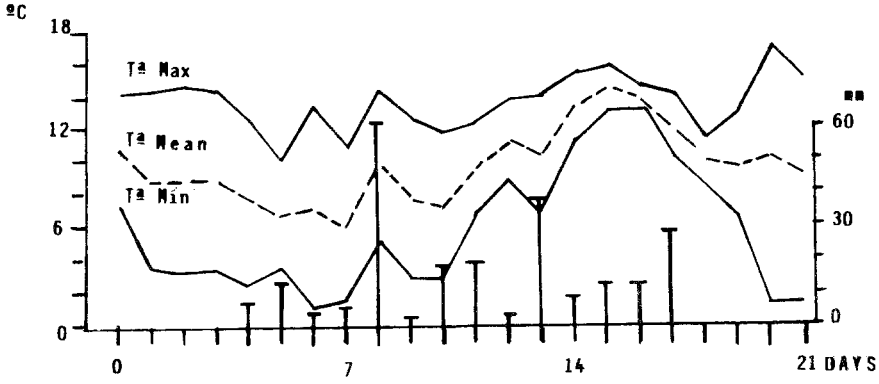

FIG. 2. Temperature and rainfall (vertical bars) during exposure of *R. obtusifolius*.

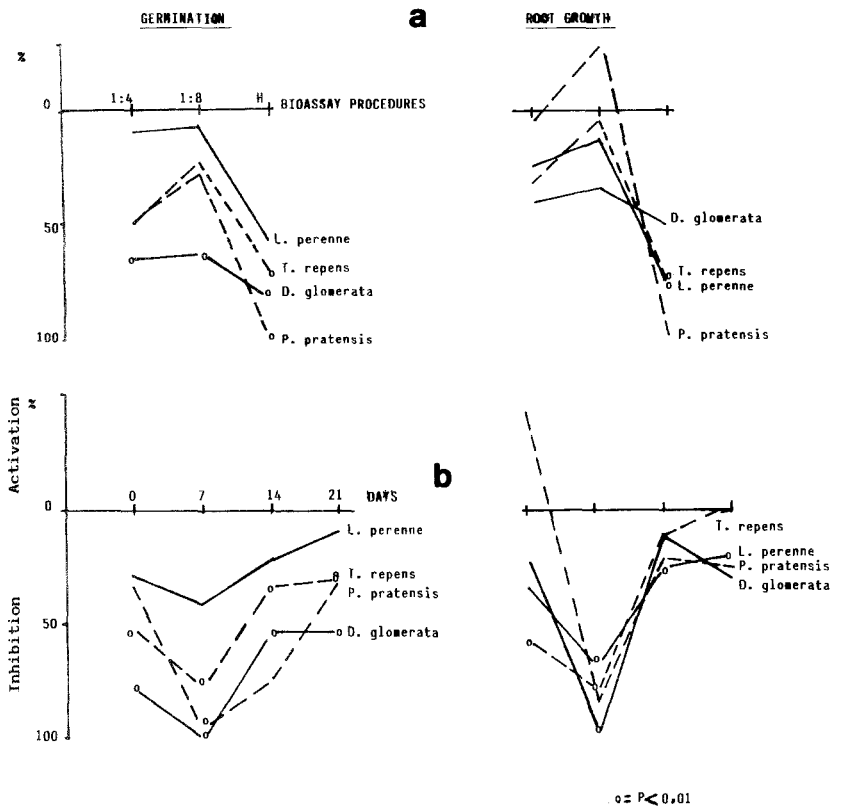

FIG. 3. Bioassay results (split-plot analysis). (a) Effects of three bioassay procedures on the germination and root growth of meadow species (average for leaf material obtained after 0, 7, 14, and 21 days of decomposition). Leachate concentration 1:8, 1:4, and decomposing leaf (H). (b) Effects of decomposition time on the germination and root growth of meadow species (average for three bioassay procedures).

TABLE 1. ACIDITY AND ELECTRICAL CONDUCTIVITY OF LEACHATES USED FOR BIOASSAYS

Bioassay procedure	Day			
	0	7	14	21
pH				
1:4	6.2	5.2	6.5	7.1
1:8	5.9	5.1	6.4	6.9
Conductivity (μmhos)				
1:4	930	3500	1520	1880
1:8	480	1200	840	960

leaf bioassay, germination and root growth averaged over all four meadow species was still inhibited by over 50% by material exposed for 21 days (Figure 4). The two leachates behaved very similarly at all times, in spite of their difference in concentration, and both were inactive when obtained from material exposed for 14 days or more.

The above results suggest that the compounds released by the *R. obtusifolius* leaves are very labile, and their continuous low-level application (as in the decomposing leaf test, the assay closest to natural conditions) is more effective than a large single dose.

Spatial Distributions. The data obtained in the field were initially analyzed

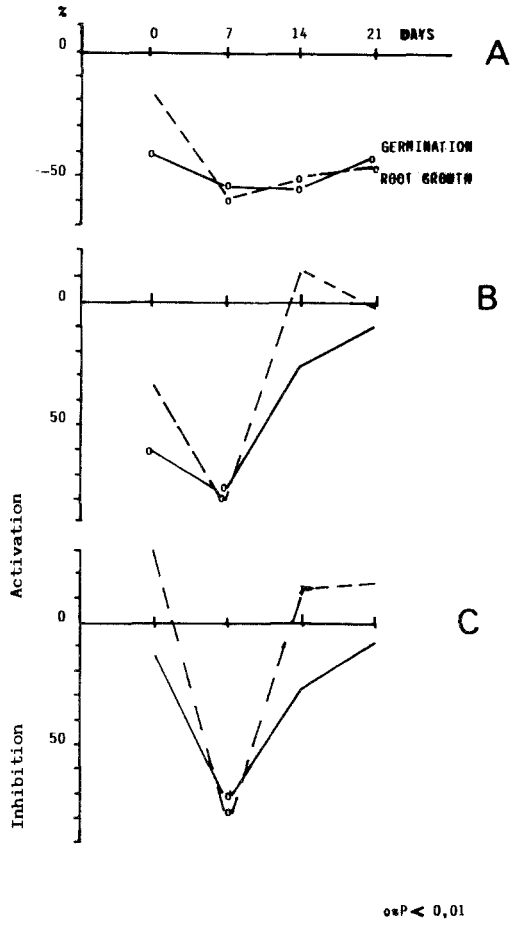

FIG. 4. Effects of decomposition time on the germination and root growth of meadow species (average for the four species studied): (A) in the decomposing leaf assay H; (B) using leachate 1:4; and (C) using leachate 1:8.

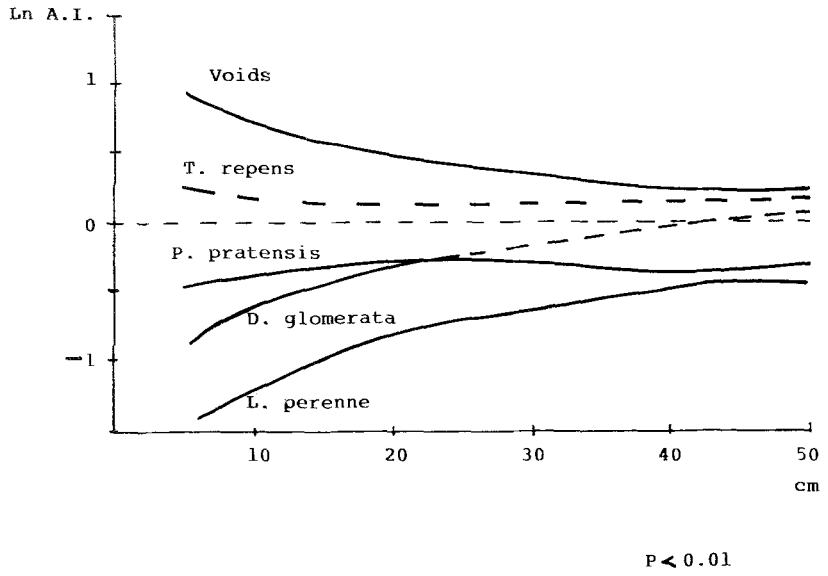

FIG. 5. Dependence of association index, *AI*, on the hypothetical radius of the domain of influence of *R. obtusifolius* as calculated by the accumulative method for four meadow species and voids.

by the "accumulative" method (Figure 5). As the radius of the hypothetical region of influence increased *AI* tended to indifference levels. Except in the case of *D. glomerata*, the weight of the inner few centimeters kept *AI* away from unity, so that it was impossible to determine the radius of the real sphere of influence. To overcome this difficulty, the data were reprocessed using the "annular" method (Figure 6), which showed that voids (absence of any of the four meadow species) were significantly associated with *R. obtusifolius* in the first 20 cm, while *D. glomerata*, *L. perenne*, and *P. pratensis* had negative affinity for *R. obtusifolius* in the inner 15, 35, and 50 cm, respectively. *T. repens* exhibited a slight tendency towards positive affinity.

DISCUSSION

For only one of the four meadow species studied did the spatial distribution results contradict the laboratory findings. *T. repens* was inhibited in the bioassays, but was largely indifferent to the presence of *R. obtusifolius* in the field, with perhaps a slight tendency to positive affinity. This contradiction may be due to the sampling procedure, which recorded all contacts with the sampling

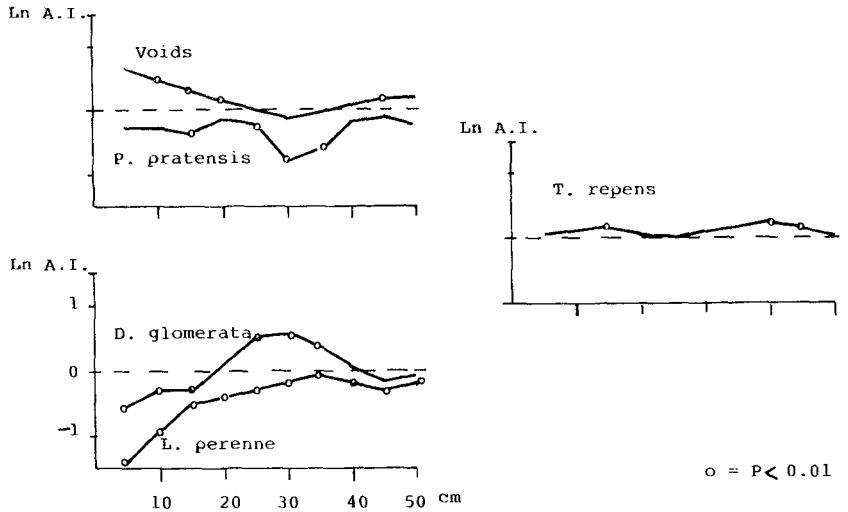

FIG. 6. Dependence of the association index, *AI*, on mean annular radius about *R. obtusifolius* as calculated by the annular method for four meadow species and voids.

needle at ground level, regardless of which part of the plant was touched. Because of the life form of *T. repens*, its aerial structures can lie close to *R. obtusifolius* without there being any competition or allelochemical interaction so long as the clover is rooted outside the influence of the other plant. The life forms of the other meadow species sutdied do not present this complication. With respect to their performance in the decomposing leaves bioassay, H (the assay most similar to natural conditions), they can be ranked by decreasing inhibition of root growth in the order *P. pratensis*, *L. perenne*, and *D. glomerata*, and by decreasing inhibition of germination in the order *P. pratensis*, *D. glomerata*, and *L. perenne*.

The range and degree of these plants' negative affinities for *R. obtusifolius* (as measured by the annular method) exhibit the same ordering as the inhibition of their root growth, which suggests that chemical interference by *R. obtusifolius* is sufficient to reduce their development but not enough to exclude them completely from the neighborhood of *R. obtusifolius* plants. This is in keeping with the current concept of allelopathic interaction, for although these mechanisms are widespread in both natural and agricultural ecosystems, their role should not be exaggerated, but considered merely complementary to other strategies (Muller, 1966). It is clear that even by causing quite small reductions in photosynthetic efficiency or nutrient uptake rates, an allelopathic agent may successfully place a potential competitor at a disadvantage.

Kershaw (1973), in listing the possible causes of patterns in plant com-

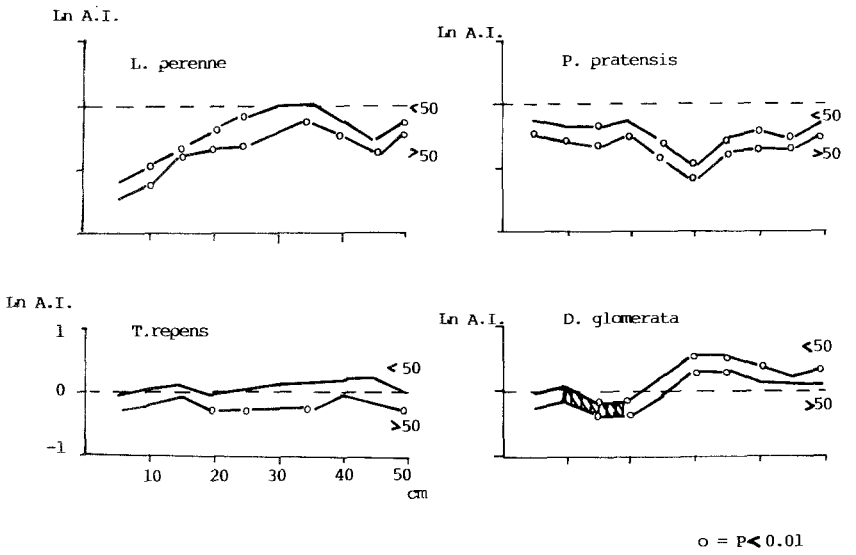

FIG. 7. Variation of association index, *AI*, with distance (annular method) for *R. obtusifolius* plants greater or less than 50 cm in diameter. Shaded area indicates statistically significant differences ($P < 0.01$).

munities as competitive capacity, environmental conditions, and allelopathic interactions, pointed out that the release of toxins may depend on the age of the individual. To investigate this question, we divided *R. obtusifolius* plants studied into two groups according to the diameter of their rosettes (73 plants of 20–50 cm diameter and 87 of 50–74 cm diameter) and performed separate analyses of the spatial distributions of the meadow species studied in the neighborhood of plants of each group (finer classification of diameters produced very unequal class sizes or classes with too few members). The results (Figure 7) confirm that the negative influence of *R. obtusifolius* on the meadow species increases with the size of the *R. obtusifolius* plant, although the difference between the two size classes considered was only statistically significant for the presence of *D. glomerata* between 10 and 20 cm from *R. obtusifolius*.

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Journal of Chemical Ecology, Vol. 14, No. 9, 1988

ASYMMETRIC SMALL-SCALE DISTRIBUTION AND ALLELOPATHY: Interaction between *Rumex obtusifolius* L. and Meadow Species

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Abstract—Analysis of small-scale distribution may allow allelopathic interaction among plant species to be distinguished from competitive interaction. If all environmental factors except slope are uniform, allelopathy produces spatial asymmetry in the association with a hypothetical receptor species and the corresponding hypothetical emitter species in the neighborhood of the latter. The interaction between *Rumex obtusifolius* and the meadow species *Lolium perenne*, *Poa pratensis*, *Dactylis glomerata*, and *Trifolium repens* is analyzed by way of example.

Key Words—Allelopathy, small-scale spatial distribution, slope, *Rumex obtusifolius*, meadow species.

INTRODUCTION

It is well known that in the field it is extremely difficult to distinguish allelopathic interactions among different plant species from competitive relationships. Studies of allelopathy are accordingly usually carried out in the laboratory, and their results are assumed to hold under natural conditions; however, the validity of such a generalization is questionable (Harper, 1977; Stowe, 1979). At what concentrations should extracts be bioassayed? How do the soil microorganisms affect the compounds released by the plants studied? Do laboratory procedures create artifacts?

A necessary condition for the valid extrapolation of laboratory findings to the field is that they be compatible with the spatial microstructure of the vegetation observed under natural conditions. Thus, of all the possible combinations of bioassay response and observed association, all those in the bottom group in

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Table 1 imply that the laboratory results are probably irrelevant to the processes predominating under natural conditions. However, compatibility is by no means sufficient to guarantee the reliability of bioassay-based conclusions. These will only be valid if the assay conditions adequately reflect natural conditions and, since uncertainty in this regard is the rule, there is a considerable risk of mistaking allelopathic interference for competition or vice versa.

These difficulties would be avoided if it were possible to tell allelopathic from nonallelopathic interactions without recourse to laboratory bioassays. Dekker et al. (1983) suggested that it is sufficient to observe the association between a hypothetical allelopathic species and its hypothetical receptors when they are grown together under conditions which ensure the absence of competition by providing an abundance of all resources. However, this method is often impractical because of costs in time or money or because of technical difficulties associated with the cultivation of noncommercial species. In any case, the results are not necessarily applicable to natural conditions, because it is known that in certain species the release of allelopathic agents is triggered or increased by stress, which may likewise affect the sensitivity of receptors (Rice, 1984).

Here we describe a method for distinguishing allelochemical interactions by analyzing the relative spatial distribution of the hypothetical emitter and receptor under natural conditions. The only limitation to its application is that the site studied should be uniform as to shade, availability of water, depth of soil, etc., but steep enough for gravity to have a significant effect on any allelochemical agent. The ideal location is a hilltop from which shallow slopes descend in all directions. The hypothesis on which the method is based is that under these conditions, gravity should favor downhill flow of aerial leachates, litter, and root exudates. In the neighborhood of the plant from which they

TABLE 1. IMPLICATIONS FOR INTERSPECIES INTERACTIONS OF CORRELATION BETWEEN BIOASSAY RESULTS AND OBSERVED SMALL-SCALE SPATIAL DISTRIBUTION

Response of supposed receptor in bioassay	Observed spatial association	Inferred interaction
Indifference	indifference	no interaction
Activation	positive	allelochemical stimulation
Inhibition	negative	allelopathy
Indifference	negative	competition
Inhibition	positive	bioassay results invalidated
Activation	negative	
Indifference	positive	
Inhibition	indifference	
Activation	indifference	

originate, this may give rise to a difference between uphill and downhill areas as affected by any allelochemical agents produced. Any such uphill–downhill asymmetry in the index of association between a hypothetical receptor and the corresponding hypothetical emitter in the neighborhood of the latter is therefore evidence in favor of the existence of allelochemical interaction (which may, of course, be superimposed upon simultaneous competitive interaction).

ASSOCIATION INDICES

The method used to measure the association between the hypothetical emitter and its receptors is a variant of the "species-region" technique proposed by Stowe and Wade (1979). This technique compares the relative frequencies of occurrence of a secondary species, B, with respect to the average plant population density inside and outside the domain of influence, R_1, of a primary species, A. Specifically, the tendency of B to be associated with A is measured by an association index, $AI = (ad)/(bc)$, where: a is the number of occurrences of B within R_1; b is the number of occurrences of species other than B or A in R_1; c is the number of occurrences of B in R_2, a region assumed to be free of A's influence; and d is the number of occurrences of species other than B or A in $R_2 \cdot AI < 1$ indicates negative affinity of B for A, $AI > 1$ positive affinity, and $AI = 1$ indifference. A χ^2 test may be used to establish the statistical significance of the observed values of AI. The dependence of association on the distance d from the primary species A may be investigated be defining as R_1 an annular region with a central ring of radius d, and plotting AI against d (since the regions R_1 and R_2 are in practice usually segments of transect, the "annulus" is in such cases a segment centered at distance d from A).

SPATIAL ASYMMETRY IN RESPONSE TO ALLELOCHEMICALS

In analyzing natural spatial distributions in accordance with the hypothesis set forth in the Introduction, the substance whose effects are being investigated is, of course, the aggregate of compounds released by the emitter plant. The overall activity of this aggregate may be considered as hormonal if, at some biologically realistic concentration, it stimulates the receptor species, and as toxic if its only action at biologically realistic concentrations is to depress the receptor species. Idealized concentration–response curves for these two types of activity are shown in Figure 1A and B. When the effect of slope in increasing the concentration of the allelochemical downhill from the plant is taken into account, it may be deduced that there are 13 possible kinds of distribution patterns manifesting the difference between uphill and downhill areas. These patterns are illustrated schematically in Figure 2, and the range of hormone or toxin

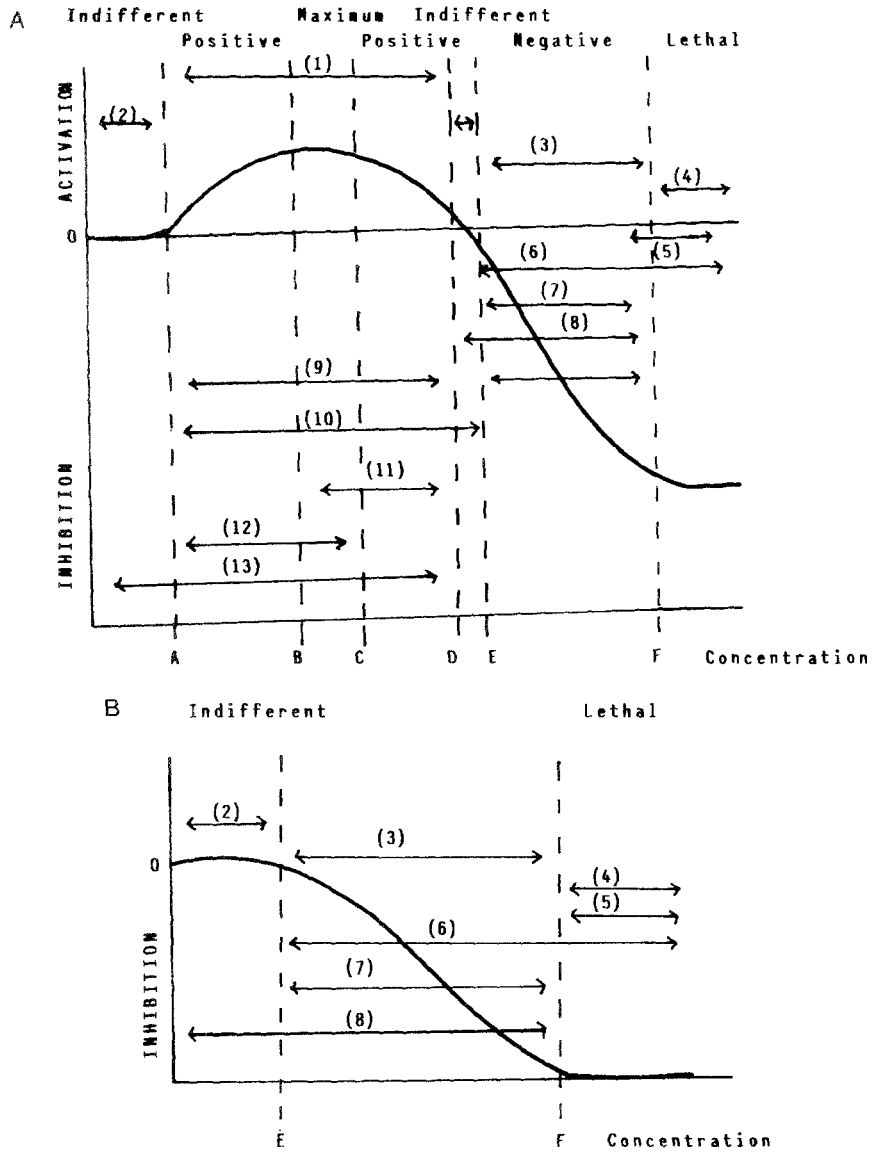

FIG. 1. Idealized response to hormonal (A) and toxic (B) allelochemical agents. Numbers in parentheses indicate the distribution pattern types (Table 2) produced by the corresponding concentration ranges.

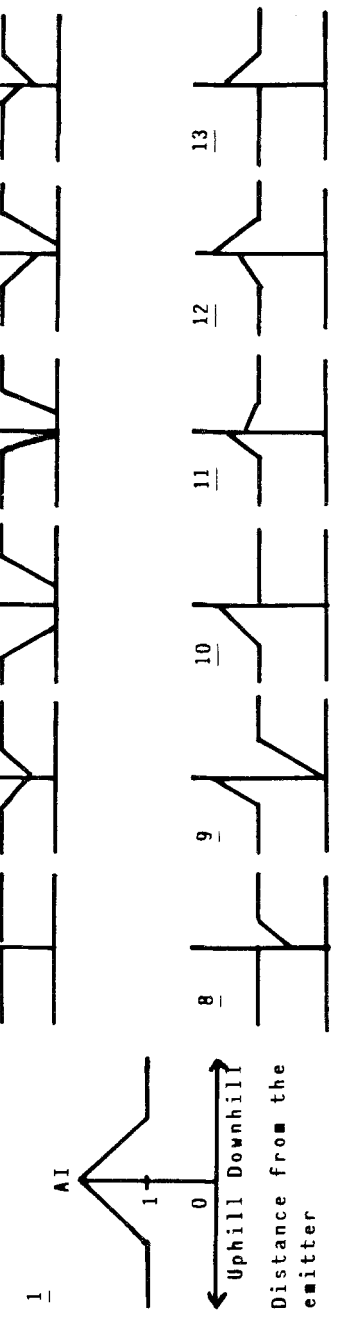

FIG. 2. Schematic representations of the 13 types of interference pattern (Table 2).

concentrations responsible for each is indicated on Figure 1A and B. Pattern 4, for example, may be produced by both hormonal and toxic agents as long as the concentrations in both downhill and uphill directions are greater than those corresponding to level F in Figure 1A and B. Table 2 lists the 13 patterns together with their implications for the type of interspecies interaction (allelopathy, sympathy, competition, or indifference) and, in the case of allelochemical interactions, for the kind of agent (hormonal or toxic). Patterns 2–8 can result from the action of either hormonal or toxic agents, whereas pattern 1 and patterns 9–13 can only be explained as the effect or hormonal allelochemicals.

EFFECT OF DISTANCE ON *AI*

If the interaction between two plant species is mediated by allelochemicals, the intensity of this interaction, and hence of the observed association index, depends not only on the intrinsic affinity of the secondary species for the primary species, but also on the distance between the specimens observed. In the diagrams of Figure 2, for example, it has been tacitly assumed that for a given

TABLE 2. INFERENCE OF INTERSPECIES INTERACTION FROM UPHILL–DOWNHILL ASSOCIATION PATTERN

Type	Association uphill–downhill	Type of interaction	Type of chemical agent
Symmetric			
1	Positive–positive	sympathy[a]	hormonal
2	Indifference–indifference	indifference	
3	Negative–negative	allelopathy or competition	hormonal or toxic
4	Exclusion–exclusion	allelopathy or competition	hormonal or toxic
Asymmetric			
5	Exclusion < exclusion	allelopathy	hormonal or toxic
6	Negative–exclusion	allelopathy	hormonal or toxic
7	Negative > negative	allelopathy	hormonal or toxic
8	Indifference–negative	allelopathy	hormonal or toxic
9	Positive–negative	sympathy–allelopathy	hormonal
10	Positive–indifference	sympathy–allelopathy	hormonal
11	Positive > positive	sympathy	hormonal
12	Positive < positive	sympathy	hormonal
13	Indifference–positive	sympathy	hormonal

[a] Sympathy = allelochemical stimulation.

intrinsic affinity, the observed association depends linearly on distance within the area influenced by the primary species. This is not necessarily so, of course.

Let us consider an emitter plant, the radius of whose projection area is $r = 20$ cm (roughly the radius of the *R. obtusifolius* plants discussed in the next section). Let d be the distance from the receptor plant to the edge of the emitter's projection area, $s = \Pi r^2$, $s' = \Pi[(r + d)^2 - r^2]$, $v = \Pi r^3 2/3$, and $v' = \Pi[(r + d)^3 - r^3]2/3$. Then at distances $d = 4$, 10, 20, and 40 cm the ratio $r/d = 5$, 2, 1, and 0.5, respectively; the ratio $s/s' = 2.27$, 0.8, 0.3, and 0.25; and the ratio $v/v' = 1.37$, 0.43, 0.14, and 0.038. For the purposes of this idealized example, we shall suppose that the biological response to an allelochemical agent is proportional to its concentration, and we shall define the affinity between two species as "intense" when at a distance $d = r/5$, $AI > 10$ (for positive affinity) or $AI < 0.1$ (for negative affinity). Then the AI threshold for intense affinity, which depends on distance, will approach unity at distances $d = 40$, 25, or 20 cm, depending on whether the dispersion of the allelochemical takes place in one, two, or three dimensions. Figure 3 illustrates, for several such arbitrary levels of affinity, the theoretical dependence of the allelochemi-

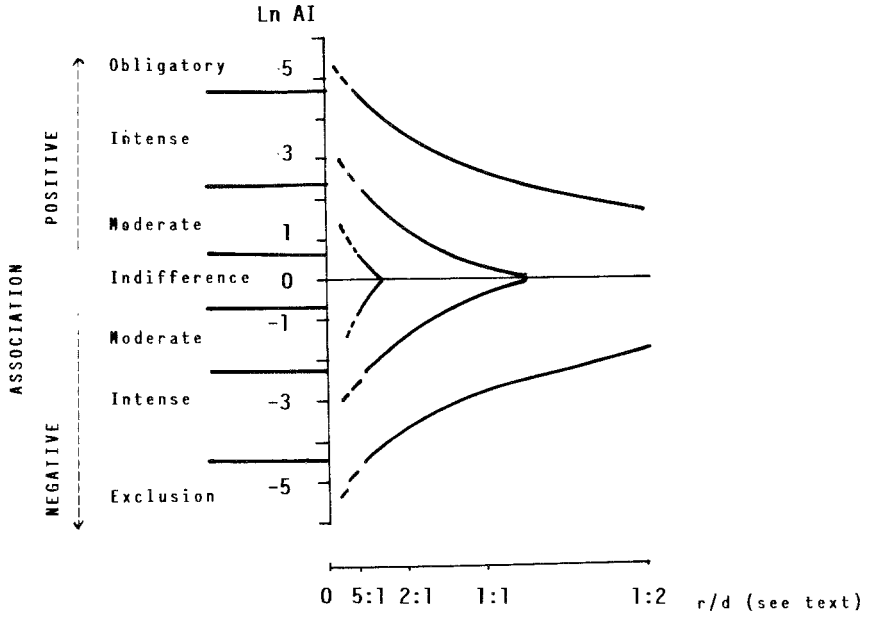

FIG. 3. Theoretical variation of AI with distance from the emitter plant in the case of two-dimensional dispersion at a slope site.

cal's activity on distance under the assumption that dispersion takes place in two dimensions (s/s').

In the idealized model considered in the previous paragraph, the decline in the activity of the allelochemical is due simply to the reduction in its concentration as the area or volume to be impregnated increases. If losses due to infiltration, retention by soil, and degradation by microflora are also taken into account, it seems very unlikely that allelochemical agents transported through the soil can have any appreciable effect at distances greater than one emitter plant radius from the edge of the emitter's projection area. Agents dispersed by other means, such as animals or wind, could of course be effective over much larger areas (Lamont, 1985).

Reasoning along the lines sketched above explains how the association between two species may change sharply within very short distances (Figure 4) and why it is necessary to use sampling intervals of the order of 1 cm in order to analyze the spatial distribution of herbaceous or shrubby vegetation.

INTERACTION BETWEEN *R. obtusifolius* AND MEADOW SPECIES

The theory set forth above was tried out using the field observations of Carral et al. (1988), who, on comparing the results of laboratory bioassays with

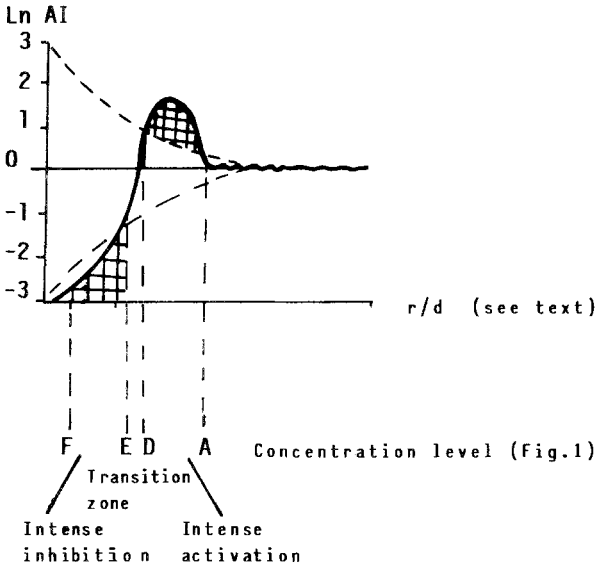

FIG. 4. Illustration of how a radical change in *AI* might be produced over a very short distance (E–D) in response to a hormonal allelochemical.

the observed association between the primary species *R. obtusifolius* and four meadow species, concluded that the invasion of artificial meadow by *R. obtusifolius* and its predominance therein is due to allelopathic mechanisms. The site studied was an artificial meadow located in Santiago de Compostela (42°50′N; 8°27′W) at an altitude of 250 m. According to Papadakis' classification the area has a warm maritime climate with a humid humidity regime. The meadow faces 218°N with a slope of 26 : 100, and its soil is a humic andosol (FAO). Established five years ago with a mixture of *Lolium perenne*, *Trifolium repens*, *Dactylis glomerata*, and *Poa pratensis*, it is periodically treated with slurry and with mineral fertilizer rich in nitrogen and phosphorus.

The spatial distribution of meadow species relative to 160 randomly chosen *R. obtusifolius* plants greater than 20 cm in diameter was studied by considering two 1-m transects drawn radially from the outer edge of each plant's projection area, one in the direction of maximum upward slope and the other diametrically opposite. A 1-m needle frame was placed over the transects, and the vegetation at ground level sampled at 1-cm intervals. The R_1s used to investigate the dependence of *AI* on distance were overlapping 10-cm segments of the transects (0–10, 5–15, 10–20 cm, etc.). R_2 was the segment of the transect comprising the 50 cm furthest from the *R. obtusifolius* plant (the validity of this was checked in preliminary experiments).

Figure 5 shows the ratio between total cover at various distances from *R. obtusifolius* and the mean total cover of the site. *L. perenne* accounts for 48% of the latter figure, *T. repens* for 18%, *D. glomerata* for 13%, and *P. pratensis* for 8%. The remaining 13% is provided by *R. obtusifolius* and the minority species *Stellaria media*, *Plantago lanceolata*, *Hypochaeris radicata*, *Capsella*

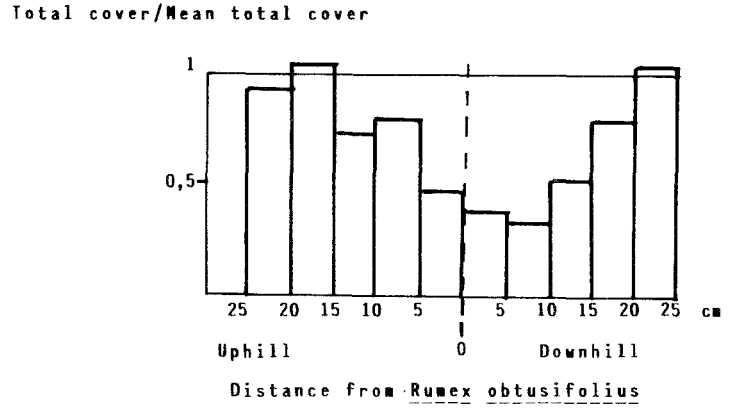

FIG. 5. Variation of relative cover with distance uphill and downhill from *Rumex obtusifolius*.

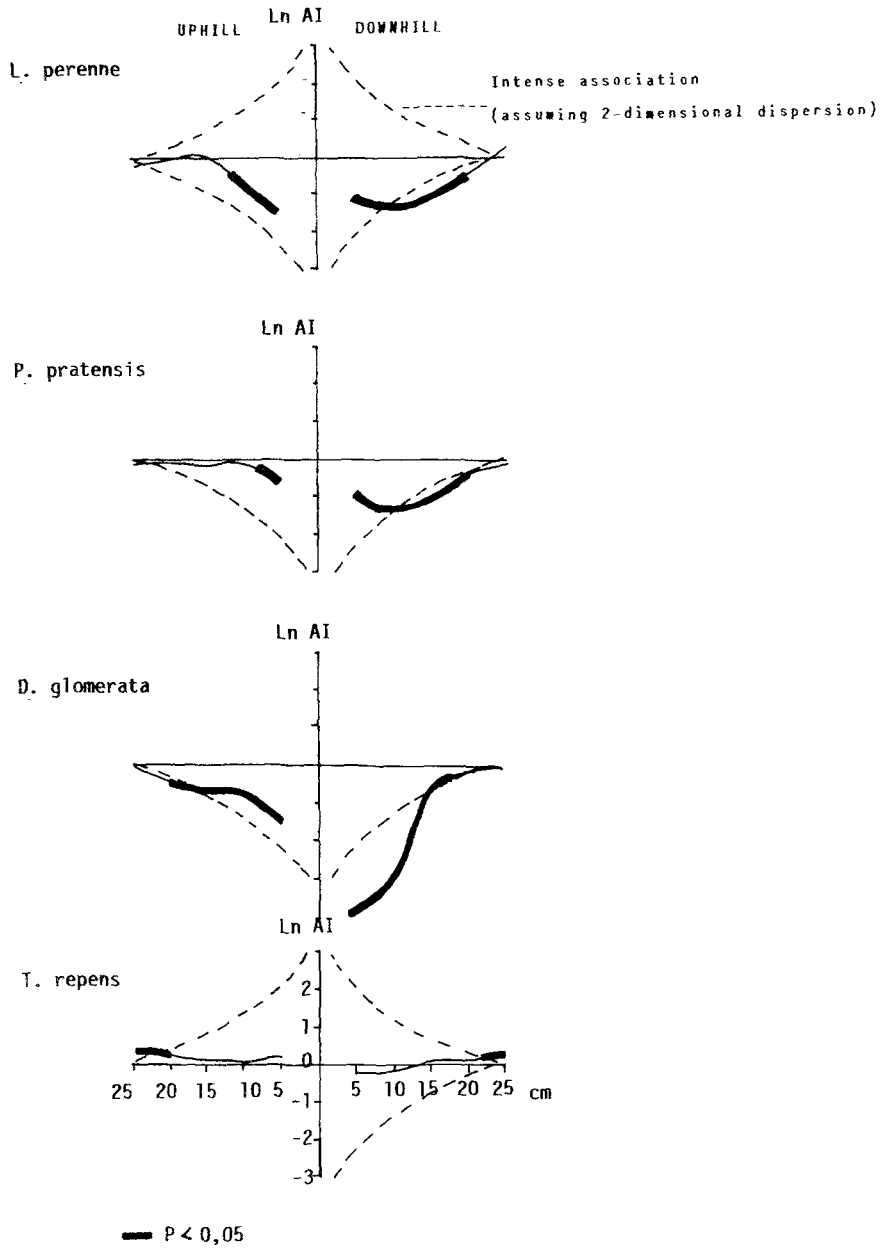

FIG. 6. Influence of distance uphill and downhill from *Rumex obtusifolius* on the *AI* between this primary species and four meadow species. Bold segments indicate that the *AI*s are statistically significant ($P < 0.05$), and dashed curves show the theoretical threshold of intense affinity for two-dimensional dispersion in the absence of slope (see Figure 3).

bursa-pastoris, *Cerastium* sp., and *Geranium* sp. In keeping with the hypothesis being tested, the thinning produced by *R. obtusifolius* can be seen to be of greater intensity and range downhill than uphill and to be limited in either case to a radius of 25 cm (the mean radius of the *R. obtusifolius* plants was 21 cm, with a minimum of 10 cm and a maximum of 36 cm).

Figure 6 shows graphs of *AI* against distance up or downhill for each of the four meadow species studied by Carral et al. (1988). The remaining species were not studied because of their low density, and indeed, it is questionable whether the density of *P. pratensis* was sufficient for the results for this species to be very meaningful. Figure 7 shows the distances for which there was a statistically significant difference between uphill and downhill *AI* (irrespective of the statistical significance of *AI*s compared). According to the classification of Table 2, it can be seen that the pattern presented by *T. repens* was type 2 (indifference–indifference, implying no interaction), with perhaps a slight tendency to type 9 (positive–negative, implying hormone-like interaction). The other species exhibit evident negative affinity for *R. obtusifolius*, with uphill–downhill asymmetries of type 7 in the cases of *L. perenne* and *P. pratensis* (negative > negative, implying hormonal or toxic allelopathy) and, in the case of *D. glomerata*, type 7 or 6 (negative–exclusion, with the same implication).

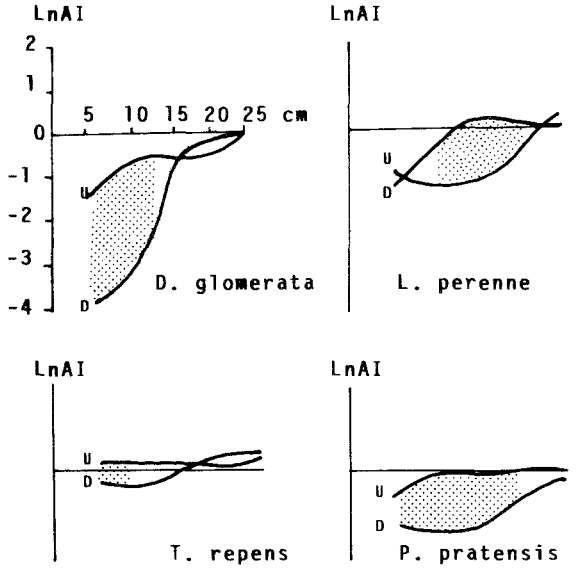

FIG. 7. Influence of distance uphill (U) and downhill (D) from *Rumex obtusifolius* on the *AI* between this primary species and four meadow species. Shading indicates distances at which the difference between the two curves is statistically significant ($P < 0.05$).

In view of the above results, we conclude that analysis of the small scale slopewise distribution of hypothetical receptors relative to a hpothetical emitter species is a simple, valid means of detecting the existence of allelopathic processes prior to subsequent research on the routes by which allelochemical agents are released, their chemical composition, the relationship between environmental factors and allelopathic efficiency, or the development of bioassays which adequately reflect natural conditions.

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Journal of Chemical Ecology, Vol. 14, No. 10, 1988

Editor's Note

The fourth annual meeting of the International Society of Chemical Ecology was held on July 13–17, 1987, at the University of Hull, England. This represented the first meeting of the society outside of North America since its founding in 1983. In keeping with the practice of the previous meetings, four symposia were featured, highlighting recent progress in diverse aspects for chemical ecology. These were: The Veracity of Bioassays in Chemical Ecology, Chemical Ecology and Plant Protection, The Applications of Chemical Ecology, and Biochemical Mechanisms and Defense Compounds. As official journal of the society, the *Journal of Chemical Ecology* is dedicating this issue to the proceedings of those symposia.

Each invited symposium paper has undergone peer review in accordance with the standard review policy of the Journal. We are grateful to the Chairman of the meeting, D. A. Jones, for his assistance in coordinating receipt of the manuscripts and to the following reviewers: G. J. Bakus, M. R. Berenbaum, U. Blum, J. M. Bradow, J. P. Bryant, B. C. Campbell, J. Cane, P. J. Canney, P. F. Dowd, S. R. Gliessman, A. E. Hagerman, M. B. Isman, K. F. Haynes, J. A. Juvik, I. Kubo, A. F. Moldenke, D. H. Netzly, F. Plapp, T. Robinson, J. R. Shann, C. M. Smith, J. N. M. Smith, J. F. Sutcliffe, N. M. Targett, and E. W. Underhill.

We acknowledge the cooperation of Plenum Publishing Corporation in producing extra copies of this issue to accommodate registrants of the meeting and others interested in the proceedings under this single cover.

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Journal of Chemical Ecology

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Proceedings Coeditors

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PLANT POLYPHENOLS (*syn.* VEGETABLE TANNINS) AND CHEMICAL DEFENSE—A REAPPRAISAL

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Abstract—Many of the hypotheses adumbrated to rationalize the role of natural products in plant–herbivore interactions have focused attention on plant polyphenols (syn., vegetable tannins). Contemporary interpretations of the importance of plant polyphenols rest largely on the assumption that they act via their capacity to bind to proteins. The central proposition of plant–herbivore interactions, namely that plants, as a response to environmental pressures, have evolved the strategem of a chemical armory appropriate to the challenges they face, is examined in the context of plant polyphenols—their ability to complex to protein and their possible function as structural polymers.

Key Words—Plant polyphenols, vegetable tannins, biosynthesis, structure-activity relationships, protein complexation, proanthocyandidins, structural polymers.

INTRODUCTION

In comparison with the ubiquitous intermediates of primary metabolism, an infinitely greater body of natural products such as alkaloids, phenols, polyenes, polyacetylenes, terpenes, and polyphenols (tannins) occurs sporadically throughout the plant kingdom. Despite a great deal of thought, speculation, and research, they still frustrate all attempts to fit them readily into the general framework of higher plant metabolism and hence the economy of the producing organism. Because of their apparently secondary role, these substances are frequently referred to as secondary metabolites; as Bu'Lock (1980) perceptively noted, they express the individuality of species in chemical terms.

Over the past 30 years, a substantial literature has accumulated that sug-

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gests a great many of these secondary substances are intimately concerned in the complex interactions between plants and their environment and that this is the raison d'dêtre for their existence. Various workers, most notably Fraenkel, Ehrlich and Raven, Feeny, Rhodes, Janzen, and Cates, among others, were responsible for the emergence of this view that secondary substances play the leading role in the determination of patterns of plant utilization by herbivores. This theory, which has an immediate intuitive appeal, has gained increasing prominence and has stimulated an exponential increase in work in this area over the past decade. However, the development of these ideas into a comprehensive theory of plant and animal coevolution sit, in the intellectual sense, far less comfortably, in particular the central proposition that plants, as a response to environmental and ecological challenges, have evolved the strategem of a chemical armory appropriate to the pressures that they face.

The intellectual effect of this theory has been hypnotic. However, should the metabolic significance of particular secondary metabolites be sought exclusively in terms of its relationship with predators, competitors, and its environment or in the context also of the plant's internal economy? This alternative perspective is given prominence here in relationship to the purported role of plant polyphenols (syn., vegetable tannins) in chemical defense. There is, as yet, no compelling evidence that tannins have any function in the physiological processes of plants, and, since Feeny's seminal papers (1968, 1970) on the deleterious effects of dietary tannins on the feeding of the larvae of the winter moth (*Operophtera brumata*) on oak, the view has developed that tannins constitute a unique quantitative defense. Feeny surmised that polyphenols are characteristic of the chemical defense of apparent plants and act as dosage-dependent barriers even to predators that may, in other circumstances, feed on such plants. The relevant physiological effects of polyphenols upon herbivores derive, it has been assumed, from their ability to complex with proteinaceous materials. Polyphenols thus have a harsh, astringent taste and produce in the mouth a feeling of constriction, dryness, and roughness. These effects are thought to be due specifically to polyphenol complexation with superficial glycoproteins within and without the epithelium and, it is believed, that they thereby render many plant tissues repellent and unacceptable as food sources to potential predators. The same interactions also undoubtedly influence and decrease the dietary value of nutritionally important forage crops such as sorghum and various categories of fodder tree leaves. This, it is believed, constitutes a second mode of chemical defense mediated by vegetable tannins.

The past decade has not only witnessed an explosive growth of interest in this area, but it has also quite clearly shown that the situation in nature, "on the ground," is much more complex than had been anticipated. Experiments on insects have thus demonstrated that, while plant tannins may deter many insects from feeding, others may be quite impervious to their deleterious effects.

Most significantly, however, their chemical complexity and heterogeneity means that they do not lend themselves to ready quantitative assessment, and this, in the author's view, has produced a confused picture of their real significance—both evolutionary and ecological.

If tannins are so generalized in their action, it is very difficult to rationalize this generality with the very wide structural diversity found among the plethora of plant polyphenols—particularly those of the hydrolyzable tannin class based on gallic acid and its derivatives (Figure 1). If these particular metabolites are the end products of energy-demanding syntheses and are not a capricious whim of nature, then it is reasonable to assume that there has been positive selection for their production. If it is the interaction of tannins with proteins and other biological macromolecules, particularly their effect on enzymic processes, that underlies their widely assumed ecological significance, then some correlation should exist between polyphenolic metabolites and their ability to complex with proteins. This proposition is discussed below as is a reinterpretation of Feeny's classic papers on the seasonal changes in oak (*Quercus robur*) leaf tannins.

DISCUSSION

The word tannin has a long established and extensive usage, particularly in the botanical literature, but a firm definition of what constitutes a vegetable tannin is not easy to give. The original implications of the word clearly indicate a plant material that produces leather from hide. Probably the most acceptable definition is still that of Bate-Smith and Swain (1962). They adopted the earlier ideas of White and classified vegetable tannins as "water-soluble phenolic compounds having molecular weights between 500 and 3000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins." Many still prefer the description "tannin," which they find valuable simply because of its lack of precision. Scientifically and terminologically plant polyphenol is to be preferred for this class of higher plant secondary metabolite.

Plant polyphenols are broadly divisible into two major groupings—the proanthocyanidins and the polyesters based on gallic and/or hexahydroxydiphenic acid and their derivatives (Haslam, 1981). As they were recorded in the earlier literature, the former category corresponds to that of the condensed tannins and the latter to the hydrolyzable tannins. Plant polyphenols possess several distinctive molecular characteristics that set them apart. Not only do they carry a multiplicity of phenolic groups, but their molecular weights encompass a wide range. Proanthocyanidins have been described up to 20,000 in molecular weight, and esters of gallic acid and its derivatives are found with molecular weights in the region of 3000 daltons. Bate-Smith and Swain (1953) and Bate-

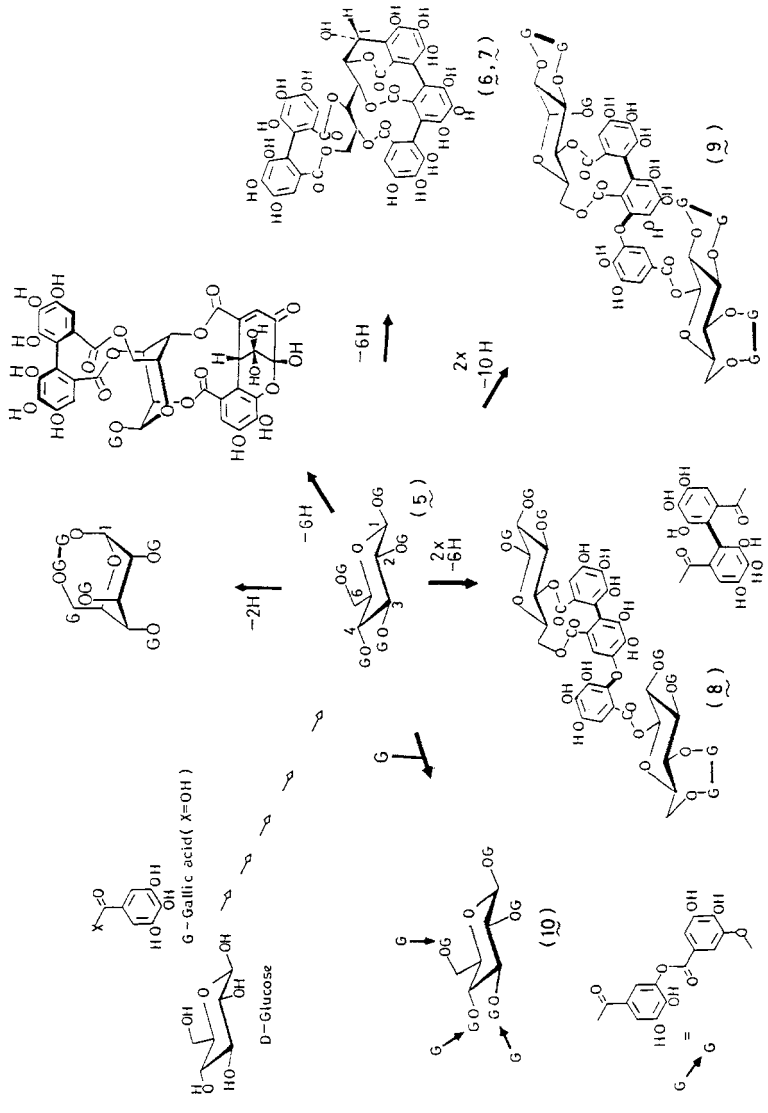
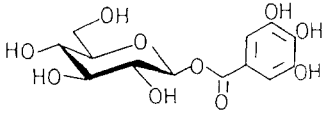


FIG. 1. Gallic acid metabolism in higher plants.

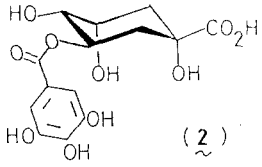
Smith and Metcalfe (1957) first drew attention to the very close similarity in the recorded systematic distribution between leucoanthocyanins (as proanthocyanidins were generally referred to at that particular time) and the diverse class of substances known in the botanical literature as "tannins." These authors suggested that proanthocyanidins were most commonly responsible for the range of reactions which, up until that point, had been attributed to the presence of tannins in plants. Subsequent detailed work, in which polyphenolic metabolites have been isolated from plant materials and identified, has given credence to that view.

Polyphenol–Protein Complexation. Although C_6–C_1 phenolic acids are found in plants and microorganisms, their occurrence is nevertheless sporadic and often constitutes something of a taxonomic speciality. In this respect, one of the most familiar examples is the willow family (Salicaceae) in which derivatives of salicyclic acid are found. Hydroxybenzoic acids and benzoic acid itself are consistently located throughout the plant kingdom esterified to both terpenoid and alkaloid structures. Likewise, although the various hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic) are ubiquitous in higher plants, they are normally found only as mono- and occasionally as bisesters with polyols (e.g., chlorogenic acid). In direct contrast to the hydroxybenzoic acids and the hydroxycinnamic acids, gallic acid and the biosynthetically derived hexahydroxydiphenic acid, are both widely distributed and found in a range of esterified forms. The occurrence of gallic acid has been noted in some 20 or so plant families and one freshwater alga in ester forms which vary from the very simple monoesters such as β-D-glucogallin (**1**), theogallin (**2**), and the flavan-3-ol gallates (**3**, **4**)—all found, for example, in young green tea shoots (*Camellia sinensis*)—to the complex soluble polyesters with D-glucose, whose molecular weights span the range 500–3000 (Figure 1). These polyphenols constitute the hydrolyzable tannins, and they are almost uniquely confined to dicotyledons. They are, in general, freely soluble plant metabolites and as such may be expected to contribute to taste and palatability and nutritional effects of plant materials in which they occur.

A routine feature of gallic acid metabolism in a great many plants is the oxidative coupling of appropriately oriented galloyl ester groups in a particular metabolite to give derivatives of hexahydroxydiphenic acid—usually referred to generically as ellagitannins. Intramolecular covalent bonding of this type produces, from a conformationally flexible and mobile galloyl ester precursor, a more rigid and compact molecular structure. The apotheosis of this effect is seen in the derivation of the diastereoisomeric phenolic metabolites—vescalagin and castalagin, in *Quercus* and *Castanea* sp., by oxidative transformation of the pivotal biosynthetic intermediate β-pentagalloyl-D-glucose (**5**). The latter has a flexible disk-like structure in which the galloyl ester groups are displayed on the periphery of the molecular surface; vescalagin (**6**) and castalagin (**7**) have



(1)



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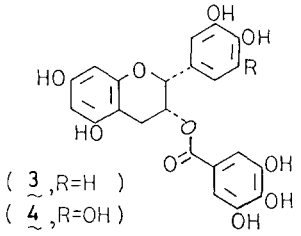


(3, R=H)
(4, R=OH)

SCHEME 1.

propeller-shaped conformations, compact and rigid. Where intermolecular oxidative coupling of galloyl ester groups occurs, this also produces phenolic metabolites of increased molecular size (dimers, trimers, etc.). It is perhaps significant that the major biosynthetic thrust in plants is towards the formation of these highly convoluted structures (e.g., vescalagin, castalagin, geraniin) (Figure 1) or the dimers and trimers. Thus, for example, in the Rosaceae plant family, the leaves of *Rosa* sp. and *Filipendula ulmaria* (meadowsweet) contain as the principle phenolic metabolite rugosin-D (**8**). Similarly in *Rubus* sp. (blackberry and raspberry), *Geum* sp., and *Potentilla* sp., the dimer (**9**) predominates. Likewise, the familiar polygalloyl ester, tannic acid (**10**), in which additional galloyl ester groups are linked as depsides to β-pentagalloyl-D-glucose (**5**), dominates the metabolic profile of *Rhus* sp., *Cotinus* sp., and some *Acer* sp. The astringency of all these plant tissues, it is assumed, is attributable in very large part to these polyphenolic galloyl esters.

The primary reaction whereby astringency develops in the palate is, according to Bate-Smith (1954), by precipitation of glycoproteins in the mucous secretions of salivary glands. The efficacy of polyphenol binding to protein derives from the fact that polyphenols are multidentate ligands able to bind simultaneously (via different phenolic groups) at more than one point to the protein surface. When polyphenols cause precipitation of proteins from solution, two situations may be envisaged. At low protein concentrations, the polyphenol associates at one or more sites on the protein surface, to give a monolayer

that is less hydrophilic than the protein itself (Figure 2a). Aggregation and precipitation then ensue. Where the protein concentration is high, the relatively hydrophobic surface layer is formed by complexation of the polyphenol onto the protein and by cross-linking of different protein molecules by the multidentate polyphenols (Figure 2b). Precipitation then follows as above. This tendency to cross-link protein molecules at higher protein concentrations explains the changing stoichiometry of the aggregates with changing protein concentrations—observation first hinted at by Sir Humphrey Davy. More polyphenol is thus required to precipitate proteins from dilute solution than from concentrated solutions.

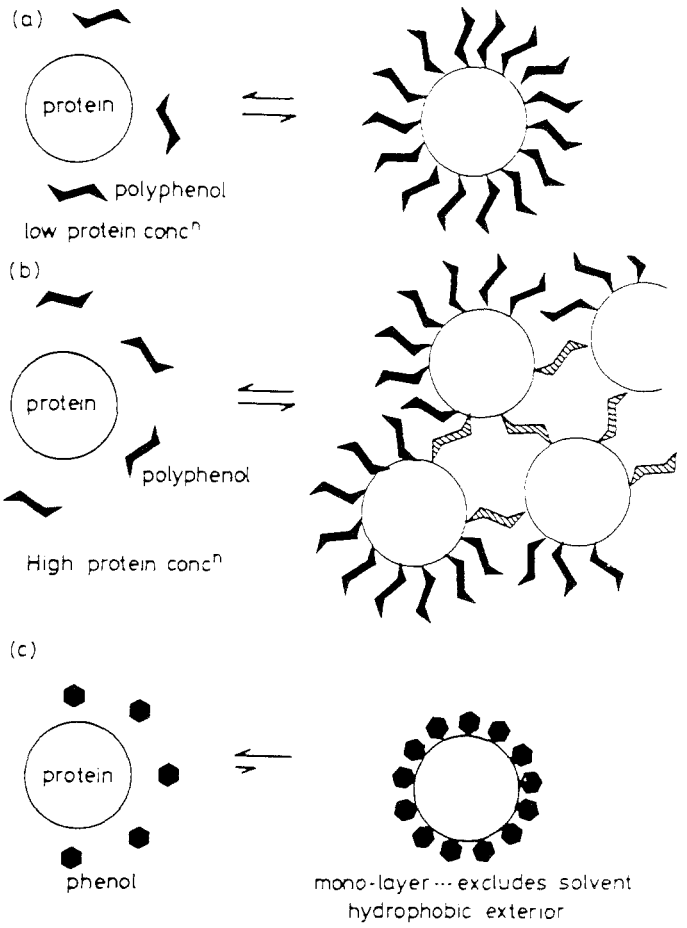


FIG. 2. Protein precipitation by phenols and polyphenols.

An interesting corollary of this hypothesis is that simple phenols such as pyrogallol should also be capable of precipitating proteins from solution if they can be maintained in solutions at concentrations sufficient to push the equilibrium in favor of the protein–phenol complex and thus form a hydrophobic layer of simple phenol molecules on the protein surface (Figure 2c). For many simple phenols, the limit is provided by their solubility in water, but it can be achieved with, for example, bovine serum albumin (BSA, 3×10^{-5} mol/kg) and pyrogallol (1 mol/kg). Clearly, such factors are important when assessing the total astringency of some plants rich in comparatively simple phenolic compounds.

Various studies of polyphenol complexation and, in particular, the delineation of structure–activity relationships have been carried out in recent years. Although as a subgroup the proanthocyanidins are most commonly responsible for the range of reactions generally attributed to tannins in plants, many of these particular studies have been most conveniently pursued with a series of biosynthetically interrelated esters of gallic acid (Figure 1). They are accessible in homogeneous forms and differ systematically in phenolic content, solubility, molecular size, and conformation. Polyphenol complexation with proteins may be studied in solution or by an investigation of the precipitation process which ultimately ensues after extensive complexation (Figure 2). Various physiocochemical techniques are appropriate to such analyses (Figure 3), and there is a broad, although not exact, comparability in the information derived from the various types of measurement (Figure 3). They show that a given polyphenol may exhibit substantially different affinities for different proteins. Hagerman and Butler (1981) have shown for example that flexible "open" proteins and those rich in the amino acid proline bind polyphenols more effectively than compact globular proteins. However, for a given series of polyphenols, their comparative behavior with one protein generally parallels the behavior with other quite different proteins. On the basis of present information, the associative process appears to be a surface phenomenon. Whether there are preferred modes of association and preferred binding sites is not yet entirely clear.

The effectiveness of plant polyphenols as complexing agents derives from the fact that they are polydentate ligands with a multiplicity of potential binding sites provided by the numerous phenolic groups and aryl rings on the periphery of the molecule (Beart et al., 1985). Because of the propinquity of these groups in the polyphenol, cooperativity of complexation to the protein surface is observed. Likewise, because of their molecular size and structure, polyphenols form stable cross-linked structures with different protein molecules (Figure 2). Molecular size is critically important as a determinant of the ability of a polyphenol to bind to protein—as inferred in Bate-Smith and Swain's definition of a vegetable tannin. Thus in the galloyl-D-glucose series, the efficacy of association with protein is enhanced with the addition of each galloyl ester group (di → tri → tetra → penta) and reaches a maximum in the flexible disklike

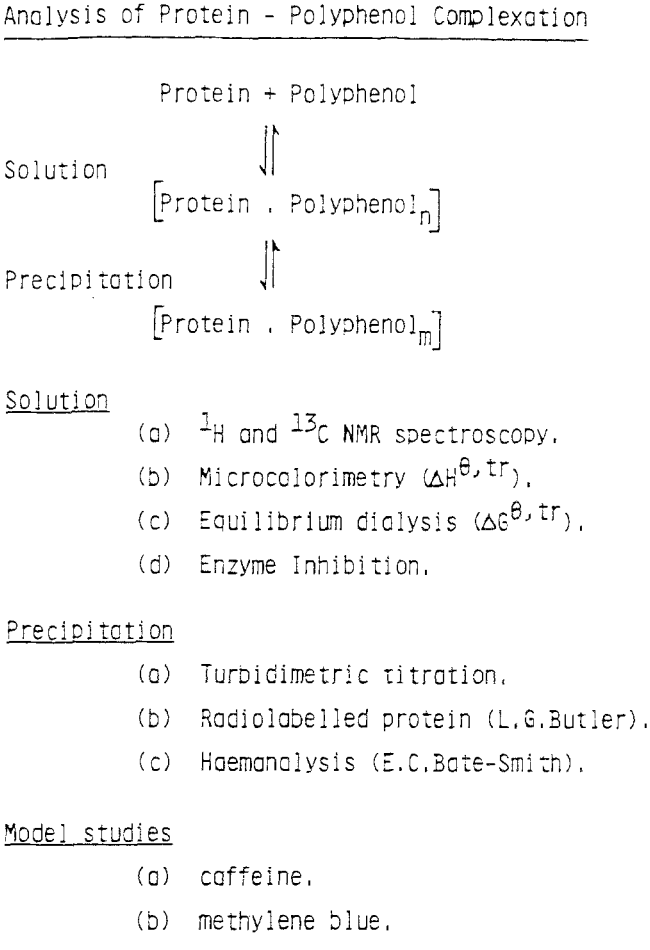


FIG. 3. Protein–polyphenol association.

structure of β-penta-*O*-galloyl-D-glucose (**5**). Further metabolism of this intermediate often substantially lowers its powers of association. Equally significant as molecular size of the polyphenol is conformational mobility and flexibility. In the galloyl D-glucoses, when vicinal galloyl ester groups are constrained by the biosynthetic intramolecular formation of biphenyl linkages and the generation of hexahydroxydiphenoyl ester groups, the loss in conformational freedom is reflected in a reduced capacity to bind to protein. The apotheosis of this effect is seen in the case of the unique open-chain D-glucose derivatives vescalagin and castalagin, metabolites of *Quercus* sp. (**6**, **7**). These rigid virtually inflexible, propeller-shaped molecules are in a sense analogs of β-penta-*O*-galloyl-D-

glucose (Figure 1), but on a molar basis they are bound much less effectively to protein than (**5**). Parenthetically, in this context, the observed "relatively lower astringency" of the proanthocyanidins compared to other polyphenols may be explained, in part, in terms of the conformational restraints imposed by restricted rotation about the repeating 4, 8 or 4, 6 interflavan bonds. Collectively, these results fully complement those of Hagerman and Butler (1981), who showed that proline rich and conformationally mobile proteins have high affinities for polyphenols. Thus, complementarity between the polydentate ligand (polyphenol) and the receptor (protein) is maximized by conformational flexibility in both components. What is not yet clear is whether there is specificity in the association with the protein (surface) or whether the binding is entirely random in nature.

The results show, however, that there is a very wide variability in the protein-complexing capabilities of the polyphenolic biosynthetic "end products" (**6–10**). There is no clear consistent pattern nor is any correlation discernible between the apparent metabolic cost to the plant of its synthesis of a particular polyphenol and the subsequent capacity of that polyphenol to complex with protein and hence that polyphenol's astringency. The dimer (**8**) binds more strongly to protein than (**5**), but frequently the protein-complexing ability of the polyphenols (e.g, **6**, **7**, **9**, and **10**) is diminished when compared to that of the key biosynthetic intermediate, β-penta-*O*-galloyl-D-glucose (**5**) from which they are presumably derived. In such cases, the plant's subsequent chemical manipulation, its embroidery of the intermediate (**5**), is quite clearly counterproductive vis-à-vis the synthesis of metabolites with enhanced astringency. If, as Rhoades (1979) has suggested, there has been positive selection for the production of such metabolites, then this does not appear to be based solely, if at all, on the capacity of these polyphenols to bind to protein.

While it may well be argued that polyphenol complexation with protein is not, as has been assumed, the critical property of polyphenols operative in plant defense, this work does suggest, at the very least, some reappraisal of the earlier hypotheses outlined above. The patterns of gallic acid metabolism outlined (Figure 1) represent a classic example of the biosynthetic prodigality evident throughout secondary metabolism. One key metabolite (gallic acid) is visualized as being formed. This then undergoes a wide range of chemical modifications (often oxidative in character), leading to a plethora of secondary metabolites, each one only slightly different from the next. The evidence presently available suggests that although the retention of polyphenolic synthesis such as this in certain taxa may confer an advantage, a secondary benefit, on the plant and may well be the basis of selective pressures, it does not appear to support the proposition that the purpose of polyphenol metabolism is to generate specifically agents for the plant's defense. On the basis of this reasoning, protection, if it exists, would be a consequence of polyphenol formation, not a cause.

Polymeric Proanthocyanidins and Structure of Tissues. The classical experiments of Feeny in the 1960s on the feeding of larvae of the winter moth on leaves of oak underpin the view that tannins play a major role in plant–herbivore interactions. In the intervening period, however, defining the exact status of tannins as defensive chemicals in plants has proved more difficult to establish. Feeny (1970) and Feeny and Bostock (1968) studied the changes in tannin content of oak (*Quercus robur*) leaves throughout a growing season. This plant species is heavily infested by insects in spring but is rarely subject to serious predation after mid-June. Feeny and Bostock (1968) showed that the level of hydrolyzable tannins remained roughly constant throughout the season but that condensed tannins (proanthocyanidins) did not appear until late May. They concluded that the period of highest insect attack on oak leaves (early spring) corresponds to the time when the total tannin content is at a minimum and condensed tannin is virtually absent. Scalbert and Haslam (1987) have recently reexamined this problem and have provided an alternative interpretation of the experimental data.

The predominant polyphenols of the vegetable tannin class in the leaf of *Quercus robur* L. are, at all stages of growth, undoubtedly those of the hydrolyzable (ellagitannin) group (Figure 4), tellimagrandin 2 (**11**), casuarictin (**12**), peduncalagin (**13**), vescalagin (**6**), and castalagin (**7**). In terms of the ability of soluble extracts of oak leaves to complex and precipitate dietary and other proteins, this group of compounds (**6**, **7**, **11–13**) is undoubtedly of greatest significance. If quantitative changes in the vegetable tannins of oak leaf tissue have a direct causal relationship with changes in insect predation, then it might be anticipated that these changes would be quantitatively reflected in the prinipal group—those of the hydrolyzable class (**6**, **7**, **11–13**). Minor qualitative changes occurred, but major qualitative and quantitative changes in the principal ellagitannins of oal leaf were not observed in two growing seasons.

Consistent with the results of Feeny (1970) and Feeny and Bostock (1968), the most significant changes among the polyphenols (tannins) takes place among the comparatively minor group (in oak)—the various proanthocyanidins of which by far the greater proportion are bound, apparently indissolubly, to plant tissue. The early observations of Sir Robert and Lady Robinson (1935) on the "insoluble" proanthocyanidins of plant tissues, confirmed by subsequent workers in this field, are directly relevant to this question. Thus, for example, Hillis and Swain (1959) have shown that the "leuco-anthocyanins" of plum leaves can be divided into three classes, the first two being successively extractable with absolute, followed by aqueous methanol, and the third, remaining in the residue, being nonextractable by these or other neutral solvents. Likewise, Bate-Smith (1973a, b), in an investigation of the proanthocyanidins of herbaceous Leguminosae, noted that in most of the species examined the amount extracted, even under the most favorable conditions, is only a small proportion of the total present. These observations are of considerable relevance to the questions sur-

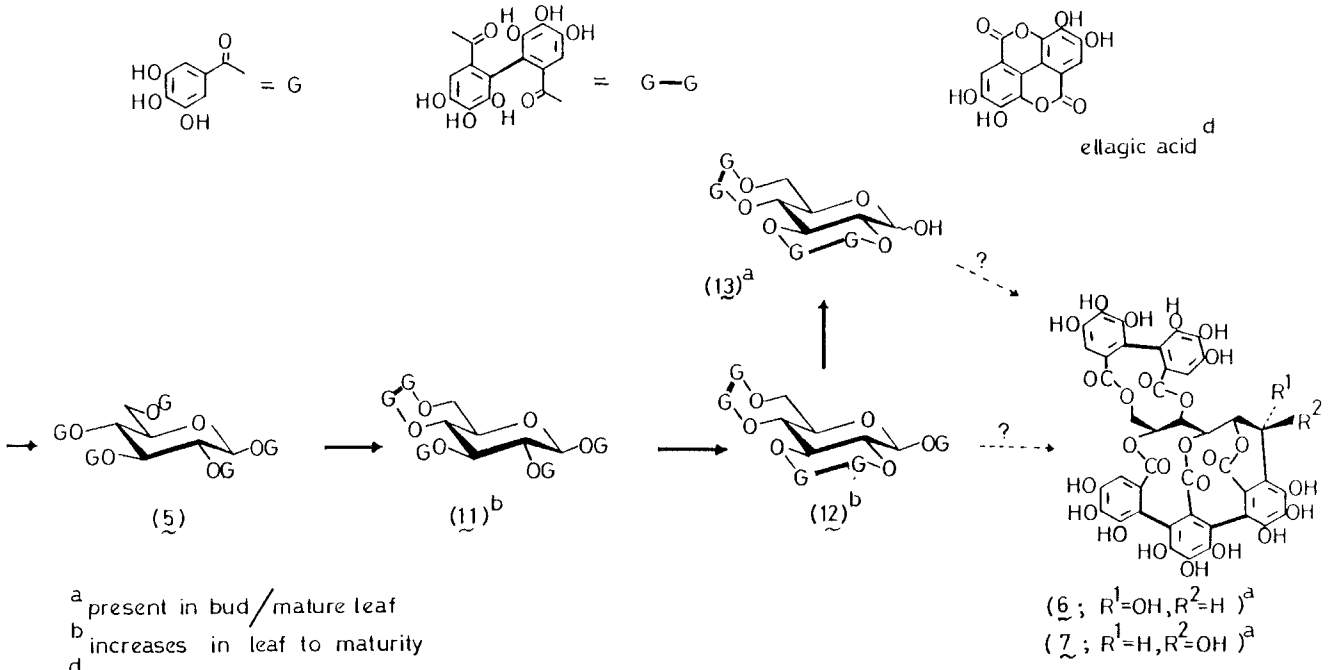
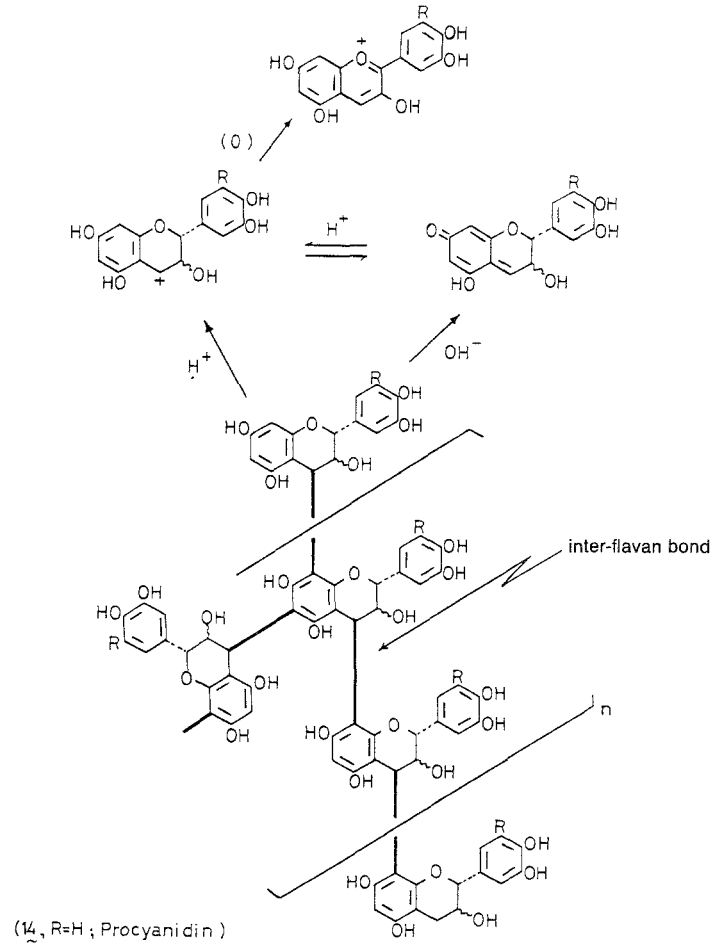


FIG. 4. Oak polyphenols—ellagitannins.

rounding the purported relationships between polyphenols and chemical defense. In a recent paper (Shen et al., 1986), it was suggested that these insoluble forms of plant proanthocyanidins are bound by covalent linkages to polysaccharide (or other) structures in the developing cellular matrix. Attention was also drawn to the possible analogies with the structure of lignin and the process of lignification in plant tissues.

Plant proanthocyanidins are based structurally on a polyflavan-3-ol structure (**14**) and, from a phylogenetic viewpoint, they first appeared as plants developed a vascular character—in ferns for example. The nomenclature proan-



SCHEME 2.

thocyanidin does not imply any biogenetic relationship, but it represents a terminology which is chemically derived. Thus, when heated with acid, the interflavan carbon–carbon bonds of proanthocyanidins are ruptured, and the flavan units, released initially as a carbocation species, are converted by aerial oxidation to characteristic anthocyanidins.

One current hypothesis suggests that the proanthocyanidins are formed as by-products of the processes in which the parent flavan-3-ols (**15**, **16**) are biosynthesized in the plant tissue (Figure 5). A key step visualized in this synthesis is the formation of the carbocation intermediate (**17**). Soluble dimeric, trimeric, tetrameric, . . . , oligomeric proanthocyanidins are thought to derive in circumstances in which the supply of biological reductant (say NADPH) required to convert (**17**) to the flavan-3-ol is limited. If the supply of reductant is rate limiting in these circumstances, the transient carbocation is envisaged to escape from the active site of the enzyme and to react with the end-product flavan-3-ol to produce first dimers and then the spectrum of higher oligomeric forms (Figure 5). In any plant tissue where proanthocyanidin synthesis occurs, there is invariably found this range of molecular species from the monomeric flavan-3-ols (the catechins and the gallocatechins) to the polymeric forms. For each plant tissue, the balance between these various kinds of molecules is probably determined by the corresponding balance between the metabolic flux to the carbocation (**17**, Figure 5) and the rate of supply of the biological reductant (say NADPH). Tissues in which the flux is low and the NADPH supply is high will contain a range of proanthocyanidins of all molecular sizes. Conversely, those tissues in which the supply of NADPH is limited and the metabolic flux is high will contain predominantly the higher oligomeric forms. With increasing degrees of polymerization, the proanthocyanidins become more difficult to solublize in aqueous and alcoholic media. It has been suggested that those which can be coaxed into solution may have molecular weights up to 7000, corresponding to an accumulation of up to 20 flavan-3-ol units in the polymer.

In addition to these various "soluble" forms of proanthocyanidins, plant tissues invariably contain polymeric forms that are entirely resistant to all forms of ready solublization. Proanthocyanidins of this class often predominate over the more freely soluble forms, typically by as much as 5 : 1 or 20 : 1. Indeed, in the tissues of some plants, e.g., ferns and the persimmon fruit, they overwhelmingly predominate. It is pertinent in this context to recall the original observations of Sir Robert and Lady Robinson (1935). These pioneers drew attention to various categories of proanthocyanidins (then referred to as leucoanthocyanins) and in particular to a group (a): "those which are insoluble in water and the usual organic solvents." While noncovalent forces may be involved in the complexation of polyphenols with polysaccharides, present evidence strongly supports the view that polymeric proanthocyanidins which fall within the category (a) defined by the Robinsons are covalently bound to a carbohydrate matrix

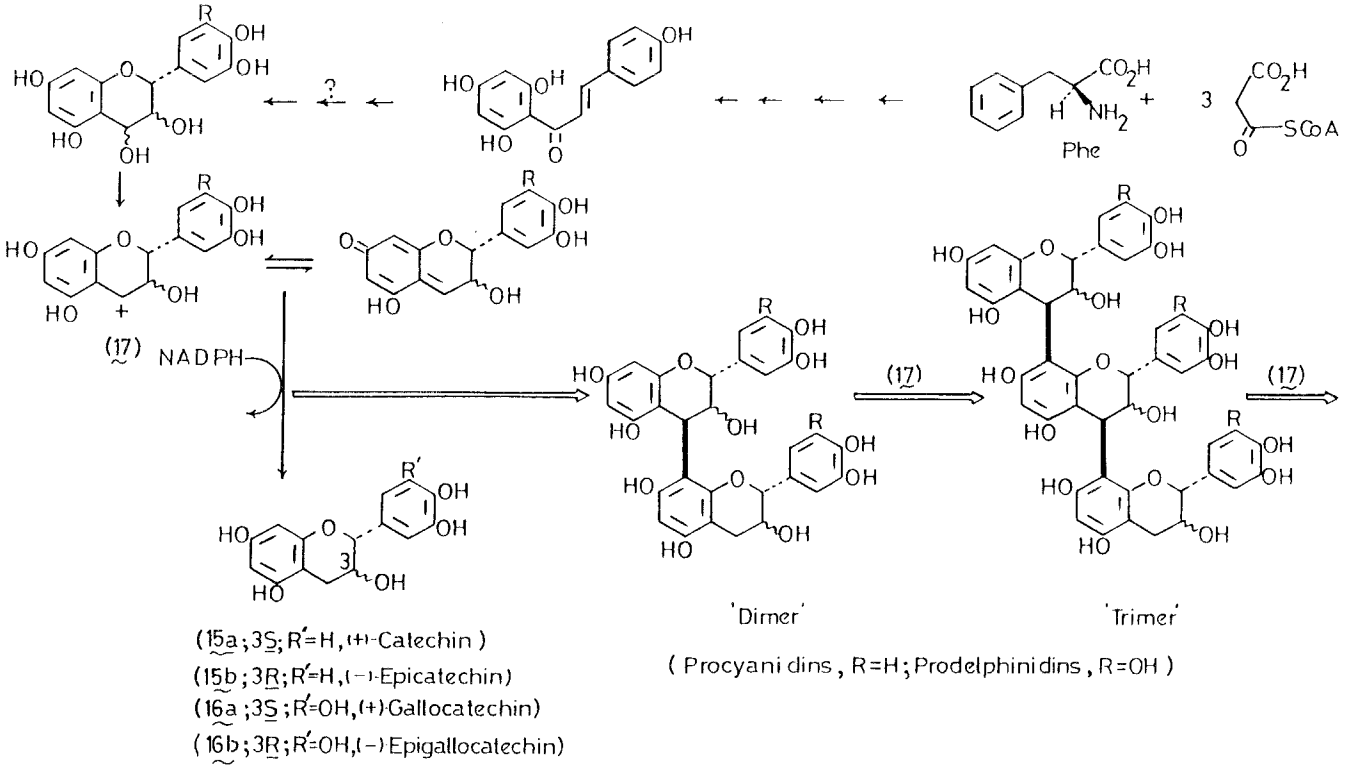


FIG. 5. Proanthocyanidin biosynthesis.

within the plant cell. Such proanthocyanidins, it has been suggested, may well result from the capture of the putative carbocation intermediate (**17**) during biosynthesis by hydroxy groups of saccharide structures in the plant cell (Figure 6).

In this context, it now seems entirely appropriate and pertinent to consider again some of the early observations of Bate-Smith and Lerner (1954) on the botanical significance of plant proanthocyanidins. Bate-Smith noted that these polyphenolic substances were frequently associated with the character and quality of "woodiness" in plants. He also strongly hinted at a possible relationship between proanthocyanidin metabolism and lignification and hence to a putative structural role in the plant for these oligomeric polyphenolic metabolites. In his studies, Feeny (1970) concluded that leaf toughness is the chief proximate factor in deterrence to insect predators of oak leaf. This opinion is now also entirely consistent with an alternative interpretation of the apparent correlation of declining insect predation with proanthocyanidin formation, namely, that leaf toughness develops simultaneously with the initiation of the development of a cell structure which has enhanced "woody" characteristics and in which concomitantly proanthocyanidin oligomers and polymers are deposited as an integral part of these structures (Figure 6). The suggested interpretation of decreased insect predation in oak in early summer is that this is related to changes in cell structure, texture, and toughness of the leaf and in the development of which the formation of proanthocyanidin polymers play an intimate role.

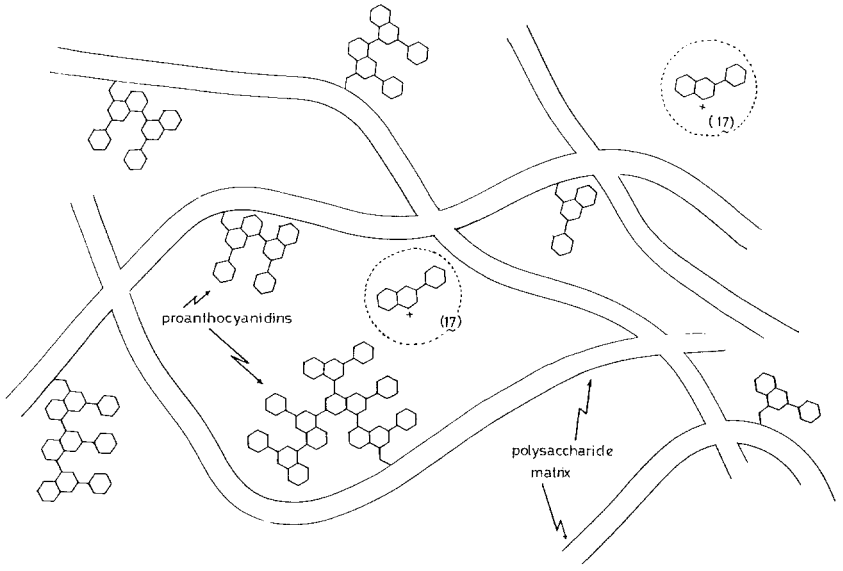


FIG. 6. Polymeric proanthocyanidins—a structural role?

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Periplaneta americana PERCEPTION OF PHYTOCHEMICAL NAPHTHOQUINONES AS ALLELOCHEMICALS

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Abstract—Electrochemistry of male *Periplaneta americana* perception of naphthoquinones as allelochemicals is presented. Importance of the oxidation–reduction potential and hydrogen-bonding capabilities of the naphthoquinone messenger to its exchange of energy with the information-encoding, redox protein of the chemosensory neuron is considered. Evidence that the required information is initially encoded by an electroreactive protein in dendritic membrane is analyzed. A sulfhydryl–disulfide protein-based electrochemical mechanism for the demonstrated linear conversion of a molecular messenger energy state into whole insect behavior (avoidance or feeding inhibition) is elucidated. Even though the information-encoding mechanism is genetically conserved (kept electrochemically similar) within the species, behavioral biotypical differences regarding sensitivity to naphthoquinones are allowed through a group of proteins in the outer aqueous medium in the chemosensory sensillum. Such proteins electrochemically interface the dendritic membrane of the chemosensory neuron in the sensillum with the environment external to the sensillum. These interfacing proteins bind (complex) with chemical messengers (e.g., naphthoquinones) and/or variously degrade them, and thus determine the amount (moles) that is required in the environment to result in stimulation of the underlying sensory neurons and in a changed behavior.

Key Words—Perception, avoidance, deterrence, naphthoquinones, *Periplaneta americana*, Orthoptera, Blattidae, allelochemicals, phytochemicals, insects, energy transduction, electrochemistry, information-encoding protein, behavioral biotypes.

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INTRODUCTION

Allelochemicals have long (Jermy, 1966; Gilbert et al., 1967; Gilbert and Norris, 1968) been considered as major parameters in insect rejections of plants or animals as hosts. Among the more than dozen families of secondary plant or animal products that have allelochemical activity against one or more species of organism, alkaloids, flavonoids, and terpenoids apparently dominate numerically (Swaine, 1977). However, other smaller classes, e.g., benzoquinones or naphthoquinones, are also extremely important in a variety of plants and animals, including insects (Morton, 1965; Rodriquez and Levin, 1976). Some of the better-known animal-derived quinone (i.e., benzoquinone and naphthoquinone) allelochemicals are found in Coleoptera, and especially in tenebrionids (e.g., *Eleodes*) (Tschinkel, 1975). Benzoquinones are also produced by approximately 20 families of diverse plants, whereas naphthoquinone allelochemicals are found in at least 20 angiospermous families and especially members of the Juglandaceae (Rodriquez and Levin, 1976).

The above facts, although only selected examples, clearly indicate that understanding the modes of action of quinones as allelochemicals would be a significant contribution to the whole of chemical ecology. In our laboratories we have researched such mechanisms of quinone actions as they occur in chemoreceptive peripheral neurons of several insects, especially the American cockroach (*Periplaneta americana* L.). The overall findings from our long-term efforts are interpreted here.

REACTIVE CHEMISTRY OF QUINONES

In considering quinones as allelochemicals and as especially odors or tastants in animal (e.g., insect) chemoreception, knowledge of their reactive chemistry is vital. Such quinone chemistry has been detailed by several specialists (e.g., Morton, 1965), so only some basic dynamic characteristics of their reactivities are presented here. Special considerations are focused on the importance of their (1) oxidative–reductive properties, (2) attributes as diketones with α,β-unsaturation, and (3) selective reactions with sulfur compounds.

Quinones as Oxidation–Reduction Systems. The oxidation–reduction (redox) potentials of quinones are important in many biochemical systems (Morton, 1965), including their reactions in *P. americana* neurochemistry (Norris, 1985, 1986). The involved overall oxidative–reductive process is presented in Figure 1 and consists of two parts (A and B); the latter, B, component is dependent on the environmental pH. The redox potential of a mixture of a quinone and its redox couple quinol at any pH is measured against the standard hydrogen electrode (SHE), taken as zero. A positive potential signifies that

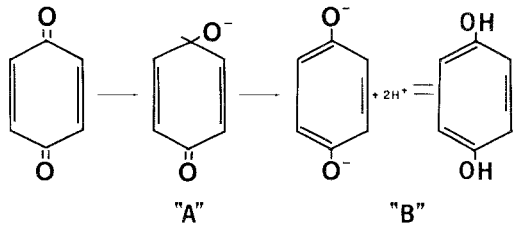

FIG. 1. Oxidative–reductive sequence for a quinone–quinol system: (A) the oxidized quinone and (B) the reduced quinol.

under the SHE conditions the combined quinone–quinol system has an oxidizing effect towards the $H^+ \rightarrow H_2$ system. Thus, one quinone–quinol system will oxidize another having a lower potential, i.e., will displace the equilibrium of the latter system in the direction of the quinone. *o*-Quinones have higher redox potentials than *p*-quinones, and therefore oxidize *p*-quinone–quinol systems. Effects of substituents on quinone reactivities cannot be readily generalized, but some specific situations are clear. For example, substitution in position 2 in 1,4-naphthoquinones reduces the redox potential much more than in any other position.

Quinones as Diketones with α,β-Unsaturation. *p*-Benzoquinones and related *p* substances belong more to aliphatic compounds than to aromatic ones. They functionally are unsaturated diketones exhibiting conjugation, i.e., α,β-unsaturation. In such quinonoid diketones both carbonyl (C=O) groups and any hydroxyl group (e.g., 5-hydroxy of juglone) exhibit hydrogen-bonding capabilities. Such bonding may be either intra- or intermolecular and usually renders the involved quinone (e.g., 5-hydroxy-1,4-naphthoquinone) more active biologically than the unsubstituted 1,4-naphthoquinone, even though the latter compound has the higher redox potential (Norris, 1969, 1970, 1986).

Because of the α,β-unsaturation of diketonic quinones, nucleophilic 1,4-addition reactions may readily occur. Thus, the nucleophiles ($\ddot{N}^-$), sulfhydryls, amino groups, imidazole residues, or hydroxyls, may compete for this covalent substitution on such quinones (Wallenfels and Eisele, 1970).

Quinone Reactions with Sulfur Compounds. Some sulfur compounds have long been known to react especially quickly with given quinones, e.g., in the absence of solvent, 1,4-benzoquinone will oxidize thioglycolic acid and become reduced to its hydroquinone (quinol couple). In this case, the sulfur, as a nucleophile, adds across a conjugated double bond in the quinonoid ring with the resultant reduction of the quinone carbonyls (Finley, 1974). Although quinones react with other nucleophiles as indicated above, they show a "selectivity" or "specificity" for some sulfhydryl groups by reacting with the latter at least 100-fold more rapidly (Wallenfels and Eisele, 1970). Thus, for practical

biological and biochemical considerations, *p*-quinones may be considered as sulfhydryl reagents.

p-Quinones may not react equally with individuals of a group of sulfhydryls because the latter may be differentially influenced, through cooperation, by hydrogen bridges, coulomb interactions, and steric hindrance or preference. In fact, such combined microenvironmental influences render sulfhydryls in proteins a very heterogenous group regarding accessibility to, and reactivity with, given *p*-quinones.

STRUCTURE-ACTIVITY RELATIONSHIPS

Insect perception of 1,4-naphthoquinones as allelochemicals is readily expressed and bioassayed behaviorally as an avoidance or feeding reduction. Such behavioral analyses of the relative allelochemical effects of several 1,4-naphthoquinones on the very different insects *Scolytus multistriatus* (the smaller European elm bark beetle) and *Periplaneta americana* (the American cockroach) indicated the same following order of relative activity: 5—OH > 1,4— > 2—OH > 2—CH_3. This order of experimentally determined behavioral effects is largely attributable to the relative combined redox potential and hydrogen-bonding capabilities of these compounds as discussed in the previous section of this paper and as detailed by Norris (1969, 1970). This same order of relative activity was also observed experimentally based on (1) electrophysiology (i.e., naphthoquinone inhibition of a standardized insect electroantennogram, EAG) (Norris and Chu, 1974); (2) competitive ligand (naphthoquinone) binding to an electrophoretically purified redox-complex, dendritic-membrane-associated protein (Singer et al., 1975); and (3) naphthoquinone-induced electrochemical (*U* 1/2) shift in a standardized 100-μg aliquot of the above protein (Rozental and Norris, 1973, 1975; Norris, 1985). Our overall experimentally determined relationships between naphthoquinone structure and behavioral, electrophysiological, biochemical, or biophysical activities in these two insects seem well explained by the known reactive chemistry of quinones.

ULTRASTRUCTURAL CONSTRAINTS

Physicochemical interpretation of the energy transduction (transfer) involved in an insect's perception of a ligand (e.g., molecule) as an allelochemical must accommodate the ultrastructural and cytochemical constraints of the in vivo chemoreception system of that insect (Norris, 1976a, 1986). Naphthoquinones as allelochemicals interact with the insect's sensory neurons through ultrastructure and cytochemistry associated with sensilla, especially on the antennae, mouthparts, or tarsi.

Olfactory (volatile) stimulants (e.g., juglone) function especially through multiporous sensilla, e.g., sensilla basiconica, which are abundant on *P. americana* antennae. These hollow hairs have thousands of pores through their cuticular wall, and stimulants may exchange energy with involved sensory neurons through cuticular pore–pore tubule interconnections (Norris, 1976a, 1986).

Contact chemoreceptors, e.g., sensilla trichodea, frequently are uniporous, i.e., have a single terminal opening through which to expose the involved sensory neuron(s) in the lumen of the sensillum to chemical messengers in the external environment. Such sensilla thus are especially important in decisions regarding feeding, gustation, or ingestion. At least some insects can physiologically control the opening of such a uniporous sensillum, and therefore "control" significantly when, and the extent to which, stimulants may enter or exit the liquor (i.e., outer aqueous medium, Figure 2) in the lumen of the sensory hair (Bernays et al., 1972).

The combined ultrastructure of an insect chemoreceptive sensillum (e.g., hollow, multiporous hair) and the dendritic branches of the involved sensory neuron(s) provides the essential elements of a fuel cell, a device which produces a continuous flow of electricity by means of chemical reaction (Figure 2) (Norris, 1986). The cytoplasmic contents in the neuronal dendritic branches within the lumen of the sensillum constitute the relatively low-protonic-potential, inner aqueous medium of the fuel cell (Figure 2, I), whereas the liquor in the sensillum cavity is the relatively high-protonic-potential, outer aqueous medium (Figure 2, E). These two differently charged aqueous media are effectively separated ultrastructurally by the relatively hydrophobic lipid bilayer dendritic membrane (Figure 2). Chemiosmotic theory of membrane bioenergetics (Mitchell, 1979) as applicable to energy transduction (transfer) in insect chemical senses states that only three molecular components need to be added into the insulating lipid bilayer dendritic membrane of Figure 2 to effect the required energy transduction (transfer). These are (1) an ATP synthase, along with reversible Na^+,K^+-ATPase activity; (2) a redox chain (complex) with characteristic H^+ and e^- stoichiometry; and (3) proton-linked (or hydroxyl ion-linked) solute porter (pump) systems. Such essential components have been experimentally demonstrated and analyzed in dendritic membranes of male *P. americana* chemosensitive neurons with regard to this insect's perception of 1,4-naphthoquinones as allelochemicals (Norris, 1981, 1986).

ENERGY TRANSDUCTION AND INFORMATION ENCODING IN EXPOSED PRIMARY CHEMOSENSITIVE INSECT NEURON

Electroreactive Receptor Protein. The electroreactive receptor protein for repellent and antifeedant 1,4-naphthoquinones in male *P. americana* antennal primary chemosensory neurons is a redox-chain (complex) polypeptide (Roz-

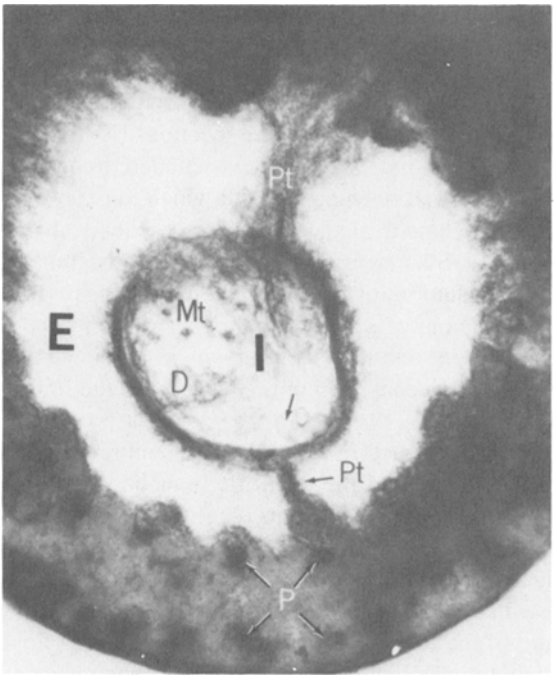

FIG. 2. High-voltage electron micrograph revealing the "fuel cell" ultrastructure of a sensillum basiconicum on the antenna of the american cockroach (*P. americana*). External (E) and internal (I) hydrophilic media surrounding the insulating dendritic (i.e., hydrophobic) membrane. D, Dendrite; Pt, pore tubules; Mt, microtubules.

ental and Norris, 1973, 1975; Norris, 1976a, 1979, 1985). This energy-transducing and information-encoding protein in the dendritic membrane of involved neurons is a sulfhydryl–disulfide lipoprotein. It is readily solubilized from the dendritic membrane with 0.6% Triton X-100 in 0.9% NaCl and can be purified (e.g., 57-fold) in terms of micrograms of naphthoquinone bound per microgram of receptor protein, by affinity chromatography using *p*-chloromercuric benzoic acid (PCMB) as a ligand on a 16-carbon sidearm attached to Sepharose 4B (Norris et al., 1977). SDS-disk electrophoresis of this purified 68,500-dalton protein resolved three subunits with molecular weights estimated at 19,000, 23,500, and 26,000 daltons (Norris, 1979, 1986).

Energy-Transduction Mechanism. The sulfhydryl–disulfide, information-encoding protein binds reversibly with, and reduces electrochemically, the allelochemical 1,4-naphthoquinone ligands (i.e., chemical messengers) (Rozental

and Norris, 1973, 1975; Norris, 1979, 1985). The involved energy-transduction interaction of the messenger naphthoquinone with the redox receptor protein reversibly produces a fluorescent product, the reduced hydroquinone (QH_2) couple of the naphthoquinone. Major reaction steps in this energy-transfer interaction are presented in Figure 3.

Such sulfhydryl–disulfide proteins may be effective redox-complex components in the involved energy-transduction machinery because they are extremely sensitive to changes in the oxidation state in the outer or inner aqueous medium surrounding the insulating dendritic membrane (Figure 2). As the oxidizing messenger 1,4-naphthoquinone enters the outer aqueous medium (i.e., contents of the cuticular pore, pore tubule, and sensillum liquor), it becomes reduced (Figure 3), which withdraws hydrogens from the outer medium and the redox-complex protein in the dendritic membrane (Figure 2). The involved hydrogen withdrawal may occur directly from this energy-transducing protein or indirectly by the 1,4-naphthoquinone allelochemical reacting with the contents of the outer aqueous medium.

Each allelochemical 1,4-naphthoquinone, at a molarity which does not saturate the electroreactive energy-transducing protein, induces in vitro a quantitative shift in that protein's *U*1/2 electrochemical parameter. This resultant quantitative shift is the basis for the observed information encoding (Norris, 1985).

Primary Peripheral Neuron Encoding of Insect Chemical Senses Information. As discussed earlier, the observed order of relative behavioral effects

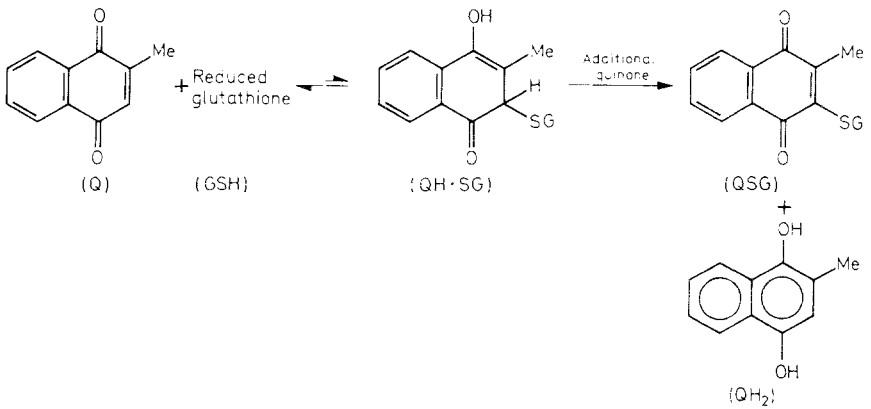

FIG. 3. Reaction that occurs between the redox chain (complex) information-encoding protein (reduced glutathione in this model system) and an allelochemical naphthoquinone (e.g., menadione, 2-methyl-1,4-naphthoquinone) as studied by fluorescence spectrophotometry. Q, menadione; GSH, reduced gluttahione; QH·SG, neutral semiradical; QSG, oxidized quinone adduct; QH_2, hydroquinone.

to *P. americana* elicited by given moles of several 1,4-naphthoquinones is the same, whether measured (1) behaviorally as percent (%) insects showing avoidance, (2) electrochemically as mV shift in the in vitro information-encoding protein's *U*1/2 value, or (3) electrophysiologically as the percent (%) inhibition of a standardized amyl acetate-elicited electroantennogram (EAG), i.e., 5—OH > 1,4— > 2—OH > 2—CH_3. The relationship between any two of these three experimental parameters is described ($P < 0.01$) by a linear equation (Norris, 1986). The correlation coefficient between the mean mV values for the observed maximal electrochemical *U*1/2 shifts in the information-encoding protein and the mean maximal percent inhibitions of the standardized electrophysiological EAG is so high (Norris, 1986) that only one of these two quantitative output parameters needs to be utilized in obtaining a reasonable mathematical description for the energy exchange (energy transduction) involved in the male *P. americana* perception and behavioral avoidance of 1,4-naphthoquinone allelochemicals. Because only two (one input and one output) of the three measured (one input and two output) variables need to be considered, an adequate quantitative (millivolt) description of the involved energy exchange is $\log y = 3.40 - 0.112 \log x$, which describes a linear relationship (Norris, 1986). y is considered as the primary measured output (efferent) variable, i.e., the mean maximal millivolt (mV) shift in the electrochemical *U*1/2 value of the in vitro information-encoding protein, whereas $x = 2 \times 10^{-5}$ mol, and pertains to the primary measured input (afferent) variable, i.e., the mean moles of messenger (allelochemic) 1,4-naphthoquinone required in the standardized avoidance bioassay to repel > 99% of the *P. americana* males from the treated tub "arena" (Rozental and Norris, 1975; Norris, 1986) (Figure 4).

The described energy-exchange relationship involved in male *P. americana* behavioral perception and avoidance of allelochemical 1,4-naphthoquinones is proof that the needed (coded) electrical information to elicit the observed avoidance behavior in the whole cockroach is already encoded in the primary chemosensory neuron and more specifically by the electroreactive redox protein in the dendritic membrane of this neuron. O'Connell (1981) stated most succinctly that, "A code, simply stated, is the set of rules which governs the mapping of the input variable onto the output variable.... Encoding mechanisms require only that rules are applied uniformly and that they lead to distinctive outcomes." He further stated that a satisfactory code in insect chemical senses can be a simple one-to-one relationship between a discriminable chemical stimulus and an electrophysiological response. Our elucidated code, $\log y = 3.40 - 0.112 \log x$, describes such a simple linear mapping of input stimulus onto output effect. Therefore, the CNS (central nervous system) of male *P. americana* may be doing many manipulations with the information it receives from stimulated peripheral primary chemosensory neurons exposed to these allelochemical 1,4-naphthoquinones, but the essential neural-impulse-based

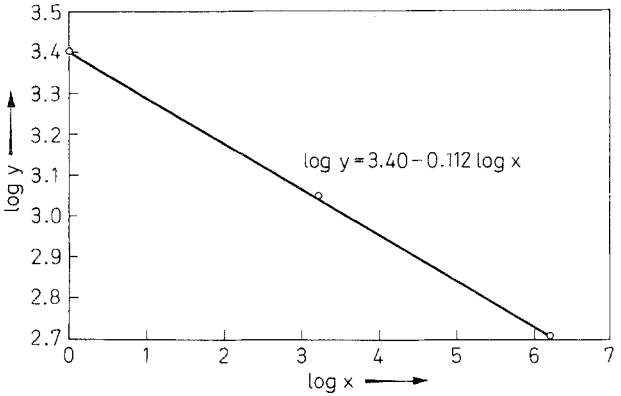

FIG. 4. Linear relationship that exists between the log of the primary input (afferent) variable, x, where $1X$ equals 2×10^{-5} moles with regards to the number of moles of a naphthoquinone required in a standardized bioassay to yield greater than 99% avoidance behavior by male *P. Americana*; and the log of the primary output (efferent) variable, y, which is the maximum in vitro electrochemical $U1/2$ shift, in millivolts (mV), by the information-encoding protein elicited by the indicated naphthoquinone.

information to elicit the prescribed insect avoidance behavior is originally processed (transduced) in the primary neuron from energy exchanged between the messenger naphthoquinone and the redox protein in that primary cell.

GENETIC CONSERVATION OF REDOX PROTEIN-BASED INFORMATION-ENCODING MECHANISM

The male *P. americana* perception of naphthoquinones as allelochemicals is dependent upon quantitative electrochemical reaction of these oxidative–reductive chemical messengers with the electroreactive sulfhydryl–disulfide information-encoding protein in the dendritic membrane of the primary insect chemosensory neurons. There are three distinct classes of electroreactive naphthoquinone-binding sites in this protein (Rozental and Norris, 1975; Norris, 1985). Each of these classes of sites involves sulfhydryl-group (−SH) dependency, and the electroreactivity of the involved sulfur atoms is readily quantifiable by dropping-mercury-electrode (DME) polarography (Rozental and Norris, 1975; Norris, 1985). Naphthoquinone saturation of the redox protein of the two behavioral *P. americana* biotypes, LAB versus WARF, causes a similar ($P < 0.01$) maximal mean millivolt shift in the electrochemical half wave ($U1/2$), e.g., for 2-methyl-1,4-naphthoquinone, LAB (15 mV) and WARF (13 mV). This maximal 13 to 15-mV inducible electrochemical shift in the infor-

mation-encoding protein of each biotype is interpreted as the summation of three equal units of electrochemical shift, each consisting of 4–5 mV (i.e., 3 units × 4–5 mV = 12–15 mV total) (Norris, 1985, 1986). Each 4 to 5-mV unit of $U1/2$ shift thus involves the electroreactivity of those sulfur atoms associated with one of the three distinct classes of binding sites for naphthoquinones in each biotype's redox protein. Thus, this electrochemically quantified, protein sulfur-based, information-encoding mechanism in male *P. americana* chemical senses has been conserved ($P < 0.01$) in these two behavioral biotypes. It seems quite reasonable that once a molecular-based mechanism for information encoding was evolved in the neurons of a species such as *P. americana* that it would be conserved at least within that species (Norris, 1985).

INTERFACING PROTEINS: MOLECULAR PROVISION BY WHICH INDIVIDUAL *P. americana* MAY EVOLVE DISTINCT PHENOTYPIC SENSITIVITIES TO A CHEMICAL MESSENGER

Even though the electrochemically based energy-transduction and information-encoding mechanism of the redox protein of male *P. americana* behavioral biotypes LAB and WARF is conserved (Norris, 1985, 1986), these biotypes still show a twofold difference in their behavioral sensitivity to the allelochemical 2-methyl-1,4-naphthoquinone (menadione). Greater than 99% avoidance behavior by male WARF cockroaches requires their exposure to 2×10^{-2} M of menadione, whereas such behavior by male LAB only requires 10^{-2} M. This twofold difference is attributable largely to differences in quantities and reactivities of specific soluble proteins, termed "interfacins" (Norris, 1976b, 1986), in the outer aqueous medium (i.e., sensillum liquor, pore tubule and cuticular pore contents) of the chemosensory sensillum (Figure 2).

These soluble proteins physically and chemically interface the dendritic membrane of each chemosensory neuron with the environment external to the sensory hair's outer cuticular surface. Certain of these proteins bind (complex) with a chemical messenger, e.g., 2-methyl-1,4-naphthoquinone, as it enters the outer aqueous medium (Figure 2) of the chemosensory sensillum (Norris, 1976a, 1986). The amount of messenger that is complexed or degraded by these interfacing proteins, soluble components of the outer aqueous medium in the chemosensory sensillum, significantly determines the amount (moles) of the given compound (messenger) required to stimulate the information-encoding protein in the dendritic membrane of the involved underlying sensory neurons. In male *P. americana*, such naphthoquinone-binding interfacins are sulfhydryl–disulfide proteins (Norris, 1976a, 1986). Some interfacins also are enzymes, especially various esterases (Kasbohm and Norris, 1989). Specific electrophoretically resolved enzymatic activities among male *P. americana* interfacing proteins include a PMSF (phenylmethylsulfonyl fluoride) -sensitive serine protease; an

arylesterase, inhibited by both PMSF and the classical sulfhydryl reagent PHMB (*p*-hydroxymercuribenzoate); and a carboxylesterase (Figure 5).

Interfacing proteins pass outward onto the environmentally exposed cuticular surface of the chemosensory sensillum through cuticular pores (Figure 2) (Norris, 1986; Kasbohm and Norris, 1989). Such proteins thus may be extensively exposed to messenger compounds which impinge on this cuticular surface. Especially through this initial exposure, the interfacing proteins readily intercept and variously interact with messenger entities.

CONCLUSIONS

There are at least two distinct molecular levels in the evolution of the chemoreception mechanism by which male *P. americana* perceive naphthoquinones as allelochemicals. First, electroreactivity has evolved in a dendritic-membrane protein whereby energy exchange between a messenger (odor) molecule and this protein is linear, as readily quantified through several experimental windows on the overall chemoreceptive process, e.g.: (1) behavioral assay, (2) electrochemistry of this electroreactive dendritic protein, or (3) EAG. This evolved molecular information-encoding device is conserved between two

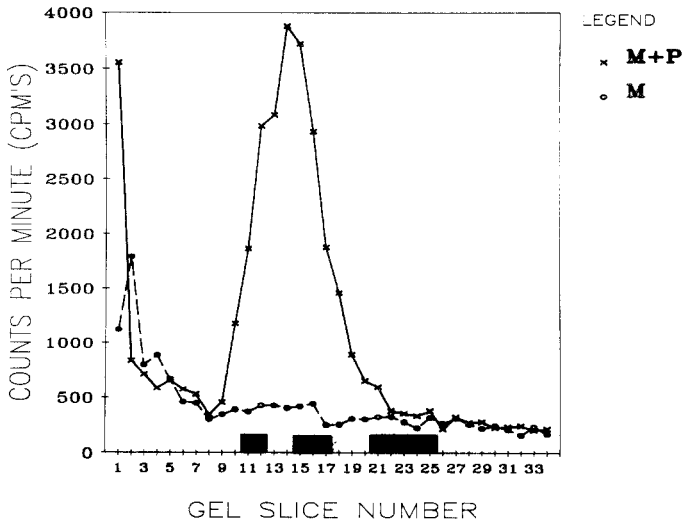

FIG. 5. Electrophoretically resolved "interfacin" proteins (bands 1, 2 and 3) from the outer aqueous medium (i.e., sensillum liquor) of male *Periplaneta americana* with indicated naphthoquinone (i.e., [^{14}C]Menadione) binding (counts per minute, CPMs, per 1.5-mm gel slice) and degradative enzymatic activities (1, serine protease; 2, arylesterase; 3, carboxyesterase).

genetically distinct behavioral biotypes, LAB and WARF, of the species *P. americana*.

This elucidated molecular mechanism of *P. americana* chemoreception explains why EAGs have been used so effectively to monitor whole insect responses to messenger molecules including pheromones; the reason is that the involved exchanges of energy through the several described molecular levels have linear relations to each other. Primary encoding of the input (afferent) information required to dictate a distinct efferent response (i.e., EAG, avoidance behavior, or feeding inhibition) thus may occur peripherally in the redox protein in the dendritic membrane of the primary chemosensory neuron.

The elucidated second level in the evolution of the molecular mechanism by which male *P. americana* perceive naphthoquinones as allelochemicals involves interfacing proteins in the sensillum liquor. Whereas the above-described redox protein is at the information-encoding level, and the species apparently may best maintain its fitness through conservation of this evolved vital mechanism among individuals (e.g., LAB versus WARF males), the second level of the sensillum-liquor chemoreceptive mechanism apparently assures that individuals within the species, e.g., *P. americana*, may evolve distinctly in their behavioral sensitivity to given stimuli, e.g., naphthoquinones. Thus, LAB males are behaviorally twice as sensitive as WARF males to 2-methyl-1,4-naphthoquinone, especially because the two biotypes have differing amounts and reactivities among one or more interfacing proteins in their sensillum liquor (i.e., the outer aqueous medium of the chemosensory sensillum, Figure 2). Our best knowledge indicates strongly that both endogenous and exogenous factors alter the quantities and functionalities of these interfacing proteins. Such assured variability would seem to guarantee individual *P. americana* dynamic day-to-day chemical senses.

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BIOASSAY OF NATURALLY OCCURRING ALLELOCHEMICALS FOR PHYTOTOXICITY

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Abstract—The bioassay has been one of the most widely used tests to demonstrate allelopathic activity. Often, claims that a particular plant species inhibits the growth of another are based entirely on the seed germination response to solvent extracts of the suspected allelopathic plant; few of these tests are of value in demonstrating allelopathy under natural conditions. The veracity of the bioassay for evaluating naturally occurring compounds for phytotoxicity depends upon the physiological and biochemical response capacity of the bioassay organism and the mechanism(s) of action of the allelochemicals. The possibility that more than one allelochemical, acting in concert at very low concentrations, may be responsible for an observed allelopathic effect makes it imperative that bioassays be extremely sensitive to chemical growth perturbation agents. Among the many measures of phytotoxicity of allelochemicals, the inhibition (or stimulation) of seed germination, radicle elongation, and/or seedling growth have been the parameters of choice for most investigations. Few of these assays have been selected with the view towards the possible mechanism of the allelopathic effect.

Key Words—Allelopathy, bioassay, mechanism of action, seed germination, radicle growth, seedling growth, *Lemna* bioassay.

INTRODUCTION

We have recently reviewed the use of bioassays in the study of allelopathy (Leather and Einhellig, 1986). In that review, we discussed the nature and types of bioassays used by investigators to demonstrate the phytotoxicity of leachates, exudates, extracts, etc., from plants suspected of being allelopathic to other

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plants or microorganisms. Yopp's (1985) treatise, "Bioassays for Plant Hormones and Other Naturally Occurring Plant Growth Regulators," is an excellent source of information on assays for specific chemicals that control plant growth and function. Many of these bioassays may be useful in the study of allelopathy when the mechanisms of action are well understood.

Our purpose here is to evaluate the veracity of the most commonly used bioassays in allelopathy research, their value in determining allelochemical mechanism(s) of action, and the suitability of those bioassays to detect the presence and phytotoxicity of allelochemicals.

DISCUSSION

Mechanism of Action. Allelochemicals that are phytotoxic have been identified from many genera of plants and belong to many classes of chemical compounds (Rice, 1984). Einhellig (1986) and Putnam (1985) have reviewed the chemistry and mechanisms of action of allelopathic agents. Table 1 is a summary of our current knowledge of the possible mechanisms by which the allelochemicals inhibit plant growth and development. Proposed mechanisms

TABLE 1. MECHANISMS OF ACTION OF ALLELOCHEMICALS IN PLANT GROWTH AND DEVELOPMENT[a]

Mechanism	Allelochemical	Reference
Cell extension	Phenolic acids, tannins	Lee and Skoog (1965), Lee et al. (1982), Lee (1980), Ray et al. (1980)
Cell division	Volatile terpenes, coumarins	Muller (1965), Jankay and Muller (1976), Avers and Goodwin (1956)
Membrane permeability	Phenolic acids	Harper and Balke (1981)
Nutrient uptake	Phenolic acids	Harper and Balke (1981), Kobza and Einhellig (1987)
Chlorophyll synthesis	Coumarins Phenolic acids	Einhellig et al. (1970), Einhellig and Rasmussen (1979)
Photosynthesis	Phenolic acids	Nyberg (1986), Scholes (1987)
Protein synthesis	Phenolic acids, coumarins	Van Sumere et al. (1971)
Enzyme activity	Phenolic acids	Jain and Srivastava (1981), Sato et al. (1982), Schwimmer (1958)
Respiration	Juglone, volatile terpenes, phenolic acids	Scholes (1987), Koeppe (1972), Muller et al. (1969)
Water relations	Phenolic acids	Einhellig et al. (1985), Blum and Dalton (1985), Blum et al. (1985a)

[a] Condensed from Einhellig (1986), and Putnam (1985).

encompass most major plant functions, including regulation of growth by interfering with cell division or cell extension either directly or through interaction with hormones, effects on respiratory metabolism, photosynthesis, and altered water balance.

It can be noted from Table 1 that a great deal of research has involved phenolic acids and that these compounds act on a number of plant processes. It is unlikely that these compounds would have such a wide range of primary action, and it is probable that the results observed may be secondary and/or tertiary responses to the compounds. This range of responses could be due to the lack of veracity from improper bioassays. Indeed, most purported allelopathic agents have not been tested in bioassays with the goal of determining their mechanisms of action.

The biochemical and/or physiological response of a bioassay organism to allelochemicals is not always linear over a range of concentrations. Many allelochemicals are inhibitory at millimolar concentrations but stimulate the measured parameters in bioassays at micromolar concentrations. This anomaly confounds efforts to determine mechanisms of action in bioassays and to relate the results to in situ concentrations of allelochemicals.

Seed Germination Bioassays. The inhibition (or stimulation) of seed germination has been the most widely used bioassay for the determination of allelopathic activity. However, the seed germination process is probably the least understood of all plant functions (Leather, 1987). Seed germination begins with imbibition of water and ends with the protrusion of the radicle through the testa. Radicle elongation is by cell extension only and does not involve cell division. The biochemical events associated with germination are not well defined and may only be preparatory for the mobilization of reserves for seedling growth. Thus, definitive conclusions of allelopathic mechanisms in seed germination bioassays are limited but may involve membrane alteration, resulting in loss of metabolites and the ability to establish the necessary osmotic potential for cell elongation (Koller and Hadas, 1982). Other processes, such as alteration of the phytochrome control of germination, may also be effected. We found that some naturally occurring volatile compounds stimulated the dark germination of *Rumex* sp. that normally require postimbibitional light (French and Leather, 1979). Other perturbations from allelochemicals of seed germination processes may be involved, but we must await further knowledge of the biochemical events that occur during seed germination that are directly related to the germination process.

The greatest problem that affects the veracity of seed germination bioassays results from the manner in which the bioassay is conducted. Anderson and Loucks (1966) emphasized the importance of the solution osmotic potential when testing plant extracts; however, few reports on allelopathic effects consider this precaution (Leather and Einhellig, 1986). Weidenhamer et al. (1987)

reported that the number of seeds relative to the solution volume used in a seed germination bioassay was a factor in the results obtained. They found that the amount of ferulic acid available to each seed influenced the germination, rather than the concentration of chemical in the test solution. In conducting this research, care was taken to prevent anaerobic conditions by submersion of the seeds in water. We have found reports in the literature of allelopathic action where the investigators germinated the test species in volumes of solution that were 20 times the amount required for optimum germination without anaerobiosis.

Blum et al. (1984) offer recommendations for the standardization of germination bioassays. However, their results are based on radicle growth subsequent to germination and perturbations at any of the stages of germination and growth may have effects on any subsequent stage. Nonetheless, their observations regarding pH, microbial contamination, photolability of allelochemical, and loss of test compound are very important when conducting germination bioassays. Our recent review on this subject outlines additional precautions that should be observed when conducting seed germination bioassays (Leather and Einhellig, 1986).

Radicle Elongation Bioassays. Radicle elongation is a more sensitive assay for allelochemicals than seed germination (Leather and Einhellig, 1985; Einhellig, 1986). Like seed germination, radicle elongation is extremely sensitive to high (100 mosmol) osmotic potentials of solutions, and concentrations of purified extracts must be evaluated prior to assay (Bell, 1974). Generally, the radicle is completely dependent upon the seed (cotyledon) reserves for growth in the dark, and precautions must be taken to separate effects upon the seed and the mobilization of storage material by the allelochemical during or immediately following germination. Blum et al. (1984) reported that surface sterilization of seeds with sodium hypochloride modified radicle growth. Thus, as previously noted, care must be taken to minimize early effects of the allelochemical upon the seed that may alter subsequent radicle growth.

The accuracy of results obtained from the measurement of radicles elongating in Petri dishes is questionable. Few such radicles elongate on a straight course, and precise measurements are difficult. We have found that removing the radicle from seed germinated in Petri dishes and thoroughly drying to a constant weight gives accurate results with small statistical error (Leather and Hurtt, unpublished). Parker (1966) described a method to determine herbicide uptake and effects on radicle elongation. We modified this method to use pregerminated seed placed between chromatography sandwich plates that are maintained at a 45° angle, thus having straight radicles for measurement. Additionally, this method allows only the radicle to be in contact with the test chemical solution (Leather and Einhellig, 1985).

Radicle elongation does afford greater possibilities for mechanism studies

than seed germination. It is particularly suited for determining effects of allelochemicals on hormones responsible for cell growth. Radicle elongation also allows evaluation of allelochemical effects on respiration and cell division.

Seedling Growth Bioassays. Seedling growth bioassays are extremely versatile but require a greater quantity of chemical than is usually available during initial isolation and identification of allelochemicals. These bioassays usually have greater sensitivity and provide the basis for a variety of mechanism studies, such as nutrient uptake, water relations, and photosynthesis, but here again, it is difficult to determine the primary sites and mechanisms of action of the allelochemical.

Blum and colleagues (Blum and Dalton, 1985; Blum et al. 1985a,b), used leaf expansion of cucumber seedlings grown in nutrient culture to determine the mechanism of action of ferulic acid and its microbial metabolic products. In these reports, they stress the importance of monitoring the loss of allelochemical through absorption, microbial breakdown, or other mechanisms, including dissociation of the chemical in solutions of changing pH values.

Using a sorghum (*Sorghum*) seedling bioassay, we found that [^{14}C]salicyclic acid was rapidly taken up from the nutrient solution in which the seedling was growing, and it was distributed throughout the seedling within 24 hr after treatment (unpublished results). Such rapid, widespread translocation severely limits the utility of seedling growth bioassays for pinpointing primary sites or mechanisms of allelochemical action.

Lemna *Bioassays.* The *Lemna* bioassay developed in our laboratories is a versatile and extremely sensitive assay for allelochemical phytotoxicity (Einhellig et al., 1985). It meets many of the criteria for the assay of naturally produced phytotoxins (Duke, 1986) and can be used in the study of mechanisms of allelochemical action (Leather and Einhellig, 1985). Although only recently reported (Einhellig et al., 1985), this bioassay is now used by numerous laboratories for the detection of phytotoxic natural substances, including those produced by microorganisms. A drawback of this bioassay may be in relating the results obtained to the allelochemical effect on terrestrial plants. This relationship needs to be further investigated.

Lemna species (duckweeds) are angiosperms and offer a variety of parameters, including flowering (Cleland and Tanaka, 1982), which can be used as indicators of phytotoxicity. We measure the effects of allelochemicals on the dry weight, frond reproduction, chlorophyll content, and anthocyanin production. Anthocyanin production by *L. obscura* is the most sensitive to inhibition by selected allelochemicals (Leather and Einhellig, 1985). *Lemna gibba* affords other parameters such as chlorophyll content and flowering as indicators of allelochemical phytotoxicity, but because it is among the largest of the duckweeds, it is not suitable for low-volume assays (Ramirez-Toro et al., 1988).

Photosynthesis and overall respiratory metabolism of *Lemna* plants are par-

ticularly sensitive to perturbation and can thereby provide insight to the potential mechanisms of allelochemical action. Nyberg (1986) used *L. minor* to evaluate the effects of 22 different allelochemicals on chlorophyll content, photosynthesis, and respiration. Using a photorespirometer in conjunction with the 24-well assay plate, the tests could be conducted using very small quantities of allelochemical. Scholes (1987) evaluated the effects of compounds from six classes of allelochemicals on photosynthesis and respiration in *L. minor*. She found, for example, that juglone depressed the photosynthesis of duckweed at very low concentrations; this is in contrast to previous reports (Koeppe, 1972) that this allelochemical acted by interfering with respiration of isolated corn (*Zea mays* L.) mitochondria. However, caution must be observed when comparisons of two different systems are made.

CONCLUSIONS

Bioassays are essential tools for the identification of allelochemicals with phytotoxic properties. Because these chemicals are generally produced in small amounts and probably exert their effects in concert with other allelochemicals, it is desirable that the selected bioassay be extremely sensitive and that some indication of the mechanism of action of the allelochemical(s) can be determined. Nearly all reports of allelopathic activity indicate the response of the suspect leachate, exudate, or extract from a plant in some bioassay. With a better understanding of the perturbations elicited, advances in identification technology, and extremely sensitive bioassays, we can now begin to evaluate the integrity of bioassays that have formed the basis for the growing science of allelopathy.

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POTENTIALS FOR EXPLOITING ALLELOPATHY TO ENHANCE CROP PRODUCTION

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Abstract—Strategies for utilizing allelopathy as an aid in crop production include both avoidance and application protocols. There are immediate opportunities for management of weed and crop residues, tillage practices, and crop sequences to minimize crop losses from allelopathy and also to use allelopathic crops for weed control. Varieties of grain and forage sorghums (*Sorghum* Spp.), sunflower (*Helianthus annuus* L.), oats (*Avena sativa* L.), wheat (*Triticum sativum* L.), rye (*Secale cereale* L.), and others may provide weed control and in some instances crop stimulation from their residues. Our four-year field study with cultivated sunflower resulted in no differences in weed biomass between plots with and without herbicide (EPTC) applications. Strip cropping that included sorghum showed that in the subsequent year weed density and biomass were significantly lower in the previous-year sorghum than in soybean strips. Possibilities exist for modification of crop plant metabolism to alter production of allelochemicals. Allelochemical–environmental interactions must be considered in efforts to benefit from allelopathy. Under greenhouse conditions, joint application of low levels of atrazine, trifluralin, alachlor, or cinmethylin with a phenolic allelochemical showed that these two categories of inhibitors acted in concert to reduce plant growth. Allelochemicals may also be adapted as yield stimulants or environmentally sound herbicides, such as cinmethylin and methoxyphenone. Isolation of bialophos, tentoxin, and others shows that bacteria and fungi are good sources of biologically active compounds.

Key Words—Allelochemicals, allelopathy, autotoxicity, bioregulators, crop production, crop residue, herbicides, no-tillage, phytotoxins, weed control, weed interference

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INTRODUCTION

Biochemical interactions among plants (allelopathy) appear to be fairly ubiquitous phenomena, occurring to some extent in most natural and agricultural ecosystems. The science of allelopathy has progressed to the point that its descriptive and experimental foundations provide a base that can be used to aid crop production. In our focus on this topic both plants and microorganisms will be considered as sources and recipients of chemicals that may modify their growth, development, and distribution. Agricultural weeds and crops produce these allelochemicals, and microbial metabolism further contributes to the diversity of compounds present (Einhellig, 1985). Typically, these allelochemicals cycle through the soil matrix, and their impact on a crop is through root contact.

The natural products that cause allelopathy are a subset of the array of secondary compounds synthesized by plants and microorganisms. Most of the currently identified compounds are products of the shikimic acid and acetate pathways (Rice, 1984). Those frequently found include a diverse group of phenolic compounds, including cinnamic and benzoic acids, coumarins, tannins, and flavonoids; many terpenoids; and a few alkaloids, steroids, and quinones. Many that contribute to allelopathic interference have not been identified. It is extremely important to dispel the illusion that situations of allelopathic inhibition are caused by a single compound. Generally, interference arises from the combined action of a number of allelochemicals, and this impact is also influenced by other environmental stresses (Einhellig, 1987).

The flourish of recent activity in allelopathy has been partially driven by hopes for ways to reduce agricultural production costs and diminish some of the current reliance on synthetic chemicals that degrade environmental quality. Strategies to capitalize on allelopathy can be categorized as (1) avoidance of negative impacts, (2) exploitation of stimulatory effects, (3) management and development of allelopathic crops to suppress weeds, (4) development of allelochemicals as herbicides or growth regulators, and (5) combinations of these approaches (Einhellig, 1985). Space requirements preclude an extensive review of the literature supportive of capitalizing on allelopathy, and only exemplary evidences that focus on agronomic crops are noted.

ALLELOPATHIC INTERACTIONS IN AGRONOMY

Weed Interference. Total crop losses from weeds are estimated at $9–10 billion annually in the United States (Chandler, 1985; Putnam and Weston, 1986). Historically, investigators have viewed weed problems only in terms of competition, and no investigations have divided economic losses in a weed-

infested crop according to allelopathic vs. competitive interference. However, Putnam and Weston (1986) list about 90 species of weeds where allelopathic potential has been inferred, and this list is expanding. Allelopathic potential has typically been evidenced by plant growth performance following residue amendments to soil and/or bioassays using extracts, leachates, or exudates.

Our own investigations have focused on aggressive weeds common to the sorghum [*Sorghum bicolor* (L.) Moench] and soybean [*Glycine max.* (L.) Merr.] fields of the North Central Plains. Seedling bioassays using these crops demonstrated inhibition from *Kochia* [*Kochia scoparia* (L.) Schrad], Jerusalem artichoke (*Helianthus tuberosus* L.), cocklebur (*Xanthium strumarium* L.), velvetleaf, giant ragweed (*Ambrosia trifida* L.), and curly dock (*Rumex crispus* L.). Aqueous weed extracts reduced the growth of sorghum seedlings when material from 1 g fresh weight of leaf was incorporated into 60 ml of the nutrient medium. *Kochia*, Jerusalem artichoke, and cocklebur residues in soil caused no effect on sorghum germination, but after two weeks sorghum grown in soil containing 0.63% (w/w) of a residue weighed significantly less than controls. Disruption of water balance was one mechanism of action associated with allelopathic inhibition (Einhellig and Schon, 1982; Einhellig et al., 1985).

Allelochemicals have been identified from some weeds, but seldom has a thorough analysis of the chemical basis of interference been conducted. Some studies on the allelopathic aspects of a few species have included field-based analyses (Table 1). Reports on quackgrass over the last 50 years show that foliage and rhizomes of living plants and decomposing residues are phytotoxic. Putnam and Weston (1986) reported both plowed-under residues and those left in no-tillage systems reduced crop growth. Alfalfa grown in no-till plots had

TABLE 1. AGRICULTURAL WEEDS WITH ALLELOPATHIC POTENTIAL DEMONSTRATED BY FIELD AND LABORATORY INVESTIGATIONS

Species	Allelochemical classes implicated	Recent reference
Abutilon theophrastic Medic. (velvetleaf)	phenolic compounds	Sterling and Putnam, 1987; Colton and Einhellig, 1980
Agropyron repens (L.) Beauv. (quackgrass)	organic and phenolic acids; flavonoids	Weston et al., 1987; Putnam and Weston, 1986
Amaranthus palmeri S. Wats (Palmer amaranth)	sesquiterpene lactones; methyl ketones; alcohols	Menges, 1987; Connick et al., 1987
Parthenium hysterophorus L. (ragweed parthenium)	phenolic acids; sesquiterpene lactones	Kanchan and Jayachandra, 1979, 1980; Jarvis et al., 1985; Kumari et al., 1985
Sorghum halepense L. Pers. (Johnsongrass)	phenolic acids; cyanogenic glycosides	Menges, 1987; Rice, 1984

only 3.7% of the biomass of controls and nodulation was suppressed. Hence, some of the allelopathic effect of quackgrass may be indirect by action on *Rhizobium*. Crop plants associated with living quackgrass often exhibit symptoms of mineral deficiency, and added fertility does not overcome these effects. It is probable that allelochemicals impair uptake of some nutrients (Einhellig, 1986; Kobza and Einhellig, 1987).

Field experience in the warm, moist conditions of the southwestern United States demonstrated that soil-incorporated residues of Palmer amaranth inhibited the growth of carrot (*Daucus carota* L.) 49% and onion (*Allium cepa* L.) 68%. Volatile methyl ketones and alcohols released into the soil atmosphere markedly diminish germination and establishment of these small-seeded crops. Ragweed parthenium is a serious problem in India (references: Table 1). All the organs of this weed are inhibitory in bioassays and more than a dozen allelochemicals have been identified. Leaves mixed into the soil reduced the yield of four crops, while they stimulated growth of bajra (*Pennisetum typhoideum* Rich.). Thus, crops may differ markedly in their sensitivity.

Decomposition time, climatic conditions, soil type, and relative placement of residue with respect to crop plants are critical. Allelopathic effects are often more severe with light-textured soils, but poorly drained, heavy soils can also accentuate a problem. Residue location dictates allelochemical concentration in the microenvironment of a germinating seed or growing root. Such information can be used for decisions on the timing and type of crop tillage preparations.

Crop–Crop Interference. Need for avoidance-management strategies is also apparent because allelopathic conditions generated by one crop may influence a subsequent planting. Often, evidence on crop sequence problems is either anecdotal, or at a minimum, it is conditional according to location, climate, and year-to-year variables. A few examples of large acreage crops illustrate the widespread nature of the problem (Table 2).

Alfalfa and some perennial legumes often exhibit autotoxicity and replant problems (Miller, 1983). Isoflavonoids, phenolic acids, and medicagenic acid glycosides have been implicated in the soil sickness of red clover (*Trifolium*

TABLE 2. INTERFERENCE FROM LARGE ACREAGE CROPS ON FOLLOWING PLANTING

First crop	Affected planting	Location
Alfalfa (*Medicago sativa* L.)	Alfalfa	U.S. Central Plains
Corn (*Zea mays* L.)	Corn	Iowa
Rice (*Oryza sativa* L.)	Rice, soybeans	Taiwan
Sunflower (*Helianthus annuus L.*)	Following crop	South Dakota
Wheat (*Triticum aestivum* L.)	Wheat	U.S., Australia, U.S.S.R., England

pratense L.) (Chang et al., 1969: Oleszek and Jurzysta, 1987). Terpenoids and polyphenols contribute to the decline in yield of successive crops of pigeon pea [*Canjanus cajan* (L.) Millsp] (Hepperly and Diaz, 1983).

Agroecosystems repeating the same cereal crop often experience yield declines. In Taiwan, rice is planted twice a year in continuous monoculture. With a short time interval between crops, yields are reduced about 25% in the second crop (Chou, 1986). Intense study of stubble-mulch tillage continuous wheat production in Nebraska showed that the residues provided an environment for a bloom of *Penicillin urticae* Bainer (McCalla and Norstadt, 1974). This fungus produced patulin, which is very toxic to wheat. High levels of microbially produced short-chain fatty acids contribute to wheat allelopathy in England (Lynch, 1987). Some inhibitors have been identified in these cases, but there are still many deficits in explaining their mode of action and how allelochemical–environmental interactions finally result in a diminished stand and yield (Einhellig, 1986).

Many of these agriculture problems evolve from a combination of allelopathy and factors such as plant pathogens, changes in soil structure, moisture and fertility stress, and others. We have observed that production is lower than normal in the year following a sunflower crop. Allelochemical deposition and reduction in soil moisture may both be involved. In Iowa, corn following soybeans the previous year yields about 10 bu/acre more than corn following corn even when the corn residue is plowed under in the fall and adequate nitrogen is supplied. Anderson (1984) suggested the yield differences are due to both an inhibitory effect of corn and a stimulatory action from soybeans. Fortunately, it is not necessary to have a full understanding of the causative factors in order to modify production practices and improve productivity. Crop rotations and tillage practices are obvious approaches to avoid detrimental crop–crop interactions and capitalize on stimulatory effects. Work in the U.S.S.R. is emphasizing breeding plant varieties with less allelopathic activity (Grodzinsky, 1987).

WEED CONTROL BY CROPS

Crop Interference. Some crops are effective in minimizing growth of companion weeds, and there may be a carryover weed suppression in the following season. We found a sunflower–oat rotation over five years showed significantly lower grass and broadleaf weed densities than in open control plots (Leather, 1983). Although weed density increased in all plots over the five seasons, the rate of increase was less in sunflower plots. There was little difference among the sunflower cultivars. In a follow-up study, weed biomass was equivalent in plots planted with sunflowers, whether or not EPTC (*S*-ethyl dipropyl carbamothioate) herbicide was applied, clearly showing the efficacy of sunflower-

mediated weed control (Leather, 1987). The crop was reduced in a second year of sunflower, indicating a compatible crop rotation strategy should be considered.

The allelopathic nature of sorghum (*Sorghum*) crops has been suggested from various parts of the world. For the past two seasons, we have monitored the weed biomass the year following strip cropping with grain sorghum, corn, and soybeans, all with no herbicide. Weed cover at the time of early spring emergence in former sorghum strips was less than 30% of that in prior soybean strips (first season, unpublished data). At midsummer, plots where grain sorghum had grown the year before had about 60% of the weed biomass of others. This reduced biomass resulted from the suppression of broadleaf weeds, with no effect on grasses. Laboratory work showed that grain sorghum inhibits early seedling growth and development more than germination. Some farmers in South Dakota currently strip plant grain sorghum and sunflower without herbicides and find reasonable weed control, as well as protection from wind erosion. Forney et al. (1985) reported that growing a forage sorghum–sudangrass hybrid prior to late-summer planting no-till alfalfa suppressed weeds and enhanced alfalfa growth.

Other examples of the suppressive effect of crop plants on certain weeds are sweet potatoes [*Ipomea batatas* (L.) Lam.] on yellow nutsedge (*Cyperus esculentus* L.) and soybeans on barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] (Harrison and Peterson, 1986; Maun, 1977). Hence, the concept of allelopathic crops may be one alternative in weed control strategies. If this prospect is to materialize, more extensive research is needed on applicable crop sequences, the roles of soil and climatic factors, long-term impacts on the weed population, and ways to integrate allelopathic crops with other weed control practices.

Genome Enhancement. The possibilities for exploiting allelopathy include genetic manipulation of crops to increase their capability for weed control. There is a general consensus that this capacity has been diminished during development of currently used strains, although we did not find this to be true with sunflowers. Fay and Duke (1977) reported some accessions of oats exuded three times as much scopoletin as a standard cultivar. Putnam and Duke (1974) found decided differences in toxicity among 500 cucumber accessions from 41 nations of origin. In field tests, one allelopathic accession reduced total weed fresh weight to approximately one third of the weed biomass found with Pioneer, a commonly grown cultivar (Lockerman and Putnam, 1979). However, the allelopathic effect was less under periods of increased rainfall.

Altering the genome of crops to enhance herbicidal activity could be approached through classical breeding programs or biotechnology. Major problems for the latter are identifying allelochemicals that may effect a desired change, learning how their production is regulated, and development of a genetic

engineering approach for moving a controlling gene element into an agronomic crop. ARCO Plant Cell Research Institute has such long-term objectives (Burke, 1987). An alternative to genome changes is to develop chemicals to manipulate crop metabolism to achieve an increase in allelochemical production. One limitation to enhancing crop-originated weed control is that autotoxicity must be avoided. A second concern is that changes in the chemical balance or chemical constituents of a crop must not have adverse effects on humans, livestock, or other nontarget organisms.

Cover Crop and Residue Management. Cover crops of wheat, barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), rye (*Secale cereale* L.), grain sorghum, and sudangrass [*Sorghum arundinaceum* (Desv.) Stapf.] have been used effectively to suppress weeds (Barnes and Putnam, 1983; Putnam and DeFrank, 1983; Putnam et al., 1983; Shilling et al., 1985). Three approaches for employing residues of these annual grasses have emerged in conjunction with minimum tillage: fall planting followed by winter kill, fall or spring planting and subsequent mowing or herbicide desiccation, and double-crop systems.

Some form of conservation tillage is used on approximately one third of the U.S. crop acreage. These practices are expanding because of potential cost savings, erosion control, and beneficial moisture conditions. Weed suppression is an added dimension that results from a combination of allelopathy and physical factors, such as light reduction and not elevating buried weed seeds to positions for germination. It has been demonstrated that allelochemicals make a significant contribution when planting no-till into residues by using control plots of *Populus* excelsior to alter the physical environment in the same manner as cover crop residues (Putnam et al., 1983). In addition, allelochemicals from cover crop residues that inhibit the germination and growth of weeds have been isolated.

The high biomass production and toxicity of rye make it a particularly effective weed-suppressant cereal. Decomposing residues generate phenylacetic acid, 4-phenylbutyric acid, and a complex of benzoic and cinnamic acids. Aqueous extracts of rye contain β-phenyllactic acid, β-hydroxybutyric acid, and toxic hydroxamic acids (Shilling et al., 1985; Barnes et al., 1986). DIBOA [2,4-dihydroxy-1,4(2H)-benzoxazin-3-one] was shown to maintain toxicity for an extended period following addition to soil. The collective allelochemical action is impressive. Barnes et al. (1986) reported that weed biomass in a cover crop of living rye was reduced 90% over unplanted controls and even a mulch of 40-day-old spring-planted rye gave 69% reduction. Shilling et al. (1985) found prior conditioning with either rye mulch or root residue before tobacco (*Nicotiana tabacum* L.), soybean, and sunflower crops gave good weed control. Soybeans and sunflowers planted without tillage into desiccated rye mulch (fall-planted, spring-killed) gave over 90% reduction in the biomass of common lambsquarter (*Chenopodium album* L.), redroot pigweed (*Amaranthus retro-*

flexus L.), and common ragweed (*Ambrosia artemisiifolia* L.), compared to tillage and no rye.

Experience with local conditions, crop tolerances, cost considerations, and integration with appropriate tillage practices are prerequisites to relying on crop residues for weed control. Crop injury from mulch-related weed control can be circumvented by adjustment of time between mulching and planting and using tolerant crops. Putnam et al. (1983) found that, generally, the larger-seeded food crops (corn, cucumber, pea, snapbean) were more tolerant than lettuce, carrot, and tomato. Also, carrot and tomato tolerated some residues but not others.

COMPLEMENTARITY OF ALLELOCHEMICALS AND HERBICIDES

Some of the previously noted research on weed control in Michigan and North Carolina desiccated the cover crops with glyphosate or paraquat. Although more expense is incurred, Putnam et al. (1983) reported better weed control in the vegetable crops was obtained from herbicide desiccation in late spring compared to winter-killed residue. Similarly, in apple and cherry orchards companion planting of rye or wheat in the fall and desiccation in the subsequent year gave weed control equal to or better than repeated tillage or herbicide spraying, and long-term studies have shown no effect on the trees.

Allelopathic crops and mulches may not provide total weed control. In our experience, allelopathic control of weedy grasses is generally poorer than for most broadleaf weeds, and some of these are more resistant than others. Shilling et al. (1985) found rye mulch in tilled systems did not significantly reduce common ragweed, whereas it depressed several other broadleaf weeds. An herbicide applied along with allelopathic conditions could have supportive action, affecting the same or different species.

We reported that when a level of atrazine that did not inhibit oat seedlings was applied in combination with ferulic acid, the net inhibition was greater than from ferulic acid alone (Einhellig, 1987). Additional work using sorghum and wheat seedling bioassays showed that low levels of trifluralin, alachlor, and cinmethylin will act in concert with phenolic acid allelochemicals (Hamm, 1984; Wegher, 1986). For example, there is an additive deleterious effect on sorghum after 10 days growth with trifluralin and salicylic acid in the medium (Table 3). In another investigation, sorghum was not altered with either 100 ppb alachlor or 0.2 mM ferulic acid, but a combination of these reduced growth by more than 50%. The experimental design was a matrix of five levels of alachlor and four levels of ferulic acid, and a two-way ANOVA showed a significant interaction term. As the treatment of either type of chemical alone became quite inhibitory, the action of the second stress was masked (Wegher, 1986). These

TABLE 3. EFFECTS OF TRIFLURALIN (TR), SALICYLIC ACID (SA), AND THEIR COMBINATION ON GROWTH OF GRAIN SORGHUM SEEDLINGS

Treatment (ppm)		Dry wt.[a] in mg ± SE (and % of control)		
TR	SA	Root	Shoot	Plant
—	—	58.9 ± 6.8a	92.9 ± 9.4a	151.8 ± 16.0a
0.1	—	44.3 ± 3.2b (75%)	66.1 ± 4.4b (71%)	110.4 ± 6.9b (73%)
—	20	32.2 ± 3.2c (55%)	55.0 ± 5.6b (59%)	87.2 ± 8.7b (58%)
0.1	20	19.4 ± 2.5d (33%)	39.0 ± 2.2c (42%)	58.4 ± 4.1c (39%)

[a] Values ($N = 12$) in a column not followed by the same letter are significantly different, $P = 0.05$, ANOVA with Duncan's multiple-range test.

data suggest that a reduced level of herbicide may be feasible to provide weed control when it operates simultaneous with allelopathic conditions. However, caution should be exercised that dual herbicide–allelopathic methods do not cause crop damage or have other negative environmental impacts.

A variety of stresses can initiate an increase in allelochemical content of plants (Rice, 1984), and allelopathic content also varies according to plant maturity (Lehle and Putnam, 1982). It is probable that manipulation of timing, formulation, and application rate of a herbicide used to kill or supplement a cover crop can enhance crop phytotoxicity, improving weed control. Herbicide-mediated suppression of some allelochemical pathways may also be possible, and eventually chemicals may be used to shift microbial populations or their metabolism to regulate the production and release of phytotoxins.

ALLELOPATHY MANAGEMENT

By definition, agroecosystems are manipulated systems. Many items that have previously been discussed allude to the need for management considerations to include the impact of allelopathic plants, whether they are weeds or crops. The goal is to control the system to avoid production losses created from allelopathy and to take advantage of beneficial aspects of allelopathic plants. The research cited on utilization of cover-crop residues is a start in this direction. Similarly, Chou (1987) described ways to eliminate phytotoxins to improve crop yield in several production systems of Taiwan. They included improvement of drainage, removal of phytotoxins by flooding, detoxification by nutrient dressing, and rotating crops. In a unique approach to allelopathy management,

Syamasundar and Mahadevappa (1987) have shown that interference from *Cassia sericea* Sw., a plant with several economic uses in India, can be used to control ragweed parthenium.

However, it is rare that planting and tillage decisions are made with regard to the natural consequences of allelopathy. Likewise, allelopathy management cannot be considered in isolation from other management criteria, such as decisions on the appropriate crop, fertility needs, control of disease, insects, and weeds. All these factors must be balanced with economic and long-term environmental considerations. There is a strong need for research on how these multiple factors, including allelopathy, will be integrated into crop production management.

COMMERCIALIZATION OF ALLELOCHEMICALS

Public demand for environmentally safer pesticides, herbicides, and growth regulators has given renewed emphasis to development of natural products for these purposes. Typically, their residence time in an ecosystem is shorter, and their toxicities are more narrowly targeted. Many crop production systems are now showing an increase in herbicide-resistant weed strains as well as changes in the composition of weed populations toward species quite closely related to the crops they infest. These factors, coupled with the increasing difficulty of developing new herbicides by traditional chemistries, indicate that new weed-control strategies must emerge (Duke, 1986a).

The number of compounds produced by plants and microorganisms probably exceeds the number of synthetics that have been prepared in the last century. Using Swain's (1977) estimate of 400,000 secondary metabolites, only about 3% of this storehouse has been identified, and only a fraction of those identified have been evaluated for herbicidal or bioregulator activity. Some plant and microbial metabolites should have direct application potential, whereas others will provide novel chemicals that can be modified to enhance biological activity (Figure 1).

Herbicides from Higher Plants. Although the inspiration that led to the development of a herbicide is seldom clear from the literature, some plant compounds have obviously served as structural templates. Several phenoxy herbicides are auxin analogs. Benzoic acid compounds are frequently implicated in allelopathy, and the herbicidal utility of their halogenated derivatives (TBA, TIBA, Dicamba, etc.) is well known. Shettel and Balke (1983) reported the common allelochemicals salicylic acid, *p*-hydroxybenzoic acid, hydroquinone, and umbelliferone effectively suppressed the growth of several weeds when applied as a spray. Unfortunately, they were not selective, and the rates necessary to inhibit weeds were quite high. Herbicide development of the photo-

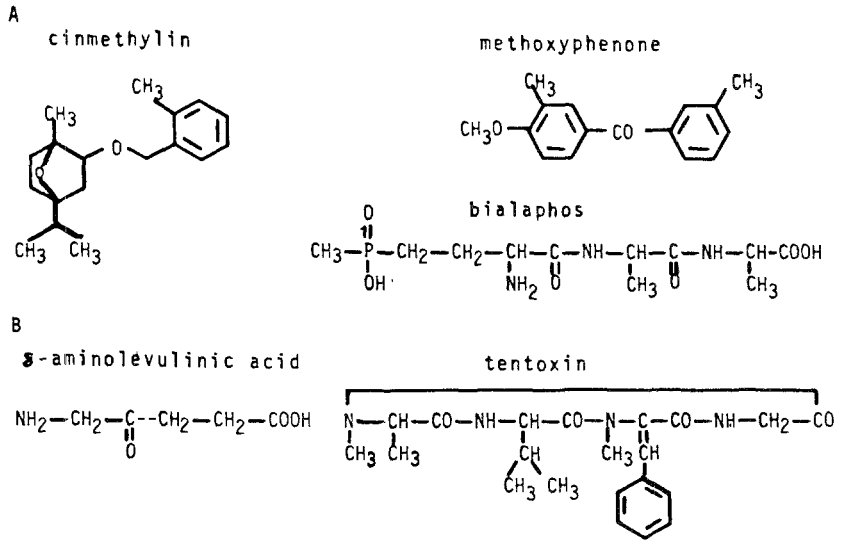

FIG. 1. Structures of (A) herbicides with a natural product basis or component and (B) plant and microbial phytotoxins with potential for development.

sensitizer Δ-aminolevulinic acid has recently been proposed (Hopen et al., 1985).

Cinmethylin (Cinch; Shell Development Company) represents a new class of herbicide (Figure 1). The molecule contains only carbon, hydrogen, and oxygen atoms, and the environmental impact should be minor. It controls many annual grasses and some broadleaf weeds with preemergence treatment. In our tests, the concentration of cinmethylin required to inhibit the growth of several grasses was comparable to that required for alachlor. Cinmethylin is a cineole alcohol with the addition of a substituted benzyl group. Its advent comes 20 years after 1,8-cineole was identified as an agent in allelopathy.

Microbial Compounds as Herbicides. Phytotoxins isolated from bacteria and fungi appear to be important sources of herbicides (Cutler, 1984, 1987; Duke, 1986b; Duke and Lydon, 1987). Purported advantages of these compounds, compared to those from higher plants, include their ease of isolation and quantities obtainable for study, potential for specificity, efficacy at low rates, and opportunities for fermentation production. Candidates include both pathogenic and nonpathogenic bacteria and fungi. The Japanese have made significant progress in this field (Otake, 1983; Cutler, 1987), and several U.S. and European firms have programs for developing herbicides from microbial metabolites. Monsanto Agricultural Co. is finishing the second year of a project screening microorganisms for phytotoxins. Several thousand isolates have been

tested in bioassays and about 3% of these have provided interesting leads (Kevin Crosby, personal communication).

The chemical skeleton of picloram (Tordon) is the microbially produced alkaloid α-picolinic acid. A product of *Streptomyces*, anisomycin, led to the development of the synthetic herbicide NK-049 (methoxyphenone) which kills barnyardgrass in rice. Bialaphos [L-2-amino-4-[(hydroxy)(methyl) phosphinoyl]-butyric acid]-was isolated from *Streptomyces viridochromogenes* and has been marketed in Japan as Herbiace (Figure 1). It kills both monocot and dicot species and is effective on perennials. Duke (1986b) lists by source and known effect 40 additional microbial non-host-specific phytotoxins that have been characterized. They include tentoxin, a cyclic tetrapeptide from *Alternaria alternata.* Cutler (1985) described several other chemical classes of toxins from *Alternaria* spp. in noting more than a dozen fungal metabolites with activity.

The selectivity of microbially produced toxins covers a wide range. Some are host-specific, affecting only one or a few species, whereas others are non-host-specific and are toxic to plants that the producing microorganisms do not infect. The potential that microbial toxins may also have a broad spectrum of effects beyond plants adds to the care that must be taken in utilization of such compounds.

Yield Enhancers. A variety of nonnutrient substances that manipulate growth, development, and composition of plants has been marketed. These exogenous bioregulators typically have been synthetic chemicals that function by some interaction with the endogenous five phytohormone groups. Even though they have made little impact on large-acreage economic crops, according to Jung (1985) plant bioregulators had a dollar market share of 3–4% among crop production chemicals. Their actions include growth retardation, flower induction, hastening maturity or senescence, enhanced biomass production, and others. Allelochemicals provide a promising source for new growth regulating compounds.

The tantalizing possibility of discovering a natural product that can be used to increase crop yield has driven several research efforts. Three compounds that have received the most attention are agrostemmin, triacontanol, and brassinolide. Rice (1984) summarized Yugoslavian work showing as little as 1.2 g of agrostemmin per hectare increased wheat yields. Its discovery followed the observation that higher wheat yields were obtained when grown in mixed stands with corn cockle (*Agrostemma githago* L.). Triacontanol, a 30-carbon primary alcohol, was isolated as a growth-promoting factor from alfalfa. Foliar applications of triacontanol have increased yields in cucumber, carrot, rice, corn, soybean, and others. Inconsistent results, perhaps due to formulation problems and to method, rate, and time of application, have reduced the efficacy of the compound (Laughlin et al., 1983). Extensive work has gone into evaluating brassinolide, a steroid isolated from rape (*Brassica repus* L.) pollen, as a yield

stimulant. Brassinolide and several analogs have been synthesized (Maugh, 1981), but the expense is currently too high for use on field crops.

The task of discovering a plant-growth regulator is substantially more demanding, perhaps by a factor of 10–100 times, than for a new herbicide (Jung, 1985). Companies are anxious to discover and develop growth regulators, as evidenced by 67 patents issued for plant-growth regulators in 1983, a threefold increase from 20 years earlier (Holmseni, 1984). However, from a commercial perspective, these efforts must produce a significant yield enhancement chemical in the next few years or resources may be deployed to more productive alternatives.

Biological Agents. No prognostication on the potential impact of allelopathy is complete without some reference to the application of organisms to aid crop production. In some cases, their actions may be the result of allelochemicals that are released. Quantum 4000 (Gustafson, Inc.) is the first of what may be several root protectant products. It is a peanut (*Archis hypogaea* L.) seed inoculant that consists of live *Bacillus subtilis*. This beneficial bacterium colonizes the roots, conferring protection against disease. Some evidence suggests that antibiotic production may be involved in the enhanced root health. Several companies are now field-testing other biological root protectants (Brosten, 1987).

Successful mycoherbicides, Collego and DeVine, have been marketed for control of northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.] and stranglevine [*Morrenia odorata* (H. & A.) Lindl.], respectively. DeVine, the first registered commercial fungus for weed control, contains a strain of *Phytophthora palmivora* (Butl.) Butl. that kills the current stand of stranglevine and controls the weed for several years to come. Whether the pathogenicity is due to toxic substances is not known. Undoubtedly, additional biological herbicides will be used successfully in weed control.

CONCLUSIONS

Sufficient information is available and production demands are opportune for exploiting allelopathy to benefit crop production. Modern agriculture is in a period of transition toward tillage reduction and curtailment of environmental damage from agrochemicals. Although information is incomplete on the specific allelochemicals and mechanisms involved, some crops can effectively control many competing weeds, and cover crops, in conjunction with reduced tillage, can supplement herbicides. Crop rotations, compatible sequences, residue management, timing of operations, and other approaches should be guided by local conditions and experience. More research is needed on ways to manage allelopathic weed and crops within the larger context of management in agro-

ecosystems. In the longer term, adaptations of biotechnology may be used to elevate or reduce production of allelochemicals by crops.

Screening for biological activity and the isolation and identification of allelochemicals must be done by the agrochemical industry to capitalize on natural products. Bacterial and fungal metabolites are good sources of chemicals for development of herbicides and bioregulators. Commercial formulations, including biological agents whose activity may be mediated by allelochemicals, have also shown success. Marketing useful allelochemicals and their analogs to enhance crop production is a challenging and potentially rewarding new frontier.

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ACTIVITY OF DRIMANE ANTIFEEDANTS AND RELATED COMPOUNDS AGAINST APHIDS, AND COMPARATIVE BIOLOGICAL EFFECTS AND CHEMICAL REACTIVITY OF (−)- AND (+)-POLYGODIAL

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Abstract—A series of natural drimanes and related synthetic compounds was tested for antifeedant activity against aphids. Polygodial and warburganal were the most active. The synthetic compounds methyl 9α-hydroxydrimenoate and 9α-hydroxydrimenal, although active against lepidopteran larvae, were inactive against aphids. Natural (−)-polygodial and the synthetic (+) isomer showed similar levels of activity as aphid antifeedants and in phytotoxicity, fish toxicity, and human taste tests, but reacted at different rates with enantiomers of 1-phenylethylamine.

Key Words—Antifeedant, sesquiterpenoid, drimane, dialdehyde, polygodial, 1-phenylethylamine, *Myzus persicae*, aphid, Homoptera, Aphididae, phytotoxicity, taste.

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INTRODUCTION

Natural sesquiterpenoid dialdehydes in the drimane series, such as (−)-polygodial (1) from the temperate herbaceous weed water-pepper *Polygonum hydropiper* (Polygonaceae) (Barnes and Loder, 1962) and (−)-warburganal (12) from the East African tree *Warburgia ugandensis* (Canellaceae) (Kubo et al., 1976), show various biological effects. These include potent antifeedant activity against lepidopteran larvae such as the armyworms *Spodoptera exempta* and *S. littoralis* and against other insect pests (for recent review see van Beek and de Groot, 1986). A wide range of related synthetic compounds has been tested behaviorally and electrophysiologically against larvae of *Spodoptera* and *Heliothis* spp., and dimethyl polygodioate (2)and methyl 9α-hydroxydrimenoate (18) were as active as (−)-polygodial and (−)-warburganal (Blaney et al., 1987). In laboratory tests, (−)-polygodial has been shown to inhibit feeding and colonization by the aphid *Myzus persicae* and to decrease the transmission of potato virus Y and beet yellows virus, even by aphid variants highly resistant to insecticides (Gibson et al., 1982). In the field, (−)-polygodial, obtained from commercially grown water-pepper by extraction with liquid carbon dioxide, has been used to reduce transmission of barley yellow dwarf virus by the aphid *Rhopalosiphum padi* (Dawson et al., 1986; Pickett et al., 1987). However, the activity against aphids of related compounds, particularly those known to be active against lepidopteran larvae, has not been reported.

Synthesis of drimane antifeedants and related compounds has been recently reviewed (Ley, 1987; de Groot and van Beek, 1987). Polygodial is readily synthesized as the racemate by two elegant routes devised by Ley (Hollinshead et al., 1983) and Lallemand (Jalali-Naini et al., 1983) and can also be synthesized as the separate enantiomers (Mori and Watanabe, 1986). Kubo and Ganjian (1981) have claimed that specific absolute stereochemistry determines antifeedant activity and also other properties such as hotness to human taste. In addition, preliminary studies suggested that the (+) isomer of polygodial was more phytotoxic than the (−) isomer (Pickett, 1985). We have therefore compared the biological activities of the (−) and (+) isomers of this compound. Furthermore, since D'Ischia et al. (1982) have suggested that the biological activity of polygodial is related to its ability to react with amino groups, we have investigated the differential reactivities of the polygodial enantiomers with a chiral amine.

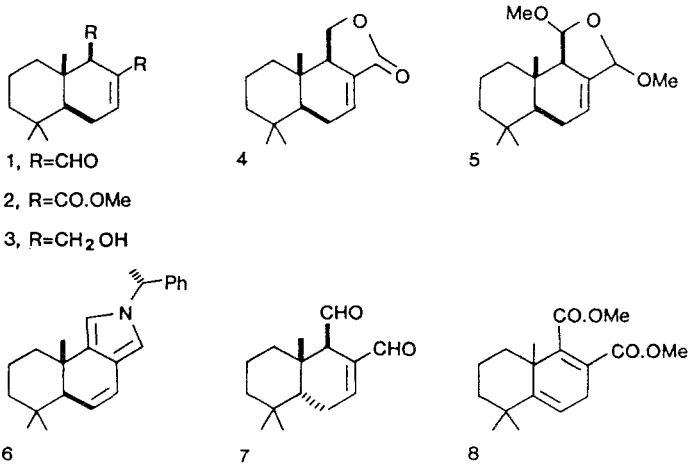
METHODS AND MATERIALS

Compounds. Natural (−)-polygodial (1) was isolated by standard methods (Barnes and Loder, 1962) from water-pepper, *Polygonum hydropiper*, collected

in Hertfordshire, U.K. (for aphid antifeedant and phytotoxicity tests) and in Tokushima, Japan (for hotness to taste and piscicidal tests).

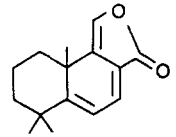
Synthesis of (±)-polygodial (1 and 20) was by a combination of two methods (Hollinshead et al., 1983; Jalali-Naini et al., 1983), but with a modification to the transformation of compound 8 to 2. Compound 8 was treated with lithium di*iso*propylamide (LDA) (1.2 equivalents) in tetrahydrofuran (THF) at −78°C; the anion was stereoselectively protonated using trimethylacetic acid and the product hydrogenated (Pd–C) to give compound 2 in over 80% yield after crystallization, which was then converted to racemic polygodial as before. Resolution of polygodial enantiomers was achieved as shown in Scheme 1: alkaline hydrolysis of 2 at room temperature gave the crude half-ester which was crystallized from acetonitrile (mp 164.5°C). [^{1}H]NMR JEOL PFT 100 (100 MHz) $CDCl_3$, diagnostic peaks at 3.15 (m, 1H), 3.63 (s, 3H), 7.13 (m, 1H), 10.8 (s, 1H). The half-ester formed a diastereomeric salt with (+)-(*R*)-1-phenylethylamine, which after five crystallizations from methanol–water (1 : 1) and acidic work-up gave the (−) half-ester $[\alpha]_D^{20} = -47.2°$ (c = 2.5, EtOH). Conversion with excess di*iso*butylaluminium hydride gave the diol $[\alpha]_D^{20} = -9°$ (c = 7.0, EtOH). Swern oxidation (using dimethyl sulfoxide and oxalyl chloride, followed by treatment with triethylamine at −78°C) gave the (−)-dialdehyde (mp 50–51°C, from hexane) $[\alpha]_D^{20} = -137°$.

Identical treatment of the half-ester with (−)-(*S*)-1-phenylethylamine gave the (+) half-ester $[\alpha]_D^{20} = +48.2°$ (c = 2.3, EtOH), diol $[\alpha]_D^{20} = +9°$ (c = 5.8, EtOH) and the (+) isomer 20 (mp 49–50°C) $[\alpha]_D^{20} = +138°$ (c = 0.35, EtOH).



1, R=CHO
2, R=CO.OMe
3, R=CH_2OH
4
5
6
7
8

STRUCTURES FOR COMPOUNDS 1–8.



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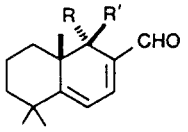
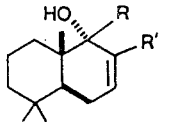


10, R=H;R'=CHO

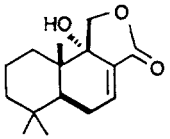
11, R=CHO;R'=H



12, R=R'=CHO

13, R=CO.OMe; R'=CHO

14, R=R' = CO.OMe



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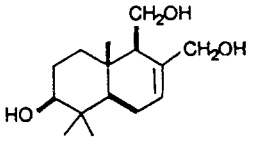

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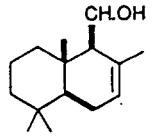

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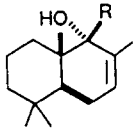
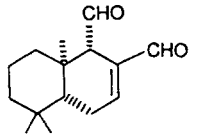


18, R=CO.OMe

19, R=CHO



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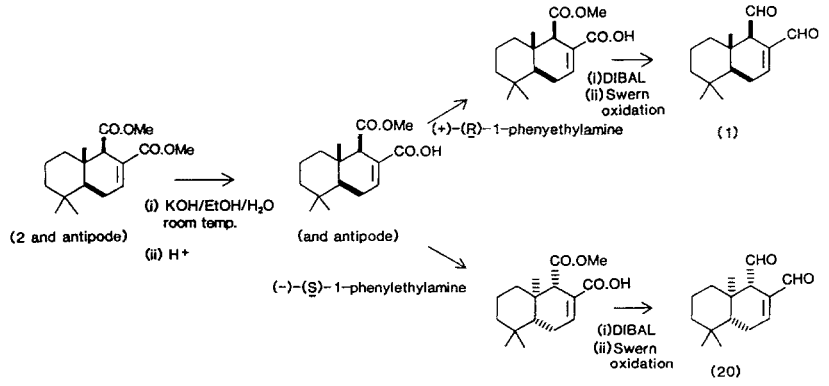
STRUCTURES FOR COMPOUNDS 9–20.



SCHEME 1.

The (−) and (+) isomers of polygodial (1 and 20) were also synthesized separately by the method of Mori and Watanabe (1986). Compounds 2, 3, 4 (cinnamolide), 8, 9, 13, 14, 15, and 16 were synthesized as racemates by methods described previously (Ley, 1987). Compound 5 was prepared as a mixture of geometric isomers from (±)-polygodial by treatment with methanol and toluene-*p*-sulfonic acid (Pezechk-Jalali, 1985). Polygodial was readily regenerated from 5 by treatment at room temperature with formic acid or an aqueous hydrochloric acid (0.5M) and diethyl ether mixture. Compound 6 (and diastereoisomer) was obtained by allowing (±)-polygodial to react with two equivalents of (+)-(*R*)-1-phenylethylamine in carbon tetrachloride overnight at room temperature, then removing solvent and heating the product at 180°C for 5 min. [^{1}H]NMR JEOL JNM-GX 400 (400 MHz) $CDCl_3$, diagnostic peaks at 1.77 (d, 3H), 2.12 (m, 1H), 5.14 (q, 1H), 5.68 (dd, 1H), 6.31 (m, 1H), 6.48 (dd, 1H), 6.53 (m, 1H). (The pyrrole protons δ6.31 and 6.53 are complicated by diastereomeric effects). Compound 7 (*cis*-polygodial) was synthesized as the racemate (Guillerm et al., 1984). Isomerization of compound 8 with LDA at −78°C in THF and kinetic protonation with 0.5 M sulfuric acid, reduction to the diol, and Swern oxidation at −62°C gave compound 10. Isomerization of 8 with 1,8-diazabicyclo[5.4.0]undec-7-ene in refluxing benzene followed by lithium aluminium hydride reduction and Swern oxidation at −72°C gave compound 11. (−)-Warburganal (12) and (−)-drimenol (17) were obtained as gifts, having been isolated from *Warburgia ugandensis* (Kubo et al., 1976) and *Drimys winteri* (Appel et al., 1959), respectively. Compounds 18 and 19 were synthesized as single isomers from (−)-drimenol (Blaney et al., 1987; P. Grice, personal communication).

Analysis. Estimation of polygodial was by high-performance liquid chromatography (HPLC) on a Spherisorb 5 μm nitrile column (25 × 0.46 cm) using ether–hexane (1 : 1) at 3 ml/min with ultraviolet detection at 224 nm. Estimation of the enantiomers of polygodial was by chiral HPLC on a β-cyclodextrin column (25 × 0.46 cm, Cyclobond I, Advanced Separation Technologies Inc.) at ambient temperature, using methanol–water (85 : 15) at 1 ml/min with ultraviolet detection at 224 nm. This method was preferred to an excellent gas chromatographic method for analyzing enantiomers of polygodial (Brooks et al., 1985) because of a requirement for derivatization with amphetamines not available commercially.

Bioassays. Antifeedant activity against aphids was tested by treating half-leaves of Chinese cabbage (*Brassica campestris* var. Chinensis, Cruciferae) with ethanolic solutions of the test compound and comparing numbers of settled aphids, *Myzus persicae*, on treated and untreated halves after 24 hr (Dawson et al., 1982). There were 10 replicates of 20 aphids at each concentration. The highest level tested was 0.1% (ca. 250 ppm compound/leaf), and the concentration was reduced by a factor of two until the difference between treated and

untreated areas was no longer significant at $P = 0.05$ (calculated by analysis of variance).

Similar antifeedant tests were done using a synthetic membrane stretched over a plastic ring. Test compounds were painted onto one half of the lower surface of the membrane, and aphids placed in the ring were allowed to feed on an artificial diet held on the upper surface (Griffiths et al., 1975). Aphids feeding on treated and untreated halves were counted after 24 hr, as before.

Antifeedant activity against the larvae of diamondback moth, *Plutella xylostella* (= *maculipennis*), was assessed by treating half-leaves of Chinese cabbage with test compound and estimating the percentage leaf area eaten after 24 hr (Pickett et al., 1987).

Phytotoxicity to Chinese cabbage leaves was scored on a scale of 0 to 5: 0, no damage to leaf surface; 1, most of leaf surface normal, some slight pitting; 2, slight pitting over whole leaf surface or dry patches on leaf; 3, slight pitting over whole leaf surface with some dry patches; 4, pitting of leaf surface, large areas dry and papery; 5, leaf shrivelled. Effects on germination were assessed using 100 wheat seeds (*Triticum aestivum* var. Norman, Graminaceae) which were dipped into ethanolic solution of test material, allowed to dry, and then grown on moist filter paper for 12 days.

Piscicidal activity was assessed at 0.4 ppm by standard methods using three killie fish, *Oryzia latipes*, per treatment (Kawazu, 1981).

Human taste tests were performed using aqueous solutions (0.045 ppm) as described previously (Kubo and Ganjian, 1981), using a panel of 14 people.

Differential Reactivity of Polygodial Enantiomers with Chiral Amine. (±)-Polygodial (23.4 mg, 0.1 mmol) in diethyl ether (5 ml) was slowly added to (+)-(*R*)-1-phenylethylamine (6.12 mg, 0.05 mmol) in diethyl ether (5 ml) and left overnight at room temperature. Solvent was removed and the absence of amine confirmed by nuclear magnetic resonance spectroscopy. The unreacted polygodial was isolated from the residue by thin-layer chromatography (silica gel, hexane–diethyl ether 1 : 1) and the specific optical rotation determined. This procedure was repeated with (−)-(*S*)-1-phenylethylamine.

RESULTS AND DISCUSSION

The influence of various drimanes and related compounds on feeding and colonization by aphids was determined by the number settled on treated and untreated halves of a leaf (Table 1). The most active compounds were the natural drimane dialdehydes (−)-polygodial (1) and (−)-warburganal (12) and the synthetic enantiomer (+)-polygodial (20). Drimanes closely related to (−)-polygodial i.e. the diester 2, the diol 3, and the lactone cinnamolide (4) were only weakly active, while compound 5, formed by addition of methanol, was

TABLE 1. APHID SETTLING TEST ON LEAVES: DRIMANES AND RELATED COMPOUNDS

Compound	Lowest concentration at which active (%)
1	0.05
2	0.1
3	0.1
4	0.1
5	N.A.[a]
6	N.A.
7	N.A.
8	N.A.
9	0.1
10	N.A.
11	N.A.
12	0.05
13	N.A.
14	N.A.
15	0.1
16	N.A.
17	N.A.
18	N.A.
19	N.A.
20	0.05

[a]N.A. = Not active at 0.1%.

inactive at 0.1%. This compound was prepared as a precursor or proantifeedant to provide extended release of the parent compound (Pickett et al., 1984). The parent compound was indeed released in weak acid, but since it was thought that this process would take place only slowly on the plant, the compound was also tested at a higher dose: at 0.5% there was significant activity. However, although at this higher level a wide range of compounds are active (Dawson et al., 1982; Briggs et al., 1983), compound 6, obtained by addition of 1-phenylethylamine but with subsequent loss of water to give the pyrrole, was significantly less active than 5, presumably because it could not readily regenerate polygodial. *cis*-Polygodial (7) and compounds 8–11, which do not have the *trans*-decalin ring of the natural drimane dialdehydes 1 and 12, were also inactive. Despite the high activity of (−)-warburganal, of the other 9α-hydroxy compounds 13–15, only compound 15, the 9α-hydroxy analog of cinnamolide (4), was active. Compound 16, formed by hydroxylation at carbon-3 in the diol 3, also lost activity. Natural (−)-drimenol (17) and the two 9α-hydroxy analogs 18 and 19 were inactive. However, compound 18 has been shown to be a highly effective antifeedant for the larvae of several species of *Spodoptera* and *Heliothis*

(Blaney et al., 1987), and compound 19, although not tested previously, proved here to be active at 0.01% against diamondback moth larvae. In these tests, 19 was even more active than (−)-polygodial and compound 18, which were only significantly active at 0.1%. Thus, it appears that the structure–activity requirements for the drimane-type antifeedants are different for aphids and lepidopteran larvae.

The similar biological activity of the synthetic (+)-polygodial (20) and the natural (−) isomer (1) (Table 2) was surprising in view of the earlier antifeedant structure–activity studies of Kubo and Ganjian (1981), and the present discovery that *cis*-polygodial was inactive. Therefore, the aphid antifeedant activity of the two enantiomers and of racemic polygodial was compared, using solutions for which the concentration and isomer content were confirmed by the reverse-phase and chiral HPLC methods. This affirmed that aphid antifeedant activity for the enantiomers was indeed similar and also demonstrated that the phytotoxic effects were similar (Table 2). At a concentration of 0.1%, the leaf damage score for Chinese cabbage was 3–4 and potato (*Solanum tuberosum*, Solanaceae) suffered scorching and yellowing of leaves, but sugar beet (*Beta vulgaris*, Chenopodiaceae) was unharmed. At this concentration, wheat seed germination was reduced only slightly from 83 to 75%, but at 0.5%, it was reduced from 92 to 69% and the number of twisted leaves was increased from 2 to 61%. (−)-Warburganal also caused similar leaf erosion of Chinese cabbage, as did other compounds with aphid antifeedant activity. To investigate the possibility that the reduction in aphid feeding was caused by damage to the leaves, the polygodial enantiomers were tested on a synthetic membrane through which the aphids could feed (Table 3). Since results on membranes were similar to those on leaves, it is unlikely that the aphid antifeedant activity is related to

TABLE 2. APHID SETTLING TEST ON LEAVES: ENANTIOMERS OF POLYGODIAL

Enantiomer	Concentration (%)	Mean number of aphids settled		P	Score of leaf damage
		Treated	Control		
(−)	0.05	3.1	11.5	<0.001	2–3
(+)	0.05	3.1	11.3	<0.001	2–3
(±)	0.05	2.0	11.0	<0.001	2–3
(−)	0.01	6.6	9.2	N.S.[a]	0–1
(+)	0.01	6.5	9.9	N.S.	0–1
(±)	0.01	8.6	6.2	N.S.	0–1

[a]N.S. = not significantly different at $P = 0.05$.

TABLE 3. APHID SETTLING TEST ON SYNTHETIC MEMBRANE: ENANTIOMERS OF POLYGODIAL AT 0.05%

Enantiomer	Mean number of aphids settled		
	Treated	Control	*P*
(−)	1.0	11.1	<0.001
(+)	1.8	9.1	<0.01
(±)	0.9	6.0	<0.01

the phytotoxic effects. Therefore, the development of nonphytotoxic drimane-type antifeedants is possible.

In view of the surprising similarity in antifeedant and phytotoxic activities for the polygodial enantiomers, two other types of biological activities were assessed. The enantiomers of polygodial showed similar piscicidal activities: (+)-polygodial, (−)-polygodial (synthetic), and (−)-polygodial (natural) at 0.4 ppm killed the test fish within 38, 36, and 30 min, respectively, whereas in the control, the fish were alive after two days. Also, these three materials had similar hot tastes to the human tasting panel.

The enantiomers of polygodial reacted differently with enantiomers of the chiral amine 1-phenylethylamine: from the reaction of (±)-polygodial with (+)-(*R*)- and (−)-(*S*)-1-phenylethylamine, the recovered polygodial gave specific rotations ($[\alpha]_D^{22}$) of −10° and +9° respectively. Although these rotations are small compared with that of pure (−)-polygodial, these results indicate a greater reactivity between the enantiomers of polygodial and the amine of like optical rotation. This initial observation makes the similar biological activity of the polygodial enantiomers even more surprising. Some of us (G.W.D., A.M., J.A.P., and Z.Z.-N.) are studying these reactions further, with Professor Charles J.W. Brooks of the University of Glasgow, U.K.

CONCLUSIONS

Of the compounds tested here, the dialdehydic drimanes are the most promising as aphid antifeedants. Further attempts to improve activity should employ different structural modifications from those described here and should involve a study of structure–activity relationships in aphids independent of lepidopteran studies. As the enantiomers of polygodial showed similar activity against aphids, the racemates, which are more readily available by synthesis, could be employed in the commercial development of drimane-type antifeed-

ants, although production by biotechnological means (Pickett, 1985) shows considerable promise for the future.

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APPLICATION OF SEX ATTRACTANTS FOR MONITORING THE PEA MOTH, *Cydia nigricana* (F.) (LEPIDOPTERA: TORTRICIDAE)

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Abstract—The use of sex attractants to monitor the pea moth, *Cydia nigricana* (F.) in the United Kingdom is decribed. Two systems are currently available: one for use in combining (dry-harvested) peas for human consumption or seed, and one for use in vining peas for freezing or canning. The development and details of both systems are reviewed and their commercial application discussed.

Key Words—Pea moth, *Cydia nigricana*, Lepidoptera, Tortricidae, sex attractant, pheromone, monitoring, peas, *Pisum sativum*.

INTRODUCTION

Peas are grown widely throughout Europe as an arable crop, and in the United Kingdom they rank fifth in value after cereals, potatoes, rape, and sugar beet. In 1986 they occupied a total area of 135,000 hectares. There are now three main types of commercial pea in the United Kingdom: (1) combining peas for human consumption or seed (harvested dry), (2) vining peas (harvested green for freezing or canning), and (3) protein peas (for animal feed). Peas grown for consumption as fresh peas now constitute a very small proportion of the market, although they are more popular in some other parts of Europe (e.g., France). Traditionally peas have been grown on the relatively dry eastern side of England, but over the last few years there has been a big increase in the pea-growing area and the crop is now grown in many parts of the country.

The pea moth, *Cydia nigricana* (F.) is a potentially serious pest of peas, the larvae boring into the pods and damaging one or more of the developing

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seeds. The economic effect of this damage and the presence of the larvae and frass depends on the type of pea crop. In combining crops, damage to peas can be removed after harvest, but the farmer will incur penalties. In vining peas, hardly any damage can be tolerated, because the peas cannot be "cleaned" mechanically after harvest. *C. nigricana* rarely has much effect on yield in the United Kingdom, so it is considered unnecessary to attempt control in crops grown for animal feed.

The life cycle of the moth is well documented (Franssen, 1954; Wright and Geering, 1948), but it is useful to summarize it here (Figure 1). There is one generation per year, and the insect passes the winter as a fully grown larva in a cocoon in the soil. Pupation takes place in late April–early May, and adults can emerge from late May onwards. The timing of emergence is very variable both between years and among different geographical locations. The adults emerge in fields that had peas the previous year, so they have to disperse to the new pea fields. Females emerge with immature ovaries, mate in the emergence field, and disperse to the peas. The ovaries mature in a matter of hours and almost all females arriving at pea fields are ready to lay eggs immediately. Males also move to the peas but in smaller numbers (Graham, 1984).

The eggs are laid mainly on the leaves and stipules towards the top of the pea plants, and hatch in ca. 10–20 days, depending on the temperature. The newly emerged first-instar larvae search for and bore into developing pea pods, where they feed on the seeds. They pass through five instars and eventually cut their way out of the pod and drop to the ground to spin a cocoon and enter diapause. The only stage that can be controlled effectively with insecticide sprays is the newly hatched first-instar larva before it enters the pod.

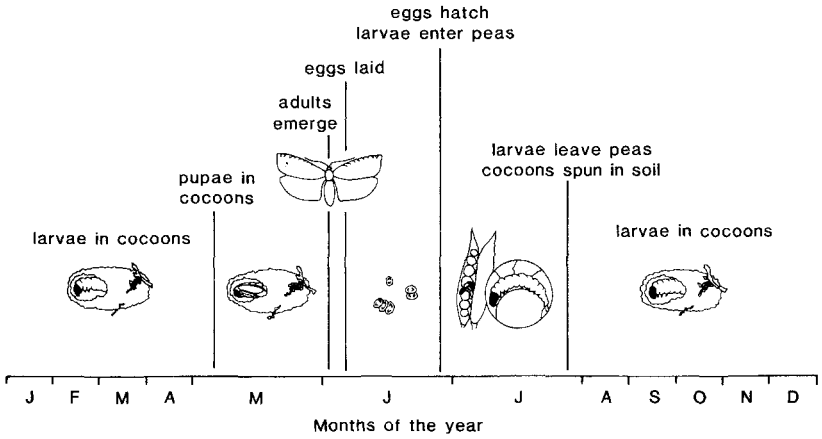

FIG. 1. The life cycle of *C. nigricana*.

THE PROBLEM

The erratic appearance of this pest means that there is a need for a local monitoring technique, which preferably can be used by the individual farmer. The only real alternative to this is to use prophylactic insecticide sprays. However, the questions to be answered by the monitoring system differ for combining peas and vining peas (Table I).

Combining pea varieties have an indeterminate growth form, producing pods sequentially for several weeks. Although most pods are probably attacked by the larvae of *C. nigricana* shortly after flower-set, it has been shown that even mature pods that are starting to dry are susceptible to attack (Graham, 1984). This means that the crop is at risk from the start of flowering to shortly before harvest. In addition, it is usually possible for the larvae to complete their development and leave the pods before harvest, thus causing maximum damage and ensuring completion of the life cycle. A local monitoring system for this crop is therefore required to provide information on (1) the arrival of moths, (2) the need to spray, and (3) the optimum timing of the spray(s).

Vining pea varieties have a determinate growth form resulting in the production of all the pods at the top of the plant over a very short period of time. Consequently, the pods mature at approximately the same time and this facilitates the harvest, which must be at a particular point in the development of the seeds to ensure the highest quality. The crops are sown sequentially so this can be achieved, and harvest often takes place only three weeks or so after flowering. Timing of the insecticide spray is not, therefore, important because the situation dictates that only one spray can be applied and the best time for this is at full flower. A monitoring system for this crop is therefore required to provide information on the need to spray. Since infesting larvae are often killed at harvest (being still in the pods) the density of *C. nigricana* in vining pea areas is generally very low. However, very low damage tolerances are set by the processors, which results in the need for a particularly sensitive monitoring technique.

TABLE 1. TYPES OF COMMERCIAL PEAS AND MONITORING REQUIREMENTS FOR *C. nigricana*

Type of pea crop	Requirements
Combining (for human consumption or seed)	Detect arrival of moths; determine need to spray; predict date of first spray
Combining (for protein)	None
Vining (for freezing or canning)	Determine need to spray

DEVELOPMENT OF SEX ATTRACTANT TRAPS

Electroantennogram screening of synthetic compounds pinpointed a number of physiologically active straight-chain acetates (Wall et al., 1976). Of these, two were subsequently shown to be very attractive to males in the field, while others acted as synergists or repellents (Greenway et al., 1982). The two most attractive compounds were (*E,E*)-8,10-dodecadien-1-yl acetate (*E,E*8,10–12 : Ac) and (*E*)-10-dodecen-1-yl acetate (*E*10–12 : Ac); the former was subsequently shown to be the female sex pheromone (Greenway, 1984). The most successful slow-release formulation for these attractants was shown to be a rubber stopper (Greenway and Wall, 1981), and dose–response and weathering field trials determined the dosage required to provide lures that remained constantly atractive for the whole of the flight period. Some difficulty was experienced initially with the diene, because in the presence of sunlight it isomerizes to produce a powerful repellent for *C. nigricana* (Wall and Greenway, 1983). It was for this reason that *E*10–12 : Ac was used as the attractant in the system developed for use in combining peas; it had the added advantage of catching fewer moths, thus avoiding trap saturation. An antioxidant was added to stabilize this attractant (Greenway and Wall, 1981, 1982). Various designs of trap and a number of retentive sticky materials were evaluated, and for practical monitoring purposes a delta-type trap with a removable sticky base was proposed (Lewis et al., 1975; Lewis and Macaulay, 1976). The sticky material has to be particularly efficient since *C. nigricana* belongs to the subfamily Olethreutinae, which tend to hold their wings in a steep rooflike form when at rest, thus presenting only the leading edges of the wing to the sticky surface. This is in marked contrast to Tortricinae, members of which have wider wings held in a "flat" profile when at rest, making it much easier to trap them on a sticky surface.

The height at which traps should be deployed in the crop was investigated (Lewis and Macaulay, 1976), and the recommendation for monitoring purposes was that the trap should be maintained at a height of two thirds the height of the crop. In the commercial version this is achieved using a sliding clip on a metal pole. The traps and sticky inserts are cardboard, thus making the whole kit lightweight and easily dispatched by mail to individual growers.

It is difficult to determine the density at which traps should be operated. Two questions need to be answered: (1) What is the minimum density to monitor the insect populations adequately? (2) At what density do traps start to interact and distort individual catches? Perry et al. (1981) showed that area spray warnings for combining peas, based on trap catches at a few sites within a large area, provide a less reliable indication of the need to spray than "on-site" monitoring traps in each field. Other work on trap interactions (Wall and Perry, 1981) and local variations in catches (Perry and Wall, 1984) confirmed

the approach of one trapping system per unit of crop unless that unit is greater than 50 hectares, in which case two systems are used. In the case of the more recent monitoring system for vining peas, traps are deployed at a maximum density of 1 per 36 hectares, which is equivalent to a spacing of 600 m.

COMBINING PEA SYSTEM

This system has been available commercially since 1978.

Description. A pair of traps is placed 100 m apart and 5 m into each field (Figure 2a) on adjacent headlands on the side of the prevailing wind (Wall et al., 1987). The traps are set at right angles to each other so that on any one day one trap will be optimally orientated to the wind, since wind direction greatly affects the catch (Lewis and Macaulay, 1976). Each trap contains 3 mg of *E*10–12:Ac on rubber, which will remain constantly attractive for at least three months (Greenway et al., 1981; Wall and Greenway, 1981). In fields larger than 50 hectares a second pair of traps is placed in the opposite corner (Perry and Wall, 1984), the whole field being sprayed if either pair indicates the need. The traps are installed in mid-May, before the flight season, and examined every other day.

Spraying is necessary only if a "threshold" catch is achieved: this is 10 or more moths in either trap during two consecutive 2-day periods (Macaulay et al., 1985). The date of the "threshold" is taken to indicate the start of substantial oviposition in the crop, and a developmental model using maximum/

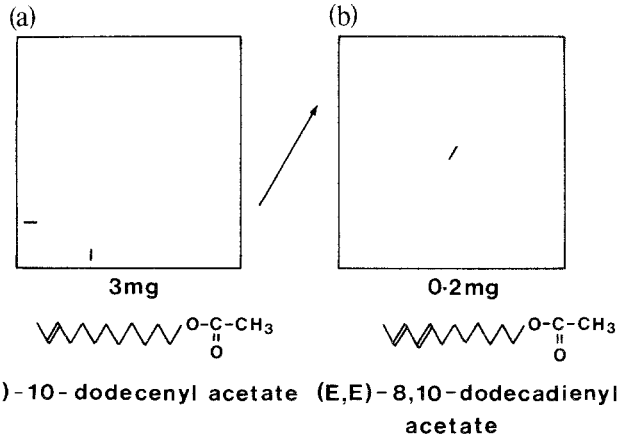

FIG. 2. Schematic representation of trap deployment in a field of peas: (a) combining peas, field <50 hectares, traps at right angles; (b) vining peas, field >36 hectares, one trap oriented to prevailing wind (arrow).

minimum daily temperatures (Lewis and Sturgeon, 1978) is used to calculate the rate of egg development and thus predict the date of the first spray. The spray is applied at the 85% development stage to ensure a kill of newly hatched larvae.

The spray prediction calculations can be done using a simple calculation derived from one described by Macaulay et al. (1985). However, it was found to be much more efficient for the advisory services to provide an interpretation service based on a computer model and temperature records from local meteorological stations. The details of the procedure are described in Wall et al. (1987) but can be summarized as follows:

A farmer who achieves a "threshold" waits three to four days, then contacts the advisory services by telephone; he is put in contact with a prerecorded message (or to the appropriate computer "page" if he has a viewdata system, known as Videotex in North America), which relays a predicted spray date based on the date of his "threshold." Since this prediction is based on both recorded and forecast temperatures, he is usually advised to telephone back a few days later to obtain an updated prediction.

As a result of this interpretative service, a very high proportion of farmers run their own traps and control the pest effectively, while applying pesticides only when needed. A five-year survey of farmers using the monitoring system, both correctly and incorrectly, confirmed its effectiveness if used correctly and has also indicated the economic gains (cf. Table 2 in Wall et al., 1987).

It seems that this system has succeeded because the threshold indicates the start of the immigration of substantial numbers of moths. Beyond this, there is no attempt to relate trap catch to population levels.

Problems. There have been remarkably few difficulties. One of the most persistent, however, is the tendency for farmers not to put traps out early enough in the season. This can lead to false "threshold" catches as the traps catch a backlog of immigrant males. Similarly, in years when the moths emerge very late, farmers may have to examine traps regularly for several weeks before catches are obtained, and some may decide to stop trapping too early. It can be difficult to persuade a farmer to keep running the trap in such circumstances.

Modifications to the system, in light of experience, are difficult to incorporate once it has become established commercially. For this reason it is still produced with pairs of traps, even though a single trap reorientated to the wind regularly and placed further into the crop (like the newer vining pea trap) might be preferable.

VINING PEA SYSTEM

This system was launched commercially in 1987 on a trial basis to assess interest among farmers.

Description. Single traps containing 200 μg *E,E*8,10–12:Ac (plus antioxidant) on rubber are placed at least 100 m into the crop (Wall et al., 1986) (Figure 2b) and at least 600 m from the nearest trap to avoid interactions (Wall and Perry, 1978, 1987). They are installed in mid-May, before the flight season, examined daily, and reorientated to the wind. A record is kept of the number of moths caught, and the cumulative catch at full flower is used to predict the maximum likely damage if the crop is not sprayed.

Wall et al. (1986) showed that there is a linear relationship between proportion of peas damaged (logit transformation) and the cumulative catch per trap at full flower. This regression was used to compute confidence limit contours for any future observations within the range of catches obtained during the experimental trial at 50 sites over three years. It is these confidence limits that form the basis of the predictive table now used to determine the need to spray (Table 2). Despite the variability of the data, meaningful predictions of the maximum damage to be expected at different probability levels can be produced. This is very different from trying to predict the damage level with reasonable accuracy, which has proved to be so difficult with sex attractant traps.

As mentioned previously, the crop is sown sequentially over a period of several weeks. Despite the findings of Graham (1984) that moths migrate into combining peas at or after flowering, no such relationship could be found between the catches in traps and crop phenology in vining peas. Males were caught up to 40 days before full flower (Wall et al., 1986). This led to the conclusion that the traps are sampling males from outside the sowing in which they are placed and that they may be indicating the presence of the moth in the area rather than the crop (sowing). Other work has shown that the sampling range of these traps is in excess of 500 m (Wall and Perry, 1987).

Problems. It is very difficult to obtain information on the levels of damage by *C. nigricana* at which vining pea crops are rejected by processors. During the three-year trial by Wall et al. (1986), damage was extremely low, averaging

TABLE 2. PREDICTED PEA MOTH DAMAGE—VINING PEAS[a]

	Upper limits of damage (% peas) at probability level:			
Cumulative moth catch	50	80	90	95
10	0.01	0.07	0.17	0.34
30	0.02	0.09	0.23	0.47
100	0.03	0.13	0.32	0.66
300	0.04	0.18	0.44	0.92
1000	0.05	0.26	0.63	1.54

[a] Data derived from Wall et al., 1987.

0.05% in unsprayed plots in 50 crops. Only 12% of the crops had damage in excess of 1%, which would probably represent an economic loss, and yet 54% of the crops were sprayed. Thus *C. nigricana* seems to have been of minor importance on vining peas in the United Kingdom during recent years. Despite this, many farmers spray prophylactically, while others hardly ever spray. The problem is to encourage both "schools" to use the traps that are now available. The "no-spray" school does not think the moth is a problem; the "spray" school regards it as pointless to trap when insecticide is so cheap and timing is not a problem.

As a result of the Common Agricultural Policy of the European Economic Community, the area of protein peas in the United Kingdom has increased considerably over the last few years. Since there is presently no need to control *C. nigricana* in protein peas, this increase could lead to a build-up of moth populations and increasing problems in both the combining-pea and vining-pea crops. There are already signs that this problem is starting in Sweden where protein peas have been grown for rather longer (B. Jönsson, personal communication). The question is: do we really have to wait until that problem hits the vining-pea industry in the United Kingdom before farmers will start to use monitoring traps extensively?

A second problem is the specificity of the attractant. Despite Greenway's (1984) identification of the pheromone as *E,E*8,10–12 : Ac and the lack of electrophysiological or behavioral evidence that there may be minor components as yet unidentified, it must be acknowledged that *E,E*8,10–12 : Ac is an attractant for males of several closely related species of *Cydia* (Table 3). Of these *C. servillana* has already turned up as a contaminant in vining-pea monitoring traps during the 1987 season in the United Kingdom. The scale of the problem will

TABLE 3. *Cydia* SPP. ATTRACTED TO *E,E*8,10–12 : Ac[a]

Species	Reference
C. americana	Stevens et al. (1985)
C. colorana	Stevens et al. (1985)
C. fagiglandana	Rotundo and Rotundo (1984)
C. illutana	Priesner (personal communication)
C. lautiuscula	Chisholm et al. (1985)
C. medicaginis	Bournoville (1979)
C. nigricana	Wall et al. (1976)
C. populana	Chisholm et al. (1985)
	Stevens et al. (1985)
C. pyrivora	Priesner (1980)
C. servillana	Priesner (personal communication)
C. splendana	Rotundo and Rotundo (1984)

[a] From Arn et al., 1986.

only become apparent with time; only then will it be possible to assess the need for further work on the identity of the sex pheromone of this species.

CONCLUSIONS

The sex attractant monitoring system for *C. nigricana* in combining peas has now been available commercially for a decade. It has worked well and is used by a large number of farmers in the United Kingdom. Its acceptance by growers is due largely to the central interpretation of trap catches provided by the advisory services, but also to its virtually unblemished record. The success with which the system monitors the occurrence and timing of moth immigration into pea crops is due to a large number of factors: the most important are (1) the insect has a discrete flight period, (2) the traps have a relatively short range of attraction and therefore sample only the crop, and (3) a simple "threshold" catch is used to determine the start of flight and predict the start of egg hatch.

The monitoring system for use in vining peas is a much later development, which has been launched commercially in 1987. Although potentially very useful because it can be used to predict the risk of damage, its uptake by the farming community is in doubt and will depend on future attitudes to minimizing pesticide usage against this pest.

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ADAPTIVE RELATIONSHIPS OF EPOXIDE HYDROLASE IN HERBIVOROUS ARTHROPODS

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Abstract—Epoxide hydrolase catalyzes a simple hydrolysis of reactive cyclic ethers that may otherwise alkylate and impair critical proteins and nucleic acids required for life. Although much less studied than the cytochrome P-450 monooxygenases that produce epoxides, differences in subcellular, tissue, pH, substrate, and inhibitor specificities argue for at least three forms of insect epoxide hydrolase. Increasing numbers of epoxides are being identified as plant allelochemicals, antifeedants, and essential hormones or precursors for herbivorous arthropods, and in many cases an associated alkene to diol pathway of metabolism is found. A role for epoxide hydrolase in arthropod–plant interactions is strongly supported by species comparisons and by age–activity and induction studies. Two major limitations for study in biochemical ecology of epoxide hydrolase are the lack of an effective in vivo inhibitor and a range of commercially available radiolabeled substrates for the enzymes.

Key Words—Epoxide hydrolase, insect, herbivore, plant epoxides, alkene, diol, coadaptation, detoxification, arthropod-plant interactions.

INTRODUCTION

Considerable effort has been invested in determining adaptive roles for detoxification enzymes in plant-feeding insects. The tens of thousands of potentially toxic secondary metabolites in plants, together with the exceptionally high levels of oxidative, hydrolytic, and conjugative xenobiotic metabolizing enzymes in insect herbivores, have stimulated much discussion of an associative role. This paradigm has been more rigorously established by recent study on specific

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dietary allelochemicals as inducers for and substrates of enzymes that metabolize foreign compounds in the respective herbivore (Ahmad, 1986; Brattsten, 1979; Brattsten and Ahmad, 1986; Dowd et al., 1983; Hodgson, 1985). Most work has concentrated on cytochrome P-450-dependent monooxygenases (EC 1.14.14.1) because of their pivotal role in initiating the concerted enzymatic reactions by which lipophilic, nonnutrient compounds are rendered into water-soluble and more excretable metabolites. Among the reactions catalyzed by these polysubstrate monooxygenases (PSMOs) are π-bond oxygenations forming epoxides. These important reactive intermediates can then be hydrolyzed to diols by epoxide hydrolases (EC 3.3.2.3) or conjugated to glutathione by glutathione transferases (EC 2.5.1.18). The three aforementioned enzyme systems constitute, within arthropods, the major enzymatic pathways by which epoxides are formed and degraded, of which the epoxide hydrolase is the least studied.

Many epoxides are strong electrophiles and can undergo rapid nucleophilic additions during both routine isolation and in vivo with water, sulfhydryl, and amino groups; they also participate in acid-enhanced rearrangements and polymerization. The high chemical reactivity of epoxides is often responsible for their high toxicity, mutagenicity, carcinogenicity, and other biological activities (Casida and Ruzo, 1986; Hutson, 1983). In turn, the presence of the usually less toxic *trans* 1,2-diol in an organism is good evidence that a PSMO-initiated alkene to diol pathway exists (Hsia, 1982–1983). There are presently no useful in vivo inhibitors of epoxide-metabolizing enzymes that can facilitate the isolation of the epoxide intermediate.

Investigators had recognized much earlier the occurrence of *trans*-diol metabolites from some aromatic hydrocarbons and alkenes, but it was not until 1950 that an enzyme responsible for their generation from epoxides was first postulated (Boyland, 1950). A few years later, a search for soil microbes capable of growth on an epoxide of fumaric acid as their sole carbon source led to the discovery that cell-free preparations from both a *Flavobacterium* spp. and the fungus *Aspergillus fumigatus* were able to convert *trans*-L-epoxysuccinic acid (**I**, Figure 1) to *meso*-tartaric acid (Martin and Foster, 1955). Subsequently, Breuer and Knuppen (1961) reported the *trans*-hydration of epoxyestrogens in rat liver slices. In plants, the simultaneous occurrence in ironweed (*Vernonia anthelmintica*) seeds of the appropriate epoxide and diol provided a base for postulating an enzyme that converts *cis*-12,13-epoxyoleic acid (**II**) to *threo*-12,13-dihydroxyoleic acid (Scott et al., 1963). Also, high levels of epoxidized fatty acids together with very active epoxide hydrolases for these substrates were found in spores of plant rusts (Hartmann and Frear, 1963; Tulloch, 1963).

The first demonstration of epoxide hydrolase in insects occurred with a synthetic cyclodiene insecticide. In vivo studies in dieldrin-resistant southern house mosquitoes (*Culex pipiens quinquefasciatus*) (Oonnithan and Miskus,

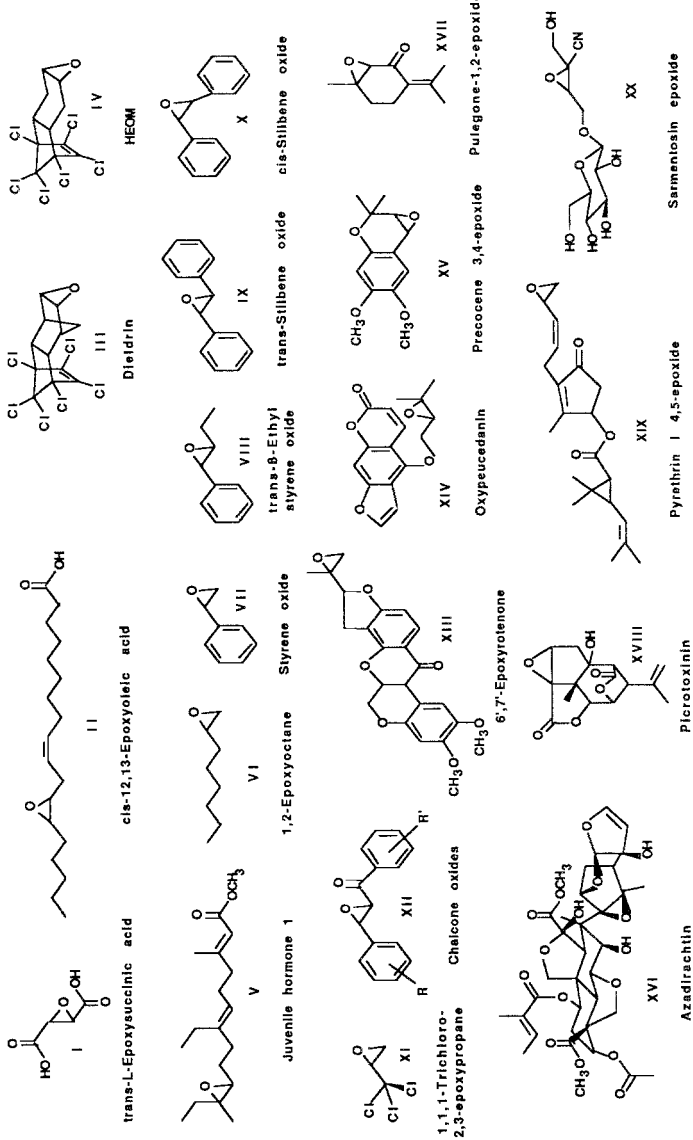

FIG. 1. Some epoxides associated with arthropod–plant interactions.

1964; Tomlin, 1968) had previously established that dieldrin (**III**) was metabolized to a product cochromatographing with aldrin *trans*-diol; similarly, house flies topically treated with chlordene epoxide formed compounds thought to be chlordene glycols (Brooks and Harrison, 1965). The enzymatic nature of this epoxide hydrolase activity towards dieldrin, its analog HEOM (**IV**), and other cyclodiene epoxides was finally verified in vitro in preparations from the house fly, and in rat and pig liver microsomes (Brooks, 1966; Brooks et al., 1970). Epoxide hydrolase has subsequently been found in all living organisms investigated. Comprehensive reviews emphasizing mammalian (Oesch, 1973; Lu and Miwa, 1980; Seidegard and DePierre, 1983; Wixtrom and Hammock, 1985) and insect (Brooks, 1977; Hammock, 1985) epoxide hydrolases are available. The consequence of epoxide hydrolase in herbivorous arthropods is now addressed.

PROPERTIES OF ARTHROPOD EPOXIDE HYDROLASE

Epoxide hydrolase has been purified from only one insect source, that of the southern armyworm, *Spodoptera eridania*, a polyphagous herbivore (Mullin and Wilkinson, 1980a). The larval midgut enzyme has an extraordinarily wide specificity for substrates and hydrolyzes a range of epoxides from simpler monosubstituted compounds to more sterically hindered compounds such as juvenile hormone (**V**) and chlorinated cyclodiene epoxides. Moreover, the activities for the latter substrates were 100–30,000 times lower than activities for 1,2-epoxyoctane (**VI**), styrene oxide (**VII**), and other less-hindered substrates (Mullin and Wilkinson, 1980b). Armyworm epoxide hydrolase, the most active eukaryotic preparation so far reported, was calculated to comprise 3.7% of the total microsomal protein in the armyworm midgut. This indicates an important role for the enzyme in this herbivore, although the low activity for juvenile hormone (JH) and cyclodiene epoxides in comparison to activities in crude preparations suggests that other isozymes are present in armyworm midgut (Mullin and Wilkinson, 1980b).

Multiple Forms. Tissue and subcellular distributions, pH optima, and substrate and inhibitor specificities strongly support the existence of multiple forms of epoxide hydrolase in arthropods. Highest activities towards commonly used substrates occur in the midgut microsomes at alkaline pH. JH epoxide hydrolase appears more widely in fat body (Fox and Massare, 1976; Wing et al., 1981) and other tissues such as the Malpighian tubules, integument (Slade and Wilkinson, 1974), wing imaginal disks (Hammock et al., 1975), silk gland (Wisniewski et al., 1986), and even the corpora allata (Gadot et al., 1987). Also, considerable JH epoxide hydrolase is cytosolic rather than microsomally bound (Yu and Terriere, 1978b; Wisniewski et al., 1986), although this is not always

the case (Hammock et al., 1974, 1975). By contrast, epoxide hydrolases for the cyclodiene HEOM (Slade et al., 1975), styrene oxide, and 1,2-epoxyoctane (Mullin and Wilkinson, 1980a,b) in the southern armyworm are largely microsomal. Recent studies with the substrates *trans*-β-ethylstyrene oxide (**VIII**), *trans*-stilbene oxide (**IX**), and *cis*-stilbene oxide (**X**) (Table I) indicate that considerable activity is cytosolic, particularly for saprophagous compared to herbivorous arthropods. In the laboratory fruit fly, *Drosophila melanogaster*, even styrene oxide, a substrate normally hydrated preferentially in microsomes (Wixtrom and Hammock, 1985, and references therein), is rapidly hydrolyzed in the cytosol (Jansen et al., 1986).

Differences in pH optima also suggest the presence of multiple epoxide hydrolases. In *S. eridania* midgut microsomes, both HEOM hydrolase (Slade et al., 1975) and 1,2-epoxyoctane hydrolase (Mullin and Wilkinson, 1980a) are optimal between pH 8.5 and 9.5 in Tris and glycine buffers, while JH epoxide hydrolase has an optimum of 7.9 (Slade et al., 1975). Activity in cabbage looper, *Trichoplusia ni*, midgut microsomes peaks at pH 7.4 for *trans*-stilbene oxide and pH 8.0 for *cis*-stilbene oxide (Ottea and Hammock, 1986). In other species, there is a similar tendency for hydrolases of *trans*-disubstituted and higher substituted epoxides such as JH to have more acidic pH optima than those for monosubstituted and *cis*-disubstituted substrates (Hammock et al., 1974; Cohen, 1981).

At present there are no effective *in vivo* inhibitors of epoxide hydrolase,

TABLE I. SUBCELLULAR DISTRIBUTION OF EPOXIDE HYDROLASE IN ARTHROPODS

Feeding strategy and Species	Tissue	Relative specific activity (microsomes/cytosol)	
		trans-β-Ethylstyrene oxide	*cis*-Stilbene oxide
Herbivore			
Northern corn rootworm[a]	Midgut	9.5	9.4
Cabbage looper[b]	Midgut	8.2	7.7
Western corn rootworm[a]	Midgut	7.0	6.4
Two-spotted spider mite[c]	Whole body	4.0	2.9
Saprophage			
House fly[d]	Abdomen	1.2[f]	1.0
Laboratory fruit fly[e]	Whole body	0.4[f]	1.0

[a]B.D. Siegfried and C.A. Mullin, unpublished data.
[b]J.A. Ottea and B.D. Hammock (1986).
[c]C.A. Mullin, F. Matsumura, and B.A. Croft (1984).
[d]J.A. Ottea, F.W. Plapp, Jr., and B.D. Hammock (1987b).
[e]J.A. Ottea, L.G. Harshman, and B.D. Hammock (1987a).
[f]Substrate was *trans*-stilbene oxide.

and this greatly limits efforts to understand the toxicological role of the enzyme. The best inhibitors of HEOM and styrene oxide hydrolases in *S. eridania*, *Calliphora erythrocepala*, *Tenebrio molitor*, and *Tribolium castaneum* include 1,1,1-trichloro-2,3-epoxypropane (**XI,** TCEP), phenolic 2,3-epoxypropyl ethers, and some synergists of PSMO (Brooks, 1973, 1977; Slade et al., 1975; Cohen, 1981), and, in addition, sodium picrylsulfonate in *S. eridania* (Mullin and Wilkinson, 1980b). By contrast, JH hydrolase is more sensitive to hormone analogs than to TCEP (Hammock et al., 1974; Slade and Wilkinson, 1973; Yu and Terriere, 1978b). Differential inhibition of *trans*- and *cis*-epoxide hydrolases by TCEP and the equally or more potent chalcone oxides (**XII**) has also provided evidence of multiple isozymes in *Tetranychus urticae* (Mullin et al., 1984), *Delia antiqua*, *Diabrotica barberi*, and *D. virgifera* (Siegfried and Mullin, unpublished data). The chalcone oxides (Mullin and Hammock, 1982) and other flavonoid epoxides such as 6′, 7′-epoxyrotenone (**XIII**; Cova et al., 1986) are strongly inhibitory to mammalian epoxide hydrolases, and in the former case inhibit particularly the *trans*-selective enzymes.

Species Distribution. Epoxide hydrolase has been found in almost 70 insect species (Table 2), and many interspecific variations are apparent. For example, house flies and blow flies (*Calliphora erythrocephala*) readily hydrate the cyclodiene HEOM, but bloodsucking diptera such as the tsetse fly (*Glossina austeni*) and stable fly (*Stomoxys calcitrans*) are deficient in this activity (Brooks, 1977). Most of the early work concentrated on the epoxide hydrolysis of insecticidal chlorinated cyclodienes (Brooks, 1977) and the sesquiterpenoid JHs and their synthetic analogs (Hammock and Quistad, 1976; 1981; Hammock, 1985), and has been exhaustively reviewed. Much less is known about the role of epoxide hydrolase in the metabolism of phytochemical epoxides. Use of appropriate model substrates such as alkylstyrene and stilbene oxides (**VIII, IX, X**) for more complex epoxides in plants has provided new insight into insect–plant relationships. These radiolabeled substrates mimic epoxide metabolites formed in mammals (cf. Scheline, 1978) from phenylpropenoids such as isoeugenol (Mullin, 1985), but they lack phenolic, methoxy, and other functional groups that would result in competing and subsequent reactions that do not allow measurement of specific rates for one enzyme activity. Exceptional levels of epoxide hydrolase acting on these substrates have been found in generalist insect herbivores (Mullin and Wilkinson, 1980a; Mullin and Croft, 1984).

PLANT EPOXIDES VIA THE ALKENE TO DIOL PATHWAY

Plant Epoxides as Allelochemicals. There is now much support to the concept that epoxides contribute greatly to the allelochemical barrier for a prospective arthropod herbivore. Since the characterization of the coumarin oxypeucedanin (**XIV**) in 1933 as the first recognized epoxide in plants, improved

TABLE 2. IN VITRO OCCURRENCE OF EPOXIDE HYDROLASE AMONG INSECTS

Order and Species	Substrate (reference)[a]
Thysanura	
Thermobia domestica	JH (1)
Orthoptera	
Locusta migratoria	JH (9)
Dictyoptera	
Blattella germanica	JH (1), SO (15)
Gromphadorhina portentosa	CE (22), JH (20)
Periplaneta americana	CE (17), JH (1,8,11)
Hemiptera	
Cimex lectularius	CE (5)
Oncopeltus fasciatus	JH (1), SO (15)
Podisus maculiventris	SO (24)
Pyrrhocoris apterus	JH (1)
Rhodnius prolixus	CE (5)
Homoptera	
Aphis nerii	SO (14)
Dactynotus ambrosiae	JH (1)
Macrosiphum euphorbiae	SO (14)
Myzus persicae	SO (15)
Neuroptera	
Chrysopa carnea	SO (15)
Coleoptera	
Acalymma vittata	SO (15)
Altica woodsi	SO (15)
Chauliognathus pennsylvanicus	SO (15)
Coccinella novemnotata	SO (15)
Coleomegilla maculata	SO (15)
Crioceris asparagi	SO (15)
Diabrotica barberi	SO (15)
D. undecimpunctata howardi	SO (15)
D. virgifera virgifera	SO (15)
Epicauta pennsylvanica	SO (15)
Epilachna varivestis	SO (15)
Hippodamia convergens	JH (1), SO (15)
Leptinotarsa decemlineata	JH (1,12), SO (15)
Plagiodera versicolora	SO (15)
Tenebrio molitor	CE (4), JH (1,11)
Tribolium castaneum	SO (6)
Trirhabda virgata	SO (15)
Lepidoptera	
Antheraea pernyi	JH (1), SO (13)
Anticarsia gemmatalis	SO (24)
Argyrotaenia citrana	SO (7)
Choristoneura rosaceana	SO (15)
Galleria mellonella	CE (5), JH (23)

TABLE 2. Continued

Order and Species	Substrate (reference)[a]
Heliothis virescens	SO (24)
H. zea	SO (14,24)
Hyalophora cecropia	JH (1,20)
Hyphantria cunea	SO (15)
Lymantria dispar	SO (14)
Malacosoma americanum	SO (15)
Manduca sexta	CE (5), JH (1,11,20,21)
Philasamia ricini	CE (5)
Pieris brassicae	CE (5)
P. rapae	SO (15)
Samia cynthia	JH (1)
Spodoptera eridania	CE (22), JH (20), SO (16)
S. frugiperda	SO (15,24,26)
Trichoplusia ni	JH (11), SO (18)
Diptera	
Aedes aegypti	CE (5), SO (15)
Anopheles stephensi	CE (5)
Calliphora erythrocephala	CE (4)
Chaoborus americanus	JH (1)
Culex fatigans	CE (5)
Delia antiqua	SO (15)
Drosophila melanogaster	JH (1), SO (2,10,19)
Glossina austeni	CE (4)
Musca domestica	CE (3), JH (1,11,25), SO (15)
Phormia regina	JH (25)
Sarcophaga bullata	JH (11,25)
Stomoxys calcitrans	CE (4)
Hymenoptera	
Apis mellifera	JH (1), SO (26)
Atta texana	JH (1)
Oncophanes americanus	SO (7)
Pediobius foveolatus	SO (15)
Solenopsis invicta	JH (1)

[a]CE = chlorinated cyclodiene epoxide, JH = juvenile hormones and analogs, SO = styrene and stilbene oxides and analogs. References:

1. Ajami and Riddiford, 1973
2. Baars et al., 1979
3. Brooks, 1966
4. Brooks, 1973
5. Brooks, 1979
6. Cohen, 1981
7. Croft and Mullin, 1984
8. Fox and Massare, 1976
9. Gadot et al., 1987
10. Hallstrom and Grafstrom, 1981
11. Hammock et al., 1974
12. Kramer et al., 1977
13. Mullin, 1979
14. Mullin, 1985
15. Mullin and Croft, 1984
16. Mullin and Wilkinson, 1980a
17. Nelson and Matsumura, 1973
18. Ottea and Hammock, 1986
19. Ottea et al., 1987a
20. Slade and Wilkinson, 1974
21. Slade and Zibitt, 1971
22. Slade et al., 1975
23. Wisniewski et al., 1986
24. Yu, 1987
25. Yu and Terriere, 1978a
26. Yu et al., 1984

methods for isolation and identification have led to discovery of hundreds of epoxides in higher plants (Cross, 1960; Dean, 1963; Mullin, 1985). The occurrence of an epoxide substrate for hydration in eukaryotic organisms is often predicted based on presence of the more stable vicinal diol and its respective unsaturated or aromatic hydrocarbon. Plant-derived epoxides such as the allatotoxin precocene 3,4-epoxide (**XV**) (Pratt et al., 1980; Soderlund et al., 1980) and various arene oxides (Brooks, 1979) are so reactive that spontaneous hydrolysis to inactive diols occurs without the intermediacy of epoxide hydrolase.

Surprisingly, alkaloid epoxides such as some pyrrolizidines are sufficiently stable to survive the acid treatments routinely used by alkaloid chemists. The high concentration of acid-stable jacobine with its respective olefin and diol in *Senecio jacobaea* indicates that plants use an enzymatic alkene-to-diol pathway to biosynthesize these alkaloids (Figure 2). Tiger moths that specialize on *S. jacobaea* are known to preferentially sequester the olefin at the expense of the epoxide and diol (Rothschild et al., 1979). The same alkaloids are potent inducers of mammalian epoxide hydrolase (Miranda et al., 1980).

Numerous insecticidal and antifeedant epoxides have been found in plants and may be substrates for the appropriate epoxide hydrolases. Among the plant terpenoids, many insect feeding deterrents including the highly potent azadirachtin (**XVI**) are epoxides (Burnett et al., 1974; Norris 1986; van Beek and de Groot, 1986; Yamasaki and Klocke, 1987), and hydration of the epoxide group can lead to loss of deterrent activity (Kubo and Matsumoto, 1985). Other epoxyterpenoids such as the anticholinesterase monoterpene, pulegone-1,2-epoxide (**XVII**), from *Lippia steochadifolia* (Grundy and Still, 1985), and the sesquiterpene picrotoxinin (**XVIII**) from *Anamirta cocculus* (Miller et al., 1979) are directly insecticidal. Unsaturated monoterpenes of wide occurrence in plants, including limonene (Brattsen, 1983) and α-pinene (White et al., 1979), are readily converted in mammals to *trans*-diols via epoxides; presumably the diols formed from the highly neurotoxic pyrethrins (**XIX**) of *Chrysanthemum ciner-*

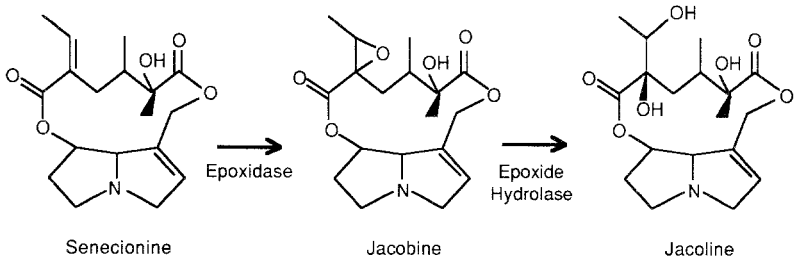

FIG. 2. Possible alkene-to-diol pathway for biosynthesis of pyrrolizidine alkaloids in *Senecio*.

ariaefolium are the result of epoxidation in the alcohol side chain followed by hydrolysis (Casida et al., 1971). Interestingly, epoxidation of synthetic chrysanthemates in the acid side chain results in neuroactive and insecticidal metabolites (Ruzo et al., 1984).

Similarly, epoxy fatty acids make up to 60% of the monomers in cuticular layers of plants and are copolymerized with polyol products of the epoxidase–epoxide hydrolase pathway to give the protective waxy surfaces (Croteau and Kolattukudy, 1974; Holloway and Deas, 1973; Holloway et al., 1981; Kolattukudy, 1985). Insect herbivores may use a *trans*-selective epoxide hydrolase to metabolize these epoxides, as is the case in mammals (Gill and Hammock, 1979).

If increased impact on a herbivore occurs via the more labile epoxide of an unsaturated plant allelochemical, it may be critical that epoxidation in the exposed herbivore occurs at or near the target site of action for the allelochemical. This should maximize the adaptive benefit of synthesizing the respective alkene in the protected plant since epoxide-to-diol detoxification in other tissues of the plant (Banthorpe and Osborne, 1984) or herbivore will reduce efficacy.

Plant aromatics are metabolized in herbivorous insects via the alkene-to-diol pathway. The allatotoxic chromenes from *Ageratum houstonianum*, precocene I and the more active precocene II, undergo bioactivation to cytotoxic 3,4-epoxides in the corpora allata of susceptible insects, such as the large milkweed bug, and thereby impair juvenile hormone biosynthesis (Bowers and Martinez-Pardo, 1977; Pratt et al., 1980; Feyereisen et al., 1981). These epoxides are reactive alkylators of macromolecules. The inactive diol metabolites formed in the corpora allata or peripheral tissues of the insect are largely formed by spontaneous hydrolysis with water (Soderlund et al., 1980) and not by epoxide hydrolase. The insecticidal isoflavonoid, rotenone, is readily detoxified in cockroaches and house flies to a dihydrodiol metabolite (Fukami et al., 1967, 1969) presumably through an epoxyisopropyl intermediate (**XIII**).

Alkene-to-Diol Pathway in Hormone Regulation. Herbivorous insects require epoxidases to synthesize both molting hormones from dietary phytosterols (Fujimoto et al., 1985) and juvenile hormones from the appropriate precursors (Feyereisen and Farnsworth, 1987, and references therein). Some evidence in the former (Rees, 1985) and much for the latter (Hammock, 1985) implicates epoxide hydrolase in regulation of titers of these epoxides. Thus, midgut microsomes of *Spodoptera littoralis* hydrolyze fucosterol-24,28-epoxide to stigmast-5-en-3β,24,28-triol which interrupts the dealkylation sequence and cannot satisfy the sterol requirement of the insect (Figure 3).

ROLE IN HERBIVORY

Evaluating the adaptive role of an enzyme in herbivore toxicology requires its dissimilar expression among species, action on potentially toxic substrates,

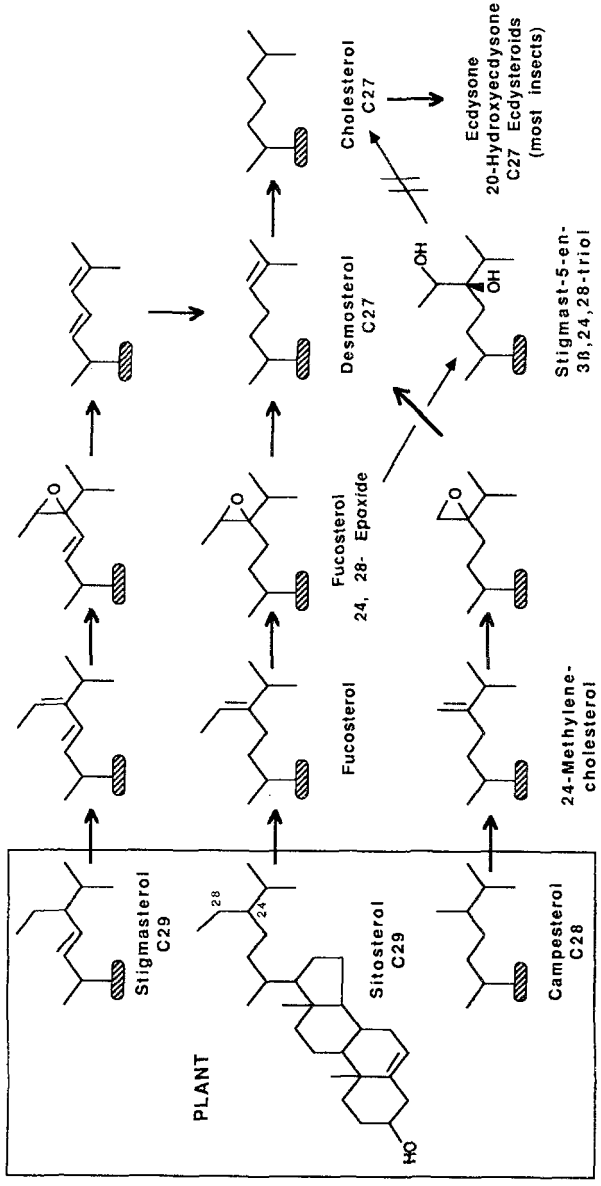

FIG. 3. General scheme for phytosterol dealkylation in herbivorous insects.

and ease of measurement under conditions that do not markedly change from species to species. Epoxide hydrolase shares with PSMO and glutathione transferase a wide and varying distribution among insect species and the ability to detoxify phytochemicals. Moreover, rapid and sensitive assays that easily allow measurements in microarthropods are available for epoxide hydrolase (Wixtrom and Hammock, 1985). Based on energetics, epoxide hydrolase should be less costly to maintain in an organism than PSMO or glutathione transferase since its catalytic mechanism is a simple hydrolysis requiring no coenzymes. By contrast, PSMO is the result of a multienzyme–hemoprotein complex that consumes NADPH and oxygen, while glutathione transferase requires a tripeptide as a cosubstrate. Consequently, epoxide hydrolase measurements should facilitate the study of arthropod–plant coadaptations.

Age–Activity Relationships. A clue to the importance of epoxide hydrolase in metabolism of dietary chemicals comes from age–activity relationships. In the southern armyworm, there is a direct association of high epoxide hydrolase activity with periods of high consumption of plant-based diets (Figure 4). Midgut 1,2-epoxyoctane hydrolase increased sevenfold in total activity during the first 60 hr of the late larval instar before undergoing a dramatic decrease in activity after cessation of feeding and preparation for pupation occurs (Mullin and Wilkinson, 1980a). A similar profile has been noted in this (Slade et al., 1976) and other lepidopteran species (Wing et al., 1981) for HEOM and a juvenile hormone analog. The single peak of JH epoxide hydrolase in the last

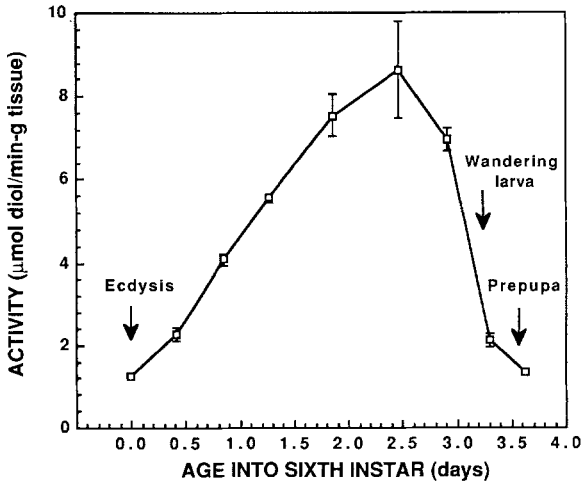

FIG. 4. Age-dependent changes in 1,2-epoxyoctane hydrolase in midgut homogenates from the sixth larval instar of the southern armyworm. Standard error bars for activities shown.

larval instar of Lepidoptera is not as finely synchronized with the biphasic needs for JH degradation as that of JH-specific esterase, which has two distinct peaks of activity. Moreover, epoxide hydrolase activity for JH is an order of magnitude less than the esterase activity (Hammock, 1985), and it is quite likely that the JH epoxide hydrolase is a nonspecific enzyme that can metabolize this and similar sesquiterpenoid substrates that are *trans*- and higher substituted epoxides.

More epoxide hydrolase was found in larvae of both house flies (Yu and Terriere, 1978b) and a braconid ectoparasitoid *Oncophanes americanus* (Croft and Mullin, 1984) than the adults. Larvae process more food than adults and expectedly would encounter more dietary epoxides. However, in Coleoptera very high levels of epoxide hydrolase occur in herbivorous adults (Cohen, 1981), particularly those that are generalists (Mullin and Croft, 1984).

Inducibility. Induction studies also support the contention that epoxide hydrolase is involved in arthropod herbivory. Microsomal epoxide hydrolase activity in southern armyworm midguts was enhanced nearly twofold by feeding larvae for one day on 0.1% pentamethylbenzene in an artificial diet (Mullin and Wilkinson, 1980a). Phytochemicals such as indole 3-carbinol and peppermint oil also induce the enzyme (Table 3). Up to a 3.4-fold increase in *trans*- and *cis*-epoxide hydrolase activities ensues in two-spotted spider mites when fed various host plants (e.g., cotton, lettuce) as compared to snapbean (Mullin and Croft, 1983), although host-related inductive effects were not seen in the fall armyworm with styrene oxide as substrate (Yu and Hsu, 1985). Classical PSMO inducers including phenobarbital in both house fly (Yu and Terriere, 1978b) and *Tribolium castaneum* (Cohen, 1982), and polychlorinated biphenyls and β-naphthoflavone in *Drosophila* (Hallstrom and Grafstrom, 1981) all significantly induce insect epoxide hydrolases. While epoxide hydrolase is, overall, considerably less inducible than PSMO (Brattsten, 1979; Yu, 1986), this may be related to the higher cost of maintaining the multienzyme–coenzyme complex

TABLE 3. INDUCTION OF MICROSOMAL EPOXIDE HYDROLASE IN ARMYWORM MIDGUT

Compound	Percent in diet (wet wt.)	Percent of control activity[a]
Pentamethylbenzene[b]	0.1	196
Phenobarbital[c]	0.2	150
Indole 3-carbinol[c]	0.2	142
Peppermint oil[c]	0.2	134

[a] Styrene oxide as substrate.
[b] Southern armyworm (C.A. Mullin and C.F. Wilkinson, 1980a).
[c] Fall armyworm (S.J. Yu and E.L. Hsu, 1985).

of PSMO in an insect. Thus, the less costly epoxide hydrolase could be kept at sufficient levels to detoxify plant-derived epoxides without a highly primed induction system.

Relationships with Feeding Strategy. Comparison of epoxide hydrolase between herbivorous and nonherbivorous species of arthropods provides additional evidence of its adaptive role. Hydrolase activity for sytrene oxide is higher in chewing herbivores such as lepidopteran larvae than in a predaceous spined soldier bug or insect species that feed on limited portions of the plant (Table 4). However, this substrate efficiently measures only the microsomal activity and may miss other isozymes more selective for plant-derived epoxides.

Use of *trans*-β-ethylstyrene oxide and *cis*-stilbene oxide as substrates in a screen on epoxide hydrolase in 36 arthropod species (cf. Table 2) has led to discovery of more robust differences between ecological groups. Chewing herbivores, on the average, have 21 times more *trans*-epoxide hydrolase and 10 times the ratio of *trans*- to *cis*-epoxide hydrolase than arthropod predators and parasitoids (Mullin et al., 1982; Mullin and Croft, 1984, 1985; Croft and Mullin, 1984; Mullin, 1985). Moreover, phloem-feeders have, on average, 80 times lower *trans*-epoxide hydrolase than herbivores that consume entire plant parts (Mullin, 1986). Further comparisons with chewing and sucking herbivores were made using species from the same insect family. Among 10 species of leaf-feeding beetles (Chrysomelidae), ratios of *trans*- to *cis*-epoxide hydrolase consistently increased ($r > 0.92$) with either number of plant families or plant genera consumed by the species (Mullin and Croft, 1984; Mullin, 1985). In the Aphididae, the highly polyphagous *Macrosiphum euphorbiae* and *Myzus per-*

TABLE 4. MICROSOMAL ACTIVITIES OF STYRENE EPOXIDE HYDROLASE IN SOME INSECTS

Species	Tissue	Styrene epoxide hydrolase (nmol/min/mg protein)	Feeding strategy
Southern armyworm[a]	Midgut	79	Generalist
Fall armyworm[b]	Midgut	26	Generalist
Antheraea pernyi[a]	Midgut	26	Specialist
Spined soldier bug[b]	Midgut	13	Predator
Honeybee[a]	Midgut	8.6	Nectar, pollen
Laboratory fruit fly[c]	Whole	4.3	Saprophagous
Red flour beetle[d]	Whole	2.7	Seed

[a] C.A. Mullin (1979); C.A. Mullin and C.F. Wilkinson (1980a).
[b] S.J. Yu, F.A. Robinson and J.L. Nation (1984); S.J. Yu and E.L. Hsu (1985); S.J. Yu (1987).
[c] A.J. Baars, M. Jansen and D.D. Breimer (1979).
[d] E. Cohen (1981).

sicae had 5–14 times higher epoxide hydrolase activity than the oleander and milkweed specialist, *Aphis nerii*. These results strongly implicate the *trans*-epoxide hydrolase in the metabolism of plant-derived epoxides and support the view that phloem-feeders such as aphids have less epoxide hydrolase than chewing herbivores, since concentrations of secondary plant epoxides in phloem should be less than those of external plant tissues (Mullin, 1986). Coincidently, the plant allelochemicals that may undergo epoxidation, such as unusual fatty acids, cinnamic acids, chalcones, phenylpropenoids, and terpenoids, tend to have a *trans* geometry or are trisubstituted olefins, whereas constitutive olefins common to both animal and plant tissues, such as fatty acids, tend to have a *cis* configuration (Mullin, 1985). Hence, epoxides resulting from epoxidation of available plant allelochemicals in either the plant or its consumer could explain the presence of a high *trans*-selective epoxide hydrolase in herbivorous species.

Epoxide hydrolase may occasionally be counteradaptive to a herbivore. For example, rat liver microsomes greatly facilitated the release of cyanide from a cyanogenic glycoside, sarmentosin epoxide (**XX**), probably by hydration to an unstable cyanohydrin (Nahrstedt et al., 1982). Other diols such as the diterpenoid antifeedant ajugarin III (van Beek and de Groot, 1986) and the synthetic aldrin *trans*-diol (Brooks, 1977; Singh and Singh, 1984) are sufficiently lipophilic to exert near or equal biological activity as the epoxide. However, the net result of increasing water solubility and excretability of an alkene makes the diol conversion a distinct detoxification pathway.

The maintenance of a tightly coupled epoxidase–epoxide hydrolase complex will generally have beneficial consequences for an arthropod herbivore since harmful epoxides will then be immediately dispatched by hydration. It is fortunate that much of cytochrome P-450 and epoxide hydrolase resides together on the endoplasmic reticulum (Brattsen and Ahmad, 1986). Usually the epoxidase is rate-limiting and produces epoxides much slower than the subsequent hydration to diols. Some epoxides such as dieldrin are refractory to hydration and serve as useful products for epoxidase measurements. Hence, the aldrin-to-aldrin diol pathway is an unusual case where epoxide hydration is rate-limiting. Dieldrin and other cyclodiene epoxides are generally recognized as the insecticidal agents in this pathway (Schroeder et al., 1977; Miyazaki et al., 1979; Singh and Singh, 1984), thus a high epoxidase-to-epoxide hydrolase ratio may be disadvantageous to a herbivore exposed to aldrin. Work with corn rootworms, the two-spotted spider mite, and aphids supports this hypothesis in that increased aldrin epoxidase to *cis*-epoxide hydrolase activity correlates with increased susceptibility to aldrin (Mullin, 1986; Mullin et al., 1984) with the potato aphid being less susceptible than the polyphagous northern corn rootworm. Nevertheless, the higher epoxidase and epoxide hydrolase activities of chewing herbivores than phloem-sucking aphids should benefit the former group

since their encounter with olefinic and epoxide phytochemicals is expectedly greater.

ROLE IN CHEMICAL CONTROL OF HERBIVOROUS PESTS

The bulk of insecticides used today lack an epoxide or an unsaturated grouping that will be readily epoxidized, and these groupings are largely removed from promising new insecticides so that improved biostability will be achieved. Chlorinated hydrocarbons where epoxide metabolites are known, including the cyclodienes (Brooks, 1979), DDT (Gold and Brunk, 1982), and lindane (Fitzloff and Pan, 1984), have received increased worldwide disfavor, and no longer are major use insecticides. Aromatic insecticides that undergo ring oxidation to form diols such as the carbamate carbaryl (Kuhr and Dorough, 1976) may do so by spontaneous hydrolysis of the intermediate epoxide, and not necessarily through an epoxide hydrolase. Thus epoxide hydrolase, by design, has a modest role in detoxification of presently used synthetic pesticides. Strategies for overcoming the resistances by arthropods to most available synthetics should not be based on this enzyme. Levels of epoxide hydrolase similar to those of susceptible strains were found in organophosphate-, carbamate-, DDT-, and methoprene-resistant strains of house fly (Hammock et al., 1977; Yu and Terriere, 1978b; Ottea et al., 1987b), organophosphate-resistant two-spotted spider mite (Mullin et al., 1982), carbaryl-resistant fall armyworm (McCord and Yu, 1987), and DDT- and organophosphate-resistant strains of *Drosophila* (Baars et al., 1979; Hallstrom and Grafstrom, 1981). For the latter case, styrene oxide was used as the substrate, whereas in other strains of *Drosophila* with *trans*- and *cis*-stilbene oxides more heterogeneity in epoxide hydrolase was observed (Ottea et al., 1987a). Similar modest increases in epoxide hydrolase were seen in a pyrethroid-, DDT-, and organophosphate-resistant predatory mite, *Amblyseius fallacis* (Mullin et al., 1982), although resistance to juvenoids in the red flour beetle has been associated with a 4.4-fold increase in styrene epoxide hydrolase (Cohen, 1981). Nevertheless, a new insecticide that is detoxified via the epoxide hydrolase should have little cross-resistance in pests that maintain metabolic resistances to other conventional pesticides. Indeed, house flies with multiple resistance to commercial alkoxide juvenoids, such as methoprene, remain susceptible to an epoxide-containing juvenoid (Sparks and Hammock, 1983).

That elevated epoxide hydrolase has not been heavily selected by commercial insecticides, yet is important to a herbivorous pest for detoxification of dietary epoxides, suggests this enzyme as a useful target for future pest control agents. Chalcone oxides and other inhibitors of epoxide hydrolase should synergize the activities of many plant defensive chemicals against herbivorous pests

and have little impact on entomophagous species, since the latter encounter and presumably need to detoxify fewer plants epoxides (Mullin and Croft, 1985).

Chalcones are regarded as universal flavonoid precursors in plants and might serve as endogenous synergists of other plant defenses if oxidized to inhibitors of herbivore epoxide hydrolase. Alternatively, they can direct the synthesis of in vivo inhibitors of epoxide hydrolase that might control populations of herbivorous pests. Additional use of these inhibitors may come through disruption of pheromone perception and deactivation in increasing numbers of herbivore species where major components are epoxides (Ahmad et al., 1986; Bell and Meinwald, 1986; Prestwich and Blomquist, 1987). These studies should give insight into how herbivores successfully coadapt with an epoxide-laden environment.

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BULLFINCHES AND ASH TREES
Assessing the Role of Plant Chemicals in Controlling Damage by Herbivores

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Abstract—Wild bullfinches (*Pyrrhula pyrrhula*) feed heavily on the seeds of ash trees (*Fraxinus excelsior*). Field and laboratory studies show that individual trees with high levels of fat and low levels of phenolic chemicals in their seeds are especially liable to predation. This paper discusses (1) the complexity of effects that need to be examined in order to identify the role of secondary chemicals in determining herbivore preferences, and (2) the consequences of seed predation for the dispersal of seeds. Because of bullfinches' habits, and the many factors influencing seed dispersal and germination, it is not clear whether the birds' activities are necessarily damaging. This study emphasises the value of understanding the whole ecology of both plants and herbivores in order to interpret their interactions.

Key Words—Chemical defense, plant–bird interaction, herbivory, seed predation, bullfinch, *Pyrrhula pyrrhula*, European ash, *Fraxinus excelsior*, secondary compounds.

INTRODUCTION

In the study of plants and animals that feed on them, the notion that secondary chemicals confer protection against herbivore damage has become widely accepted. It has been invoked to explain herbivores' preferences at many levels, from antelopes that select appropriate types of herbage from mixed-species swards (e.g., Cooper and Owen-Smith, 1985), to monkeys that choose particular trees of a plant species (e.g., McKey et al., 1981), and birds that select within the fruit crop of an individual plant (e.g., Herrera, 1982).

For vertebrate herbivores, evidence has been drawn chiefly from one of

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two approaches: either field data on feeding preferences and behavior are compared to measurements of chemicals in representative samples of plants, or plant material is extracted and applied to food experimentally to test its effect on palatability. Those studies following both avenues (e.g., Reichardt et al., 1984; Tahvanainen et al., 1985) provide the most compelling evidence for an influence of chemicals such as tannins, alkaloids, and terpenes on herbivores' food selection.

However, deterrence by the presence of a particular chemical is not enough to demonstrate a significant effect on the reproductive success of the plant. Fitness is governed by a complex array of interacting factors that require broader studies than most plant–herbivore investigations. In this paper it is argued that it is necessary to consider the whole ecology of both plant and herbivore in order to reach reliable conclusions about the role of particular plant chemical components.

There are three principal issues. First, one must identify chemical influences on behavior, while recognizing that statistical or experimental control of confounding variables obscures the context in which feeding choices actually occur. Second, it is important to recognize that chemicals may be proximate cues on which acceptance or rejection of food is based, or ultimate reasons behind a preference, or both. Third, the consequences to the plant of damage by a herbivore may not be simple or obvious, especially in long-lived species.

As an example of the complexity in these interactions, I shall discuss the effects of feeding by bullfinches (*Pyrrhula pyrrhula*) on the seeds of the European ash tree (*Fraxinus excelsior*). These studies form part of a broad investigation by MAFF into the feeding ecology of bullfinches in relation to their status as pests of fruit orchards in the south of England (e.g., Greig-Smith and Wilson, 1984; Wilson, 1984; Greig-Smith, 1985a,b). Through field, aviary, and laboratory investigations, we have explored the role of phenolic chemicals as deterrents to bullfinches' feeding. The purpose of the present paper is to explore connections between the presence of phenolics and the preferences shown by bullfinches for or against the seeds of individual ash trees. Data selected serve to demonstrate the kind that is needed to justify conclusions about the causes, and consequences for the trees, of bullfinches' behavior.

FRUIT PRODUCTION AND PREDATION

Ash trees produce crops of fruits that vary enormously in number, both from year to year and from tree to tree. Fruits gradually ripen between August and November (Figure 1) to leave a mass of dark brown, dry fruits in dense clusters over the whole tree canopy. A large flattened wing aids wind dispersal and, during winter, fruits are progressively lost from the tree by the actions of wind and seed predators. The rate of removal varies greatly, however; in some years trees may have no fruits left by December, while at other times substantial

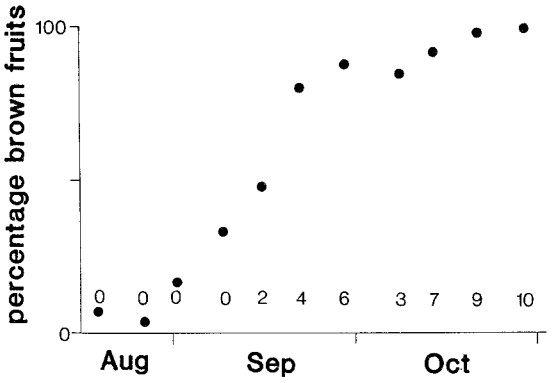

FIG. 1. Timing of ripening of ash seed, shown by average percentages of fruits that were brown on 10 trees in 1982. Figures from 0 to 10 indicate the number of trees with 100% of brown fruits.

numbers may remain until the following autumn, when they are borne alongside the next year's crop (Newton, 1964; personal observation).

Physiological studies have shown that the seeds require two winters before their dormancy is broken, so that fruits that fall shortly after ripening may lie on the ground for 18 months before germinating (Villiers and Wareing, 1964). During the six months after they have ripened, ash seeds on the tree remain stable in chemical composition (Figure 2). The trees therefore present a non-renewing food resource of fixed quality to winter herbivores.

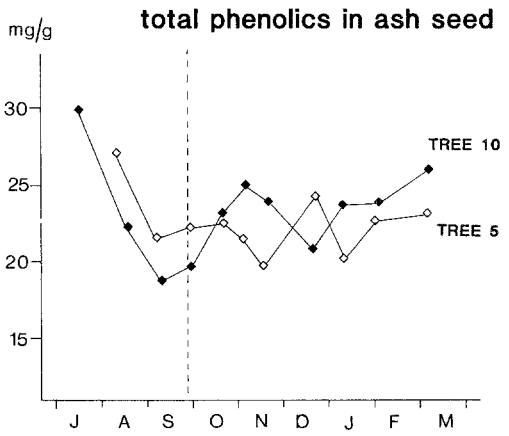

FIG. 2. Examples of stability of phenolic components in ash seed after ripening. The dotted line indicates the time at which 100% of fruits were brown. Data for six trees produced no consistent trends in the concentrations of phenolics (Folin-Denis assay), fat, total soluble protein, or total glucose, between October and March.

Ash seeds are large (20–60 mg), and rich in fat and protein (Greig-Smith and Wilson, 1985). This, and their conspicuous availability over the course of winter, makes them an attractive food source for bullfinches. In some years, ash seed is the major item in their diet, occurring in the gut contents of nearly all birds examined (Newton, 1967; Summers, 1981; Greig-Smith, 1985b). It is likely that almost the entire fruit crop of certain trees is removed by bullfinches. The availability of natural foods such as ash seed is apparently the most important factor determining whether bullfinches cause damage in fruit-tree orchards during any particular winter (see Newton, 1968). Fruits that fall to the ground may also be taken by predators, for the seeds are attractive to small mammals such as wood mice (*Apodemus sylvaticus*), which can account for the loss of much of trees' seed production in certain years (e.g., Gardner, 1977).

CAUSES OF BULLFINCH PREFERENCES AMONG ASH TREES

In all field studies, observers have noted that bullfinches have distinct preferences for certain ash trees and ignore others (Newton, 1967; Matthews, 1983; Greig-Smith and Wilson, 1985). This is not simply related to the size of the trees, their locations, or the amounts of fruits they bear, but might perhaps have something to do with the fruits themselves. To investigate these preferences, we placed nets underneath the tree canopy to collect falling fruits and seeds (Greig-Smith and Wilson, 1985). The total numbers of damaged and intact fruits can be used to assess the impact of predation on the tree's reproduction, while the ratio of these numbers provides an index of preference. In a survey of 22 trees within a single study area, 0–95% of fallen fruit was damaged by bullfinches, demonstrating the wide extremes of the birds' preferences. Seed and fruit collection in nets gives a measure of many birds' feeding combined activity. Direct observation and radio tracking was used to trace the behavior of individual birds and has confirmed the broad range of acceptability of different trees (Greig-Smith and Wilson, 1985; Greig-Smith, 1985b).

To account for the birds' behavior, I searched for correlations between preferences and features of fruit size and composition. Size varies more between trees than within trees (Figure 3) and is of importance for two reasons. First, it affects the quantity of food a bullfinch gains by eating a given number of seeds, and second, along with shape, it may dictate the ease with which birds can remove the pericarp to obtain the seed (Greig-Smith and Crocker, 1986). The handling time for a seed must be weighed against the food content (Krebs and McCleery, 1984), and we have shown that bullfinches are liable to drop fruits—deliberately or accidentally—during efforts to extract the seeds (Greig-Smith and Wilson, 1985). At the start of winter, the birds' efficiency is so poor that they successfully eat seeds from only half the fruits they pluck and handle.

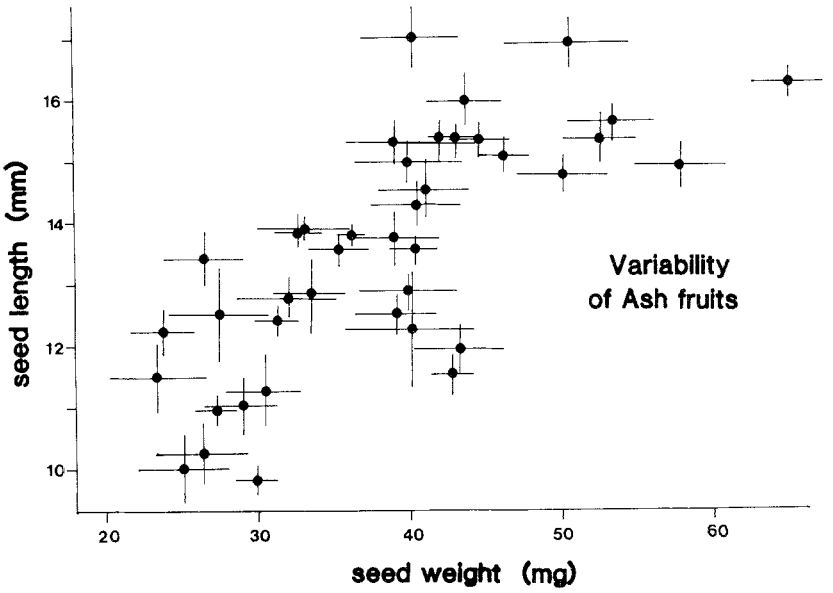

FIG. 3. Scale of variation in size of ash seed, within and among trees. Data are means ± standard errors for samples of 25 seeds from 50 trees, collected on the same day in November.

The chemical composition of ash seed also varies greatly (Table I), although no single feature stands out as being obviously correlated closely with the birds' preferences. In a previous analysis, Greig-Smith and Wilson (1985) found that if the confounding variations in other features were held constant in a partial correlation analysis, statistically significant relationships emerged with fat content ($r_p = 0.45$, $N = 17$) and total phenolic content ($r_p = -0.51$), but not with protein content ($r_p = 0.16$). This implies that preferences are governed by a balance between beneficial nutritional components and aversive secondary chemicals.

The fact that this conclusion is evident only when other features are held equal is a sign that intercorrelation of fruit features may override these effects in the field. Thus, the data matrix from which Table 1 is drawn reveals that these seed components do not vary independently; for example, fruit width (a measure of the difficulty with which bullfinches can extract seeds) is positively correlated with fruit weight ($r = 0.52$, $N = 20$), whereas fat concentration of the seed tissue is inversely correlated with phenolic concentration ($r = -0.44$). Intercorrelations of this kind may offer clues to the evolutionary pressures affecting plant populations, but they present problems for researchers trying to unravel causes and effects in herbivore behavior.

TABLE 1. LEVELS OF SELECTED CHEMICAL CONSTITUENTS IN SEEDS OF 20 ASH TREES AT SINGLE SITE[a]

Tree	Ranked bullfinch preference	Average seed weight (mg)	Concentration (mg/g)			
			Total phenolics	Fat	Total soluble protein	Total glucose
58	1	35.2	15.6	157.8	114.2	7.2
26	2	44.2	21.2	109.0	44.4	6.7
12	3	32.8	30.8	151.0	84.5	11.4
54	4	25.0	21.1	132.5	74.9	11.4
63	5	30.9	22.7	137.7	92.2	10.9
49	6	27.4	19.3	152.9	61.5	11.2
101	7	25.4	22.2	105.3	52.4	14.2
45	8	20.4	23.9	105.7	49.0	8.1
16	9	29.7	23.9	116.0	89.9	13.7
57	10	25.6	13.5	108.2	89.0	10.9
5	11	59.1	21.1	88.4	125.6	15.3
9	12	34.1	16.0	122.5	50.8	13.9
64	13	32.4	27.4	125.7	80.8	11.7
53	14	35.8	16.2	135.3	51.5	11.0
48	15	19.0	48.2	78.1	45.8	11.8
10	16	38.6	25.2	109.8	54.1	15.1
2	17	58.0	29.5	69.7	84.4	20.3
68	18	43.4	21.4	122.2	103.4	7.2
32	18	53.7	24.5	149.4	86.7	10.9
86	18	12.4	13.5	128.8	62.1	7.4

[a] Variability within samples (standard error as % of mean) was approx 5–10%.

A further difficulty lies in knowing which parameters to measure, since the chemical components of a seed may be expressed in different ways. Figure 4 compares the variation in fat and phenolic composition of ash seed in terms of concentration (milligrams per gram of tissue) and content (milligrams per seed). The resulting histograms provide only slightly different distributions, but the ordering of trees depends heavily on which index is used. Correlations between the two rankings were only 0.46 for fat and 0.51 for phenolics. It is not possible to identify one measure as generally "better" or more appropriate than the other, for it depends on how the chemicals affect herbivores. Some components have characteristic sensory effects (e.g., the astringency of tannins; Joslyn and Goldstein, 1964), whereas others will only have later effects during digestion. It is important to distinguish the role of chemicals as cues in the formation of learned aversions or attractions (e.g., Arnold et al., 1980; Mason and Reidin-

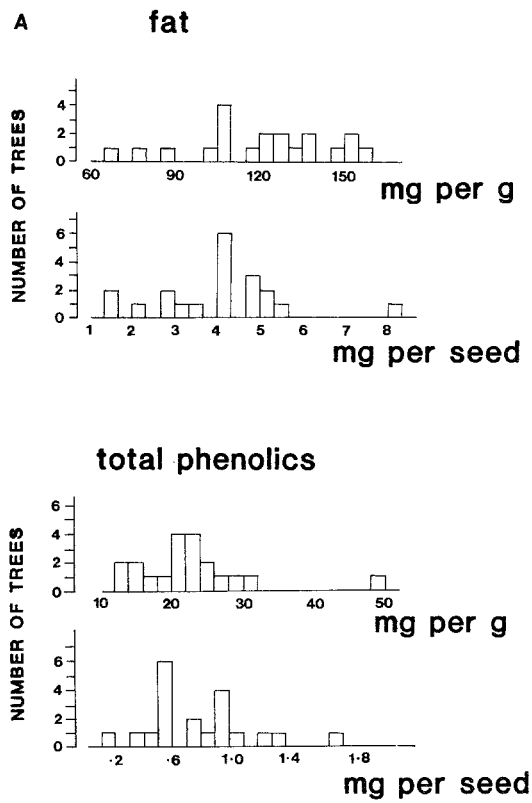

FIG. 4. (A) Variations in the fat content and phenolic content of seeds from 20 ash trees, measured as milligram per gram of tissue, and as milligram per seed. Correlations refer to the correspondence between rankings of individual trees obtained by the two methods. (B) Correlations between bullfinch feeding preferences for particular trees and their fat content measured by the two indices in (A). Crosses indicate trees in which bullfinches never fed and are excluded from the calculation of correlation coefficients.

ger, 1983; Stewart et al., 1983) from metabolic properties related to food processing mechanisms. In the case of fat (Figure 4), preference scores are more strongly correlated with concentration in mg per gram than with total seed content, although this need not imply an influence on sensory detection.

An example of the importance of feeding cues comes from examining the phenolic component of ash seed in more detail. One characteristic component is fraxin, a compound present in the seed chiefly in the form of its hydrolyzed derivative, fraxetin (Figure 5A). In contrast to the total phenolic complement, variation in this substance is positively correlated with bullfinch preferences

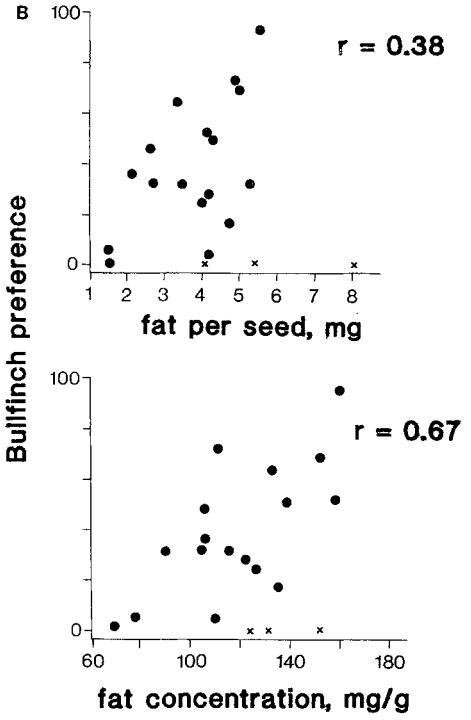

FIG. 4. Continued.

(Figure 5B), the likely reason being that it varies in parallel with fat and is therefore available to birds as a potential flavor cue indicating the presence of fat-rich seed. Confirmation that fraxin/fraxetin can be used as a positive food selection cue by bullfinches was obtained from laboratory feeding trials in which seeds were adulterated with fraxin, and birds' behavioral responses measured in detail (see Greig-Smith, 1985a, for methods). Of 10 bullfinches tested, four showed statistically significant evidence of detecting the presence of fraxin (either in comparison of successive trials with and without the chemical, or within trials between food dishes having treated or untreated food), and all four revealed an attraction towards the substance (ate more food in a standard interval or rejected fewer pieces of food during handling) (see Figure 5C).

CONSEQUENCES OF SEED LOSS FOR TREES' REPRODUCTION

For a species such as ash, producing a single annual crop of seed, it seems obvious that the tree's loss of reproductive potential should be proportional to the number of fruits removed by herbivores. Greig-Smith and Wilson (1985)

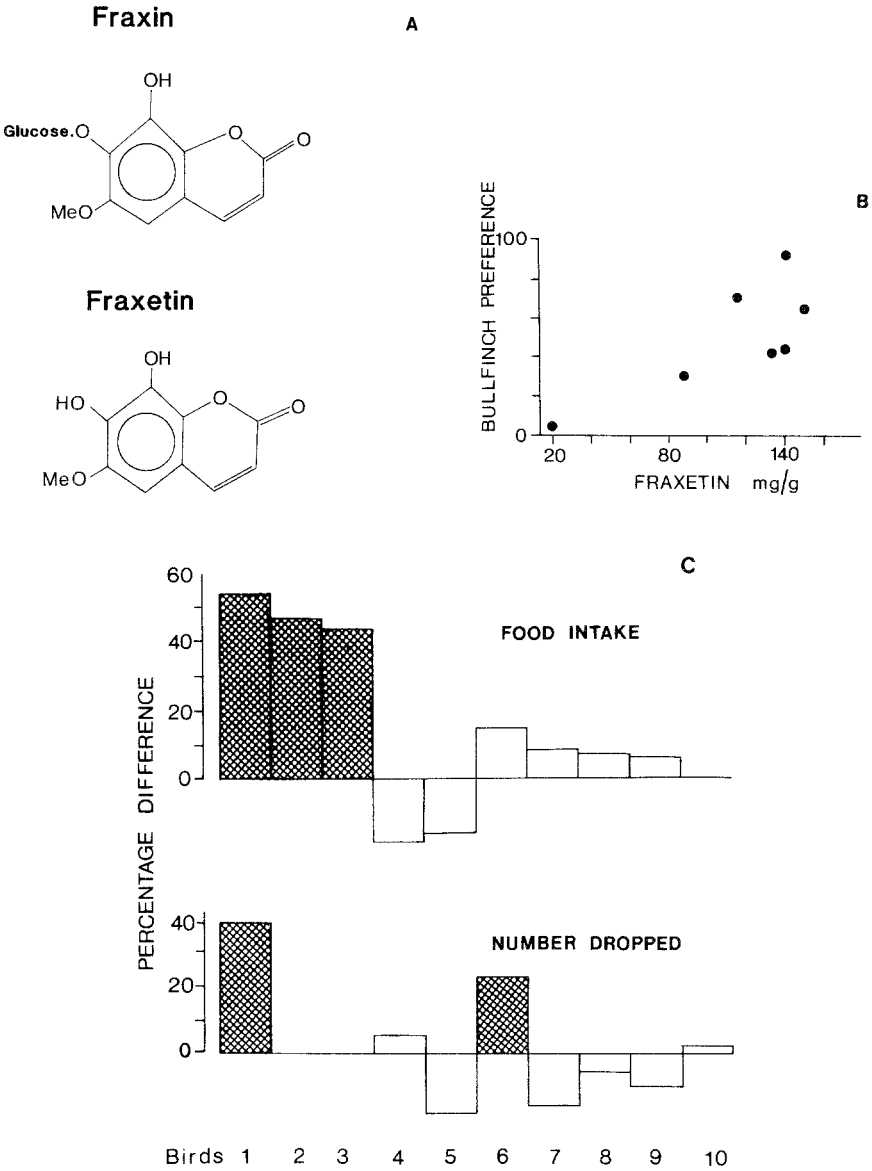

FIG. 5. (A) Formulas of fraxin and fraxetin, two components of ash seed. (B) Correlation between the fraxetin content of seeds and bullfinch feeding preferences among seven ash trees; $r = 0.82$, $P < 0.05$. (C) Results of a laboratory feeding trial in which 10 bullfinches were given choices between food adulterated with fraxin and untreated food, and their behavior was monitored on videotape. Columns indicate differences between behavior at the two types of food, those above the line representing attraction to fraxin. Shaded columns indicate statistically significant differences.

found that trees with higher levels of phenolics in their seeds retained fruits longer into the following year. This suggests an adaptive protection bestowed by the plant's secondary chemistry. However, the consequences of seed loss are not so straightforward.

There are several routes by which seeds can be transported from tree to ground and survive to eventually germinate (Figure 6); wind dispersal, accidental or deliberate dropping of intact fruits by feeding bullfinches, and escape from predation by small mammals. Fruits may also be attacked by insect larvae (Wardle, 1961). The fate of fruits is greatly affected by three aspects of the dispersal process: the number that fall, the distances to which they are carried, and the time at which they are removed from the tree. Together, these parameters influence the exposure of fruits to bird and small mammal predation and affect the competitive environment in which seedlings develop and grow (McCanny and Cavers, 1987). Without knowledge of local predator populations and behavior, and information on edaphic factors and seed density, it is not easy to predict whether fruits' prospects will be better near or far from the parent tree, after falling early or late in the winter.

The characteristics of individual trees may be important; for example, the distance to which falling fruits are carried depends on fruit size and shape (unpublished data; cf. Augspurger and Franson, 1987). However, the activities of bullfinches also play a role, expressed in four distinct ways. First and most obviously, their feeding removes or damages some of the seed crop, although predation cannot totally prevent dispersal, because substantial numbers of seeds are dropped intact. Second, the dispersal of fruits that are dropped is earlier than would occur by wind alone. These fruits are not a random sample of those available, since bullfinches feed selectively within trees on the basis of fruit size (Greig-Smith, 1987). Third, bullfinches feed in the canopy of ash trees

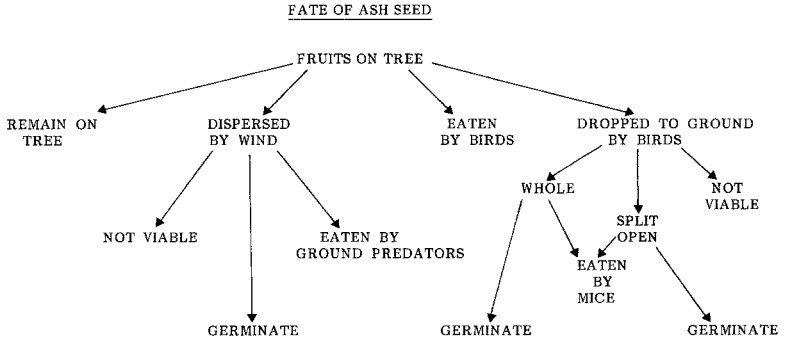

FIG. 6. Fate of ash fruits, indicating alternative routes by which fruits may reach the ground, survive, and germinate.

only when weather conditions are favorable, particularly at low wind speeds; dropped fruits therefore travel less far than fruits blown from the trees by wind (Figure 7A). Fourth, many fruits fall when partly split open, but with the seed undamaged. This is beneficial for seed development as rupture of the pericarp allows freer gas exchange, promoting embryonic enlargement (Villiers and Wareing, 1964) (see Figure 7B). As a result, split fruits dropping early may be more likely to germinate in their first winter than those that fall intact.

Clearly, bullfinches can modify the dispersal pattern in a variety of adverse and beneficial ways. However, our field studies do permit a preliminary explo-

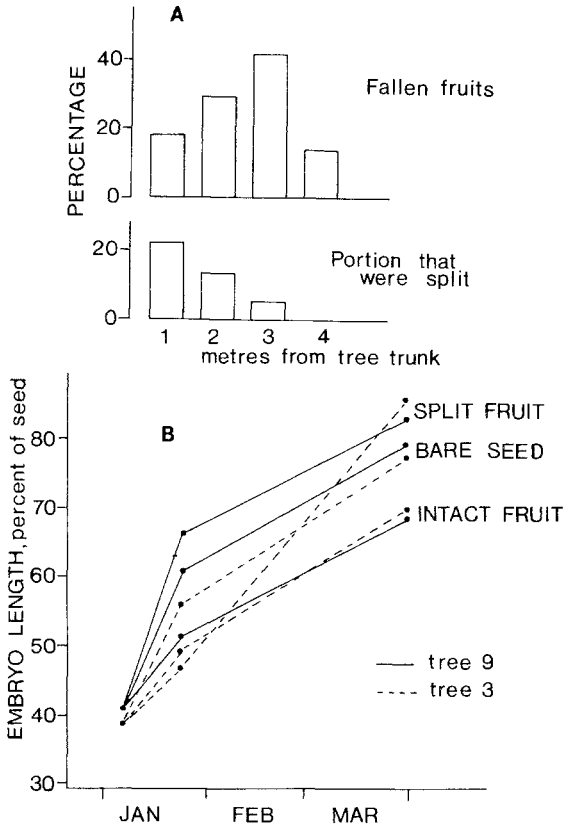

FIG. 7. (A) Example of the distribution of fruits fallen around an ash tree shown separately for intact fruits (largely wind-dispersed) and those split open and dropped by bullfinches. (B) Differences in embryonic elongation in seeds with an intact fruit coat, no fruit coat, or a fruit coat split open as by a feeding bullfinch. Data are taken from a laboratory germination experiment in which seeds and fruits were placed on moist filter paper for three months.

TABLE 2. FATE OF FRUITS REMOVED FROM CANOPY OF SIX ASH TREES IN SINGLE WOOD[a]

Tree	Bullfinch preference	Wind dispersed	Eaten	Dropped		Total onto ground
				Whole	Split	
26	1	0	417	204	75	279
54	2	531	321	54	39	624
49	3	672	546	105	129	906
63	4	51	54	0	0	51
57	5	363	117	63	84	510
48	6	579	27	3	3	585

[a] Figures represent numbers of fruits per m^2 below the center of the canopy, over the entire winter. Bullfinch preference was ranked on the basis of the proportion of fruits affected by the birds' feeding.

ration of how the birds' preferences among trees influence the fates of fruits. Table 2 estimates the numbers of fruits falling per square meter that had been wind-dispersed, dropped whole, or dropped split, and the number eaten by bullfinches, in six trees growing close together in a small wood. This shows that in tree 26, ranked as most preferred by bullfinches (i.e., where the largest proportion of fruits had been plucked by the birds), no fruits were wind-dispersed. Nevertheless, a substantial number reached the ground intact or with the pericarp split but probably still viable. Overall, there was certainly not a close correspondence between bullfinches' feeding activity and the amount of viable fallen seed accumulated by the end of the winter.

Seed chemistry and seed size can be incorporated into this approach. Table 3 lists correlations across a sample of 19 trees, between variations in the four

TABLE 3. CORRELATIONS OF SEED SIZE AND THREE CHEMICAL COMPONENTS OF SEEDS WITH LEVEL OF BULLFINCH ACTIVITY AT 19 ASH TREES AND WITH FALL OF VIABLE SEED OVER WINTER PERIOD

	Fruits attacked by bullfinches (%)	Total seed fall	Fall of whole seed
Seed weight	−0.16	−0.53	−0.32
Total phenolics	−0.12	−0.06	−0.04
Fat	+0.35	−0.25	−0.02
Fraxetin	+0.28	+0.10	+0.15

most relevant seed features and measures of bullfinch preference and seed survival. As discussed above, the birds' attentions were related most strongly to fat content and its correlated cue, fraxetin. In contrast, the eventual cumulative seed fall was related more strongly to different features, particularly the size of seeds. None of the correlation coefficients is large, indicating that many factors are involved in determining these patterns.

CONCLUSIONS

The theme of this essay has been that a complex interaction of many factors must be studied to understand how plant chemicals influence a vertebrate herbivore and what this means for the plant. For bullfinches feeding in ash trees, the habit of dropping whole fruits contributes greatly to the complexity. Even without this, however, plants may be able to avoid the apparently adverse consequences of seed predation by animals that do not contribute to dispersal. One of the most widespread means of escaping these effects, among plants whose growing parts are damaged, is compensation by increased growth. For example, removal of up to 90% of fruit-tree buds results in much less reduction in the yield of fruit, particularly if the damage is sustained for several years (Summers, 1982). A growing number of studies are also considering the existence of "induced" chemical defenses that appear after the onset of herbivore attacks, although the evidence is not conclusive (Fowler and Lawton, 1986) and the mechanisms involved are the subject of active debate (see Haukioja and Neuvonen, 1985).

The ecological issues discussed in this paper call for caution in interpreting facts that seem to suggest a simple protective role for plant secondary chemicals, in light of the wide range of confounding effects. This is not to deny the possible importance of chemical defenses; however, it will be valuable for future studies to broaden their scope to examine these issues in the context of the whole ecology of both plant and herbivore.

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CHEMICAL ECOLOGY AND THE SEARCH FOR MARINE ANTIFOULANTS
Studies of a Predator–Prey Symbiosis

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Abstract—The gorgonian octocoral *Leptogorgia virgulata* (Phylum Coelenterata, Class Anthozoa) is rarely overgrown by fouling organisms and is avoided by most predators. Laboratory experiments suggest that secondary metabolites and calcium carbonate spicules interact synergistically to provide *L. virgulata* with an effective defense against predatory fish. In spite of these defenses, *L. virgulata* is consumed by the symbiotic, trophically specialized gastropod *Neosimnia uniplicata*, which closely mimics the appearance of the gorgonian. *Neosimnia uniplicata* is readily eaten by fish and appears not to acquire an effective antipredator defense from its gorgonian host. Extracts of *Leptogorgia virgulata* and *Neosimnia uniplicata* strongly inhibited the settlement of the barnacle *Balanus amphitrite*. Bioassay-directed purification of the more potent antifouling agents from *L. virgulata* led to the isolation of two previously described, diterpenoid hydrocarbons, known as pukalide and epoxypukalide. A third inhibitor of barnacle settlement, whose structure is presently unknown, was obtained from both *L. virgulata* and *N. uniplicata*. When assayed for ability to inhibit barnacle settlement, these three compounds possessed EC_{50} values ranging from 19 to 55 ng/ml. These secondary metabolites may prevent the overgrowth of *L. virgulata* by fouling organisms in nature. The allelochemicals of *L. virgulata*, *N. uniplicata*, and other marine organisms may provide nonpolluting alternatives to existing, commercial antifoulants based on derivatives of tri-*n*-butyltin.

Key Words—Chemical defense, biofouling, marine antifoulants, symbiosis, predator–prey interactions, gorgonians, gastropods, *Leptogorgia virgulata*, *Neosimnia uniplicata*.

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INTRODUCTION

Marine chemical ecology encompasses a broad range of topics, including chemoreception and chemical signaling, chemical defenses against predation, allelopathic interactions between competitors, the biochemical control of larval settlement and metamorphosis, and marine symbioses (Bakus et al., 1986; Rittschof and Bonaventura, 1987). The basic knowledge obtained in such investigations has begun to provide new insight into problems of practical importance, such as the management of marine fouling (Colwell, 1983).

Fouling is one of the most important problems currently facing marine technology (Costlow and Tipper, 1984). Presently, marine fouling is controlled by the use of surface coatings that contain long-lived, broad-spectrum poisons such as tri-*n*-butyltin (TBT). Although effective, the active agents of these coatings have become serious pollutants in some estuaries. The use of TBT-based paints has been banned recently in France and Great Britain and is being closely scrutinized in other countries.

Fouling organisms usually do not colonize the surfaces of sessile marine invertebrates such as sponges (Phylum Porifera) and octocorals (Phylum Coelenterata: Class Anthozoa). Sponges and octocorals contain a wealth of secondary metabolites (Goad, 1978; Tursch et al., 1978; Faulkner, 1977, 1984; Fenical, 1982; Scheuer, 1985), which may inhibit fouling (Ciereszko, 1962; Burkholder, 1973; Cimino and DeStefano, 1978; Hadfield and Ciereszko, 1978). These compounds could provide alternatives to the metal-derived toxins currently used in most commercial antifoulant coatings.

One octocoral, the gorgonian *Leptogorgia virgulata* Lamarck, 1815), is abundant in the shallow waters near Beaufort, North Carolina. Gorgonians are colonial coelenterates that are comprised of a proteinaceous axial skeleton covered by a fleshy cenencyme that contains symbiotic zooxanthellae and needle-like spicules of calcium carbonate (Bayer, 1961). Gorgonians can regenerate lost tissue and thus can undergo partial predation (Silveira and Van't Hof, 1977; Harvell and Suchanek, 1987). Like most other octocorals, *L. virgulata* is rarely fouled, even though other surfaces near it are covered in a period of weeks by a thick growth of barnacles, bryozoans, hydroids, algae, and other organisms. Secondary metabolites may play an important role in the protection of *L. virgulata* from overgrowth by fouling organisms (Standing et al., 1982; Rittschof et al., 1985; Targett et al., 1983).

A highly specific assemblage of symbiotic organisms is associated with *Leptogorgia virgulata* (Patton, 1972). One of these symbionts, the gastropod *Neosimnia uniplicata*, feeds upon the cenenchyme of *L. virgulata* and closely mimics the appearance of the gorgonian (Osborn, 1885; Patton, 1972). *Neosimnia uniplicata* is a member of the family Ovulidae, a group of gastropods that often live in close association with corals (Robertson, 1970). Behavioral evi-

dence suggests that secondary chemicals may function in many roles in the relationship between *L. virgulata* and *N. uniplicata* (Rittschof and Gerhart, unpublished observations).

The secondary chemicals of *Leptogorgia virgulata* and *Neosimnia uniplicata* are interesting from two standpoints. Similarities and differences in the chemical content of these organisms provide insight into their ecological interaction, since *N. uniplicata* may sequester or modify the secondary metabolites of its prey (Coll et al., 1983; Faulkner and Ghiselin, 1984). The comparison also could provide data of applied importance, since metabolic alteration of *L. virgulata* metabolites by *N. uniplicata* could lead to more effective antifoulants or could provide clues to structure–activity relationships of antifouling molecules.

In this paper, we examined the possible roles of secondary metabolites in the ecological interaction between *Leptogorgia virgulata* and *Neosimnia uniplicata*. Investigative tools used in this study included biological assays of palatability and antifouling potential, coupled with thin-layer chromatography (TLC).

METHODS AND MATERIALS

Collection. Leptogorgia virgulata and *Neosimnia uniplicata* were collected in Bogue Sound, Morehead City, North Carolina, USA at a depth of approximately 5 meters. *Leptogorgia virgulata* was collected April 3, 1987. Individuals of *N. uniplicata* were collected April 3 and 24, 1987. After collection, the tissues were cleaned, weighed, and then frozen and lyophilized. To measure the water content of the gorgonian tissue, the material was weighed before and after lyophilization.

Extraction and Analysis. Tissues of *Leptogorgia virgulata* or *Neosimnia uniplicata* were soaked overnight in HPLC grade methanol. The extract was decanted, and the tissue was extracted a second time in fresh HPLC grade methanol. The filtered extracts were combined and reduced to a total volume of approximately 25 ml in a rotary evaporator. The concentrated extract was partitioned between HPLC grade methylene chloride and HPLC grade water to remove salts. The methylene chloride layer was evaporated to dryness using a rotary evaporator, and the residue was transferred to a preweighed vial in 2 ml of HPLC grade methylene chloride. The solvent was removed using a vacuum desiccator, and the weight of the vial and its contents was determined.

Analytical TLC was performed using Whatman MK6F silica gel plates (25 $\times$ 75 mm). Samples were applied in a small volume of methylene chloride to the plates. Plates were developed in 1 : 9 acetonitrile–methylene chloride, and visualized using long-wave ultraviolet light, short-wave ultraviolet light, and by treatment with vanillin–sulfuric acid spray.

Spicules of *Leptogorgia virgulata* were prepared by soaking cenenchyme in a dilute solution of sodium hypochlorite, as recommended by Bayer (1961). Axial skeleton was prepared by manually stripping it of cenenchyme and rinsing the skeleton with water. The spicules and axial skeleton were dried in a vacuum desiccator and weighed.

Fish-Feeding Experiments. The acceptability of *Leptogorgia virgulata* and *Neosimnia uniplicata* was determined by offering sections of tissues to individual pinfish, *Lagodon rhomboides*, that had been collected in Beaufort Harbor, North Carolina. *Lagodon rhomboides* was selected as assay organism because it is omnivorous and occurs sympatrically with *L. virgulata*. Fish were offered 2 × 4-mm sections of *L. virgulata* cenenchyme, stripped from the axial skeleton, since fish of the size used in the experiments (30–45 mm standard length) cannot sever the axial skeleton but could remove pieces of tissue from the axial skeleton if feeding on a gorgonian. Fish were also offered crushed *Neosimnia uniplicata*. To ensure that each fish used in the assay was not satiated, control food items were also offered.

Feeding experiments were used to determine the influence of gorgonian spicules and secondary metabolites on the acceptability of artificial food pellets to *Lagodon rhomboides*. Spicules and extract were incorporated, either alone or in combination, into 2% alginate (w/w) food pellets that were flavored with 5% (w/w) powdered, lyophilized fish. To produce alginate containing *L. virgulata* extract, compounds were delivered to a vial in a small volume of methylene chloride, and the solvent removed by vacuum desiccation. Alginate and fish powder, with or without spicules, were then added to the vial and the contents stirred to disperse the materials uniformly throughout the alginate. To produce alginate without extract, pure methylene chloride was added to a vial and evaporated. Alginate and fish powder, with or without spicules, were then added and stirred. Ten-microliter aliquots were taken from each type of alginate mixture, and hardened in a 1 M solution of calcium chloride. The final concentrations of spicules and extract in the pellets corresponded to the concentrations naturally found in the gorgonian (see Results).

In all runs of the experiments, pellets were offered to individual *L. rhomboides* in the following sequence: one pellet of commercial fish food, two experimental pellets, one pellet of commercial fish food. Fish were scored as eating a pellet, mouthing and rejecting a pellet, or ignoring a pellet. A new, previously untested fish was used in each trial to ensure maximum independence between runs of the experiment. Data were not included for fish that did not consume both the initial and final pellets of commercial fish food; this ensured that satiated fish were not included in the experiment.

Antifouling Assays: Barnacle Settlement Inhibition. Cyprid (settling stage) larvae of the barnacle *Balanus amphitrite* were cultured following methods described in Rittschof et al. (1984). Three-day-old cyprid larvae were added to

clean glass vials containing either 5 ml of aged, filtered seawater (30 parts per thousand salinity), or seawater containing various concentrations of test substances. The vials were then incubated at 28°C for 24 hr, with a 15:9 light–dark cycle. After incubation, the vials were removed, and the larvae were examined under a dissecting microscope to determine if they were still alive. The larvae were then killed by addition of several drops of 10% formalin solution. The attached and unattached cyprids were then counted (Rittschof et al., 1984). The number of attached larvae was expressed as a percentage of the total number of larvae in the vial. These data were used to generate dose–response curves; EC_{50} values for settlement inhibition were obtained by probit analysis (Lieberman, 1983).

Bioassay-Directed Isolation of Antifouling Components. Preparative TLC was performed using Whatman PK6F plates (20 × 20 cm). Compounds were dissolved in methylene chloride and applied to the plates with capillary pipettes. Plates were developed in 9:1 methylene chloride–acetonitrile. Compounds were visualized using short-wave and long-wave ultraviolet light. Regions of interest were scraped from the plate using a clean metal spatula, and the compounds were eluted from the silica with methylene chloride. The solution of purified components was then filtered and evaporated to dryness in a vacuum desiccator. These materials were then employed in barnacle settlement assays. This procedure was repeated to obtain highly purified components that inhibited barnacle settlement.

Field Assay of Antifouling Activity. A plastic structure consisting of vertical rods and horizontal surfaces was used for field experiments. One section of this structure was sprayed with an extract of *Leptogorgia virgulata* in methylene chloride to give an average of 600 μg extract per square centimeter of surface. An adjacent section of the structure was treated in a similar fashion with methylene chloride alone. The structure was placed subtidally in Beaufort Harbor, North Carolina, for 24 hr, and then retrieved. Settled barnacles, bryozoans, or hydroids attached to the structure were counted.

RESULTS

Composition of Leptogorgia virgulata. Water made up more than 50% of the wet weight of colonies of *Leptogorgia virgulata* used in this study. Carbonate spicules comprised over 20% of the wet weight, while axial skeleton and chemical extract made up 8% and 1% of the wet weight, respectively.

Acceptance/Rejection of Leptogorgia virgulata *and* Neosimnia uniplicata. In initial laboratory experiments, *Lagodon rhomboides* readily consumed all sections of *Neosimnia uniplicata* ($N = 10$). In subsequent laboratory experiments that incorporated controls for satiation, *L. rhomboides* accepted 9.1% (3

of 32) of all sections of *Leptogorgia virgulata*, while accepting 97% (31 of 32) of all control pellets that were offered to them. These rates of acceptance were significantly different (Williams' adjusted $G = 58.3$; $P < 0.001$).

Acceptance/Rejection of Alginate Pellets. Lagodon rhomboides accepted control alginate pellets at a rate that was not significantly different from their rate of acceptance of commercial fish food pellets (10 of 12 control pellets eaten vs. 12 of 12 commercial fish food pellets eaten; $G = 2.360$ using Williams' correction; $P > 0.1$).

Since these experiments involved feeding two pellets to each tested fish, transition matrices could be constructed to examine the effect of choice of the first alginate pellet on choice of the subsequent pellet. These transition matrices were examined for each treatment. Analysis of the transition probabilities showed no statistically significant facilitation or inhibition ($P > 0.1$ for each treatment), indicating that the choices of the two trials could be considered statistically independent (Legendre and Legendre, 1983).

A G test for independence between the treatments showed that treatment significantly affected the proportion of pellets that were accepted (Williams' adjusted $G = 18.6$; $P < 0.001$; Table 1). The data for alginate pellets of different types were then analyzed using a posteriori tests, and employing an experiment-wise error rate of 0.05 (Sokal and Rohlf, 1983). One maximally nonsignificant subset consisted of pellets with spicules and pellets containing both spicules and *L. virgulata* extract. The second maximally nonsignificant subset was comprised of control pellets, pellets containing spicules, and pellets containing *L. virgulata* extract.

Settlement Inhibition by Extracts of Leptogorgia virgulata *and* Neosimnia uniplicata. Extracts of *L. virgulata* and *N. uniplicata* were potent inhibitors of barnacle settlement. Probit analysis of data from a dilution series experiment indicated that the EC_{50} for settlement inhibition of *L. virgulata* extract was 5 μg/ml, with a 95% confidence interval of 3.7–7.3 μg/ml. For *N. uniplicata* extract, the EC_{50} was 4 μg/ml, with a 95% confidence interval of 2.0–3.9 μg/ml (Figure 1).

TABLE 1. ACCEPTANCE AND REJECTION OF ALGINATE PELLETS BY PINFISH, *Lagodon rhomboides*

Treatment	Number eaten	Number not eaten
Control	10.0	2.0
+ Spicules	8.0	6.0
+ Extract	13.0	1.0
+ Spicules and extract	3.0	11.0

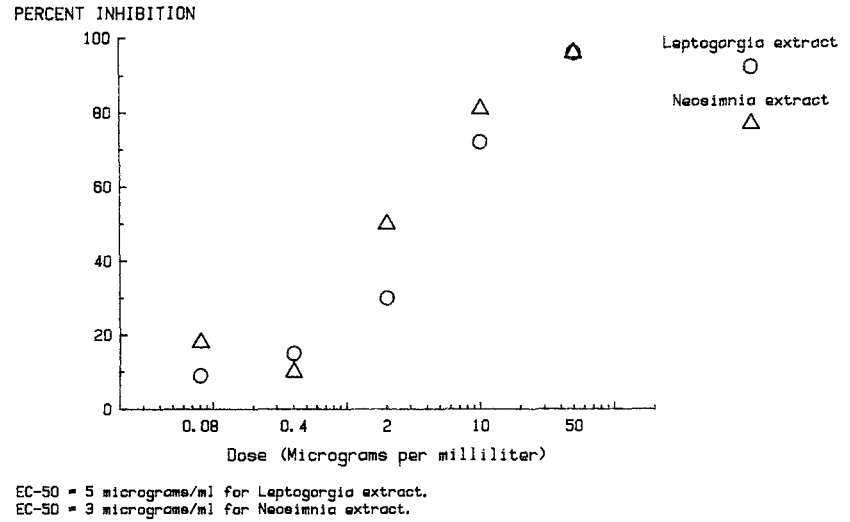

FIG. 1. Inhibition of barnacle settlement by crude extracts of *Leptogorgia virgulata* and *Neosimnia uniplicata*.

Settlement Inhibition by Pure Compounds. The bioassay-directed separation of antifouling compounds from the extract of *Leptogorgia virgulata* led to the isolation of three components. One of these compounds was found to have an EC_{50} value of 19 ng/ml, with a 95% confidence interval of 11–27 ng/ml (Figure 2). The second compound possessed an EC_{50} value of 55 ng/ml, with a 95% confidence interval of 28–78 ng/ml (Figure 3). Structural analysis of these compounds by two-dimensional proton NMR, FAB mass spectrometry, and other techniques, indicated that LV1 and LV2 were previously described diterpenes, known as epoxypukalide and pukalide, respectively (Keifer, unpublished data). A third active component possessed an R_f of approximately 0.78 in 1:9 acetonitrile–methylene chloride. This compound caused cyprids to stick to glass surfaces, and thus was dubbed "cyprid flypaper." Dose–response experiments indicated that cyprid flypaper inhibited the settlement of *Balanus amphitrite* with an EC_{50} of 42 ng/ml, with a 95% confidence interval of 17–90 ng/ml (Figure 4). Pukalide, epoxypukalide, and cyprid flypaper did not kill the cyprid larvae of *B. amphitrite* but inhibited settlement by a nontoxic mechanism.

Field Antifouling Experiment. Four larvae (two barnacle larvae and two bryozoan larvae) settled on the area of the plastic structure that was treated with *Leptogorgia virgulata* extract. The control area of the structure, which had been treated with only pure methylene chloride, had been settled upon by 111 larvae

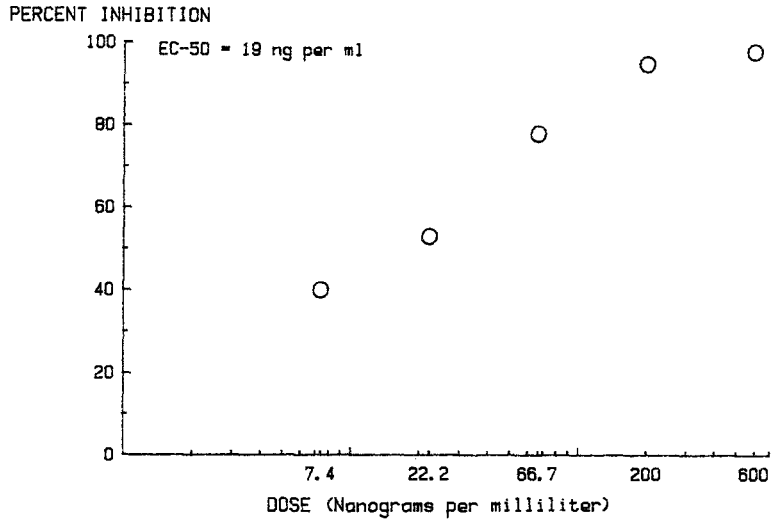

FIG. 2. Inhibition of barnacle settlement by the diterpene pukalide.

(16 barnacle larvae, 93 bryozoan larvae, and 2 hydroid larvae). This result was significantly different from a distribution that assumed larvae settled without preference on either area (G test for goodness of fit; $G = 124.2$ using Williams' correction; $P < 0.001$).

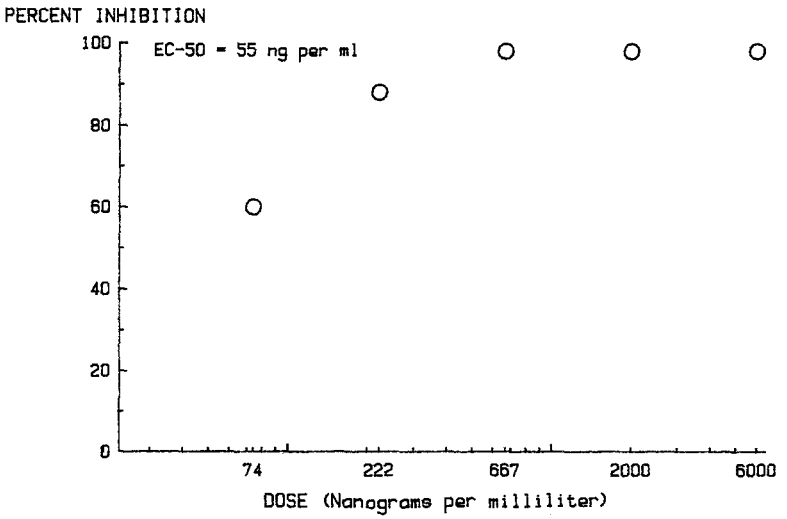

FIG. 3. Inhibition of barnacle settlement by the diterpene epoxypukalide.

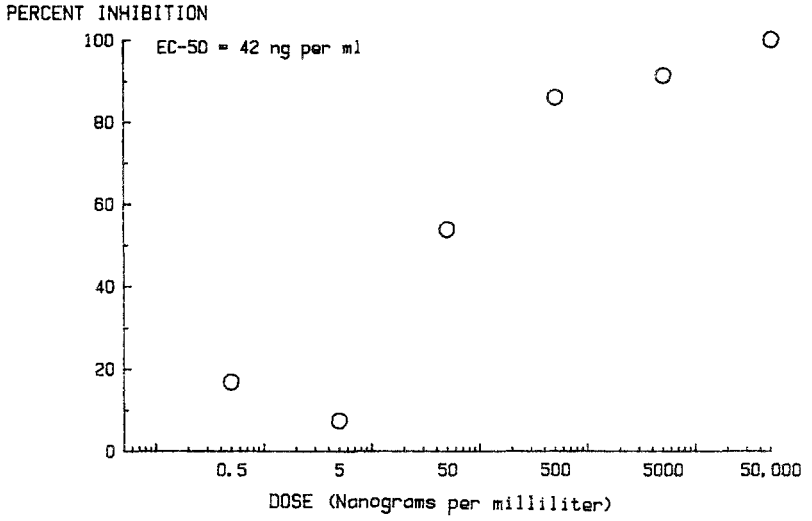

FIG. 4. Inhibition of barnacle settlement by cyprid flypaper.

DISCUSSION

Leptogorgia virgulata was not consumed in the laboratory by *Lagodon rhomboides*. These data corroborate field observations, indicating that *L. virgulata* is rarely eaten by predatory fish. Spicules appear to be important in the protection of *L. virgulata* from fish predation, since incorporation of spicules into alginate pellets was accompanied by greatly reduced acceptance by *Lagodon rhomboides*. When delivered alone in alginate, the chemical extract of *L. virgulata* had no significant affect on pellet avoidance by *L. rhomboides*. The combination of spicules and extract, however, was particularly effective in reducing pellet acceptance. The antipredator defenses of *Leptogorgia virgulata*, therefore, appear to be based upon a synergistic interaction between secondary chemicals and morphological structures. The mechanism of this interaction is unknown. The supraadditive response may have been a behavioral reaction to the simultaneous presence of two negative stimuli. Alternatively, when *L. rhomboides* attempted to ingest pellets containing both spicules and extract, the spicules may have pierced or abraded the mouth and pharynx of the fish, providing a route of entry for defensive compounds that had little or no effect on palatability in the absence of spicules. A similar interaction of physical structures and chemical defenses has been postulated for some terrestrial plants, in which raphides (needle-like crystals of calcium oxalate) may provide a route of entry for secondary metabolites into the predator. Many octocorals, sponges,

and opisthobranchs contain siliceous or carbonate spicules (Bayer, 1961; Paine, 1963; Bergquist, 1978; Thompson, 1976; Thompson and Brown, 1984), as well as high concentrations of secondary metabolites. The synergistic interaction of secondary metabolites and physical structures may, therefore, be important in the antipredator defenses of a variety of sessile marine invertebrates.

Neosimnia uniplicata was readily accepted by *Lagodon rhomboides* in the laboratory. The thin shell of this gastropod affords physical protection from attack by some predators. Thin-layer chromatography demonstrated that *N. uniplicata* contained some compounds that were also present in *Leptogorgia virgulata*. These compounds did not, however, reduce the initial acceptance of the gastropod by predatory fish. The question of whether *N. uniplicata* actively sequesters the secondary metabolites of *L. virgulata* remains to be answered. *Neosimnia uniplicata* may modify some of the terpenes that it ingests, in a manner that has been suggested for another ovulid gastropod that feeds upon octocorals (Coll et al., 1983). The data obtained in our experiments suggest that *N. uniplicata* is a palatable organism that gains protection by closely mimicking an undesirable prey. A similar refuge against predation has been described for palatable algae that live on gorgonians (Littler et al., 1987).

The crude extract of *Leptogorgia virgulata* and *Neosimnia uniplicata* effectively inhibited settlement by cyprid-stage larvae of the barnacle *Balanus amphitrite* in the laboratory. The crude extract of *L. virgulata* also proved to be effective in the field at inhibiting settlement of fouling organisms. Two of the active antifouling agents from *L. virgulata*, described previously as LV1 and LV2 (Rittschof et al., 1985), proved to be two diterpenes, pukalide and epoxypukalide (P.K. Keifer, unpublished data). These compounds were first isolated from specimens of the alcyonacean octocoral *Sinularia sp.*, which occurs on shallow reefs through the tropical Indo-Pacific (Missakian et al., 1975). Traces of pukalide have also been found in Pacific gorgonians of the genus *Lophogorgia* (Fenical et al., 1981). Pukalide proved to be significantly more active in settlement assays than epoxypukalide. The unsaturated lactone ring of pukalide appears to be important for antifouling activity; epoxidation of this ring may be an initial step in the detoxification of pukalide.

The compound known as "cyprid flypaper" appeared to interact with the integument of the cyprid larvae of *Balanus amphitrite*, preventing them from settling. When cyprids were added to vials containing this compound, they adhered to the walls of the vial and were thus immobilized. Nauplius stage larvae, however, were unaffected. The structure of cyprid flypaper is not yet known.

Pukalide, epoxypukalide, and cyprid flypaper inhibited the settlement of barnacle cyprids without killing the larvae. The mechanism or mechanisms involved in this nontoxic settlement inhibition are not yet understood.

Defense against predators has been a prime target of chemical ecological

research with gorgonians and other octocorals (Stoecker, 1980; Bakus, 1981; Coll and Sammarco, 1983; Gerhart, 1984, 1985). An alternative, although not necessarily mutually exclusive, *raison d'être* for secondary metabolites is the inhibition of overgrowth by competitors (Stoecker, 1978; Targett et al., 1983; Gerhart, 1986). Overgrowth is an important source of mortality for gorgonians (Kinzie, 1974). Pukalide, epoxypukalide, and other secondary metabolites from octocorals (Rittschof et al., 1986; Keifer et al., 1986) can significantly inhibit barnacle settlement at micromolar to nannomolar levels. The antifouling effectiveness of these metabolites suggests that they may have evolved as an adaptation to counter fouling. Much additional research must be performed, however, to demonstrate that these secondary metabolites actually provide *L. virgulata* with antifouling protection in nature. The concentrations of metabolites on the surface of the gorgonian are not known, and the importance of other factors, such as mucous cast-off (Patton, 1972), has not been determined. At present, we can only conclude that pukalide, epoxypukalide, and cyprid flypaper are of potential ecological importance as antifoulants in *L. virgulata*.

Although the ecological roles of pukalide, epoxypukalide, and cyprid flypaper are still debatable, the importance of these agents to marine technology is without question. *Leptogorgia virgulata* and other marine organisms have evolved mechanisms to combat fouling. By identifying the active agents that may contribute to this antifouling activity, marine chemical ecology may provide new, nonlethal alternatives to commercial antifoulants based on toxic metals. Thus, while providing new insight into the basic problem of how secondary metabolites influence overgrowth, marine chemical ecology may also yield new solutions to an age-old problem: marine fouling.

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ENZYMIC ADAPTATIONS IN LEAF-FEEDING INSECTS TO HOST-PLANT ALLELOCHEMICALS

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Abstract—Herbivorous insects have the capacity to develop behavioral, physiological, and biochemical resistance mechanisms in response to chemical selection pressures. Among natural insect-plant associations, there are several cases of target-site insensitivity to and enhanced metabolism of plant allelochemicals. There are also known instances of physiological defenses such as extra rapid excretion or storage of toxic compounds. Multiple defenses seem to be prevalent in natural insect-plant interactions that involve toxic compounds, possibly reflecting the long time these interactions have had to evolve compared to insect-synthetic insecticide interactions. Synthetic insecticides were introduced about 45 years ago. Until recently they have been used as single-active-component preparations. As such, they have been and are very effective in producing insect populations with enhanced detoxification ability and target-site insensitivity. Most insecticide-resistant insect populations have one major defense mechanism. This feature makes the synthetic insecticides very useful tools for studies of insect defenses against toxic chemicals. Information gained from studies with insecticides can shed light on the capabilities of insects to adapt to toxicants in their environment. In assessing the validity of work with synthetic insecticides for natural systems, the fundamental differences between these substances and allelochemicals, and in their presentation to the insects, must, however, be considered. The prevalence of multiple defenses and reliance on modified physiological processes in natural interactions may reflect different properties of the natural chemicals in being generally highly biodegradable and often less acutely toxic than synthetic insecticides. In many cases, the plant allelochemicals are presented to the insects as mixtures. It is, however, to be expected that pest insects will evolve effective multiple defenses against synthetic insecticides. About 20% of all resistant populations have already developed multiple defenses, in most cases combinations of enhanced metabolism and target-site insensitivity. This implies that current crop protection practices need to be modified to ensure the continued usefulness of synthetic insecticides. To

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achieve this, it is important to study intensively not only insect-insecticide interactions but also the interactions operating in natural insect-plant associations.

Key Words—Plant-insect interaction, herbivory, enzymic adaptation, allelochemicals, detoxification.

INTRODUCTION

Plants rely primarily on fitness-reducing and/or antifeedant chemicals as defenses (Fraenkel, 1959; Scriber, 1981; Frazier, 1986) and only occasionally, and perhaps then by chance, do they contain compounds with high acute toxicity (Metcalf, 1977). Synthetic insecticides used for crop protection, on the other hand, are by design highly and acutely toxic. It is to a large extent thanks to studies elucidating the abilities of insects to adapt to insecticides by identifiable resistance mechanisms that their defenses to toxic chemicals in their environment have begun to be appreciated and understood. This is because it is easier to detect the mechanism of an effect that can be traced to a discrete, acute interference than to one which causes gross symptoms that may be derived from many relatively small effects on several or many biochemical systems. Also, funding is easier to obtain for the study of a problem with fairly direct practical implications. However, it is now obvious that studies of natural interactions involving plant allelochemicals are essential. They benefit us in terms of improved ability to predict adaptive responses of the insects to new insecticides, opportunities to model new insecticides on bioactive plant compounds, design of new uses for insecticides on strategies that plants have used successfully for millions of years, and guidance for efforts in breeding or genetically producing resistant crop cultivars. These studies are also essential for understanding basic adaptive and evolutionary mechanisms.

Even though pest insects have adapted with fantastic speed to most synthetic insecticides, the evolutionary "endpoint" is not yet at hand. It is possible to discern evolutionary stages of insecticide resistance development. At first, in some cases very shortly after the introduction of a class of synthetic insecticides, resistance could be ascribed to a single mechanism, most often enhanced ability to detoxify the toxicant (see Brattsten, 1988). Less than 40 years after large-scale synthetic insecticide applications began, more than 20% of resistant pest insects rely on more than one resistance mechanism (Georghiou, 1981). This is, in most cases, accomplished by a combination of enhanced metabolism and target site insensitivity. In contrast, in the large majority of known cases of insects that feed on plants containing toxic allelochemicals, multiple physiological and biochemical defenses are present. It is evident that insects have

many physiological and biochemical options for dealing with toxic chemicals. The lesson from the study of natural systems is the existence in many successfully adapted insects of effective combinations and permutations of discrete defense mechanisms.

The following is a review of the major defense mechanisms employed both against synthetic insecticides and plant allelochemicals. It draws primarily from information obtained in the course of insecticide-resistance studies because, with few exceptions, the underlying mechanism(s) involved in the fate of plant allelochemicals in insects is not identified or characterized in chemical ecology studies.

There are three general categories of defenses to toxic chemicals, namely behavioral adaptations, modified physiological processes, and biochemical resistance mechanisms. Behavioral adaptations and modified physiological processes will be treated very briefly, whereas biochemical defenses, in particular metabolic mechanisms, are emphasized here. This does not imply that metabolism of toxicants is the most important mechanism in each and every case of insect–plant interaction in nature and agricultural fields. There is, however, a good likelihood that metabolic adaptations are of primary importance in the development of the patterns of host utilization by insects.

BEHAVIORAL RESISTANCE

A behavioral defense against a toxic chemical may be conceived as a behavior that allows an insect to feed on a plant despite the presence of toxicants in or on it. There are two outstanding examples of such behavior. One is the trenching by the squash beetle when feeding on cucurbits containing moderate to high concentrations of cucurbitacins (Tallamy, 1985, 1986). The other is the vein-cutting by several insect species adapted to feed on latex-containing plants (Dussourd and Eisner, 1987). In the latter case, the behavior is directed against a mechanical obstacle to feeding (gumming of the mandibles), but the authors point out that it may also block an influx of allelochemicals to the feeding site. It can be conjectured that this sort of defense takes a long time (at least hundreds of years) to be transmitted uniformly throughout a species. It may also depend on non-acutely acting toxicants. Aphids restrict their feeding to phloem, a plant part known to contain only low concentrations of allelochemicals (Mullin, 1986; Dreyer and Campbell, 1987). It is probably common also for chewing insects to feed selectively to minimize allelochemical intake and optimize nutrient intake (Schultz, 1983; Tallamy, 1986), especially in early instars.

Insects exposed to highly toxic, synthetic insecticides have not developed any equivalent defensive behavior, although generally increased irritability and

simple avoidance of treated surfaces have been observed in several cases (Georghiou 1972; Pluthero and Singh, 1984; Gould, 1984). The stalk- or fruit-boring feeding habits of several agricultural pest insects probably evolved in response to nonchemical selection pressures.

PHYSIOLOGICAL RESISTANCE

Adaptations in physiological processes can take the form of extra rapid excretion of ingested toxicants; their sequestration or storage in body compartments, specialized spaces, by enhanced concentrations of binding and storage proteins; or fortification of the integument to slow down the penetration of toxicants. These mechanisms are poorly understood and reflect in each case one or more altered, discrete biochemical functions that have not been identified and characterized. These modified physiological processes may be insignificant by themselves in protecting an insect from poisoning and are probably always combined with other defenses.

There are many known cases of physiological resistance against both synthetic insecticides and plant allelochemicals. For instance, the tobacco hornworm excretes 93% of an ingested dose of nicotine in 2 hr. The housefly, not adapted to feed on nicotine-containing plants, excretes 10% unmetabolized nicotine in 18 hr (Self et al., 1964). The tobacco hornworm has several other defenses against nicotine (Brattsten, 1986). The large milkweed bug and the monarch butterfly are well-documented examples of adapted insects that store cardenolides taken in with their food (Scudder et al., 1986; Brower and Glazier, 1975). Houseflies resistant to DDT can store in their bodies enough DDT to kill susceptible flies (Perry and Hoskins, 1950). The green peach aphid has a protein with carboxylesterase activity that binds insecticides with ester bonds with high affinity but hydrolyzes them very slowly (Devonshire and Moores, 1982) and is, thus, an effective storage protein. The southern and fall armyworms have a similar protein in their fatbodies (Brattsten, 1988). Several insect species including lepidopterans, dipterans, and orthopterans contain hemolymph proteins. These are, characteristically, very large (about 500,000 daltons), have a high content of aromatic amino acids, contain a significant portion of lipids, occur only in immature insects, and reach very high concentrations during the last instar of caterpillars. They may participate in toxin storage, although such a role for them does not seem to have been considered widely (Levenbook, 1985). Lipophorin and arylphorin isolated from caterpillars, including the tobacco hornworm (Shapiro et al., 1984) and the corn earworm (Haunerland and Bowers, 1986a), store certain allelochemicals and insecticides of suitable lipophilicity (Haunerland and Bowers, 1986b).

BIOCHEMICAL RESISTANCE MECHANISMS

Two broad categories of enzymes are involved in insect–plant interactions: target enzymes and digestive enzymes. The receptor proteins that insects use to locate and recognize plants suitable for feeding and/or oviposition are also highly involved but will not be included in this review. The two categories of enzymes are to some extent overlapping; digestive enzymes are inhibited by both plant allelochemicals and synthetic compounds that in a few cases have found practical use as insecticide synergists. The inhibition of a digestive enzyme can be serious enough for survival; the insect may suffer malnutrition, be poisoned by a compound that would normally be detoxified by the inhibited enzyme, or be weakened and so fall an easier prey. But inhibition of a digestive enzyme is never as acutely fatal as inhibition of a so called target enzyme, that is, one which plays a crucial role and without which the insect cannot survive for more than minutes.

Target Site Insensitivity

The nervous system contains many important target enzymes or proteins. Figure 1 shows a simplified sketch of a synapse and an axon and illustrates several specific targets and corresponding inhibitors. In the presynaptic cell, the nerve impulse transmitter acetylcholine is biosynthesized and released; cyclodiene insecticides interfere with these processes (reviewed by Corbett et al., 1984). The acetylcholine receptor located in the postsynaptic membrane is activated by nicotine (Eldefrawi et al., 1970), which cannot be released quickly

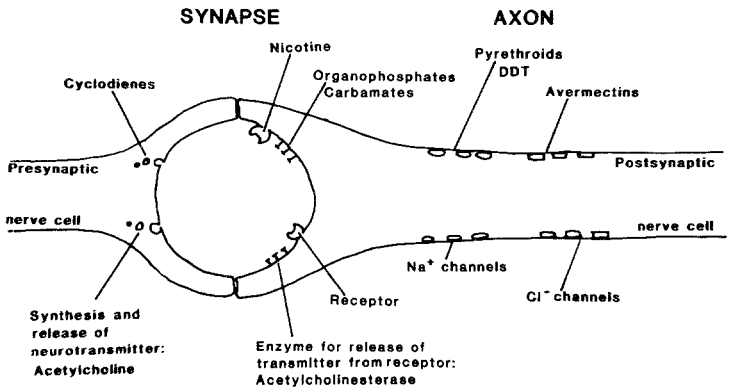

FIG. 1. Scheme of the major factors in nerve impulse transmission and the major inhibitors of each factor. See text for explanation.

enough to restore the membrane resting potential. The crucially important enzyme acetylcholinesterase is also bound to the postsynaptic membrane in close proximity to the acetylcholine receptor; this enzyme releases acetylcholine from the receptor so that the next transmitted impulse can be received. This enzyme is inhibited by organophosphorous and carbamate insecticides (reviewed by Corbett et al., 1984). Beyond the synapse, the nerve impulse is transmitted in the axonal membrane by a rapid wave of successive depolarizations and repolarizations, brought about by influxes and effluxes of ions controlled by the sodium and chloride channels; these processes are inhibited by DDT, pyrethroids, and avermectins (reviewed by Lund, 1985). More than 90% of all commercially used insecticides are aimed at these targets in the nervous system; yet, insects have developed adaptations that render these targets so substantially less sensitive to inhibition that control with synthetic insecticides is often no longer feasible. The only exception is the chloride channel complex, which has not been exposed long or intensively enough.

The sodium–potassium ATPases that catalyze ion transport across cell membranes are the target for inhibition by cardenolides. The monarch caterpillar and the large milkweed bug are good examples of insects that have developed insensitive or less-sensitive ATPases, presumably in response to the presence of cardenolides in their host plants (Vaughan and Jungreis, 1977; Moore and Scudder, 1985).

An insensitive target is the most powerful defense and, being a "built-in" defense (Berenbaum, 1986), does not require the energy expenditure always associated with metabolism. It is, however, probably harder to come by, because it requires a subtle genetic modification of an essential and critical function. This must be slight enough not to impair the normal function, and yet substantial enough to decrease or eliminate binding to the toxicant (Brattsten et al., 1986; Brattsten, 1988). Target-site insensitivity is a widely occurring defense against both plant allelochemicals and synthetic insecticides (Berenbaum, 1986; Brattsten et al., 1986). It affords the most complete protection against acute poisoning, but may not prevent fitness-reducing effects unless combined with other defenses.

Metabolic Defense Mechanisms

It should be clear from the foregoing that metabolism is only one of many possible defenses; in most cases it augments and complements other defenses. Characteristically, a large number of enzymes operate in concert in metabolic defenses.

Two kinds of digestive enzymes are highly involved in all insect–plant interactions: those that metabolize nutrients and those that are specialized to metabolize allelochemicals. Again, these two kinds overlap considerably. Some

of the foreign compound-metabolizing enzymes also participate in the metabolism of endogenous substances, such as hormones and pheromones; and, depending on the toxicant, some of the nutrient-digesting enzymes play important roles in detoxification, e.g., in the case of toxic amino acids.

Metabolism of foreign compounds has traditionally been represented by a scheme (Figure 2) that emphasizes the conversion of lipophilic compounds, which often are highly toxic, to water-soluble metabolites that can easily be excreted. This scheme was designed with drugs and insecticides in mind (Williams, 1974) and applies very well to such chemicals, most of which are lipophilic. Since the enzymes involved in allelochemical metabolism are the same as those metabolizing drugs, and insecticides and the end products of allelochemical metabolism are of the same kind (often water-soluble conjugates) as those of drug or insecticide metabolism, the scheme is convenient for an overview and introduction to the enzymes. However, since many allelochemicals are quite water soluble, several of the enzymes assigned to the so-called secondary or phase II metabolism may be of primary importance in the metabolism of plant allelochemicals, as suggested in Ahmad et al. (1986). Many excellent reviews of the enzymes metabolizing foreign compounds and their properties have been published recently (Agosin, 1985; Ahmad, 1986; Ahmad et al., 1986; Dauterman, 1985; Heyman, 1980; Hodgson, 1983, 1985; Mullin, 1986; Oppenoorth, 1985; Terriere, 1983, 1984; Wilkinson, 1980, 1983, 1984a,b, 1986; Yu, 1986). These should be consulted for details and for information on enzymes not discussed in this paper.

Cytochrome P-450. The cytochrome P-450 system, also known as microsomal monooxygenases or polysubstrate monooxygenases (PSMO), is the most important mechanism for metabolizing lipophilic chemicals. This system, capa-

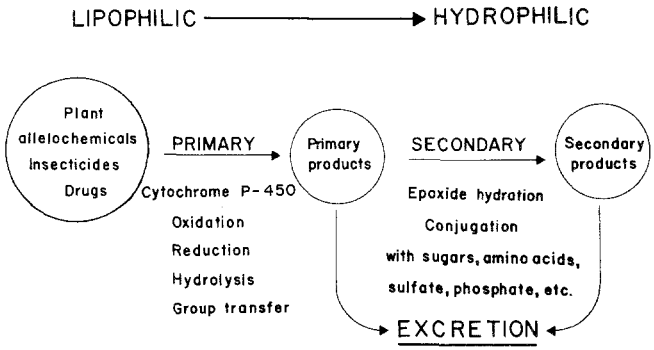

FIG. 2. Schematic representation of the overall events in foreign compound metabolism based on R.T. Williams (1964). Reprinted with permission from Plenum Publishing Corporation.

ble of attacking something as highly lipophilic as a polycyclic aromatic hydrocarbon, also accepts as substrates relatively water-soluble compounds such as the organophosphate insecticides, as long as they contain a lipophilic site to which the enzyme can bind. By virtue of its reaction mechanism, essentially a combination of a lipophilic substrate binding interaction and an oxygen free radical attack, cytochrome P-450 can attack molecules with widely varying structures. Also, a suitable molecule can be attacked at several different molecular sites or appear to undergo several different reactions, including carbon hydroxylations, *N*- and *O*-dealkylations, or epoxidations (see Ahmad et al., 1986), all of which are monooxygenations. Figure 3 illustrates sites at which cytochrome P-450 can attack several allelochemicals.

The versatility in accepting many different compounds as substrates is enhanced by the occurrence of several different molecular forms of cytochrome P-450 with broadly overlapping properties. Some of these forms are constitutive and, possibly, characteristic of tissues or even of cells. Others may appear as a result of induction by foreign chemicals (see later). The composition of cytochrome P-450 molecular species has been elucidated only in rat and rabbit liver

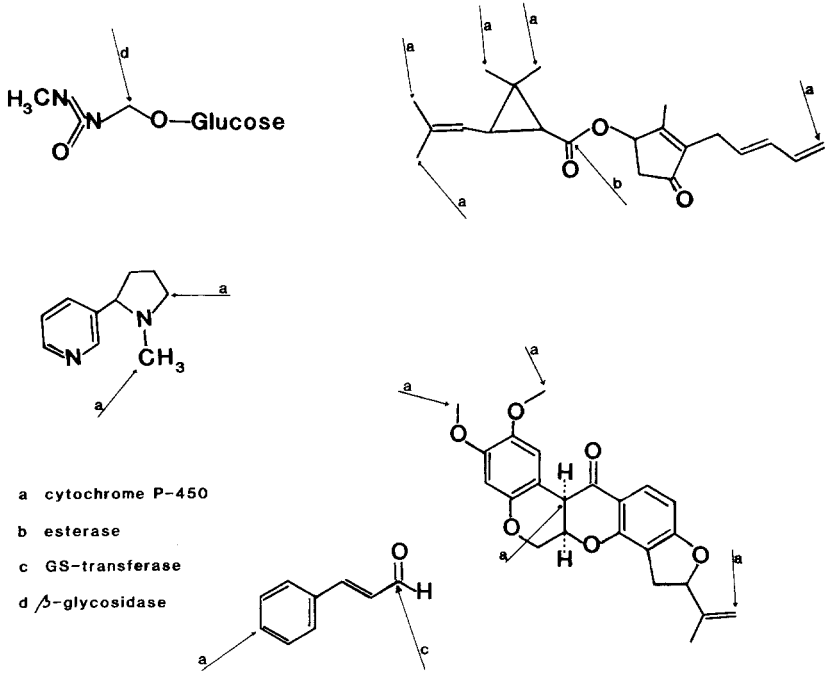

FIG. 3. Sites of enzymatic attack on selected plant allelochemicals.

(Ryan et al., 1982; Coon and Persson, 1980). It has not yet been possible to do similarly detailed studies in insects.

To fully appreciate the effectiveness of metabolic defenses, we must remember that cytochrome P-450 does not operate in splendid isolation. There are several other enzymes located in close proximity to the cytochrome that also attack incoming foreign molecules at the same time or within milliseconds of the cytochrome P-450 action. Also, other enzymes use cytochrome P-450 metabolites as substrates, forming short metabolic pathways (Mullin, 1985, 1988) that may lead to conjugated end products. The relatively more water-soluble molecules are more likely to be attacked by several enzyme simultaneously. For instance, cytochrome P-450 is the only enzyme known to attack rotenone, a lipophilic substance, whereas certain organophosphorous insecticides can be attacked by carboxylesterases and glutathione transferases in addition to cytochrome P-450.

This concerted action is the more important since primary metabolites are, in many cases, more reactive and, therefore, potentially more toxic than the parent molecule. The epoxide metabolite of precocene II is a good example of a toxic cytochrome P-450 metabolite that alkylates and thereby destroys macromolecules. When it is formed in the corpora allata, the gland is destroyed because of absence of other enzymes to detoxify the epoxide (Ellis-Pratt, 1983). However, precocene II does not necessarily fatally damage other tissues that have epoxide hydrolases or glutathione transferases ready to convert the epoxide to a hydroxylated or conjugated product. Molecular form(s) of cytochrome P-450 in fatbodies of European corn borer larvae oxidize precocene II to several metabolites in addition to the epoxide (Soderlund et al., 1980), the net outcome of which may be detoxification.

Transferases. Group transfer enzymes catalyze the conjugation of a foreign compound or its metabolite to a small, highly water-soluble endogenous group by a covalent bond. In most cases, a high-energy intermediate is a required cofactor or cosubstrate (Williams, 1974; Caldwell, 1982; Dauterman, 1985; Wilkinson, 1986; Ahmad et al., 1986). With exceptions, conjugations usually result in detoxification (Paulson, 1982). The glutathione transferases are probably the most important conjugating enzymes in animals, rivaled only by the hexose transferases. Other transferases may be very important in special cases.

Glutathione Transferases. Unlike cytochrome P-450, which is embedded in the membranes of the endoplasmic reticulum, the glutathione transferases (GSTs) are floating in the soluble fraction of the cells. Even though all the molecular forms of GSTs prefer lipophilic substrates, their subcellular location helps them to attack considerably water-soluble molecules also. The GSTs are less versatile than cytochrome P-450 and accept as substrates compounds with an electrophilic center, such as α,β-unsaturated carbonyl compounds, electronically mimicked by some of the organophosphate insecticides, thiocyanates, and

isothiocyanates (Scheline, 1978). Many plant allelochemicals satisfy these substrate requirements, including cinnamaldehyde, other unsaturated aliphatic aldehydes, and the glucosinolates. GSTs from midguts of the fall armyworm metabolize several unsaturated carbonyl allelochemicals and allyl and benzyl isothiocyanate in vitro (Wadleigh and Yu, 1987). The GSTs also play an important role in conjugating epoxyhydrocarbons (Boyland and Williams, 1965; Fjellstedt et al., 1973), such as may result from cytochrome P-450 catalyzed epoxidation of olefinic allelochemicals.

Insect GSTs have received considerable attention because of their involvement in the detoxification of organophosphorous insecticides (Oppenoorth et al., 1972; Levine and Murphy, 1977; Motoyama and Dauterman, 1980). They also detoxify lindane and other halogenated aromatic and aliphatic hydrocarbon compounds (Cohen et al., 1964; Menguelle et al., 1985; Clark et al., 1986). In houseflies, a GST form catalyzes the dehydrochlorination of DDT (Clark and Shaaman, 1984) and is identical to the enzyme previously called DDT dehydrochlorinase; dehydrochlorination to DDE is, in many insects, the major metabolic fate of DDT and represents one important resistance mechanism to DDT.

The GSTs are multifunctional proteins with catalytic activity to combine the foreign compound or its primary metabolite with reduced glutathione (GSH) by a covalent bond. They are, however, also important as storage proteins for foreign compounds or their metabolites and function in this capacity as soluble binding proteins. As enzymes, the GSTs require and are, except in some plants, specific for GSH. In many cases, the foreign compound combines spontaneously with GSH, which occurs at millimolar concentrations in some cells, e.g., rat liver cells. The role of the GST enzymes is thought to be primarily to assure close proximity and orientation of the reactive centers of the foreign compound and the activated thiolate ion, GS^-, to facilitate the reaction between them (Jakoby and Habig, 1980). In the case of DDT dehydrochlorination, GSH is used only in catalytic amounts by a reaction thought to encompass an E2 elimination with an initial abstraction of a benzylic hydrogen followed by the removal of one of the chlorine atoms (Wilkinson, 1986).

GST activities have been isolated and purified from a few insect species. Four forms with similar properties and overlapping substrate preferences were found in fatbodies of the American cockroach (Usui et al., 1977). Three forms, functionally somewhat different, were isolated from houseflies (Clark et al., 1985). Four forms were found in porina moth (*Wiseana cervinata*) larvae (Clark and Drake, 1984). Common laboratory animals (rats, mice, etc.), as a rule, have multiple GST forms (Jakoby and Habig, 1980). However, only one form could be isolated from larvae of the greater wax moth (Chang et al., 1981). GST activity towards commonly used model substrates have been recorded from several insect species (see Ahmad et al., 1986, for review).

The ultimate fate of glutathione conjugates in mammals is excretion as a mercapturic acid derivative formed by subsequent enzymatic cleavage of the tripeptide (Ahmad et al., 1986). Practically nothing is known about the enzymes in insects that catalyze these subsequent reactions, and there are very few demonstrated cases of excretion of mercapturic acid derivatives in insects; 2,4-dinitrophenyl mercapturic acid is excreted by the American cockroach (Dykstra and Dauterman, 1978) and the housefly (Abd-Elraof and Dauterman, 1981).

Hexose Transferases. Glucose is the sugar most commonly used for conjugation. It is activated by linkage to uridine diphosphate (UDP) via a high-energy phosphoester bond. Insects use the activated glucose directly and form *O*-glucosides in most cases. In mammals, the extra step of oxidizing the glucose to glucuronic acid, which is activated to UDP-glucuronic acid, is inserted in the enzymatic conjugation sequence for reasons that are not obvious; in consequence, mammals make predominantly glucuronide conjugates. Plants use a wide variety of sugars to make glycosides. Although all sugar conjugates are easily excretable, glucosides are in some cases also a storage form in insects. The cyanogenic glycosides biosynthesized by some insects are good examples (Davis and Nahrstedt, 1985). Storing the toxicant as an inactive glycoside enables the insects to use it as a predator deterrent. The UDP-glucosyl transferases are likely to be important in insect–plant interactions, and many insects excrete glucose conjugates of insecticide metabolites; however, the enzymes have not been extensively studied in insects.

Hydrolases. The hydrolases of general importance in foreign compound metabolism include the esterases, the epoxide hydrolases, the enzymes that hydrolyze glycosidic and thioglycosidic bonds (glycosidases and glucosinolases), and a few hydrolytic enzymes that are important in special cases.

Among the esterases, the carboxylesterases are intensively studied in insects (see Ahmad et al., 1986) due to their importance in insecticide and juvenile hormone metabolism and in nerve impulse transmission mediated by the acetylcholinesterases; they may also participate in the digestion of nutrients and in other areas of endogenous metabolism, although in these cases their precise roles are not known. Enhanced carboxylesterase activity is a major resistance mechanism against synthetic insecticides. Apart from their importance in insecticide and juvenile hormone metabolism, very little is known about insect carboxylesterases or their participation in the metabolism of plant allelochemicals. Atropine, cocaine, reserpine, other drugs, and plant-derived esters are hydrolyzed by many different mammalian tissues (Scheline, 1978).

Carboxylesterases, like other hydrolases, cleave a covalent bond with the aid of a molecule of water and without any cofactorial energy requirement. These enzymes only attack molecules containing an ester bond, although some carboxylesterases also hydrolyze phosphoester and amide bonds. There are many

different forms of carboxylesterases, some of which are soluble, whereas others are bound to the endoplasmic reticulum of cells. Substrate specificities seem to be quite variable between different carboxylesterase forms. A form in the midgut of the soybean looper hydrolyzes *trans*-permethrin but not naphthyl or nitrophenyl esters (Dowd and Sparks, 1986), whereas in the green peach aphid, the E4 form hydrolyzes pyrethroids, organophosphates, and carbamates with a very broad substrate specificity (Devonshire and Moores, 1982). In some cases substrate specificities can be widely overlapping between forms; the cattle tick has as many as 15 forms, all of which hydrolyze 1-naphthyl acetate (DeJersey et al., 1985), and the cabbage looper has at least two forms that hydrolyze pyrethroids (Ishaaya and Casida, 1980).

Epoxide hydrolases are the subject of another paper in this issue (Mullin, 1988) and, therefore, will not be reviewed here.

Glycosidases hydrolyzing sugars that are β-bonded to allelochemical aglycones have not been studied widely in insects. Sugar-cleaving enzymes have been studied in most detail in wood-boring insects because of their role in nutrition. The harvester termite has a β-glycosidase in the gut tissues which digests cellulose to cellotriose (Potts and Hewitt, 1974). Larvae of *Rhagium inquisitor* contain many α- and β-glycosidases (Chipoulet and Chararas, 1985). In the case of larvae of *Sirex cyaneus*, the sugar-hydrolyzing activity was shown to reside in the tissues of fungal symbionts ingested along with the wood (Kukor and Martin, 1983). β-Glycosidase activity related to the metabolism of foreign compounds has been implied in a few insect species including the migratory locust, a water boatman, a mealworm, the American cockroach, the bean aphid (Robinson, 1956), and in larvae of *Seirarctia echo* (Teas, 1967).

Since many, if not most, aglycones are more toxic than the glycoside, it is unclear how specific β-glycosidases could evolve in insects. If the insect is adapted to a glycoside-containing host plant, the selection pressure would favor deletion of the activity; if the insect is a generalist feeder, it may retain nonspecific glycosidases, provided that it is well equipped with other enzymes to metabolize the aglycone. In either case, it would be safest not to have the activity. Besides plants, bacteria and other microorganisms are well known to have β-glycosidase activities (Scheline, 1978; Goldman, 1982); it is possible that the activity observed in the insects derived from their gut microorganisms. Although microbes are extensively and intimately associated with insects (Jones, 1984), the extent to which they participate in foreign-compound metabolism in insects is not well known.

Factors Affecting Enzyme Activities

Enzymes specialized to metabolize compounds that may not always be present are likely to occur in large amounts, represented by high activities, only

when they are needed. The biosynthetic energy cost in maintaining a high amount of constantly turning-over enzyme proteins may be considerable. The regulation of such enzymes may thus be susceptible to external factors. However, the activities of these enzymes are probably also regulated by internal, hormonal factors. Measurement of cytochrome P-450 activities in different life stages and larval instars, in male and female insects, and in different tissues, e.g., guts and fatbodies, show inherent differences in activities that cannot be ascribed to external factors (Krieger and Wilkinson, 1969; Wilkinson and Brattsten, 1972; Benke et al., 1972; Brattsten et al., 1980; Feyereisen and Farnsworth, 1985; Ahmad, 1986; Ahmad et al., 1986). There are also differences in response to external factors, especially between tissues.

It is well known that the foreign-compound-metabolizing enzyme activities are modulated temporarily by ingested chemicals, including plant allelochemicals. Many such compounds induce or inhibit cytochrome P-450, glutathione transferase, epoxide hydrolase, and carboxylesterase activities (Terriere, 1983, 1984; Yu, 1986; Brattsten, 1988).

Induction, in which enzyme activities are elevated only as long as a sufficient concentration of the inducing chemical remains in the insect tissues and then return to "normal" levels (Brattsten and Wilkinson, 1973), is well documented in herbivorous insects, most of which have happened to be generalist feeders. The velvetbean caterpillar and the boll weevil are the only specialist or adapted feeders studied in any detail so far. Both species have higher activities of cytochrome P-450 and glutathione transferases after the insects have fed on diets containing inducers (Yu, 1986; Wadleigh and Yu, 1987; Brattsten, 1987).

The examples we have so far do not show clearly the extent to which induction is important in natural insect–plant interactions. Even though the enzymes in both specialists and generalists are inducible, induction may be more important in generalist feeders. These, after having depleted one food source, may, on short notice (hours or minutes), have to "adapt" to a completely different allelochemical load of a new food source. A specialist would not be faced with this biochemical challenge, having accepted a food source on the basis of taste receptor inputs. A specialist may have "permanent" activity levels that reflect the allelochemical content of its host plant. For example, many species of caterpillars that feed on plants rich in terpenoids have very high cytochrome P-450 activities (Rose, 1987). Terpenoids are generally good inducers of cytochrome P-450 activities. Black swallowtail caterpillars have high cytochrome P-450 activities (Brattsten, 1979; Bull et al., 1984), perhaps attributable to the allelochemical content of their umbelliferous host plants. On the other hand, a specialist may have permanently depressed activities if its host plant contains inhibitory allelochemicals. For instance, the larvae of *Utetheisa*

bella have very low cytochrome P-450 activities, perhaps inhibited by pyrrolizidine alkaloids in their *Crotalaria* host plants (Brattsten, 1979, 1986).

IMPLICATIONS OF INSECT DEFENSES AGAINST TOXICANTS

Generally valid statements about insect survival on plants that contain toxic allelochemicals are very difficult, or even impossible, to make. This could be a function of our limited stock of thoroughly investigated and well-documented cases from which to derive patterns and principles. It could also reflect a reality of almost limitless combinations of discrete adaptive mechanisms optimized for each individual interaction in time and space. The foregoing, brief discussion of some of the major defense mechanisms should have shown the multitude of possibilities that insects have available to them.

Possession of defenses of very impressive variety and biochemical sophistication has obvious implications for the distribution of insects on plants in the patterns observable today. It also has implications for how they may be controlled in agroecosystems by methods that are economically and socially acceptable and pose a minimal risk to the environment. It is likely that methods for insect control to meet these criteria will have to be highly specific for each combination of factors constituting the agroecosystem in need of protection.

It is difficult or impossible to reconstruct the evolutionary, biochemical, and behavioral events that led to association of a certain insect species with a certain plant species. It may, however, be possible to complement our understanding of the current associations of insects with certain plants but not with others by improved understanding of how their defensive enzymes operate. For instance, the southern armyworm, regarded as a generalist feeder, clearly prefers to feed on certain cyanogenic plants such as the lima bean (SooHoo and Fraenkel, 1966; Brattsten et al., 1983). It has high activity of the enzyme β-cyanoalanine synthase (Brattsten, unpublished), which is known to catalyze the first step in the series of biotransformations that incorporate cyanide into the amino acid pool, detoxifying it in the process (see Davis and Nahrstedt, 1985; Brattsten, 1986, for recent reviews). The southern armyworm caterpillars also have rhodanese activity (Long and Brattsten, 1982), although this enzyme is probably of minor, if any, importance in cyanide detoxification (Brattsten, 1986). On the other hand, the southern armyworm has never been documented feeding on mint plants in nature. The terpene content of mints may deter or kill early instars but is not deterrent, at concentrations occurring in plants, to last instars (Gunderson et al., 1985). The armyworm midgut cytochrome P-450 that metabolizes one of the monoterpenes, pulegone, is induced by another monoterpene, α-pinene, to a molecular form that hydroxylates pulegone to a precursor of menthofuran, more toxic than pulegone (Trammell, 1982; Gunderson et

al., 1986). This could contribute to the absence of mints among southern armyworm host plants.

In the case of insects that store toxic compounds, it is interesting to speculate whether a target site insensitivity was the prerequisite for the development of the ability to store the toxicant (Berenbaum, 1986). Once stored, the insect could take advantage of the toxicant in defense against predators. Alternatively, the ability to store was a genuine but, perhaps imperfect, defense against the toxicant. The latter case then conferred two potentials: it provided the insect with the opportunity to develop an insensitive target site (a risky proposition and undoubtedly costly in terms of individuals) (Brattsten, 1988) and the sequestered toxicant could be exapted (Gould and Vrba, 1982) as defense against predators.

Synthetic insecticides have played an important role in food production and in the combat against insect-vectored diseases such as malaria. They have also caused many serious problems with environmental pollution, destruction of nontarget species and, in some cases, direct or indirect adverse health effects. The chemicals per se are effective in doing what they were designed to do and have contributed significantly to the production of affordable commodities. Their excessive and haphazard use is the major cause of their associated problems. Since it is no longer easy, scientifically or economically, to invent and develop new insecticides, it is important that the ones we have be used so as to preserve their usefulness, in particular by avoiding resistance problems. Their incorporation in control strategies that also rely on available cultivation and biological control methods, i.e., in integrated pest management programs (IPMs), is the most promising way to take advantage of their benefits and minimize their undesirable side effects (Georghiou, 1983).

There is currently a large effort to develop pest-resistant crop plants, either by conventional plant breeding programs or by genetic engineering. This approach holds considerable promise and will likely result in some commercial successes within the decade. However, endowing crop plants with genetic capacities to biosynthesize their own chemical defenses against insects is really only a different way of exposing insects to toxic chemicals. It will be important to have on hand a number of different cultivars with the ability to biosynthesize different defensive chemicals with different modes of action before the use of any one cultivar starts, so that the insect population can be exposed to multiple chemicals. If not, the insects will soon adapt to the cultivars, one after the other, just as they do when chemicals are sprayed on fields. The most effective chemical plant defenses are the so-called inducible defenses, such as the injury-induced increase in monoterpene production in pine trees in response to invading pine beetles (Raffa, 1986). Insects in nature and in model systems have the most difficulty in adapting to unpredictable and multiple toxicants (Dolinger et al., 1973; Pimentel and Bellotti, 1976). Other potential problems with resistant

crop plants, such as residues, will be similar to those of conventional insecticide spray use; these problems (e.g., toxic hay) may be lessened by the tendency of plant allelochemicals to be highly biodegradable, unlike many synthetic chemicals.

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BIOASSAYS OF SEGREGATING PLANTS
A Strategy for Studying Chemical Defenses

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Abstract—*Solanum chacoense* is a wild potato species resistant to the Colorado potato beetle, *Leptinotarsa decemlineata*. Most genotypes of *S. chacoense* synthesize the glycoalkaloids solanine (sol) and chaconine (chac) and are hosts of the beetle. A few rare genotypes have a gene(s) for acetylation of carbon-23 of the steroid aglycone of sol and chac. Laboratory bioassays and replicated field tests of clones differing in the presence or absence of the acetyl moiety showed that acetylation of sol and chac markedly affects the response of both adults and larvae to the foliage. Adult feeding deterrency conferred by acetylated forms of sol and chac (leptines) in leaf-disk preference tests was consistent with the degree of antixenosis measured in the field. Development of larvae on foliage of clones with leptines was also inhibited. The studies support the validity of using laboratory bioassays of plants segregating for levels of a suspected defense compound to determine the role the compound has in defending the plant from attack by an insect predator in the field.

Key Words—Glycoalkaloids, insect resistance, Colorado potato beetle, leptines, solanine, chaconine, *Leptinotarsa decemlineata*, *Solanum chacoense*, *Solanum tuberosum*.

INTRODUCTION

Knowledge of chemical defense against insect pests may be useful in developing resistant cultivars of crop plants. The breeding process could certainly become more efficient if the nature of a crop's natural defenses were understood and assessments of resistance could be made in the laboratory or greenhouse at early growth stages using reliable biological or chemical assays. Laboratory

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assays of breeding populations will, of course, only be useful to the plant breeder if they are valid measures of resistance and are highly correlated with field results.

Investigating chemical defense by isolating compounds and testing their effects on pests with in vitro bioassays can be inefficient and sometimes ineffective. Assays for chemical composition differences among plants segregating for resistance, an often overlooked alternative, can be a more efficient and sometimes the only effective strategy for identifying defense compounds. David Jones et al. (see Rosenthal, 1986) used this approach in a series of ingenious experiments that established the role of cyanogenic glycosides in the defense of bird's foot trefoil and white clover against slugs and snails. Segregates producing both cyanogenic glycosides and the enzymes to liberate cyanide when the leaves are attacked are invariably more resistant to damage than segregates lacking either the cyanogenic glycosides, the enzymes, or both.

GLYCOALKALOIDS AND RESISTANCE

Chemical assays of segregating plants were used first to identify and then establish the role of nitrogen-containing steroid glycosides (glycoalkaloids) in the defense of wild Solanaceous species against the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say. Using field tests and bioassays of detached foliage, European breeders and entomologists found wide segregation for CPB resistance among wild potato and tomato species in the 1930s and 1940s. Chemical defense was suspected because of the strong aversion of larvae and adults to the foliage of highly resistant plants. Analyses for possible allelochemicals revealed the presence of higher glycoalkaloid levels in foliage from resistant plants (see Dimock and Tingey, 1985; Tingey, 1984; Bongers, 1970, for reviews and references).

The apparently simple correlation between glycoalkaloid concentration and level of resistance became somewhat clouded, however, as new chromatographic and spectrophotometric methods of determining glycoalkaloid composition came into use. With the new analytical methods, new glycoalkaloids were discovered in some potato species previously thought to contain only a single glycoalkaloid, solanine (sol).

Some resistant species had very high levels of sol and chaconine (chac), the glycoalkaloid pair (Figure 1) found in low levels in susceptible *Solanum tuberosum* L. Some had high concentrations of tomatine (tom), the glycoalkaloid in tomatoes. Other resistant species had moderate to high concentrations of new glycoalkaloids, demissine and leptines.

Large differences among the glycoalkaloid forms in their CPB feeding-deterrency activity were discovered when *S. tuberosum* foliage infiltrated with

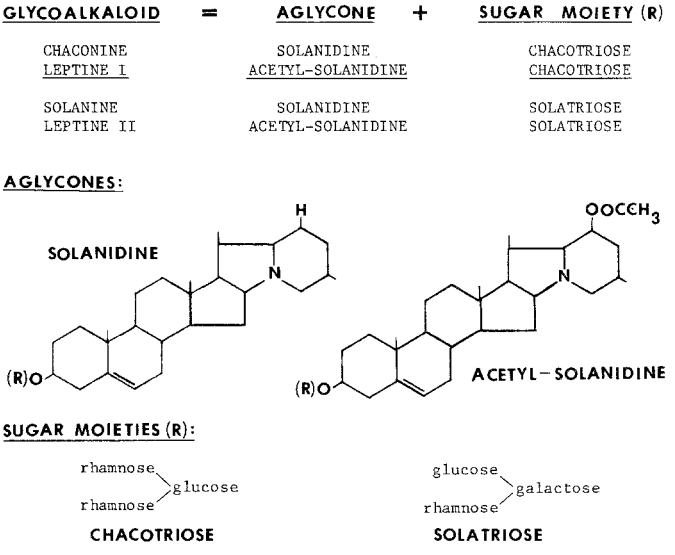

FIG. 1. Structures of leptines I and II, chaconine and solanine.

graded concentrations of individual glycoalkaloids was fed to CPB in laboratory bioassays. Of seven different glycoalkaloids tested against adult CPB (Sturckow and Low, 1961), leptine I and leptine III were by far the most potent (Figure 2). Similar wide differences in activity among glycoalkaloid forms were found in bioassays for effects on larval development.

Almost all studies of the response of CPB adults and larvae to glycoalkaloids suggest feeding inhibition as the primary and perhaps only effect of the different glycoalkaloids. How CPB sense the presence and concentration of a particular glycoalkaloid is not fully understood. Mitchell and Harrison (1985) showed that both tarsal and galleal sensilla respond with bursting activity to tom, sol, and chac at concentrations well within the range of those found in plant foliage. There is, however, no difference in threshold levels for tom and sol, even though tom is about fivefold more potent than sol in deterring adult feeding.

LEPTINES

Leptines I and II are acetylated forms of chac and sol, which are the two glycoalkaloids found in *S. tuberosum* and most other potato species. Potent leptine I (Figure 2) is identical in structure to weakly deterrent chac, except for the substitution of an acetyl on carbon-23 of the steroid aglycone (Figure 1).

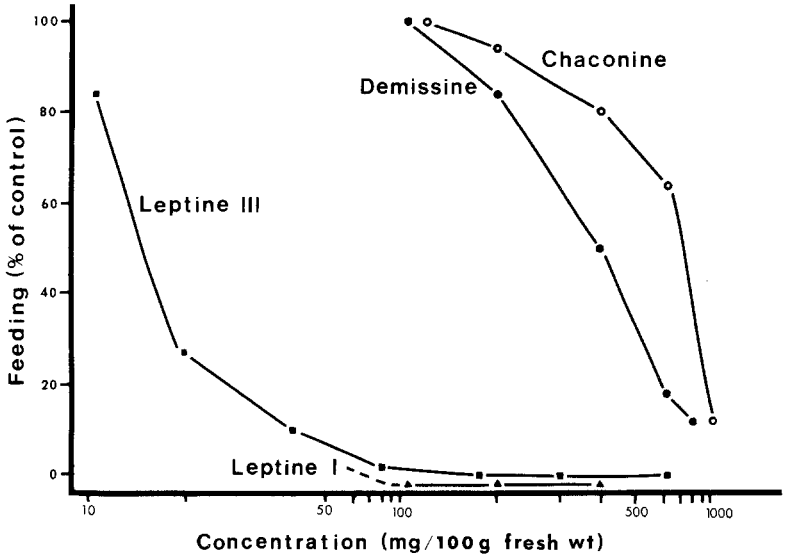

FIG. 2. Feeding rates of Colorado potato beetle on potato leaf disks infiltrated with acetylated (leptines I and III) and nonacetylated forms of chaconine and demissine. Redrawn from Sturckow and Low (1961).

Leptine II is the C-23-acetylated analog of sol. Two other leptines, III and IV, have been detected but not chemically characterized (Kuhn and Low, 1961). Leptine III is probably the acetylated analog of demissine and leptine IV the acetylated analog of commersonine. Foliage from resistant F_2 *S. chacoense* Bitter segregates that synthesized demissine and commersonine also synthesized small amounts of acetylated forms of the two glycoalkaloids (Sinden et al., 1980).

The biosynthetic pathway leading to C-23 acetylation is not known. However, microsomal fractions derived from leaves of leptine-synthesizing plants convert [^{14}C]solanidine to 23-OH-solanidine in the presence of NADPH (Osman et al., 1987). Acetylation of 23-OH-solanidine, catalyzed by an esterase synthetase, might then easily occur in intact cells. Acetylation was not, however, detected in cell-free incubations.

Leptines are difficult to isolate and purify because they are somewhat soluble in ammonia and because they are found in mixtures containing 6–12 major glycoalkaloids. Extracts containing an acetylated form of a particular glycoalkaloid always also contain 23-OH-substituted and -unsubstituted forms of the glycoalkaloid. Kuhn and Low (1961) isolated leptines I and III from a complex glycoalkaloid mixture containing major or minor amounts of at least 12 different

glycoalkaloids, a remarkable accomplishment at that stage of development of separation chemistry.

Because of the difficulties in isolating them, only two of the four known leptines have been tested for activity against CPB in infiltration bioassays. The threshold level for activity of infiltrated leptine I against CPB is not known because the concentrations that could be tested with adults (Figure 2) and larvae were limited by scarcity of the compound. Leptine III has not been tested for activity against larvae, and leptine II has not been tested against either adults or larvae. Even though leptines are not synthesized in tubers (Sinden et al., 1986a), their mammalian toxicity should probably be tested before they are introduced into the foliage of a food crop. Obviously, there is a need for further leptine isolations to provide purified compounds for additional tests of their effects on insects and other animals. In spite of the limited testing of leptines for activity against CPB, it is clear from the available data that infiltrated leptines I and III are at least five- to tenfold more potent in deterring adult feeding than their nonacetylated analogs. Acetylation of carbon-23 apparently converts weak or impotent glycoalkaloids such as demissine and chaconine into potent feeding deterrents (Figure 2).

BREEDING FOR RESISTANCE

Breeding for CPB resistance in potatoes began in Europe in the 1940s, before chemical defense by glycoalkaloids was known. Resistant hybrids were obtained (Torka, 1950) but resistance levels rapidly declined in backcross generations. Breeding for CPB resistance in Western Europe was discontinued when effective pesticide control became available in the 1950s, before the information on chemical defense could be tested for possible application in breeding. There is now new information on host selection by CPB (Bongers, 1980), new information on potato genetics and glycoalkaloid inheritance (Sanford and Sinden, 1972), and new sources of resistance (Dimock et al., 1986). Chemical and bioassay techniques have been improved, and there are new means of transferring genes between species and genera. Therefore, breeding for CPB resistance may now become more efficient.

Dimock and Tingey (1985) suggested using knowledge of chemical defense in a new approach to CPB resistance breeding, i.e., selecting parents and progeny on the basis of specific glycoalkaloids, rather than total glycoalkaloid concentration, should be more efficient because the inheritance of individual glycoalkaloids is likely to be less complex and assays for specific compounds less variable. Selection for more potent glycoalkaloid forms in *S. tuberosum* hybrids rather than for higher levels of weak deterrents such as sol and chac could also help in maintaining tuber glycoalkaloid concentration at present safe

levels (Sanford and Sinden, 1972). We are currently testing this approach with assays of hybrids between *S. tuberosum* and *S. chacoense* selected for leptine contents.

The remarkably potent leptines are found in only a few rare *S. chacoense* accessions (Sinden et al., 1986a) among more than 200 *Solanum* species analyzed for glycoalkaloid composition (Schreiber, 1968). Most of the 60 *S. chacoense* accessions we analyzed for leptines did not contain plants that synthesized leptines at levels high enough to detect with sensitive gas chromatographic methods. Both nonleptine and high-leptine genotypes were, however, sometimes found in the same accession, e.g., 458313-4 and 458313-1 from accession 458313 (Table 1).

S. chacoense is highly polymorphic for both resistance level and glycoalkaloids. Resistance levels in the species range from susceptibility comparable to that of host *S. tuberosum* to nonhost immunity (Torka, 1950), apparently because of the wide segregation for glycoalkaloid levels and structural forms in the species (McCollum and Sinden, 1978). Most *S. chacoense* genotypes synthesize the nonacetylated *S. tuberosum* glycoalkaloids, sol and chac. Both the leptine and nonleptine sibs we selected within individual accessions (sib-clones), and clonally propagated for our study of leptines and resistance, synthesize sol and chac. The sib-clones differ, however, in the portion of the total glycoalkaloid that is acetylated. In foliage of the leptine clones, a major portion of the total glycoalkaloid is present as acetylated forms of sol and chac, i.e., leptines I and II (Table 1 and Figure 1).

The wide segregation for leptine synthesis among *S. chacoense* genotypes made it possible to verify the major role for leptines I and II in the chemical defense of the species. Laboratory bioassays and field measurements of the resistance of three pairs of sib-clones selected within accessions for wide differences in leptine level between the two clones in each pair (Table 1) confirmed the high potency for infiltrated leptines found in leaf-disk bioassays (Figure 2) by Sturckow and Low (1961). The close relationship between the field resistance and endogenous leptine level in the clones clearly demonstrates the important role of leptines in the chemical defense of *S. chacoense* plants in the field. The sib-clone that synthesized primarily sol and chac, but little or no leptine, was invariably more susceptible than the leptine-synthesizing sib within the pairs in both laboratory bioassays and field tests. The clone that synthesized a relatively low level of sol and chac (320287-2, Table 1), and no leptines, was nearly as susceptible as *S. tuberosum* (Sinden et al., 1986b), whereas the three clones that synthesized high levels of acetylated forms of chac and sol (leptines I and II) were nearly immune.

Nonleptine and leptine synthesizing clones from the same accession, e.g., sib-clones 458313-4 and 458313-1, have similar total glycoalkaloid levels. The sib-clones from a single accession are also morphologically similar. Since gly-

TABLE 1. RESPONSE OF COLORADO POTATO BEETLE TO FOLIAR LEPTINES IN SEGREGATES OF *S. chacoense*.[a]

	Glycoalkaloids (mg/100 g fresh wt)		Field resistance		Laboratory bioassays	
Clone	Leptines	Solanine + chaconine	Adults (No./plant)	Plant damage (rating)[b]	Adult feeding rate (mg/hr/adult)	Larval survival (%)[c]
320287-2	0	167	38	4.4	5.6	53
320287-1	120	11	10	2.4	1.6	5
458310-3	51	253	14	2.1	1.8	35
458310-1	271	69	4	0.6	1.1	3
458313-4	0	704	14	3.0	2.3	48
458313-1	306	243	2	0.5	1.2	8

[a] Modified from Sinden et al. (1986b).
[b] Damage ratings of 0 (no visible damage) to 5.0 (complete defoliation).
[c] Percent of larvae surviving to adult eclosion.

coalkaloid content is the only factor in *S. chacoense* that has been associated with CPB resistance, the differences in resistance between sib-clones from a single accession are probably caused by differences in the forms of glycoalkaloid (primarily sol + chac in nonleptine sibs vs. primarily leptines I + II in leptine sibs), and not by total glycoalkaloid levels or differences in morphology.

Total glycoalkaloid level, regardless of glycoalkaloid forms, is also an important determinant of resistance in *S. chacoense* (Sinden et al., 1980) and other *Solanum* species (see Tingey, 1984). Although sol and chac are only weak deterrents of adult feeding (Figure 2) and may not affect larval development even at concentrations as high as 900 mg/100 g fresh weight (Hsiao, 1974), very high concentrations of sol and chac can confer considerable resistance to adult feeding, e.g., clone 458313-4 vs. 320287-2 (Table 1).

MEASURING RESISTANCE

Field testing for CPB resistance in large breeding populations is impractical, primarily because of the wide variation in density of infestations and resulting CPB damage. It may be prudent to select for moderate resistance levels resulting from feeding deterrents in the foliage that cause less adult feeding, slower larval development, and reduced fecundity. These subtle effects may be important in integrated pest management programs and less likely to be rapidly overcome by CPB. To measure such resistance levels in field tests would require extensive replication. Replication such as we had in field tests of the *S. chacoense* segregates (Sinden et al., 1986b) is usually not possible in early breeding generations when few seed tubers of individual segregates are available.

In assaying *S. chacoense* segregates, we measured the effects of leptine level on three components of field resistance using field infestation tests and laboratory bioassays: (1) antixenosis of plants in the field, (2) adult feeding deterrency and, (3) inhibition of larval development. We were also searching for efficient laboratory bioassays well correlated with field results that might be effective in screening large numbers of progeny in early breeding generations. Since we expect lower resistance levels in interspecific hybrids than in *S. chacoense*, bioassays sensitive enough to detect small differences in resistance level are needed.

Antixenosis was measured by placing adult CPB between rows of test plants in the field and recording the number of adults per plant the following day. Recording of plant damage severity (Table 1) also measured antixenosis, but probably measured the combined effects of adult attraction and feeding deterrency. Differences in feeding deterrency caused by endogenous contents of leptines were easily measured in a two-way preference test using leaf disks and adults collected from the field. The high correlation ($r = 0.99$; $P < 0.01$)

between numbers of adults on the field plants and feeding rates in leaf-disk bioassays suggests a close relationship between antixenosis in field plantings and feeding deterrency in laboratory bioassays. Feeding rates on leaf disks were also highly correlated ($r = 0.88$; $P < 0.01$) with the estimates of field antixenosis by rating damage severity. Thus, leaf-disk bioassays may be an efficient means of detecting the presence of feeding deterrents in segregating progenies (Schalk and Stoner, 1976).

Only a few larvae survived to adult eclosion on detached leaves from leptine-synthesizing clones (Table 1). Measuring CPB development from larval hatching to adult eclosion is, however, too time-consuming to evaluate resistance of progeny in breeding populations. Cantelo et al. (1987) found that recording development stage of neonate larvae after feeding for only four days on detached foliage was more sensitive in detecting differences among two of our *S. chacoense* segregates and a susceptible *S. tuberosum* cultivar than leaf-disk bioassays with adults or other measurements of larval development. All of the bioassays we tested differentiated the nonleptine from the leptine-synthesizing sib-clones. However, the leaf-disk preference tests with adults and the four-day test with neonate larvae appeared to be the most efficient of the various laboratory bioassays.

CONCLUSIONS

Measuring the resistance of plants segregating for suspected defense compounds can be an effective means of determining their role in the defense. Chemical assays, selecting and cloning desired segregates, or even breeding to create desired genotypes may be necessary. The availability of cloned or inbred plants that produce suspected defense compound(s) in a range of levels can greatly facilitate investigations of the compound(s) importance in conferring resistance to plants in the field.

The reason for the remarkable increase in glycoalkaloid potency against CPB as a consequence of glycoalkaloid acetylation remains to be determined. This will require testing pure forms of leptines for their electrophysiological, membrane, and biochemical effects.

If we are successful in transferring an *S. chacoense* gene(s) for acetylation of sol and chac to *S. tuberosum*, leptine concentrations may be lower and therefore may not confer the same high level of CPB resistance. However, even somewhat lower resistance levels could mean fewer egg masses, fewer larvae, and less plant damage. If the proposed breeding approach appears feasible after leptine inheritance and the correlation between resistance and leptines are determined in small populations of hybrids, we plan to assay large numbers of hybrid and backcross progenies for leptines and then bioassay the leptine segregates

that also have desirable horticultural characters. This approach could reduce the need for large field tests in the early generations of breeding.

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BIOASSAY TECHNIQUES
An Ecological Perspective

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Abstract—Research in plant-herbivore interactions relies heavily on bioassays as analytical tools. Successful bioassay experiments are difficult to design because of the need to reconcile the exacting requirements of hypothesis testing with the dynamic nature of the plant and insect test organisms. Several research protocols are discussed that utilize ecologically flawed techniques to answer ecologically important questions.

Key Words—Bioassay design, bioassay flaws, plant-insect bioassays.

Bioassays are an important research method for testing hypotheses concerning plant-herbivore interactions. In plant-herbivore research, "bioassay" is defined in broad terms: any assay in which a living organism is permitted to declare whether a specific variable (e.g., presence or absence of an allelochemical, age of the plant tissue, concentration of a nutrient, presence of a plant pathogen) "makes a difference" as to how the organism fares; i.e., does the variable have an impact on the herbivore's life history parameters? Bioassays have been used extensively to discover plant characteristics that have been, or could be, of either ecological or evolutionary importance to herbivores. Many studies have used bioassays to full advantage to describe the role of the variable being studied, but this has not always been the case. Bioassays have also been designed and interpreted improperly. In some instances, researchers have been so focused on the question at hand that they seem to have lost sight of the ecological framework in which they were working.

Some recent review articles have dealt with bioassays of insect feeding preferences (Lewis and van Emden, 1986), insect oviposition preferences

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(Singer, 1986), and postingestive effects of phytochemicals on insects (Berenbaum, 1986). In this discussion, I focus on bioassays performed to evaluate the impact of a plant characteristic on the growth and development of herbivorous insects. It is more logical to assess the impact of tannins on herbivorous insects by feeding tannin-containing material to the herbivore in question than by evaluating the protein-precipitating quality of the tannin. Similarly, assessing the impact of a proteinase inhibitor on a herbivore by rearing the insect on food containing a proteinase inhibitor is preferable to *in vitro* assays of trypsin inhibitory activity. While the logic of testing hypotheses with the relevant organisms is intuitive, designing the proper experiment is difficult.

Currently, there are no guidelines on the best way to design a bioassay in any particular instance, but I have observed some common types of errors. Bioassays are often designed to answer questions by manipulating and simplifying a complex system. The manipulation and simplification can involve making assumptions that are often never specifically addressed and may be inappropriate. The complexity of the test system often prohibits controlling all relevant factors; it is the rare researcher who has total control over such things as the genetics of both plant and insect test organisms, the environment to which these organisms have been exposed, or a complete understanding of how the testing environment interacts with the test. Such uncontrolled sources of variation often enter into an experimental design; they become a major problem only when their possible impact on the experiment is not considered. It is possible for an experiment to be well executed yet for the results to have no relationship to the ecological context of the original question.

I will present some "typical" problems of the type described. In this brief discussion I make no attempt to cover all types of experiments or all types of problems. My purpose is to generate discussion and reconsideration of certain bioassay methodologies, methodologies which purport to answer ecological questions but which can have important ecological flaws.

Laboratory vs. Field Populations

Many researchers depend on laboratory populations of insects for their bioassays. Laboratory populations are available as needed and are usually disease- and parasite-free, but they have also been subjected to laboratory selection. Selection for survival under laboratory conditions can alter the genetic composition of the population as can genetic drift from inbreeding or population crashes. Such changes can dramatically alter the biology of a laboratory population. Lab populations have been known to lose their ability to grow successfully on their original host plants (Guthrie and Carter, 1972) and to develop fertility problems (Bledsoe, personal communication). Since such major changes in biologically relevant characteristics are possible under laboratory rearing,

broad extrapolation to natural populations from experiments on lab populations could certainly be inappropriate. This is not to suggest that the use of laboratory populations should be abandoned but rather, that we should be aware of their limitations.

If a bioassay is intended to determine, for example, the biological activity of different formulations of a compound, then using a laboratory population can be appropriate. Even though the lab population may no longer be representative of the wild population, it is still a living system. On the other hand, a bioassay carried out with a laboratory population could be inappropriate to test a hypotheses about a type of insect, i.e., generalist herbivores, even if the species is considered a generalist feeder. The physiological adaptations that contribute to being a successful generalist could have been lost or altered under laboratory rearing. Lab populations of insects can be very useful, but before making generalizations to wild populations from experiments performed with lab populations, the relationship between the lab population and its wild counterparts must be monitored (Huettel, 1976; Reese and Field, 1986).

Complexity of Interactions

Most ecologists doing research in plant–herbivore interactions are interested in understanding the relationships between plants and their herbivores. They are attempting to answer such questions as: why is this plant species eaten by this insect and not by another?, why at this time of year and not later or earlier?, why only in this habitat and not elsewhere? An ecologist walking in the woods and seeing some individual trees with leaves just expanding while most of the population has fully mature leaves, or some insects hatching or flying about while others are still dormant, would note that the observed species or the aberrant individuals were different from their cohorts. Yet some incautious researchers wanting to determine, for example, whether leaf age is important for an insect species might use the plants with different times of leaf expansion as a source of material for a "natural experiment" on the effects of leaf age. They would use aberrant plants whose leaves are expanding one or even two months later than their congeners to provide test material to be compared with the abundant mature leaves. This experimental protocol, intended to measure the effect of leaf age, tacitly assumes that leaves from these two types of trees differ only in age; that is, leaf material from individual trees which are obviously different from most of the population are representative of young leaves from the main population in which leaf expansion occurred two months earlier. Unfortunately, a comparison of insect growth rates between insects growing on "typical" trees and insects growing on "abnormal, late-leafing trees" does not necessarily say anything about the effect of leaf age on insect growth. If the delay in time of leaf expansion was related to herbivory during

the previous year, or a pathogen, or root-infesting insects, or some site-specific soil characteristic, the bioassay evaluates the impact of the stress on leaf quality, but does not answer the question posed. The leaf-age question posed by the ecologist is not trivial and is difficult to answer. Proper testing of such a question requires the imposition of appropriate controls; to test for the effects of leaf age, only leaf age should differ between treatments. Some methods that have been used successfully involved using altered leaf material [frozen leaf tissue (Feeny, 1970), freeze dried leaf tissue (Broadway et al., 1986)] or altered insects [manipulating time of egg hatch such that larvae are available to feed on leaves when those leaves are at the appropriate age, (Schroeder, 1986)].

Another type of problem seen with "natural" experiments has been pointed out by Neuvonen and Haukioja (1985), namely, that it is impossible to sort out cause and effect associated with natural levels of herbivore infestation. A hypothesis associating differences in plant quality with differences in levels of herbivory (e.g., do plants with high-quality foliage have greater amounts of herbivory?) cannot be tested by assessing the levels of herbivory under natural field conditions. Herbivory can cause herbivore-induced chemical changes (more to this point later) which will alter leaf quality. Secondly, herbivores are very selective in their oviposition and food preferences and can detect subtle differences in plant chemistry. Therefore the differences in chemistry observed in plants that are undergoing different natural levels of herbivory could be either the result (herbivore-induced changes) or the cause (oviposition or feeding stimuli) of the observed levels of herbivory. In addition, plant chemistry can affect the behavior of parasitoids and predators of the herbivores so that the distribution of herbivores observed in the field could be different from the distribution that the "plant quality" characteristics of the plants would have evoked from the herbivores (Vinson, 1974).

Experimentally Induced Changes in Host Plants

The past few years have seen a dramatic increase in research documenting changes induced in host plant quality vis-à-vis insect herbivores as a result of herbivory or disease. Plant pathogens can induce feeding deterrents (Rowan and Gaynor, 1986), lower host-plant quality (Karban et al., 1987), or can be associated with increased food preference (Lewis, 1979). Physically damaging leaves, whether through mechanical means, artificial defoliation, or natural or controlled herbivory can alter the chemical characteristics of the leaf tissue. Both increases and decreases in leaf nutritional quality have been reported in response to herbivore damage as have alterations in leaf acceptability (Carroll and Hoffman, 1980; Edwards et al., 1985; Tallamy, 1985; Wagner and Evans, 1985; Williams and Myers, 1984). The negative induced effects can be very rapid (within hours) (Ryan and Green, 1974; Carrol and Hoffman, 1980), mod-

erately rapid (two days to two weeks) (Haukioja and Niemela, 1977; Karban and Carey, 1984; Edwards et al., 1986), or long lasting (three to five years) (Neuvonen and Haukioja, 1984; Wratten et al., 1984; Wallner and Walton, 1979). Although the final chapter on the importance of induced responses has certainly not been written (Fowler and Lawton, 1985), any bioassay utilizing field collected materials needs to consider the possible role of induced changes in leaf quality. It would seem essential that the specific history of field collected material be known; plants that appear similar could be very different. In addition, extreme care has to be taken to control for changes associated with the experimental regime (damaging leaves while collecting or sampling, etc.).

In addition to induced changes resulting from leaf damage or disease, plant quality can be affected by various environmental factors such as flooding and root cutting (Louda, 1986), light intensity (Woodhead, 1981; Khan et al., 1986), drought (Mattson and Haack, 1987), available nutrients (Wolfson, 1982; Wermelinger et al., 1985), or interactions among these variables (Waring et al., 1985). (See White, 1984, for discussion on the impact of "stress" on plant quality.) Although all of these sources of variation cannot necessarily be avoided in field-grown materials, in a bioassay experiment we can control for the phenotypic plasticity of plants by using appropriate controls. Additionally, with modern environmental chambers, we can grow plants using a defined growing regime under controlled conditions so as to minimize environmental variation. Environmental-chamber grown plants are not "natural" but they are "definable."

Over 30 years ago Beck (1956) advised abandoning the use of excised leaf tissue in studies attempting to delineate host plant relationships of herbivores (i.e., which plant species can or cannot support growth of an herbivore and why). His recommendation was based on data from plant physiologists indicating that within hours of excision leaves undergo biochemical degradation and changes in water relationships. He concluded that because of the changes in the host plants, it was difficult, if not impossible, to define host-plant relationships with experiments that used excised plant tissues. How can one sort the insect's response to the plant from the insect's response to changes in the plant that resulted from excision? Unfortunately many researchers continue to use excised tissues with, apparently, little appreciation of the complexity of plants. In some research protocols, plants are treated as if they are inert insect food substrates (but see Dabrowski and Bielak, 1978). I can cite the example of a PhD student who was working on host-plant resistance in a major crop. He was using one of that crop's important herbivores to bioassay his test cultivars, and he thought he had identified resistant germ plasm. His bioassay consisted of comparing growth rates of insects reared on excised leaves in moist Petri dishes which were held in the dark in a temperature chamber. The leaves were changed only every five days. I tried to explain that with his protocol he could not possibly

differentiate between levels of host-plant resistance among his cultivars and the insect's response to the, presumably independent, differential rates of leaf senescence among the cultivars.

Plants should be expected to respond to manipulations and mutilations. Why ever assume that comparisons between excised leaves maintained in water to excised leaves out of water compare leaf water content and nothing else. While all leaves change after excision, a leaf kept in water is probably less different from its original state than a leaf allowed to desiccate. Similarly, leaves cultured in a toxin solution to foster toxin uptake or infused with the toxin could have been stressed by presence of the toxin. Therefore the experimental leaves differ from the control leaves not only by the presence of the toxin but also by the metabolic stress. While it is certainly not necessary to abandon all experimental protocols using excised leaf tissue, problems inherent in the method need to be kept in mind, and controls designed with great care. It might be preferable to evaluate the importance of leaf water content or the effect of a toxin by using artificial diets or experiments designed with stepwise changes.

Defining Proteins

The statistical association between the presence or absence of specific leaf chemicals and host plant quality has given rise to some specific hypotheses about the ecological importance of the chemicals, e.g., late-season forest herbivores are protein-limited. Some attempts to test such hypotheses have assumed that different compounds within a class of compounds were effectively the same vis-à-vis the insect. A test of protein limitation might utilize casein, hemoglobin, or egg white protein, for example, to supplement leaf protein, or a test on the effect of an undefined host-plant tannin might use quebracho tannin, tannic acid, or sorghum tannin. Recently several researchers have been using "ecologically relevant" plant-extracted tannins (Berenbaum, 1983; Manuwoto et al., 1985) in preference to commercially available tannins, acknowledging the differences among tannins documented by biochemists (Asquith and Butler, 1986; Mole and Waterman, 1987). The inability, for example, of a herbivorous beetle to respond to protein supplementation when the protein supplement is unrelated to its normal dietary protein can shed little light on the protein limitations of the beetle. Although most insects require protein, not all proteins have the same amino acid composition, and it is ultimately the amino acids derived from proteins that are limiting. By testing the "protein limitation" hypothesis with an ecologically irrelevant protein, the researcher is assuming that the test insect has the appropriate proteinases for digesting the test protein, that the amino acid ratio of the test protein is appropriate for the insect, that the change in protein concentration did not place the insect under metabolic stress, that the

protein would not be bound by tannins and would remain available for nutritional purposes, etc. A better way, perhaps, to test this hypothesis would be to determine the amino acid composition of the natural food and supplement with a balanced amino acid mixture. Recently Murdock and colleagues (personal communication) have evaluated the impact of a proteinase inhibitor on a bruchid beetle. They were able to reverse the inhibitor's negative impact on growth and development by supplementing the inhibitor-containing diet with a mixture of amino acids that reflected the ratio found in the host. In contrast to protein supplementation, this amino acid supplement method could bypass the problem of tannin precipitation, the possible stress of increased proteinase production, and would provide the insect with nearly the same amino acid mix as would normal protein digestion. Another possibility might be to extract the plant protein or specific protein fractions and then supplement the diet with this material (Nawrot et al., 1985).

Artificial Diet is Just That—Artificial

Artificial diets were crucial for the development of insect dietetics and are essential for the economic maintenance of many insect laboratory populations, but they can have their shortcomings in bioassays intended to assess the ecological importance of plant allelochemicals. Artificial diets usually have one of two problems: they are suboptimal or they are superoptimal. Compared with even the most susceptible host plants, artificial diets often foster more rapid growth (Beck, 1974; Reese and Field, 1986). The luxuriant growth often observed on these "super foods" could make insects much less susceptible to the allelochemical being tested. As recently discussed by Berenbaum (1986), tests of chemicals in artificial diets could be compromised by eliminating naturally occurring synergistic interactions (Berenbaum and Neal, 1985) or by fostering unnatural interactions between nutrients and allelochemicals. Nowhere is this made more clear than in Berenbaum's observations (1986) regarding the potential importance of tannins to herbivores; she notes that in all cases in which tannins are reported to reduce digestibility, artificial diets were used, whereas in those experiments in which tannins did not reduce digestibility, the test material was natural leaf materials.

Not all insects are easily reared on artificial diets, and in some instances, suboptimal diets have been used to test specific chemicals. By suboptimal, I mean diets that permit some development but that cannot be used to rear larvae from hatch to pupation or diets on which developmental rates and survival are markedly inferior to the natural food. Such a suboptimal diet is a source of stress for the insects even without any addition of test chemicals. The problems of testing allelochemicals in this situation are clear: how can the impact of a test chemical be evaluated on a highly stressed insect? What does a change from

50% mortality on the diet to 75% mortality when the test compound is incorporated into the diet indicate about the compound's possible importance under natural conditions? It is difficult to determine.

Experimental Biases Large and Small

We Prefer to Measure What We Can Easily See. Researchers choose late instar larvae for bioassays of selected compounds presumably because larger larvae are easier to handle and measure than their younger counterparts. It is much easier to measure such things as consumption rate, growth rate, or frass production with large larvae. There are several potential problems associated with the use of older larvae, but their relative importance depends on the questions posed in the research. Young larvae are generally much more sensitive than older larvae (Reese et al., 1982; Rossiter et al., 1986), and plant characteristics, either physical or chemical, of potential importance to insects could be overlooked if only older larvae are tested. Compounds or concentrations of compounds that produce little or no acute effect on older larvae could have chronic effects, but testing for chronic effects requires longer assays, which, in turn, would necessitate starting with small, young, difficult-to-handle, larvae (Reese and Beck, 1976). Another problem associated with testing only older instars is the impact of changing food on the results of the bioassay. In some protocols, a cohort of the test insect is reared on an optimal diet, either artificial or natural, and then, when the appropriate stage is reached, only the experimental insects face a radical change to a new test diet (the control insects might have a slight change such as the incorporation of solvent into their diet). For some insects, changing food plants during larval development, even if the new host plants are within their host plant range, can negatively impact growth and development (Scriber, 1979). Therefore, when using older larvae in a bioassay, we need to control not only for the treatment, but also for the trauma of imposing the treatment.

Time Goes on Even When Growth Doesn't. When the test chemicals included in bioassay prove effective but do not cause acute toxicity, they often impact the insect's growth and development. Larvae in the different treatments are, therefore, of different sizes. This difference in size will affect ease of handling during weighings or transfers onto fresh food. Since smaller larvae are much more susceptible to injury in a bioassay (e.g., drowning in a water droplet, being pierced by a hair on a paint brush, falling from a leaf), mortality data should not be gathered if the bioassay procedure involves considerable handling of larvae. Even though under natural conditions smaller larvae would probably suffer greater mortality from both biotic (e.g., predation) and abiotic factors (e.g., wind or rain), these natural sources of mortality are not the same as those

found in the laboratory, and the laboratory mortality observed, although associated with the treatment, is not necessarily a result of the treatment.

A Leaf can be More than the Sum of Its Constituent Parts. Allelochemicals found in leaf tissue naturally cooccur with a complex of other materials. Synergists have been described in some plants (Berenbaum and Neal, 1985) and might exist in others. Similarly, some allelochemicals, e.g., glucosinolates and cyanogenic compounds (Kjaer, 1960; Jones, 1972) cooccur with their hydrolytic enzymes. The activity of the toxin in the absence of the enzyme would be expected to be very different than in its presence (but see Erickson and Feeny, 1973). Therefore, evaluation of isolated chemicals in artificial diets could be misleading.

Wherein Lies the Solution?

The preceding is not intended to be a condemnation of all planned bioassay research. Rather, my intention was to stress the necessity, in bioassay experiments, of focusing clearly on the specific question being asked and to inquire as to whether the planned bioassay can be used to answer that question: i.e., do the controls and the treatments differ only by the variable in question? In the examples cited above, the answer often would have been no.

I have recently been involved in the development of two bioassay systems that appear to withstand many of my earlier criticisms. In these systems, the parameters are well defined and the results repeatable (Murdock et al., 1988; Wolfson and Murdock, 1987b). These bioassay systems are not based on artificial diets; rather, they are based on the natural food turned into an artificial substrate. Both systems were developed to assess the impact of proteinase inhibitors, either proteins or peptides, on a herbivore, and the bioassays work well for that purpose. They have not been tested with other classes of chemicals. The system for leaf-feeding insects has been used extensively with herbivorous beetles and Lepidoptera and involves incorporating the test chemical into a gelatin solution that is then painted onto the leaf surfaces (either upper, or lower, or both) (Wolfson and Murdock, 1987a,b). The compounds remain active while on the leaf and, since the compounds are in gelatin solution rather than a solvent, there is little chance of their entering the leaf and interacting with the leaf at the cellular level. Excised leaves from the insect's host plant are the substrate used for the gelatin, but the plants from which the leaves are taken are grown in environmental chambers, under controlled and prescribed conditions, and only plants of defined age classes are used. This procedure helps us avoid some of the inherent variability in plants associated with age and environmental variation. In addition, leaves in all of our bioassays are changed daily so that changes associated with senescence remain constant and are evenly distributed over all

treatments. In these assays, we are assessing the impact of the test compound on the insect and changes in the leaf associated with excision would be present in all treatments.

The second bioassay system involved the development of an artificial seed system for use with bruchid beetles (Shade et al., 1986), primarily *Callosobruchus maculatus*. Our confidence in this system rests in the ability of our test insects to grow and develop in the artificial seeds made from their natural host beans (cowpeas) at rates and with developmental characteristics that are statistically indistinguishable from intact seeds. In a comparison between the artificial seeds and intact cowpea seeds, there was no significant difference in larval developmental time, mortality, prereproductive adult dry weight, rate of adult emergence, adult longevity, postreproductive adult weight, and fecundity. Test materials can be easily incorporated into the artificial seeds. Infested seeds are then monitored for adult emergence, and comparisons in developmental time, adult weights, and fecundities can be made between growth in the presence and absence of the test chemical.

Other bioassay systems have been developed to answer specific research questions because the researchers were dissatisfied with previously developed tests or were working with an insect–plant system that had no described bioassay. Bioassays directed to answering ecological questions are difficult to design and, as researchers, we are often faced with choosing the lesser of evils. Compromises have to be made, but we need be aware that we are making them and make clear to our colleagues that we understand the possible implications of those compromises.

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PLANT–INSECT COEVOLUTION AND INHIBITION OF ACETYLCHOLINESTERASE

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Abstract—The theory of plant–insect coevolution provides for diffuse coevolution and the expectation that plants evolve broad-spectrum chemical defenses with which some insects coevolve by detoxifying and using the compounds as host-location cues. Specific biochemical modes of action have been assigned to relatively few such defense chemicals and one major class, the terpenoids, is investigated here. Six terpenoids inhibited the enzyme acetylcholinesterase (derived from electric eel) and elicited the appropriate in vivo effects of insect paralysis and mortality. The diterpene gossypol was a reversible uncompetitive inhibitor. Five monoterpenes, representing a range of functional groups, were reversible competitive inhibitors apparently occupying at least the hydrophobic site of the enzyme's active center. Such data suggest the involvement of acetylcholinesterase in the coevolved insect response to terpenoids.

Key Words—Herbivory, pheromones, chemical defense, monoterpenes and diterpenes, insect paralysis and mortality, enzyme evolution, coevolution, acetylcholinesterase inhibition, plant–insect coevolution.

INTRODUCTION

The study of coevolution has generated much interest since Ehrlich and Raven coined the term with the statement "one approach to what we would like to call coevolution is the examination of patterns of interactions between two major groups of organisms with a close and evident ecological relationship, such as plants and herbivores" (Ehrlich and Raven, 1964). Although a key influence on present-day studies of coevolution, Ehrlich and Raven were not the first to recognize its existence. Darwin was aware of the special relationships between

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insects and the plants they pollinated: ". . . thus can I understand how a flower and a bee might slowly become simultaneously or one after another, modified and adapted in the most perfect manner to . . . each other" (Darwin, 1859). A comprehensive review of contributions subsequent to Darwin's would be rather inappropriate here, but the following seem especially significant.

Janzen (1980) defined coevolution as when "an evolutionary change in a trait of the individuals of one population is followed by an evolutionary response by a second population to the change in the first." By this definition, the evolution of each trait is due to the others, a reciprocal condition that views plant–insect interactions as one to one. A more general definition is that coevolution has occurred when the interaction between two or more ecologically interacting species involves an adaptive response to genetic change in the other(s) (Futuyama, 1983). This is also less restrictive, allowing for the interaction of several and not specifically two species. Known as diffuse coevolution (Janzen, 1980), this occurs where many plants have evolved chemical and physical defenses against a diverse group of insects and also where many insects have acquired the ability to detoxify a wide range of plant chemicals (Futuyama, 1983).

One consequence of the theory of diffuse coevolution is the expectation that plants would select a broad spectrum of defense, i.e., one or a few plant chemicals that would be antagonistic to a wide range of herbivorous insects. This is an economical measure, as the production of secondary chemicals is at the expense of other areas of metabolism. For example, tobacco plants especially rich in alkaloids are stunted, suggesting that energy allocated to the manufacture of nicotine has been abstracted from energy available for growth (Whittaker and Feeny, 1971). Also it would be metabolically impractical for any plant to evolve a new chemical against every insect species attacking it. Accordingly, some secondary plant chemicals should act as broad-spectrum insecticides, and indeed some have been used as such for centuries. The dried flower heads of *Crysanthemum cinerariaefolium* (Compositae) and *C. coccineum* contain pyrethrins, potent insecticides with a rapid knockdown action.

A chemical class conspicuous among plant secondary compounds and containing chemicals inimical to insects are the terpenoids (Mabry and Gill, 1979). The cyclic monoterpene pulegone, an irritant commonly found in mint oils, deters feeding by the slug, *Ariolimax dolichophallus* (Rice) and by the fall army worm, *Spodoptera frugiperda* (Smith) and repels the German cockroach, *Blattella germanica* (L.) (Gunderson et al., 1985). Added to diet, it killed the larva of the fall armyworm and decreased pupation by the southern armyworm (Brattsten, 1983). Resistance of western red cedar wood, *Thuja plicata* (D.), to insect attack is attributed to the presence of monoterpenes, of which methyl thujate is toxic to larvae of the black carpet beetle, the furniture beetle, and the case-making beetle (Becker, 1963). Two other monoterpenes produced by western red cedar, β-thujaplicin and γ-thujaplicin, are both highly insecticidal against

the larva of the old-house borer, *Hylotrupes bajulus* (Cerambycidae), a common pest of structural timber in Europe (Becker, 1963). The diterpene gossypol, an abundant diterpene in the lysigenous glands of cotton, decreases weight gain in larvae of the tobacco budworm and pink bollworm (Hedin et al., 1983) (for many other examples of terpenoid–insect interactions, see Mabry and Gill, 1979). However, specific biochemical modes of action have been assigned to few such compounds.

A common structural feature of terpenoids is their hydrocarbon skeleton, which in turn confers upon them a common property, hydrophobicity. Many hydrophobic compounds are associated with protein deactivation and enzyme inhibition, and one enzyme particularly susceptible to hydrophobic interactions is acetylcholinesterase (Hansch and Deutsch, 1966), present in the neuromuscular junction. This enzyme also functions in the peripheral sensory nervous system of the insect antenna (Sanes et al., 1977). The present report quantifies the toxicity of a range of plant terpenoids to a nonadapted insect and provides what seems to be the first evidence that they are reversible inhibitors of acetylcholinesterase.

METHODS AND MATERIALS

Compounds that inhibit or inactivate acetylcholinesterase (AChE), cause acetylcholine to accumulate at the cholinergic site. This produces continuous stimulation of cholinergic nerve fibers throughout the central and peripheral nervous system, followed by paralysis and death (Corbett et al., 1984). Accordingly, we used an in vivo assay established as appropriate to quantify insect mortality and an in vitro assay for AChE inhibition.

The following five monoterpenes, representing a range of functional groups, were selected for investigation: citral (aldehyde), pulegone (ketone), linalool (alcohol), (−)-bornyl acetate (ester), and cineole (ether); for comparison a single diterpene gossypol (alcohol) was included (Figure 1). As these monoterpenes are characteristic constituents of plant leaves and as gossypol is associated with pigment glands of cotton seed, the test insect, *Tribolium castaneum*, a pest of stored grain, may be viewed as a nonadapted species. The insecticide-susceptible *Tribolium* colony was derived from stocks originally supplied by the University of Chicago.

Insect Mortality. Mortality was assessed by an F.A.O. contact method devised to measure the resistance of agricultural insect pests to insecticides (Anon., 1970) with the modification that acetone served as solvent in place of *Risella* oil. Usually four concentrations of each test compound were used within a range established by previous experiment as eliciting mortality (for specific concentrations see Figure 2). There were two replicates of each treatment and

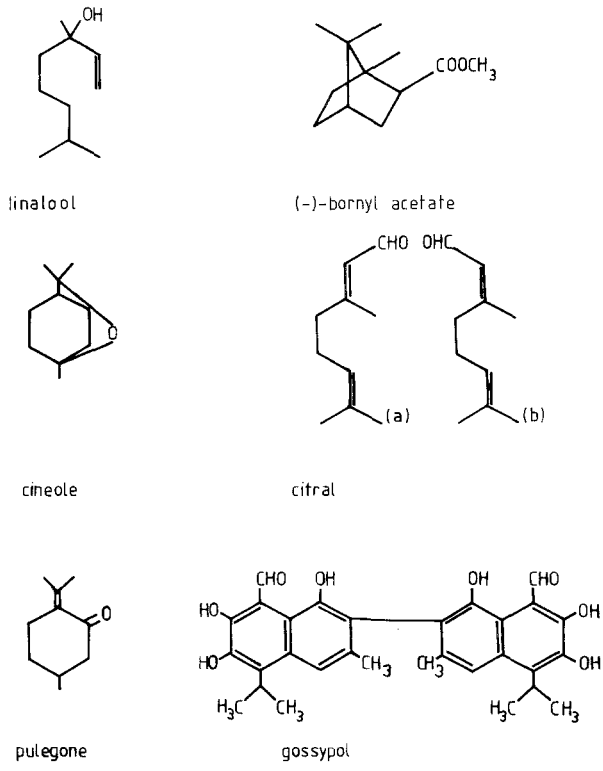

FIG. 1. Configurations of the five monoterpenes and the diterpene (gossypol) selected for investigation: citral is a mixture of geranial (a) and neral (b).

of the control, i.e., solvent only. Solutions were pipetted in 0.5-ml aliquots onto filter papers (5.5 cm diam.) that were allowed to dry (approx. 1 min). Then batches of 20 beetles, prestarved for 24 hr, were transferred to each treated paper where they were confined by plastic arenas, sealed on top by glass plates, that were placed in an incubator (28°C). Mortality was estimated from knock-down, i.e., the inability of the insect, after 5 hr in the arena, to stand or walk after a gentle push forwards with a forceps. Correction for control mortality was made using the Abbott formula (Abbott, 1925), $P_T = [(P_0 - P_c)/100 - P_c] \times 100$, where P_T is corrected percentage mortality, P_0 is observed mortality, and P_c is control mortality. LC_{50} values (concentrations eliciting 50% mortality), were derived by interpolation from lines representing dosage-mortality trends drawn on logarithmic probability paper; such lines were constructed using the regression formula for best fit.

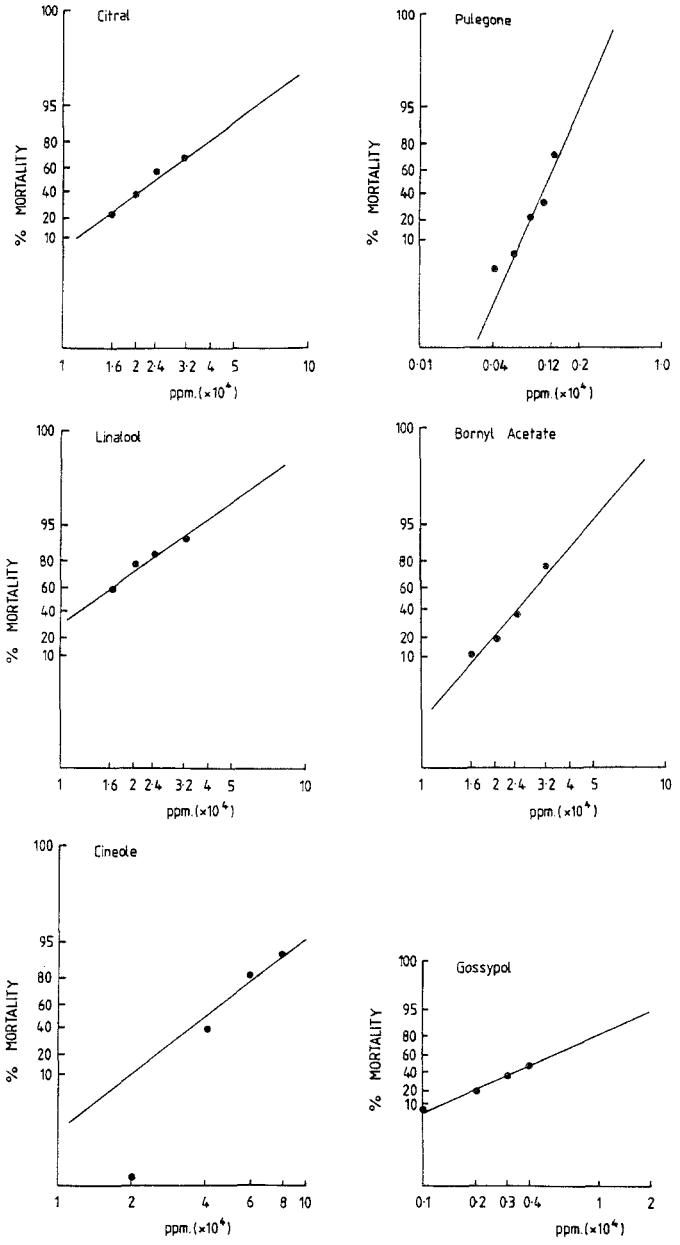

FIG. 2. Dosage–mortality relationships for the six terpenoids studied.

Enzyme Assays. Inhibition of AChE was assessed by the Ellman (1961) assay. Monoterpenes were dissolved in 5 ml absolute alcohol and made up to 50 ml in 0.1 M phosphate buffer, pH 8, to give a stock solution of 0.09 M. Gossypol is a paste at 0.09 M so the stock solution was 0.009 M. Serial dilution yielded the assay concentrations with the result that the concentration of ethanol never exceeded 2%, which we experimentally assessed as inhibiting the enzyme by less than 4% or negligible. The substrate was acetylthiocholine iodide and the color reagent was 5,5′-dithiobis-2-nitrobenzoic acid (DTNB).

Each assay was duplicated, and a typical assay mixture contained 40 μl substrate, 20 μl DTNB, 1 ml terpene solution, and 100 μl electric eel AChE (0.5 unit). Hydrolysis was measured at 25°C in a Pye Unicam SP8-100 spectrophotometer at 412 nm, and all test and control assays were corrected by blanks for nonenzymic hydrolysis.

Chemicals. Citral (99% pure), pulegone (85%), linalool (99%), (−)-bornyl acetate (97%), and cineole (99%) were supplied by Aldrich, Gillingham, Dorset, England. Gossypol, AChE (c-3389, electric eel), DTNB, and acetylthiocholine iodide were supplied by Sigma, Poole, Dorset, England.

RESULTS AND DISCUSSION

All six terpenes killed *T. castaneum*, and this mortality was dose-responsive (Figure 2). Pulegone was the most potent (LC_{50}, 0.12×10^4 ppm) followed by gossypol (0.45×10^4), citral (1.5×10^4), linalool (2.5×10^4), (−)-bornyl acetate (2.7×10^4), and cineole (4.3×10^4). It was evident that beetles became paralysed prior to death.

All six terpenoids reversibly inhibited AChE. Gossypol was an uncompetitive inhibitor, as indicated by decreasing inhibition associated with decreasing substrate concentrations and by the parallelism of the Dixon plot (Figure 3). The five monoterpenes were competitive inhibitors, as indicated by increasing inhibition associated with decreasing substrate concentration and by the intersections in the Dixon plots (Figure 3). Inhibition constants (K_i) were: cineole 2.5×10^{-2} mM; pulegone 8.5×10^{-1} mM; gossypol 1.5 mM; linalool 5.5 mM; citral 7.0 mM, and (−)-bornyl acetate 21.3 mM.

The present evidence from both in vivo and in vitro experiments consistently indicates that the terpenoids tested are effective inhibitors of acetylcholinesterase: specifically, they paralyzed and killed a nonadapted insect and also inhibited electric eel AChE. There is little doubt that terpenoids may act as plant defense compounds or allomones, but this seems to be the first demonstration that a relevant biochemical–physiological mechanism is inhibition of cholinesterase. As the five monoterpenes tested represent five distinct functional groups, such inhibition may be a widespread property of monoterpenes.

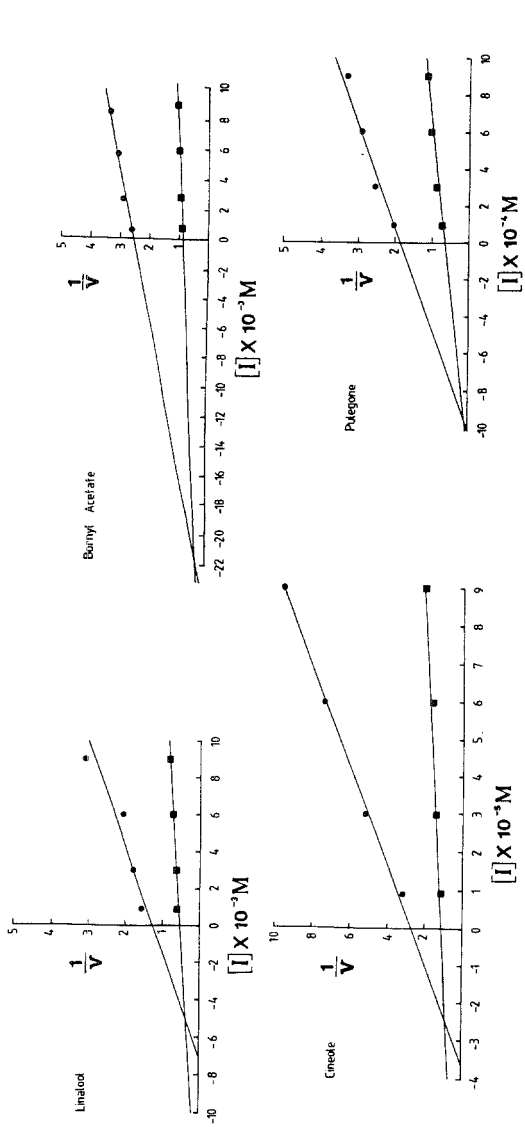

FIG. 3. Dixon plots derived from the inhibition of acetylcholinesterase by the six terpenoids. In each plot the concentrations of substrate (acetylthiocholine iodide) are 0.066 mM (● —— ●) and 0.5 mM (■ —— ■). The additional substrate concentration for gossypol is 0.2 mM (▲ —— ▲).

An apparent anomaly is that the order of potency for the compounds differs according to whether mortality or inhibition is assayed; for example, pulegone, the most potent as an insecticide, ranks second as an AChE inhibitor. It is relevant that vertebrate and insect AChE may differ in their susceptibility to insecticides (Hollingworth et al., 1967). Also it is widely recognized that in vitro K_i values are a rather poor indicator of insecticidal potencies, as they cannot reflect differential abilities of compounds to penetrate cuticle and gut barriers or their differential solubilities in insect hemolymph: each factor influences migration rates to the cholinergic sites (Corbett et al., 1984). For example, three carbamates gave K_i values against housefly brain AChE of 4×10^{-4} mM, 2×10^{-1} mM and 5×10^{-2} mM, respectively, representing a 500-fold range; their potencies against housefly using topical application were 50, 500, and 100 μg/g, respectively, or a 10-fold range (Kolbezen et al., 1954). Another study ascertained no correlation between LD_{50} values of carbamate insecticides and in vitro K_i values with honeybee AChE (Abdel-Raof et al., 1977). Accordingly, K_i values are taken here as adequate to establish compounds as AChE inhibitors but without the expectation of a direct relationship with toxicity.

Nevertheless, it is noteworthy that *m*-nitrophenyl-*N*-methylcarbamate has a K_i value of 2.0×10^{-1} mM (Kolbezen et al., 1954) as compared with 8.5×10^{-1} mM for pulegone. Also, pulegone's LC_{50} potency is 0.12×10^4 ppm as compared with LC_{50} values ranging from 0.007 to 0.027×10^4 ppm for the commercial insecticide and cholinesterase inhibitor, malathion, using the same assay and various susceptible strains of *T. castaneum* (Champ and Campbell-Brown, 1970). Pulegone's potency, 4.4- to 17.1-fold less than this insecticide's, clearly indicates that its action as an AChE inhibitor is relevant to its function as a plant defense compound; the same rationale may be applied to the other terpenoids tested.

Gossypol is an uncompetitive inhibitor of AChE, i.e., it binds not to the enzyme but to the enzyme–substrate complex thus preventing product formation. The five monoterpenes, however, are competitive inhibitors, i.e., they compete with the substrate for its active center on the enzyme. The interaction of the substrate (acetylcholine) with AChE's active center is usually represented as involving three subsites, the anionic, esteratic, and hydrophobic sites (Figure 4). As none of the five monoterpenes possesses a charged region and only one possesses a carbonyl group, this suggests by elimination that they take effect mainly by hydrophobic binding. Their configuration reinforces this, as it is consistent with a good fit to the enzyme's hydrophobic site (Figure 4). The in vivo insecticidal activity of various methyl carbamate and organophosphate insecticides is closely correlated with the extent of their hydrophobic bindings to AChE (Hansch and Deutsch, 1966).

Despite the ability of these terpenoids to inhibit cholinesterase and kill a nonadapted insect, they are not deleterious to all insect species. Specifically,

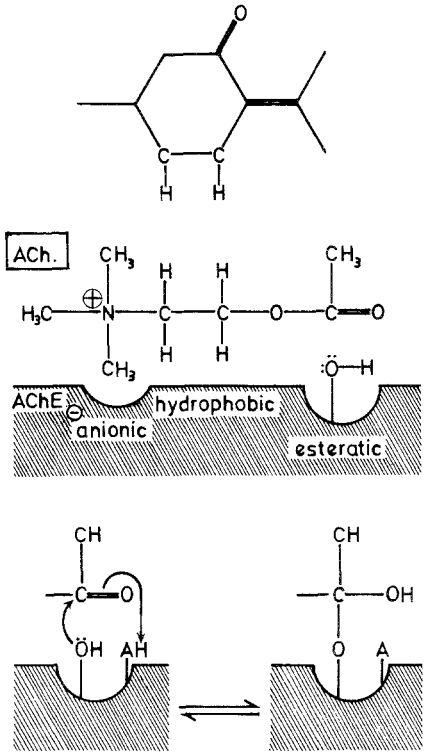

FIG. 4. Diagrammatic representation of the active center of acetylcholinesterase (AChE) indicating the anionic, hydrophobic, and esteratic sites together with the structural formula of acetylcholine (ACh). At bottom is a representation of ACh hydrolysis involving the carbonyl group of ACh and the enzyme's esteratic site: OH represents a hydroxyl group on a serine residue and HA represents a hydrogen donor (after Corbett et al., 1984). As competitive inhibitors, all five monoterpenes must bind to the enzyme's active center. At top is the configuration of pulegone with two CH groups designated to indicate the precise fit possible with the enzyme's hydrophobic site: the structures of the other four monoterpenes are also consistent with such binding.

linalool, a constituent of pine, oils of Ceylon, cinnamon, sassafras, etc., is an attractant for the silkworm (Brattsten, 1983). (−)-Bornyl acetate is a host-location cue for the carrot fly larva, and it seems particularly relevant that strong and statistically significant attraction was elicited by a concentration on filter paper of 9.8×10^4 ppm (Ryan and Guerin, 1982); this concentration was associated with 97% mortality of *Tribolium*. Thus, the present data are not assignable to concentration effects. (+)-Bornyl acetate is a mimic of the sex

pheromone of the American cockroach (*Periplaneta americana*) (Manabe and Nishino, 1983). Citral, a constituent of citrus fruits, is also an alarm and defense pheromone of ants but an attractant and assembly pheromone for the honeybee worker (Wilson, (1971).

The foregoing effects are consistent with the principles of diffuse coevolution but the compounds probably were detoxified by recipients before use as attractants. Detoxifying enzymes of insects belong to the general categories of oxidases, hydrolases, transferases, and reductases (Ahmad et al., 1986). Insect success in detoxifying cholinergic inhibitors, as evidenced by strains with developed resistance to organophosphate and carbamate insecticides, is assigned to specific enzymes. One is a modified carboxyesterase that apparently lost most of its esterase activity, acquiring phosphatase activity instead (Sawicki, 1973); another is gluthathione-*S*-transferase (Lewis, 1969). In addition, AChE has evolved in resistant strains to be more slowly inhibited than AChE in susceptible ones: 1.2- to 6-fold less in organophosphate-resistant strains and 17- to 1570-fold less in carbamate-resistant strains (Hemingway and Georghiou, 1983).

The process by which detoxified terpenoids subsequently become attractants is not easy to visualize, but the involvement of AChE in antennal sensory transmission seems a further reason for considering a role for this enzyme in plant–insect coevolution as mediated by terpenoids.

Acknowledgments—Is is a pleasure to thank Michael A. Raftery, California Institute of Technology, Pasadena, and E. O. Wilson, Harvard University, for kind and stimulating sabbatical hospitality to MFR in 1982 and 1986, respectively.

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Journal of Chemical Ecology, Vol. 14, No. 10, 1988

Erratum

ASTRINGENCY OF DOUGLAS-FIR FOLIAGE IN RELATION TO PHENOLOGY AND XYLEM PRESSURE POTENTIAL

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Due to publication errors that occurred after the author's approval of page proofs, several errors were printed on page 1233 (Results and Discussion). The second through fifth sentences on this page should read: "In contrast to unperturbed trees, tannin content of prebudbreak foliage from trenched trees was significantly lower than that of mature foliage (3.21 $\pm$ 0.15 and 7.12 $\pm$ 0.68, respectively; $P < 0.05$).

Treatment-Related Differences in Xylem Pressure Potential. ANOVA revealed significant treatment effects on both predawn ($P < 0.0001$) and daytime ($P < 0.03$) xylem pressure potentials (Table 2). However, differences between the experimental groups were not consistent throughout the season, as indicated by significant date $\times$ treatment interactions ($P < 0.0002$ and $P < 0.02$ for predawn and daytime measurements, respectively)."

The first and second sentences of the last paragraph on this page should read:

"*Astringency in Relation to Tissue Age and Xylem Pressure Potential.* Analysis of covariance revealed significant ($P < 0.05$) treatment effects on foliage astringency."

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Finally, the second sentence of the footnote in Table 2 should read: "Means in rows followed by different letters are significantly different ($P < 0.05$) between treatments."

REFERENCE

HORNER, J.D. 1988. *J. Chem. Ecol.* 14:1227–1237.

Journal of Chemical Ecology, Vol. 14, No. 11, 1988

Introduction

This special publication of the *Journal of Chemical Ecology* is dedicated to Dr. James H. Tumlinson, the 1986 recipient of the Burdick and Jackson International Award in Pesticide Chemistry, the highest research recognition award sponsored by Baxter International, Inc., Burdick and Jackson Division, and the Division of Agrochemicals of the American Chemical Society (ACS).

The invitational papers in this issue are based on talks presented by the authors in the symposium "Insect Chemical Communication: Unifying Concepts," held at the 194th ACS National Meeting, New Orleans, Louisiana, August 30–September 4, 1987. These eleven papers present recent concepts and supporting research information on insect behavioral biochemistry.

The papers by Tumlinson, Teal, and Roelofs and Wolf discuss biosynthesis of insect pheromones. Teal and Tumlinson describe the isolation and properties of a cuticular primary alcohol oxidase that catalyzes the conversion of the stored *Heliothis zea* female sex pheromone to the active but relatively unstable aldehyde. Remarkably, this enzyme retains its activity both in dichloromethane and hexane.

Roelofs and Wolf report on the chemical events in lepidopteran species leading to the biosynthesis of specific sex pheromones through unique chain-shortening involving $\Delta 9$, $\Delta 10$, and $\Delta 11$ desaturase systems. However, the mechanism governing the formation of the precise blend of *Z* and *E* acetates in tortricid species is as yet not understood but is presumed to proceed through a final reduction sequence from acyl intermediates. Apparently, these regulatory events are genetically controlled and differentially expressed in geographically isolated but related species and/or subspecies.

Klun and Huettel analyze the genetic regulation of the ratios of *Z* and *E* pheromone isomers in sympatric populations of wild-type females of the European corn borer, *Ostrinia nubilalis*. Circumstantial evidence from this work suggests that a male moth can carry an allele coding for production of one isomer ratio, but may respond to another ratio.

Ding and Prestwich, studying receptors and perception of pheromonal signals in antennae of *Heliothis virescens*, synthesized several classes of potential inhibitors of aldehyde-oxidizing enzymes (ADEs) that oxidize and inactivate the major *Z*11–16 aldehyde component of the pheromone. Cyclopropanols were found to be inhibitors of ADEs. However, the most potent irreversible inhibitors were α,β-unsaturated carbonyl mimics of the aldehyde.

Neuroregulation studies of pheromone biosynthesis promise to enlarge our knowledge of pheromone production, release, and perception. Raina reports on the factors regulating release of the neuropeptide (PBAN) that activates pheromone biosynthesis in *H. zea*. These factors include stimulation of the brain by light and by plant cues. Raina also provides indirect evidence for the occurrence of a hemolymph-borne, male PBAN inhibiting factor that is transferred to the female during mating and blocks pheromone production in the mated female.

Visser and DeJong analyze the patterns of olfactory semiochemical perception in *Pieris brassicae* larvae and *Leptinotarsa decemlineata* adults. Signals for mating and food consist of blends of chemicals.

The subject of chemical blends is also addressed by Oehlschlager and coworkers who studied aggregation pheromones in several sympatric cucujid grain beetles. These species respond either to single pheromonal compounds or to combinations of compounds, and show enantiomeric specificity.

Blum and Fales report on the multiplicity of semiochemicals modifying honey bee behavior with special attention to those chemicals that govern caste specificity.

Silverstein reviewed the requisites a natural product chemist must possess in order to study and determine the role of chiral molecules in insect communication.

These topics highlight major contemporary frontiers in insect semiochemical research. This new knowledge may provide the insights and tools to manipulate insect behavior, to trap or disorient pests, and to attract beneficial insects.

JULIUS J. MENN
PAUL A. HEDIN
Co-Chairs of the Symposium and Guest-Editors

Journal of Chemical Ecology, Vol. 14, No. 11, 1988

CHIRALITY IN INSECT COMMUNICATION

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(Received October 15, 1987; accepted February 3, 1988)

Abstract—In dealing with chiral molecules, the natural-products chemist must be knowledgeable in several areas of stereochemistry. This brief survey will serve for simple situations and as a source of references for more complex molecules.

Key Words—Chirality, enantiomers, stereochemistry, structure-bioactivity.

INTRODUCTION

Chirality, as one aspect of stereochemistry, "expresses the necessary and sufficient condition for the existence of enantiomers" and thus for optical activity (Cahn et al., 1966). Enantiomers are nonsuperposable mirror images. The ultimate test for a chiral molecule is thus nonsuperposability of its mirror image.

"Specification of Molecular Chirality" is the title of the last of three landmark papers by Cahn, Ingold, and Prelog (1966) in which the CIP rules formulate a comprehensive, extremely useful, although arbitrary nomenclature system for stereoisomers; each succeeding document elaborates on, and amends, the preceding document. Cahn (1964a) wrote a summary paper at the introductory level in the *Journal of Chemical Education*. This is required reading; only the dedicated stereochemist will read completely through the three CIP documents. Even Cahn (1964a) lost his bearings in the sequence rules and was forced to publish an Errata note (1964b, see below).

To establish a comprehensive, rigorous system of nomenclature, Cahn, Ingold, and Prelog attempted to expand their rules and interpretations thereof to cover all of organic chemistry, and they come remarkably close to succeeding. Inevitably, however, the papers became tomes of "legalistic" scholarship

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and reference sources for myriads of succeeding papers, as well as reviews on an almost annual basis in *Topics in Stereochemistry* (Allinger and Eliel).

Natural-products chemists, whom I address, must be knowledgeable in the following areas: (1) the basic stereochemical conventions needed to draw and name the structures likely to be encountered; (2) symmetry criteria in NMR spectrometry for structure elucidation; (3) methods for the determination of enantiomer composition and for enantiomer isolation; (4) methods for stereoselective synthesis (references only given herein); (5) principles and methods of biosynthesis (references only given herein); (6) the relationships between structure and bioactivity.

I consider these topics *seriatim* and hope that this brief survey will serve for simple situations and as a source of relevant references for more complex situations.

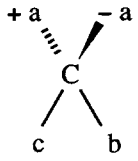
BASIC STEREOCHEMICAL CONVENTIONS

Basic definitions and rules are as follows:

1. A molecule is *chiral* when it is not superposable on its mirror image; *achiral* if superposable. A conformational-mobile molecule is chiral only if all of its possible conformations are chiral.

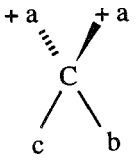
2. A molecule is *asymmetric* if it has no element of symmetry. All asymmetric molecules are chiral, but not all chiral molecules are asymmetric, molecules with simple axes of rotation only being chiral. A chiral atom (a chiral center) may possess no element of symmetry other than a simple axis or axes.

3. An *asymmetric carbon atom* is bonded tetrahedrally to four "different" atoms or groups, none of the groups being the mirror image of any of the others. Thus $C_{a,b,c,d}$ is asymmetric (chiral), whereas $C_{+a,-a,b,c}$ is not, even though +a and −a appear to be "different"; they are different only in being mirror images (enantiotopic). Note that the latter structure cannot be termed asymmetric or chiral since it has a plane of symmetry. The dilemma of having four apparently "different" substituents on a carbon center that has a plane of symmetry was resolved by assigning the rather cumbersome term "pseudoasymmetric" (Scheme 1).



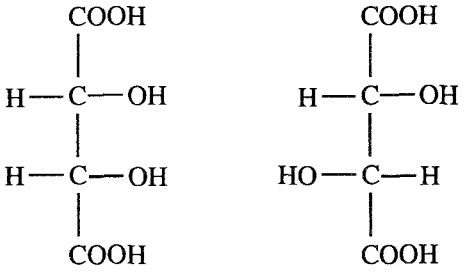
SCHEME 1.

How then do we treat $C_{+a,+a,b,c}$ (Scheme 2)? At first glance, +a and +a substituents on a tetrahedral carbon would seem identical, but they fail the test for identity: interchange through a simple axis of rotation (see below under Symmetry Criteria in NMR). Since they cannot be interchanged through any symmetry operation, they are diastereotopic. Again, we have a dilemma; both dilemmas will be discussed below in connection with the recent challenging paper by Mislow and Siegel (1984).



SCHEME 2.

4. Stereoisomers that are not enantiomers are *diastereomers*, which may be chiral or achiral (Scheme 3). Parenthetically, note that the term "geometric" isomers for *cis* and *trans* (*Z* and *E*) olefins and *cis* and *trans* cyclanes has been dropped in favor of the designation diastereomers (Eliel, 1971).



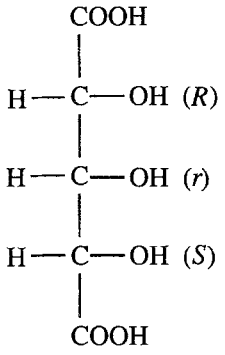
Achiral Diastereomer Achiral Diastereomer

(Fischer projections)

SCHEME 3.

5. Compounds with chiral centers are designated by prefix *R* or *S* (*r* or *s* for pseudoasymmetric centers) assigned by the CIP sequence rules (see below) preceded when necessary by locants (Scheme 4).

In one of the giant constructs of organic chemistry, Cahn et al. (1966) developed the sequence rule with six subrules (0–5) for specifying molecular chirality for each enantiomer in terms of the familiar symbols *R* and *S* (*r* or *s*). The following sequence rule, subrules, and discussion will cover most common situations. Application to more complex molecules is by no means trivial, and



(2*R*,3*r*,4*S*)-Trihydroxyglutaric acid
(Fischer projection)

Note that the pseudoasymmetric center is (*r*) as a consequence of the CIP sequence rule (below) that assigns priority of (*R*) over (*S*). Relative configurations are differentiated by (*R**), (*S**) (spoken *R* star, *S* star) preceded by locants when necessary; the lowest numbered locant is arbitrarily assigned (*R**).

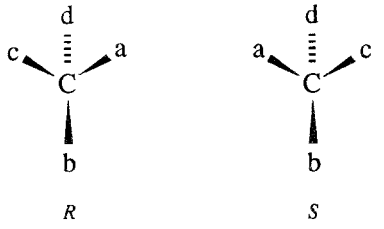
SCHEME 4.

it will be necessary to refer to the 1966 paper by Cahn, Ingold, and Prelog for further details.

The sequence rule itself is a "method of arranging atoms or groups (including chains and rings) in an order of precedence, often referred to as an order of preference [a priority sequence]; for discussion, this order can be generalized as a > b > c > d where > denotes 'is preferred to'." The three possible chiral elements of a molecule are considered in the sequence: chiral center, chiral axis, and chiral plane, of which by far the most common is the chiral center. For convenience, the sequence rule and the six subrules are here reproduced verbatim.

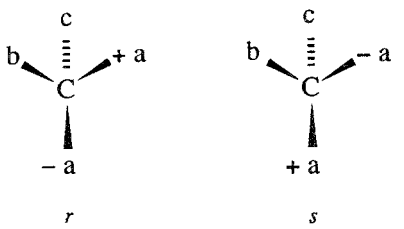
> Sequence Rule. The ligands associated with an element of chirality are ordered by comparing them at each step in bond-by-bond explorations of them, from the element, along the successive bonds of each ligand, and, where the ligands branch, first along branch-paths providing highest precedence to their respective ligands, the explorations being continued to total ordering by use of the following Standard Subrules, each to exhaustion in turn, namely:
> (0) Nearer end of axis or side of plane precedes further.
> (1) Higher atomic number precedes lower.
> (2) Higher atomic mass-number precedes lower.
> (3) Seqcis precedes seqtrans [seq is the abbreviation of sequence rule].
> (4) Like pair *R*,*R* or *S*,*S* precedes unlike *R*,*S* or *S*,*R*; and *M*,*M* or *P*,*P* precedes *M*,*P* or *P*,*M*; and *R*,*M* or *S*,*P* precedes *R*,*P* or *S*,*M*; and *M*,*R* or *P*,*S* precedes *M*,*S* or *P*,*R*; also *p* precedes *s*.
> (5) *R* precedes *S*; and *M* precedes *P*. [*M* and *P* are helicity designators].

The priority sequence having been established by applying the subrules, the chirality rule is applied as follows: "Among ligands [substituents] of highest precedence, the path of their sequence is followed from the preferred side of the molecule, that is the side remote from the group of lowest precedence, and, according as the path turns to right or left, the element is assigned the chiral label *R* or *S*, or, if pseudo-asymmetric, *r* or *s*." We thus have the familiar picture of a generalized tetrahedral asymmetric molecule $C_{a,b,c,d}$; where a > b > c > d, the label is *R* if the sequence abc is clockwise (right, *r*ectus), and *S* if counterclockwise (left, *s*inister) (Scheme 5).



SCHEME 5.

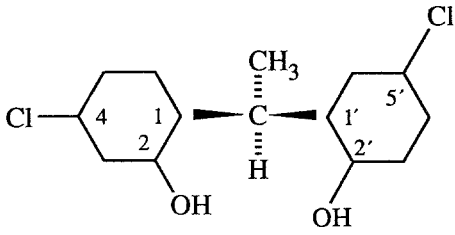
For the generalized pseudoasymmetric molecule $C_{+a,-a,b,c}$ mentioned above, where +a is an *R* substituent, −a is the mirror image (hence *S*), and b and c are in decreasing order of priority, we have the assignments as in Scheme 6. The assignments are based on subrule 5: *R* > *S*. [In subrules 4 and 5, *M* and *P* (*M* for *M*inus and *P* for *P*lus) are less familiar designators for helices.]



SCHEME 6.

In the vast majority of cases, the designators are decided by application of subrule 1, and simple examples of its application are given in all textbooks of organic chemistry. However, it may be worthwhile to discuss here in detail the difficulty that caused Cahn (1964b) to publish his correction and that continues to bedevil students and experts alike, namely how to handle ring systems.

Cahn used the molecule with two rings (Scheme 7) to illustrate the establishment of the priority sequence. The correct procedure as stated in the sequence

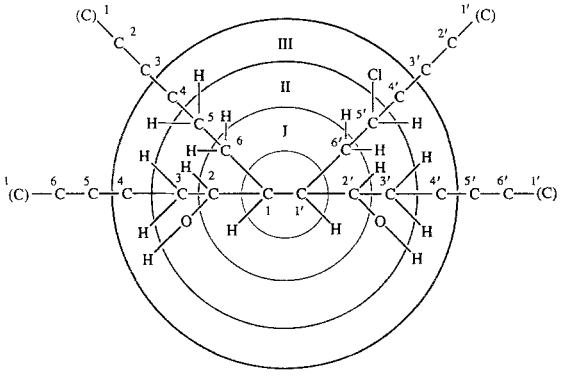


SCHEME 7.

rule is to place the ligands in priority sequence "by comparing them *at each step in a bond-by-bond exploration* of them, from the [chiral] element, along the successive bonds of each ligand, and, *where the ligands branch, first along branchpaths providing highest precedence to their respective ligands*, the explorations being continued to total ordering. . . ." [italics added]. Thus Cahn may have reasoned that the H substituent has the lowest priority (d), CH_3 is next lowest (c), and no decision can be made between the ring CH groups. At the branch in each ring, the lower branch (CHOH) takes precedence over the upper branch (CH_2). In the lower branch of the left ring, C-3 is CH_2, and in the lower branch of the right ring, C-3′ is also CH_2—no decision. In the left ring, C-4 is CHCl and in the right ring, C-4′ is CH_2. The left ring, therefore, is given priority over the right and is labeled "a"; the right ring is labeled "b," and the molecule is labeled "*S*." Cahn then published an Errata note (1964b) in which he reversed the priority assignments with the statement: "The correct procedure is to work outward by single groups along all branches simultaneously; at the first step along the branches, one finds CHOH and CH_2 in each ligand; at the second step, one finds CHCl, CH_2 on the right hand and CH_2, CH_2 on the left. Thus the right-hand ring has precedence (not the left as stated), the Cl being decisive." True, although a bit cryptic.

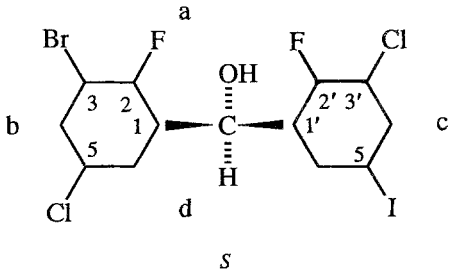
To ensure that the investigator proceeds "along all branches simultaneously," Prelog and Helmchen (1982) specified that cyclic ligands be converted into branched structures, a "tree graph," and that atoms be assigned to "spheres"; atoms of equal topological distance lie in the same sphere. One progresses outward through the spheres, analyzing all the atoms in each sphere before going on to the next. (Note that each branch is terminated by a supernumerary C atom representing C-1 or C-1′.) The ranking of each atom within an *n*th sphere "depends in the first place on the rank of the atom within the (n − 1)th sphere to which it is bonded, and only then on its rank according to the sequence rules." Cahn's molecule is treated as follows (see tree graph, Scheme 8).

In sphere I, there is no distinction between the atoms of the right-hand main branch and the left-hand main branch. The same holds true for sphere II,



SCHEME 8.

but now it is possible to distinguish between the upper and lower subbranches; in each main branch, the lower subbranch takes precedence because of the O atom. In sphere III, the higher ranking subbranches are compared with one another, there being one C and three Hs in each and thus no difference. Still in sphere III, the lower ranking subbranches are compared; the right-hand subbranch with one Cl, one C, and one H takes precedence over the left-hand subbranch with one C and two Hs. Therefore, the right-hand main branch takes precedence over the left-hand main branch—i.e., right ring > left ring— and the overall ranking around the chiral center is (a) right ring, (b) left ring, (c) CH_3, and (d) H. The configuration is *R*. Cahn apparently selected the correct subbranches on the basis of the OH groups at C-2 and C-2′ and continued along these subbranches incorrectly selecting the left-hand ring with Cl at C-4 over the right-hand ring with Cl at C-5. Construction of a tree graph and concentric spheres helps the investigator to "proceed along all branches simultaneously" (Cahn 1962b). Subsequently, Prelog and Helmchen (1982) assigned priorities to the substituents in a molecule similar to that used by Cahn (Scheme 9).

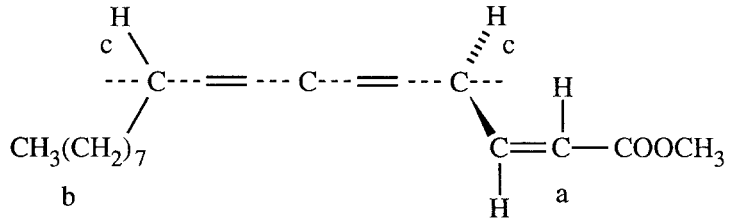


S

SCHEME 9.

Thus far, all chiral molecules in this presentation have been characterized by one or more chiral centers, but the CIP rules were extended to cover the chiral axis for such molecules as allenes and biaryls, and further to cover chiral planes. A molecule is first "factorized" by chiral centers, then, if necessary, by chiral axes and chiral planes.

The chiral axis was regarded as an extended chiral center, "the giving of one dimension to an original chiral center" (Cahn et al., 1966). For example, the putative pheromone of the "dried bean weevil" was identified by Horler (1970), and the stereochemistry was specified by Pirkle and Boeder (1978). If it is drawn as in Scheme 10, the designator is *R*.

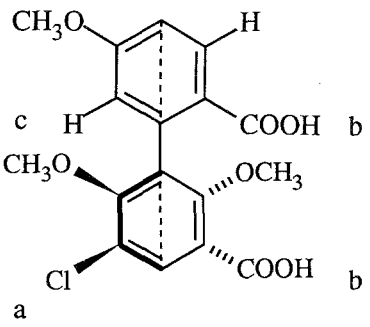


R

SCHEME 10.

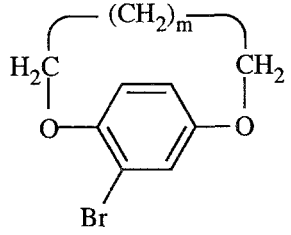
For a chiral axis to exist, it is only necessary that the substituents at each end differ from one another. Priority is assigned as usual to the ligands: a > c at the right, and b > c at the left. In accordance with subrule zero (near side precedes far side), we view down the chiral axis (dotted line) from a to c at the near side, thence to b at the far side. The spiral path is clockwise and the designation is *R*. The near side is chosen arbitrarily; the sequence b to c (near) to a (far) gives the same result. A similar treatment is used for hindered biaryls, which have not yet been described as a semiochemical, and a modified subrule zero is invoked (Cahn et al., 1966, p. 399): groups nearest together along the axis take precedence. In the example in Scheme 11, H and COOH and the two OCH_3 groups are the near groups (to the center), but since the OCH_3 groups cannot be distinguished from one another, the far groups Cl and COOH are used. The spiral sequence "a" to "b" (same ring) to "b" (other ring) stipulates *S*.

The first step in the selection of a plane of chirality is to locate the "natural" plane of symmetry that has been "desymmetrized" (Cahn et al., 1966). Such a "natural" plane of symmetry in the following simple example (Cahn, 1964a) lies in the plane of the benzene ring and is considered to be desymmetrized (faces made different) by the CH_2 chain (Scheme 12). The sequence-rule



S

SCHEME 11.



R

SCHEME 12.

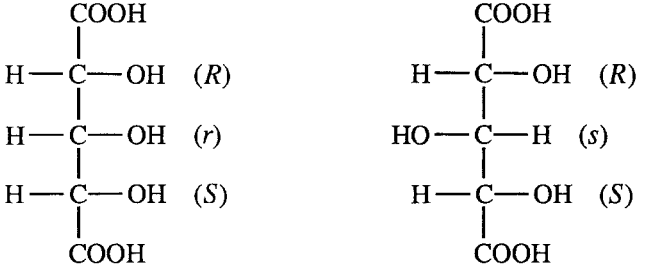
preferred atom directly attached to the plane is chosen as the pilot atom. This is determined to be the CH_2 attached to the left-hand O atom since $H_2C-O-C-CBr$ takes precedence over $H_2C-O-C-CH$. The preferred sequence in the ring from the branch point is thus through the ring atom bearing the Br atom. This path, O—C—CBr, rotates clockwise when viewed from the pilot atom, and the molecule is labeled *R*. Cahn et al. (1966, pp. 400–401) should be read for guidance in more complex molecules.

Problems in applying the CIP rules for chiral axes and chiral planes are discussed by Mislow and Siegel (1984, footnote 65), Prelog and Helmchen (1982), and Hirschmann and Hanson (1983).

The practical contributions of the CIP empirical procedures notwithstanding, a number of difficulties have been pointed out, not least of all by the three authors themselves during the long course of development of their concepts. In particular, Mislow and Siegel (1984) in a seminal paper in stereochemical theory propose that some of the basic CIP concepts are flawed, although acknowl-

edging the enormous utility of the procedures. In particular, they propose that the CIP concept of chirality actually embodies two separate and distinct concepts: local geometry (symmetry properties) and stereoisomerism. Mislow and Siegel use the term "chirotopicity" to refer to local geometry: Atoms that reside in a chiral environment are termed "chirotopic," if in an achiral environment, "achirotopic," the distinction resting on symmetry considerations. (In fact, all atoms and spaces in a chiral molecule are chirotopic.) They use the term "stereogenicity" to refer to the effect of interchange of two ligands of a tetrahedral carbon atom in a molecule: If such interchange results in stereoisomers (enantiomers or diastereomers), the carbon atom is referred to as "stereogenic," the converse term being "nonstereogenic." Thus, the carbon atom in the chiral molecule CHBrClF is chirotopic and stereogenic, whereas the carbon atom in the achiral molecule CH_2BrCl is achirotopic and nonstereogenic. It is this remarkable linkage of properties in most, but not all, tetrahedral carbon atoms that accounts for the enormous practical success of the CIP procedures.

Difficulties in the CIP concepts arise with the definition of "different" in the familiar description of an asymmetric atom as having four "different" substituents. In the CIP definition (see under Basic Stereochemical Conventions, No. 3), "different" means constitutionally different or diastereotopically different; enantiotopic differences are specifically excluded. The term "pseudoasymmetry" was coined to describe an atom such as C-3 in the two *meso* forms of trihydroxyglutaric acid in which the designators *r* and *s* are used for C-3 bearing enantiotopic substituents (Scheme 13). The molecules in Scheme 13 are achiral by virtue of a plane of symmetry through C-3. By definition, C-3 cannot be described as chiral or asymmetric; yet it has the property whereby an interchange of ligands results in a diastereomer.



Achiral Molecule Achiral Molecule

(Fischer projections)

SCHEME 13.

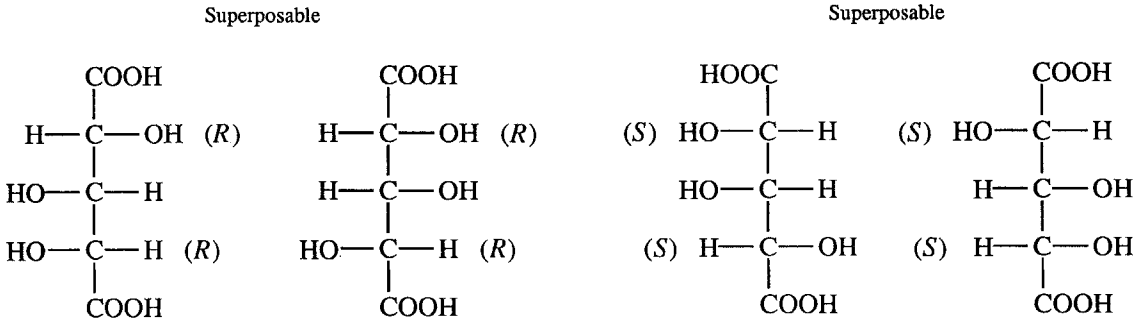
Note that the terms diastereotopic and enantiotopic refer to "attached" substituents (ligands), that is, within the molecular environment. The terms diastereomorphic and enantiomorphic have been used by other authors to refer to substituents in "isolation," that is, free of the molecular environment. The terms diastereomeric and enantiomer refer to whole molecules. Note further that, although enantiotopic substituents in $C_{+a,-a,b,c}$ are not "different" in the sense of producing an asymmetric center, they are nonetheless distinguishable by chiral reagents (such as enzymes) or by NMR in a chiral environment.

In the two mirror image (enantiomeric) chiral forms of trihydroxyglutaric acid (Scheme 14), the molecules have no element of symmetry. C-3 is chiral (asymmetric) since C-2 and C-4 are not interchangeable through a symmetry operation and are therefore diastereotopic ("different"). Yet interchange of H and OH (or of the other substituents) on C-3 gives an identical molecule for each of the two chiral enantiomers (superposable by in-plane rotation). The CIP procedures do not yield an *R* or *S* (or *r* or *s*) designator for C-3, nor is there need for one since interchange results in identical molecules.

These inconsistencies and points of perennial confusion are resolved by the proposal of Mislow and Siegel (1984) to use the separate properties of chirotopicity and stereogenicity (see above). Thus, in the achiral (*meso*) molecules of trihydroxyglutaric acid, C-3 is stereogenic since interchange produces the diastereomer, but it is not chirotopic. In the chiral molecules, C-3 is not stereogenic, but it is chirotopic. In both cases the usual linkage of these two properties fails. Mislow and Siegel further comment that "the term 'pseudoasymmetric' therefore lacks any meaningful reference to symmetry and geometry. It is seen to be an artifact of an unwarranted superposition of stereogenicity onto local chirality." They argue further that *R* and *S* in the CIP system "solely serve to identify isomers and have no bearing whatever on symmetry relationships among or within molecular models. It is therefore inappropriate to refer to them as 'chiral descriptors'." Thus, in the *meso* trihydroxyglutaric acids, "the chiral descriptors *r* and *s* are applied even though the atoms are achirotopic. These are really nomenclatural devices that bear no relationship to the local symmetry of the atoms to which they refer." Prior to the Mislow and Siegel paper, Prelog and Helmchen (1982) identified these elements as stereogenic units. Mislow and Siegel emphasize the point that "paradoxically, the selection of 'elements of chirality' in a molecule do not depend on local or molecular chirality."

This brief description hardly does justice to the paper by Mislow and Siegel, which should be read in its entirety, along with the interpretive article by Maugh (1984).

In a recent paper, Eliel (1985) proposed a convenient mnemonic for assigning *R* or *S* descriptors to three-dimensional formulas. He also discussed several


Superposable
COOH
H—C—OH (R)
HO—C—H
HO—C—H (R)
COOH
COOH
H—C—OH (R)
H—C—OH
HO—C—H (R)
COOH
Identical on C-3 interchange
Superposable
HOOC
(S) HO—C—H
HO—C—H
(S) H—C—OH
COOH
COOH
(S) HO—C—H
H—C—OH
(S) H—C—OH
COOH
Identical on C-3 interchange
(Fischer projections)

SCHEME 14.

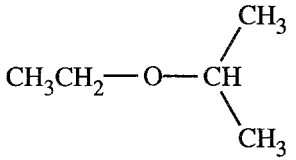
of the changes—many of them esoteric—in the CIP system proposed by Prelog and Helmchen (1982).

The related topic of prochirality (prostereoisomerism) cannot be covered in this review. Reference is made to the extensive discussion by Eliel (1982).

SYMMETRY CRITERIA IN NMR FOR STRUCTURE ELUCIDATION

In their general chapter entitled "Stereoisomeric Relationships of Groups in Molecules," Mislow and Raban (1967, pp. 12–30) included a section on symmetry criteria in NMR spectrometry, which together with subsequent papers by Eliel (1980), Silverstein and Silberman (1973), and Silverstein and LaLonde (1980), provide the chemist involved with interpretation of NMR spectra with the basic concepts; these are briefly summarized in the present section as a series of definitions as concisely as possible.

The familiar Pople notation for spin systems—AB, AX, AX_3, etc.—is based on the concept of sets of nuclei within a spin system. An AX type system consists of "loosely coupled" sets (the ratio of the chemical shift difference to the coupling constant J, both in Hz, is much greater than about 10), ($\Delta\nu/J >> \sim 10$), whereas the AB type consists of tightly coupled sets ($\Delta\nu/J << \sim 10$). A *set* of nuclei consists of chemical shift equivalent nuclei. A *spin system* consist of sets of nuclei that spin couple only with one another. Spin systems are "insulated" from one another (e.g., the ethyl protons and the isopropyl protons in ethyl isopropyl ether comprise two spin systems) (Scheme 15).



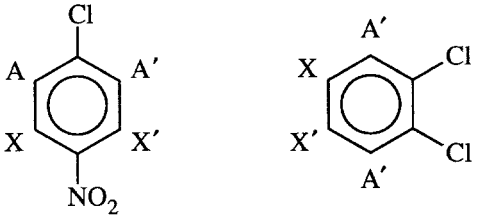
SCHEME 15.

Chemical shift equivalent (*isochronous*) nuclei are interchangeable through a symmetry operation or a rapid mechanism (i.e., rapid on the NMR time scale). They have the same chemical shift in an achiral environment. Nuclei are interchangeable if the structures before and after the operation are indistinguishable. The symmetry operations are rotation around a simple axis of symmetry (C_n), reflection through a plane of symmetry (σ), inversion at a center of symmetry (i), or rotation around an axis of symmetry followed by reflection in a plane normal to this axis (S_n), the alternating axis of symmetry. Actually, σ and i are

equivalent to S_1 and S_2, respectively. The symmetry element must be one for the entire molecule.

Nuclei that are interchangeable through a simple axis of symmetry (homotopic) have the same chemical shift in both achiral and chiral environments. Nuclei that are interchangeable through any other symmetry operation but not in addition to a C_n operation (enantiotopic), are chemical shift equivalent only in an achiral environment. Nuclei that are not interchangeable through a symmetry operation or through a rapid mechanism (either diastereotopic or constitutionally heterotopic) are not chemical shift equivalent in any environment. Nuclei that are interchangeable through a rapid mechanism are chemical shift equivalent in any environment. Nuclei that fortuitously absorb at the same shift are sometimes classified as chemical shift equivalent, but such coincidence is neither absolute nor fundamental, depending as it does on instrumental resolution.

A further refinement involves the concept of *magnetic equivalence*. If nuclei in the same set (i.e., chemical shift equivalent nuclei) couple equally to any (probe) nucleus in any other set in the spin system, they are magnetic equivalent, and the simple Pople designation (A_2X_2 for example) applies. If not, the designation AA'XX' is used in place of A_2X_2, and one should not necessarily expect first-order spectra. The proton spectra of *p*-chloronitrobenzene or *o*-dichlorobenzene (the common agent for determining instrument resolution) are complex, and the ring proton spin system is described as AA'BB' or AA'XX', depending on instrument resolution and on the difference in chemical shifts (Scheme 16).



SCHEME 16.

METHODS FOR DETERMINING ENANTIOMER COMPOSITION AND ISOLATION

A plant or an animal may produce a chiral compound as a single enantiomer or as a mixture of enantiomers in any ratio. A laboratory synthesis using achiral starting materials and reagents produces a racemate that must, with rare

exceptions, be resolved with a chiral reagent, which may be in the form of a substrate of a gas or liquid chromatographic column. A stereoselective synthesis yields a predominance of one of the enantiomers. The chapter on "the significance of chirality" by Mori (1984) is suggested reading; a comprehensive table of chiral insect pheromones is included.

Determination of enantiomeric composition of a minute quantity of a chiral compound isolated as an insect pheromone, for example, is not a trivial operation. Frequently the operation is avoided in favor of synthesizing the pure enantiomers and challenging the insect with a gamut of ratios, on the reasonable assumption that the insect will respond most favorably to the ratio perceived under natural conditions; however, this procedure is neither accurate nor rigorous. The synthetic approach may also yield the absolute configurations of the chiral elements.

The methods for determining enantiomeric composition are (Silverstein, 1985):

1. Polarimetry
2. NMR (^{1}H and ^{13}C) with chiral shift reagents, chiral derivatizing agents, or chiral solvating agents
3. Achiral chromatography (gas or liquid) of diastereomeric derivatives
4. Chiral chromatography (gas or liquid) of the enantiomers directly
5. X-ray crystallography of a derivative prepared with a chiral reagent
6. Bioassay of synthetic enantiomers (as mentioned above neither accurate nor rigorous)

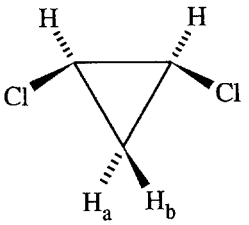
The basic requirements for polarimetry are a relatively large sample, a relatively high specific rotation, and a knowledge of the specific rotation of the pure enantiomer under identical conditions of temperature, concentration, and solvent; these are severe limitations for semiochemical studies. Comparison of determined values with those in the literature is often risky.

At the other extreme—small sample size, no derivatization, and no need for pure reference samples—is gas or liquid chromatography by complexation on chiral columns. However, these procedures are quite new. Column choice is limited, temperature constraints (for GC columns) are severe, and resolution is often modest and limited to particular chemical classes. On the other hand, successful resolution of compounds that would be difficult to derivatize, such as bicyclic acetals (Weber and Schurig, 1981), makes this an extremely useful approach that continues to undergo development.

Gas and liquid chromatography on achiral columns of derivatives with chiral reagents are the most widely used techniques. A functional group and an appropriate derivatizing reagent are necessary. As a single example, Mosher's reagent for alcohols, (+)- or (−)-α-methoxy-α-trifluoromethylphenylacetyl

chloride (MPTA Cl) is available at high purity (as the carboxylic acid) for both enantiomers and conspicuously lacks an epimerizable center that makes most other acid chloride reagents less attractive. However, the low vapor pressure of many of these derivatives places limitations on gas chromatography. Liquid chromatography, of course, is used for such low-vapor-pressure derivatives, and the development of HPLC techniques in recent years has been rapid. Two very useful reviews are available for determination of enantiomeric composition and isolation of enantiomers by gas (Schurig, 1983) and liquid (Pirkle, 1983) chromatography.

Plummer et al. (1976) and Stewart et al. (1977) reported the first use of a chiral shift reagent and of a chiral derivatizing agent with [^{1}H]NMR spectrometry to determine the enantiomeric composition of several isolated pheromones. Use of a chiral shift reagent requires a functional group that is a strong enough base to form a stable complex, sufficient resolution to separate the corresponding peaks in each diastereomeric complex, and freedom from interfering peaks. The ideal protons are those of an uncoupled CH_3 group near the functional group; a similarly situated, uncoupled CH group, although less intense, is also useful. Protons of a methylene group can be troublesome; they may be enantiotopic as in 1-nitropropane, for example, and thus chemical shift equivalent in an achiral environment but not so in the presence of a chiral shift reagent. Protons of a methylene group may also be diastereotopic and thus not chemical shift equivalent in any environment (Scheme 17, protons a and b). Separation



SCHEME 17.

of absorptions of chiral derivatives is further enhanced by addition of an achiral shift reagent; for example see Iwaki et al. (1974). It is worth mentioning that use of the Mosher derivative in NMR spectrometry has a unique advantage since the fluorine NMR spectrum shows the CF_3 peaks free of interferences. Note that the small coupling with the methoxy protons results in slight broadening for the CF_3 group in each enantiomer.

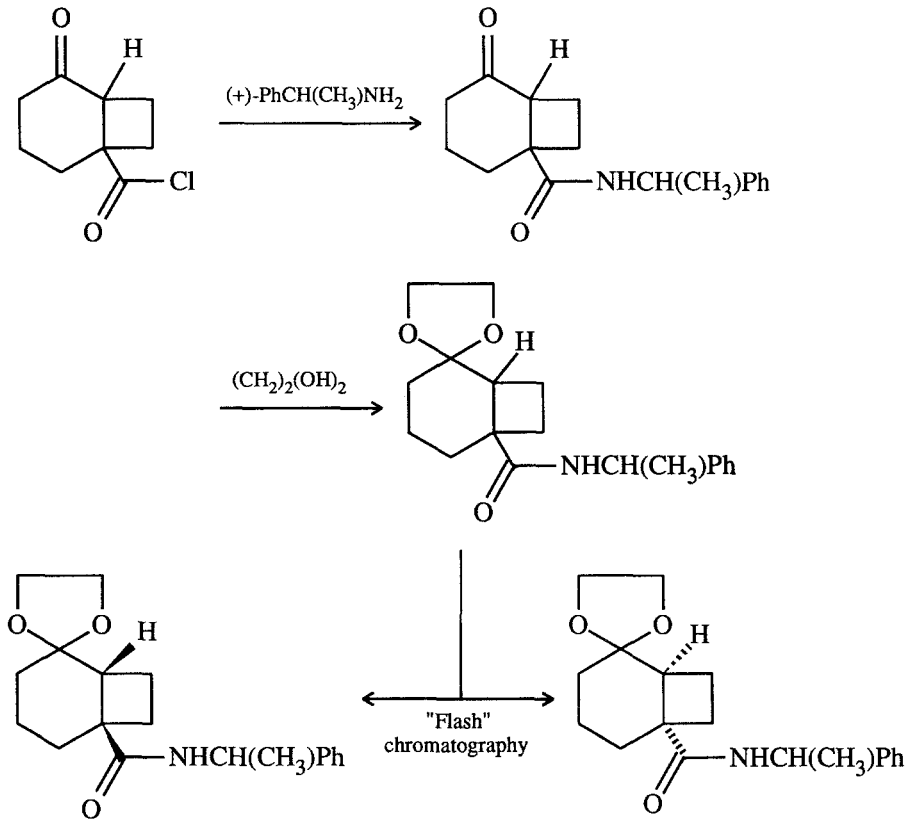
Chiral solvating reagents (Gaudemer, 1977) in NMR spectrometry have been less widely used than chiral shift reagents. However, Ravid et al. (1978)

employed this technique to determine enantiomeric composition of a γ-lactone pheromone.

X-ray crystallography was recently used by Mori et al. (1982) on a crystalline derivative produced with a chiral derivatizing reagent to determine the absolute configuration of a pheromone. Generally, however, absolute configuration is determined by synthesis from starting materials whose absolute configuration is known.

Enantiomers can be separated (resolved) on a preparative basis by scaling up the chromatographic techniques for determining enantiomeric composition. The limitations are, of course, column capacity and the need to regenerate the enantiomers from their diastereomeric derivatives. Details will not be discussed here, but four comprehensive references on resolution are cited (Newman, 1987; Wilen et al., 1977; Klyne and Buckingham, 1978 and its supplement by Buckingham and Hill, 1986). It might be mentioned here that, on a large scale, resolution of a carboxylic acid by recrystallization of its salt formed with an amine enantiomer is frequently the method of choice. Regeneration of the salt to recover both the enantiomer and the amine is simple and economical, but following the course of resolution by regenerating the enantiomer at each step and determining the optical rotation is tedious and unreliable. Webster et al. (1982) demonstrated the utility of [^{13}C]NMR spectrometry of the amine salt directly to follow the course of resolution; this is a direct procedure that does not depend on a pure enantiomer as a reference compound. The recrystallization procedure is not applicable, of course if the salts do not crystallize, or if the recrystallization process is inefficient.

One example of large-scale resolution and proof of enantiomeric purity will suffice to illustrate some of the problems encountered (Scheme 18): During the preparation of optically pure enantiomers of grandisol, Webster and Silverstein (1986) were unable to obtain satisfactory crystalline salts of the intermediate racemic carboxylic acid. Treatment of the corresponding acid chloride with (*S*)-(−)-α-methylbenzylamine gave the diastereomers of the keto amide which, however, could not be resolved by either recrystallization or HPLC. Finally, the acetal was readily resolved by large-scale flash chromatography followed by facile recrystallization to >99% purity for each diastereomer (HPLC analysis). Although this procedure was very satisfactory, it was necessary to devise a novel procedure for cleavage of the highly hindered amides, standard procedures having failed (Webster and Silverstein, 1986). The secondary amides were cleaved with Li/NH_3 to the primary amides, which were then hydrolyzed to the carboxylate with base. The latter step was acceptable since there was no risk of epimerization of the quaternary bridgehead carbon atom alpha to the carboxyl group. Olah's reagent, nitrosonium tetrafluoroborate in acetonitrile (Olah and Olah, 1965), proved very useful for cleavage of a hindered epimerizable amide (Webster et al., 1986).



SCHEME 18.

METHODS FOR STEREOSELECTIVE SYNTHESIS

It is impossible in this brief chapter to cover the rapidly developing field of stereoselective synthesis. Reference is made to the continuing series edited by Morrison (1983–1985), to a recent review by Masamune et al. (1985), and to a recent textbook by Coppola (1987).

PRINCIPLES AND METHODS OF BIOSYNTHESIS

This subject is thoroughly addressed in several chapters in a very recent book edited by Prestwich and Blomquist (1987).

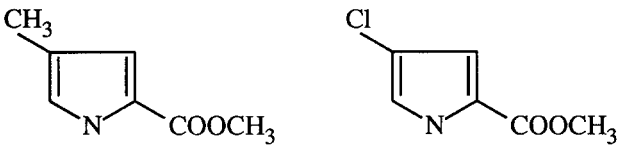
THE RELATIONSHIP BETWEEN STRUCTURE AND BIOACTIVITY

Chemists and biologists have long been intrigued by the relationships between chemical structure and bioactivity. The chemist classifies structures hierarchically from most to least "different":

I. "Different" compounds—different molecular formulas
II. Isomers—same molecular formula
  A. Constitutional isomers—atoms joined in different orders
  B. Stereoisomers—atoms joined in the same order but arranged differently in space
    1. Diastereomers—not mirror images
    2. Enantiomers (optical isomers)—nonsuperposable mirror images

But this graduation of "different" is dubious, possibly resulting from the chemist's inability to distinguish enantiomers with achiral laboratory tools. Insofar as bioactivity is concerned, one would a priori predict that the receptor cells with their chiral elements should distinguish between enantiomers. It turns out that insects, on which some elegant studies have been done, distinguish between compounds at every level, including enantiomers (see the very recent chapter by Tumlinson, 1987). It also seems reasonable to predict that within each category it may not be possible with present devices and techniques to detect whatever differences in bioactivity exist if the differences are small. Thus, at the enantiomer level, a long-standing controversy over the odors to humans of (+)- and (−)-carvone persisted because of the presence of impurities or because of poor experimental procedures. The issue was finally settled by overkill in 1971 when three groups, using highly purified samples, determined independently that (*S*)-(−)-carvone has a spearmint odor and is detectable at a lower threshold than the antipode, which has a caraway odor (Friedman and Miller, 1971; Russell and Hills, 1971; Leitereg et al. 1971).

In the first category, compounds grossly different in structure—*D*-glucose and propane for example—surely elicit different responses. On the other hand, the trail-following pheromone isolated from an ant and identified by Tumlinson et al. (1980) was mimicked in a laboratory bioassay by the 4-chloro analog (Sonnet and Moser, 1972) (Scheme 19). One may, of course, argue justifiably that a laboratory assay is not the same thing as a field bioassay in the natural context of the insect population and that, with more extensive testing, differences would have been found. Very likely. In summary then, an organism may, and usually does, distinguish between two compounds in any category. We usually can perceive the difference by means of a bioassay, but sometimes we do not. The usual but not inevitable consequence of a small change from the



Pheromone Analogue

SCHEME 19.

natural molecule in molecular structure in any category is a marked decrease in the sought response.

These effects at the level of enantiomers have been of great interest in the field of insect pheromones (Mori, 1984; Silverstein, 1979, 1984, 1985). If an insect produces a single enantiomer, the following responses elicited by the other enantiomer (an artifact) are possible (Silverstein, 1979): (A1) less active or inactive; (A2) equally active; (A3) more active; (A4) synergistic; and (A5) inhibitory.

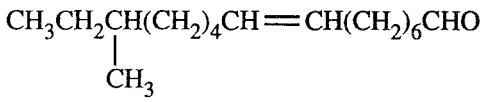
If both enantiomers are present, the following responses are possible: (B1) the insect responds optimally to the natural ratio; (B2) the insect responds more strongly to one enantiomer than to the other or to the natural ratio; (B3) the insect responds equally to each enantiomer and to all ratios; and (B4) one enantiomer inhibits the response to the other.

Where the insect produces a single enantiomer, one would expect response A1 to be most common, response A5 possible, and a priori, the other responses to be unlikely. In fact, almost all examples do fall under responses A1, and there are several examples (unexpected at the time) under response A5. Tumlinson et al. (1980) showed in field tests that only a few percent of the wrong enantiomer greatly decreased the response to the naturally occurring enantiomer in the Japanese beetle. Lanier et al. (1980) and Birch et al. (1980) demonstrated that the Idaho population of *Ips pini* produces and responds to (−)-ipsdienol; this response is inhibited by (+)-ipsdienol, which occurs in the New York population in a 65% (+):35% (−) ratio, and also occurs as a pheromone component of *Ips paraconfusus*, a competing species of the Western population of *Ips pini*. Only 5% of (+)-ipsdienol virtually blocked the response of the Idaho population to (−)-ipsdienol. It thus appears that pheromonal specificity at the enantiomeric level is a principal isolation mechanism among sympatric species of *Ips* (Birch et al., 1980). The optimal response of the New York population was to the natural ratio (category B1).

The boll weevil response has been reported to be in A2 (Mori et al. 1978), but this claim has been disproved by a recent thorough investigation of electrophysiological and field responses to samples of chemically and enantiomerically pure (+)-grandisol and (−)-grandisol; field studies were carried out in the pres-

ence of the other pheromone components (personal communication from Joseph C. Dickens, Aug. 10, 1987).

Early experiences with enantiomers involved compounds whose chiral center was directly attached to, or close to, the functional group. Thus, it was of some interest to compare the biological activity of the stereoisomers (14*R*, 8*Z*; 14*S*, 8*Z*; 14*R*, 8*E*; 14*S*, 8*E*) of trogodermal against four *Trogoderma* species (Silverstein et al., 1980) (Scheme 20).



SCHEME 20.

Briefly, the results demonstrated the high specificity associated with the chiral center far distant, in terms of number of bonds, from a functional group. The *R* enantiomer(s) was more active by two orders of magnitude. Our mild surprise was probably conditioned by experience with chiral shift reagents where distance (and angle) between the chiral center and the functional group is valid for predicting the effect. Obviously, such extrapolation to a chiral molecule at a chiral antennal receptor is not useful. Removal of the branched methyl group resulted in a 100- to 1000-fold loss of activity in comparison with the racemic branched compound.

In addressing the topic of "chirality," I have reviewed briefly several areas of stereochemistry that are essential for the natural-products chemist. The pedagogical approach was adopted to impart some appreciation of the complexities involved and especially for the extraordinary contributions of Cahn, Ingold, and Prelog. A brief section introduces some recent advances in stereochemical theory.

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OLFACTORY CODING IN THE PERCEPTION OF SEMIOCHEMICALS[1]

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Abstract—Information processing in the olfactory pathway underlying the perception of semiochemicals by insects is discussed. Both the chemical message for mates and the message for food consist of blends of chemicals. Olfactory receptors in an insect species are tuned to the detection of those compounds which comprise such chemical messages for that species. The classification of receptors as specialists or generalists coincides with two concepts of information processing, i.e., labeled lines and across-fiber patterns, respectively. The olfactory code coming from antennal receptors in *Pieris brassicae* larvae is a combination of labeled lines and across-fiber patterning. When antennae of adult Colorado potato beetles, *Leptinotarsa decemlineata*, are stimulated by binary mixtures of leaf odor components, the pattern of neural activities in the olfactory receptors shows some separation into two channels, quantitative versus qualitative detection. The separation is complete in the antennal lobe of this beetle.

Key Words—Colorado potato beetle, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, *Pieris brassicae*, Lepidoptera, Pieridae, olfactory coding, information processing, olfactory receptors, antennal lobe, response profiles, stimulus mixtures, pheromones, host plant odor, semiochemicals.

INTRODUCTION

The insect's perception of semiochemicals underlies the message for finding mates or food. On their release in the atmosphere from a calling female or a

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plant, volatile semiochemicals are transported by the wind and comprise chemical messages for insect receivers. Downwind of the source one insect species responds obviously to the message, as observed in individuals which fly or walk upwind towards the source (Visser, 1988). On the other hand, other insect species do not respond to the same message and maintain their original courses. Although all chemical messages occupy the same information channel, i.e., the air, the "tune" of a particular message is not immediately drowned out by "background music."

Both the chemical message for mates and the message for food consist of a blend of chemicals. Sex pheromones are the class of semiochemicals that has been predominantly studied. Their multicomponent nature is well known (see Arn et al., 1986). Host-plant odors possess an even more complicated blend composition than these odors and, to a large extent, consist of compounds which are generally distributed among plant species (Visser, 1986).

It should be realized that an insect searching for mates or food is facing a complex chemical world. The complexity resembles a puzzle with the many words being the individual compounds, and only some words constitute a phrase, i.e., the proper blend composition. Moreover, an insect solves the puzzle in just a few seconds; that is the time one generally observes an insect takes to change its motor pattern in response to an attractive odor.

The present paper will discuss features of information processing, starting in the olfactory receptors that are thought to be essential for the recognition of a chemical message.

THE OLFACTORY PATHWAY

Upon binding of molecules with acceptor sites on the dendritic membranes of an olfactory receptor cell, its spontaneous neural activity shows an increase (excitation) or decrease (inhibition) (Visser, 1986). The olfactory receptor cells in insect antennae send their axons directly to the brain, where the first relay station is found in the glomeruli of the antennal lobe (Figure 1) (Boeckh et al., 1984; Matsumoto and Hildebrand, 1981; Boeckh and Ernst, 1987). Thus, on stimulation with an odor, the neural activity in the whole array of antennal olfactory receptor cells changes. The change of activity in all receptors is considered as the olfactory code which contains information about odor quality, i.e., the blend composition, as well as odor intensity, i.e., the overall concentration.

The information is further processed in the antennal lobe by a complicated network of interneurons. In adult insects, the number of output neurons leaving the antennal lobe is relatively small compared to the number of input antennal fibers. This input convergence results in amplification of the original signal as

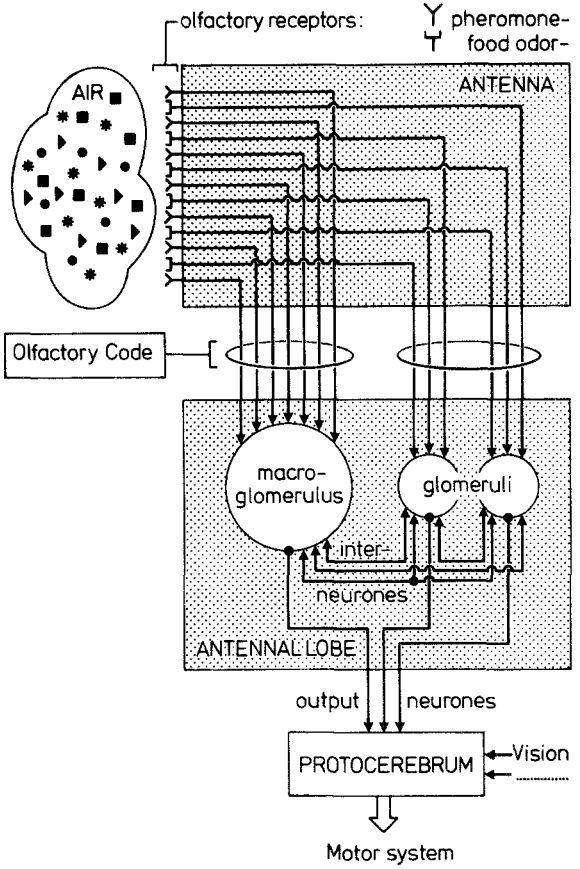

FIG. 1. The olfactory pathway in adult insects. See text for further explanation.

well as a reduction of background noise (Boeckh et al., 1984; Visser, 1986). In other words, at the level of the antennal lobe the neural activities of individual neurons are already modified at stimulus concentrations which are 100- to 1000-fold lower than those needed for a significant change in neural activities of peripheral receptors. In the protocerebrum, the olfactory information is integrated with other sensory modalities, and output elements are connected with the motor system controlling the insect's motor patterns (Figure 1) (Boeckh and Ernst, 1987).

The present discussion will focus further on the specificity of olfactory receptor cells. Their characteristics for the detection of relevant compounds, the relevant "words," underlie the recognition of the blend composition, the chemical message.

SPECIALISTS AND GENERALISTS

In early studies on the neural responses of individual olfactory receptors, the conclusion was reached that receptor neurons could be classified as either specialists or generalists (Boeckh et al., 1965). Specialists are narrowly tuned to the reception of one particular compound, whereas broadly tuned receptors, those neurons showing responses to several compounds, are called generalists.

Specialists. After the discovery of the classical specialist, the bombykol receptor of *Bombyx mori*, other pheromone receptors were treated in the same way. One class of olfactory receptors in males of the redbanded leafroller moth, *Argyrotaenia velutinana*, responds to *cis*-11-tetradecenyl acetate; the second class of receptors responds mainly to the second pheromone component *trans*-11-tetradecenyl acetate (O'Connell, 1975). A similar conclusion was reached for the detection of the two main pheromone components of the summerfruit tortrix moth, *Adoxophyes orana*: one class responding to *cis*-9-tetradecenyl acetate, the other class responding mainly to *cis*-11-tetradecenyl acetate, and, to a much smaller extent, to *cis*-9-tetradecenyl acetate (Den Otter, 1977).

In the Noctuidae and Tortricidae, families whose sex pheromones are studied most extensively, it appears that an N component pheromone blend is detected by N different receptors (Priesner, 1986). The specificity of these moth antennae to sex pheromone components even permits their application as an electroantennographic detector for the identification of pheromone components of other moth species (Guerin et al., 1985). In addition, specialist receptors are found in other insect groups. For example, in males of the American cockroach, *Periplaneta americana*, two receptor types are present in the same sensillum: one type specialized for the detection of the sex pheromone component periplanone A, and the second type responds to periplanone B (Sass, 1983).

Generalists. Generalist receptors respond to food odors. In contrast with the uniformity of specialists, the generalists show a wide differentiation in their reaction spectra. Receptor cells in the sensilla basiconica of the silk moth, *Antheraea pernyi*, possess unique response profiles, although their spectra overlap considerably (Schneider et al., 1964). Generalist receptors of the blowfly, *Calliphora vicina*, were divided into meat- and flower-odor receptors, and a further separation revealed six and three types, respectively (Kaib, 1974).

Occasionally, response profiles of the same receptors were interpreted differently. Thus the neurons in sensilla placodea on antennae of the honeybee, *Apis mellifera*, which were reported as true generalists (Lacher, 1964), were later arranged into seven reaction groups since the spectra of each showed little or no overlap (Vareschi, 1971). Cluster analysis of the response spectra of antennal receptors in the Colorado potato beetle, *Leptinotarsa decemlineata*, separated these generalists into five receptor types (Ma and Visser, 1978). However, on close observation of the neural activities in these receptors to three

components of potato leaf odor, the interpretation had to be altered. The sensitivities for these components is gradually distributed over the population of olfactory receptors (Visser, 1983). Generalist receptors in *Periplaneta americana* were also divided into reaction groups, like the pentanol, hexanol, octanol, decanol, and dodecanol groups, although their reaction spectra clearly overlapped (Selzer, 1984).

Both the analysis of electrophysiological data and the selection of odorous stimuli contain arbitrary elements. Furthermore, in most cases the classification of receptor cell responses showing certain degrees of specialization is hampered by the absence of knowledge concerning the food-odor compounds (and their concentrations) that are biologically relevant for the insect species under study. Our knowledge about the chemistry of food odors is very incomplete, and, thus, behavioral experiments with food-odor blends of defined compositions were rarely performed. Hence, the characterization of the response profiles of olfactory receptors to represent a specialist or a generalist should proceed with some restraint (Vareschi, 1971; O'Connell, 1975; Selzer, 1984; Visser, 1986).

Nevertheless, it appears that in several insect species pheromone- and food-odor information are processed through two different channels up to the level of the protocerebrum. Pheromone receptors in moths and the American cockroach are exclusively connected with the macroglomerulus, whereas food-odor receptors contact interneurons in the smaller glomeruli (Figure 1) (Boeckh et al., 1984; Matsumoto and Hildebrand, 1981; Christensen and Hildebrand, 1987; Boeckh and Ernst, 1987).

OLFACTORY CODE

The perception of a chemical message starts in the olfactory receptors. The peripheral receptors in an insect species are tuned to the detection of those compounds which comprise a chemical message for that species and thus release vital behaviors like mate and food finding. In addition, the receptors in a species should neglect irrelevant messages such as those used by other species. The response profiles of individual receptors, as discussed in the foregoing section, indicate these abilities. Furthermore, the classification of receptors as specialists or generalists coincides with two concepts of information processing in the olfactory pathway. Besides the detection of relevant compounds by the receptors, the perception of blend composition in the central nervous system underlies the recognition of a chemical message.

The concept of labeled lines involves specialist receptors, each responding to one compound of the chemical blend (Figure 2). The discrimination of blend composition in the central nervous system would be a simple comparison between neural activities in separate channels. On the other hand, an across-

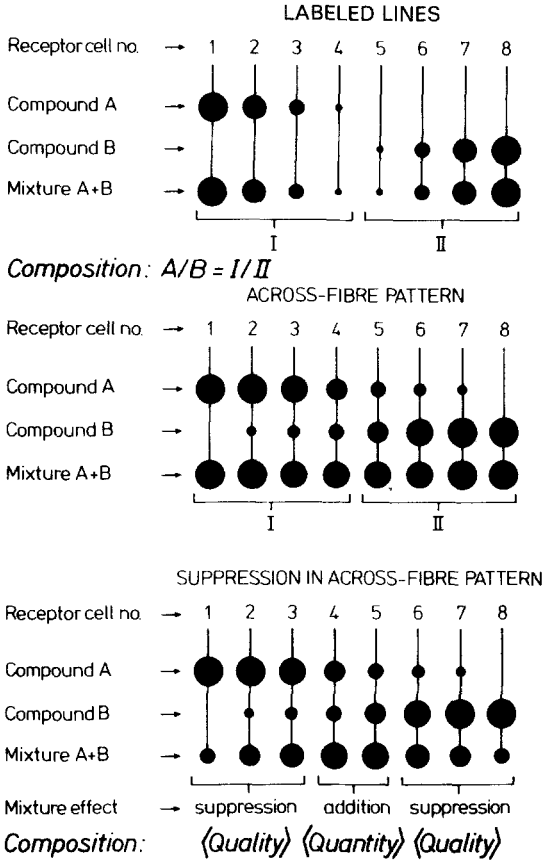

FIG. 2. Concepts of information processing in the olfactory pathway. The neural activities of olfactory receptors in response to compound A or B, and to their 1 : 1 mixture are visualized in the areas of circles. See text for further explanation.

fiber pattern would require a complicated evaluation by the central nervous system of neural activities in the whole array of olfactory receptors.

Figure 2 contains a rather simplified representation of an across-fiber pattern: generalist receptors responding to two compounds. Actually, the pattern becomes complicated when the addition of a third compound generates a certain distribution of neural activities in the same olfactory receptors. Hence, each compound adds a new dimension to the profile of activities. A natural food odor thus generates a particular pattern of neural activities across the fibers of olfactory neurons running towards the brain. The composition of such a blend would

be recognized when its across-fiber pattern matches some template present in the central nervous system.

In adult insects, the recognition of a chemical message involves the concerted action of thousands of olfactory receptors (Boeckh et al., 1984; Visser, 1986; Boeckh and Ernst, 1987). Therefore, we may wonder whether we can grasp eventually the complete olfactory code. Chemoreception in caterpillars, on the contrary, is based upon a small number of sensory neurons.

Olfactory Code in Pieris brassicae *Larvae.* A caterpillar antenna bears, on its second segment, two large sensilla basiconica, i.e., the medial and lateral sensillum basiconicum. In addition, its third segment bears a large sensillum basiconicum. The number of neurons associated with these three sensilla is seven, five, and four, respectively (Schoonhoven and Dethier, 1966). Electrophysiological recordings in larvae of *Manduca sexta*, *Hyalophora gloveri* (Schoonhoven and Dethier, 1966; Dethier and Schoonhoven, 1969), and *Malacosoma americanum* (Dethier, 1980) indicated that these 16 neurons represent the full complement of olfactory receptors in one antenna.

Previous studies on responses of these olfactory receptors involved stimulations with chemically undefined leaf odors and some individual chemicals at high doses. These kinds of experiments were repeated in fourth-instar *Pieris brassicae* larvae (Visser and Schepers, unpublished data) with a set of biologically relevant and chemically defined stimuli, i.e., constituents of the "green odor" (Visser et al., 1979), and allylisothiocyanate, a host-specific compound (Figure 3).

Methods of odor delivery were as previously described (Ma and Visser, 1978), and chemicals were diluted at the source in paraffin oil (10^{-2} and 10^{-1} v/v). Glass electrodes were inserted into antennae either at the base of the medial sensillum basiconicum on the second antennal segment (Figure 3, I) or at the base of the large sensillum basiconicum on the third antennal segment (II). Individual units were identified by measuring their spike amplitudes. On stimulation of the preparation with the series of single compounds, individual olfactory neurons showed various degrees of excitation or inhibition of their spontaneous firing activities. Temporal patterns like those reported for *Manduca sexta* (Schoonhoven and Dethier, 1966; Dethier and Schoonhoven, 1969) were never observed in the recordings; stimulations caused regular phasic–tonic responses.

The changes in neural activities of 23 neurons in response to the test chemicals were measured for the stimulation period (0.5 or 1 sec) and were expressed relative to the response of the cell's best stimulant (Figure 3). Identical response profiles were grouped in a spectral type. The grouping resulted in six spectral types for the medial sensillum (A–F) and two spectral types for the sensillum on the antennal headpiece (G and H).

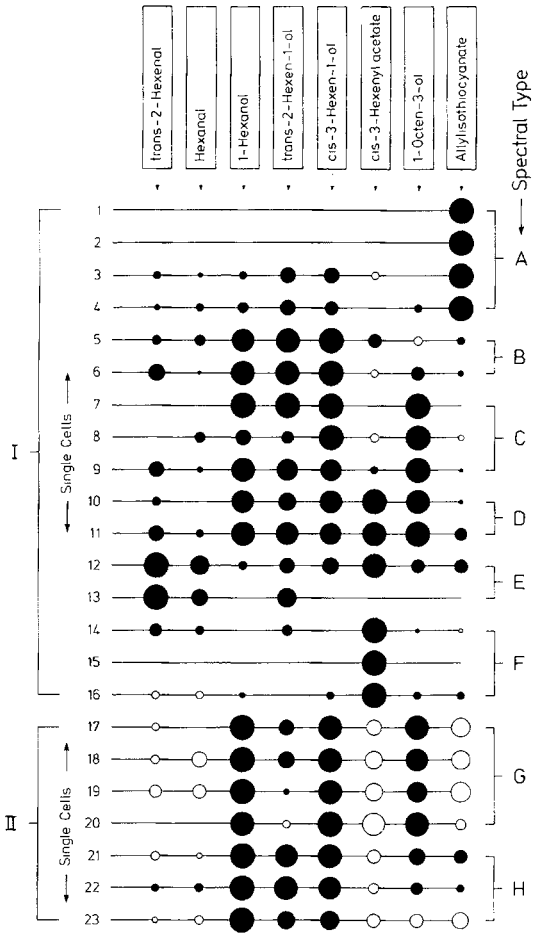

FIG. 3. Relative response spectra of olfactory receptors in *Pieris brassicae* larvae, visualized in the areas of circles. Excitation shown as filled circles, inhibition as open circles. Neurons are associated with either the large medial sensillum basiconicum on the second antennal segment (I) or the large sensillum basiconicum on the third antennal segment (II). See text for further explanation.

Since those sensilla in one antenna possess seven and four neurons, respectively (Schoonhoven and Dethier, 1966), each spectral type is considered as an individual neuron, its response being recorded repeatedly. Thus, the eight spectral types represent an across-fiber pattern in half of the total neuron population. The pattern contains specialist receptors for allylisothiocyanate (Figure 3A) and

cis-3-hexenyl acetate (F), as well as generalist receptors, e.g., the alcohol receptor (C). Hence, the olfactory code coming from this simple receptor system is a combination of labeled lines and across-fiber patterning.

Although most research on olfactory coding has involved the assessment of response profiles in populations of sensory neurons to chemicals applied singly, it remains to be determined whether such patterns of neural activities resemble olfactory codes produced on stimulation with complete blends. In an effort to answer this, we recorded the neural activities of olfactory receptors on the Colorado potato beetle's antenna, but now included stimulations with binary mixtures.

Olfactory Code in Adult Colorado Potato Beetles. The Colorado potato beetle is now a very suitable model to study olfactory coding of food-odor blends because of our present understanding of its behavior, chemoreceptors, and the plant-odor components involved in host finding.

This insect is specialized to feed on solanaceous plant species; potato, *Solanum tuberosum*, is its most common host (Visser, 1983). Beetles respond to airborne potato plant odor by walking upwind. This behavior is generally referred as an odor-conditioned positive anemotaxis and has been studied in much detail for this insect (Visser, 1988). The potato leaf odor was isolated, and the components identified included *cis*-3-hexen-1-ol, *trans*-2-hexenal, *cis*-3-hexenyl acetate, *trans*-2-hexen-1-ol, and 1-hexanol (Visser et al., 1979; Visser, 1983). The beetles' antennal olfactory neurons are selectively tuned to the detection of these so-called green-odor components (Visser, 1979). The green odor, in addition, constitutes an essential part of the host-plant odors for other phytophagous insects (Visser, 1983, 1986). The release of odor-conditioned anemotaxis in the Colorado potato beetle depends on the ratios between the green-odor components (Visser and Avé, 1978). Thus, mixing of host- with nonhost-plant odor prevents upwind orientation of the beetles (Thiery and Visser, 1986, 1987). Response profiles of the beetles' olfactory neurons to compounds applied singly were initially characterized by the grouping into five receptor types (Ma and Visser, 1978). Further analysis of receptor responses to three potato leaf-odor components revealed an across-fiber patterning over the array of olfactory receptor cells studied (Visser, 1983).

We reexamined the activities of olfactory neurons in the Colorado potato beetle's antenna in response to the five potato leaf-odor components applied singly and to three binary mixtures (1 : 1) composed of *trans*-2-hexenal, *cis*-3-hexenyl acetate, and 1-hexanol. A complete description of experimental methods and results is presented elsewhere (De Jong and Visser, 1988b).

The relative response spectra in a receptor population of 39 neurons to the compounds applied singly clearly showed a gradual differentiation of individual receptors that ranged from generalists to specialists. This conclusion was further verified by calculating the degrees of specialization for the array of receptors.

However, receptor responses to binary mixtures, in most cases, were quite different from what one would expect.

Figure 4 shows neural activities in two different receptors on stimulation with 1-hexanol, *cis*-3-hexenyl acetate, and their 1 : 1 mixture. In the first record, the response to the mixture equals the sum of responses to the constituents when applied singly. One would expect this outcome when it is assumed that different test compounds in a mixture contribute to the neural response in a simple additive manner (Figure 5). Nevertheless, the vast majority of neuronal responses to mixtures actually showed various degrees of suppression; the second record represents an example of such suppression (Figure 4). In case of suppression, the receptor response to a binary mixture is smaller than the sum of its responses

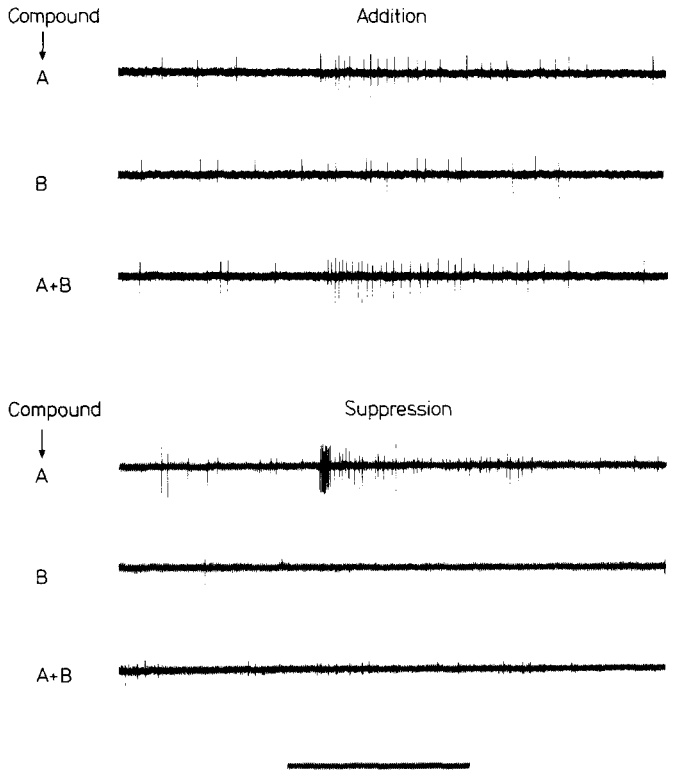

FIG. 4. Neural activities of two olfactory receptors in the Colorado potato beetle's antenna in response to 1-hexanol (A), *cis*-3-hexenyl acetate (B), and their 1 : 1 mixture (A + B), showing addition (top) and suppression (bottom). Chemicals were diluted at the source in paraffin oil (4×10^{-2} v/v). Line at bottom indicates stimulation period (2 sec).

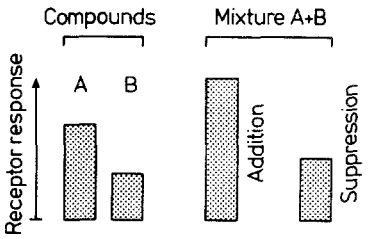

FIG. 5. Schematic illustration of addition and suppression in the receptor responses to mixtures.

to the individual components (Figure 5). It is noteworthy that mixture effects like suppression and synergism have also been reported for pheromone receptors in males of the cabbage looper moth, *Trichoplusia ni* (O'Connell, 1985; O'Connell et al., 1986) and the bark beetle, *Dendroctonus pseudotsugae* (Dickens et al., 1984).

Further analysis of the beetles' receptor responses to mixtures revealed a highly significant negative correlation of the neurons' mixture responses with their degrees of specialization. In other words, specialized receptors respond with less excitation to a mixture than their generalist counterparts. Hence, we adapted our thoughts on the olfactory code as an across-fiber pattern and included suppression as a mixture effect (Figure 2) (De Jong and Visser, 1988b).

When the antennae are stimulated by a blend, the pattern of neural activity across the fibers running towards the brain shows some separation into two channels (Figure 2). The neural activities in the first channel, consisting of generalist receptors, arise from addition of the neurons' responses to individual components. This channel is mainly affected by the quantities of components present in an odor blend. The second channel, consisting of specialized receptors, responds to a mixture by suppression. Thus, the change in neural activities in that channel depends on the interaction of components in a stimulus mixture and their quality, that is, the ratios between components of an odor blend (De Jong and Visser, 1988b).

This concept for the recognition of the blend composition, in the way the information is processed by the peripheral receptors, is further supported by a study on the responses of neurons in the antennal lobe of this beetle (De Jong and Visser, 1988a).

The neural activities of 22 neurons in the beetle's antennal lobe were recorded intracellularly. The relative response spectra of these neurons to the five potato leaf-odor components were classified into four spectral types (Figure 6). Two spectral types showed responses only when antennae were stimulated with either *cis*-3-hexenyl acetate (I) or 1-hexanol (III). The other two types

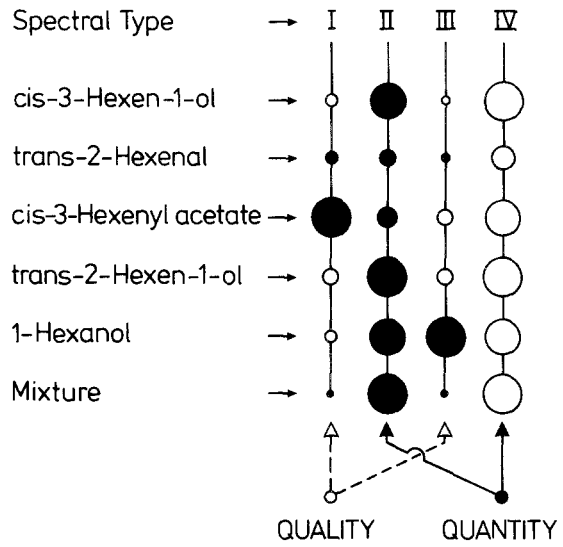

FIG. 6. Mean relative response spectra of interneuron types in the antennal lobe of the Colorado potato beetle, visualized in the areas of circles. Excitation shown as solid circles, inhibition as open circles. Antennae were stimulated with single compounds and a mixture of all potato leaf odor components (De Jong and Visser, 1988a). The mixture response of each type is indicated as either large or small. See text for further explanation.

responded to several leaf-odor components: type II showed excitations and type IV showed inhibitions of their spontaneous firing activities. On stimulation of antennae with a mixture of all leaf-odor components, such as a paraffin oil extract of potato leaves or an artificial mixture (1 : 1 : 1 : 1 : 1), the spontaneous neural activities were changed in the generalist (excitation in II and inhibition in IV) but not in the specialist interneurons (I and III).

We see that at the level of the antennal lobe the separation in two channels is complete. This suggests that host-plant odor recognition in the Colorado potato beetle relies upon the first channel responding to the presence of leaf-odor components (the quantity) and the second channel tuned to detect any unbalance in the ratios between these components (the quality) (De Jong and Visser, 1988a).

CONCLUSIONS

The recognition of a chemical message, the perception of the blend composition, starts in the olfactory receptors. The neural activities in the array of peripheral neurons, the across-fiber pattern, are affected by interactions between

the components of a stimulus mixture. Receptor responses to mixtures do not simply reflect addition. The unraveling of the olfactory code, the way the information about blend composition is processed in the olfactory pathway, certainly deserves more attention. Our understanding of the recognition of semiochemicals by insect receivers is crucial for the manipulation of such signals in programs on insect pest control.

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PHEROMONE BIOSYNTHESIS IN LEPIDOPTERA

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Abstract—Pheromone components for many lepidopteran species are produced by the use of unique chain-shortening and Δ9, Δ10, and Δ11 desaturase systems. Correlations in the Tortricidae indicate that the pheromone components derived from Δ9 and Δ01 desaturases are found in the more primitive species (those possessing morphological plesiomorphies). The precise blend of *Z* and *E* acetates in a number of species is regulated in the final reduction sequence from acyl intermediates. Preliminary research has been conducted on the characterization of the various desaturase enzymes used and on the important blend regulating sequence. Initial purification work on the Δ11 desaturase enzyme found in the cabbage looper moth is reported.

Key Words—Lepidoptera, desaturases, pheromone, enzymes, detergents, fatty acids, biosynthesis.

INTRODUCTION

Sex pheromones have received much attention in the past because these olfactory cues can be used in insect monitoring and control programs. However, this chemical communication system also makes possible studies on fundamental questions of behavior, olfaction, genetics, biosynthesis, etc. The specificity of sex pheromone production and perception in moth species constitutes an excellent system to study discrimination among the various mixtures by the male's olfactory system. Such studies also can address questions of regulation by the female of precise *E*/*Z* ratios of components in the glands, how the biosynthetic pathways are genetically controlled, and how the unique blend of components evolved. In this paper we discuss research conducted on a number of pheromone

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biosynthetic pathways and some key enzymes in moth species, particularly in the Tortricidae.

COMMON Δ11 DESATURASE PATHWAYS

Previous research (reviewed in Roelofs and Bjostad, 1984; Bjostad et al., 1987) has shown that a large number of moth species can produce pheromone components by utilizing various combinations of two unusual enzyme systems: limited chain shortening through β-oxidation and a Δ11 desaturase. The starting material for these routes is palmitic acid (16:COOH), which is prepared from acetate in the pheromone glands via the fatty acid cycle involving fatty acid synthetase. If chain shortening occurs first, then the saturated acyl derivatives of 18:COOH, 14:COOH, and 12:COOH can be produced as substrates for the desaturation step. Interaction of the Δ11 desaturase enzyme with these precursors can produce various *E* and *Z* blends of Δ11-18:COOH, Δ11-16:COOH, Δ11-14:COOH, or Δ11-12:COOH. These in turn can undergo chain-shortening steps to produce the following series: (1) Δ11-18:COOH to Δ9-16:COOH to Δ7-14:COOH to Δ5-12:COOH to Δ3-10:COOH; (2) Δ11-16:COOH to Δ9-14:COOH to Δ7-12:COOH to Δ5-10:COOH; (3) Δ11-14:COOH to Δ9-12:COOH to Δ7-10:COOH; and (4) Δ11-12:COOH to Δ9-10:COOH. They also can undergo chain elongating steps, for example to produce Z13-18:COOH from Z11-16:COOH. Monounsaturated pheromone components are produced by reducing the above acyl intermediates to alcohols or acetates and, in some cases, converting these to aldehydes (Teal and Tumlinson, 1986; Morse and Meighen, 1986).

Additional components can be produced by converting a monounsaturated into a diunsaturated intermediate by one of several different methods. With the domestic silkworm, *Bombyx mori*, a Δ11 monoene precursor is converted to the conjugated Δ,Δ-10,12 diene system through some unknown mechanism. A large amount of Z11-16:COOH intermediate was found in the gland (Yamoaka and Hayashiya, 1982; Bjostad Roelofs, 1984), which evidently (Yamaoka et al., 1984; Ando et al., 1986) is further reacted to generate the conjugated double-bond system of the pheromone component (*E*10, Z12-16:OH). Another method of generating the conjugated double-bond system is to have the desaturase enzyme interact with an intermediate both before and after chain shortening or chain elongation. An example of this is in the production of a diene pheromone component for the light brown apple moth, *Epiphyas postvittana*. The two pheromone components for this species had been found to be *E*11-14:OAc and *E,E*-9,11-14:OAc (Bellas et al., 1983). An in vivo study (Foster and Roelofs, 1988a) using deuterium-labeled palmitic and myristic acids showed that this species uses an *E*11 desaturase system to produce the *E*11-

16:COOH, *E*11–14:COOH, and *E*,*E*-9,11–14:COOH intermediates necessary for production of the two pheromone acetates (Figure 1).

The various combinations of (1) limited chain shortening and (2) Δ11 desaturation steps are used to produce a great proportion of the known lepidopteran pheromone components. Although the Δ11 desaturase system is common in these moth species, it has not been reported in any other animal species. Attempts to characterize these enzymes will be discussed later.

Δ10 DESATURASE PATHWAY

A review of sex attractants and pheromones of almost 150 species in the Tortricidae (Roelofs and Brown, 1982) shows that a number of the components that are used, such as *Z*8–12:OAc, *Z*8–14:OAc, and *Z*10–14:OAc, could not be produced via the Δ11 desaturase. One example is the New Zealand leafroller species, *Planotortrix excessana*, which was found to use *Z*8–14:OAc as its main pheromone component (Galbreath et al., 1985). Analysis of the fatty acid intermediates in the female pheromone gland of this species showed that there was an abundance of *Z*8–14:COOH and *Z*10–16:COOH (Löfstedt and Roelofs, 1985). These intermediates suggested that the pheromone acetate was produced by action of a Δ10 desaturase on palmitic acid followed by limited chain shortening from 16C to 14C. This pathway was confirmed (Foster and Roelofs, 1988b) by in vivo studies using deuterium-labeled palmitic and myristic acid precursors. There was incorporation of label from the d_3-16:COOH precursor into *Z*10–16:COOH, *Z*8–14:COOH, and 14:COOH. With the myristic acid precursor (d_3-14:COOH), however, label was incorporated only into 14:OAc and, to a much lesser extent, into *Z*10–14:OAc. These results showed that *Z*8–14:OAc was produced by involvement of a Δ10 desaturase in the pheromone pathway, and not by a Δ8 desaturase.

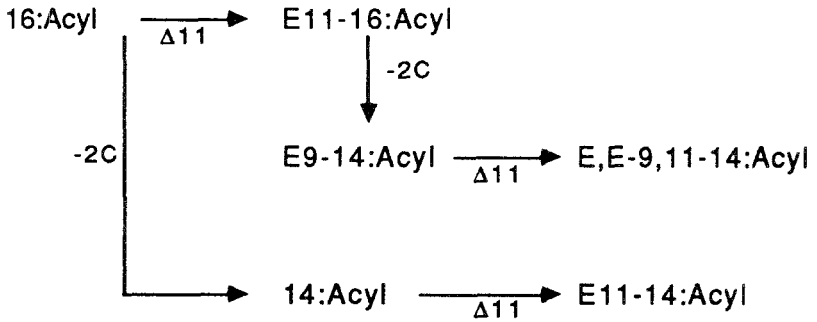

FIG. 1. Biosynthetic pathways for producing a diunsaturated and monounsaturated pheromone component in the light brown apple moth with *E*11 desaturation.

Δ9 DESATURASE PATHWAY

Although most of the pheromone components in the Tortricidae can be accounted for by the pathways described above, there still remain a few whose structures suggest the functioning of a Δ9 desaturase enzyme. One example of this is provided by the brownheaded leafroller, *Ctenopseustis obliquana*, of New Zealand, whose main pheromone components have been reported to be Z5–14 : OAc and Z8–14 : OAc (Young et al. 1985). Analysis (Löfstedt and Roelofs, 1985) of the fatty acyl intermediates in the female pheromone gland showed presence of ubiquitous oleic (Z9–18 : COOH), linoleic, linolenic, and palmitoleic (Z9–16 : COOH) unsaturated acids, as well as the unusual Z7–16 : COOH, Z10–16 : COOH, and Z8–14 : COOH acids. Studies (Foster and Roelofs, 1988c) with deuterium-labeled precursors showed that the Z8–14 : OAc component was prepared from palmitic acid, but that the Z5–14 : OAc component was not. Label incorporation into the Z5 component was obtained only when labeled oleic acid was used as the precursor. These data suggested that this component is produced in the gland from oleic acid that is synthesized somewhere else in the insect or obtained from dietary sources. Therefore, the pathway for this pheromone component involves the ubiquitous Δ9 desaturase enzyme in the production of the oleic acid, but this enzyme probably is not active in the sex pheromone gland in this case.

Another example involving extracellular oleic acid as the pheromone percursor is the pink bollworm pheromone, which consists of Z7,Z11– and Z7,*E*11–16 : OAcs (Hummel et al., 1973). Studies (Foster and Roelofs, 1988d) with various deuterium-labeled precursors showed that no label incorporation was obtained with stearic or palmitic acid, but there was incorporation with oleic acid into Z7–16 : COOH and the pheromone components. The results support a pathway in which oleic acid present in the gland is shortened to Z7–16 : COOH and then desaturated with Δ11 desaturases to produce the Z7,Z11– and Z7,*E*11–16 : COOH precursors.

EVOLUTIONARY SIGNIFICANCE OF PHEROMONE PATHWAYS

In defining pheromone pathways utilizing different desaturase systems in the Tortricidae (Figure 2), we have developed some ideas on the evolution of these pheromone systems. The most striking trend in these data is that species using pheromone components from extracellular oleic or palmitoleic acid (Δ9 desaturase) or from the Δ10 desaturase pathway usually possess plesiomorphic characters and are classified by taxonomists as quite primitive relative to other tortricids. In a previous review of tortricid sex attractants (Roelofs and Brown, 1982), these components were listed in the primitive Olethreutinae genera of

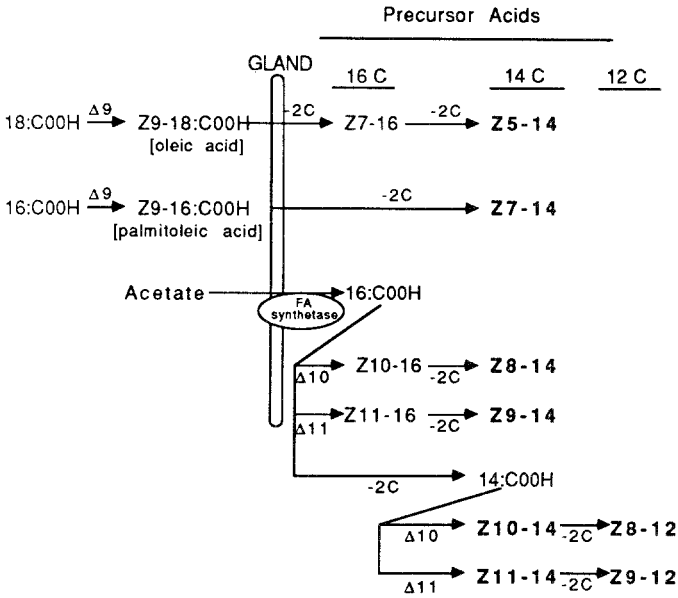

FIG. 2. Summary of biosynthetic pathways in the Tortricidae for producing most of the known pheromone component precursors.

Microcorses, *Endothenia*, and *Apotomis*. Subsequently, another primitive Olethreutinae species in the genus *Bactra* was shown to produce Z10–14:OAc as the main pheromone component (Bjostad and Roelofs, 1983). In an interesting survey of 19 Tortricinae species throughout New Zealand (Foster and Dugdale, 1988), it was learned that nine of these species are distinct from the rest by possession of a variety of plesiomorphic characters. All of these species also were found to use pheromone components that have biosynthetic pathways involving either Δ10 desaturation or Δ9-unsaturated precursors. The remaining apomorphous species all have pheromone components derived from a pathway involving Δ11 desaturation.

Further support for the idea that the use of Δ9- or Δ10-desaturated precursors represents a plesiomorphy for tortricids comes from a survey of 30 tortricid species in Australia (Horak et al., 1988). In this study, over half the species used pheromone components derived by way of Δ11 desaturation, but none of the plesiomorphic members of the Tortricinae, or Olethreutinae tribes, used pheromone components involving Δ11 desaturation. Again, the species possessing plesiomorphic characters used pheromone components with unsaturation is the 5, 7, or 9 position from Δ9 desaturation, or in the 8 or 10 position from Δ10 desaturation.

The general trend of pheromones in the Tortricidae suggests that perhaps limited chain shortening in the microsomes evolved first to produce unique components from available oleic and palmitoleic acids. The ubiquitous Δ9 desaturase system then could have been activated in the gland to produce some additional precursor acids, such as Z9–14:COOH, which could account for the occurrence of Z9–14:OAc in some of the plesiomorphic species described in the New Zealand and Australian surveys. Mutation of the Z9 desaturase then could account for the occurrence of the other unique Δ10 and Δ11 desaturase systems found commonly in the Lepidoptera.

PRODUCTION OF SPECIFIC GEOMETRIC ISOMER BLENDS

Research on the biosynthetic precursors of the redbanded leafroller moth aided in pinpointing the critical step for regulation of the *E*/*Z* mixture of pheromone components. Two of the main components, *E*11- and Z11–14:OAc, are held in a tightly controlled 9:91 ratio (Miller and Roelofs, 1980; Roelofs et al., 1986). At first we assumed that this ratio would be controlled in the desaturation step, which produces a mixture of *E*11- and Z11–14:COOHs. However, base-catalyzed methanolysis of glandular lipids produced a mixture in which the *E* isomer predominated at a 58:42 *E*/*Z* ratio (Bjostad et al., 1981). Analysis of several lipid classes found in the gland showed that there was an abundance of these fatty acyl groups in the triacylglycerols in a 61:39 *E*/*Z* ratio, and smaller amounts of these acyl groups in the choline and ethanolamine phosphatides in a 31:69 ratio. Moreover, analysis of the choline phosphatides with phospholipase A_2 showed that the *E*11- and Z11–14:Acyl groups were found only at the sn-2 position, but analysis of the triacylglycerols with lipase showed that they were not preferentially distributed at particular positions. Hence, the 9:91 *E*/*Z* ratio of acetates must be generated in the reduction sequence from acid precursors to acetates. Other studies with radiolabeled precursors showed that an isomerase was not responsible for the final ratio of *E*/*Z* isomers (Bjostad and Roelofs, 1984).

Research with various radiolabeled triacylglyerols containing either 14:Acyl, *E*11- or Z11–14:Acyl in the sn-2 position showed that triacylglyerols probably do not serve as acyl donors n the production of pheromone components (Bjostad et al., 1987). It is possible, however, that the phosphatides are early intermediates in the pathway and that their *E*/*Z* ratio reflects the initial production of these unsaturated precursors by the Δ11 desaturases. It has been suggested (Bjostad et al., 1987) that the fatty acyl groups are selected from the phosphatides to produce the 9:91 *E*/*Z* ratio found in the acetates and that the remainder, which would be high in *E* isomer, is dumped into the triacylglycer-

ols. It is the specific production of the 9:91 ratio in the reduction sequence that remains a mystery.

Research on several races of European corn borer moths has provided some insight into this final reduction sequence. Genetic studies (Klun and Maini, 1979; Roelofs et al., 1987) have been carried out with races that have opposite blends of the two pheromone components, *E*11- and *Z*11-14:OAc, also used by the redbanded leafroller. In analyzing crosses and backcrosses with these races, it was determined that the blend of *E*/*Z* isomers (99:1, 65:35, and 3:97 for the *E* race, hybrid, and *Z* race, respectively) is controlled by a single autosomal genetic factor. However, analysis of the fatty acyl intermediates showed that they all had about the same ratio (70:30) of *E*/*Z*11-14:COOH precursors, regardless of which acetate ratio was finally produced (L. Sreng and W. L. Roelofs, unpublished). This implies that the single genetic factor is operative on some enzyme system in the final reduction sequence. Therefore, although the biosynthetic pathway to these pheromone components has many steps, it takes only a change in one enzyme system to generate a different component ratio. However, studies so far have shown that it takes more than intense selection pressure to modify this highly canalized enzyme system (Roelofs et al., 1986).

EVIDENCE FOR A FAMILY OF Δ11 DESATURASES

In studying the biosynthetic pathways of several lepidopteran species, it became obvious that there were a number of different Δ11 desaturases present (Wolf and Roelofs, 1986, 1987). In the cabbage looper, the enzyme produces only the *Z* isomer of 16- and 18-carbon chain acids, and very little desaturated 14-carbon acid. Conversely, in redbanded leafroller moths, the enzyme produces both *E* and *Z* isomers of 14-carbon chain acids, but no unsaturated 16- or 18-carbon acid. When we investigated the spruce budworm moth pheromone pathways to prove that the *E*11-14:OAc was indeed produced by Δ11 desaturation rather than by some other pathway as previously reported (Morse and Meighen, 1984), we found (Wolf and Roelofs, 1987) that spruce budworm females did produce *E*11 and *Z*11 isomers from 14-carbon acid in a 10:1 *E*/*Z* ratio and that they also had an enzyme that produces *Z*11-16:COOH. This apparent combination of three enzyme systems also is found in the European corn borer populations, which exhibit a 70:30 *E*11/*Z*11 ratio of 14-carbon acids, and an abundance of pure *Z*11-16:COOH. Interestingly, the corresponding acetate of *Z*11-16:COOH has not been found to be part of the pheromone blend in the corn borer. Research discussed above (Figure 1) also shows that the light brown apple moth possesses a Δ11 enzyme that produces only *E* isomer with

both 16- and 14-carbon chain acids. The evidence suggests a family of Δ11 enzymes with slightly different specificities and product stereochemistry.

CHARACTERIZATION OF CABBAGE LOOPER MOTH Δ11 DESATURASE

The Δ11 desaturase enzyme from cabbage looper moth female glands was partially purified by ultracentrifugation, and the pellet obtained at 100,000*g* containing the microsomal fraction was found to contain the Δ11 desaturase activity (Wolf and Roelofs, 1986). Some of its properties were characterized and compared with those of the ubiquitous Δ9 desaturase enzyme (Wang et al., 1982). Similarities between the two systems include subcellular location (microsomal), substrate specificity (16- and 18-carbon acids), and lack of sensitivity to carbon monoxide, while differences include cofactor preference (NADH rather than NADPH), sensitivity to cyanide ion, pH optimum (7.4–7.8 vs. 6.8–7.2), inactivation by ferrocyanide, and location in the organism (in the pheromone gland compared to generally distributed).

The enzyme uses palmitoyl-CoA as a substrate much more efficiently than palmitic acid. However, the product is not simply the desaturated CoA derivative. The workup procedure employed partitions the product between organic and aqueous layers. Coenzyme A and its derivatives remain in the water layer, but analysis of the palmitoyl-CoA desaturation reaction shows that all the desaturated acid is in the organic solvent. Separation of the lipid classes by thin-layer chromatography (Bjostad and Roelofs, 1984) followed by fatty acid analysis of each class showed the desaturated acid to be mostly in three regions of the plate: phosphatidylcholines (27%), ethanolamines (7%), and free fatty acids (66%) (Wolf and Roelofs, unpublished). In addition, a small amount of acid was found in the triglyceride region. Since it is known that the glands contain very little free fatty acid (Bjostad and Roelofs, 1984) and that the CoA derivatives are not very stable under our assay conditions, it is assumed that most of the free acid represents material that would be found on some ester (CoA or lipid) in the natural system. At this time it is not known which derivative of Z11–16:COOH is used for the next chain-shortening sequence—a coenzyme A or a phosphatidylcholine derivative.

PURIFICATION OF CABBAGE LOOPER MOTH Δ11 ENZYME

Attempts are being made to purify the cabbage looper moth Δ11 desaturase further. The enzymatic assay being used to follow the purification is the same as previously reported (Wolf and Roelofs, 1986). Since microsomal enzymes are generally resolubilized before purification, we decided to investigate the effects of various detergents on our crude microsomal fraction. Initially, it

seemed reasonable to assume that the structure of the Δ11 enzyme would be similar to that of the ubiquitous Δ9 desaturase (Jeffcoat, 1979). Thus, we observed the effects of sodium deoxycholate and Triton X-100, both of which had been used to purify the Δ9 desaturase (Strittmatter et al., 1974; Joshi et al., 1981), as well as CHAPS, a reportedly mild sulfonic detergent (Womack et al., 1983). However, the addition of 0.1–1.0% of any of these detergents to the assay buffer resulted in the loss of over 90% of the enzymatic activity, indicating that they are not suitable for this system.

We next tried nonionic alkyl detergents. Both octanoyl-*N*-methyl glucaide (Hildreth, 1982) and *n*-octyl-β-D-glucopyranoside enhanced the reactivity of the system when crude enzyme (supernatant of 10,000*g* centrifugation) was used, but only the glucamide retained this effect when the microsomal fraction was assayed (Table 1).

Suspension of the microsomal fraction (pellet from 100,000*g* centrifugation for 1 hr) in buffer containing these detergents followed by recentrifugation for hr at 100,000*g* showed that most of the activity in the glucamide-containing buffer remained in the supernatant (Table 2). Longer (3-hr) centrifugation resulted in more activity in the pellet, so the solubilization cannot be termed complete.

Purification of the microsomal fraction was attempted using Blue Sepharose, an affinity chromatography substrate that binds a variety of NAD^+- and $NADP^+$-requiring enzymes. Column chromatography gave a single broad peak of activity. Batch treatment of the enzyme solution with the Sepharose followed by reelution of the bound proteins showed that most of the desaturase activity did not bind to the substrate (it stayed in the supernatant). Thin-layer chromatograph of the enzyme assays showed the the Blue Sepharose supernatant had fewer side products than did the control. In addition, gel electrophoresis

TABLE 1. CABBAGE LOOPER Δ11 DESATURASE SOLUBILIZATION[a]

	10,000*g* supernatant[b]	100,000*g* pellet[b]
Control	100	100
Octanol-*N*-methyl glucamide (1%)	160	190
n-Octyl-β-D-glucopyranoside (1%)	185	90

[a] Protein solution was divided into three parts and added to cofactors, radiolabeled palmitoyl-CoA, and detergent in separate assay tubes. After 1-h, the assays were extracted and the extracts transmethylated and subjected to TLC on silver-impregnated silica. The radiolabel was located and counted using a scintillation counter (see Wolf and Roelofs, 1987).

[b] Reactivity relative to control. Absolute values for the control were: 10,000*g* supernatant, 10,823 dpm; 100,000*g* pellet, 1576 dpm.

TABLE 2. DETERGENT SOLUBILIZATION OF Δ11 DESATURASE[a]

	Relative activity[b]	
	Supernatant	Pellet
Control	60	40
Glucamide[c]	200	30
Pyranoside[c]	50	40

[a] 100,000*g* pellet suspended in buffer with detergent and recentrifuged at 100,000*g*.
[b] Relative to control (total control activity = 100).
[c] Detergents same as in Table 1.

(SDS-PAGE) showed that this fraction contained fewer proteins, especially in the molecular weight range expected for the proteins that make up the desaturase (25,000–50,000 daltons) by analogy to the Δ9 enzyme (Jeffcoat, 1979). After this much manipulation, the total activity of the enzyme fractions is low, but further attempts at stabilization and purification are proceeding.

ENZYMES IN REDBANDED LEAFROLLER MOTH

Sex pheromone biosynthesis is the redbanded leafroller moth involves chain shortening (16:COOH to 14:COOH) followed by Δ11 desaturation (to give a mixture of *E* and *Z*11–14:COOH) (Bjostad et al., 1987). Previous studies in cell-free systems prepared from pheromone glands of the orange tortrix moth, *Argyrotaenia citrana*, showed that the chain-shortening reaction could be observed when the following reactants were supplied: NADH, NADPH, BSA, coenzyme A, ATP, and magnesium chloride (Wolf and Roelofs, 1983). It is assumed that the latter three aid in the synthesis of the CoA ester, which could be the preferred substrate for the chain-shortening step. In the redbanded leafroller, the same assay conditions also allow chain-shortening to occur. Omission of BSA or NADPH from the reaction does not reduce the amount of 14:COOH produced, but omitting NADH or CoA effectively stops chain-shortening (Wolf and Roelofs, unpublished).

The Δ11 desaturase produces both geometric isomers from 14:CoA, but in a *Z* to *E* ratio of 60:40, which differs both from that of the fatty acids present in the gland (42:58) and of the pheromone (91:9) (Wolf and Roelofs, 1987). Attempts to use 1,2-dimyristoyl-L-3-phosphatidylcholine as substrate for this reaction were unsuccessful (Wolf and Roelofs, unpublished), giving more

weight to the idea that the CoA derivative is the natural substrate for the desaturase.

SUMMARY

Many species of Lepidoptera utilize pheromone components that are produced with a combination of unique chain-shortening and desaturase steps. It appears that the chain-shortening step was used initially to convert existing oleic and palmitoleic acids to pheromone components of a shorter carbon length. The evolution of Δ10 and Δ11 desaturase enzymes then allowed the moths to generate a host of unique chemicals to be used in species-specific pheromone blends. The purification and characterization of some of these unique enzymes is a big challenge to biochemists, but should provide the information needed to take the next step in isolating the gene that encodes for these enzymes.

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CHEMICAL STUDIES OF PROTEINS THAT DEGRADE PHEROMONES: Cyclopropanated, Fluorinated, and Electrophilic Analogs of Unsaturated Aldehyde Pheromones[1]

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Abstract—Aldehyde components of lepidopterous pheromones are converted to carboxylic acids by aldehyde oxidizing enzymes (AOEs) that are present at high levels in antennal tissues of adult moths. The AOEs may include O_2-requiring aldehyde oxidases as well as nucleotide-cofactor-requiring aldehyde dehydrogenases. Three classes of inhibitors were synthesized and examined in vitro for AOE inhibition using *Heliothis virescens* antennae: (1) cyclopropanols, (2) α-fluorinated aldehydes, and (3) α,β-unsaturated carbonyls. First, cyclopropanated analogs of (Z)-11-hexadecenal (Z11-16:Al), a common unsaturated aldehyde component of many species' pheromone blends, were synthesized as isosteric pheromone analogs and as potential inhibitors of the moth AOEs. NMR assignments are reported for the *cis*- and *trans*-cyclopropanols. Cyclopropanols appear to act as oxidase-activated AOE inhibitors, perhaps via the unstable cyclopropanones. Second, α-fluoro and α,α-difluoro substituted analogs of Z9-14:Al were synthesized and shown to be modest inhibitors of both the alcohol oxidase and AOE activities. Finally, the most potent inhibitors were α,β-unsaturated carbonyl mimics of the Z11-16:Al. The α-methylene aldehyde was 1000-fold less effective than the vinyl ketone Z1,11-16:3-oxo. This inhibition appears irreversible and is postulated to involve electrophilic modification of an active site sulfur nucleophile.

Key Words—Cyclopropanation, cyclopropanol, enzyme inhibitor, pheromone analog, vinyl ketone, *Heliothis virescens*, *Plutella xylostella*, α-fluoroaldehyde, (Z)-11-hexadecenal, (Z)-9-tetradecenal, Lepidoptera, Noctuidae, Plutellidae.

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INTRODUCTION

Pheromone analogs offer important insights into the binding interactions necessary for recognition by the molecular acceptor sites located in insect antennae (Prestwich, 1987a,b). Typically, important functional groups are mimicked with electronically different isosteric substitutions or by electronically similar but sterically divergent replacements. In order to study the aldehyde-metabolizing enzymes of moths, we have synthesized and tested a number of potential inhibitors of the aldehyde-oxidizing enzymes (AOEs). These AOEs convert the aldehyde pheromone components to the carboxylic acids, which lack the ability to produce olfactory stimulation. We had previously designated these aldehyde-oxidizing enzymes from *H. virescens* as aldehyde dehydrogenases (Ding and Prestwich, 1986; Prestwich, 1987a,b; Prestwich *et al.*, 1987b). However, recent experiments with enzyme-staining in electrophoretic gels in our labs (M.L. Tasayco J., unpublished results) and from workers examining *Manduca* antennal enzymes (M. Lerner and R. Ripchinsky, unpublished results) have suggested that the catabolic enzymes are more likely to be aldehyde oxidases. We now present three examples in which reactive or latently reactive electrophilic functionalities have been incorporated into variants on the aldehyde functional group. Selected experimental details have been included (Ding, 1987).

SELECTED ALDEHYDE MIMICS

Cyclopropanated Pheromone Analogs

Background. Surprisingly, cyclopropyl-containing analogs of pheromones have received scant attention. Two primary motivations are important in examining cyclopropyl analogs. First, the cyclopropanation of an internal (*Z*)-alkene preserves the "bent" nature of the alkenyl chain, but alters local pi electron density and bond angles. It also confers a new stereogenic element, such that two enantiomers of the *cis*-cyclopropanated analogs exist. Second, introduction of cyclopropanol or cyclopropanone moieties at the oxygen-functionalized end of the pheromone offers the opportunity to produce targeted inhibitors of pheromone processing enzymes (Prestwich, 1987a; Prestwich et al., 1987b).

Synthesis of Internal Cyclopropyl Analogs. To prepare the internally cyclopropanated analogs (Figure 1), Z11–16:OH (**1**) was first protected as the *t*-butyldimethylsilyl ether (**2**) and then cyclopropanated (95°C, 16 hr, benzene–toluene) with excess diiodomethane and diethylzinc (Rousseau and Slougui, 1983) to give silyl ether **3**. The *cis*-cyclopropyl analogs were prepared by ether cleavage with excess tetra-*n*-butylammonium fluoride (TBAF) in THF (0–25°C, 1 hr) to give alcohol **4**. Acetylation (Ac_2O, Py, 16 hr, 20°C) or pyridinium dichromate oxidation (1.5 equiv., CH_2Cl_2, 5 hr, 20°C) of alcohol **4** produced acetate **5** or aldehyde **6**, respectively. Each compound was purified by silica gel

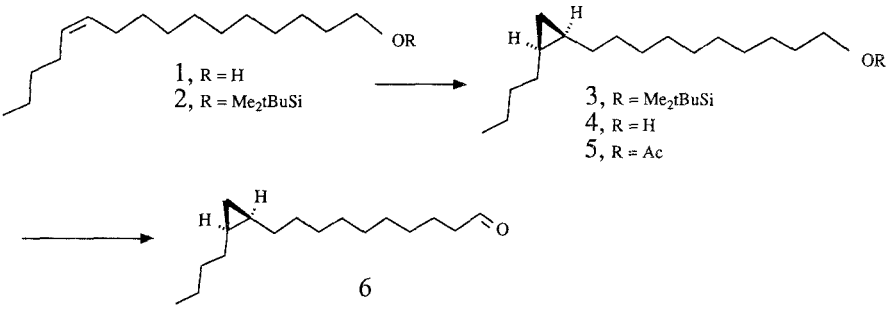

FIG. 1. Synthesis of internally cyclopropanated Z11–16 analogs.

flash chromatography, and homogeneity was ascertained by TLC and by capillary GC (Ding, 1987). We will refer to these *cis*-cyclopropyl analogs using the shorthand "*cc*"; thus, the two analogs are *cc*11–16 : Ac (**5**) and *cc*11–16 : Al (**6**).

Synthesis of Cyclopropanols. Cyclopropanols were prepared by cyclopropanation of the trimethylsilyl enol ethers of the unsaturated aldehyde Z11–16 : Al as shown in Figure 2. Thus, Z11–16 : Al (**7**) was converted to a 62 : 38 mixture

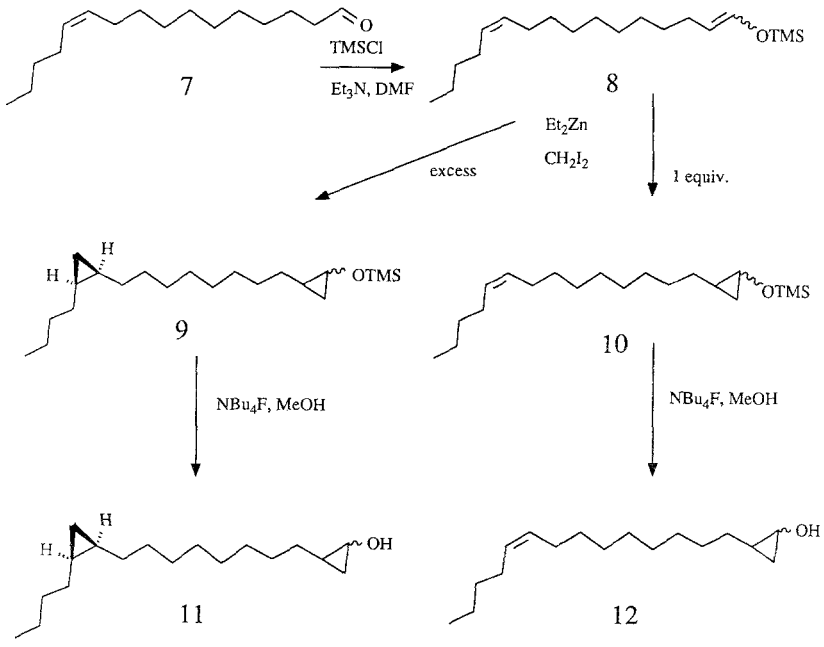

FIG. 2. Synthesis of cyclopropanol analogs of Z11–16 : OH.

of the *Z/E* enol TMS ethers **8** using 2.4 equiv. of trimethylchlorosilane and 4.8 equiv. of triethylamine in dry DMF (reflux, 16 hr) (Ito et al., 1979). The proton NMR signals (300 MHz) for the enol ether vinyl protons allowed assignment of stereochemistry and relative proportions: *Z*-isomer, δ 4.46 (dt, J = 6.1, 7.1 Hz), 6.11 (dt, J = 6.1, 1.3 Hz); *E*-isomer, δ 4.96 (dt, J = 12.0, 7.1 Hz), 6.16 (dt, J = 12.0, 1.3 Hz). Monocyclopropanation could be accomplished using 1.6 equiv. of diethylzinc and 2.0 equiv. of diiodomethane by adding the diiodomethane to the remainder of the reagents in dry ether at −78°C, and then stirring for 12 hr at 20°C with careful monitoring of the reaction progress by capillary GC. The cyclopropanol TMS ethers **10** were obtained as a 62:38 *cis*:*trans* mixture as seen by the diagnostic proton NMR signals for the carbinyl hydrogen of each isomer: *cis*, δ 3.36 (ddd, J = 6.6, 6.6, 3.3 Hz); *trans*, δ 3.02 (ddd, J = 6.1, 2.6, 2.6 Hz). Addition of excess diethylzinc–diiodomethane reagent and warming to 95°C for 12 hr readily produced the biscyclopropanated ethers **9**. Cleavage of the silyl ether with tetrabutylammonium fluoride in methanol (Wasserman and Dion, 1982) followed by column chromatography afforded the pure cyclopropanols **11** and **12**. Proton NMR signals of the cyclopropyl protons (Wiberg et al., 1973) were readily assigned for the *cis*- and *trans*-substituted cyclopropanols: δ 3.18 (ddd, *trans* H-1, J = 6.2, 2.7, 2.7 Hz), 3.50 (ddd, *cis* H-1, J = 6.5, 6.5, 3.1 Hz). We have adopted a shorthand for the cyclopropanol terminus "Cpo" which *excludes* the extra carbon, such that the Z11–16:OH analogs are named as Z11–16:Cpo (**12**) and *cc*11–16:Cpo (**11**). In addition, the Z9–14:OH analogs Z9–14:Cpo and *cc*9–14:Cpo were prepared following the same procedures (Ding, 1987).

Electrophysiological Activity. Cyclopropanated compounds were examined as analogs of Z11–16:Ac, Z11–16:OH, and Z11–16:Al in electroantennogram (EAG) assays using antennae of male diamondback moths, *Plutella xylostella* (E.M. Giblin and E.W. Underhill, personal communication). The *P. xylostella* antennal system has previously been used to evaluate the relative EAG responses to haloacetate analogs of Z11–16:Ac (Prestwich and Streinz, 1987). The results for the cyclopropanols and *cis*-cyclopropyl chain analogs are presented in Table 1. It is surprising that the *cis*-cyclopropanation alone lowers the EAG response to a much greater extent than does the cyclopropanol moiety; both modifications together essentially eliminate EAG activity. Indeed, it was also found that the 2,3-cyclopropyl-Z13–18:OH (a cyclopropyl carbinol) still showed a relative EAG potency of 0.78 relative to Z11–16:OH (Ding, 1987; E.M. Giblin and E.W. Underhill, personal communication).

Enzyme Inhibitory Activity. The internally cyclopropanated aldehyde *cc*11–16:Al was examined as a competitive inhibitor of the aldehyde dehydrogenase of *Heliothis virescens* antennae, using [^{3}H]Z11–16:Al as the substrate. As expected, it appeared to act as an alternative substrate and no inhibition was observed at concentrations up to 10 μM, which is two to four times the apparent K_M for the crude enzyme preparation (M.L. Tasayco J., unpublished results).

TABLE 1. EAG ACTIVITY OF CYCLOPROPANATED ANALOGS WITH *Plutella xylostella*

Compound	Mean rel. EAG response[a]	
Z11-16:Al	1.01 a	(N = 5)
Z11-16:Ac	1.00 a	
*cc*11-16:Ac	0.174 c	
*cc*11-16:Al	0.298 b	
*cc*11-16:Cpo	0.044 d	
Z11-16:Al	1.00 a	(N = 3)
Z11-16:OH	0.483 b	
Z11-16:Cpo	0.416 b	

[a]Responses with different letters are statistically different at the $P < 0.05$ level using a multiple range test (Duncan, 1955).

The substituted cyclopropanols were prepared with the expectation that they would function as alcohol-oxidase-activated inhibitors of antennal AOEs, as shown schematically in Figure 3. We hypothesized that a cyclopropanol (e.g., **12**) would be oxidatively activated by the oxidase activity present in antennae to an unstable cyclopropanone (**13**) which would be capable of acting as transition state analog inhibitor of the aldehyde dehydrogenase by formation of a stable tetrahedral adduct such as **14** with an active site cysteine of the antennal AOE (Walsh, 1982; Wiseman and Abeles, 1979; Wiseman et al., 1980). Covalent modification of an aldehyde oxidase could also be envisioned. Direct preparation of the substituted cyclopropanones (Wasserman et al., 1974) had been unsuccessful (Ding, 1987). It was not possible to overcome the extreme

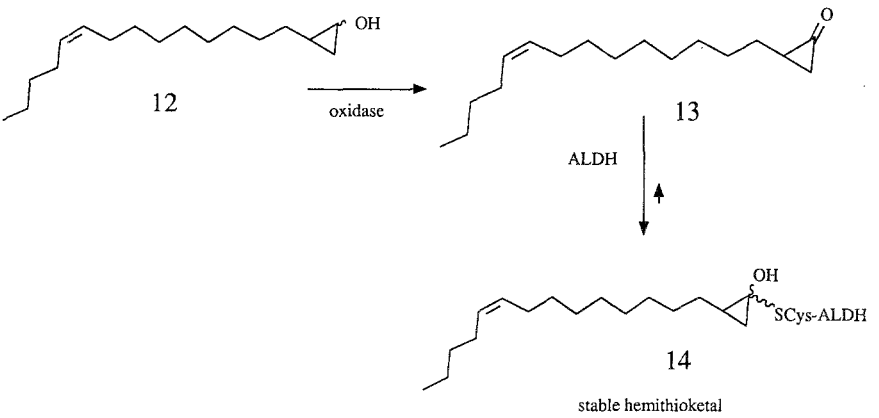

FIG. 3. Proposed oxidase activation of cyclopropanol **12** to produce cyclopropanone **13**, and formation of stable hemiketal adduct **14** with the aldehyde dehydrogenase activity.

instability of monoalkylsubstituted cyclopropanones (Dowd et al., 1984) even long enough to obtain NMR spectra of cleavage products of the protected hemiketals.

Earlier, we demonstrated alcohol oxidase activity in homogenates of the legs, antennae, and glands of adult *H. virescens* for oxidation of [^{3}H]Z9–14:OH and for [^{3}H]Z11–16:OH (Prestwich, 1987a; Ding, 1987; Ding and Prestwich, 1986). Recently, capillary GC analysis has been used to show that the oxidase activity associated with the cuticle of female glands is relatively nonspecific for saturated vs. unsaturated primary alcohols, but will not oxidize secondary alcohols (Teal and Tumlinson, 1986; Tumlinson and Teal, 1987).

The assays for both oxidase (substrate was [^{3}H]Z11–16:OH) and AOE activities (substrate was [^{3}H]Z11–16:Al) were performed after a preincubation (35 min, 20°C) of the cyclopropanol inhibitors (1 μM and 10 μM) with the enzyme preparation. As shown in Table 2, significant inhibition of both AOE and alcohol oxidase was observed when 0.2 equiv. female gland was assayed, but not when 2 gland equiv. per assay were used. The female pheromone gland has been shown to possess both high AOE and high oxidase activity in vitro (Ding and Prestwich, 1986), and at higher enzyme concentration, the inhibitory effect is negligible.

Table 2 also illustrates the results of the inhibition of the AOE activity of male *H. virescens* antennae by the alkenyl cyclopropanol (**12**) and the cyclopropylalkyl cyclopropanol (**11**). The effect of the internal cyclopropyl is neg-

TABLE 2. INHIBITION OF *Heliothis virescens* ENZYMES BY CYCLOPROPANOLS

		Inhibition (%)[b]	
	Enzyme source[a]	1 μM	10μM
A. Enzyme comparison for cyclopropanol **12**			
Enzyme assayed[c]			
AOE	2 FG	—	0
AOE	0.2FG	23	32
OX	2 FG	0	0
OX	0.2FG	10	60
B. Inhibitor comparison for male antennal AOE			
Compound			
12 (mono)	0.2MA	50	85
11 (bis)	0.2MA	45	60

[a] FG = female gland; MA = male antenna.
[b] Means of duplicate or triplicate assays.
[c] AOE = aldehyde oxidizing enzymes, with 3 mM NAD^+ and 0.3 μM [^{3}H]Z11–16:Al as substrates; OX = alcohol oxidase, with [^{3}H]Z11–16:OH as substrate.

ligible; it neither hinders nor enhances the inhibition due to the cyclopropanol moiety. This might be expected from the results of Teal and Tumlinson (1986) since the oxidase activity and perhaps also the AOEs activity appear to act on a variety of nonphysiological substrates.

The time dependence of the inhibition of AOE activity was explored briefly using 0.2 male antenna/assay (Ding, 1987). In this experiment, the Z9–14:Cpo homolog was preincubated for 10, 20, 30, 40, 50, or 60 min with the enzyme homogenate prior to assay with [^{3}H]Z11–16:Al. Inhibition of AOE activity increased from 80% to nearly 100% during this time course, and then declined to below 80% at the longest preincubation time. This suggests that the "slower" enzyme (alcohol oxidase) was producing an unstable inhibitory species for the "faster" AOEs. After the optimal preincubation time and during the subsequent assay period, the reduction in inhibition could be attributed to dissociation of the covalent tetrahedral adduct and decomposition of the cyclopropanone to a simple noninhibitory carboxylic acid.

α-Fluorinated Aldehyde Analogs

The substitution of fluorine for hydrogen often produces analogs of bioactive natural compounds with altered chemical or physical properties (Walsh, 1983). In particular, we (Prestwich, 1986) and others (Camps et al., 1983) have been interested in the effects of fluorination on insect steroid metabolism and on pheromone binding and catabolism. ω-Fluorinated fatty acid derivatives can be pheromone analogs as well as potential toxicants (Prestwich, 1987a; Carvalho and Prestwich, 1984). Modification of the reactivity of the aldehyde functionality itself involves replacement of the α-hydrogens or the aldehydic hydrogen by fluorine. The latter has been shown to produce a hyperagonist aphrodisiac in the case of the Z11–16:Acf and Z9–14:Acf analogs of the *H. virescens* aldehydes (Prestwich et al., 1986). More recently, we showed that although the α,β-unsaturated acyl fluorides are poor electrophysiological mimics of the boll weevil cyclohexylidene aldehydes, the α-fluoro compounds showed biological activity equivalent to the natural components (Prestwich et al., 1987a). Replacement of an α-hydrogen by fluorine stabilizes the tetrahedral hydrate form of the aldehyde, and can lead to an effective enzyme inhibitor (Gelb et al., 1985). Similarly, trifluoromethyl ketones can act as potent transition-state analog-type esterase inhibitors (Hammock et al., 1982). Since both esterase and aldehyde dehydrogenase (but not aldehyde oxidase) mechanisms involve acyl enzyme and tetrahedral adduct intermediates, we examined these α-fluorinated analogs as AOE inhibitors.

Synthesis. We required mild syntheses of the α-fluorinated aldehydes which would not compromise the internal double bond; however, compounds with both remote olefinic bonds and α-fluorinated aldehydes were unknown when

we began in 1985. The successful scheme is shown in Figure 4 for the Z9–14:Al analogs. The Grignard prepared in dry ether from (Z)-7-dodecenyl bromide (**15**) and Mg metal was added slowly to a −78°C solution of excess diethyl oxalate in ether, stirred 1 hr (20°C), and worked up to give a 60% yield of the homogeneous α-ketoester **16** after chromatography (Huet et al., 1973). The difluoro analog was readily obtained by treatment of an ice-cooled CH_2Cl_2 solution of the ketoester **16** with 1.2 equiv. diethylaminosulfur trifluoride (DAST) and stirring at 20°C for 3 hr (Middleton and Bingham, 1980). Workup with bicarbonate solution avoided free HF; attempted silica chromatography of the α,α-difluoroester **17** resulted in 50% decomposition to the α-fluoro-α,β-unsaturated ester (δ 6.11, H-3, dt, $^3J_{HF}$ = 33.3 Hz, $^3J_{HH}$ = 7.8 Hz). The difluoroester **17** in ether was reduced by addition of 1.2 equiv. of DIBAL-H in toluene (Erni and Khorana, 1980) at −78°C, warming to 20°C for 3 hr, and isolation of the α,α-difluoroaldehyde **18** as the hydrate after chromatography on neutral alumina (activity III). The free aldehyde could be obtained by evaporation distillation (0.5 mm, 100°C) of the hydrate.

The monofluoroaldehyde was prepared using an analogous sequence beginning with the α-hydroxyester **19**, obtained in ca. 50% yield by reduction of the α-ketoester **16** (20–35°C, 3 hr) with a reagent prepared from vacuum-dried silica gel and lithium aluminum hydride in ether (Kamitori et al., 1982). Addition of the hydroxyester **19** in CH_2Cl_2 to 1.2 equiv. DAST in CH_2Cl_2 at −78°C gave, after aqueous workup, a quantitative recovery of the crude α-fluoroester **20** which was used without further purification: [^{1}H]NMR, δ 4.87 (dt, $^2J_{HF}$ = 49.6 Hz, $^3J_{HH}$ = 5.5 Hz). Following the same DIBAL-H reduction, a 6:4 mixture of aldehyde to overreduced alcohol was obtained. Swern oxidation (Mancuso and Swern, 1981) of the crude mixture gave, after purification on alumina, a 98% yield of the α-fluoroaldehyde **21** as the hydrate.

Enzyme Inhibition. The two fluoroaldehydes were tested as potential inhibitors of the alcohol oxidase and aldehyde dehydrogenase activities in tissue

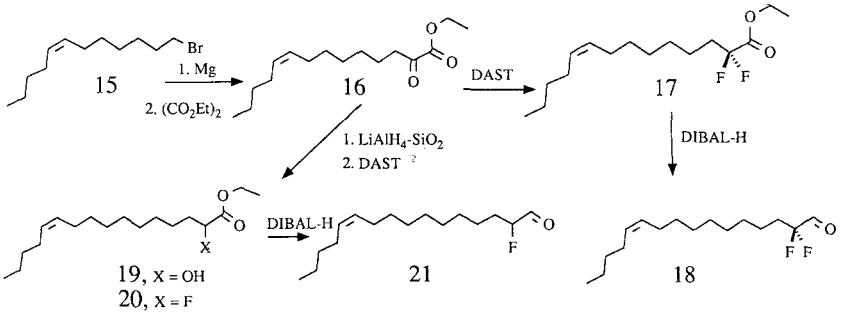

FIG. 4. Synthesis of α-fluorinated aldehyde analogs of Z11–16:Al.

homogenates of *Heliothis virescens* using [^{3}H]Z9–14 : OH and [^{3}H]Z9–14 : Al, respectively. The tissue preparation, incubation, TLC separation of product and substrate, and correction for nonenzymic degradation was performed as described earlier (Ding and Prestwich, 1986), except that the inhibitor (as an ethanolic stock solution) was preincubated with the enzyme solution for 90 min prior to addition of substrate. The final ethanol concentrations were below 1%, and ethanol blanks were included. Substrate concentrations were 0.1 μM of carrier-free [^{3}H]Z9–14 : Al or [^{3}H]Z9–14 : OH.

The results are summarized in Table 3. The male antennal AOE activity is only inhibited by the difluoroaldehyde **18** and shows no inhibition by the monofluoroaldehyde **21** at the highest tested concentration of 10 μM. When less enzyme is present, as in the female gland AOE, inhibition is higher; when more enzyme is present, as in the use of two antennal equivalents, no inhibition is observed. By comparison, the oxidase activity of the female gland is effectively completely blocked by the difluoroaldehyde **18** and the oxidase activity of the male antennae shows substantial inhibition by the monofluoroaldehyde **21**. The results are consistent with the absence of carbonyl form under aqueous conditions, such that little interaction with an AOE can occur. The hydrate, on the other hand, may be acting as a substrate for the oxidase; the precise mode of inhibition by such carbonyl hydrates would require further investigation.

TABLE 3. INHIBITION OF *Heliothis virescens* OXIDATIVE ENZYMES BY α-FLUORINATED ALDEHYDES[a]

Enzyme assayed		Inhibition (%)[b]	
		1 μM	10 μM
A. α,α-Difluoro aldehyde **18**			
AOE	2 MA	—	0
	0.2 MA	16	33
	2 FG	—	0
	0.2 FG	43	69
	0.02FG	—	100
OX	2 FG	—	19
	0.2 FG	—	100
B. α-Fluoro aldehyde **21**			
AOE	0.2 MA	—	0
OX	2 MA	10	29
	0.2 MA	50	75

[a] Abbreviations as in Table 2. Preincubation 90 min; incubation 40 min at 20°C.
[b] Means of duplicate or triplicate assays.

A preliminary evaluation of the two fluorinated aldehydes showed essentially no stimulatory activity at all in EAGs using *P. xylostella* male antennae.

α,β-Unsaturated Carbonyl Analogs

Our search for effective AOE inhibitors then focused on electrophilic functionalities capable of covalent modification of the putative active-site thiol residue, which is required for enzymatic activity (Flynn and Weiner, 1986). To this end, we chose to synthesize the α-*exo*-methylene aldehyde **25** and the vinyl ketone **23** as analogs of *Z*11–16:Al and to examine their potencies as AOE inhibitors in vitro. These electrophilic analogs could act as potent, irreversible inhibitors of *H. virescens* antennal AOE.

Synthesis. The synthesis of the vinyl ketone is shown in Figure 5 and follows procedures we had employed earlier for making ^{14}C-labeled vinyl ketone to study detoxification of a termite defense secretion (Spanton and Prestwich, 1981). Thus, addition of *Z*9–14:Al (**22**) in THF to a 0°C solution of 5 equiv. of vinylmagnesium bromide in THF followed by stirring 1 hr at 20°C, aqueous workup, and chromatography afforded an 80% yield of the allylic alcohol. Oxidation of the allylic alcohol with activated MnO_2 in hexane (24 hr, 20°C) gave, after chromatography, an 86% yield of the vinyl ketone: [^{1}H]NMR, δ 5.81 (H-1*E*, dd, J = 10.4, 1.2 Hz), 6.21 (H-1Z, dd, J = 17.6, 1.2 Hz), 6.35 (H-2, dd, J = 17.6, 10.4 Hz).

The *exo*-methylene analog **25** was prepared as indicated in Figure 6. The TMS cyclopropyl ether **10** (prepared as described above) was dissolved in CH_2Cl_2 and added to a 1 M solution of stannic chloride in methylene chloride, stirred for 30 min 15°C, and concentrated in vacuo to give the trichlorostannyl adduct **24** as a yellowish semisolid (Ryu et al., 1986) which was used without further purification. The crude adduct **24** was stirred with DMSO in hexane or chloroform. The reaction could also be performed in d_6-DMSO-$CDCl_3$ and monitored by [^{1}H]NMR at 300 MHz. Within 45 min, the signal at δ 9.70 for the starting stannyl-complexed aldehyde disappeared and a new peak at δ 9.52 had appeared for the unsaturated aldehyde. After removal of solvents, the residue was chromatographed to give a 50% yield of the reactive α-methylene aldehyde **25**: [^{1}H]NMR: δ 2.21 (H-3, t, J = 7.5 Hz), 5.96 (s, H17*E*), 6.22 (s, H-17Z), 9.52 (s, H-1).

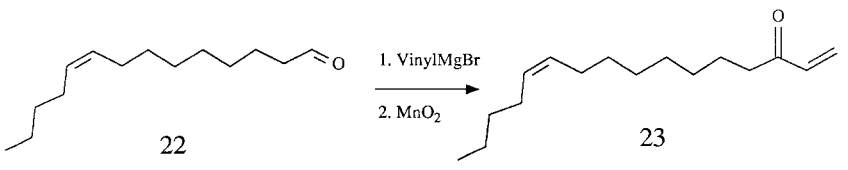

FIG. 5. Synthesis of vinyl ketone analog of *Z*11–16:Al.

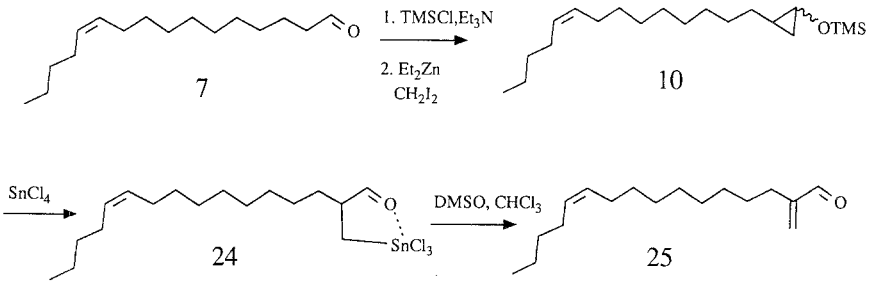

FIG. 6. Synthesis of the *exo*-methylene analog of Z11–16:Al.

Enzyme Inhibition. The preliminary in vitro assays of AOE inhibition by each of the two α,β-unsaturated carbonyls were conducted in duplicate with *H. virescens* male antennal homogenates, using a 40-min preincubation time (26°C) for inhibitor concentrations of 0.5, 1, 2, 5, 10, and 20 μM. Figure 7 summa-

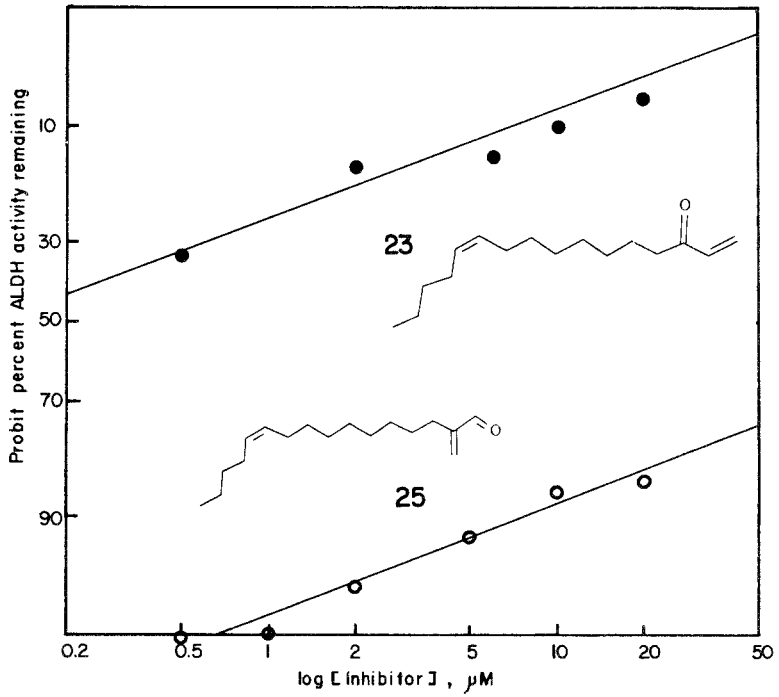

FIG. 7. Concentration dependence of inhibition of *H. virescens* male antennal AOE by vinyl ketone **23** and *exo*-methylene aldehyde **25**.

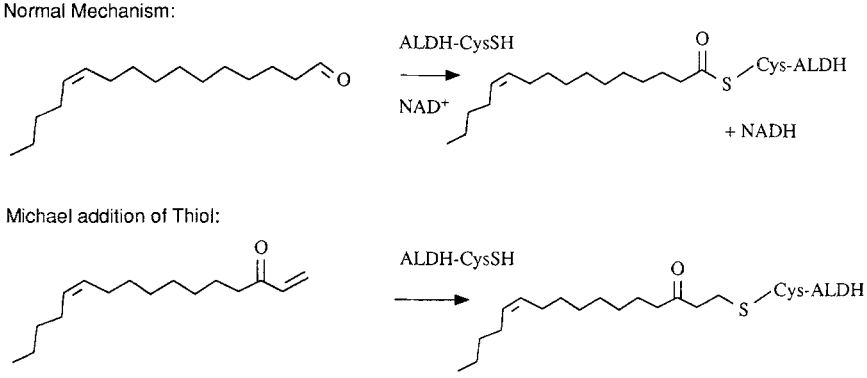

FIG. 8. Proposed mode of inhibition of AOE by vinyl ketone **23**.

rizes the results for these two inhibitors using 0.2 antennal equiv. per assay and 0.1 μM of the substrate, [^{3}H]Z11-16:Al. It is clear that the vinyl ketone **23**, with an I_{50} value below 0.1 μM under these conditions of low substrate concentration ($<0.1\ K_M$), is a minimum of 1000 times more potent as an AOE inhibitor than the α-methylene aldehyde **25**, which shows only 15% inhibition even at 20 μM. Full inhibition with the vinyl ketone is reached in less than 10 min preincubation, and the inhibition appears to be irreversible.

The observed inhibition is consistent with the model shown in Figure 8. Thus, the proximity of the β-carbon of the vinyl ketone to the thiol group at the AOE active site enables facile conjugate addition to the enone system in competition to direct carbonyl addition. This gives an adduct unable to undergo further processing by the enzyme. In contrast, the *exo*-methylene aldehyde **25** appears to lack the correct spatial orientation of the β-carbon to allow this conjugate addition. We believe this may be a substrate-specific modification of the specific pheromone-processing AOE. Although the action of vinyl ketones on aldehyde dehydrogenases has precedent, the effects of vinyl ketones on aldehyde oxidases are poorly known. Our current studies (M.L. Tasayco J., B. Latli, and M. Handley, unpublished results) show (1) a rapid time course of inactivation and (2) a distinct optimal length (C_{14}, C_{16}) and requirement for unsaturation for the carbon chain of the inhibitory vinyl ketones. The electrophysiological effects of these potent enzyme inhibitors toward *P. xylostella* and for *H. virescens* sensory cells are also being examined.

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GENETIC REGULATION OF SEX PHEROMONE PRODUCTION AND RESPONSE: Interaction of Sympatric Pheromonal Types of European Corn Borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae)

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Abstract—The sex pheromone communication system of the European corn borer moth varies intraspecifically. Analyses of pheromonal extracts of wild females, collected in a region where the types (each producing a different isomeric proportion of *Z*- and *E*-11-tetradecenyl acetate) are sympatric, showed that the *Z* pheromone-production allele frequency was ca. 4 times greater than the *E* allele. The paucity of *E* production and response alleles in the population indicates that moths inheriting those alleles concomitantly inherit some undefined disadvantage. The types interbreed, but the frequency of heterozygous pheromone-production types among wild females was less than predicted by Hardy-Weinberg equilibrium and was evidence of positive assortative mating. Rates of male captures in field traps baited with females of the three pheromonal types also evidenced assortative mating in the population. Progeny tests with males captured in the traps provided circumstantial evidence that pheromone response and production functions in the species are regulated by separate genetic loci and that the loci are not always complementary; i.e., a male can carry an allele coding for production of one pheromonal isomer ratio but can be genetically predisposed to respond to another.

Key Words—11-Tetradecenyl acetate, population genetics behavioral genetics, *Ostrinia nubilalis* European corn borer, Lepidoptera, Pyralidae.

INTRODUCTION

Examples of intraspecific communications involving tactile, auditory, visual, or chemical cues are abundant throughout the animal kingdom; however, our understanding of how they might have evolved or the genetic determinants of these communication channels is surprisingly limited. The inception of any communication system requires the evolution of two novel capacities, that of production of a signal and that of an appropriate receiver. The improbability of the coincident and de novo occurrence of the two events staggers the imagination (Kitteredge and Takahashi, 1972). Despite the improbability, the channels exist, and are a source of fascination.

The sex pheromone communication systems of insects have been of particular interest over recent years, and their chemistry and behavioral aspects have been fairly well elucidated. However, there have been few studies relevant to the issue of genetic control of production and receiver functions in these communication channels. Partly responsible for this fact is that examples of intraspecific pheromonal variability, in which genetic regulation of these functions might be studied, are rare (Mitter and Klun, 1987). The European corn borer (*Ostrinia nubilalis*) is one of the rare examples. In this species, the isomeric composition of the female sex pheromone, 11-tetradecenyl acetate, varies intraspecifically and is regulated by simple Mendelian inheritance involving a pair of autosomal alleles (Klun and Maini, 1979). The pheromone production alleles involved can be designated as *Z* and *E*; the *ZZ* genotype secretes ca. 97:3 (*Z*:*E*), *EE* secretes ca. 3:97 (*Z*:*E*), and the *ZE* heterozygote secretes ca. 35:65 (*Z*:*E*). These alleles were previously designated as *a* and *A* by Klun and Maini. Thus, the species is comprised of three female sex pheromone-production types; the heterozygote arises by hybridization and subsequent recombination.

The European corn borer is distributed throughout much of the holarctic, and male trapping surveys, using various geometrical mixtures of 11-tetradecenyl acetate, have indicated that the *Z* allele predominates in most populations while the *E* allele predominates only at select locations in Switzerland, Italy, and the northeastern United States (Klun and cooperators, 1975; Buechi et al., 1982; Anglade et al., 1984). In some instances where male trap captures were monitored over successive years at the same location, Anglade et al. (1984) noted that the male capture rate in traps baited with 3:97 (*Z*:*E*) and 35:65 11-tetradecenyl acetate often declined from one season to the next while captures in 97:3-baited traps increased. They speculated that the change in apparent phenotypic frequency might be related to variations in environmental conditions (climatic and trophic) that selectively favor the *ZZ* type. Because of its worldwide prevalence, it appears that the *ZZ* type may enjoy some selective advantage; however, no basis for it is known.

Voltinism in the European corn borer is variable, depending primarily on geographic location, and most studies (Klun and cooperators, 1975; Anglade et al., 1984; Roelofs et al., 1985) indicate that the pheromonal type in a population and voltinism are independent variables. Glover et al. (1987) speculated that females of the *EE* type store more 11-tetradecenyl acetate in their pheromone glands than females of the *ZZ* type; analyses of the pooled ovipositor extracts from homozygous laboratory colonies of the two types showed that the *EE* type females stored ca. 3 times more pheromone than *ZZ*. Aside from this observation, there are no known differences between types, nor are there any morphological or physiological characters that permit their differentiation.

Although the genetics of female sex pheromone production in the European corn borer have been known for some time, the genetics regulating male olfactory response was only recently revealed (Hansson et al., 1987), and the relationship between production and response loci is unknown. For purposes of this study, we hypothesize the existence of a complementary locus in males with two alleles (Z' and E') that promote sexual response to products of the Z and E alleles of females, respectively. Response and production functions are usually coordinated to permit functional communication channels in the species. As evidence of this coordination, Klun and Maini (1979) showed that heterozygous F_1 males, from a cross of *ZZ* and *EE* genotypes, responded preferentially to the 11-tetradecenyl acetate isomer ratio that was characteristic of female siblings (35 : 65, Z : E) and less effectively to either of the parental ratios. Thus, the results showed that males track changes in the female pheromone signal, and the electrophysiological studies by Hansson et al. (1987) indicate that the tracking process is related to inherited changes in the action potential response patterns of specific male antennal chemosensory cells to the geometric isomers of 11-tetradecenyl acetate. These changes in the sensory cell response patterns to the isomers are apparently regulated, like sex pheromone production in the female, by a Mendelian autosomal gene. Roelofs et al. (1987) have indicated that, although discrimination of geometric composition at the sensory level is controlled by the autosomal gene, male behavioral response to the geometric ratios is also influenced by a sex-linked gene. This apparent redundancy of genes controlling male response warrants further study.

In the summer of 1984, we conducted a preliminary male European corn borer trapping study in the vicinity of Beltsville, Maryland, and found that the pheromonal types in this bivoltine population were sympatric. This finding prompted us to study the population in an effort to determine the relative abundance of pheromonal types, to gain insight into the extent to which they interbreed, and to better understand the organization of the species' mating system. We report that the Z allele is the most abundant in the population. Results indicate that sympatric pheromonal types mate assortatively; however, interbreeding does take place at a low level. Gene flow is apparently facilitated by

males in the population that respond sexually to one geometric proportion of 11-tetradecenyl acetate, but they carry female-limited pheromone production alleles that code for a dissimilar geometric proportion.

METHODS AND MATERIALS

Insect Cultures. Colonies of European corn borer, homozygous for *ZZ* and *EE* pheromone production genes, were established in the laboratory by collecting 60 gravid females with sweep nets from the headland vegetation of cornfields at the Beltsville Agricultural Research Center over the course of the 1984 and 1985 growing seasons. Isofemale lines were reared on artificial diet (Reed et al., 1972), and female progeny in each family were screened by gas chromatographic analysis of their ovipositor extracts to identify the lines that were homozygous for pheromone production. Forty-seven progeny lines were identified as *ZZ*, six were *EE*, and seven were heterozygous. The homozygous lines were combined to establish *ZZ* and *EE* colonies, and these were maintained in incubators (80% relative humidity; 16 hr light–8 hr dark, 26°C:20°C) on separate floors of our laboratory to ensure their homozygous condition. F_1 hybrids were prepared as needed by placing 40 *ZZ* females and 40 *EE* males together in a screened cage fitted with a sheet of waxed paper at its top. The insects mated, egg masses were collected, and the progeny were reared at the conditions described above. Pheromonal heterozygosity in the F_1 progeny was always verified by chromatographic analyses.

Live-Trapping Males and Progeny Tests of Trapped European Corn Borer Males. Virgin *ZZ*, *ZE*, and *EE* female types, used to bait traps in the field, were caged individually in 5 × 5 cm cylindrical screen cages that were each fitted with a cotton dental roll saturated with water. The females were always ca. 24 hr old when they were deployed in the insect traps. The conditions in the laboratory incubators, in which they were held prior to deployment in the traps, were the same as described previously and the photoperiod was synchronous with the diurnal cycle outdoors. Throughout the course of the trapping, test sets of females in the traps were provided water daily and were replaced with fresh females after three or four nights or whenever a female died.

The cone-trap design (*Heliothis* Scentry trap) used in the study was identical to that described by Webster et al. (1986) with the exception that the containment portion of the trap was constructed of a finer fabric mesh size (1.5 × 1.5 mm) to prevent the escape of captured males. The entire containment portion of the trap was attached to the top of the cone with a Velcro fastener for easy recovery of trapped males. Each trap was baited with a caged virgin female by attaching the cage to a string that was strung across the center of the lower opening of the trap.

Sets of 10 traps were deployed at the perimeter of cornfields at three sites on the Beltsville Agricultural Research Center that were at least 2 km apart. Traps were deployed in line 30 m apart and positioned with their lower openings 1 m from the ground. Six traps at each location were baited randomly with *EE* females, two traps were baited with *ZE* females, and the remaining two with *ZZ* females. A larger number of *EE* female-baited traps was used in the experiment because earlier trapping tests showed that *EE*-responding males were least abundant in the population. Thus, the extra *EE* female-baited traps at each location ensured that a sufficient number of *EE*-responding males would be captured for subsequent progeny tests.

Males were trapped from July 21 through August 9, 1986, during the summer flight of the borer. Each morning, the number of males captured the previous night in response to each female type was recorded. Approximately 10 males, captured in traps baited with each female type, were collected individually in sealed plastic cups and labeled to designate the genotype causing its capture. Each male was then paired with a virgin female from the laboratory colony that was homozygous for pheromone production. Specifically, males captured in traps baited with *ZZ* females were mated with *ZZ* females and males captured in *EE* or *ZE* female-baited traps were paired with *EE* females. Subsequent chromatographic analysis of the pheromone produced by female progeny resulting from these pair-matings allowed determination of the pheromone production genotype of captured males. To achieve this end, each mating pair was retained for ca. 5 successive nights in a 5 × 5 cm cylindrical screened cage with a sheet of waxed paper at its top in incubators operated at conditions described previously. Females that mated layed eggs on the waxed paper and egg masses from each pair were placed over artificial diet in a ca. 500-ml screw-cap jar that was fitted with a cylinder of corrugated paper as a pupation site for the insects and sealed with a 80 × 60 mesh/2.54 cm^2 copper screen. The progeny lines were reared under reverse photoperiod, and pupae from each line were isolated individually in jelly cups to await adult emergence. Female progeny were held for 48–72 hr after emergence, and then the individual ovipositors were excised under a low-intensity red light at mid-scotophase. Each ovipositor was transferred to a screw-cap microvial having a conical interior and containing 10 μl heptane. The excised ovipositor and the associated pheromone gland of each female were allowed to soak in the solvent for ca. 30 min before the tissue was removed. Extracts were stored in sealed vials at −15°C until 11-tetradecenyl acetate geometrical compositions could be determined chromatographically.

Capillary gas chromatography (GC) with flame ionization detection was performed using four Hewlett-Packard gas chromatographs that were operated using splitless injectors (225°C; split-valve open 0.4 min after injection). Two of the instruments, models 5830 and 5840, were fitted with 60 m × 0.25 mm

ID DB-1, nonpolar columns (J&W Scientific, Inc., Folsom, California 95630) and the other two instruments (both model 5840A) were each fitted with 60 m × 0.25 mm ID Supelcowax polar columns (Supelco, Inc., Bellefonte, Pennsylvania 16823). Hydrogen was the carrier gas with a linear velocity of 55 cm/sec at initial oven temperature. The DB-1 and Supelcowax columns were operated using oven temperature programs: 120°C at injection, heating at 15°C/min 0.45 min after injection to 175°C, and 90°C at injection, heating at 30°C/min 0.45 min after injection to 180°C, respectively. Operating at these conditions, the GC columns fully resolved (*E*)- and (*Z*)-11-tetradecenyl acetate.

Routinely, each ovipositor extract was concentrated to 2–3 μl, injected into a gas chromatographic column, and the *Z*:*E* proportion of 11-tetradecenyl acetate in the extract was determined from the area or height of peaks representing the respective geometrical isomers in the chromatographic run. Extracts of line progeny females containing 0:100 to 10:90 *Z*:*E* 11-tetradecenyl acetate isomer ratios were scored phenotypes of the *EE* genotype, 20:80 to 50:50 *Z*:*E* were scored *ZE* genotype, and extracts with 100:0 to 85:15 *Z*:*E* ratios were scored as *ZZ* genotype (Klun and Maini, 1979). A minimum of 10 individual female analyses was obtained from each progeny line to determine the pheromone-production genotype of the male parent. Knowing which female type had caused capture of each male parent allowed development of correlations between the apparent sexual response preference of the males and the female limited pheromone-production allele frequencies within each male behavioral class.

Frequency of Female Pheromonal Forms by GC. Wild adult females (n = 177) were collected during the day in the vicinity of the insect traps. These insects were placed individually in jelly cups and held in an incubator until the 2–3 hr of the next scotophase. At that time, the mating status of each female was determined by inspection for the presence or absence of a spermatophore (Drecktrah and Brindley, 1967), and the ovipositor was excised and extracted with heptane. The extracts were subsequently analyzed by GC to determine the pheromonal phenotype of each female and to permit genotypic designation.

Flight Tunnel Assay of Response of Field-Collected Males to 11-Tetradecenyl Acetate Isomers. Six cone traps were deployed 30 m apart in a line at the edge of a cornfield; four were baited with a rubber septum treated with 100 μg (*Z*)-11-tetradecenyl acetate and two were baited with septa treated with 100 μg 11-tetradecenyl acetate (97:3, *Z*:*E*). Freshly treated septa were placed in the traps every third day. Each morning, from July 23 to August 7, 1987, samples of males captured the night before in respective pure *Z* and 97:3 *Z*:*E* baited traps were collected individually in capped jelly cups and transferred to an environmental chamber that was programmed to simulate the existing seasonal day length and temperatures. Between 2300 hr and 0200 hr of the night following collection from the traps, pure *Z*-, *Z*:*E*-captured males, and males from our *ZZ* colony were assayed for their responsiveness in a flight tunnel to rubber septa

treated with 100 μg pure Z, 97:3 Z:E, or 33:67 Z:E 11-tetradecenyl acetate. The flight tunnel and conditions of the assay were identical to those described by Schwartz et al. (1988). The criterion for a positive male response was that the moth flew 3 m upwind in the stimulus plume and came within 1 cm of the septum or landed on it. The assay was replicated 31 times using a randomized block design, and the percentage of positive responses among male types to each isomer mixture was calculated.

RESULTS AND DISCUSSION

Pheromone-Production Allele Frequencies among Females. Table 1 shows that unequal numbers of females were collected at each of three locations for gas chromatographic determination of the frequency of female pheromonal types. The unequal number of samples reflects the time that was spent collecting insects at each location and is not a measure of insect density. Inspections for spermatophores indicated that most of the females collected had mated but pheromone titer in a majority of the females (121) was sufficient to determine the 11-tetradecenyl acetate isomeric proportions of their pheromone. We calculated the expected pheromonal genotypic frequencies, Z and E allele frequencies, and tested (chi-square) the observed genotypic frequencies at each location for conformity to the Hardy-Weinberg expectations at equilibrium (Hartl, 1980). The results (Table 2) show that the Z allele frequency was higher than the E frequency at each location. The frequency of genotypes observed at locations 2 and 3 deviated significantly from the expectations of the Hardy-Weinberg equilibrium. At these locations, a deficiency in the observed number of heterozygotes indicated that pheromonal types in the population mate assortatively.

TABLE 1. SAMPLES OF WILD FEMALES AT THREE LOCATIONS ON BELTSVILLE AGRICULTURAL RESEARCH CENTER, JULY 16–18, 1986[a]

	Number of females		
Location	Collected	Mated	Positive phenotype determinations
1	15	8	12
2	61	54	31
3	101	92	78

[a] Locations 1, 2, and 3 were on the so-called South, West, and East farms, respectively, at the Beltsville Agricultural Research Center.

TABLE 2. FEMALE SEX PHEROMONE PRODUCTION ALLELE FREQUENCY ACCORDING TO GAS CHROMATOGRAPHIC (GC) ANALYSIS OF FERAL FEMALES AND MALE RESPONSE ALLELE FREQUENCY ACCORDING TO MALE TRAP-CAPTURES AT THREE LOCATIONS[a]

	Frequency of female types by GC			Pheromone production allele frequency			$\bar{X}$ males/trap/female type			Male response allele frequency		
	ZZ	*ZE*	*EE*	χ^2	PZ	QE	*ZZ*	*ZE*	*EE*	χ^2	PZ′	QE′
Location 1												
Observed	10	1	1	0.7	0.88	0.12	382	21	39	256.1[b]	0.89	0.11
Expected	9	3	0.1				348	88	6			
Location 2												
Observed	21	5	5	6.9[b]	0.76	0.24	93	153	32	6.8[b]	0.61	0.39
Expected	18	11	2				103	132	42			
Location 3												
Observed	54	13	11	18.3[b]	0.78	0.22	112	84	25	2.2	0.69	0.31
Expected	47	27	4				107	93	20			

[a] $\chi^2 = \{[|(ZZ_o - ZZ_e)| - 0.5]^2\}/ZZ_e + \{[(ZE_o - ZE_e)| - 1]^2\}/ZE_e + \{[|(EE_o - EE_e)| - 0.5]^2\}/EE_e$. *ZZ* and *EE* are the assumed homozygous male responders and *ZE* are assumed heterozygous male responders. o = observed, e = expected. The expected values were calculated from the expectations of the Hardy-Weinberg equilibrium model for random mating, with a correction for continuity.

[b] $P < 0.05$.

Samples from location 1 did not differ significantly from Hardy-Weinberg equilibrium, but this may be a result of the small sample size from that location.

Frequency of Male Response Types. Male captures in female-baited traps across all locations during the 1986 summer flight of the insect are shown in Figure 1. Arrows in the figure indicate the nights when fresh females of each type were placed in the traps. The data show that captures were maximal at two different times during the course of the flight and that capture rates in traps among female types rose and fell synchronously, indicating that the fluctuations in capture rate were independent of female type. Captures/trap/female type (Table 2) varied from one location to another, indicating that male pheromone response preferences of populations differed parochially. Assuming that the capture rate in each female-type baited trap is a measure of the frequency of three genetically determined male behavioral classes (*Z′Z*, *Z′E′*, and *E′E′* genotypes) at each location, and that male response is regulated, like pheromone production, by a pair of alleles at a single locus, we calculated male response allele frequency and tested the observed frequency of male genotypes for conformity to Hardy-Weinberg equilibrium. Results (Table 2) show that the putative *Z′* male-response allele frequency was also greater than *E′* at all locations.

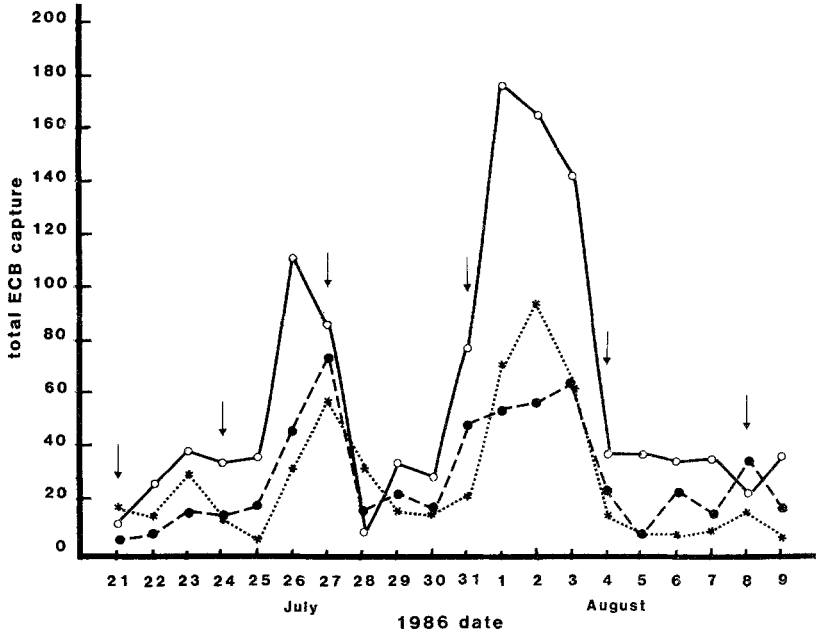

FIG. 1. Total daily captures of European corn borer (ECB) males in traps baited with female pheromonal biotypes during the second brood flight in 1986. Arrows indicate the nights when fresh virgin females were positioned in traps. Open circles represent data points for captures in *ZZ* female-baited traps, solid circles represent *EE* female traps, and asterisks represent data for *ZE* female traps.

Thus, trap capture data and GC analyses of wild females, respectively, indicated a high frequency of *Z'* alleles for response and *Z* alleles for pheromone production in the population. At locations 1 and 3, the high frequency of *Z'* alleles is attributable to an abundance of *Z'Z'* homozygotes, while the high *Z'* allele frequency at location 2 is owed to the relatively large number of presumed heterozygotes observed at that site. Thus, the variability in the frequency of types of male responders indicates that populations separated by 1–2 km are not entirely panmictic. At two of the three locations, the frequency of male types deviated significantly from Hardy-Weinberg equilibrium. This deviation is an indication that males select mates assortatively. Moreover, the male trapping test results also have practical implications. In instances where one might wish to monitor quantitatively European corn borer populations that are made up of three pheromonal types, such monitoring will require the use of at least three traps, each baited with a lure attractive to each type; 97:7, 35:65, and 3:97 *Z*:*E* 11-tetradecenyl acetate.

Mating Efficiency of Trapped Feral Males and Laboratory-Culture Females. The incidence of successful matings of individual field-trapped males with homozygous females of the laboratory culture was generally low: 59 progeny lines were established from 223 *ZZ*-responding males, 27 progeny lines from 179 *ZE* responders, and 61 lines from 261 *EE* responders (Table 3). Reasons for the low mating efficiency (15–26%) are difficult to ascribe, but male age, previous mating experience, behavioral incompatibility of cultured females and feral males, handling of the males, and/or inappropriateness of the laboratory setting for mating by feral males, etc., could have impacted adversely upon the mating process.

Sex Pheromone Production Allele Frequency in Male Responders. Results of the progeny tests are shown in Table 4. These results represent the outcome from more than 1240 individual female GC analyses. The data show that pheromone-production allele frequencies among males captured in traps baited with *ZZ* females were $Z = 0.90$ and $E = 0.10$, while the allele frequencies were $Z = 0.16$ and $E = 0.84$ in *EE* female-baited traps. Thus, as might be predicted, most (85%) of the males attracted to *ZZ* females were *ZZ* pheromone-production genotypes and, in the reciprocal, most (72%) males attracted to *EE* females were *EE* genotypes. The data clearly reflect the assortative nature of the mating system and coordination of response and production functions. However, a small percentage (11–24%) of males heterozygous for pheromone production and a smaller percentage (4%) homozygous for pheromone production and, genotypically opposite to the female to which they were attracted, were also found in the *ZZ* and *EE* female-baited traps.

TABLE 3. MATING SUCCESS OF FIELD-TRAPPED MALES PAIRED WITH LABORATORY-CULTURED FEMALES IN PROGENY LINE TESTS

	Female genotype causing male capture		
Site	*ZZ*	*ZE*	*EE*
Location 1			
No. males paired	100	13	90
No. mated	34	2	27
Location 2			
No. males paired	61	99	103
No. mated	14	18	21
Location 3			
No. males paired	62	67	68
No. mated	11	7	13
Total pairs	223	179	261
Total mated	59	27	61

TABLE 4. SEX PHEROMONE PRODUCTION ALLELE FREQUENCY OF MALES CAPTURED IN TRAPS BAITED WITH *ZZ*, *ZE*, AND *EE* FEMALE GENOTYPES ACCORDING TO PROGENY TESTS[a]

Female causing capture	Sex pheromone production genotype of male (%)			Sample allele frequency	
	ZZ	*ZE*	*EE*	P(Z)	Q(E)
ZZ (*N* = 47)	85	11	4	0.90	0.10
EE (*N* = 50)	4	24	72	0.16	0.84
ZE (*N* = 27)	22	33	44	0.39	0.61

[a] *N* = number of families.

The occurrence of heterozygous and opposite homozygous pheromone production types in the *ZZ* and *EE* female-baited traps might be attributed to chance or to mistakes being made by the males. However, we prefer to hypothesize that these males more likely represent individuals that are genetically predisposed to respond to a specific 11-tetradecenyl acetate geometric ratio, but they carry a pheromone production gene on a separate locus that codes for an entirely different geometric ratio. The basis for forming this hypothesis is vested in the observed frequency of pheromone production genotypes captured in traps baited with hybrid females. The pheromone-production allele frequency among males captured in the hybrid-baited traps was $Z = 0.39$ and $E = 0.61$ and, unlike the frequency of pheromone-production genotypes captured in *ZZ* and *EE* female-baited traps, there was a proportionately large number of the "wrong" (22% *ZZ* and 44% *EE*) pheromone-production genotypes in hybrid-female traps. If chance or male error were to account for the appearance of "wrong" pheromone-production genotypes in any female-baited trap, one would have expected the frequency of male types in the hybrid-female traps to be similar to the distribution observed in the traps baited with homozygous females, that is, a low frequency of *ZZ* and *EE* types with a high *ZE* zygote frequency. This was not the case; the zygotic ratio was 22 : 33 : 44 *ZZ* : *ZE* : *EE*. Therefore, neither chance nor male mistakes satisfactorily accounts for the frequency of pheromone-production types, but the idea of separate loci controlling response and production with *Z* or *E* polymorphism at each locus serves as a tenable model to explain the observed frequencies.

Using *Z* and *E* to denote production alleles, *Z'* and *E'* to represent male response alleles, and assuming that simple Mendelian inheritance governs response, the production–response genotypes of males captured in the hybrid-female baited traps would be *ZE/Z'E'*, *EE/Z'E'*, and *ZZ/Z'E'*. Correspond-

ingly, males captured in *EE*-female traps would be *EE/E'E'*, *ZE/E'E'*, and *ZZ/E'E'*, and males in the *ZZ*-female traps would be *ZZ/Z'Z'*, *ZE/Z'Z'*, and *EE/Z'Z'*. These types could arise as result of a rare mating of *EE/E'E'* and *ZZ/Z'Z'* types to yield heterozygotes which would mate assortatively and produce a segregated group composed of the nine possible genotypes. In the next generation, the expected frequency of *Z'E'* male-response types carrying *ZZ*, *ZE*, or *EE* production alleles would be twice that of either *Z'Z'* or *E'E'* response types carrying the alleles. This fact can account, in part, for the proportionately high frequency of *ZZ* and *EE* pheromone-production types that were observed in the hybrid-female baited traps as compared to the proportionately lower frequency of "wrong" pheromone-production types that were detected in *ZZ*- and *EE*-female baited traps.

Response of Field-Captured Males to 11-Tetradecenyl Acetate Isomers in Flight Tunnel. During the course of the trapping, an average of 72 males/trap/night were captured in traps baited with *Z* isomer compared to the 267 males/trap/night captured in 97:3 *Z*:*E*. The higher capture rate in 97:3-baited traps was an expected result inasmuch as it is known that males of a *ZZ*-type pheromonal population exhibit response over a narrow range of ratios of geometric isomers and that 97:3 is an optimum (Klun et al., 1973). Similarly, data in Table 5 show that all field-captured males responded better in the flight tunnel to 97:3 *Z*:*E* 11-tetradecenyl acetate than to pure *Z* isomer, independent of which isomer combination caused their capture in the field. The *ZZ*-colony males also responded best to 97:3 *Z*:*E*. Most interestingly, results show that 13% of males captured in the field, specifically with 97:3, responded ambivalently to 33:67 (an isomeric proportion characteristic of hybrid females) in the flight tunnel. However, neither males captured with *Z* alone in the field nor *ZZ*-colony males exhibited this ambivalence of response. It is plausible that the moths, exhibiting response to 97:3 and 33:67 isomer ratios, represent a special class

TABLE 5. PERCENTAGE UPWIND FLIGHT RESPONSE OF EUROPEAN CORN BORER MALES CAPTURED IN FIELD TRAPS BAITED WITH PURE *Z* AND 97:3 *Z*:*E* 11-TETRADECENYL ACETATE TO THREE ISOMERIC MIXTURES OF 11-TETRADECENYL ACETATE IN FLIGHT TUNNEL ($N = 31$)[a]

Isomer causing field capture	Percentage upwind to *Z*:*E*		
	100:0	97:3	33:67
Z alone	16	61	0
97:3	48	65	13
Control (*ZZ* colony males)	52	94	0

[a]See Methods and Materials, p. 2050.

of males that could be attracted to and mate with either *ZZ* or *ZE* females in nature. Evidence of their existence suggests that inheritance of sexual response in males may involve more than simple Mendelian inheritance.

Moreover, indications are that the genetic determinants of the European corn borer sex pheromone communications include response and production genes that occur at separate loci in the genome. Our previous study of the inheritance of pheromone production (Klun and Maini, 1979) and the recent electrophysiological research on the pheromone receptor cells (Hansson et al., 1987) indicate that both genes are autosomal, and evidence presented here shows that they are most often coupled in a complementary fashion so that response and production are coordinated to effect positive assortative mating. However, this coordination can be uncoupled as a consequence of rare matings among sympatric opposite homozygous types; the resulting progeny are heterozygous at the response and production loci. These polymorphic forms undoubtedly contribute to gene flow among types. However, pheromonal allele frequency in a sympatric population appears to be dependent upon undefined factors that selectively favor the *ZZ/Z'Z'* genotype. It will be interesting to monitor these allele frequencies in the Beltsville population in the future to see if they shift from their present values.

Comparison of our findings to other studies of the genetic regulation of pheromone communications in other insects is difficult because most of them involved investigation of the effects of interspecific hybridizations. As examples, Lanier (1970) studied hybrids formed between three bark beetle species of the genus *Ips* and speculated that production and response were controlled by autosomal loci; Sanders et al. (1977) hybridized moths of the genus *Choristoneura* and obtained results that indicated pheromone production was controlled by both X-chromosomal and autosomal loci; Grant et al. (1975) hybridized two species of tussock moths (*Hemerocampa* spp.) and inferred that the hybrid's pheromone channel was more like the female parent than the male; however, they were not able to make the reciprocal cross to verify the inference. A similar parental effect has also been observed in hybridization of *Heliothis subflexa* female moths with *Heliothis virescens* males; the F_1 females produced sex pheromone that was identical to the array of compounds produced by *H. virescens* females (Klun et al., 1982). Thus, in this case, the pheromone-production alleles of the male parent showed complete dominance over those of the female. The response characteristics of the F_1 male have not been determined, but male progeny resulting from a backcross of the F_1 female with the *H. virescens* male responded sexually to backcross females and, in turn, backcross females were attractive to *H. virescens* males (Raulston et al., 1979). Thus, *H. virescens* male response alleles also expressed dominance over *H. subflexa*. The pheromone production and response characteristics of the reciprocal-cross hybrid and a more detailed study of F_1 male response is currently

underway in Gainesville, Florida (J.H. Tumlinson, personal communication), and a clearer picture of this system should be forthcoming.

As a final example, the mode of inheritance of pheromone communications in the sulfur butterflies was investigated by Grula and Taylor (1979). They prepared hybrids between *Colias eurytheme* and *C. philodice* and found that production of male pheromone in *C. philodice* is controlled by autosomal genes while the X chromosome carried most of the genes controlling pheromone production in *C. eurytheme*. They, however, did not address the issue of inheritance of female response.

In general, the evidence indicates that the genes regulating the pheromone communication systems of the insects can be carried on either autosomal or X chromosomes, but the mechanisms by which response and production functions are linked remain obscure. We are aware of at least three species of insects that exhibit intraspecific pheromonal variability: the bark beetles, *Ips pini* (Lanier et al., 1972) and *I. calligraphus* (Lanier, 1972), and a tortricid moth, *Amorbia cuneana* (Bailey et al., 1987). These species represent prime candidates for study and expansion of our understanding of the organization of insect chemical communications at the genetic level. In the European corn borer, indications are that the genetic determinants of its sex pheromone system involve autosomal response and production genes at separate loci.

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SELECTED FACTORS INFLUENCING NEUROHORMONAL REGULATION OF SEX PHEROMONE PRODUCTION IN *Heliothis* SPECIES

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Abstract—Sex pheromone production and release in females of *Heliothis* species exhibit a diel periodicity. Phermone production is controlled by a hormone, the pheromone biosynthesis activating neuropeptide (PBAN). Release of PBAN to activate pheromone production follows a circadian rhythm. In *H. zea* females, mating terminates pheromone production. An unidentified hemolymph-borne factor is transferred from the male to the female during mating. It is speculated that this factor interacts with the release mechanism of PBAN to prevent further production of the pheromone following mating. Wild females of *H.* phloxiphaga (reared from larvae collected in the field) did not produce or release the sex pheromone unless kept in association with the host plant. Pheromone production could be induced in these females by the injection of PBAN. It is suggested that a signal from the host plant is essential to trigger the release of PBAN to induce pheromone production.

Key Words—*Heliothis zea, Heliothis phloxiphaga*, Lepidoptera, Noctuidae, sex pheromone, neuropeptide hormone, mating, host plant factors.

INTRODUCTION

A majority of moth species, including those in the genus *Heliothis*, are nocturnal. In order to find their mates, they usually rely on chemical signals or sex pheromones. In most cases the sex pheromone is produced by the female and is composed of a blend of several organic compounds is very specific proportions. The blend composition is species specific and functions in part to maintain reproductive isolation among closely related species. Moths are also short-

lived as adults and often possess mature oocytes at eclosion. It had been earlier speculated that among the Lepidoptera there was no need for neurohormonal regulation of sexual activity (for a review, see Raina and Menn, 1987). However, even among the short-lived moths, there are events and circumstances that warrant the neurohormonal regulation of sex pheromone production.

As nocturnal species, the reproductive activity of these moths is usually gated, following an endogenous rhythm that is modified by environmental cues (Cardé and Webster, 1980). It would be a waste of resources and energy to maintain a higher titer of sex pheromone during the photophase. Diel periodicity of pheromone production and release has been reported in a number of moths such as *Trichoplusia ni* (Sower et al., 1970), *Platyptilia carduidactyla* (Haynes et al, 1983), and *H. zea* (Pope et al., 1984; Raina et al., 1986). Some form of neurohormonal regulation would appear to be essential to control the periodicity of their pheromone production. Neurohormonal control of pheromone release in the Lepidoptera had been suggested earlier (Riddiford and Williams, 1971). However, it was not until recently that conclusive evidence for neurohormonal control of pheromone production in *H. zea* was presented (Raina and Klun, 1984). The neuropeptide PBAN originates in the suboesophageal ganglion (SOG) and is released through the corpora cardiaca (CC) to activate pheromone biosynthesis in *H. zea* (Raina and Menn, 1987; Raina et al., 1987). The hormone has a molecular weight of 3899 and contains 33 amino acid residues (Raina, unpublished).

Females of most species of moths do not exhibit discrete reproductive cycles as seen, for example, in the cockroaches (Barth, 1965). Even though a single mating could provide the necessary sperm to fertilize a full complement of eggs, females in the majority of moth species are known to mate more than once. In most of these cases a successful mating is followed by the termination of their readiness to engage in further sexual activity (Truman and Riddiford, 1974). For example in *H. zea*, mating is followed by a significant drop in the pheromone titer (Raina et al., 1986), which can be considered to be an expression of the loss of sexuality in mated females. It has been reported that sexuality in *Hyalophora cecropia* is suppressed after mating by the release of a substance from either the spermatheca or the bursa into the hemolymph and its reaction with a hypothetical neural center in the brain (Truman and Riddiford, 1971; Riddiford and Ashenhurst, 1973). It is entirely possible that the neuroendocrine system is also involved in the termination of pheromone production and loss of sexuality following mating in *Heliothis* species.

Another factor that may interact with the neurohormonal regulation of pheromone production and release involves the host plant. A suggestion to that effect had been made in the case of the polyphemus moth, *Antheraea polyphemus* (Riddiford and Williams, 1967a,b; Riddiford, 1967). It was reported that *trans*-2-hexenal, a volatile from oak leaves, produces a sensory input from the

antennae to the brain which is relayed through the neuroendocrine system of the females to its receptor site (Riddiford and Williams, 1967a).

The above factors and their interaction with PBAN to control pheromone production were investigated in *H. zea* and *H. phloxiphaga*.

METHODS AND MATERIALS

First-instar larvae of *H. zea* placed on artificial diet were regularly obtained from the Southern Field Crops Management Laboratory of USDA in Stoneville, Mississippi. The larvae were kept in environmental chambers maintained at 16:8 light (26°C)–dark (20°C) cycle and 60 ± 5% relative humidity, with lights off at 8 AM and on at 4 PM. Upon pupation, males and females were kept in separate chambers. *H. zea* larvae were also collected on corn in Mississippi and shipped to Beltsville. The resulting adults constituted the wild population. *H. phloxiphaga*, a nonpestiferous species, was collected on Texas paint brush (*Castilleja indivisa*) around College Station, Texas, and reared in the laboratory on soybean–wheat-germ diet (Shaver and Raulston, 1971). Pheromone titers were determined as described by Raina et al. (1986).

H. zea females were mated during the third scotophase of their adult life and pheromone titer determined 2 hr after the termination of mating. Hemolymph from mated females was drawn 10 and 100 min after mating, mixed with cold saline, and injected into virgin females. Pheromone titers of the recipient females were determined 2 hr after the injection. Mated females were also injected with brain–SOG homogenates, and their pheromone titers were determined 3 hr postinjection.

H. phloxiphaga females reared from field-collected larvae as well as females reared in the laboratory for 3 and 10 generations were observed for calling behavior in the absence and presence of the host plant. Pheromone titers of these females were also determined. The experiments were also repeated with laboratory-reared and wild *H. zea*, using corn as the host plant. Wild females of both species were injected with brain–SOG homogenates and their pheromone titer determined.

RESULTS AND DISCUSSION

The pheromone titer [measured as nanograms of (*Z*)-11-hexadecenal (Z11–16:Ald)] in mated females of *H. zea* dropped from a premating average of 120 ng to <5 ng within 2 hr following the termination of mating. Since mated females were not observed to call, the drop in the pheromone titer is probably due to the termination of pheromone production and metabolism of the existing pheromone. Pheromone production in virgin females was terminated when they

were injected with hemolymph from mated females obtained soon after the termination of mating. The effect was less pronounced when hemolymph was obtained from females 100 min after the termination of mating and injected into virgin females. The effect was also associated with hemolymph of both virgin and mated males. These results indicate that a factor or factors transferred from the male to the female at the time of mating is responsible for the termination of pheromone production. This sexuality-suppression factor (SSF) is not related to the sperm, because when castrated males (testes removed in the larval stage) were mated to normal females, they transferred a spermatophore devoid of any sperm but suppressed pheromone production. It is speculated that the SSF may be a component of the accessory gland secretion. The pheromone titer of a mated *H. zea* female could be restored to the premated level by the injection of either brain–SOG homogenate or partially purified PBAN.

In the case of the housefly *Musca domestica*, it was reported that the injection of secretions from male ejaculatory ducts into a virgin female effectively inhibited its sexuality (Rieman et al., 1967). A water-soluble mating inhibitor was subsequently isolated from mated female housefiles (Adams and Nelson, 1968). When a male housefly whose accessory gland substances were radiolabeled was mated, the labeled substances were transferred to the female, absorbed through its vaginal pouches within 10–40 min, and carried in the hemolymph to the head (Leopold et al, 1971). Similar interaction of the SSF with PBAN release in mated *H. zea* females is speculated (Figure 1).

Whereas laboratory-reared females of *H. phloxiphaga* (reared on artificial diet for ca. 10 generations) produced and released the sex pheromone, females reared from larvae collected in the field produced very low amounts of the pheromone, and none of them was observed to call (Table 1). When Texas paint brush plant was introduced into the cage, the females started producing and releasing the pheromone. When these wild insects were reared in the laboratory on artificial diet, the requirement for the presence of a host plant as a prerequisite for pheromone production was reduced or lost after three to four generations. However, only a small proportion of the females were observed to call, and calling in these females was significantly increased by the presence of the host plant.

Results from preliminary experiments have indicated that pheromone production in wild females can be induced even in the absence of a host plant by the injection of brain–SOG homogenates or PBAN. Also, the PBAN titer in the brain–SOG of wild females was higher when they were kept without a host plant as compared to females with a host plant. These observations suggest that a signal from the host plant causes the release of PBAN to activate pheromone production. A similar host factor or the pheromone titer itself could cause the production and transmission of a neural signal to the pheromone gland to induce calling.

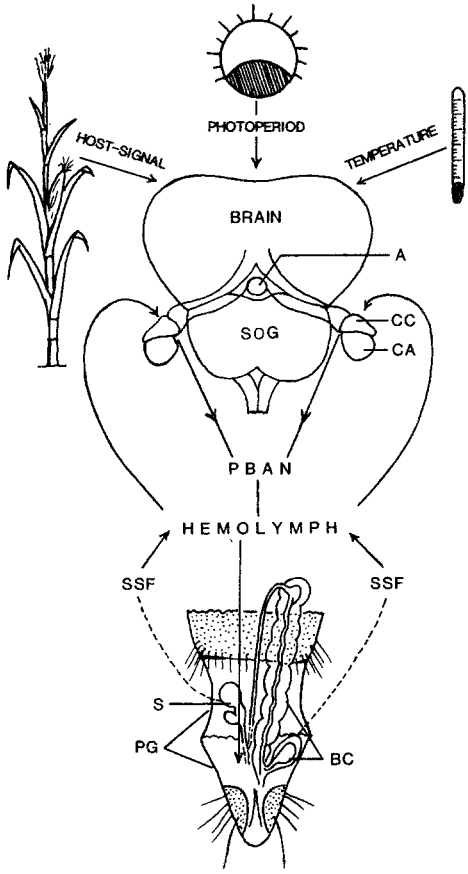

FIG. 1. A diagrammatic representation of the neurohormonal control of sex pheromone production in *H. zea*, and various interactions with external and internal stimuli. A, aorta; BC, bursa copulatrix; CA, corpus allatum; CC, corpus cardiacum; PBAN, pheromone biosynthesis activating neuropeptide; PG, pheromone gland; S, spermatheca; SOG, suboesophageal ganglion; SSF, sexuality suppression factor.

Preliminary tests with *H. zea* indicate that volatile factor(s) from the corn silk may act as a signal for the release of PBAN to initiate pheromone production (Figure 1). Apparently the host plant signal can override the circadian rhythm of PBAN release. Experiments to isolate and identify the SSF as well as the volatile factor(s) from corn that impact on pheromone production in *H. zea* are in progress. Identification of these factors are essential to study their interactions with PBAN in the control of pheromone production and release.

TABLE 1. EFFECT OF HOST PLANT ON PHEROMONE PRODUCTION AND CALLING IN *H. phloxiphaga*

Source of females	Calling (%)		Z11-16:Ald/female (ng)	
	Without host	With host[a]	Without host	With host
Wild[b]	3.0 (32)	57.5*[c] (36)	2.2	103.4*
Lab-reared				
3rd generation	16.6 (8)	66.3* (9)	67.8	89.7 NS
10th generation	64.6 (18)	72.2 NS (20)	97.1	83.8 NS

[a] Texas paint brush plants were used as hosts.
[b] Wild females were reared from larvae collected in the field on Texas paint brush.
[*c] Asterick: statistically significant differences between values obtained without host and with host in each category are indicated for $P < 0.05$. Figures in parentheses represent the number of insects observed. The same numbers were used for the determination of pheromone titers.

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CHEMICAL COMMUNICATION IN CUCUJID GRAIN BEETLES[1-3]

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Abstract—Males of five sympatric species of economically damaging cucujid grain beetles, *Cryptolestes ferrugineus* (Stephens), *C. pusillus* (Schönhen), *C. turcicus* (Grouvelle), *Oryzaephilus mercator* (Fauvel), and *O. surinamensis* (L.), produce macrolide aggregation pheromones especially in the presence of food. Work leading to the isolation, identification, and establishment of biological activity of these semiochemicals is reviewed. The trivial name "cucujolide" is proposed and used to identify these compounds that are characteristic of the Cucujidae. The two *Oryzaephilus* share species share a common cucujolide pheromone, while *Cryptolestes* species use cucujolides that are either enantiomeric, unique to the genus, or released in trace quantities by *Oryzaephilus* spp. and not used as pheromones by the latter species. The major mechanisms for species specificity in chemical communication are: (1) presence of a unique pheromone (*C. ferrugineus* and *C. pusillus*); (2) use of pheromones that are inactive alone but synergize response to cucujolides unique to a species (*C. pusillus*, *C. turcicus*, and *O. surinamensis*); (3) response to only one enantiomer of a pheromone (*C. ferrugineus*, *O. surinamensis*, and *O. mercator*); and (4) synergism between enantiomers of a pheromone (*C. turcicus*). The only species for which cross-attraction was evident was *O. mercator* to *O. surinamensis*. Both sexes of *Oryzaephilus*

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spp. produce (*R*)-1-octen-3-ol, which highly synergizes response to the cucujolide pheromones. Similar synergism occurs between hexanal, octanal, and nonanal and the cucujolide pheromones of *Oryzaephilus* spp. The males of a sixth cucujid species, *Cathartus quadricollis* (Guér) produce a different aggregation pheromone, (3*R*,6*E*)-7-methyl-6-nonen-3-yl acetate. Trapping of *Cryptolestes* and *Oryzaephilus* spp. in cardboard traps baited with pheromones is efficient in environments mimicking food-storage areas. Pheromone-baited plastic probe traps are the most efficient at capturing these species in infested grain.

Key Words—Grain beetles, Coleoptera, Cucujidae, *Cathartus quadricollis*, *Cryptolestes ferrugineus*, *C. pusillus*, *C. turcicus*, *Oryzaephilus mercator*, *O. surinamensis*, macrolide aggregation pheromone.

Hormones and pheromones are potent regulators of insect growth, development, reproduction, and behavior. Many new, safe, and specific approaches to insect control are based on manipulation of insect populations using these regulators. Knowledge of the chemical communication systems of economically important, grain-infesting insects (Levinson and Levinson, 1979; Burkholder, 1981, 1982) contributes to the development of semiochemical-based control programs. In the present article, we review the isolation, identification, and biological activity of the pheromones of six species of grain beetles in the family Cucujidae, for which we have unraveled much of the chemical communication systems.

IMPORTANCE OF CUCUJID GRAIN BEETLES

The species of beetles investigated are important stored-product pests of worldwide distribution. In the United States, the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), ranked first in importance as a pest of stored products and processed food and second as a program on raw grain (Mueller, 1982). The sawtoothed grain beetle cannot penetrate dry intact kernels and, when found in grain, is usually an indication of a prior infestation. The merchant grain beetle, *O. mercator* (Fauvel) is more affected by low ambient temperatures (Howe, 1956). It is an established household pest in North America, especially on cereal products and processed food (Loschiavo and Smith, 1970; Loschiavo and Sabourin, 1972). The rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), is a severe pest of stored wheat throughout the world (Reid, 1942; Howe and Lefkovitch, 1957; Banks, 1979). In Canada, the beetles infest wheat during storage (Loschiavo, 1975) and transportation (Monro, 1969; Loschiavo, 1974). Pockets of damp grain subject to heating are attractive to these insects (Smith, 1983). Infested wheat suffers loss of germinating ability (Campbell and Sinha, 1976). The flour mill grain beetle, *Cryptolestes turcicus* (Grouvelle), is a secondary feeder on broken and milled grain (Howe, 1956; Olsen et al., 1987). The flat grain beetle, *Cryptolestes pusillus* (Schönherr), is a seri-

ous pest of diverse stored products worldwide (Reid, 1942; Howe, 1956) and is especially severe in the grain-producing Midwestern states (Mueller, 1982; Barak et al., 1981). The square-necked grain beetle, *Cathartus quadricollis* (Guér.) is abundant in the southern United States and infests a variety of stored commodities such as corn and peanuts (Anonymous, 1955).

BIOLOGY OF CUCUJID GRAIN BEETLES

All of these species are long-lived, adult-feeding beetles (Anonymous, 1955; Sinha and Watters, 1985). They survive at temperatures between 18°C and 40°C and 40–90% relative humidity but are optimally maintained between 31°C and 35°C and 70–90% relative humidity. Females of these species lay several hundred eggs in a lifetime by dropping them loosely among foodstuff and crevices of grain. Eggs hatch in three to five days, and lavae crawl about feeding until they construct delicate cocoonlike coverings by joining together small kernels or fragments of foodstuffs. Within this cell, the larva pupates over a week and emerges as an adult. Development from egg to adult requires three to four weeks. Adults live and feed an average of 6–10 months but can live much longer under ideal conditions.

ISOLATION OF CUCUJID PHEROMONES

Borden et al. (1979) and A.M. Pierce et al. (1981) developed procedures for laboratory rearing of the target species on whole wheat and wheat germ (95:5) or rolled oats and brewer's yeast (95:5) that provide populations of uniform characteristics for biological and semiochemical investigations. Insects and frass for aeration are sieved from the medium, and the insects are collected by aspiration as they walk away from the tailings. Fine sieving of the tailings provides frass (boring dust and fecal pellets) (Borden et al., 1979; Millar et al., 1985a,b).

H.D. Pierce, Jr. et al. (1984) modified (Figure 1) the procedure of Byrne et al. (1975) for capture of the volatiles of insects, frass, and food on Porapak Q. In the presence of food and moisture, volatiles are collected continuously over long periods (7–10 days) with little or no mortality of the beetles. Volatiles emitted at very low rates (picograms per hour) are accumulated until sufficient amounts are available for bioassay and structural study. Captured volatiles are extracted from the Porapak Q (Soxhlet) with pentane, which is removed by slow distillation (Dufton column) to provide stock solutions of volatiles for bioassay or fractionation. Bioassays of volatiles are conducted using an open arena air-flow (Figure 2) or a two-choice pitfall olfactometer (Figure 3). The former was originally developed to investigate the activity of volatiles from scolytid bark beetles (Wood and Bushing, 1963), but it is labor-intensive and does not allow

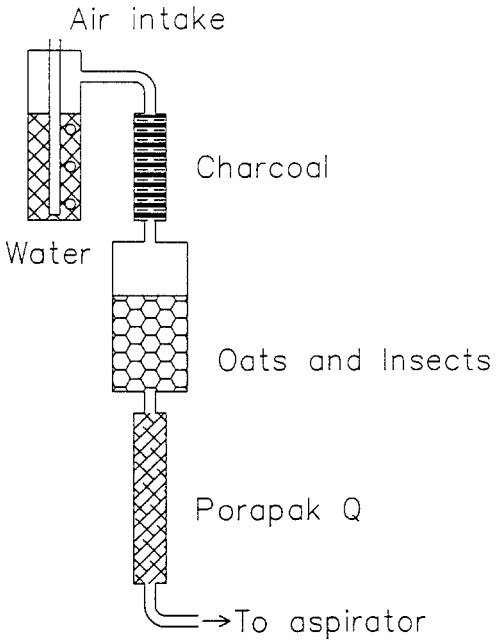


FIG. 1. Aeration apparatus in which volatiles from insects alone or on food are collected by drawing air (2 liters/min) with a water aspirator through water and an activated-charcoal scrubber, then over the source material, and finally through Porapak Q.

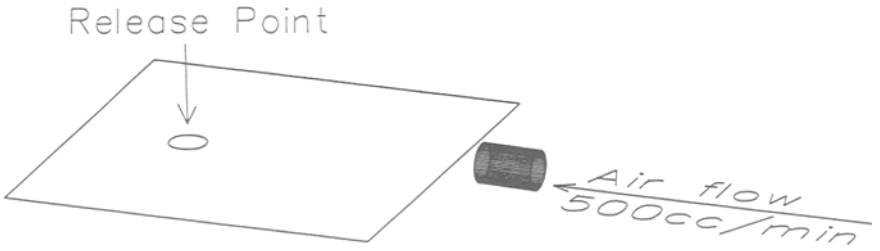


FIG. 2. Arena olfactometer with a 15 × 15-cm paper arena and an air nozzle (flow rate, 500 ml/min) in the middle of one side. A glass tube containing a rolled paper impregnated with stimulus is slipped into the air nozzle, and the air flow was directed toward the insect release point. In a typical bioassay, a vial containing 15 insects is inverted onto the center of the arena and left in place so that the insects could not escape while the test stimulus is placed in the air stream. Removal of the vial allows the insects to walk freely. Insects reaching a 3 × 1-cm-wide area in front of the stimulus tube are counted as responders. Insects reaching the arena edge and insects still walking around on the arena at the end of an allotted 2-min time limit are counted as nonresponders (Borden et al., 1979).

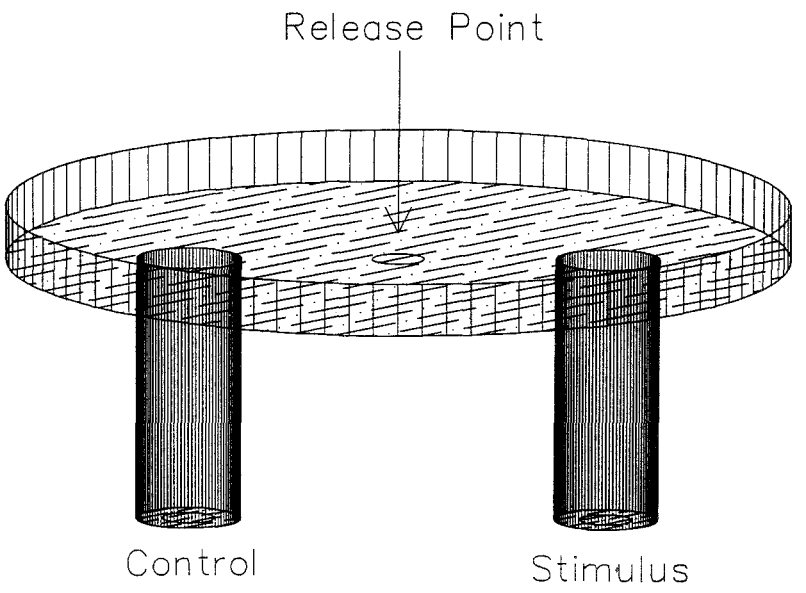


FIG. 3. Pitfall olfactometer with two 8-ml glass vials suspended from holes cut in the bottom of a 15-cm plastic dish. A filter paper disk treated with a pentane solution of the stimulus is placed in one vial, and a disk treated with the same volume of pentane is placed in the other. Typically 12–15 insects are used and each bioassay is replicated 6–12 times for each stimulus. A lid (not shown) prevented beetle escape. Bioassays are run in the dark at 23°C and ~60% relative humidity. At the end of a 2-hr period, the numbers of insects in the control and stimulus vials are recorded. To ensure uniform conditioning, beetles for bioassay are sifted from cultures, aerated in darkness for 18–48 hr with clean, moist air, and counted into holding vials ~2 hr before testing. After a bioassay, insects are allowed to feed at least four days before being reused.

for simultaneous replication. It has been replaced in our laboratory by the two-choice pitfall olfactometer (Figure 3) (A.M. Pierce et al., 1981), which is more discriminating and less labor-intensive.

Extensive investigations revealed the volatiles of mixed-sex beetles or their frass are attractive to both sexes of conspecifics (Borden, et al. 1979; A.M. Pierce et al., 1981, 1984, 1985; Wong et al., 1983; H.D. Pierce, Jr. et al., 1984, 1988; Millar et al., 1985a,b). Bioassay of the volatiles of the separated sexes disclosed that this attraction is due to emissions of the males of each species. Thus, all five species utilized male-produced aggregation pheromones. Gas chromatographic analysis of the volatiles obtained from aerations of *C. ferrugineus*, the first species investigated, revealed the presence of several late-eluting components in the beetle volatiles that are not present in food volatiles (Figure 4). Wong et al. (1983) demonstrated these late-eluting components were

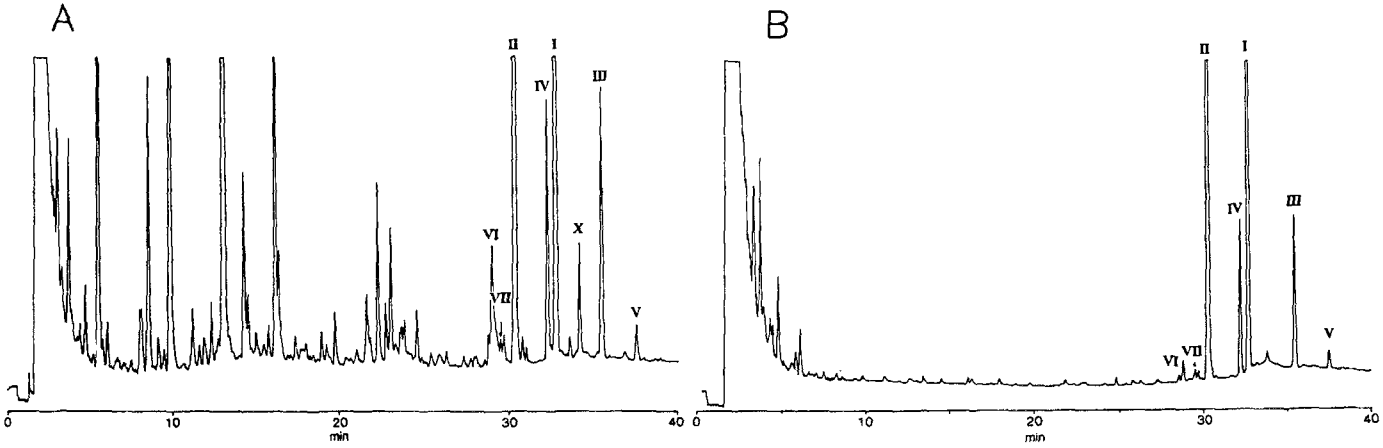
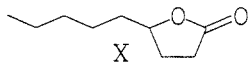


FIG. 4. Gas chromatogram of *C. ferrugineus* frass volatiles (A), and mixed-sex beetle volatiles (B). Volatiles are captured on Porapak Q, extracted with pentane, and analyzed on a 33-m × 0.66-mm ID glass open-tubular column coated with SP-1000, programmed at 4°C/min from 70–200°C.

produced specifically by males and not by females. The two major late-eluting components were isolated by preparative gas chromatography and found to be attractive at nanogram doses. These components were identified as cucujolides I and II[6] (Table 1) by [^{1}H]NMR, mass spectroscopy (Wong et al., 1983), and chemical synthesis (Oehlschlager et al., 1983). The minor components eluting in the same gas chromatographic range are lactones III–VII (Table 1) and X (Scheme 1). Gas chromatographic analysis of diastereomeric (*S*)-*O*-acetyllactate derivatives of natural and synthetic chiral isomers of II by Oehlschlager et al. (1987) revealed that *C. ferrugineus* produces only (*S*)-II (Figure 5).

Wong et al. (1983) determined that threshold responses of *C. ferrugineus* to synthetic I and II are equivalent to those of volatiles of feeding beetles containing similar quantities of I and II (21 and 18 ng, respectively). This suggests that these two components were responsible for all the activity in this odor source (Figure 6). Synergism between I and II is evident in that response to mixtures of I and II is higher than to either pheromone alone. That (*R*)-II is an inactive enantiomer is suggested by the observation that the threshold response elicited by (*S*)-II is about 100-fold lower than for the *R* enantiomer. Although the natural ratio of I:II is ~1:1, this mixture elicited a higher threshold (40 ng, 20 ng of each component) than either 3:1 (17.5 ng) or 9:1 (4.7 ng) mixtures.

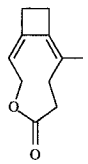
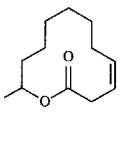
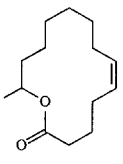
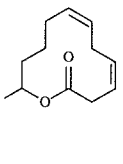
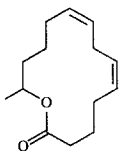
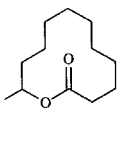
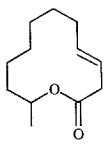
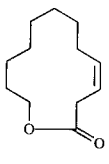
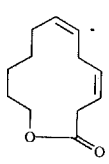


SCHEME 1.

Millar et al. (1983, 1985a) disclosed that volatiles of *C. pusillus* contained cucujolides III, VIII, and IX (Table 1). Adults are attracted to synthetic VIII alone but neither racemic III nor IX individually. Cucujolide IX elicits a threshold response about 100-fold higher than that observed for VIII alone and is not synergistic with VIII in ratios (100:1 to 10:1) spanning the natural ratio of ~75:1. When added to VIII, racemic III elicits a small synergism that is maximal near the natural ratio (~20:1). In this test, *C. pusillus* responds equally to either enantiomer of III, although Oehlschlager et al. (1987b) found feeding adults produce only (*S*)-III.

[6]Because of their prevalence in the Cucujidae and their characteristic structure, we propose that the macrolides discovered in the genera *Cryptolestes* and *Oryzaephilus* be given the trivial name cucujolides. This trivial name is for convenience and does not ignore the fact that these compounds have other natural sources (Wong et al., 1983). Cucujolides I and II replace the common names ferrulactone I and II originally proposed for the two pheromones of *C. ferrugineus* (Wong et al., 1983)

TABLE 1. DISTRIBUTION OF CUCUJOLIDES IN *Cryptolestes* AND *Oryzaephilus* SPECIES[a]

	 I	 II	 III	 IV	 V	 VI	 VII	 VIII	 IX
C. ferrugineus	38–60P[b]	34–42P	3.5–5	2–4	0.5	1.5	<0.5		
C. pusillus			2.5P_{syn}[c]					96P	1.5
C. turcicus			28P_{syn}		72P				
O. mercator		45P	<0.1	55P	<0.1				1
O. surinamensis			<0.1	16P	51P_{syn}				33P

[a] I, (*E*, *E*)-4,8-dimethyl-4,8-decadien-10-olide; II, (*Z*)-3-dodecen-11-olide; III, (*Z*)-5-tetradecen-13-olide; IV, (*Z*, *Z*)-3,6-dodecadien-11-olide; V, (*Z*, *Z*)-5,8-tetradecadien-13-olide; VI, dodecen-11-olide; VII, (*E*)-dodecen-11-olide; VIII, (*Z*)-3-dodecenolide; IX, (*Z*, *Z*)-3,6-dodecadienolide. Values are given as percent of total macrolides.

[b] P = pheromone.

[c] P_{syn} = synergyst, inactive alone.

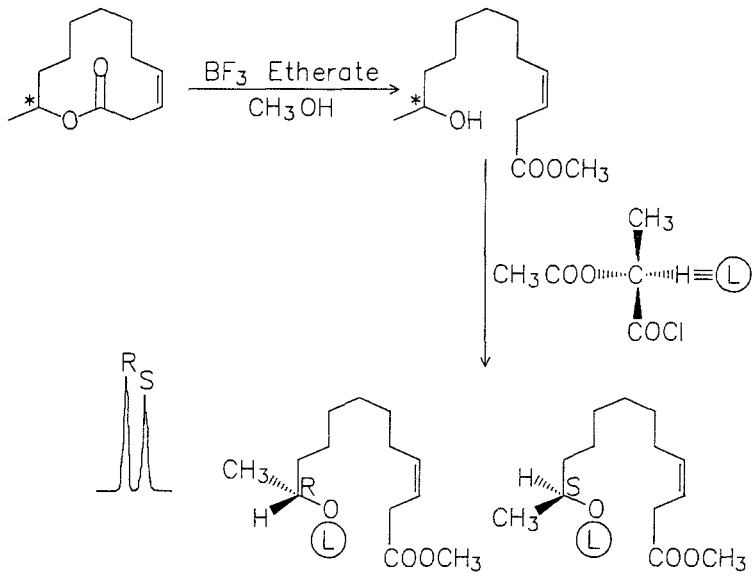


FIG. 5. Preparation and analysis of (*S*)-*O*-acetyllactate derivatives of cucujolides. Chiral cucujolides are reacted first with BF_3 in methanol. The hydroxy esters derived from this reaction are reacted with (*S*)-*O*-acetyllactyl chloride in the presence of pyridine to form the diastereoisomeric derivatives which are separated by capillary gas chromatography.

Millar and Oehlschlager (1984) and Millar et al. (1985b) isolated cucujolides III and V from the volatiles of *C. turcicus* and established both as aggregation pheromones. Response to racemic III and V revealed that only V was active alone. A test for synergism between racemic III and V over a range of ratios from 1:25 to 2:1 showed that all mixtures used are attractive. Ratios of 1:3 and 2:3 of III:V are significantly more attractive than V alone, indicating that III synergizes V when used in approximately the natural ratio (~ 1:5). The threshold for significant response for racemic V was between 240 ng and 24 μg, considerably higher than for *C. pusillus* to its singly active pheromone.

Oehlschlager et al. (1987b) determined that cucujolides III and V from *C. turcicus* are a 33:67, *R*:*S* and a 85:15, *R*:*S* mixture, respectively (Table 1). Millar et al. (1985b) demonstrated *C. turcicus* is attracted to mixtures but not the pure enantiomers of V at the 5 μg level. When the dose of V is reduced to 1 μg, only (*R*)-III and not (*S*)-III elicited synergism even though naturally produced III is rich in the *S* enantiomer. Attraction to mixtures of enantiomers of V is maximal near the natural ratio (*R*:*S*, 80:20). This is the first evidence of

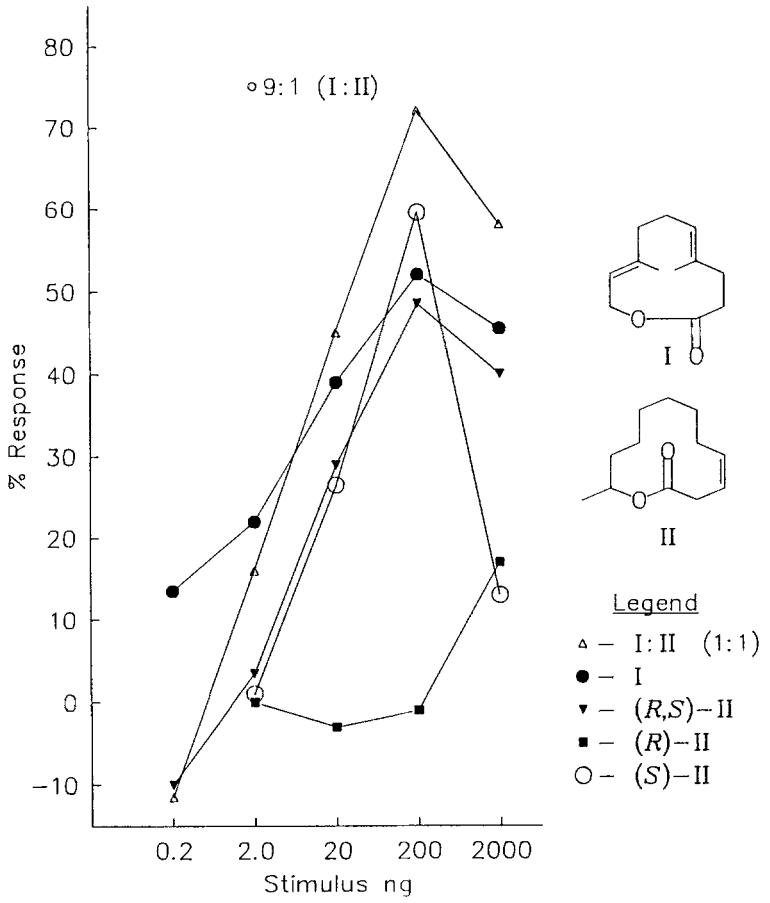


FIG. 6. Response of *Cryptolestes ferriguneus* to cucujolides I and II in arena olfactometer.

enantiomeric synergism outside of the Scolytidae, in which it occurs in *Gnathotrichus sulcatus* (Borden et al., 1976) and *Ips pini* (Lanier et al. 1980).

It would be expected that cucujids such as *O. mercator* and *O. surinamensis* would use cucujolides similar to I–IX as aggregation pheromones. Initial experiments by A.M. Pierce et al. (1981) revealed olfactory responses of both these species to volatiles of mixed-sex beetles and frass. Thresholds of response to volatiles of conspecifics of both species are ~20 bh (where 1 bh = the volatiles emitted by 1 beetle in 1 hr), roughly equivalent to those observed

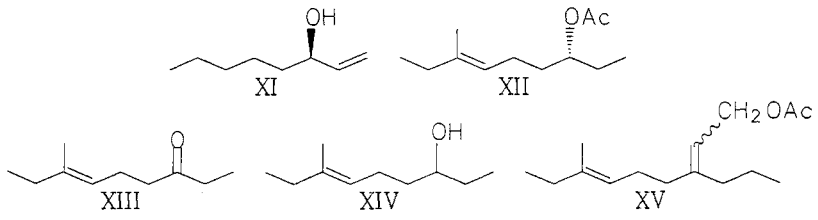
(~30 bh) for *C. ferrugineus* by Wong et al. (1983) and for *C. pusillus* by Millar et al. (1985a). Surprisingly, the only detectable (coupled gas chromatography–selected ion mass spectrometry) cucujolide in the volatiles of *O. surinamensis* was present in trace amounts (V, ~0.01 pg/bh). *O. mercator* produced only trace (~0.01 pg/bh) quantities of cucujolides II and IV. The attractive volatiles derived from the frass of these insects contained 6–18 pg/bh of these cucujolides, 1000 times less than the amounts of I and II found in *C. ferrugineus* frass by Wong et al. (1983).

The low quantities of suspect cucujolides in the beetle-derived *Oryzaephilus* volatiles coupled with Burkholder's (1982) hypothesis that long-lived, stored-product Coleoptera require food for pheromone production, prompted A.M. Pierce et al. (1984) to aerate mixed-sex insects on food. Both *Oryzaephilus* spp. produce dramatically more cucujolides when fed. Of the four cucujolides produced by feeding *O. mercator* (Table 1), significant response at the nanogram level was elicited by only II and IV (A.M. Pierce et al., 1984, 1985, 1987). Maximum response to these pheromones alone and in combination occurs when they are combined in a ratio of 1:1, which is near the natural ratio of 1:1.25. Males and females respond differently to II and IV. The threshold for response of males to II is lower (1 ng) than that of females (10 ng), while the reverse is observed for cucujolide IV. Over the range 0.2–20,000 ng, II and IV are not synergistic. A systematic examination of the attractancy of the enantiomers of II and IV by A.M. Pierce et al. (1987) revealed that only the naturally produced (Oehlschlager et al., 1987) *R* enantiomers of each cucujolide are attractive.

A.M. Pierce et al. (1984, 1985, 1987) also found that of the four cucujolides produced by *O. surinamensis* (Table 1) only racemic or (*R*)-IV and IX elicits significant activity alone (threshold ~10 ng). The dose at which racemic IV and IX elicit significant response is lowered by an order of magnitude when they are combined in a 2:1 or 1:1 mixture. A third cucujolide (racemic V, threshold >300 ng) slightly increases activity of racemic IV and IX when added to either and is synergistic in combination with both racemic IV and IX when the ternary mixture is composed in the nearly natural ratio 1:3:1, IV:V:IX). The naturally produced *R* enantiomers of IV and V are attractive, while the *S* enantiomers are inactive.

The low quantities of cucujolides present in the volatiles of nonfeeding *Oryzaephilus*, coupled with threshold responses to these volatiles that are similar to those for volatiles of *Cryptolestes* spp., provided the initial suggestion that components other than the cucujolides in the *Oryzaephilus* volatiles are responsible for attraction. Components that might contribute to the attractancy of these volatiles are low-molecular-weight carboxylic acids and aldehydes apparently derived from oxidation of long-chain fatty acids. Additional attrac-

tants are obtained when mixed sex beetles of either *Oryzaephilus* species are aerated while feeding on rolled oats. Aeration of each sex of each species revealed that (*R*)-1-octen-3-ol (XI) and (*Z*)-2-octen-1-ol are produced one to two months posteclosion by both sexes of both species (Scheme 2). While the



SCHEME 2.

latter alcohol is devoid of biological activity in the pitfall bioassay at doses from 1 to 10,000 ng, both chiral isomers and the racemic mixture of the former are strong attractants to both sexes of both species. When combined with threshold quantities of the ternary cucujolide mixture for *O. surinamensis* or the binary mixture of *O. mercator*, a strong (1000-fold) synergistic response is obtained.

H.D. Pierce et al. (1988) found that the volatiles of mixed-sex *Cathartus quadricollis* captured on Porapak Q contain several unsaturated acyclic branched-chain compounds (Scheme 2, XII–XV) but no macrolides. Only XIII (quadrilure) is attractive in the pitfall olfactometer at the nanogram level. The *R* enantiomer of this aggregation pheromone is produced naturally and is the attractive enantiomer. When males or females are aerated separately (*R*)-1-octen-3-ol (XI) is produced. Racemic and (*R*)-XI but not (*S*)-XI are repellent to both sexes alone at the 10 ng level but did not diminish attractancy to XIII.

CROSS-ATTRACTION AND SPECIES RECOGNITION IN CUCUJIDS

Cross-attraction to semiochemicals produced by sympatric species might be expected in situations where the food resource to be utilized is spacious and abundant (A.M. Pierce et al., 1987). For example, ambrosia beetles that inhabit the spacious sapwood of conifer trees elicit primarily cross-attraction (Borden et al., 1981), while inhibition of response occurs between two species of bark beetles, *Ips pini* (Say) and *I. paraconfusus* (Lanier), that compete for an essentially two-dimensional resource, the inner bark of trees (Birch and Wood, 1975).

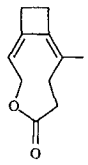
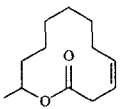
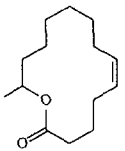
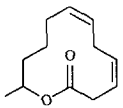
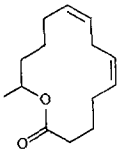
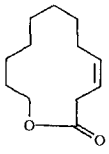
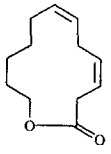
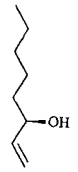
One mechanism by which species specificity can be maintained is through differential resource utilization. Within the *Oryzaephilus* species studied, several observations point to *O. surinamensis* as a primary colonist and *O. mercator* as a secondary colonist. Taxonomic differences between the two species suggest that the former would be better able to feed on undamaged grain (Slow, 1958). *O. surinamensis* is also the more dispersive of the two species (Howe, 1956; A.M. Pierce et al., 1983). In addition, A.M. Pierce et al. (1983, 1984) found *O. surinamensis* to be sensitive to crowding by conspecifics as evidenced both by decreased responsiveness to pheromone and decreased pheromone production, whereas at high population densities, *O. mercator* is not. Finally, A.M. Pierce et al. (1987) observed that one of the cucujolide pheromones of *O. surinamensis* (IV) is strongly attractive to *O. mercator*, allowing the latter to be attracted to habitats occupied by the former. The differential colonization roles proposed for the *Oryzaephilus* parallel those proposed for colonization of new habitats by *Tribolium* species (Ghent, 1963; Ziegler, 1976). Structures and activities of the cucujolides produced by *Cryptolestes* and *Oryzaephilus* spp. provide information on taxonomic relationships and the chemical basis for cross-attractancy and species recognition. A close taxonomic relationship between *O. mercator* and *O. surinamensis* (Slow, 1958) is supported by the observation that they share a common cucujolide pheromone (IV). A more distant relationship between *Cryptolestes* and *Oryzaephilus* spp., established by chromosomal comparisons (Robertson, 1959), is supported by the observation that *Cryptolestes* spp. use as aggregation pheromones cucujolides that are either unique to the genus, are released in trace quantities and not used as pheromones by *Oryzaephilus* spp., or are of opposite chirality to those used by *Oryzaephilus* spp.

The information in Table 1 is reinterpreted and supplemented in Table 2 to illustrate the major mechanisms identified by Oehlschlager et al. (1987b) in this sympatric group that are responsible for species specificity in chemical communication. These major mechanisms are: (1) presence of a unique pheromone (*C. ferrugineus*, I; *C. pusillus*, VIII); (2) use of synergistic pheromones that are inactive alone but synergize response to other pheromones (*C. pusillus*, *C. turcicus*, III; and *O. surinamensis*, V); (3) response to only one chiral isomer of a pheromone (*C. ferrugineus* and *O. mercator*, II); and (4) synergism between enantiomers of a pheromone (*C. turcicus*, V).

C. ferrugineus is not expected to elicit cross-attraction to other cucujids for two reasons. First, only *C. ferrugineus* produces I. Second, although both *C. ferrugineus* and *O.mercator* share II as a pheromone, the former produces and responds only to (*S*)-II whereas the latter produces and responds only to (*R*)-II.

C. pusillus cross-attraction to other cucujids is not expected since VIII, a singly active component, is not produced by any other species. Although III is

TABLE 2. MECHANISMS OF SPECIES SPECIFICITY IN SEMIOCHEMICAL COMMUNICATION BETWEEN SIX CUCUJID SPECIES[a]

	 I	 II	 III	 IV	 V	 VIII	 IX	 XI	 XIII
C. ferrugineus	Unique Synergist	*S*[b], Synergist	+[c]	+	+				
C. pusillus			Synergist *R*:*S* 30:70			Unique	+		
C. turcicus			Synergist		*R*:*S* 85:15				
O. mercator		*R*, Pheromone	+	*R*, Pheromone	+		+	Synergist	
O. surinamensis			+	*R*, Pheromone	*R*, Synergist		Unique	Synergist	
C. quadricollis									Unique

[a] For IUPAC names, see Table 1.
[b] Designates configuration of chiral center.
[c] + Trace

a synergist to VIII and is common to most cucujids investigated, it is not attractive alone to *C. pusillus*.

C. turcicus is not expected to be attracted to *C. ferrugineus*, even though both III and V are produced by the latter. In *C. turcicus* the III : V ratio is 0.38, whereas in *C. ferrugineus* it is 13. Furthermore, III and V are only minor components of *C. ferrugineus* volatiles. Attraction to *C. pusillus* is not expected since they share only III, which is inactive in both species. Attraction to *O. mercator* is unlikely because both III and V are produced by the latter only as minor components. Attraction to *O. surinamensis* is not expected because the ratio of V to III produced by *O. surinamensis* (>510) is drastically different from that of *C. turcicus*. Furthermore, V produced by and attractive to *C. turcicus* is a mixture of *R* and *S* enantiomers, whereas *O. surinamensis* produces (*R*)-V of high chiral purity.

O. mercator produces and responds to (*R*)-II and (*R*)-IV of high chiral purity. This species is not expected to be attracted to *C. ferrugineus* because the latter produces and responds only to the *S* enantiomer of II. Cross-attraction of *O. mercator* to *O. surinamensis* is possible since both species produce (*R*)-II, which is attractive alone for *O. mercator*.

O. surinamensis produces and responds only to the *R* isomers of IV and V. Cross-attraction to *C. ferrugineus* due to production of minor amounts of IV and V is not likely since the IV : V ratio in *C. ferrugineus* does not approximate that found in *O. surinamensis*. Cross-attraction to *C. pusillus* is not likely since the only *O. surinamensis* pheromone produced by *C. pusillus* is IX and this is a minor component in the volatiles of the latter. Attraction to *C. turcicus* is not expected to occur even though these two species produce major amounts of V. This component of the *O. surinamensis* pheromone blend is not attractive unless IV and IX are present. Both of the latter components are absent in *C. turcicus* volatiles. Attraction of *O. mercator* to *O. surinamensis* is likely since each species uses (*R*)-IV as an active pheromone component. In addition, *O. mercator* produces a minor amount of IX, which is a singly active pheromone for *O. surinamensis*. The only cucujids investigated to date that elicit significant cross-attraction are *O. mercator* and *O. surinamensis* (A.M. Pierce et al., 1987).

FACTORS AFFECTING PHEROMONE PRODUCTION AND RESPONSE TO PHEROMONES

In all species investigated, A.M. Pierce et al. (1984) observed that newly emerged grain beetles produced little or no pheromone. During the first few weeks after emergence, pheromone production increases, reaching a maximum around four weeks, and remains rather constant to 10 weeks posteclosion. Dur-

ing the first few weeks posteclosion, crowding by conspecifics decreases pheromone production for *O. surinamensis* but not *O. mercator*. The variation from a smoothly increasing pheromone production with age evident in Figure 7 is probably due to temperature variations during the course of pheromone collection, as pheromone production for *Oryzaephilus* spp. generally increases with temperature.

It is hypothesized that aggregation pheromones arose as a mechanism to cause aggregation at a suitable food source (Shorey, 1973). In bark beetles (Borden, 1985), production of aggregation pheromones is often enhanced or commences upon arrival of the producing sex at a food source. Similarly, A.M. Pierce et al. (1984) found that production of pheromones by grain beetles is enhanced when the insects are feeding. When *Oryzaephilus* spp. are aerated without food, cucujolide pheromone production levels is in the subpicogram/bh range. For *Cryptolestes* spp. they are in the low nanogram/bh range. When the beetles are aerated while feeding (Table 3), pheromone production in *Oryzaephilus* spp. increased ~40,000-fold, while in *Cryptolestes* spp. it increased by ~50-fold. A.M. Pierce et al. (1987) interpreted these differences to indicate that the cucujolide pheromones may serve as more critical signals of food sources for *Oryzaephilus* than *Cryptolestes* spp.

Vanderwel and Oehlschlager (1987) have shown through radio- and stable isotope tracer experiments that cucujolides II–IX are produced by cucujids both de novo and via modification of dietary fatty acids. Cucujolide I is derived from farnesol. Pheromone XII, and *C. quadricollis*, is presumed to be terpenoid (Vanderwel and Oehlschlager, 1987). The cucujolide compositions in Table 1

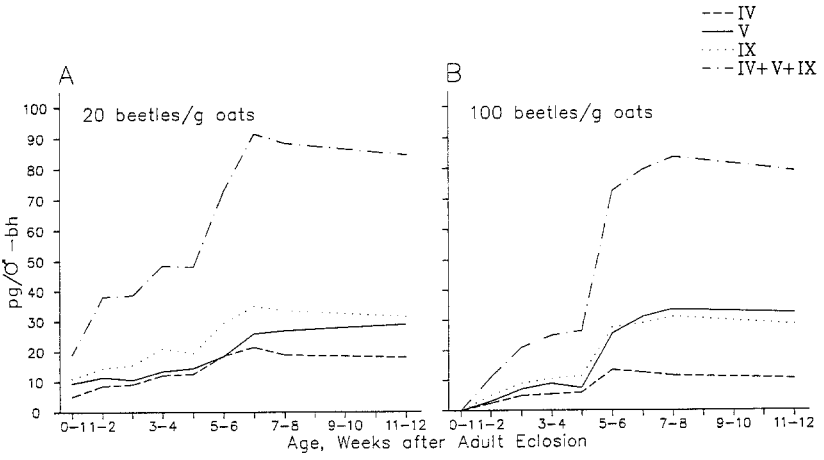


FIG. 7. Production of cucujolides IV, V, and IX by mixed-sex *O. surinamensis* aerated for five-day intervals at different culture densities. Volatiles captured on Porapak Q.

TABLE 3. COMPARISON OF PRODUCTION OF CUCUJOLIDES BY FEEDING AND UNFED *Cryptolestes* AND *Oryzaephilus* SPECIES

Species	Feeding regime	Production rates (pg/beetle-hr)						
		I	II	III	IV	V	VIII	IX
C. ferrugineus	unfed	36	42					
	feeding	990	640					
C. pusillus	feeding			30			1300	15
O. mercator	unfed		0.022		0.030			
	feeding		770		200			
	unfed					0.013		
O. surinamensis	feeding			10		28		25

are all obtained from mixed-sex adults of each species feeding on the same diet of rolled oats. Although it would be expected that the distribution of cucujolides within a species would vary slightly with food source, this has not been investigated.

Small variations in cucujolide composition are observed for several laboratory strains of *Oryzaephilus* spp. For example, the ratio of IV : V : IX for laboratory strains of *O. surinamensis* varies from 1 : 3 : 1 (A.M. Pierce et al., 1984, 1987) to nearly 1 : 1 : 1 (A.M. Pierce et al, 1985). An English strain of *O. surinamensis* has a different cucujolide composition than the 1 : 3 : 1 IV : V : IX composition (Chambers et al., 1986). In the case of *C. ferrugineus*, feeding insects produce I and II on a ratio of 1.6 : 1, but this changes to 0.86 : 1 when the insects are starved (Lindgren et al., 1985).

The gradual increase in cucujolide production exhibited by young feeding adults (Figure 7) shows that maximal production of aggregation pheromones by *Cryptolestes* and *Oryzaephilus* spp. requires both food and reproductive maturity. In the Scolytidae, production of aggregation pheromones is regulated by juvenile hormones (Borden, 1985); it has been demonstrated in several species that administration of juvenile hormones to mature adults stimulates pheromone production (Hughes and Renwick, 1977; Harring, 1978). A.M. Pierce et al. (1986) found that when adult *C. ferrugineus*, *O. mercator*, and *O. surinamensis* (15–21 days posteclosion) are fed on rolled oats containing 20 ppm of the juvenile hormone mimic, methoprene, pheromone production is stimulated by factors of 1.5–4.7. Enhancement is in the same range as observed for all four components of the pheromone complex of adult boll weevils, *Anthonomous grandis* Boheman, fed on JH-III-treated medium (Hedin et al., 1982). Stimulation of cucujolide production by methoprene suggests that its addition to food-

baited, live traps would be beneficial in pheromone-based monitoring in the Cucujidae (A.M. Pierce et al., 1986).

Responsiveness to pheromones and food volatiles as a function of age and culture density is known only for *Oryzaephilus* spp. (A.M. Pierce et al., 1983). Newly emerged adults are unresponsive to both sources of volatiles. Sensitivity to pheromones and food volatiles appears soon after eclosion, reaching a maximum after four weeks and remaining high throughout the long adult life of these beetles (A.M. Pierce et al., 1983). Neither species exhibits diel periodicity in responsiveness even when reared in constant darkness (A.M. Pierce et al., 1987). There is a significant decrease in responsiveness for mature *O. surinamensis* but not *O. mercator* when reared in high-density cultures (A.M. Pierce et al., 1983). When *O. surinamensis* reared in high-density cultures (~40,000 beetles/kg diet) are recultured at low density (1,000 beetles/kg diet), responsiveness during the next week steadily increases to "low-density rearing" levels (A.M. Pierce et al., 1983).

H.D. Pierce et al. (1988) observed that when *C. quadricollis* respond to pheromone and enter the capture vials used in the pitfall bioassay, they readily climb out. Placing food (an oat flake) or frass in the capture vial arrests this behavior. Thus, the aggregation pheromone apparently functions both by luring the insect to the point of emamation and by stimulating a pattern of searching for food. If a food stimulus is not provided, the responding insect will then leave the site.

ATTRACTION OF CUCUJIDS TO FOOD VOLATILES

Volatiles from several food sources are attractive to *Oryzaephilus* spp. (Freedman et al., 1982; Mikolajczak et al., 1983, 1984; O'Connell et al., 1983; Stubbs et al., 1985). Volatiles from rolled oats and brewer's yeast are attractive to both species of *Oryzaephilus* (A.M. Pierce et al., 1981). The threshold of significant response for *O. mercator* to volatiles of rolled oats (45 gh) and brewer's yeast (26 gh) are several orders of magnitude higher than to volatiles of frass (0.006 gh). For *O. surinamensis* the response thresholds to volatiles of rolled oats (4.5 gh), yeast (0.26 gh), and frass (0.64 gh) are of the same order of magnitude. These food-derived volatiles are very complex mixtures comprised predominantly of fatty aldehydes, acids, and other carbonyl compounds. Surveys of these types of compounds for attractancy have revealed none that is attractive at the nanogram levels elicited by the pheromones (Mikolajczak et al., 1984).

The commercial attractant for attraction of *Oryzaephilus* spp. and other species of grain-infesting Coleoptera to corrugated cardboard traps is oil derived from solvent extraction of oats and wheat germ (Trece Inc., 1986). Mikolajczak

et al. (1984) have analyzed for attractancy to *O. surinamensis* 16 of the more than 100 carbonyl-containing components derived from solvent extraction of rolled oats. Only (*E*)-2-nonenal and (*E*,*E*)-2,4-nonadienal are significantly attractive at the 0.1 μg level. These oat oil constituents elicit good attraction at 10-μg doses, but at the 100-μg level they are repulsive. Surprisingly hexanal (30%), octanal (2%), and nonanal (5%) are not significantly attractive until doses of 10–100 μg are applied in a pitfall bioassay (Mikolajczak et al., 1984).

Volatiles obtained from aeration of feeding or unfed *O. mercator* typically contain hexanal, octanal, and nonanal. In volatiles obtained from unfed *O. mercator*, the levels of these presumptive fatty acid oxidation products are much higher (20-, 20-, 3-fold, respectively) than cucujolides (A.M. Pierce and H.D. Pierce, 1985). Since volatiles derived from these unfed beetles are attractive at doses significantly higher than expected based on the amounts of cucujolide pheromones present, the aldehydes have been examined for attractancy. All three aldehydes are attractive at nanogram doses in the pitfall bioassay. Hexanal and octanal are the least attractive (threshold of significant activity, 100 ng), whereas nonanal is active at doses one order of magnitude lower. All three aldehydes elicit decreasing attractancy above 1 μg. The narrow range of concentrations in which these aldehydes elicit significant attraction for *O. mercator*, if also operational for *O. surinamensis*, may explain why they were not identified as strong attractants for the latter species in earlier studies (Mikolajczak et al., 1984). The aldehydes act synergistically to give a ternary (1 : 1 : 1) mixture that is attractive to *O. mercator* at the 100-ng level (A.M. Pierce and H.D. Pierce, 1985). Addition of nonanal (100 ng) or a ternary mixture (100 ng) of aldehydes to a 1 : 1 mixture (10 ng) of cucujolides I and II also elicits a synergistic response. These mixtures (I,5 ng : II,5 ng : aldehydes, 100 ng) elicit responses roughly equivalent to those elicited by 2000 ng of a 1 : 1 mixture of (*R*,*S*)-I and (*R*,*S*)-IV in the pitfall bioassay (A.M. Pierce and H.D. Pierce, 1985).

FIELD TESTS OF PHEROMONES

Traps baited with the pheromones listed in Table 1 are expected to be more attractive than food and to be effective monitoring devices in food storage areas. Since the unnatural enantiomers of chiral cucujolies are inactive, the less expensive racemates can be used in traps. Futhermore, in a few cases examined by A.M. Pierce et al. (1987), nonpheromone cucujolides produced by one species have no repulsive effect on response by other species to their respective pheromones. Thus, it is expected to be possible to combine pheromones of several species in a single trap, a technique that would greatly reduce the cost of pheromone-based monitoring programs for these pests.

Several studies have been conducted to develop protocol for field utiliza-

tion of cucujid pheromones in monitoring for these insects. Three types of traps have been examined for their efficiency in capturing grain beetles from grain and areas mimicking food storage areas. All rely on pheromone and food attractants to lure the insects to dark enclosures from which escape is difficult. For obvious reasons, none of the traps utilize insecticidal chemicals.

Two types of traps have been examined for efficiency at recapturing grain beetles released in environmental chambers. One trap (Lindgren et al., 1985) is constructed from a perforated plastic weighing dish (Figure 8A) containing hollow plastic fibers (Conrel) filled with pheromone as the attractant release device. The test species were *C. ferrugineus* and *Tribolium castaneum*. The bait for *C. ferrugineus* was a 13:16 mixture of cucujolides I and (*R*, *S*)-II, which was released at 1.25 μg/day at 22°C. For *T. castaneum*, 4,8-dimethyldecanal (DMD) (Suzuki, 1980) was synthesized from (*R*)-citronellol (Suzuki, 1981) and contained approximately 4.5% of the active 4*R*, 8*R* and 4*R*, 8*S* isomers (Suzuki et al., 1984; Levinson and Mori, 1983). It was released at 0.08 μg/day. The recapture experiments involved the release of 3000 beetles within a controlled environment chamber. Paired baited and control traps are placed around the periphery of the floor of the chamber with 15 cm within pairs and 35 cm between pairs.

Both species are readily caught in single-baited traps. The recapture rate for *C. ferrugineus* was 76% but only 21% for *T. castaneum* (Lindgren et al., 1985). The lower recapture rate for the latter insect is probably due to the low release rate of the pheromone and the unfavorable 1:1 ratio of the active isomers. The natural ratio of isomers of (4*R*, 8*R*)- to (4*R*, 8*S*)-DMD is 4:1, and the latter acts synergistically with the former (Suzuki et al., 1984). Both species are captured equally in traps containing their own aggregation pheromones regardless of the presence of the pheromone of the other species. There is slight cross-attraction of *T. castaneum* to the pheromones of *C. ferrugineus* but not vice versa. By placement of known numbers of *C. ferrugineus* or *O. mercator* within the trap, Lindgren et al. (1985) demonstrated that the trap was essentially escape-proof.

Corrugated cardboard traps (Figure 8B) baited with oat-wheat germ oil attractant are commercially available (Trece Inc., 1986) and recommended for monitoring *Oryzaephilus* spp. These traps were used as controls in tests designed to examine the effectiveness of cucujolide pheromones of *O. mercator* and *O. surinamensis*. Insects were released near the center of an arena and recapture rates examined. Responses of *O. surinamensis* to traps baited with both pheromone and oat-wheat germ oil are significantly higher than to traps baited only with oat-wheat germ oil at doses above 0.5 μg of the pheromone mixture (1:1:1, IV:V:IX, Figure 9A). Females detect the pheromone at doses at least an order of magnitude lower than the males, but response of males to increasing pheromone dose is higher than that of females.

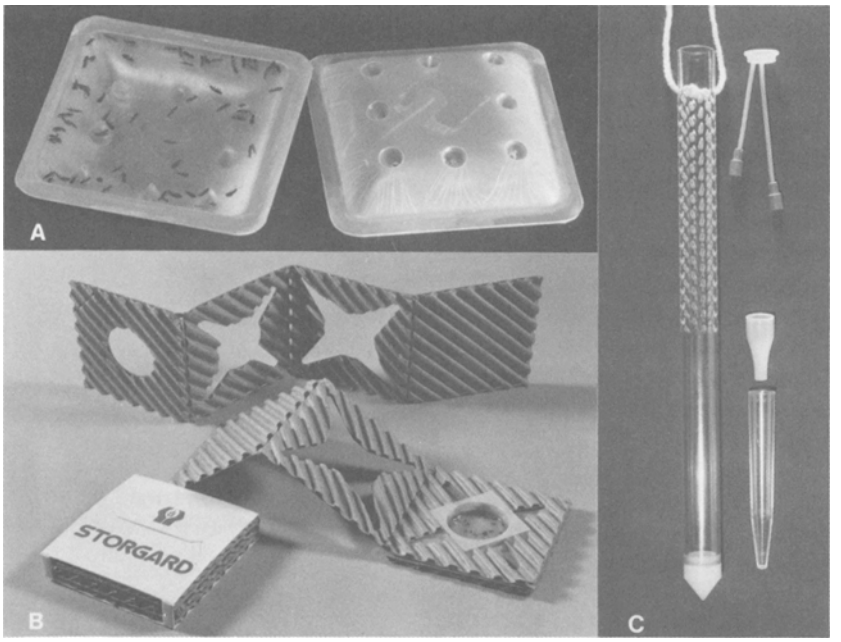


FIG. 8. Three traps used in tests of aggregation pheromones. (A) Inverted plastic weighing boat (4.1 × 4.1 × 0.8 cm) with eight inward-pointing conical depressions (basal idameter 3 mm, length 3–4 mm) each with a 1- to 2-mm wide hole at the bottom. The bottom of each trap consists of transparent packaging tape. Pheromone is released from 0.2 mm ID Conrel plastic fibers (Albany International Co., Needham, Massachusetts). (B) Corrugated paper trap adapted from an original design by Burkholder (1981) consisting of four layers of corrugated paper and containing a cup with an absorbant pad in the bottom. Food-derived oil added to the cup attracts and captures the insects (Trece Inc., 1986). (C) Plastic grain probe trap, adapted by Burkholder (1984) and Trece Inc. (1986) from an original design by Loschiavo and Atkinson (1967), consists of a transparent, hollow, cylindrical tube 36 × 2.5 cm with 186 evenly spaced 3-mm-diam. holes drilled downwards toward the inside at an angle of 60°. Insects fall through these holes into a removable collection tube housed in the bottom of the trap. Suspended inside the upper end of the trap is a nylon rod to which an attractant lure is attached. A rope attached to the top of the trap allows recovery from bulk grain.

Both sexes of *O. mercator* are responsive to low doses (4 μg) of their pheromones (1:1, II:IV) added to oat–wheat germ oil attractant (Figure 9B). As the dose increases to 2000 μg, female response increases while male response decreases relative to oat–wheat germ oil alone. Increasing the pheromone dose in oat–wheat germ oil increases capture efficiency for both *O. mercator* and *O.*

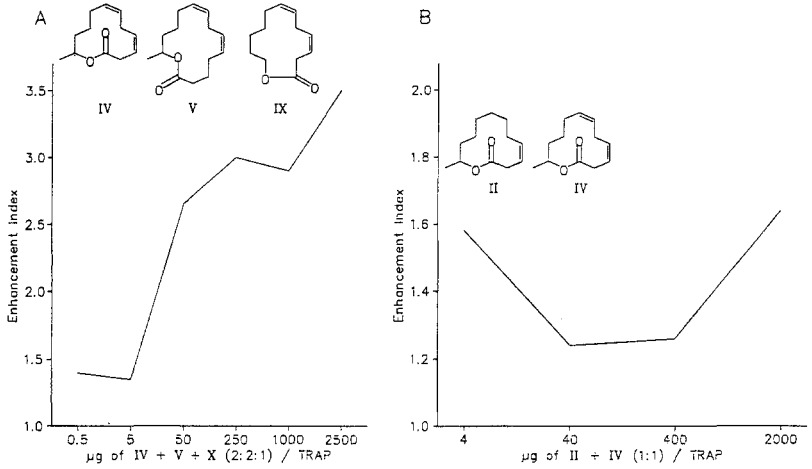


FIG. 9. Enhancement of efficiency of corrugated cardboard traps (Trece Inc., 1986) in recapturing mixed-sex *O. surinamensis* (A) and *O. mercator* (B) using pheromones added to oat–wheat germ oil attractant. Enhancement index to oat–wheat germ oil = 1.0.

surinamensis, but enhancement is higher for *O. surinamensis* than for *O. mercator*. For both species, both sexes exhibit similar sensitivities toward their pheromone except at higher dose rates to which the females are more sensitive than males.

Metal grain probe traps (Figure 8C) designed by Loschiavo and Atkinson (1967) have been tested in grain annex bins and in farm bins to ascertain if addition of pheromones in Conrel fibers (Albany International Co., Needham, Massachusetts) increases efficiency of capture of *C. ferrugineus* (Figure 10) (Wong, 1982; Loschiavo et al., 1986). Efficiency of both control and baited traps decreases dramatically as they are positioned deeper in the grain. Traps at a depth of 30–60 cm catch 15 times more beetles than those placed at 150 cm or lower. Near the surface, traps baited with one pheromone-filled fiber catch 10 times as many beetles as unbaited traps, while traps baited with two fibers catch approximately 20 times as many insects as controls. In deep (~244 cm) traps, the efficiency of control and baited traps is not significantly different. In bins ranging in temperature from 28.5 to 32°C, approximately 10 times as many insects are caught as in bins where the temperature ranged between 21.5 and 24°C. In farm bins, both baited and unbaited probe traps are relatively ineffective (less than one insect average/trap/week) if the grain temperature is below 14°C. At the highest temperature to which grain in farm bins normally rises during a season (~19–20°C) baited probe traps placed near the surface are only marginally effective (~4 insects/trap/week).

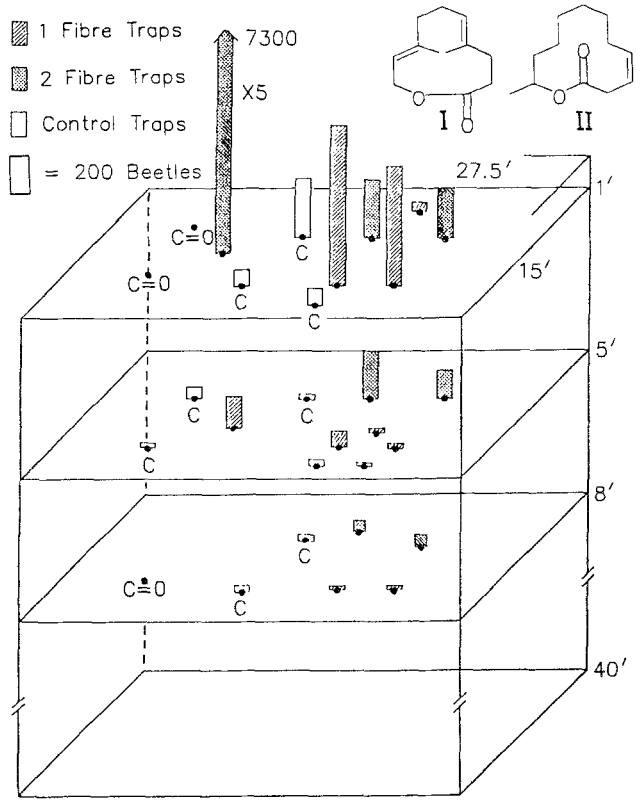


FIG. 10. Capture of *C. ferrugineus* by pheromone-baited and control traps placed in bins of wheat and left for one week (adapted from Loschiavo and Atkinson, 1967, in Wong, 1982). Pheromone (9 : 1, I : (*R*, *S*)-II) is released from one or two (0.2 mm ID) Conrel (Albany International Co., Needham, Massachusetts) polyester fibers. The release rate in the probe traps is about 14 times lower than the 1.25 μg/day/fiber determined under laboratory conditions and corresponds to a rate 23-230 times that of a feeding male *C. ferrugineus*. Grain temperature was between 24 and 28.5°C.

The relative efficiencies of the three types of traps described in Figure 8 to recapture insects released in grain in small bins have been examined (A. Javer et al., unpublished). The tests were conducted with introduced populations of adult *C. ferrugineus*, *C. pusillus*, *O. mercator*, and *O. surinamensis* at ~2 insects/kg of grain. Pheromone baiting increased the efficiency of all traps.

Pheromone-baited probe traps are the most efficient for the capture of all species. Corrugated paper traps and plastic dish traps are only efficient in capturing *O. mercator*. Unbaited probe traps are as attractive as pheromone-baited

traps to *O. mercator*, but not *O. surinamensis*. Possibly, the presence of food particles in the recapture vials stimulated pheromone production by *O. mercator*. These results indicate that field tests with different trap types, baited and unbaited, are necessary for each target species in order to develop optimally integrated and effective trapping regimes.

CONCLUSIONS

During the last five years, semiochemicals for many species of long-lived coleopteran pests of stored products have been discovered. It has been shown that, in addition to the cucujids discussed herein, species in several other families, e.g., Bostrichidae (grain borers), Curculionidae (weevils), and Tenebrionidae (flour beetles), also respond to food volatiles and male-produced aggregation pheromones (Burkholder and Ma, 1985). The latter are invariably mixtures of several chemicals that are produced in greatest amounts when the beetles are feeding, indicating a close relationship between the population of pheromones and the exploitation of scarce food resources. Moreover, the potency of aggregation pheromones is enhanced by their combination with food volatiles. While various mechanisms of species specificity exist, inhibition of attraction of one species to its pheromone due to the presence of the pheromone of another has not yet been detected in stored-product coleopterans. Thus, it should be possible to utilize combinations of pheromones and host volatiles in multiple-species monitoring programs for these remarkable insect pests.

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ECLECTIC CHEMISOCIALITY OF THE HONEYBEE: A Wealth of Behaviors, Pheromones, and Exocrine Glands

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Abstract—A dazzling variety of honeybee behaviors are triggered by pheromones produced by disparate exocrine glands. A multiplicity of chemical releasers of social behavior has been demonstrated to regulate a diversity of societal interactions, and many of these compounds are synthesized with great caste specificity. Recent investigations have resulted in the identification of a host of new compounds that are products of either worker or queen honeybees. This report fractionates these newly identified exocrine products according to their glandular proveniences and focuses on both the structural and behavioral eclecticism that characterizes these chemical signaling agents.

Key Words—Honeybee, *Apis mellifera*, Hymenoptera, Apidae, alarm pheromones, wax compounds, antiaggression pheromone, exocrine glands, chemisociality.

INTRODUCTION

Among invertebrates, the honeybee, *Apis mellifera*, may represent the zenith of chemisociality. A large variety of behaviors has been demonstrated to be regulated by pheromonal signals originating from diverse exocrine glands of either worker or queen bees (Gary, 1974), and many of these signaling agents have been structurally elucidated (Free, 1987). As the pheromonal world of the honeybee has been probed, it has become increasingly evident that this hymenopteran has evolved a dazzling behavioral repertoire, many components of which are controlled by exocrine products. Indeed, it is not unreasonable to conclude

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that the elegant exploitation of sociality by *A. mellifera* is a consequence of its ability both to biosynthesize these compounds programatically—in a variety of glands—and to exhibit distinctive behaviors in their presence.

Research in the last three decades has illuminated the biosynthetic emphases that characterize several of the social organs utilized by honeybees in the colonial milieu. In particular, the mandibular glands of queens have been demonstrated to produce a large variety of aliphatic acids (Pain and Barbier, 1963; Callow et al., 1964), and more recently Crewe and Velthuis (1980) and Crewe (1987) reported that workers can also synthesize diverse aliphatic acids in these cephalic organs. An abdominal structure of similar biosynthetic versatility, the Nasonov gland, has emerged as the worker's monoterpene-producing gland par excellence (Boch and Shearer, 1962, 1964; Shearer and Boch, 1966), and a sesquiterpene has now been identified as a product of this isoprenoid factory (Pickett et al., 1980). Another abdominal product, the blend of alarm pheromones liberated from the extruded sting shaft of workers, is probably derived from the functionally associated Koschewnikow glands (Mauchamp and Grandperrin, 1982; Grandperrin and Cassier, 1983), which emphasizes the biosyntheses of a large variety of aliphatic acetates and alcohols (Boch et al., 1962; Blum et al., 1978; Collins and Blum, 1983).

In the present review, we wish to discuss briefly the significance of a variety of newly identified compounds derived from different abdominal glands of honeybees. In so doing, we hope to focus on the virtuosity of this insect as a synthesizer of exocrine compounds while further emphasizing that the natural products potential of this wondrous hymenopteran is far from exhausted.

ALDEHYDIC WAX GLANDS

While the wax scales produced by worker bees are derived from abdominal glands, they appear to be modified with cephalic secretions during manipulation with the mouthparts (Hüber, 1792). Beeswax is reported to contain a large series of highly volatile constituents (Ferber and Nursten, 1977), but it is very likely that many, if not most of these compounds, are plant natural products that have been absorbed by the wax. To determine what relatively low-boiling compounds are associated with beeswax uncontaminated by plant volatiles, samples of wax produced by caged swarms were analyzed.

Bees were fed only sucrose solution, and the newly made comb was extracted and analyzed by gas chromatography–mass spectrometry. Aside from an expected series of normal alkanes, six oxygenated volatiles were consistently detected, five of which were aldehydes (Blum et al., 1989). The major compound identified was decanal, and it was accompanied by its corresponding

alcohol, 1-decanol. Nonanal, which was also a quantitatively important constituent, was detected along with a minor aldehydic product, octanal. Furfural and benzaldehyde were concomitants of the aliphatic aldehydes.

Neither the functions nor the precise origins of these oxygenated volatiles have been unambiguously established. Although none of these compounds have been previously identified as honeybee natural products, they clearly contribute significantly to the characteristic odor of freshly constructed comb. Efforts to determine whether these aldehydes are responsible for the observed hoarding behavior of workers stimuated with volatiles from empty comb (Rinderer, 1981) yielded ambiguous results, although it was established that these compounds affect the intensity of hoarding (Blum et al., 1989). Indeed, wax may constitute a product whose exocrinological significance could be considerable in terms of honeybee biology.

A WEALTH OF ALARM PHEROMONES

The everted sting of a mature and aroused worker is a veritable magnet for sister workers that are attracted to the pheromonal releasers of alarm behavior emanating from the sting shaft. Originally identified with isopentyl acetate (Boch et al., 1962), the alarm signal was subsequently shown to be triggered by a blend of compounds comprised primarily of esters and alcohols (Blum et al., 1978; Pickett et al., 1982; Collins and Blum, 1983). Twenty alcohols and acetate esters were detected as sting-shaft volatiles, and the different components were demonstrated to possess a wide range of activities as releasers of alarm behavior for worker bees (Collins and Blum, 1982, 1983; Pickett et al., 1982; Grandperrin, 1983; Free et al., 1983). Surprisingly, the signal appears to be even more complex and may be qualitatively distinct for different populations of *A. mellifera*.

At least 30 relatively short-chain oxygenated compounds have been detected as sting-associated volatiles of different populations of honeybees (Blum and Fales, unpublished data). *n*-Octanoic acid and 3,3-dimethylacrylic acid are produced by a few populations of *A. mellifera*, but only the latter is a quantitatively important constituent. On the other hand, 13 alcohols have been identified in sting extracts from different populations, and some of these carbinols are major constituents of great functional significance.

The major alcohol present in all populations is (Z)-11-eicosen-1-ol, a compound that has been demonstrated to be important in the generation of an alarm signal (Pickett et al., 1982). Another alcohol, 2-nonanol, which has also been shown to be highly active as an alarm pheromone (Collins and Blum, 1982), is a major constituent of several populations of *A. mellifera*, particularly those of

African origin. 2-Nonanol is sometimes accompanied by two other secondary alcohols, 2-heptanol and 2-undecanol, both of which constitute minor or trace constituents.

Eight primary alcohols have been identified as sting-shaft volatiles, but several of these compounds have only been detected as products of selected populations of honeybees. For example, 1-butanol and 1-heptanol were only detected in *A. m. scutellata* and *A. m. capensis*, whereas the only branched alcohol identified, isopentyl alcohol, was most frequently identified in populations of *A. m. ligustica*. Among the short-chain primary alcohols, 1-octanol, a moderately active alarm pheromone (Collins and Blum, 1983), was consistently detected in all populations as a quantitatively significant constituent. However, with the exception of 2-nonanol (see above), the major carbinols identified are unsaturated constituents. (*Z*)-11-Eicosen-1-ol, the major alcohol present (Pickett et al., 1982; Blum, 1982), is accompanied by (*Z*)-9-octadecen-1-ol and nonadecen-1-ol, both of which are major constituents. Unlike (*Z*)-11-eicosen-1-ol, neither (*Z*)- nor (*E*)-9-octadecen-1-ol are reported to possess any activity as releasers of alarm activity when tested alone or in combination with another pheromone, isopentyl acetate (Pickett et al., 1982). However, since (*Z*)-9-octadecen-1-ol was not tested in combination with (*Z*)-11-eicosen-1-ol and isopentyl acetate, its possible synergistic role as an alarm releaser cannot be ruled out completely. Indeed, (*Z*)-9-octadecen-1-ol is reported to give as strong an electroantennographic response as (*Z*)-11-eicosen-1-ol (Pickett et al., 1982), demonstrating that the former can be perceived by bee workers with the great acuity that characterizes insects' responses to their alarm pheromones.

Although 14 esters have been identified as string-shaft volatiles (Blum and Fales, unpublished data), only one of these compounds, *n*-eicosyl acetate, contains an alcoholic moiety with a chain length greater than 10 carbon atoms. Two minor constituents, butyl *n*-butyrate and isopentyl *n*-butyrate, constitute the first nonacetate esters identified in these blends, but as in the cases of other compounds, their occurrence appears to be limited to certain populations. Similarly, isohexyl acetate, the only other branched acetate detected besides isopentyl acetate, has only been found in one population of honeybees (Blum and Fales, unpublished data).

From a quantitative standpoint, 2-nonyl acetate and 2-decenyl acetate can constitute major constituents along with isopentyl acetate. Hexyl and octyl acetates are also quantitatively significant, as is another ester of a secondary alcohol, 2-heptyl acetate.

As pointed out previously, the release of alarm behavior in *A. mellifera* was originally identified with only one ester, isopentyl acetate (Boch et al., 1962), but it has subsequently been demonstrated that several other esters identified as sting-shaft volatiles are highly active alarm pheromones. Collins and Blum (1982) showed that hexyl acetate and butyl acetate were effective stimu-

lators of alarm behavior, and Free et al. (1983) also reported that butyl acetate was a powerful stimulator of attack and stinging by honeybee workers under field conditions. One of the unsaturated esters, 2-nonenyl acetate, also exhibits high activity as an alarm pheromone (Collins and Blum, 1983). Another unsaturated ester, 2-decenyl acetate, which has been reported to be an alarm pheromone of other species of *Apis* but had not been identified as product of *A. mellifera* (Veith et al., 1978), has been detected by us in several populations of *A. m. ligustica*.

AN ANTIAGGRESSION PHEROMONE IN QUEEN FECES

Virgin queen bees have evolved an elegant strategy for both repelling hostile workers and reducing their level of aggressivity. During hostile confrontations with workers, these queens excrete a highly odorous anal exudate, which, under experimental conditions, effectively reduces worker aggression and ultimately releases pronounced autogrooming in the latter (Page and Erickson, 1986; Post et al., 1987). In effect, queens have converted their intestines into a social organ whose secretion—pheromone-fortified feces—plays a key role in protecting the virgin female from injuries that might be inflicted by the worker siblings.

Recently, a major fecal pheromone was identified as *o*-aminoacetophenone, a compound that repels honeybee workers as effectively as the feces of virgin queens (Page et al., 1988). However, the aromatic ketone does not stimulate autogrooming behavior, which is probably released by other constituents in this complex secretion. Indeed, *o*-aminoacetophenone is a minor constituent in the secretion, which is thoroughly dominated by esters and hydrocarbons.

Significantly, virgin queens are programmed to produce this ketone during a particularly vulnerable period in their lives. During the first 24 hr of adult life when virgin queens are not likely to be challenged by workers, no *o*-aminoacetophenone is detectable in the feces. Similarly, after about 14 days of age, when queens are mated and are likely to function as mother queens, the ketone cannot be detected in the feces. Therefore, the biosynthesis of this key pheromone is of great temporal adaptiveness, ensuring that this worker repellent will be readily available during the period in the virgin queen's life when agonistic interactions are most frequent. In addition, as emphasized by Post et al. (1987), fecal pheromones may play a key role during aggressive interactions between virgin queens.

The fecal exudate of these virgin queens contains a complex mixture of constituents that includes several other relatively volatile products whose possible ethopotencies remain to be established. For example, octanoic acid and 1-dodecanol, which are present in considerably greater concentration than *o*-

aminoacetophenone (Blum et al., unpublished data), do not repel workers at physiological concentrations, although the former does reduce autogrooming significantly (Page et al., 1988). A host of esters are present in the fecal exudate, and the possible pheromonal role of these major constituents is unknown. Several of these esters (e.g., decyl decanoate) have previously been identified as constituents of the sting shaft of queen bees (Blum et al., 1983), and the significance of their presence as major fecal constituents is not readily evident. The same can be said for octanoic acid, which has also been identified as a cephalic (=mandibular gland?) constituent of virgin and mated queens (Boch et al., 1979).

CONCLUSIONS

The three secretions described in this brief review are illustrative of the potential of the honeybee as a producer of eclectic pheromones derived from a wealth of exocrine glands. Indeed, if only the known exocrine glands of this insect are considered as pheromonally relevant, then it seems likely that a host of unidentified natural products awaits structural elucidation. It would be no exaggeration to conclude that the best is yet to come.

Gary (1974), in his seminal review on honeybee pheromones, described a variety of behaviors that are released by pheromones, most of which have not been identified yet. Workers and queens possess a host of potential exocrine glands whose secretions are believed to regulate an incredible potpourri of behaviors (Pain, 1973). In addition to these secretions from adult females, brood pheromones (Koeniger and Veith, 1983) and drone pheromones (Gerig, 1972; Lensky et al., 1985) have been described as additional examples of the exocrinological virtuosity of the honeybee. While these cases of caste-specific pheromones further document the natural products potential of *A. mellifera*, they also serve to emphasize that chemical communication in this eusocial species is identified with a multitude of factors that can be easily overlooked.

For example, the demonstration that the rectum contained virgin queen pheromones (Post et al., 1987) exposed the intestine as a social organ, emphasizing that any glandular structure that can externalize its product can be regarded as a potential pheromonal source. Furthermore, this exudate is illustrative of most of the glandular products of honeybees in being composed of a complex mixture of compounds belonging to several chemical classes. As is usually the case, the functions of most of these compounds are terra incognita, but it would be a mistake to simply conclude that these semiochemicals possess no ethopotencies. In the absence of appropriate bioassays, the roles of these exocrine products—in the colonial milieu—can hardly be illuminated.

The alarm pheromone blend associated with the extruded worker sting

offers another example of the exocrinological complexity that characterizes the honeybee. The pheromonal bouquet comprising this alarm releaser may contain up to 30 compounds, many of which are outstanding triggers of alarm behavior under both laboratory and field conditions (Collins and Blum, 1982, 1983; Grandperrin, 1983; Free et al., 1983). Significantly, since Collins and Blum (1983) have demonstrated that highly odoriferous foreign compounds do not elicit alarm behavior, it is clear that the sting-derived volatiles are specifically perceived by bee workers as alarm releasers.

Our studies of honeybee alarm pheromones also suggest that idiosyncratic natural products may characterize the pheromonal blends generated by selected populations. For example, certain esters (e.g., butyl *n*-butyrate) have only been identified as products of certain African populations of *A. mellifera*, and it is possible that such compounds may be of considerable communicative importance. It would be a mistake to overlook the possibility that populations of insects may produce characteristic pheromonal components that are highly adaptive in certain ecological contexts. These populationally distinctive compounds could be of great behavioral significance and, as a consequence, to ignore their potential roles as colonial regulators is simply not justified.

In the case of all three secretions described in this review, it has been emphasized that blends of compounds are characteristically encountered. Obviously, honeybees are perceiving multicomponent mixtures of natural products, and ultimately these chemical signals must be interpreted in terms of behavioral responses to these blends. Although it is much more convenient to monitor electrophysiological and behavioral responses in terms of one or a few compounds, the real world of the honeybee is not characterized by such odoriferous simplicity. As researchers on sex pheromones have so painfully learned, to ignore the roles of minor constituents in the sex pheromone blends often results in frustratingly puzzling lacunae in our comprehension of sexual behaviors.

Finally, it is of major importance to recognize that honeybees are programmed to produce their pheromones so as to optimize their adaptiveness in the milieu of the colony. Alarm pheromones are produced by bee workers with great temporal precision so as to exploit the great resources inherent in a colonial response (Boch and Shearer, 1966), and there is no reason to feel that this programmed biosynthesis is not similarly adaptive in terms of other pheromones. If we are to understand the communicative elegance of the honeybee, it will require our appreciating the interplay of exocrine and behavioral factors that regulate their chemisocial world.

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CONTEMPORARY FRONTIERS IN INSECT SEMIOCHEMICAL RESEARCH

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Abstract—Recent advances in analytical chemistry coupled with more definitive behavioral analyses have allowed more rigorous identification of many insect pheromones. This, in turn, has increased our understanding of the roles of pheromones in mediating insect behavior. Other semiochemicals that mediate insect behavior include those that enable parasitic and predatory insects to locate their hosts or prey. These may be produced by the host insects or by the plant on which they feed. Additionally, there are pheromones and plant-produced seminochemicals that regulate insect oviposition, a critical phase in the life cycle of insects. The challenge to chemists and biologists is to explore these areas to find new environmentally safe methods to control insect pests. One of the newer strategies used to investigate these semiochemicals is the study of the biochemistry of pheromone production and semiochemical perception in insects. These types of studies may reveal weak links in these systems that can be exploited to develop new, more effective and environmentally safe control methods.

Key Words—Identification techniques, biosynthesis, receptor-semiochemical interactions, oviposition stimulants, oviposition deterrents.

INTRODUCTION

The ultimate practical goal of research on insect pheromones and other semiochemicals [chemicals that transmit information between organisms (Law and Regnier, 1971)] is to find new biorational, environmentally safe methods for insect pest control. Before significant progress can be made toward achieving this goal, more basic knowledge in several areas must be acquired. Thus, a considerable amount of basic research remains to be done to understand the

chemistry and mechanisms that regulate insects' chemical communication systems. When we understand these systems, we should be able to develop methods to exploit their inherent weaknesses to control insects.

In the last 25 years a large number of insect pheromones have been identified (Klassen et al., 1982; Inscoe, 1982) and many of these have been used effectively in traps to monitor or detect insects (Sternlicht, 1986). In a few cases insects have been controlled successfully by using atmospheric permeation with pheromones to disrupt the insects' communication system and thus prevent mating (Klassen, 1987; Ridgway et al., 1988). However, we have not yet exploited these systems to the fullest extent possible for insect control. There are at least four areas of research related to insect semiochemicals that should prove both challenging and very rewarding in leading to new methods for pest management. All of these areas have been investigated to some extent and significant progress has been made in some. However, there is still a great amount of work to be done in each of these areas to obtain the information needed to develop effective new pest control methods. These areas are:

1. *Complete Pheromone Identification.* While pheromones have been identified for a large number of species, it is doubtful if the "complete" pheromone has been elucidated in many cases. With our present knowledge of insect behavior and the available analytical chemical technology, we should be able to identify all the compounds that comprise a given chemical signal and determine precisely and accurately the most effective release ratios and rates of these compounds and their effects on the insects' behaviors. We need this knowledge to design pheromone-based systems to efficiently suppress insect reproduction.

2. *Pheromone Biosynthesis.* Although there have been an increased number of investigations in this area in the last few years, there is still a lot to be learned about the mechanisms and pathways of pheromone biosynthesis (Prestwich and Blomquist, 1987). These systems control the production of the precise chemical signals that are required to ensure reproduction and other essential activities in a species. They are under genetic control and thus should be subject to genetic manipulation. Elucidation of the biochemical pathways of a particular system inevitably leads to a more complete knowledge of the pheromone emitted. There are obviously many facets of these systems that remain to be exposed and subsequently exploited for insect control.

3. *Semiochemical Perception.* The mechanisms by which the insects' receptors interact with the molecules of the semiochemical and the integration of these signals by the central nervous system (CNS) to decipher the message is understood in only the most rudimentary terms. The biochemistry of semiochemical–receptor interactions is very interesting and offers exciting possibilities for development of chemicals to interfere with communication. Similarly, when CNS processing is more fully understood, it may be possible to obstruct this system in several ways. Two excellent papers that appear in this symposium

series address these subjects in more detail (Ding and Prestwich, 1988; Visser and DeJong, 1988).

4. *Other Semiochemicals*. There are many types of semiochemicals other than pheromones that insects perceive and use to ensure their survival and that of their progeny. For example, the location of suitable sites for oviposition is crucial to the survival of the progeny and is governed by both pheromones and by semiochemicals produced by the plants or other hosts. These semiochemicals may be either stimulating or deterring. Prevention of oviposition or shifting oviposition to a noncrop host should be a very effective method of insect pest control. Similarly, beneficial parasitic and predatory insects use semiochemicals to locate their harmful hosts. Evidence to date suggests that the effectiveness of these organisms in biological control programs may be enhanced significantly by the application of certain semiochemicals at critical stages of their host searching.

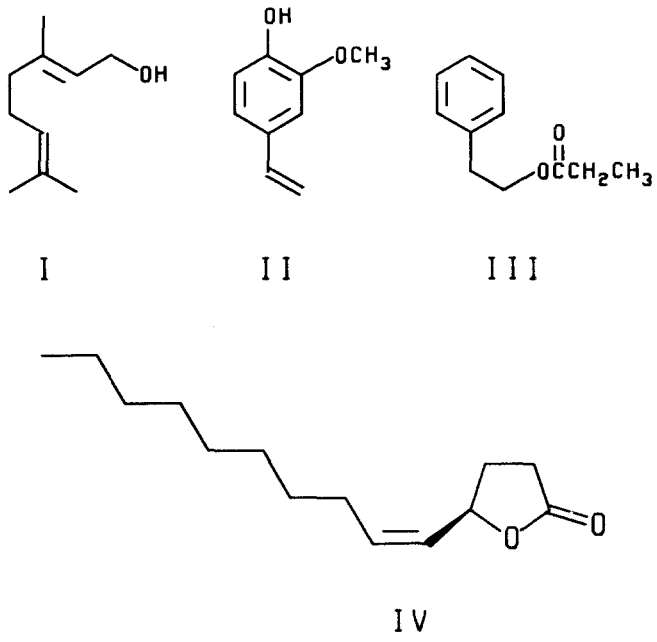
Various aspects of several of these topics are addressed by other papers in this symposium series. In the ensuing discussion, I will use selected examples from our research and from that of others to illustrate some of these points more fully.

PHEROMONE IDENTIFICATION

Many difficulties are encountered in attempting to elucidate the complete pheromone system of an insect. Most pheromones are produced and detected in nanogram or subnanogram quantities. Many pheromone systems are comprised of multicomponent blends in which the ratio of the components is controlled precisely. Usually the minor components of these blends comprise only a very few percent of the total blend, although they may be critical in eliciting key behavioral interactions among the insects. However, often only one or two of the components of the blend are ncessary to lure the insects into traps. Thus, (*Z*)-7-dodecen-1-ol acetate (*Z*7-12 : Ac)[1] is a very good trap lure for male cabbage looper moths, although there are at least five other components in the pheromone blend (Bjostad et al., 1984). Similarly, male tobacco budworm moths can be captured very efficiently with a mixture of *Z*9–14 : Al and *Z*11–16 : Al, although the complete pheromone consists of a blend of six aldehydes (Teal et al., 1986). Thus, a more effective bioassay is required to analyze the insects' behaviors and the effects of various chemicals or mixtures of chemicals on these behaviors. This usually can be accomplished for flying insects with a wind-tunnel bioassay (Baker and Linn, 1984).

[1] A standard shorthand notation for pheromone molecules of this type will be used in this paper. For example (*Z*)-9-tetradecenyl acetate is abbreviated Z9–14 : Ac; the corresponding alcohol is *Z*9–14 : OH, and the aldehyde Z9–14 : Al; tetradecan-1-ol is abbreviated S-14 : OH.

In addition to the complexities of the pheromone systems, we must also consider the effects of external factors on the insects' responses to their pheromones. Very often plant constituents have a major effect on these responses. In some cases, as in the bark beetles, plant constituents function as kairomonal components of a semiochemical system that includes the pheromone signal (Pearce et al., 1975; Birch, 1984; Tumlinson, 1985). In such cases, both the plant chemicals and the pheromone may be required to elicit attraction or the plant constituents may increase the responses to pheromones that are already very active alone. For example, when geraniol (I), eugenol (II), and phenethyl propanoate (III) are dispensed from the same trap as the Japanese beetle sex pheromone (IV), the response of both male and female beetles is increased significantly over that to either lure alone (Ladd et al., 1981) (Scheme 1).



SCHEME 1.

One of the primary considerations in identifying a pheromone is to determine precisely and accurately the composition of the pheromone released into the air by the insect during the time when it is signaling or "calling." This is important for several reasons, one being that the gland that produces the pheromone may contain many other substances, including precursors, some of which may be inhibitory (Teal et al., 1984). Also, the ratio of components released

into the air may differ from that found in the gland (Teal et al., 1986). In some cases the actual pheromone may not be stored in the gland, but may be produced only just before release.

A system used in our laboratory to collect volatiles from several species of insects is depicted schematically in Figure 1. This system and its use are described in detail by Heath et al. (1989). The pressure in the system is kept very close to atmospheric by balancing the air drawn out by a vacuum pump with compressed air forced through filters and a humidifier and then into the chamber containing the insects. Air passed over the insects is drawn out of the chamber through one of three filter traps connected in parallel in which the entrained volatiles are collected. Volatiles are eluted from the traps with solvent

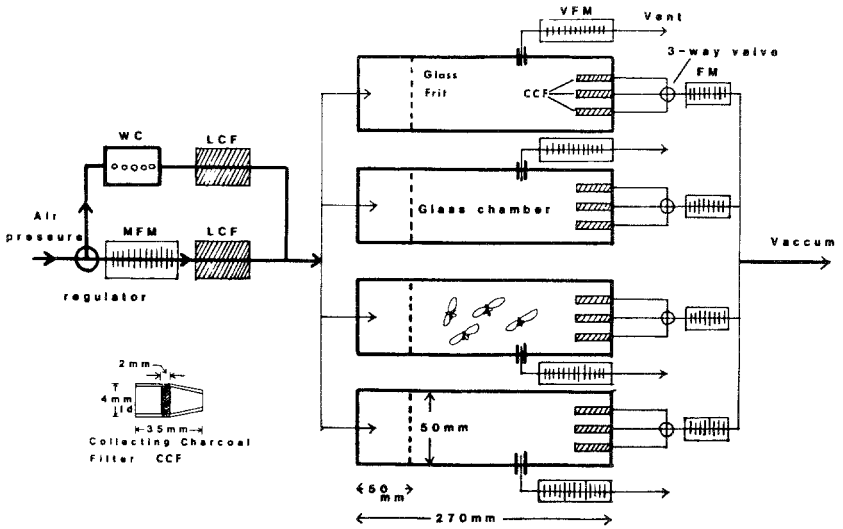


FIG. 1. Four-chamber volatile collection system. The flow of compressed air is regulated and measured by a flow meter (MFM) so that part of the stream is diverted through a water chamber (WC) to control the degree of humidity. Then both streams are passed through activated charcoal filters (LCF) before being mixed and routed through a four-way manifold to the glass volatile collection chambers. A course glass frit (dotted line) in the upwind end of each chamber disperses the air to provide a uniform flow through the chamber. Volatiles emanating from the insects are entrained in the air stream and pulled through a charcoal collecting filter (CCF) inserted in the upstream end of each exit port. Flow can be diverted by the three-way valve through any one of the ports for a desired period of time and then switched to another port when appropriate. This allows volatiles to be collected on different filters at different times or for different events during the photoperiod. Flows are regulated and balanced with the various flow meters (MFM, VFM, FM). Maintaining a slight positive flow (ca. 5%) through the vent ensures that the chamber is not under vacuum.

as described previously (Tumlinson et al., 1986). It is possible with this system to determine the composition of volatiles released by an insect for any period of time under a variety of conditions.

With this system we collected and analyzed the volatiles produced by male Caribbean fruit flies of several different ages and at three different periods (0–4 hr, 4–8 hr, 8–12 hr) of the photophase (Chuman et al., 1989). The analysis of the collected volatiles by capillary gas chromatography (Figure 2) illustrates their complexity. In addition to identifying a previously undiscovered compound, suspensolide (A), we discovered that the ratio of suspensolide to epianastrephin (D) changed as the day progressed, with the relative amount of epianastrephin being greater during the last 4 hr of the photophase. This, and the fact that suspensolide is converted to anastrephin (C) and epianastrephin (D) by gas chromatography on a packed column, suggests that suspensolide may be

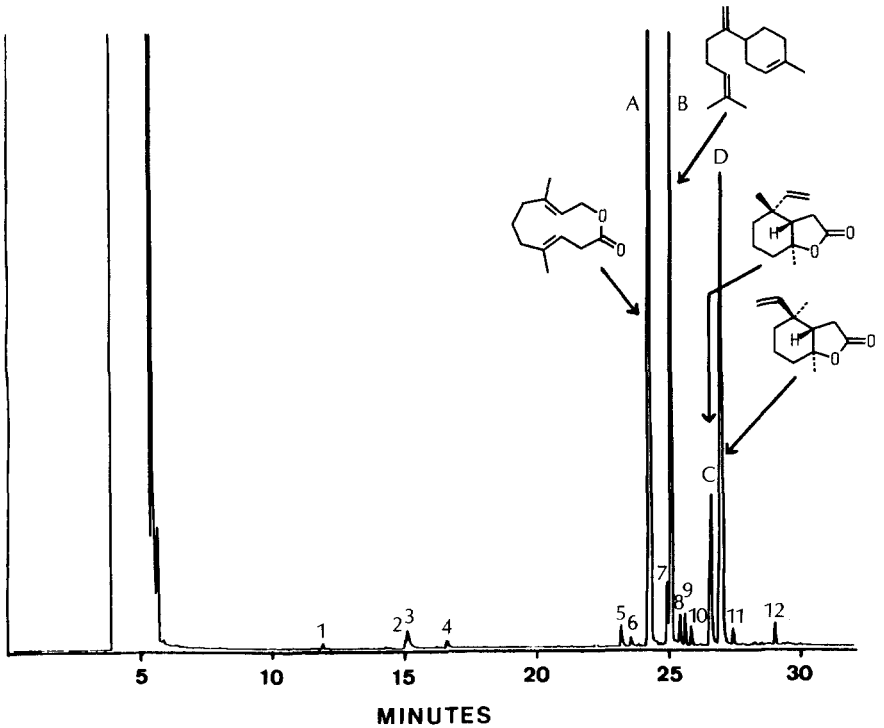


FIG. 2. Analysis of volatiles collected from Caribbean fruit fly males on a 50-m fused silica OV-101 capillary gas chromatographic column. Peaks identified were: (1) ocimene, (2) (Z,Z)-3,6-nonadienol, (3) (Z)-3-nonenol, (A) suspensolide, (B) α-bisabolene, (C) anastrephin, and (D) eipanastrephin.

a precursor to the anastrephins. Although the structures of the minor components 5–12 (Figure 2) have not been elucidated, the methane ionization and electron impact mass spectra indicate that components 5–10 are sesquiterpene hydrocarbons. More behavioral studies are needed to determine which of the components of this blend are involved in pheromonal communication. However, we now know many of the compounds and the ratios that need to be tested.

In determining the composition of a pheromone it is important to be able to separate the complex mixtures produced by the insects. It is particularly important to be able to separate isomers because often an isomer will occur in quantities of 1–2% or less of the mixture and will be essential for full activity. Additionally, it is very important to be able to establish the purity of an isolated pheromonal compound to ensure correct identification of the pheromone. Several techniques for purifying and analyzing pheromones are described in detail by Heath and Tumlinson (1984). The analysis of the volatiles produced by fall armyworm females illustrates the application of capillary gas chromatography to the identification of the components of a pheromone blend (Figure 3). In this case it was necessary to use three different types of stationary phases to ascertain with certainty the composition of the blend. One problem that further complicated this analysis was that the 12-carbon acetates were produced only in quantities of 100 pg/female/hr or less and thus were no larger than some of the background impurities from which they had to be resolved.

It is also very important that the structures of the active compounds be accurately described. The insects' receptors are very specific and slight changes in the geometry of the molecule can render the compound inactive or even inhibitory. For example, addition of as little as 1% of *Z*3,*Z*13–18 : Ac greatly reduces the activity of *E*3,*Z*13–18 : Ac as an attractant for lesser peachtree borer males (Tumlinson et al., 1974). In many cases, slight changes in isomeric composition may make a pheromone blend attractive to another species because many closely related species use different ratios or isomers of the same compound to achieve specificity in their pheromone systems. Chirality is also very important, and while some species that produce pheromones with asymmetry in the molecules appear to respond to the racemic mixture as well as to the pure active enantiomer, in other cases the opposite enantiomer may be inhibitory. Even different strains of the same species may respond to different enantiomers of the same compound (Birch et al., 1980; Lanier et al., 1980). Silverstein (1988) in another paper in this symposium discusses chirality in pheromones in more detail.

Even when the pheromones are identified completely and correctly, the importance of developing formulations that will release the synthetic pheromone at the same rate and in the same ratio as the natural pheromone is released by the insects is often overlooked. Testing of large or incorrect doses will often

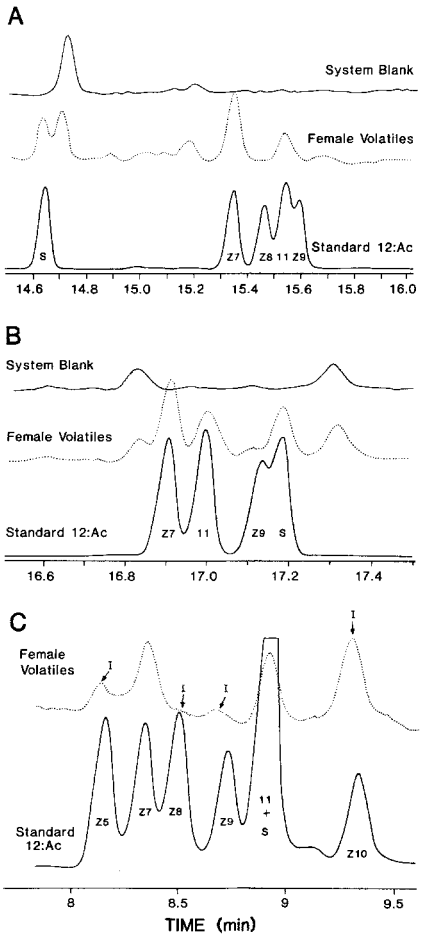


FIG. 3. Sections of chromatograms showing separation of 12-carbon acetates and analysis of volatiles collected from calling, laboratory-reared fall armyworm female moths on three capillary GC columns (A) CPS-1; (B) OV-101; (C) cholesteryl-*p*-chlorocinnamate (liquid crystal). In (C) the symbol I indicates impurities found in the system blank. As these chromatograms indicate, Z9–12 : Ac, previously identified as a fall armyworm pheromone, did not coincide with any of the candidate pheromone peaks on any of these columns. The volatile components not present in the system blank were coincidental on all three columns with S-12 : Ac, Z7–12 : Ac, and 11–12 : Ac.

obscure subtle behaviors in bioassays and may actually result in reduced captures. In other cases it can lead to the identification of compounds as pheromones when they are really only mimics that are active at doses far greater than the actual pheromone. For example, the fall armyworm (FAW) sex pheromone

was identified as Z9–12 : Ac (Sekul and Sparks, 1976; Jones and Sparks, 1979), but this compound could not be found in the gland of FAW females. Instead, the pheromone was found to consist of a blend of Z7–12 : Ac and Z9–14 : Ac released in a ratio of 3.4 : 96.6, respectively (Tumlinson et al., 1986). This blend is active in luring FAW males to traps in the field at concentrations at least 10-fold less than Z9–12 : Ac when formulated on rubber septa. Thus, although Z9–12 : Ac will attract FAW males, it is not a pheromone and is active only at doses much higher than those dispensed by the female.

In another study, a 40 : 60 blend of (Z,Z,Z)-3,6,9-eicosatriene and -heneicosatriene loaded onto rubber septa was found to capture significantly greater numbers of velvetbean caterpillar (VBC) moth males than any other ratio of these two compounds (Landolt et al. 1986). However, Mitchell and Heath (1986) found that high doses of a 40 : 60 blend of these two compounds actually decreased trap captures of VBC (Figure 4). Thus, if this pheromone had been tested at 5–10 mg/septum, which is not uncommon, it may have been dismissed as only very slightly or not active. There are several papers that discuss pher-

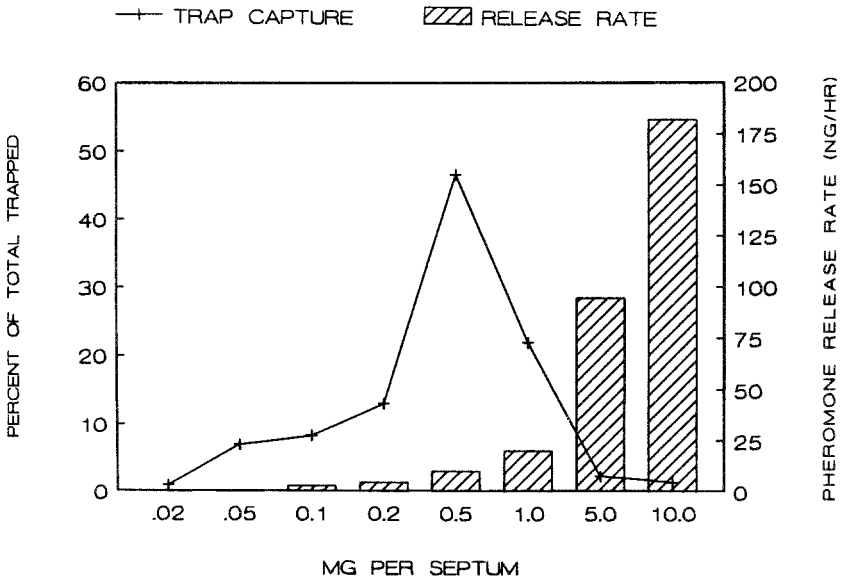


FIG. 4 Response of male velvetbean caterpiller moths to bucket traps baited with rubber septa impregnated with different doses of sex pheromone, a 40 : 60 blend of (Z,Z,Z)-3,6,9-eicosatriene and (Z,Z,Z)-3,6,9-heneicosatriene. Each dose was replicated 10 times; a total of 18,710 moths was captured in the test. Pheromone release rate data were determined as a constant wind speed of 0.225 m/sec. All release rate data were converted to nanograms of total pheromone per hour.

omone formulation in detail (Heath et al., 1986; Leonhardt and Moreno, 1982; Weatherston et al., 1982). The type of formulation will vary, depending on the volatility and other characteristics of the pheromone and the use for which it is intended. However, for meaningful results, the formulation must be designed initially to duplicate the insect's release ratio and rate. Subsequently, it may be possible to improve trap capture or other results by varying dose, composition, or ratio.

In summary, pheromone identification requires more than just the chemical characterization of compounds extracted from insects. It should be possible to make more effective use of pheromones for insect pest management if we determine precisely and accurately the composition and release rate of the pheromone produced by the insect, the identity of any plant-produced or other semiochemicals that may synergize or otherwise affect the pheromone activity, and the effects of all these chemicals on the insect's behavior. Then we must formulate a synthetic blend that will compete effectively with the natural signal or that is designed to disrupt the communication system of the insect by interfering in some way with key pheromone mediated behaviors.

PHEROMONE BIOSYNTHESIS

Pheromone biosynthesis is receiving increasing emphasis in investigations conducted by several different research groups on a variety of insects in the orders Lepidoptera, Diptera, and Coleoptera (Prestwich and Blomquist, 1987). Among the different orders of insects, the pathways and sites of biosynthesis vary considerably. While many of the flies, like the housefly, synthesize their pheromones in the abdominal cutical (Blomquist et al., 1987), the pheromones of many beetles are synthesized in their guts (Vanderwel and Oehlschlager, 1987). However, the moths studied thus far synthesize their sex pheromones in glands that are usually located near the tip of the abdomen (Bjostad et al., 1987; Morse and Meighen, 1987; Teal and Tumlinson, 1986).

The biosynthesis of the pheromones in the moths begins with the production of a polypeptide hormone in the brain–subesophageal ganglion complex in response to endogenous and external stimuli including temperature and light (Raina and Menn, 1987). The hormone then triggers the conversion of fatty acids in the pheromone gland to pheromone components by a series of enzymatic processes that may include desaturation, beta oxidation chain shortening, reduction, acetylation, hydrolysis, and/or oxidation. These processes are varied in different species to produce precise blends characteristic of each species. A more detailed discussion of the pathways of desaturation and the mechanisms that regulate *Z* and *E* ratios in pheromone blends is provided by Roelofs and Wolf (1988) in another paper in this symposium.

In some cases the pheromones are produced and stored in the gland until

the moth releases them. In others it appears that precursors are synthesized and stored and then converted to the actual pheromonal compounds just prior to release. The latter process has been found to occur when the pheromonal compounds are aldehydes. This crucial terminal step in biosynthesis, in which the insects produce the active compounds, has been of some interest to us because of its importance in the pheromone systems of the very important pests in the genus *Heliothis*.

Aldehydes play a major role in the sex pheromone systems of all the *Heliothis* species. However, extracts of the female pheromone glands of *H. virescens*, the tobacco budworm; *H. zea*, the corn earworm; or *H. subflexa* reveal the presence of alcohols corresponding in structure to the aldehydic pheromone molecules. In *H. subflexa*, we also found the corresponding acetates (Teal et al., 1981). We recently demonstrated that the aldehydic components of the tobacco budworm moth pheromone are produced by the action of an alcohol oxidase on the precursor alcohols produced in the pheromone gland (Teal and Tumlinson, 1986). Application of primary alcohols dissolved in dimethyl sulfoxide (DMSO) to the surface of the tobacco budworm moth female's sex pheromone gland resulted in the production of the corresponding aldehydes. This enzymatic system is specific for primary alcohols; it did not convert secondary alcohols into ketones. However, it is not very specific for chain length, or number of location of olefinic bonds. All primary alcohols from 12 to 18 carbons in chain length, and with 0, 1, or 2 double bonds, that were applied to the gland in DMSO were converted into the corresponding aldehydes. The ratio of aldehydes produced is governed by the ratio of alcohols in the gland. Thus, when a 3:1 ratio of S-16:OH to S-14:OH was applied to the gland, approximately a 3:1 ratio of the corresponding aldehydes was produced. Additionally, we discovered that oxygen was required for conversion of the alcohols to aldehydes. When alcohols were applied to the gland in vivo under N_2, no aldehyde was produced. Introduction of air after 15 min revived the production of aldehyde (Teal and Tumlinson, 1987). The same or a very similar enzymatic system resides in the gland of *H. zea* and *H. subflexa*. Recently, we have established that the enzyme is located in the cuticular layer covering the sex pheromone gland in these species (Teal and Tumlinson, 1988). The enzyme is active during the entire photoperiod. Thus, in *Heliothis* species, the primary alcohols are synthesized in the sex pheromone gland and subsequently oxidized to aldehydes as they pass through the cuticle. This explains why only aldehydes have been detected in volatiles collected from *H. virescens* (Pope et al., 1982; Teal et al., 1986), although the alcohols, particularly Z11-16:OH, are found in relatively large amounts in gland extracts. We are now in the process of purifying and characterizing this alcohol oxidase and in determining whether or not there are differences in this system among the three *Heliothis* species we are studying.

Investigations of pheromone biosynthesis like these and others (e.g., Bjo-

stad et al., 1987) increase our knowledge and understanding of the pheromone systems of insects. This is very important in designing programs to monitor or manage pest species. It also seems reasonable to speculate that, as our knowledge of the mechanisms that control these systems increases, we should be able to find ways to disrupt or alter pheromone biosynthesis and thus develop new techniques for pest management.

OTHER SEMIOCHEMICALS

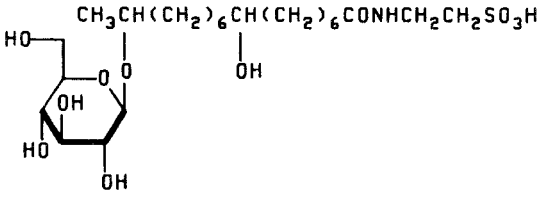
Although there are many types of semiochemicals other than sex pheromones, I have chosen to focus on those that mediate the oviposition behavior of insects and those that beneficial parasitic and predatory insects use to locate their hosts. I believe that these systems offer the greatest opportunities for exploitation for the development of pest management methods.

The location of suitable sites to deposit eggs is a very important step in any insect's life cycle. The females must ensure that their progeny will have access to proper and sufficient food and have a chance to survive in a world filled with enemies and competitiors. Thus, each species has evolved to occupy a certain niche in the ecosystem and has developed strategies to take maximum possible advantage of the available resources. These strategies vary from massing many eggs from several females in one location to dispersing single eggs evenly over all available sites or resources in a large area. It has been demonstrated in many instances that semiochemicals play key roles in these strategies. The semiochemicals that mediate oviposition may include those produced by the hosts that facilitate host location, inhibitors or deterrents produced by hosts or nonhosts as defensive mechanisms, oviposition-stimulating pheromones that aid in grouping eggs from many females in a suitable location, and oviposition-deterring or -spacing pheromones that prevent too many eggs from being deposited in one location with limited resources.

As an example of the latter type of semiochemical, Prokopy (1981) has shown that apple maggot fly females, after ovipositing in a cherry, apple, or other fruit, drag their ovipositors over the surface of the fruit and spread a substance that deters females that subsequently land on this fruit from ovipositing. In this way they distribute their eggs more evenly over all available fruit rather than placing several eggs in one fruit that may only provide enough food for one larva. The substance that they deposit on the fruit is water soluble, nonvolatile, and very stable. It can be washed from the surface of fruit on which females have oviposited and reapplied to clean fruit to achieve the deterrent effect.

Recently, Hurter et al. (1987) isolated and identified an oviposition-deterring pheromone of the cherry fruit fly, which is in the same genus as the apple

maggot fly. They reported this pheromone to be *N*[15(β-glucopyranosyl)oxy-8-hydroxypalmitoyl]-taurine (Scheme 2, V). When this compound is synthe-

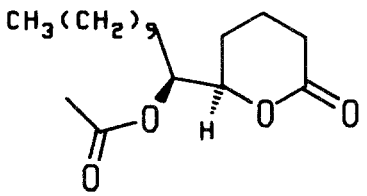


V

SCHEME 2.

sized and tested in the field, it will be interesting to find out whether or not these types of pheromones can be used effectively for pest management. We might predict that they will be very useful since Katsoyannos and Boller (1976) demonstrated in a small field test that naturally produced pheromone, when collected and applied to fruit, provided good protection from fly oviposition and damage.

In contrast to the apple maggot fly, the mosquito *Culex pipiens fatigans* Wiedemann, and others of that genus were found to release an oviposition attractant onto their eggs. This attractant induces other females of the species to lay rafts of eggs in the same location (Bruno and Laurence, 1979). The major component of this pheromone was identified as *erythro*-6-acetoxy-5-hexadecanolide (Scheme 3, VI) (Laurence and Pickett, 1982), and subsequently the nat-



VI

SCHEME 3.

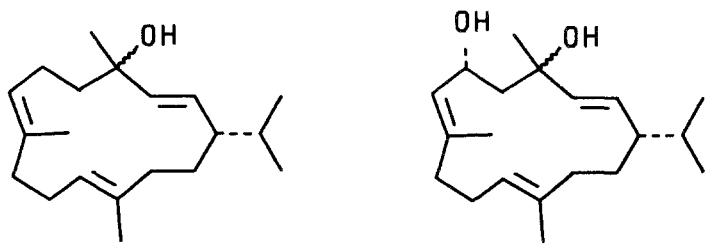
urally produced pheromone was determined to be the (−) -(5*R*, 6*S*)-enantiomer by chromatography of 6-trifluoroacetoxy analogs of the natural and synthetic compounds on a chiral capillary GC column (Laurence et al., 1985). Conceivably, this pheromone can be used to induce the female mosquitos to place their

eggs in a defined area that can safely be treated with insecticide and thus reduce the area over which insecticide must be applied for control.

The plant-feeding insects use many factors including semiochemicals to locate their hosts (Schoonhoven, 1981; Miller and Strickler, 1984). Several plants use semiochemicals to repel insects, while in many other plants the chemicals produced attract the insects to suitable oviposition sites or food sources. In some cases, the same compounds appear to be both attractants and oviposition stimulants. For example, alkyl sulfides containing a propyl thiol moiety attract and induce the onion fly to lay eggs (Miller and Strickler, 1984). However, in many cases it appears that nonvolatile compounds act as oviposition stimulants. As an example of the latter, the butterfly *Papilio protenor* is stimulated to oviposit when it contacts the flavanone glycosides naringen and hesperidin, which are found in one of its hosts, sour orange (Honda, 1986).

Since *Heliothis* species are major economic pests in the United States, it is not surprising that there is interest in semiochemically mediated plant–insect interactions that involve these species. *H. virescens* and *H. zea* attack a wide variety of plants including many important crops. In contrast, *H. subflexa*, which is not a pest in the United States, feeds exclusively on groundcherry (Mitchell and Heath, 1987). *H. subflexa* and *H. virescens* are closely related and recently have been the subjects of many hybridization studies. Thus, this group of insects provides an excellent opportunity to compare and contrast the semiochemically mediated plant–insect interactions of monophagous and polyphagous species.

Jackson et al. (1986) conducted a series of studies of the responses of *H. virescens* to susceptible and resistant varieties of tobacco. They found that the duvane diterpene stereoisomers, α- and β-4,8,13-duvatrien-1-ol (VII) and also α- and β-4,8,13-duvatrien-1-3-diol (VIII) (Scheme 4), obtained from extracts



VII VIII

SCHEME 4.

of leaves of susceptible tobacco varieties, increased *H. virescens* egg deposition when sprayed onto the leaves of a resistant variety. On the other hand, Tingle and Mitchell (1986) found that extracts prepared from elderberry leaves with

H_2O and other polar solvents deterred oviposition by *H. virescens* females in both laboratory and field cage tests. The compounds responsible for this deterrence were not isolated. It seems likely that *H. virescens*, which attacks a wide variety of plants and encounters a wide variety of chemicals may have a complex semiochemically mediated oviposition behavior. However, since *H. subflexa* is very host-specific, it may be attracted and stimulated to oviposit by a very specific semiochemical or blend. Recently, in a laboratory bioassay, methanol washes of groundcherry leaves were found to increase egg deposition by *H. subflexa* females (Mitchell and Heath, 1987). Also, Tingle et al. (1989) found that the same methanol leaf washes induced *H. subflexa* females to respond in a wind tunnel with upwind flight toward the odor source and contact with the dispenser on which the extract was placed. Interestingly, the responses of males and of virgin females were significantly less than that of mated females. It is not yet clear whether or not the females are attracted and induced to oviposit by the same compounds. It will be very interesting to discover, as these investigations progress, the similarities and differences in the semiochemically induced host-locating and oviposition behavior of these two species.

As more is learned about the behavioral mechanisms of host location and oviposition, and as more of the semiochemicals that mediate these behaviors are identified, it should be possible to devise new pest management methods based on interference with this system or manipulation of the behaviors. We should be able to prevent oviposition on valuable crops and induce the pests to deposit their eggs on noncrop plants or in locations where they can be destroyed with minimal applications of pesticides. It is conceivable that this could be accomplished either by the application of synthesized semiochemicals or by genetically engineering plants to produce or not produce certain semiochemicals.

The foraging and egg-laying behaviors of parasitic insects that ovipost in eggs, larvae or adults of other insects are similar in many respects to those of herbivorous insects and in most cases are also mediated by semiochemicals. Many of these parasitoids are considered beneficial in that their hosts are pests. Agricultural scientists generally agree that biological control of insect pests with natural enemies is a very important component of any pest management strategy and that beneficial organisms like insect parasitoids should be used to the greatest extent possible. Therefore, it is very important to understand the mechanisms by which beneficial parasitic insects locate and attack the pest insect species that are their hosts. A thorough knowledge of the foraging behavior of these parasites and the semiochemicals that mediate this behavior should enable us to develop methods to enhance the effectiveness of the beneficial organisms.

Most of the parasitic insects studied thus far have shown a great degree of adaptability and flexibility in their searching behavior. Many of them use the pheromones of their hosts as chemical cues to guide them in host foraging. For example, the oviposition-deterring pheromone desposited by apple maggot fly

females stimulates searching by *Opius lectus*, a parasitoid of the fly eggs (Prokopy and Webster, 1978). Also, female wasps in the genus *Trichogramma*, which oviposit in moth eggs, have been found to respond to the sex pheromones of several of their hosts including *H. zea* (Lewis et al., 1982), Pieris brassicae, and *Mamestra brassicae* (Noldus and van Lenteren, 1985). Similarly, insects that prey on or parasitize several species of bark beetles are attracted to the beetles' pheromones (Wood et al., 1968; Lanier et al., 1972; Vité and Williamson, 1970; Pitman, 1973; Kline et al., 1974; Dyer et al., 1975; Bakke and Kvamme, 1978). Interestingly, spined soldier bugs, *Podisus maculiventris*, voracious predators of a wide variety of insects, are themselves the targets of four parasitic species, *Hemyda aurata*, *Euclytia flava*, Telenomas spp., and *Forcipomyia crinita*, that are attached to the soldier bug pheromone, a mixture of (*E*)-2-hexenol, α-terpineol, linalool, terpinen-4-ol, and benzyl alcohol (Aldrich et al., 1984).

An example of flexibility in the foraging behavior of beneficial organisms is demonstrated by studies of a polyphagous predatory mite, *Amblyseius potentillae* that feeds and reproduces on phytophagous spider mites (Dicke et al., 1986). A searching response in this predatory mite is elicited by volatile kairomones from the two-spotted spider mite only when the predator is reared on a carotenoid-free diet. Two-spotted spider mites and others contain carotenoids required for diapause induction in the predator, but are inferior prey due to the dense webbing they produce. When predators are not deficient in carotenoids, they reject the two-spotted spider mite and search for other spider mites that are more likely to assure them reproductive success. Such a prey is the European red spider mite, the volatile kairomone of which elicits a searching response by the predator whether or not it is carotenoid deficient.

There is considerable evidence that parasitoids may use the odors of plants on which their hosts feed to guide them to possible host habitats. For example, Elzen et al. (1984) found that terpenoid components of cotton volatiles were attractive to *Campoletis sonorensis*, an ichneumonid wasp that is a parasitoid of noctuid moth larvae like the tobacco budworm, *H. virescens*. Also, Drost et al. (1986, 1988) and Eller et al. (1988) found in wind tunnel and olfactometer studies that females of *Microplitis croceipes*, a braconid parasitoid of *Heliothis* species larvae, responded to plant odors. However, in the latter case the *M. croceipes* responsiveness in the flight tunnel was influenced by preadult experience of the parasitoid species, growth phase and part of the host plant, presence of host larvae, and age and adult experience of the parasitoid. Herard et al. (1988) discovered that when *M. demolitor* was reared on *H. zea* larvae fed artificial diet, the adult females needed preflight exposure to feces from plant-fed hosts to be effective in flying to host larvae feeding on cowpea leaves. However, *M. demolitor* females reared from plant-fed hosts were generally very effective in flying to their hosts without any preflight exposure or experience.

Their results indicated that imprinting of the adult females at the time of emergence from the cocoon was occurring.

When a parasitoid's host is polyphagous, as are *H. zea* and *H. virescens*, there is an obvious advantage to the parasitoid in being able to adapt or to learn to respond to different odors associated with different host-plant complexes. We have recently demonstrated (Lewis and Tumlison, 1988) that associative learning plays a key role in the host-foraging process of *M. croceipes* females and also that it is probably involved in the host-locating behavior of *Cotesia marginiventris*, which attacks the larvae of at least 20 lepidopteran species (Turlings et al., 1989). The feces of *H. zea* larvae fed cowpea leaves contains two substances that elicit two discrete effects on the host-seeking behavior of *M. croceipes* females. First, a volatile component(s), extractable with hexane, attracts females that have been exposed previously to feces. However, exposure of the females to the hexane extract does not induce subsequent flight responses to host-related odors. Second, a nonvolatile, water-extractable material, when contacted by the parasitoids with their antennae, will induce subsequent flight responses to host-related odors. We also demonstrated that contact with the water extractable substance in the presence of a novel odor like vanilla would induce subsequent flights to vanilla, an otherwise unattractive substance.

Thus the females learn to respond to a variety of odors (conditioned stimuli) associated with the hosts by linking them with a water-extractable, nonvolatile chemical (unconditioned stimulus) in host feces, evidently a specific host-recognition kairomone. The identification and synthesis of this kairomone and the components of the volatile signals associated with various host-plant complexes should make it possible to condition laboratory-reared parasitoid females to search for hosts on particular plants. This should greatly increase the efficiency of parasitoids released for biological control.

CONCLUSIONS

The area of insect chemical communication is a very rich area for exploration. Although many pheromones and semiochemicals have been identified, we have only begun to understand the insects' chemical communication systems. There are many opportunities for investigation and possibilities for exploitation, some of which probably have not yet been discovered. We must not consider that the identifications of the semiochemicals is the end of the investigation and that we will only use these chemicals directly for insect control in ways similar to pesticides. Although it is now possible in some cases to use pheromones and other semiochemicals directly for trapping or communication distruption, the greatest value of these investigations may lie in the knowledge gained about the mechanisms of biosynthesis, preception, and CNS

processes that integrate the information and control the behavioral responses. This knowledge should lead us to new, safer, and more effective methods of insect control.

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PROPERTIES OF CUTICULAR OXIDASES USED FOR SEX PHEROMONE BIOSYNTHESIS BY *Heliothis zea*

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Abstract—Biosynthesis of the aldehydic sex pheromone components released by females of *Heliothis zea* was found to be catalyzed by primary alcohol oxidases residing in the cuticle that covers the glands. Activity, as indicated by conversion of primary alcohol to aldehyde, was as high in cell-free cuticle as it was in intact pheromone glands. Studies indicated that some activity was associated with the surface of the epicuticle and could be removed, into buffer, by sonication. However, the majority of activity lies within the inner epicuticle and exo- and endocuticular layers. The oxidase was not functional in pharate pupae that did not have mature adult cuticle but became functional just prior to adult emergence. The enzyme in individual glands was saturated at alcohol concentrations above 100 n. moles. Nonionic detergents did not affect the activity of the oxidase in the cuticle but treatment with either 7 M urea or 1% SDS resulted in total loss of activity. Studies on the effect of pH indicated an optimum at 6.4; however, activity was high throughout the range of 5–9. The oxidase was functional in both dichloromethane and hexane, suggesting that this enzyme system may have applications for organic synthesis of aldehydes.

Key Words—Sex pheromone biosynthesis, cuticle, alcohol oxidase, alcohol, aldehyde, *Heliothis zea*, Lepidoptera, Noctuidae, enzyme.

INTRODUCTION

The volatile compounds that comprise the sex pheromone blend released by females of *Heliothis zea* (Boddie) are aldehydes (Pope et al., 1984), which include (Z)-7-hexadecenal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, and hexadecanal. Extracts obtained from the pheromone glands of females not only contain the aldehydes but also the corresponding alcohols in a ratio that approx-

imates the aldehyde ratio (Teal et al., 1984, 1986). Recent studies have indicated that the alcohols are the immediate precursors of the aldehydic pheromone components used by *Heliothis* species and that the ratio of alcohols present within the gland regulates the blend ratio of aldehydes produced (Teal and Tumlinson, 1986). The enzyme responsible for the conversion of the alcohols to aldehydes has been defined as an alcohol oxidase specific for primary alcohols but which shows little or no specificity for geometry or number of double bonds (Ding and Prestwich, 1986; Teal and Tumlinson, 1986, 1987a, b).

Studies conducted using *Heliothis virescens* (F.) by Ding and Prestwich (1986) have indicated that the oxidase is present in the soluble fraction of pheromone gland homogenates. However, their studies also have indicated that homogenates of the pheromone gland contain an aldehyde dehydrogenase that has greater activity than the oxidase (Ding and Prestwich, 1986). This suggests that if both enzymes were present within the cells of the pheromone gland, then all of the aldehyde produced would be converted immediately to the acid analogs, leaving little or no aldehyde available for release as pheromone components. This is not supported by studies on pheromone production by these moths because (Z)-11-hexadecenoic acid, the acid corresponding to the pheromone component present in greatest amount, has not been found in extracts obtained from females that are releasing sex pheromone (Klun et al., 1980a, b; Pope et al., 1982, 1984; Teal et al., 1984, 1986).

Studies conducted by Morse and Meighen (1986) and Teal and Tumlinson (1986) have suggested that the oxidases responsible for production of the aldehydic pheromone components released by females of the spruce budworm and *H. virescens* moths, respectively, are present within the cuticle overlying the glands. Although unproven, these hypotheses have considerable merit because extracellular conversion of alcohols to aldehydes avoids the toxic effects of the aldehydic products and compartmentalizes the oxidase in a different area of the gland from the aldehyde dehydrogenase. This would enable the moth to use the aldehydic compounds for pheromone communication.

Although the majority of studies on cuticular enzymes have dealt with those associated with tanning, wound healing, and digestion of cuticle (see Andersen, 1985; Hepburn, 1985), enzymes that have functions in pheromone communication have been documented. These enzymes have been associated with pheromone degradation, thus enabling the insect to remove pheromone that adsorbs onto the cuticle (Ferkovich et al., 1982; Lonergan, 1986; Vogt and Riddiford, 1986). We report here the results of studies that demonstrate that the conversion of primary alcohols to aldehydic pheromone components by females of *H. zea* is accomplished by the action of a primary alcohol oxidase maintained within the cuticle overlying the pheromone gland. We also discuss the results of experiments performed to assess various functional aspects of this oxidase.

METHODS AND MATERIALS

Insect Preparation. Females of *H. zea* were obtained from cultures maintained at the Insect Attractants, Behavior, and Basic Biology Research Laboratory, ARS, USDA, Gainesville, Florida. All insects used were 2- to 4-day-old adults, and experiments were performed during the photophase, at which time no active pheromone biosynthesis occurs under natural conditions (Raina et al., 1986). Previous studies have indicated that the oxidation of topically applied alcohols was as efficient during the photophase as it was during the period of maximum pheromone production (4th to 7th hour of scotophase) (Teal and Tumlinson, 1986).

In Vivo Preparation. Studies in which various primary alcohols were applied topically to the pheromone glands were conducted using insects whose pheromone glands had been extended and clamped as described elsewhere (Bjostad and Roelofs, 1983; Teal and Tumlinson, 1986). In initial studies, using *H. zea*, we repeated earlier experiments conducted on *H. virescens* (Teal and Tumlinson, 1986), in which a 1.0-μl drop of a 0.5 μg/μl emulsion of candidate alcohol substrate in distilled water was applied to the gland surface and allowed to incubate for 30 min. After incubation, the glands were excised, placed in a conical microvial, and extracted with 5 μl of hexane containing 10 ng of pentadecanyl acetate (15:Ac) as internal standard. The total volume of the extract was then analyzed by capillary gas–liquid chromatography (GC). In a second series of topical application studies, preparations were treated with a 2.0-μl drop but were allowed to incubate for only 10 min. After incubation the water–substrate drop was pulled up carefully into a 10-μl microsyringe. The drop was then deposited into a conical microvial and extracted using two aliquots of 5 μl each of hexane, each containing 1 ng/μl of 15:Ac. The extracts were then concentrated to 5 μl and analyzed by capillary GC. Blank preparations for both experiments were conducted using glands incubated with a 1.0-μl drop of H_2O. Also a 1.0-μl drop of the substrate–H_2O emulsion was incubated for 30 min in a conical microvial prior to extraction and analysis as above.

In studies to determine if the oxidase could be dissolved from the surface of the intact glands of females, insects were first injected with 2.0 μl of a 0.25 g/ml tetrasodium ethylenediaminetetracetate by inserting a 10-μl syringe through the head and into the terminal abdominal segments as described by Pope et al. (1982). The whole abdomen was then removed from the insect at the junction of the thorax, and the majority of eggs were removed through the hole in the anterior segment of the abdomen. The pheromone gland was then everted by applying pressure to the anterior abdominal segments and a cotton thread ligature was placed around the abdomen 0.5 cm from the posterior tip. A second ligature was added 0.5 cm anterior to the first. The abdomens were

then placed in conical microvials that contained 25.0 μl of 75 mM phosphate buffer (pH 7.2) so that the pheromone gland was immersed in buffer. The vials were then placed in a sonic bath (Megason, ultrasonic instruments) and sonicated for 30 min. The initial temperature of the bath was 10°C, and the final temperature was 35°C. After sonication, the abdomens were removed, and 2 μl of the 0.5 μg/μl emulsion of alcohol and water was added. The preparations were allowed to incubate for 1.5 hr prior to extraction with two aliquots of 25 μl of hexane that contained 15 : Ac as internal standard. The hexane extract was concentrated to 5 μl under a gentle stream of N_2 prior to analysis by capillary GC.

In Vitro Preparation. Studies performed to determine the stage of development at which the oxidase becomes functional were conducted using glands that had been excised from the abdomen. After removal from the abdomen, glands were dried on a filter paper and placed into a conical microvial, 2 n. moles (424 ng) of Z11–14 : OH dissolved in hexane (see below) was then added to the microvial and the preparations incubated for 30 min. The glands were extracted for 30 sec by adding 5 μl of hexane, which contained 10 ng of internal standard, and analyzed. Glands used were obtained from prepharate pupae (pupae having unsclerotized adult cuticle), pharate pupae (having sclerotized adult cuticle), newly emerged adults, adults that had emerged 15, 30, or 60 min earlier, and 2-day-old adults. When pupal insects were used, the pupal case was carefully removed from the last four abdominal segments prior to applying pressure to the anterior portion of the abdomen, which caused the gland to be everted.

The effect of various organic solvents including acetone, acetonitrile, dichloromethane, diethyl ether, hexane, and tetrahydrofuran (THF) on the functioning of the enzyme was studied using glands that had been excised during the noncalling period. Individual glands were soaked in one of the solvents for 5 min and then dried on filter paper. Then the gland was placed in a conical microvial and 2 μl of a 212 ng/μl emulsion of Z11–14 : OH in 75 mM phosphate buffer (pH 7.2) was added. The preparation was incubated for 30 min. After incubation the tissue and medium were extracted two times with 5 μl of hexane containing 10 ng of internal standard each time. The combined hexane extracts were concentrated to 3 μl and injected onto the capillary GC. We also studied the relative efficiencies of 75 mM phosphate buffer (pH 7.2) and hexane as incubation media. In these studies, individual glands were incubated for 30 min in conical microvials with either 2 μl of the 212 ng/μl emulsion of Z11–14 : OH in phosphate buffer or 2 μl of a 212 ng/μl of Z11–14 : OH in hexane. After incubation for 30 min, the preparations were extracted and analyzed as described above.

The effect of substrate concentration was examined by incubating various amounts of Z11–14 : OH dissolved in 2 μl of hexane, containing 10 ng of octa-

decane as internal standard, with individual glands for 30 min in conical microvials. Temporal production of (*Z*)-11-tetradecenal (Z11–14 : Al) was studied by incubating individual glands in a microvial with 16.8 μg of Z11–14 : OH dissolved in 40 μl of hexane containing 320 ng of octadecane as internal standard. The vial was capped with a Teflon-faced septum to prevent solvent evaporation. At 5-min intervals up to 30 min, a syringe needle was inserted into the solution through the Teflon septum and a 2-μl sample was removed and analyzed by GC.

In order to determine if the oxidase was present within the cuticle of the pheromone gland, we made cell-free cuticle preparations from groups of glands that had been excised from the abdomen. Glands were placed in distilled H_2O (50–70 glands/ml) and were sonicated for 15 min in the sonic bath. After sonication, the H_2O was concentrated to two female equivalents (FE) per 10 μl using a Centricon-30 microconcentrator (Amicon, Danvers, Massachusetts) and tested for oxidase activity by incubating 1 FE with 2 μl of the 212 ng/μl emulsion of Z11–14 : OH in phosphate buffer for 30 min. This was followed by extraction and analysis as described earlier. The glands were then washed four times with 1 ml aliquots of distilled H_2O and then homogenized in 1-ml aliquots of distilled H_2O using a Teflon homogenizer (Wheaton 2 ml, Millville, New Jersey). The liquid was removed after 10 turns of the pestil. Then 1 ml of H_2O was added and the glands were rehomogenized. This procedure was repeated until the homogenate appeared to be clear, usually after five changes. The combined homogenates were centrifuged at 4000 g for 30 min at 5°C, and the aqueous layer was removed, combined with the other liquid fractions, and concentrated to 1 FE per 5 μl using a Centricon-30 microconcentrator. One FE was then tested for oxidase activity as indicated above.

After the final homogenization, the cuticle remained as large pieces. Pieces of this cuticle were then tested for activity by incubating them with 2 μl of a 212 ng/μl solution of Z11–14 : OH in hexane for 30 min, the extracts of these incubations being analyzed by capillary GC. The fragments of cuticle were then subjected to sonication for 15 min in distilled H_2O. The sonicate was removed, concentrated, and tested for activity, and the cuticles were rinsed twice with 1 ml of distilled water. Cuticular pieces were then dried by placing them on filter paper. Fragments of cuticles were examined under a microscope at 400× to ensure that gland cells had been disrupted. Cuticle fragments representing one gland equivalent by weight were then placed in a conical microvial to which 440 ng of Z11–14 : OH was added in a 2.0-μl drop of hexane and incubated for 30 min. Control experiments conducted in conjunction with these experiments included incubation of the intact pheromone gland, which had been excised from the abdomen, with 440 ng of Z11–14 : OH in 2.0 μl of hexane or just 2.0 μl of hexane.

Studies on the effects of various nonionic detergents (including Tween 80,

NP-40, and Triton X-100) on oxidase activity were conducted by sonicating for 15 min equal weights (8–14 FE) of cuticle in 500 μl of 1% solutions of the detergents in phosphate buffer (75 mM, pH 7.2) or phosphate buffer alone. After sonication the cuticle was rinsed 10 times with phosphate buffer, dried on filter paper, and tested for activity by incubation with 4 μl of 212 ng/μl of Z11–14:OH in hexane. Similar studies were conducted using 0.2 M Na_2SO_4 (pH 7.0), 7 M urea in phosphate buffer, and 1% sodium dodecyl sulfate (SDS). In all of the above experiments, results were compared with those obtained when untreated cuticle was incubated with the alcohol–hexane solution.

The pH dependence of the cuticular oxidase was studied by sonicating weighed amounts of cuticle in 500 μl of buffers having different pH values. Buffers of different pH were made by adding appropriate amounts of either acetic acid or sodium hydroxide to the 75 mM phosphate buffer having a pH of 7.2. The experimental protocol was the same as that used in studies on the effects of various detergents.

Reagents. All alcohol substrates were obtained from the Sigma Chemical Company (St. Louis, Missouri) and were purified by high-performance liquid chromatography (HPLC) using a 25 × 0.46 cm (ID) column packed with 5 μm silica (Altech) eluted with 20% ether in hexane. GC analysis of the purified alcohols indicated that they were 99% pure and were free of the corresponding aldehydes. Aldehydes used as analytical standards were prepared from the alcohol analogs by oxidation with pyridinium chlorochromate (Corey and Suggs, 1975) and were purified by HPLC as described above. All solvents used were Fisher (Pittsburgh, Pennsylvania) HPLC grade.

Chemical Analysis. Samples were analyzed on fused silica capillary GC columns (30 m × 0.25 mm ID SPB1 and 30 m × 0.25 mm ID Supelcowax 10, Supelco, Bellfonte, Pennsylvania) in Varian 3700 (Sunnyvale, California) gas chromatographs equipped with split/splitless capillary injectors and flame ionization detectors. Chromatographic conditions were: initial column temperature = 60°C for 2 min, then temperature programmed at 30°/min, final temperature = 175°C (Supelcowax 10), 195°C (SPB1), injector changed from splitless to split mode at 30 sec after injection. Helium was used as the carrier gas in both instruments at a linear flow velocity of 18 cm/sec. Data were acquired and analyzed with a SP4100 computing integrator (Spectra Physics, Santa Clara, California) interfaced to one GC (SPB1 column) and a Perkin-Elmer chromatographic data system (Norwalk, Connecticut) interfaced to the GC containing the Supelcowax 10 column. Retention times of peaks eluting during the analysis of experimental samples were compared with those of authentic standards and amounts were calculated based on the concentration of the internal standard used.

Chemical ionization mass spectra (MS) were obtained for samples resulting from incubations of cell-free cuticle to ensure that the compound assign-

ments were correct. This was accomplished using a Nermag R10-10 MS (Delsi Instruments, Fairfield, New Jersey) interfaced to a Hewlett-Packard 5792 GC (Palo Alto, California) equipped with a split/splitless capillary injector. Helium was used as the carrier gas, while methane was used as the reagent gas. Samples were chromatographed on a 50 m × 0.25 mm (ID) OV-1 fused silica capillary column. The oven temperature was maintained at 50°C for 2 min then increased at 10°C/min to 240°C. Injector purge occurred 1 min after sample injection. Retention times and spectra of compounds eluting during analysis of experimental samples were compared to those of authentic standards.

RESULTS AND DISCUSSION

In Vivo Assays. Analysis of extracts of glands of females of *H. zea* treated with 0.5 μg of Z11–14:OH in H_2O for 30 min (Figure 1C) indicated that the alcohol was converted to the corresponding aldehyde. An average of 16.7 ±

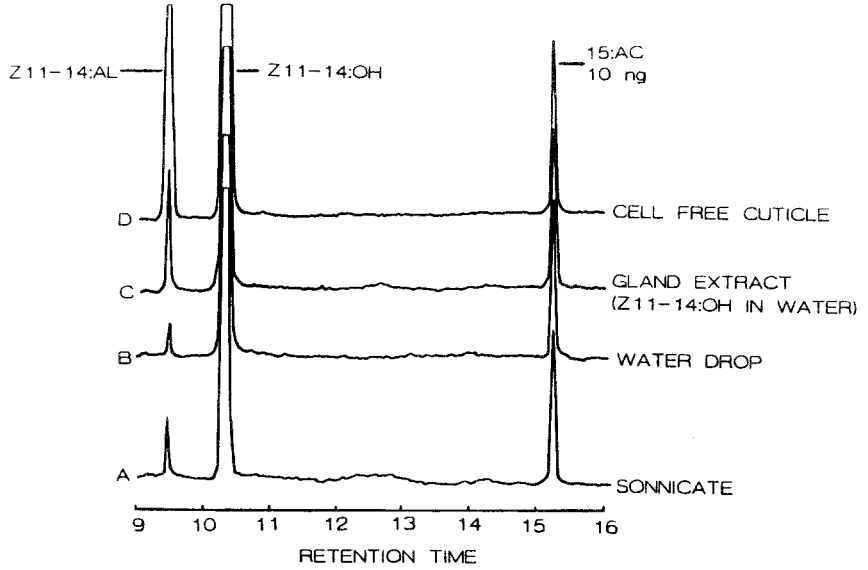

FIG. 1. Chromatograms obtained using the 30 m × 0.25 mm (ID) SPB1 (methyl silicone) fused silica capillary column. (A) Analysis of the extract after a 1.5-hr incubation with alcohol–buffer of the eluate obtained by sonicating the gland of females of *H. zea*. (B) Analysis of the extract of a drop of alcohol–H_2O removed from the surface of the cuticle of an *H. zea* female after a 10-min incubation. (C) Analysis of the extract of the pheromone gland of an *H. zea* female after a 30-min incubation with a 1-μl drop of alcohol–H_2O. (D) Analysis of the extract obtained after a 30-min incubation of the purified cuticle of the pheromone gland of an *H. zea* female in a 0.5 μg/μl solution of Z11–14:OH in hexane.

7.2 ng of (Z)-11-tetradecenal (Z11–14:Al) was recovered ($N = 5$). Analysis of extracts of water–alcohol droplets removed from the surface of the glands of *H. zea* ($N = 5$) after a 10-min incubation indicated that 2.5 ± 0.6 ng of Z11–14:Al were obtained (Figure 1B). Analysis of hexane extracts of alcohol–water drops incubated in a conical microvial without being in contact with the pheromone gland indicated that no detectable autooxidation of the alcohol to aldehyde occurred during the 30-min incubation. Similarly, no Z11–14:OH or Z11–14:Al was detected in extracts obtained from glands incubated with only a 1.0 μl drop of H_2O for 30 min.

The eluate obtained by sonicating the gland in buffer contained only limited oxidase activity. Nonetheless, the preparations obtained from *H. zea* ($N = 5$) (Figure 1A) produced 2.8 ± 1.2 ng of Z11–14:Al. Blank incubations of Z11–14:OH in phosphate buffer did not contain detectable amounts of the corresponding aldehyde.

The results of studies performed to determine the stage of development at which the oxidase became functional are shown in Figure 2. Pupal insects (*N*

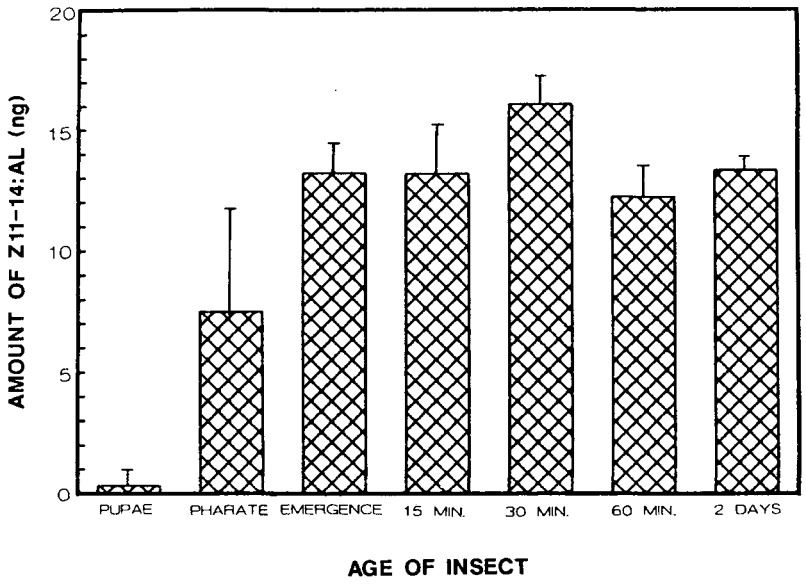

FIG. 2. Effect of insect age on oxidase activity as indicated by production of Z11–14:Al from Z11–14:OH. Prepharate insects are pupae having unsclerotized adult cuticle; pharate insects are pupae having sclerotized adult cuticle; times indicated are after adult emergence. Error bars represent standard errors of the mean. The number of insects analyzed is given in the text.

= 10), which had unsclerotized adult cuticle (prepharate), did not convert substantial amounts of alcohol to aldehyde. Pharate pupae (sclerotized adult cuticle) produced an average of 7.5 ng of aldehyde. This was intermediate between levels produced by mature adults and prepharate insects. Interestingly, nine of the 15 pharate insects analyzed produced 2–4 ng, while the other six produced amounts equivalent to adult insects. Newly emerged and older insects produced approximately equal amounts of aldehyde (N = 10, each age). These results indicate that the oxidase becomes functional during maturation of the adult cuticle just prior to emergence of the adult.

Glands converted significantly less alcohol to aldehyde after they had been pretreated by soaking in acetone, acetonitrile, THF, or ethyl ether than when they were soaked in H_2O prior to incubation in the alcohol-buffer emulsion (Figure 3). Neither hexane nor dichloromethane had any effect on oxidase activity. While we cannot explain the different effects of the solvents on enzyme activity, it is noteworthy that organic solvents of intermediate to high polarity

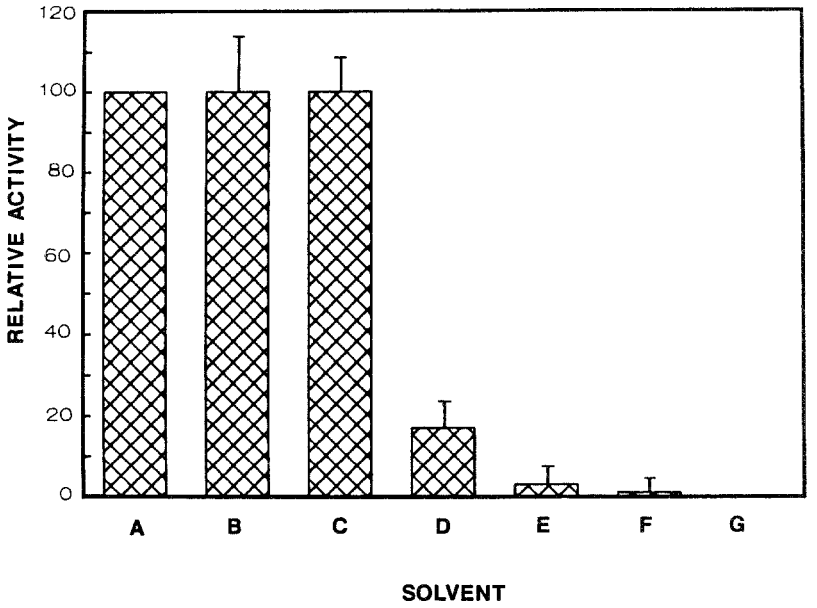

FIG. 3. Effect of soaking cuticle in organic solvents for 5 min relative to soaking in water prior to incubation in a 212 ng/μl emulsion of Z11–14:OH in PO_4 buffer: A = H_2O, B = hexane, C = dichloromethane, D = acetone, E = ether, F = THF, G = acetonitrile. Error bars represent the standard errors of the means of five replications for each solvent.

cause the activity to be lost. Therefore, the observed effects of the solvents may result from dipole interactions between the solvent and enzyme–cuticle matrix. Thus, solvents like THF and ether, which have functional groups having strong dipole moments, may interact with the enzyme, which results in a loss of activity.

We also found that the use of hexane as a carrier for the alcohol substrate resulted in a four- to fivefold increase in the amount of aldehyde recovered over that found when either phosphate buffer or water emulsions of the alcohol were used. These findings can be rationalized by the fact that hexane effectively carries the alcohol into the cuticular matrix, making it readily available for oxidation. Water does not penetrate the cuticle and therefore the only alcohol molecules available for oxidation are those that diffuse out of the emulsion and into the cuticle. The above results suggest that this oxidase may be useful for organic synthesis of aldehydes in organic solvents. Reports of the use of enzymes in organic synthesis using organic solvents are becoming more common and, in fact, a recent report by Kazandjian and Klinbanov (1985) has indicated that an oxidase that oxidizes polyphenols to quinone is much more efficient when in chloroform than in water.

Results of studies on the rate of aldehyde production and effect of substrate concentration on the enzyme activity present in individual glands are shown in Figures 4A and 4B. The amount of aldehyde produced increased in a linear fashion over time ([Z11–14:Al] = 0.311 + 3.262 time, $R^2 = 0.99$) (Figure 4A). A hyperbolic plot of substrate concentration versus aldehyde produced was obtained when glands were incubated for 30 min (Figure 4B).

No intact cells were found when pieces of cuticle obtained by homogenization and sonication of the glands in H_2O were observed under a microscope at 400×. This was expected because we anticipated that osmotic shock would cause cellular disintegration. The initial purification step of sonicating the glands resulted in a slight decrease in oxidase activity when glands were tested. However, after the final step, the cuticular fragments had somewhat more activity (Figure 1D) than intact, untreated glands (Figure 5). The water used to sonicate the glands in the initial step contained some activity and, as indicated in Figure 5, successive purification steps resulted in further solubilization of the oxidase. However, the activity present in solution was always significantly lower than that present in the cuticle fraction of that step. The above results demonstrate clearly that the majority of oxidase activity is associated with the cuticle overlying the cells of the pheromone gland, thus confirming the hypotheses of Morse and Meighen (1986) and Teal and Tumlinson (1986). However, we cannot exclude the possibility that some oxidase activity is associated with the cells. Nonetheless, cellular oxidase activity, if present, is considerably less than that associated with the cuticle.

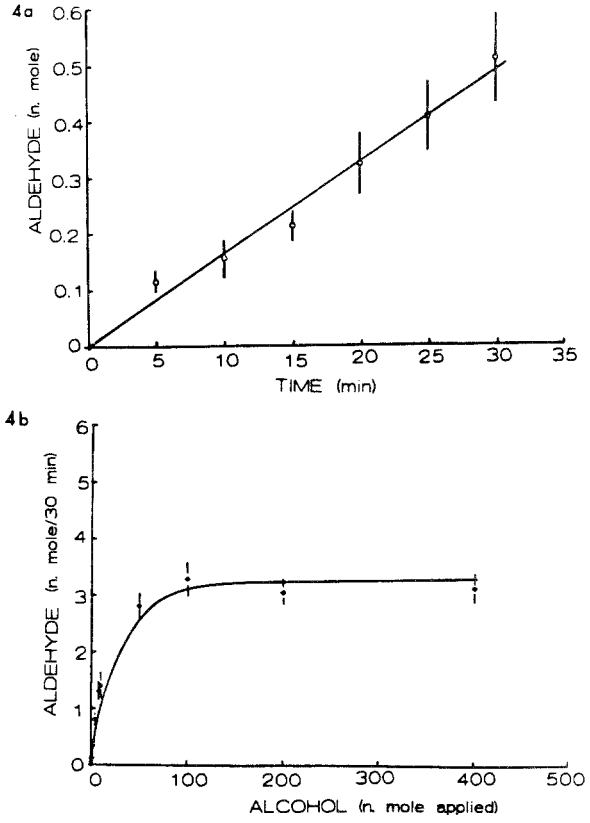

FIG. 4. Effect of incubation time on aldehyde production (a) and effect of substrate (Z11–14:OH) concentration on aldehyde production (b) in individual pheromone glands ($N = 6$ each experimental data point, bars are standard errors of means). (A) Glands were incubated in 40 μl of hexane containing 16.8 μg of Z11–14:OH. Samples (2 μl) were analyzed at 5-min intervals. (B) Various amounts of Z11–14:OH contained in 2 μl of hexane were incubated with individual glands for 30 min.

The enzyme present in cell-free cuticle was functional over a broad pH range, having an optimum at 6.4 and with only 30% reduction in activity at pH 9 (Figure 6). Similarly, no loss in activity occurred when the cuticle was treated with Na_2SO_4 or any of the nonionic detergents tested (Figure 7). No appreciable activity was found in the soluble fractions of these tests (data not shown). However, all activity was lost when the cuticle was treated with either 7 M urea or 1% SDS (Figure 7).

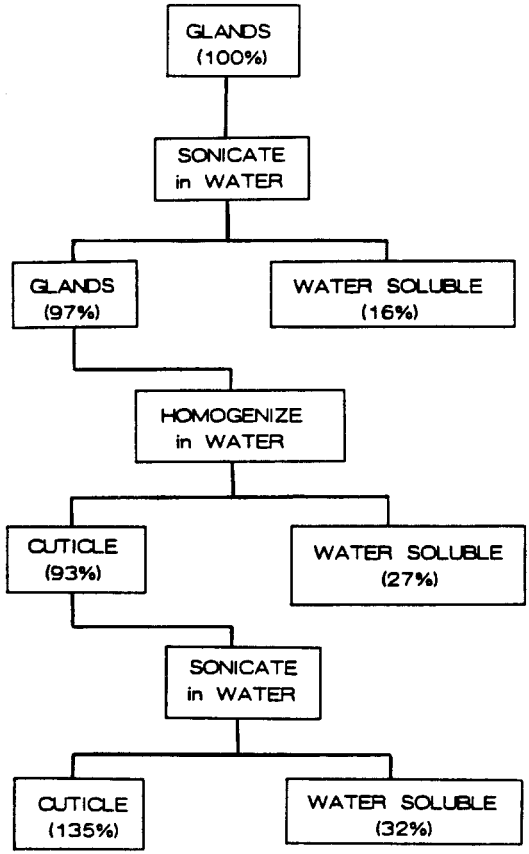

FIG. 5. Distribution of oxidase activity during steps of cuticle purification. Numbers in parentheses indicate the amount of activity in each step relative to intact glands ($N = 3$ replications of 100 glands). At each step an amount of either cuticle or extract equivalent to that present in one female was incubated (two replications each set of incubations) as indicated in the text.

Ultrastructural studies on *Choristoneura fumiferana* (Clem.) (Percy, 1974), which also appears to employ a cuticular oxidase for pheromone biosynthesis (Morse and Meighen, 1986), have indicated that epicuticular filaments extend through the pore canals of the endocuticle and terminate close to the apical microvillae of the pheromone gland cells. These filaments have been hypothe-

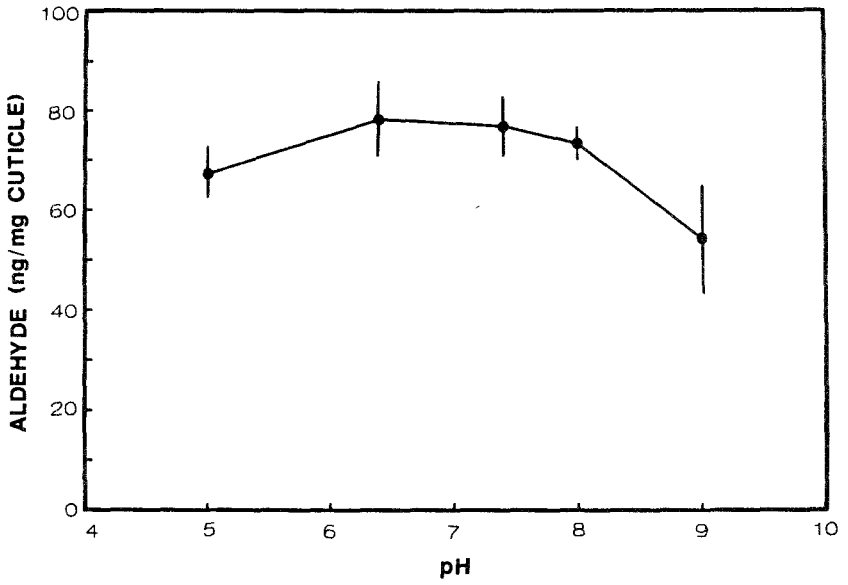

FIG. 6. Effect of sonicating purified cuticle in buffers of different pH for 15 min on oxidase activity as indicated by nanograms of Z11-14:Al produced per milligram of cuticle incubated for 30 min in 2 μl of hexane containing 840 ng of Z11-14:OH. Bars indicate standard errors of the mean for five replications at each pH.

sized as being the site of enzymes involved in pheromone biosynthesis by *C. fumiferana* (Percy, 1974). A similar situation could explain the distribution of the oxidase in *H. zea*. In *C. fumiferana*, groups of filaments coalesce to form pores in the epicuticle but are greatly dispersed and much more numerous in the endocuticular layer (Percy, 1974). For *Heliothis* species, this would explain why only limited activity was obtained in surface washes because only the enzyme at the surface of the epicuticular pores would be removed using our techniques. This would explain also why small amounts of activity could be solubilized from the cuticle during purification because as the cuticle was fragmented, more of the enzyme would be freed from the cuticular matrix. Consequently, we believe that the enzyme is limited to the cuticle. We are presently pursuing studies aimed at elucidation of the site of enzyme activity within the cuticle and studies aimed at isolating the enzyme in an active form from the cuticle.

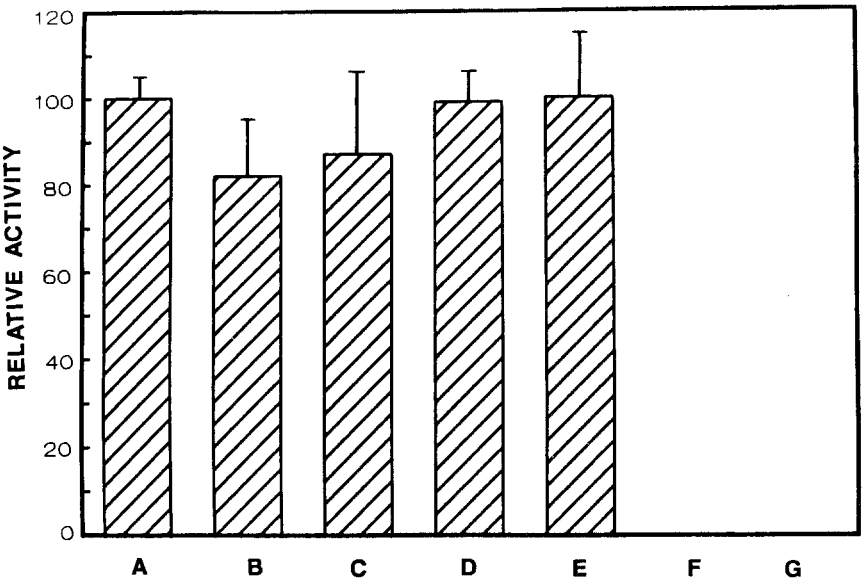

FIG. 7. Effect on oxidase activity of sonicating purified cuticle in: A = phosphate buffer, B = NP-40, C = Tween 80, D = Triton X-100, E = Na_2SO_4, F = 7 M urea, G = 1% SDS for 15 min prior to incubation in 4 μl of hexane containing 848 ng of Z11-14:OH. Values shown indicate the activity relative to sonication in phosphate buffer with error bars indicating the standard errors of the mean for five replications of each treatment.

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HOST PLANT UTILIZATION AND IRIDOID GLYCOSIDE SEQUESTRATION BY *Euphydryas anicia* (LEPIDOPTERA: NYMPHALIDAE)

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Abstract—The iridoid glycoside content of individual adult *Euphydryas anicia* butterflies from two Colorado populations was quantitatively determined. At one site (Red Hill), larval host plants were *Castilleja integra* and *Besseya plantaginea*, while at the other site (Cumberland Pass) a single host plant, *B. alpina*, was used. At Red Hill, macfadienoside and catalpol were sequestered, while at Cumberland Pass, catalpol and aucubin were sequestered. Artificial diet studies showed that larvae hydrolyzed a major iridoid of *B. plantaginea*, 6-isovanillylcatalpol, to catalpol (which was sequestered) and isovanillic acid (which was excreted). Large year-to-year and individual variation in butterfly iridoid content was established as was a female–male difference in macfadienoside vs. catalpol content. Larval host plant distributions and numbers were determined at Red Hill for two years and compared with changes in butterfly populations and sequestered iridoids.

Key Words—*Euphydryas anicia*, Lepidoptera, Nymphalidae, *Castilleja integra*, *Besseya plantaginea*, *Besseya alpina*, Scrophulariaceae, iridoid glycosides, sequestration, metabolism, plant–insect interactions, herbivory.

INTRODUCTION

Stermitz et al. (1986) reported the sequestration of iridoid glycosides by several populations of *Euphydryas anicia* (Lepidoptera: Nymphalidae). Adult *E. anicia* from a high-plains Colorado site (Red Hill) contained the iridoids catalpol, **1**, and macfadienoside, **2** (Figure 1) from larval consumption of *Castilleja integra* Gray and/or *Besseya plantaginea* (James) Rydb. (Scrophulariaceae). Macfadienoside occurred only in *C. integra*, and hence the presence of that iridoid in

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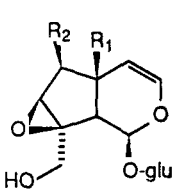
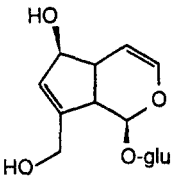
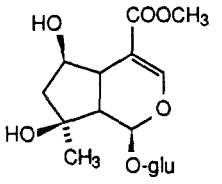




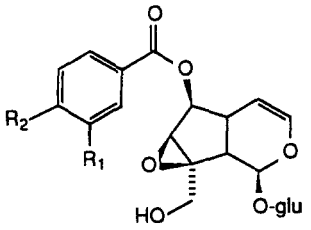


1 R_1 = H; R_2 = OH (catalpol)

2 R_1 = R_2 = OH (macfadienoside)

6 aucubin

7 shanzhiside methyl ester



3 R_1 = R_2 = H (veronicoside)

4 R_1 = OH; R_2 = OCH_3 (6-isovanillylcatalpol)

5 R_1 = R_2 = OH (verproside)

FIG. 1. Host plant iridoid glycosides.

adult butterflies was a marker for larval consumption of *C. integra*. Catalpol was not the major component of either host plant, but *B. plantaginea* contained large amounts of veronicoside, **3**, 6-isovanillylcatalpol, **4**, and verproside, **5** (Gardner et al., 1987). Only a few individual butterflies were analyzed, but there appeared to be both individual and sex differences in host-plant utilization and/or sequestration processes. The same butterfly at several alpine sites, where the host plant is *B. alpina* (Gray) Rydb., sequestered catalpol and aucubin, **6.** These were the two major alkaloids of this host plant, although it also contained **3–5.**

This preliminary investigation raised several questions about differential host plant consumption by larvae and sequestration or metabolism of host plant chemicals that we have attempted to answer in the present work. Evidence from *Euphydryas* and other butterfly studies suggests that at sites where multiple host plant use occurs, individual butterflies and larvae may select and utilize a narrower range of host-plant species than does the overall butterfly population (Fox

and Morrow, 1981; Stanton and Cook, 1984; Bowers, 1986; Singer, 1983). If macfadienoside sequestration is a marker for *Castilleja* consumption at Red Hill, could we establish the presence of a subset of larvae in the population which feeds only on that host plant or a subset which feeds only on *Besseya*? If so, one might encounter a group of macfadienoside-containing adults and a group that lack that iridoid. The preliminary study indicated that males and females contained differing relative amounts of catalpol and macfadienoside. We were interested in learning if this sex difference was a result of differential feeding between the two host plants or of differing sequestration or metabolism processes. We also hoped to quantitatively establish if there was intrapopulation variation in the amounts of sequestered defensive substances. Such variation has rarely been estimated for larval-obtained compounds, particularly in localized insect populations. Some data are available for the sequestration of milkweed cardenolides by the migratory monarch butterfly (Brower et al., 1984, and references therein; Cohen, 1985; Lynch and Martin, 1987) and for the lygaeid *Oncopeltus fasciatus* (Scudder et al., 1986, and references therein). Each of these studies encountered large individual variation in defensive substances present in the adult insect.

In order to study interpopulation sequestration differences, we also focused more attention on one of the preliminarily studied alpine sites, Cumberland Pass. Here only a single host plant, *B. alpina*, is used.

Finally, the studies at both sites were to provide chemical data as part of our long-term, broad studies on *E. anicia* biology and ecology (Odendaal et al., 1988a–b).

METHODS AND MATERIALS

Organisms and Sites. The major part of the study was conducted on the checkerspot butterfly *Euphydryas anicia* Doubleday and Hewitson at a high-plains (2900 m), essentially flat site (Red Hill) 11 miles east of Fairplay, Colorado (Park County) on the northwest side of U.S. Highway 285. Here, *E. anicia* occurs in a relatively localized demographic unit concentrated in a 700 × 1000-m area. The adult flight season is early or mid-June to mid-July. Eggs are seen on both host plants from late June to mid-July, and prediapause larvae are present from late July through August. Postdiapause larvae consume host plants from early May through early June.

Population density and residence times for adult males and females were estimated through mark–release–recapture studies and transect counting (Pollard, 1977), details of which will be reported elsewhere. Adult *E anicia* (57 females and 39 males in 1985 and 10 of each sex in 1986) were captured at varying times throughout the flight season, placed in glassine envelopes, refrig-

erated, and finally frozen. An additional three males and three females were collected, treated similarly, and then dissected for body part analyses. Abdomen, wings, and head–thorax–legs parts were combined for each of the sexes. In 1985, four additional adult males were collected just off the south side of the site near a ravine where only *B. plantaginea* was present. For larval analyses, two last-instar larvae were collected for analysis from *B. plantaginea* in the ravine, three from *B. plantaginea* on the site proper, and four from *C. integra*, also on the site.

Nine early postdiapause larvae were collected in 1986 from *C. integra* and raised in the laboratory on an artificial diet (Bowers, 1983) containing 6-isovanillylcatalpol, **4,** at a concentration approximating that present in the host plant, 3.5 mg/g dry weight diet (Gardner et al., 1987). Frass was collected for analysis beginning several days after initiation of the diet feeding, when green color no longer showed in the frass. Pupae, which were formed one to two weeks after feeding commenced, were removed to 6-oz vials containing filter paper on the bottom and sides. Adults were removed 24 hr after eclosion, having emitted meconium onto the filter paper prior to their removal.

The *C. integra* and *B. plantaginea* host plants at the site were counted along 1000-m transects by establishing two 5 × 5-m quadrats for each 50-m linear portion of the transect and counting within the quadrats. Essentially complete counts for the site (seven longitudinal transects) were made in 1985. In 1986, three transects typical of the site were counted, and these were compared with the same three transects in the 1985 counts in order to estimate year-to-year variation. Both host plants are perennials, whose first leaves usually emerge in early May. Host plant counts were made in late June. At this time the broad-leaved *B. plantaginea* and the red-bracted *C. integra* plants are more readily observable.

For the interpopulation comparison, collections of *E anicia* adults (10 males in 1985 and 10 males and 10 females in 1986) were made at Cumberland Pass, an alpine site described by the Ehrlich group (Cullenward et al., 1979), where the host plant is *Besseya alpina* (Gray) Rydb. (White, 1979).

Chemical Analyses. GLC analyses of iridoid glycosides were performed on the trimethylsilylated derivatives using a Hewlett Packard 5890 gas chromatograph equipped with a split/splittless injector, FID detector, and a Hewlett Packard 3392A integrator. The column was a DB-1 capillary (J&W Scientific), 30 m (length), 0.32 mm (ID) with a film thickness of 0.10 μm. The injector was used in the split mode, split ratio of 30:1, and at a temperature of 275°C. Carrier gas was high-grade helium (99.996%) with a column head pressure of 12 psi and a linear velocity of 30 cm/sec (1.44 ml/min). The detector temperature was 320°C, with a hydrogen flow of 38 ml/min, air flow of 200 ml/min, and a helium (make-up gas) flow of 40 ml/min. Samples were run under one of two programs: Program A: Initial column temperature 200°C for 1 min,

which was then raised to 260°C at a rate of 20°/min and held for 8 min. Program B (needed to analyze for the catalpol esters): Initial column temperature 200°C for 1 min, which was then raised to 260°C at a rate of 20°/min and held for 6 min; column temperature was then ramped to 320°C at a rate of 20°/min and held for a final time of 10 min. (Figure 2).

Individual butterflies were removed from the freezer and dried at room temperature or 55°C (Cumberland Pass collections, 1986) prior to analysis. They were weighed, placed in screw-cap test tubes, crushed and 3–5 ml of MeOH added. The samples were extracted for about 4 hr, filtered through cotton, and the filtrate evaporated to dryness. The dry residue was triturated with 2 ml H_2O and 3 ml diethyl ether and both triturates combined in screw cap test tubes. To each tube was added 1.00 ml of internal standard, phenyl-β-D-glucose (0.500 mg/ml). Samples were mixed by shaking and placed in a centrifuge to aid in separating the layers. The ether layer was removed, and the aqueous layer washed two more times with 3 ml of ether. After the final ether wash, the aqueous layer was evaporated to dryness, and the dry residue removed with two washings of 0.5 ml MeOH. The MeOH rinses were combined together in a 0.5-dram screw cap vial. A 10% aliquot was placed into a small glass sample tube (closed end 0.5 mm) and evaporated to dryness. To this sample tube was added 0.10 ml of the silylation reagent (TRI-SIL'Z' from Pierce Chemical Company)

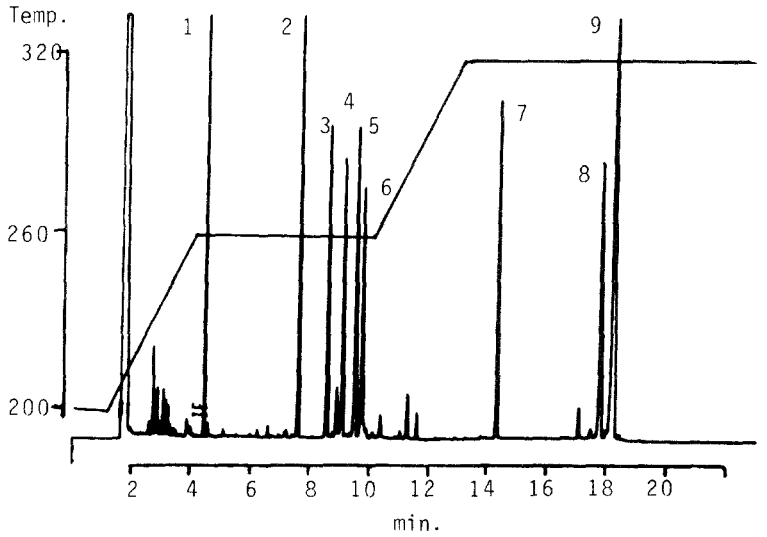


FIG. 2. GLC analysis of host plant iridoid glycoside TMS derivatives. (1, phenylglucose internal standard; 2, aucubin; 3, catalpol; 4, macfadienoside; 5 shanzhiside methyl ester: 6, adoxoside; 7, veronicoside; 8, isovanillylcatalpol; 9, verproside.

and the closed tube was heated at 70–80°C for 15 min. After the solution had cooled, 1 μl was injected onto the gas chromatograph. The remaining MeOH solution was also evaporated to dryness and stored under refrigeration for further analyses, such as TLC or [^{1}H]NMR. A similar analysis scheme was used for postdiapause larvae.

Statistical analyses of butterfly iridoid content were by analysis of variance (ANOVA) using either CLR ANOVA (Clear Lake Research, Inc.) or StatWorks (Cricket Software, Inc.) programs on a Macintosh (Apple) microcomputer. In the ANOVA analyses, all percentage values were transformed with an arcsine function and microgram by the square root of X.

Special techniques were used for chemical analyses in the artificial diet feeding studies. Adult butterflies, collected frass, pupal cases, and shredded filter paper containing the emitted meconium were extracted with methanol for 24 hr. The solutions were evaporated to dryness after filtration. To the samples was added 1.00 ml of solution containing 0.05 mg/ml of 3,4-dihydroxybenzoic acid as an internal standard. A 10% aliquot was removed for GLC analysis of iridoids as described above, and a portion was analyzed for isovanillic acid (3-hydroxy-4-methoxybenzoic acid) by HPLC. The analysis for isovanillic acid was by procedures similar to those of Anderson and Pedersen (1983) and Casteele et al. (1983). HPLC was performed on a Beckman system equipped with a 110B pump, a variable UV detector, Beckman injector (20-μl sample loop) and an Altex Ultra Sphere ODS reverse-phase Si gel column (15 × 250 mm). Elution was with 30% methanol in 0.05 formic acid at a flow rate of 1.2 ml/min and UV detection at 254 nm. Calibration and determination of the response factor for isovanillic acid was accomplished over three injections of a standard solution containing 0.05 mg/ml of 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, and isovanillic acid.

RESULTS

Butterfly and Host Plant Population Estimates (Red Hill). Transect counts yielded an estimate of 1800 butterflies present at the site on June 27, 1985, and 24,000 present on June 25, 1986. These dates were approximately at the middle of the four- to five-week flight season and were near the time of maximum population size. Other counts during both seasons were in agreement with these estimates. Qualitative observations by three researchers present on the site for most of both flight seasons substantiated the marked increase in butterfly numbers from 1985 to 1986. Details of butterfly counts and the results of an extensive mark–release–recapture study will be presented elsewhere.

Host-plant estimates and comparisons for the two years along three site-typical transects showed that in 1985 both host plants were present in nearly all quadrats, but with a clear preponderance of *C. integra* throughout (Table 1). In

TABLE 1. COMPARATIVE HOST PLANT DENSITIES OVER TRANSECTS FOR 1985 AND 1986 AT RED HILL[a]

Plot No.	Transect B				Transect D				Transect F			
	Castilleja		Besseya		Castilleja		Besseya		Castilleja		Besseya	
	1985	1986	1985	1986	1985	1986	1985	1986	1985	1986	1985	1986
0	5	0	16	0	3	0	12	0	7	2	7	2
1	34	0	3	0	17	0	7	0	31	0	14	0
2	26	0	5	0	9	0	5	0	72	0	8	0
3	52	0	20	0	5	0	6	0	61	0	4	1
4	4	0	3	0	6	0	5	0	33	2	8	1
5	5	0	10	0	3	0	2	0	34	0	5	0
6	9	0	0	0	23	0	1	0	38	0	7	0
7	34	0	2	0	8	0	2	0	7	0	4	0
8	22	0	9	0	28	0	5	0	16	1	13	0
9	9	0	3	0	23	0	2	0	30	0	10	9
10	24	0	1	0	33	0	1	0	41	0	6	0
11	80	0	1	1	73	59	3	0	1	9	2	0
12	0	8	0	0	63	0	1	0	0	0	2	2
13	44	19	0	2	20	27	0	0	0	0	4	0
14	32	0	3	1	23	51	1	0	47	0	5	0
15	70	33	2	1	12	9	0	0	76	0	21	1
16	101	69	2	4	34	38	1	1	37	4	5	0
17	160	187	0	0	74	22	2	0	78	63	7	5
18	135	37	1	1	45	1	3	0	155	47	5	3
19	121	166	1	0	27	0	0	0	72	14	5	4
20	52	34	0	0	60	1	2	0	8	0	2	0
Totals	1019	553	82	10	589	208	61	1	844	142	144	28

[a] Counts in two (of ten total) quadrats for every 50 m (1000 m total transect length).

1986, there was a substantial reduction in the abundance of both host plant species, although *C. integra* was still the most abundant. In 1985, transects B, D, and F showed 92%, 90%, and 89% *C. integra* (percentage of total host plants), while the 1986 results were 98%, 99.5%, and 83% *C. integra*. Qualitative observations made while postdiapause larvae were being collected from the plot 10-13 area on May 20, 1986, confirmed the low host plant density in this area and many larvae were seen wandering in search of host plants. Such was not the case at the same time in 1985, when all larvae were found on host plants.

Iridoid Analyses (Red Hill). A condensed summary of the 1985 results on catalpol and macfadienoside presence in adult butterflies is given in Table 2, with some statistical comparisons in Table 3. Data for individual butterfly analyses are also available (Gardner, 1987). Traces of shanzhiside methyl ester, **7**,

TABLE 2. IRIDOID GLYCOSIDE ANALYSIS SUMMARY (*E. anicia:* RED HILL)[a]

	1985						1986					
	N	Mean	MED	SD	MIN	MAX	*N*	Mean	MED	SD	MIN	MAX
Males	39						10					
Dry weight (mg)		22.4	20.2	5.3	16.2	38.0		26.6	26.1	4.9	20.3	38.0
% CAT		3.1	3.1	1.6	0.4	7.6		0.8	0.5	0.6	0.2	1.8
% MAC		1.0	0.8	0.8	0.0	3.7		0.4	0.4	0.2	0.1	0.7
% Total		4.1	4.2	1.7	0.8	9.1		1.2	1.1	0.7	0.3	2.2
CAT (μg)		630	650	280	150	1340		210	140	150	70	470
MAX (μg)		230	170	200	0.0	1100		90	95	50	10	180
Total (μg)		850	850	310	220	1610		300	245	160	80	570
MAC/CAT		0.49	0.40	0.57	0.0	3.33		0.63	0.43	0.51	0.14	1.5
Females	57						10					
Dry weight (mg)		37.9	36.4	9.4	21.8	59.5		31.1	31.2	9.3	16.9	48.7
% CAT		1.6	1.5	0.8	0.2	3.9		0.4	0.4	0.2	0.0	0.9
%MAC		1.5	1.4	0.9	0.0	3.9		0.6	0.7	0.3	0.3	1.1
% Total		3.1	3.2	0.9	0.9	5.8		1.0	1.0	0.4	0.3	1.8
CAT (μg)		620	540	360	70	1560		130	100	100	00	310
MAC (μg)		540	480	310	00	1380		200	150	140	80	520
Total (μg)		1160	1230	390	360	2280		330	250	220	90	780
MAC/CAT		1.39	1.22	1.35	0.0	8.43	9[b]	1.96	1.71	1.41	0.78	5.33

[a]KEY *N* = number samples, MED = median, SD = standard deviation, MIN = minimum, MAX = maximum, CAT = catalpol, MAC = macfadienoside.

[b]One value was eliminated because of an undectable amount of catalpol.

TABLE 3. STATISTICAL SUMMARY FOR TABLE 1[a]

		Dependent variable, *Y*						
		Dry weight (%)[b]			Dry weight (μg)[c]			
	DW	CAT	MAC	TOT	CAT	MAC	TOT	MAC/CAT
Effect								
Sex on *Y* (P)	<0.001	<0.001	<0.025	NS	NS	<0.001	<0.05	<0.001
(F)	26.306	13.483	5.574			15.892	4.248	16.444
Year on *Y* (P)	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS
(F)		63.431	12.936	97.115	56.486	14.238	91.622	
Sex in 1985 on *Y* (P)	<0.001	<0.001	<0.01	<0.01	NS	<0.001	<0.001	<0.001
(F)	89.435	32.492	10.703	9.833		39.034	18.207	15.278
Sex in 1986 on *Y* (P)	NS	NS	NS	NS	NS	NS	NS	<0.01
(F)								7.059
Year on males *Y* (P)	NS	<0.001	<0.05	<0.001	<0.001	NS	<0.001	NS
(F)		40.040	4.956	57.919	21.448		30.856	
Year on females *Y* (P)	<0.025	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	NS
(F)	6.459	24.143	8.246	39.770	36.267	13.203	65.347	

[a] DW = dry weight; CAT = catalpol; MAC = macfadineoside; TOT = total; NS = not significant.
[b] Transformed with an arcsine function.
[c] Transformed by square root of *X*.

were detectable in some adults but amounts were too small to quantify. Esters **3–5** and other iridoids present in the host plants (adoxoside, adoxosidic acid, 6-hydroxyadoxoside, shanzhiside, 8-epiloganic acid, and mussaenoside) were not found in any butterflies. To emphasize individual butterfly and sex variation, we graphically display 1985 data on dry weight (Figure 3), percent total iridoids (Figure 4), percent catalpol (Figure 5), and percent macafadienoside (Figure 6).

The 1985 male percent dry weight average iridoid content was 4.1% compared to 3.1% for females ($P < 0.01$). Because of the larger female size, total iridoid content was, however, greater for females (1.16 mg per butterfly) than for males (0.85 mg) ($P < 0.001$). There was a marked drop in total iridoid content for the 1986 collections (1.2% for males and 1.0% for females) as compared to 1985, and the male–female difference was no longer significant.

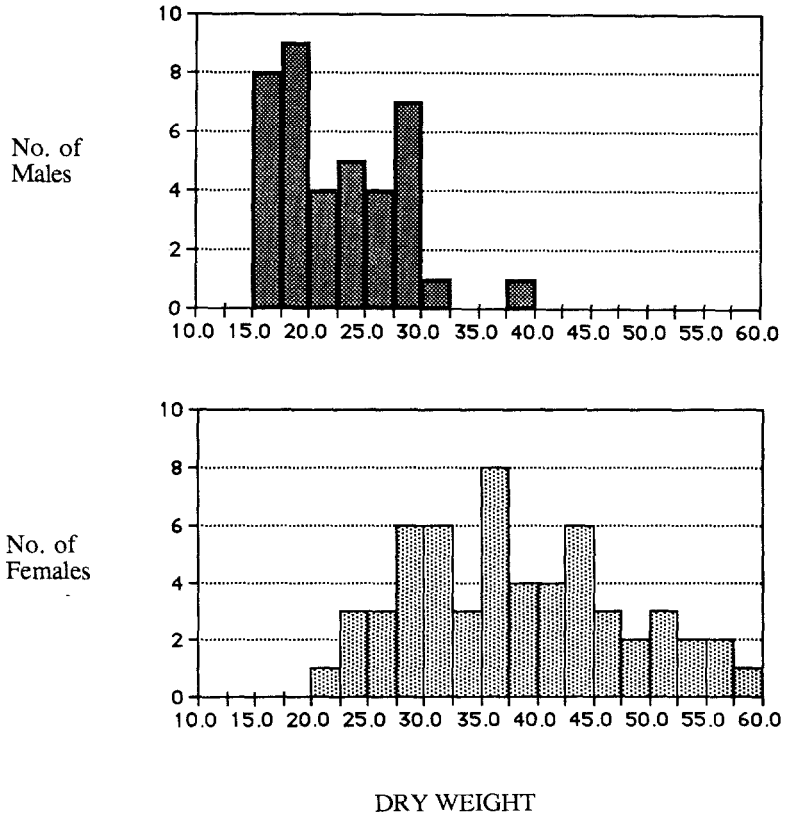


FIG. 3. Dry weight variation among male and female *E. anicia* (Red Hill, 1985).

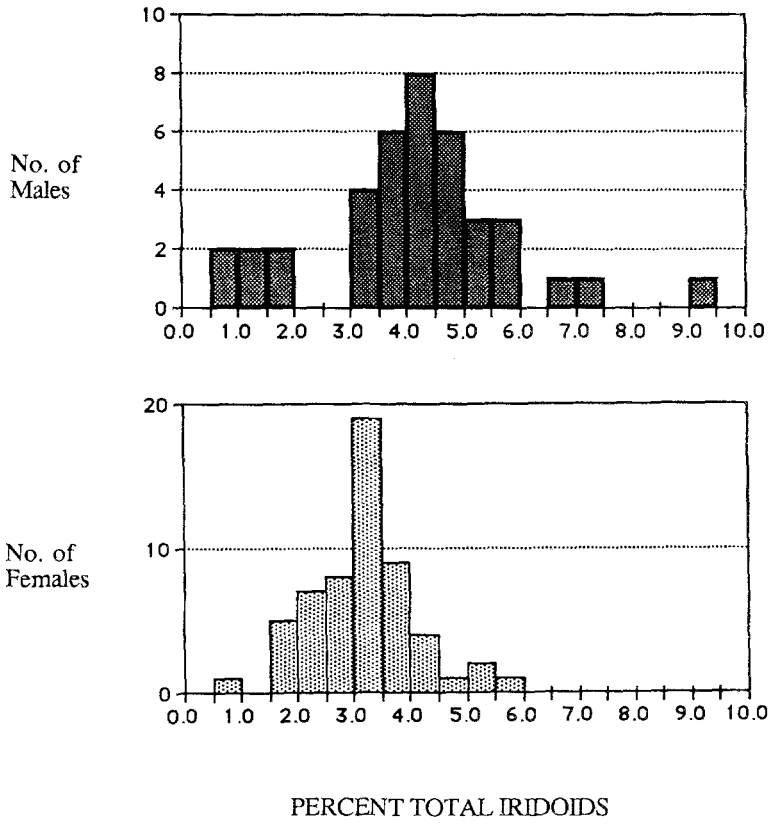


FIG. 4. Variation in total iridoid content among male and female *E. anicia* (Red Hill, 1985).

In 1985, macfadienoside was found in 55 of 57 females and 33 of 39 males. Catalpol was found in all. The four males collected near the ravine off-site (see Methods and Materials), where only *B. plantaginea* is present, contained only catalpol and are not included in the Table 2 data. In 1986, macfadienoside was found in 20 of 20 butterflies and catalpol in 19 of 20. Differences were seen in macfadienoside vs. catalpol content when comparing males and females. Macfadienoside content of males in 1985 was significantly lower than of females, both on a total ($\bar{X}$ = 200 μg for males compared to $\bar{X}$ = 540 μg for females) and percentage dry weight basis (1.0% for males compared to 1.5% for females). The macfadienoside-to-catalpol ratio (mg per butterfly) was significantly different in both 1985 and 1986 for females (1.39 and 1.96) as compared to males (0.49 and 0.63) (Figure 7).

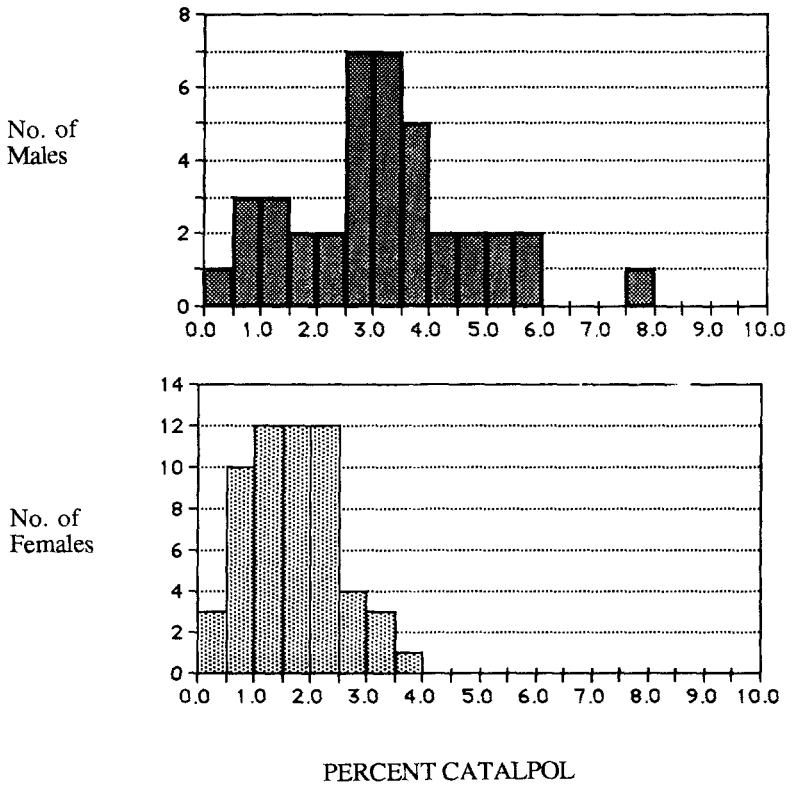


FIG. 5. Variation in catalpol content among male and female *E. anicia* (Red Hill, 1985).

Results of iridoid analyses on postdiapause larvae are given in Table 4. The two off-site larvae and one on-site larva collected on *B. plantaginea* contained only catalpol, while two on-site larvae from *B. plantaginea* contained macfadienoside. One of these, surprisingly, contained no catalpol. Since there is no macfadienoside in *B. plantaginea*, the two on-site larvae collected on *B. plantaginea* must have previously consumed *C. integra*. The larvae collected on *C. integra* were quite consistent in having considerably more macfadienoside than catalpol.

Iridoid analysis of the dissected butterflies indicated that the total iridoid content in each of the body part divisions was similar (Table 5). Iridoid content as percent dry weight in each combination was variable because of the weight difference among the parts. The female head–thorax–leg combination appeared particularly high in macfadienoside and low in catalpol.

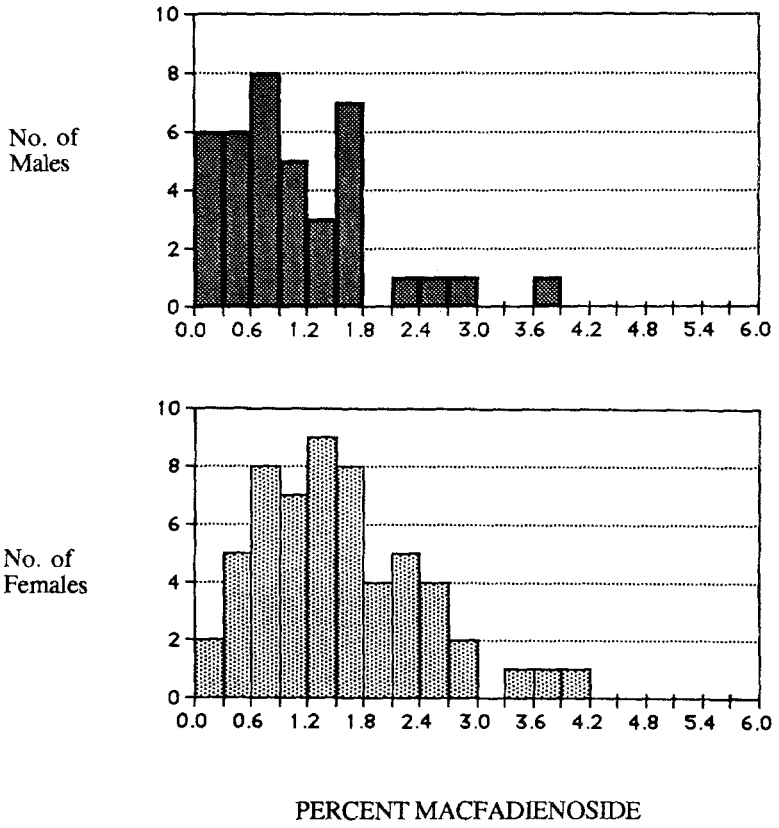


FIG. 6. Variation in macfadienoside content among male and female *E. anicia* (Red Hill, 1985).

Iridoid Analyses (Cumberland Pass). The results of 1985 and 1986 collections and analyses are given in Tables 6 and 7. Especially noteworthy was the high 1985 total iridoid content of the males ($\bar{X}$ = 7.9%, range 4.8–10.4%; 1420 μg per butterfly average). A large decrease in male iridoid content was seen in comparing these 1985 results ($\bar{X}$ = 7.9%) to 1986 ($\bar{X}$ = 3.4%). Male–female comparisons were only available for 1986, and neither total iridoid content nor the ratio of aucubin to catalpol sequestered differed significantly between the sexes. Catalpol and aucubin are present in the host plant in approximately equal amounts (Stermitz et al., 1986), but butterflies contained much more catalpol than aucubin. Large individual variations were noted, with catalpol content varying from 180 to 1700 μg per butterfly and aucubin content from 0.0 to 530 μg.

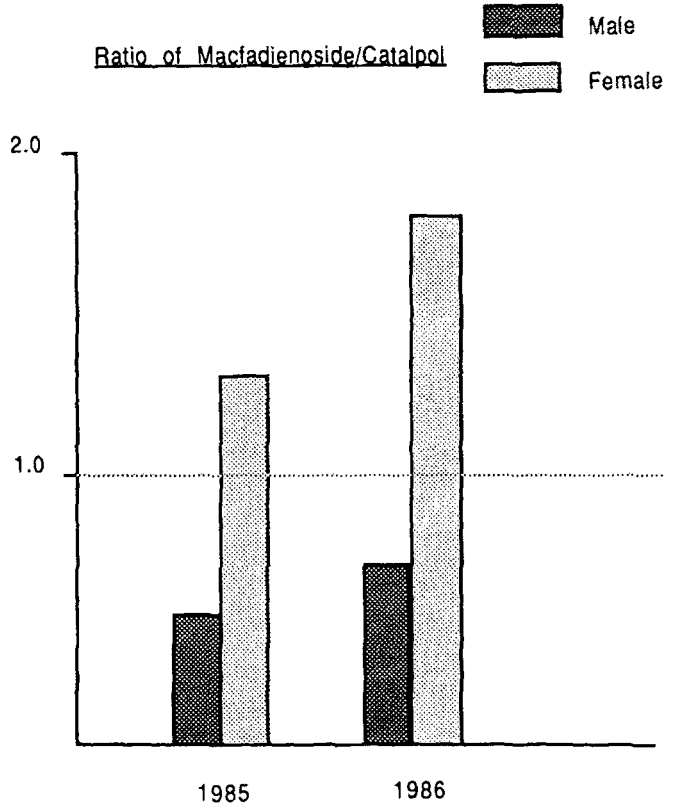


FIG. 7. Male–female difference in macfadienoside-to-catalpol ratio (Red Hill, 1985).

TABLE 4. IRIDOID CONTENT OF POSTDIAPAUSE *E. anicia* LARVAE (RED HILL)

Larvae no.	Host plant on which collected	Wet wt. (mg)	Dry wt. (mg)	Catalpol (μg)	Macfadienoside (μg)
1	*Besseya plantaginea*[a]	210	50	234	none
2	*B. plantaginea*[a]	211	51	123	none
3	*B. plantaginea*[b]	352	94	120	none
4	*B. plantaginea*[b]	281	66	none	53
5	*B. plantaginea*[b]	288	78	53	190
6	*Castilleja integra*[c]	345	90	58	345
7	*C. integra*[c]	242	57	52	373
8	*C. integra*[c]	143	48	44	254

[a] From ravine offsite; only host-plant species present.
[b] From site; *C. integra* also present.
[c] From site; *B. plantaginea* also present.

TABLE 5. IRIDOID ANALYSES OF RED HILL *E. anicia* BODY PARTS: DATA FROM COMBINED PARTS OF THREE BUTTERFLIES OF EACH SEX (1986 COLLECTION)

	Males			Females		
Iridoid	Head, thorax, legs	Abdomen	Wings	Head, thorax, legs	Abdomen	Wings
Catalpol						
μg	70	40	150	5	140	160
%[a]	0.2	0.1	1.0	<0.1	0.2	1.1
Macfadienoside						
μg	60	190	110	570	370	440
%[a]	0.1	0.4	0.7	1.3	0.5	3.0

[a] Percent of part dry weight.

Catalpol Ester Metabolism. Of the nine early postdiapause larvae collected from *C. integra* at Red Hill and then fed in the laboratory on an artificial diet containing 6-isovanillylcatalpol, seven reached adulthood, while two were found to have been parasitized by wasps. Quantitative analyses were performed on three adults, their pupal cases, emitted meconium, and frass from two of the individuals (Table 8). Frass contained isovanillic acid, but no iridoids. The pupal cases, meconium and adult butterflies contained no isovanillic acid or 6-isovanillylcatalpol, but did contain the iridoids catalpol and macfadienoside.

DISCUSSION

Differential Host Plant Utilization. At Red Hill, 92% of all butterflies in 1985 ($N = 96$) and 100% in 1986 ($N = 20$) contained macfadienoside and hence utilized *C. integra* at some time during the larval stages. Prediapause larvae weigh only about 5 mg, while postdiapause larvae reach several hundred milligrams (Table 4), so the adult iridoid content must primarily be a reflection of postdiapause larval consumption. Since we found macfadienoside-lacking adults just off the site in an area where only *Besseya* occurs, the few site butterflies that lacked macfadienoside might have moved in from such an area. In both years observational data indicated the presence of numerous *Besseya* with egg masses, but the relative use of *Castilleja* vs. *Besseya* for oviposition has not yet been assessed. Little or no catalpol or macfadienoside was present in the frass collected during the artificial diet studies (Table 8). Macfadienoside was retained from the early field consumption of *C. integra* even after a subsequent week or more of feeding on an artificial diet that lacked that iridoid.

TABLE 6. IRIDOID GLYCOSIDE ANALYSIS SUMMARY (*E. anicia:* CUMBERLAND PASS)[a]

	1985						1986					
	N	Mean	MED	SD	MIN	MAX	*N*	Mean	MED	SD	MIN	MAX
Males	10						10					
Dry weight (mg)		18.3	17.8	2.8	14.3	23.1		19.3	19.1	2.2	16.6	23.5
% AUC		1.3	0.9	1.0	0.3	3.6		0.5	0.4	0.4	0.0	1.4
% CAT		6.6	7.4	2.6	2.1	9.6		2.9	3.7	1.4	0.8	4.2
% Total		7.9	8.0	1.8	4.8	10.4		3.4	4.4	1.7	0.8	5.4
AUC (μg)		230	155	180	60	530		100	82	70	00	240
CAT (μg)		1190	1350	450	310	1730		550	680	280	180	890
Total (μg)		1420	1480	330	840	1870		640	795	320	180	970
Females	0						10					
Dry weight (mg)								41.3	40.5	11.2	26.9	62.6
% AUC								0.2	0.2	0.1	0.0	0.3
% CAT								1.9	2.3	0.9	0.6	3.2
% Total								2.1	2.5	1.0	0.7	3.5
AUC (μg)								50	50	40	00	100
CAT (μg)								810	940	380	200	1250
Total (μg)								870	1005	410	200	1350

[a]KEY *N*= number of samples, MED = median, SD = standard deviation, MIN = minimum, MAX = maximum, AUC = aucubin, CAT = catalpol.

TABLE 7. STATISTICAL SUMMARY FOR TABLE 6[a]

	Dependent variable, *Y*						
		Dry weight (%)[b]			Dry weight (μg)[c]		
	DW	AUC	CAT	TOT	AUC	CAT	TOT
Effect							
Sex in 1986 on Y (P)	<0.001	<0.025	NS	NS	NS	NS	NS
(F)	66.024	7.893					
Year on males Y (P)	NS	<0.05	<0.01	<0.001	<0.05	<0.01	<0.001
(F)		4.729	15.932	33.387	5.548	12.510	24.551

[a]DW = dry weight, AUC = aucubin, CAT = catalpol, TOT = total; NS = not significant.
[b]Transformed with an arcsine function.
[c]Transformed by square root of *X*.

TABLE 8. METABOLISM RESULTS ON *E. anicia* COLLECTED AS LARVAE ON *C. integra* AND MAINTAINED ON ARTIFICIAL DIET CONTAINING 6-ISOVANILLYLCATALPOL, **4**

	Sample					
	Initial larva[a]	Pupa[a]	Adult[b]	Pupal case[b]	Meconium[b]	Frass[b]
Female 1						
Weight (mg)	74	230	50	2.5		
Catalpol (μg)			60	20	60	
Macfadienoside (μg)			140	160	170	
Isovanillic acid (μg)			none	none	none	
Female 2						
Weight (mg)	87	189	34	2.9		42
Catalpol (μg)			90	80	20	none
Macfadienoside (μg)			440	140	450	none
Isovanillic acid (μg)			none	none	none	42
Male 1						
Weight (mg)	90	210	34	2.4		66
Catalpol (μg)			40	20	250	none
Macfadienoside (μg)			110	100	1010	none
Isovanillic acid (μg)			none	none	none	40

[a]Wet weight.
[b]Dry weight.

Since macfadienoside is retained and is the major iridoid of *Castilleja*, adults with high macfadieneoside-to-catalpol ratios probably came from larvae that had consumed relatively more *Castilleja* than *Besseya*.

The most parsimonious explanation of the high percentage of macfadienoside-containing adults may simply be that *C. integra* is more plentiful at Red Hill than is *B. plantaginea* (Table 1), that the two host plants are relatively uniformly found at the site, and that larvae eat what they randomly encounter. With the plant counts showing a 9:1 ratio of abundances of *C. integra* and *B. plantaginea* in 1985, the finding of 92% macfadienoside-containing adults is as expected. That year both host plants were available and used by postdiapause larvae. This was not the case in 1986, when there was little emergent *B. plantaginea* at the time of postdiapause larval feeding. This may account for the finding of some females with only macfadienoside and no catalpol (Table 2).

In a study of *E. editha* hosted by *C. linariifolia* near Almont in Gunnison County, Colorado (Holdren and Ehrlich, 1982), it was suggested that the host plant choice there was determined by ecological characteristics, primarily host plant phenologies, and not nutrition or biochemistry. Thus, *C. chromosa*, although present at Almont, was never used and the suggestion was made (Holdren and Ehrlich, 1982) that the early senescence of this species as compared to *C. linariifolia* was the determining factor in host plant use. At Red Hill both *C. integra* and *B. plantaginea* are generally available for both oviposition and larval consumption, although some seasonal and year-to-year variation does occur. There appear to be no phenological determinants that would preclude either host plant being utilized at Red Hill.

The interpretation of data from Cumberland Pass is simplified since here *E. anicia* uses only one host plant, *B. alpina*. The data of Table 5 show preferential sequestration of catalpol over aucubin, since these two iridoids are of approximately equal concentration in the host plant (Stermitz et al., 1986). Significantly more catalpol than aucubin was found in 27 of 29 butterflies. This result parallels that for *E. phaeton* adults from larvae fed on *Chelone glabra* and *Plantago lanceolata*, where selective sequestration of catalpol over aucubin was also reported (Bowers and Puttick, 1986). Our analyses on *B. alpina* were conducted on material collected at one time, while larvae consume host plants over a period of several weeks. In order to confirm this selective sequestration, an analysis of the relative catalpol and aucubin content in individual plants over the period of consumption must be done. In none of the 30 Cumberland Pass butterflies analyzed, nor in any of those previously studied from the alpine sites, was macfadienoside found. This confirms the suggestion (Stermitz et al., 1986) that macfadienoside, although a simple allylic hydroxylated product of catalpol, is not formed by butterfly metabolism of catalpol.

Individual Variation in Iridoid Content. Butterflies showed a highly variable iridoid content at both Red Hill and Cumberland Pass and in each season,

with individuals ranging from less than 1% to over 10% iridoid on a dry weight basis. This would result in a large range in bitterness, which is expected to be iridoid-concentration dependent, and should lead to a difference in palatability to predators. It has been shown that *E. phaeton* butterflies are unpalatable to blue jays when raised on high-iridoid-content plants, but are palatable when raised on low-iridoid-content plants (Bowers 1980, 1981). Dose–activity data have been important in monarch–cardenolide work (Brower, 1984) and need to be established for iridoids before we can make any definitive statements about unpalatability. If the low-iridoid-content butterflies in our natural populations are not sufficiently bitter to deter vertebrate predators, while the high concentrations do suffice, then the low-content butterflies could be considered automimics, as has been suggested for the monarch butterfly case. With both macfadienoside and catalpol being sequestered in major amounts by butterflies at Red Hill, it will be also important to establish the relative potencies (or bitterness) of these two iridoids. This is particularly so since there is a difference in the relative amounts of these iridoids in adult males as compared to females. This could result in different selection pressures on the sexes.

Field and laboratory observations indicate that iridoids are not protective against ants or spiders, since cases of predation by these invertebrates on larvae and adult butterflies have been seen. Wasp parasitoids are also not deterred. Parasitized larvae contain iridoids, although the wasp cocoons and pupae do not (Gardner, 1987; K. L'Empereur, unpublished work). We have not, however, looked at butterfly iridoid concentrations relative to invertebrate predation, and there could be a differential predation pressure on individuals with varying iridoid content.

In the lubber grasshopper, Jones et al. (1986) reported interindividual variations in defensive substance content of two to three orders of magnitude, as well as qualitative differences, and suggested that the grasshopper defensive secretions were of a fundamentally idiosyncratic and unpredictable nature. High variability in adult-obtained pyrrolizidine alkaloids were reported for ithomiine butterflies (Brown, 1984a,b). Our results are similar in many respects to the monarch and queen butterfly (Brower et al., 1984, Lynch and Martin, 1987) and *Oncopeltus* (Scudder et al., 1986) cases. There qualitative cardenolide content is quite closely tied to larval host-plant allelochemical content, mean quantitative content somewhat less so, but where individuals can show extremely high variability. Iridoid sequestration from hostplants is more selective and less exactly a reflection of the host plant content than is cardenolide sequestration, since we observe more metabolism (ester hydrolysis) prior to sequestration in some cases and since some host plant iridoids are not found in the butterflies. Some possible predator–prey scenarios that might influence the evolution and maintainence of individual variations in defensive chemical content in insects have been suggested (Jones et al., 1986).

Metabolism and Additional Aspects of Sequestration. The artificial diet experiment involving the catalpol ester **4** established that *E. anicia* larvae are able to hydrolyze the ester and sequester the resulting catalpol while excreting isovanillic acid. It is highly likely that they do the same with catalpol esters **3** and **5** since we have never encountered these esters in the adult butterflies. Thus, as the major iridoid components of *B. plantaginea*, the esters must provide most of the catalpol present in larvae feeding on this host plant. Esterases are common in insects, and it is unlikely that this process is a specialized one for *E. anicia*. The relatively high levels of macfadienoside found in adults, pupal cases, and meconium (Table 8) are interesting since the larvae had doubled or tripled in weight since consuming any of the macfadienoside-containing *C. integra* host plant. Particularly striking were the results with male 1 where 10 times as much macfadienoside was excreted in the meconium as was maintained into the adult stage, while the two females sequestered and excreted approximately equal amounts of that iridoid. This differential processing could be one reason for the lower macfadienoside content of males as compared to females, but additional samples are clearly necessary to confirm this possibility. A defensive role for meconium emission of iridoids has been suggested (Bowers and Puttick, 1986), and this might account for release of a substance that could otherwise provide additional longer term adult defense.

Female *E. anicia* emerge with a full complement of eggs, and the iridoid content of eggs would be important to establish, since up to 20% of the female weight can be assigned to eggs (Murphy et al., 1983). Preliminary experiments on only a few field-collected eggs or eggs from dissection (Gardner, 1987) showed them to have a high catalpol and very low macfadienoside content. If this fact could be confirmed, it would make the high female content of macfadienoside even more striking. In some insects male spermatophores can constitute a significant contribution to the weight of the male and inseminated female. We have shown (Gardner, 1987) that this does not hold for *E. anicia* since the spermatophore constitutes only about 2% of the dry weight of the male. The spermatophore iridoids represent less than 1% of the total iridoid load of the male (Gardner, 1987).

One literature report (Franke et al., 1987) suggested the possibility of metabolism other than the ester hydrolysis we have established, but the data are equivocal. The sequestration of aucubin and catalpol by *E. cynthia* was reported (Franke et al., 1987), but the adults also contained large amounts of 6-*O*-glucopyranosylaucubin, which was not present in the *Plantago lanceolata* that was being used to feed larvae in the lab. Larvae were wild-collected from *Plantago alpina* and then maintained on *P. lanceolata*. It was suggested that the 6-*O*-glucopyranosylaucubin might have been either a metabolic product or had been present in an unknown prior utilized host plant. The iridoid content of the original host plant, *P. alpina*, was, however, not reported and hence the question

of insect metabolic formation of the new glucosidic iridoid remains to be established.

The very high levels of iridoids that are reached in many of the *E. anicia* could also be important to the insect if they provide some benefit other than as vertebrate predator defenses. For example, some iridoids have been reported to have antimicrobial, antifungal, and/or antioxidant properties (Davini et al., 1986; Cameron et al., 1984; Ishiguro et al., 1986). Catalpol is, however, not known to be particularly active and macfadienoside has not been tested as far as we know. Aucubin is one of the more biologically active iridoids, especially as the aglycone.

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AGGREGATION PHEROMONE OF SQUARE-NECKED GRAIN BEETLE, *Cathartus quadricollis* (GUÉR.)[1,2]

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Abstract—When feeding on rolled oats, male square-necked grain beetles, *Cathartus quadricollis* (Guér.), produced the aggregation pheromone (3*R*,6*E*)-7-methyl-6-nonen-3-yl acetate, for which the trival name "quadrilure" is proposed. The pheromone was highly attractive to both sexes in a two-choice, pitfall olfactometer modified to retain responding beetles by placing a food stimulus (an oat flake) in the glass vials containing the experimental and control stimuli. The *S* enantiomer of the pheromone was inactive. Males also produced small amounts of (*E*)-7-methyl-6-nonen-3-one, (*E*)-7-methyl-6-nonen-3-ol, and (6*E*)-7-methyl-3-propyl-2,6-nonadienyl acetate, but these compounds were inactive in the laboratory bioassay. Segregated males and females both produced (*R*)-(−)-1-octen-3-ol, which by itself was repellent to both sexes but did not diminish beetle response to the aggregation pheromone.

Key Words—*Cathartus quadricollis* (Guér.), square-necked grain beetle, Coleoptera, Cucujidae, aggregation pheromone, (3*R*,6*E*)-7-methyl-6-nonen-3-yl acetate, (*E*)-7-methyl-6-nonen-3-one, (*E*)-7-methyl-6-nonen-3-ol, (6*E*)-7-methyl-3-propyl-2,6-nonadienyl acetate, 1-octen-3-ol, repellent.

[1] Coleoptera: Cucujidae.

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INTRODUCTION

The square-necked grain beetle, *Cathartus quadricollis* (Guér.), is a long-lived, cosmopolitan pest of stored products. In North America this polished, reddish-brown beetle is chiefly abundant in the southern United States and infests a variety of stored commodities such as corn and peanuts (Anon., 1955). The beetle is also found in the wild and attacks a large variety of plant seed pods. In morphology and habit, *C. quadricollis* is similar to other grain-infesting beetles of the genera *Cryptolestes* and *Oryzaephilus*, but it is larger and a strong flyer (Anon., 1955). As an extension of our investigation of semiochemicals in cucujid grain beetles (H.D. Pierce et al., 1984), we inititated a study of *C. quadricollis*. Herein, we report the identity of a male-produced aggregation pheromone and the structures of several related beetle-produced compounds.

METHODS AND MATERIALS

Insect Rearing. C. quadricollis were reared on large-flake rolled oats and brewer's yeast (95:5, w/w) in 3.8-l glass jars at 30°C and 65–70% relative humidity (RH). Numbers of adults were determined by mean weight (1 beetle = 0.79 mg, $N = 1047$). Adult beetles were sexed by the presence (males) or absence (females) of a small, sharply pointed process on the coxa of the hind leg.

Pheromone Collection. Volatiles from male, female, or mixed-sex beetles feeding on rolled oats contained in vertically oriented glass chambers were trapped on Porapak Q as described by H.D. Pierce et al. (1984). Trapped volatiles were recovered from the Porapak Q by extraction with pentane in a Soxhlet extractor for 24 hr. The pentane solution of the volatiles was concentrated to approx. 10 ml by distilling off the pentane through a Dufton column.

Test Beetles for Bioassay. Mixed-sex *C. quadricollis* 8–12 weeks old were harvested from rearing cultures and placed in fresh medium at a density of approx. 10,000 beetles/kg at least one week before bioassays. Approximately 48 hr before a bioassay, 1.1 g of beetles were removed from the culture and preconditioned at 23°C in darkness without food in a 6-l Erlenmeyer flask through which charcoal-filtered, humidified air was drawn by a water aspirator at 1.9 l/min. Sexed adults were also conditioned for bioassay in this manner.

Bioassay Procedures. Several hours before commencement of a bioassay, 12 conditioned test beetles were aspirated into each of several 60-ml glass vials. At this time, oat flakes were also placed in the experimental and control vials of a two-choice, pitfall olfactometer (A.M. Pierce et al., 1981). The vials of test beetles and vials for the olfactometers were then taken to an environmental chamber maintained at 24°C and 60–65% RH. This modification was neces-

sary, because *C. quadricollis* tend to climb out of the vials unless host material (an oat flake) or attractive frass is present in the experimental vial (Table 1). There was minimal response to a control vial with only an oat flake (Table 1).

After approx. 3–4 hr, a group of 12 test beetles was released into each olfactometer, and the bioassay was conducted for 2 hr (A.M. Pierce et al., 1981). Mixed-sex test beetles were used only once and discarded, while sexed beetles were recovered at the end of the bioassay and returned to their rearing jar.

Unless otherwise stated, the raw data were analyzed by the *t*-test for paired, correlated data, and results were expressed as the mean percent response of the total number of beetles per treatment.

In one bioassay, response by *C. quadricollis* in the two-choice, pitfall olfactometer was determined at selected time intervals by counting the number of beetles in the experimental and control stimulus vials. Counting the beetles in the vials necessitated picking up and slightly tilting the olfactometer dish. This process did not disturb the responding beetles if done gently. The experimental stimulus for this bioassay was frass and a pentane extract thereof. The latter was prepared by placing 3.75 g frass from a mature culture in a sintered glass funnel. Approximately 10 ml of pentane was added, and the mixture was stirred with a spatula. The solvent was then drawn off by application of a slight vacuum. This process was repeated three times. The combined pentane washes were concentrated under a stream of nitrogen to 1.5 ml.

TABLE 1. RESPONSE TO FRASS OF MIXED-SEX *C. quadricollis* IN TWO-CHOICE, PITFALL OLFACTOMETER[a]

	Response, % ($\bar{X}$ ± SE)[b]					
Time after release of beetles (min)	Experimental: 25 mg frass in vial	Control: empty vial	Experimental: pentane ext. of 25 mg frass on filter paper	Control: pentane on filter paper	Experimental: pentane ext. of 25 mg frass on filter paper and oat flake	Control: pentane on filter paper and oat flake
15	94.1 ± 3.4a	3.4 ± 1.8	79.2 ± 4.0a	5.0 ± 1.4	86.7 ± 4.8a	10.0 ± 3.9
30	94.1 ± 3.4a	3.4 ± 1.4	79.2 ± 3.6a	2.5 ± 1.3	80.8 ± 5.2a	10.0 ± 3.7
60	94.9 ± 2.4a	2.5 ± 1.3	60.8 ± 4.2b	8.3 ± 2.5	87.5 ± 4.8a	10.0 ± 3.9
120	91.5 ± 3.2a	3.4 ± 1.8	51.7 ± 4.4b	10.8 ± 2.7	88.3 ± 4.7a	10.0 ± 4.1

[a] Showing retention of beetles responding to frass volatiles when frass is present in experimental vial, lack of persistent retention when experimental vial contains frass extract on filter paper, and restoration of retention when oat flake is present in experimental and control vials. $N = 10$ replicates per treatment, 12 beetles per replicate.

[b] Response to experimental stimulus significant in all cases, *t*-test, $P < 0.001$. Means in the same row followed by the same letter not significantly different (Newman-Keuls test, $P < 0.05$).

Instrumental Methods. Hewlett-Packard 5830A and 5880A gas chromatographs equipped with flame-ionization detectors and fitted with open-tubular glass columns (36–48 m × 0.5 mm ID) coated with Carbowax 20 M, SP-1000, or SUPEROX-4 (Alltech Associates, Deerfield, Illinois) were employed for analytical gas–liquid chromatography (GLC). The micropreparative gas chromatograph has been previously described (A.M. Pierce et al., 1985). A 3.3-m × 3.18-mm OD stainless-steel column packed with 5% OV-101 on Chromosorb G (70/80) was used for separations with temperature programming.

Coupled gas chromatography–mass spectroscopy (GC-MS) was carried out with a Hewlett-Packard 5895A GC-MS-DS. The transfer line and ion source were maintained at 250°C and 200°C, respectively. The electron impact (EI) voltage was 70 eV. Isobutane was the reagent gas for spectra obtained in the chemical ionization (CI) mode. Fused silica columns (0.32 mm ID) coated with SP-1000 (30 m) or DB-1 (30 and 60 m) liquid phases (J&W Scientific Inc., Folsom, California) were coupled directly into the ion source. Helium was the carrier gas for GC and GC-MS.

Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker WM-400 spectrometer. Samples separated by micropreparative GC were rinsed from collection tubes into NMR tubes with $CDCl_3$ (99.96 atom % D) or C_6D_6 (99.6 atom % D) (Merck Sharp & Dohme, Canada Ltd., Kirkland, Quebec). Chemical shifts are reported on the δ scale.

Synthetic Chemicals for Bioassays. The syntheses of the racemate and enantiomers of the aggregation pheromone and of several related beetle-produced compounds have been reported elsewhere (Johnston and Oehlschlager, 1986). (*R*, *S*)-1-Octen-3-ol was purchased from the Aldrich Chemical Company (Milwaukee, Wisconsin). (*R*)- and (*S*)-1-Octen-3-ol were gifts from Dr. A. Mosandl (Mosandl et al., 1986).

RESULTS

Identification of Aggregation Pheromone. Comparison of a gas chromatogram of the Porapak Q-trapped volatiles from mixed-sex *C. quadricollis* feeding on rolled oats to that obtained from rolled oats established that four compounds (peaks A–D) were produced by the beetles (Figure 1). That one (or more) of the compounds was probably an aggregation pheromone was indicated by the strong response of mixed-sex *C. quadricollis* to the unfractionated volatiles in the modified two-choice, pitfall olfactometer (Figure 2).

The four beetle-produced peaks were isolated by micropreparative GC. Only peak B was attractive and accounted for most of the biological activity in the unfractionated Porapak Q-trapped volatiles (Figure 2). The remaining bee-

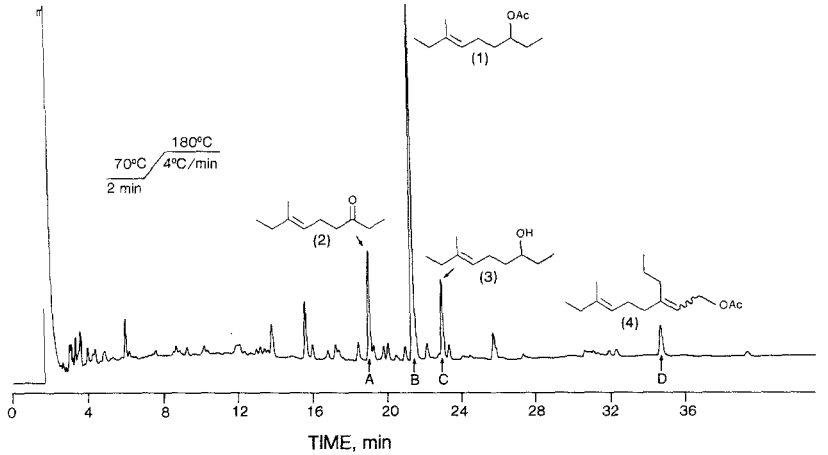

FIG. 1. Gas chromatogram of the Porapak Q-trapped volatiles from mixed-sex *C. quadricollis* feeding on rolled oats, designating beetle-produced peaks A–D and depicting structural formulae.

tle-produced peaks neither synergized nor inhibited the response of the beetles to peak B (data not shown). Thus, peak B was identified as an aggregation pheromone of *C. quadricollis*.

Structure of Aggregation Pheromone. In the mass spectrum of peak B (Figure 3), the highest observed fragment ion is *m/z* 138, which is also the highest and characteristic ion in the mass spectra of numerous volatile fatty acid esters of citronellol (Jennings and Shibamoto, 1980). The spectrum of the pheromone was similar to those of these compounds. We, therefore, suspected that the pheromone was also an ester of a C_{10} alcohol of mol wt = 156. This suspicion was confirmed by the CI mass spectrum wherein the highest observed ion was *m/z* 199 ($M^+ + H$). Thus, the *m/z* 138 ion is due to the loss of acetic acid from the molecule ion of a compound with the formula of $C_{12}H_{22}O_2$, i.e., the acetate of monounsaturated alcohol. Loss of an ethyl group from the *m/z* 138 ion would give the base peak ion at *m/z* 109.

The NMR spectrum of peak B is shown in Figure 4. The single proton triplet (J = 7.0 Hz) at δ 5.08 indicated that the double bond was trisubstituted with a methylene geminal to the olefinic proton. The chemical shift of the single proton quintet at δ 4.81 established the presence of secondary acetate (δ 2.04, 3H) with two adjacent and nearly equivalent methylenes (J = ca. 6.2 Hz). Irradiation of the carbinyl proton affected only the methylenes absorbing at ca. δ 1.56. The sharp singlet (δ 1.57, 3H) was assigned to an allylic methyl group,

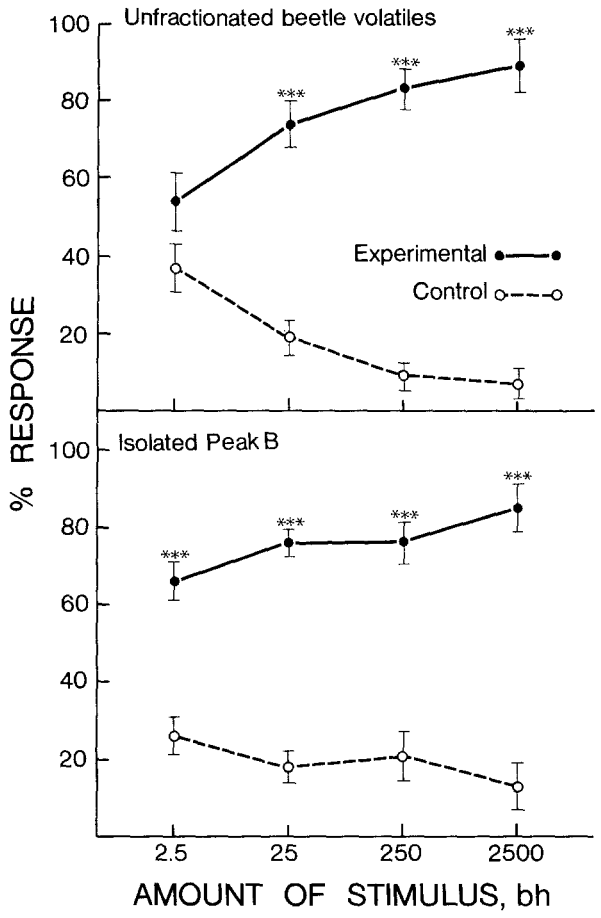

FIG. 2. Response ($\bar{X} \pm$ SE) of mixed-sex *C. quadricollis* in two-choice, pitfall olfactometer to unfractionated Porapak Q-trapped volatiles from mixed-sex *C. quadricollis* feeding on rolled oats and to isolated peak B. Significant response to experimental stimulus indicated by the following: $***P < 0.001$. $N = 12$ replicates. bh = amount of volatiles collected from one beetle aerated for 1 hr.

and the chemical shift indicated that the trisubstituted double bond was of *E* geometry (Marfat et al., 1979). Irradiation of the allylic protons at δ 1.98 established that one methyl (δ 0.97) was attached to an allylic methylene and the other (δ 0.88) was not. Irradition of the methyl group at δ 0.97 resulted in the collapse of three of the peaks of the quintet at δ 1.98 to a singlet, which was superimposed over a quartet. On the basis of the NMR spectral data, the struc-

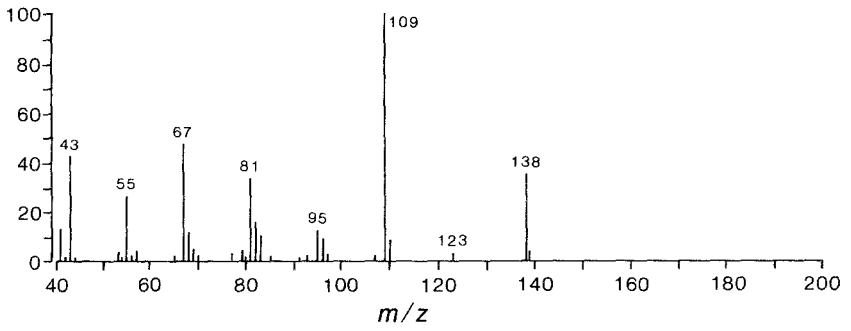

FIG. 3. Unit resolution mass spectrum of peak B.

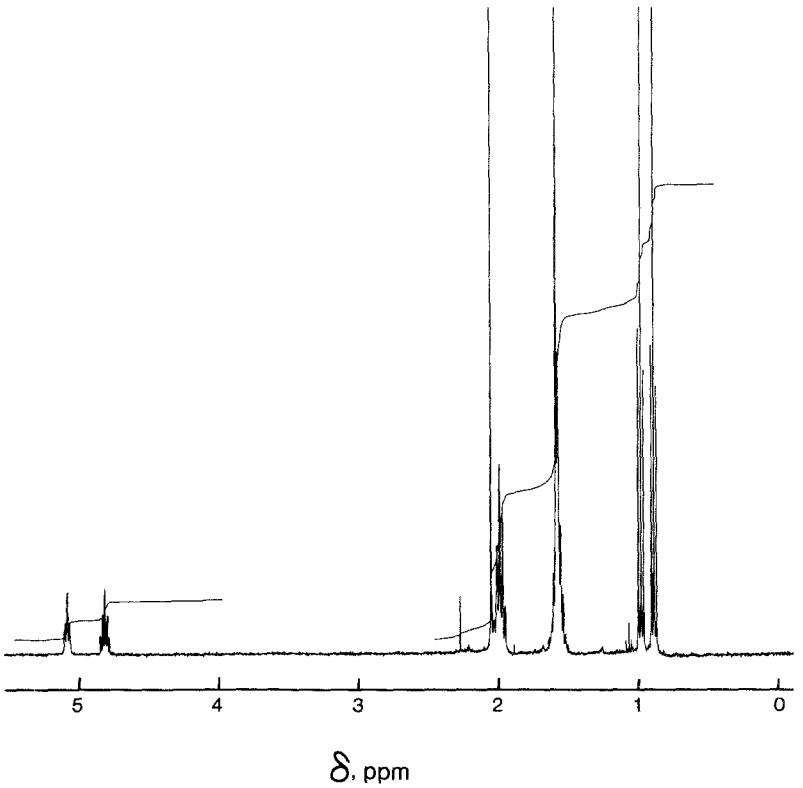

FIG. 4. [^{1}H]NMR spectrum of peak B recorded at 400 MHz in $CDCl_3$.

ture of the pheromone was formulated as (*E*)-7-methyl-6-nonen-3-yl acetate (**1**) (Figure 1). Comparison of the mass and NMR spectra and of the gas chromatographic retention time of the beetle-produced compound to those of an authentic sample (Johnston and Oehlschlager, 1986) confirmed the structural assignment. We propose the trivial name "quadrilure" for the pheromone.

Structures of Related Beetle-Produced Compounds. The mass spectrum of peak A exhibited M^+ at *m/z* 154. The NMR spectrum of peak A possessed several proton resonances that were identical to those of natural **1** but lacked the carbinyl proton and acetate methyl group resonances. The methylene resonances at δ 1.56 of natural **1** were shifted to δ 2.42. These spectral data and the shorter GC retention of peak A suggested that it was (*E*)-7-methyl-6-nonen-3-one (**2**). The structure of peak A was confirmed by comparison of spectral data and GC retention time to that of synthetic **2.** The structure of peak C was easily established as (*E*)-7-methyl-6-nonen-3-ol (**3**) (Figure 1) by comparison of spectral data to that of an authentic sample, which was an intermediate in the synthesis of **1** (Johnston and Oehlschlager, 1986).

The EI mass spectrum of peak D was not particularly informative, but the CI mass spectrum indicated that the molecular weight of the unknown was 236. In Figure 5 is shown the NMR spectrum of peak D. Comparison of Figure 5 to Figure 4 revealed that the unknown and natural **1** share several common resonances. The chemical shifts of the coupled low-field olefinic proton (δ 5.34, 1H, J = 7 Hz) and carbinyl protons (δ 4.59, 2H, J = 7 Hz) indicated the compound was a primary allylic acetate (δ 2.05, 3H) with two trisubstituted double bonds. Decoupling experiments established that the methylene at δ 1.41 (a sextet) was coupled to the high-field methyl (δ 0.89, J = 7.34 Hz) and an allylic methylene (ca., δ 2.06, J = 7.6 Hz). Irradiation of the allylic methyl at δ 1.59 removed small splittings (ca. 1.5 Hz) from the olefinic peak at δ 5.08. A structure consistent with these data is (6*E*)-3-propyl-7-methyl-2,6-nonadienyl acetate (**4**). The spectral data, however, were insufficient for assignment of the geometry of the C-2,3 double bond.

Bioassays with Synthetic Pheromone. Table 2 shows the response of mixed-sex beetles to (*R,S*)-**1** (*E*/*Z* = 3) and to natural **1.** The doses of natural **1** were 37.5% of (*R,S*)-**1** since the pheromone has one chiral center (see below). These data show that the pheromone is probably one isomer and that the presence of the other optical and geometrical isomers did not inhibit the response of the beetles. In a 12-replicate experiment, the mean percent response (± SE) of mixed-sex beetles to racemic **1** (10 ng, 98% *E*) was 80 ± 3.9 (percent response to pentane control = 16.7 ± 4.1) in the presence of an oat flake and 40 ± 4.4 (percent response to pentane control = 12.5 ± 4.2) in its absence. These results corroborate those in Table 1 and confirm that an oat flake is a sufficient requirement for sustaining the response of the beetles during the 2-hr bioassay.

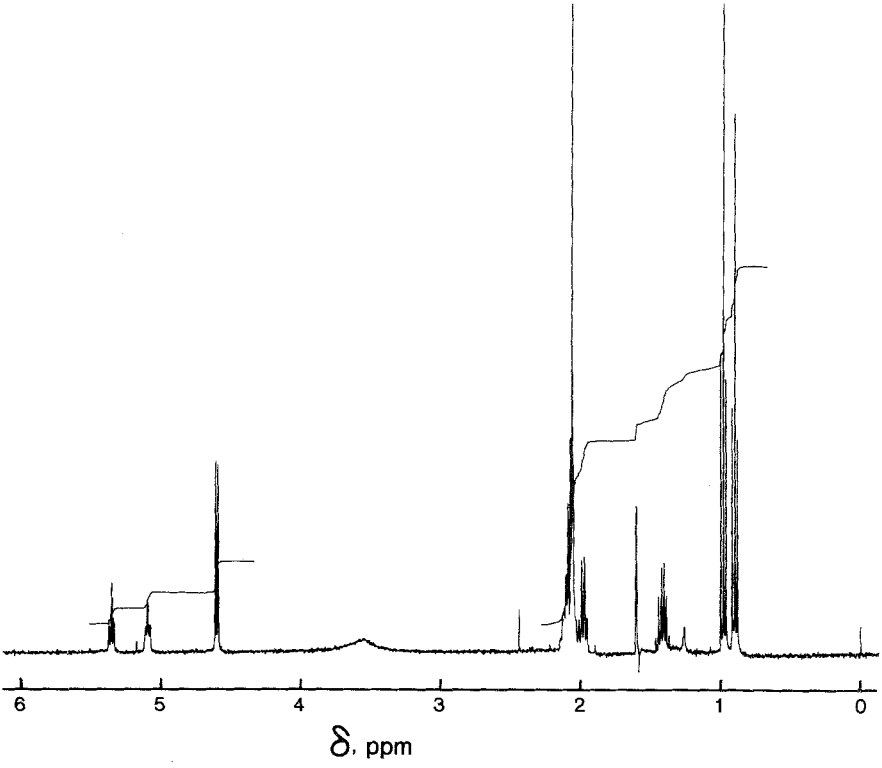

FIG. 5. [^{1}H]NMR spectrum of peak D recorded at 400 MHz in $CDCl_3$. The reduced intensity of the allylic methyl at δ 1.59 is due to irradiation of a water peak at δ 1.58.

TABLE 2. RESPONSE OF MIXED-SEX *C. quadricollis* IN TWO-CHOICE PITFALL OLFACTOMETER TO NATURAL AND SYNTHETIC, RACEMIC PHEROMONE (N = 12 REPLICATES)

		Response, % ($\bar{X}$ ± SE)[a]	
Experimental stimulus	Amount (ng)	Experimental stimulus	Pentane control
Natural pheromone	0.375	64.1 ± 4.1	25.5 ± 4.8
	3.75	79.9 ± 2.1	12.5 ± 1.9
	37.5	79.9 ± 3.5	11.1 ± 3.2
Synthetic pheromone (E/Z = 3)	1	69.0 ± 5.3	19.3 ± 3.8
	10	77.1 ± 2.2	13.2 ± 2.8
	100	85.4 ± 2.5	8.3 ± 2.0

[a]Response to experimental stimulus significant in all cases, t-test, $P < 0.001$.

The response of females and males to (*R,S*)-**1** (98% *E*) were similar (Figure 6), but the threshold for detection was lower for females than for males. At a stimulus dose of 100 μg, response to the pheromone was inhibited (Figure 6).

Determination of Pheromone-Producing Sex. Analysis by GC of the Porapak Q-trapped volatiles from cultures of segregated males or females estab-

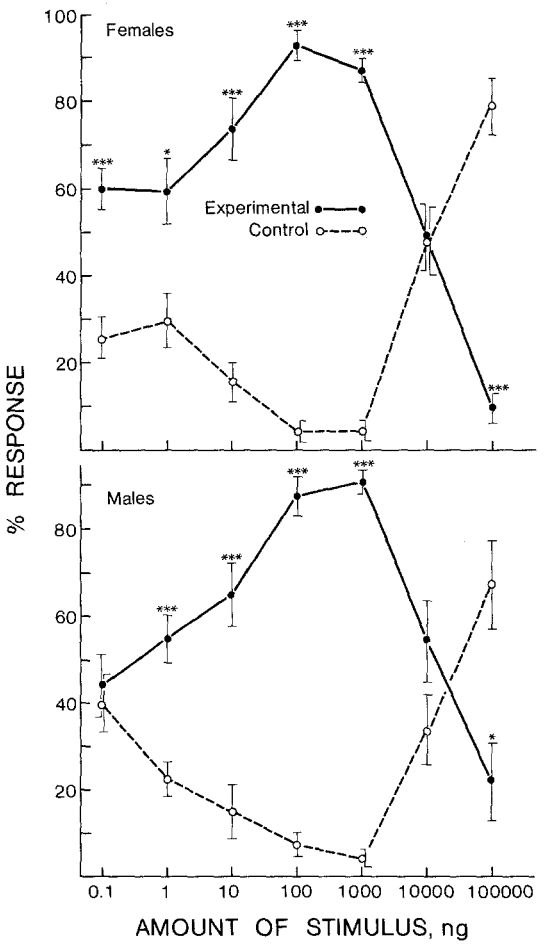

FIG. 6. Response ($\bar{X} \pm$ SE) of female and male *C. quadricollis* to racemic pheromone (98% *E*). Significant response to experimental stimulus indicated by the following. ***$P < 0.001$; *$P < 0.05$. $N = 12$ replicates.

lished that only the males produced the pheromone (**1**) and **2–4** (Figure 7). In addition to the pheromone and **2–4,** segregated male beetles apparently produced 1-octen-3-ol (**5**), which was also found in the females' volatiles. The production of **2** and **3** by males was much smaller (Figure 7) while production of **4** was unchanged from that produced by males in mixed-sex culture (Figure 1). No *Z* isomer of the pheromone was detected in this sample by GC-MS analysis on the 60-m DB-1 column.

Determination of Chirality. The enantiomeric enhancement (ee) and chirality of **1** were determined by the method of Slessor et al. (1985). Lithium aluminium hydride was used to remove the acetate group of the pheromone. Male beetles produced (*R*)-**1** (91% ee) and both sexes produced (*R*)-(−)-1-octen-3-ol (ca., 94–95% ee).

Bioassays with Chiral Pheromones. Although responses to (*S*)-**1** (>99% ee) at dosages of 10–10,000 ng were significantly different from those to control stimuli, significant responses to (*R,S*)-**1** occurred at a 100-fold lower dose (Figure 8). Conversely, the thresholds for response to (*R*)-**1** and the racemate were identical (Figure 9). Evidently, beetles in the *S* enantiomer experiment were more active than those used in the *R* enantiomer experiment. Thus, response to the *S* enantiomer might be entirely due to the presence of the optical impurity. The data indicate that the *S* enantiomer neither repels the beetles nor inhibits

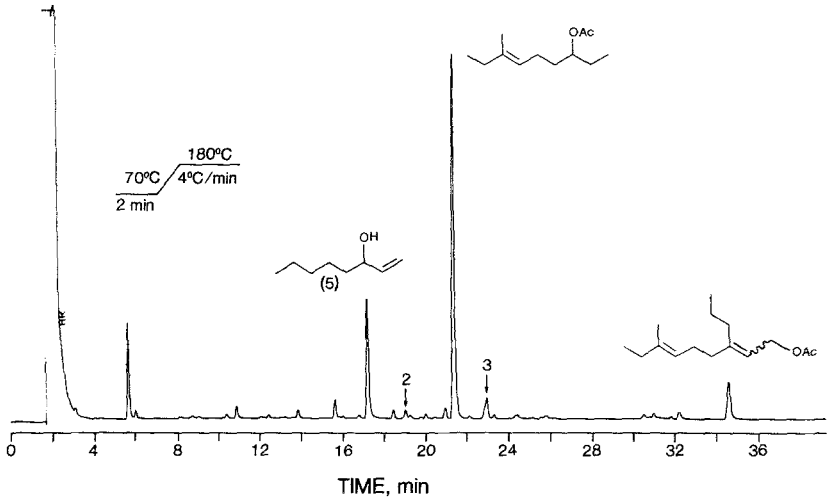

FIG. 7. Gas chromatogram of the Porapak Q-trapped volatiles from male *C. quadicollis* feeding on rolled oats. The peak at *RT* = 17.2 min. is 1-octen-3-ol.

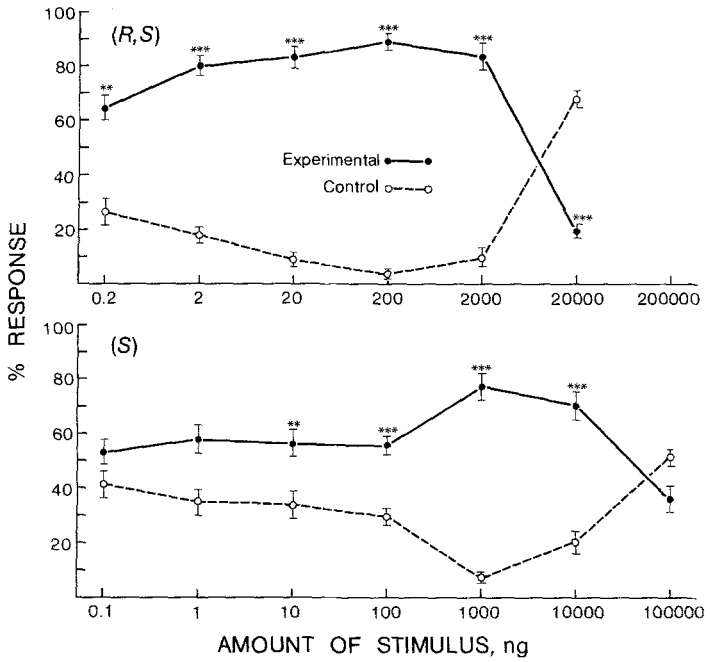

FIG. 8. Response ($\bar{X} \pm$ SE) of mixed-sex *C. quadricollis* in two-choice, pitfall olfactometer to (*S*)- and (*R*,*S*)-**1.** Significant response to experimental stimulus indicated by the following: ***$P < 0.001$; **$P < 0.01$. $N = 12$ replicates.

their response. The data (Figure 9) also suggest that the *S* isomer probably does not synergize the response of mixed-sex beetles to the *R* isomer since the threshold (1 ng) for significant response was the same (however, see Discussion).

Neither (*R*)- nor (*R*,*S*)-1-octen-3-ol was attractive to beetles of mixed sex at any dosage, and at most dosages the beetles were repelled (Table 3). (*S*)-1-Octen-3-ol was at least a 100-fold less active than the *R* enantiomer. The data suggest that the *S*-enantiomer is relatively inactive as a repellent. The response of the beetles at the higher dosages of the *S* isomer is probably due to a small amount of the antipode (ca., 1%). Although (*R*)-1-octen-3-ol apparently functions as a repellent in *C. quadricollis*, response of mixed-sex beetles to the aggregation pheromone is not reduced by a large excess of the compound (Table 3).

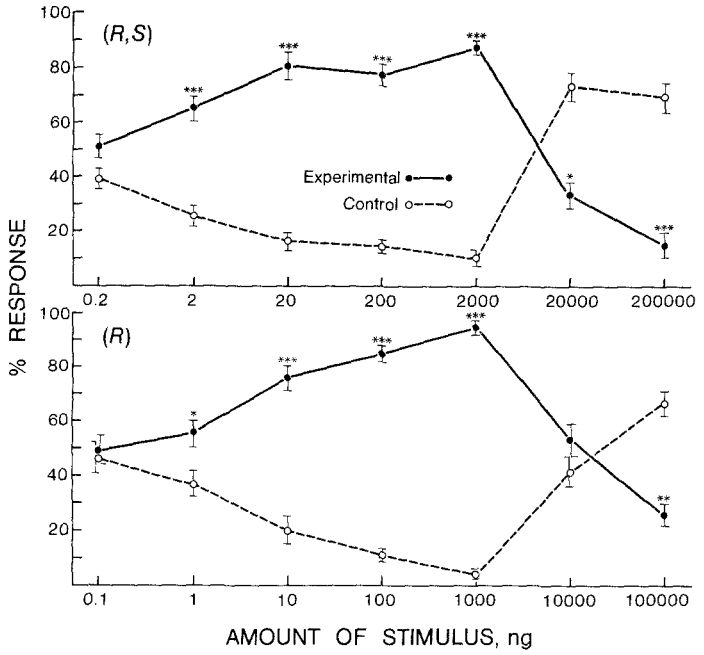

FIG. 9. Response ($\bar{X} \pm$ SE) of mixed-sex *C. quadricollis* in two-choice, pitfall olfactometer to (*R*)- and (*R,S*)-**1**. Significant response to experimental stimulus indicated by the following: $***P < 0.001$; $**P < 0.01$; $*P < 0.05$. $N = 12$ replicates.

DISCUSSION

In the laboratory, *C. quadricollis* are mobile and are excellent climbers of vertical glass surfaces. We thus hypothesized that after initially responding to a volatile stimulus, the beetles subsequently found no host-related stimuli and then crawled out of the vials, resulting in a low overall response at the end of a 2-hr bioassay (Table 1). The beetles, however, responded well to frass from the rearing culture, which is a good source of attractive semiochemicals for *Cryptolestes* and *Oryzaephilus* spp (Borden et al., 1979; A.M. Pierce et al., 1981; Wong et al., 1983; Millar et al., 1985a,b). Frass probably also provided host-related stimuli, as did the oat flake.

In their pitfall bioassay system, Phillips and Burkholder (1981) applied a thin strip of Teflon to the inner rims of the vials to prevent rice weevils, *Sitophlius oryzae* (L.), from crawling back into the chamber. Possibly, a kernel of grain in the vials might retain responding weevils. Similarly, to retain fruit flies,

TABLE 3. RESPONSE OF MIXED-SEX *C. quadricollis* TO (*R*)-, (*S*)-, AND (*R,S*)-1-OCTEN-3-OL (**5**), AND TO (*R,S*)-**1** (98% *E*) ALONE AND IN COMBINATION WITH (*R,S*)-**5** (*N* = 12 REPLICATES)

Experimental stimulus	Amount (ng)	Response, % ($\bar{X}$ ± SE)[a]	
		Experimental stimulus	Pentane control
(*R*)-1-Octen-3-ol	1	34.1 ± 4.0	41.7 ± 2.5 NS
	10	20.8 ± 4.2	63.9 ± 5.1***
	100	13.9 ± 3.3	79.2 ± 3.7***
	1000	13.9 ± 2.6	76.4 ± 3.7***
	10000	15.2 ± 3.4	71.0 ± 4.5***
(*S*)-1-Octen-3-ol	1	28.0 ± 2.8	47.0 ± 5.2*
	10	41.7 ± 5.2	38.2 ± 4.2 NS
	100	38.2 ± 4.8	47.9 ± 4.6 NS
	1000	16.4 ± 4.1	71.2 ± 5.2***
	10000	11.0 ± 1.9	78.6 ± 3.3***
(*R,S*)-1-Octen-3-ol	10	29.2 ± 4.8	57.6 ± 5.4*
	100	17.9 ± 3.1	62.1 ± 5.2***
	1000	23.3 ± 3.7	66.4 ± 4.2***
	10000	16.0 ± 2.8	66.0 ± 5.1***
(*R,S*)-**1**	1	59.0 ± 4.5	29.9 ± 3.6***
	10	81.9 ± 3.9	10.4 ± 2.7***
	100	82.6 ± 4.2	6.9 ± 3.1***
(*R,S*)-**1** + (*R,S*)-1-Octen-3-ol	1 + 10	67.8 ± 3.2	15.8 ± 3.2***
	10 + 100	57.9 ± 5.3	37.2 ± 5.9 NS
	100 + 1000	84.8 ± 3.2	9.0 ± 3.0***

[a] Significant positive or negative response to experimental stimulus indicated by the following: ***$P < 0.001$; *$P < 0.05$; NS, not significant.

Drosophila virilis, inside a semiochemical-baited vial, Bartelt et al. (1985) placed a drop of water on the vial bottom.

While the use of an auxillary substance with semiochemicals may seem to be unnecessary and adds an additional step to make possible a reliable and convenient laboratory bioassay, we suggest that these modifications better simulate the natural environment. Moreover, our results and the cited examples suggest that aggregation pheromones function not only to draw responding insects to the point of emanation but also to stimulate other behaviorial patterns such as searching for food and mates. If the secondary stimuli are not found, the responding insect then leaves the site.

In our earlier studies of cucujid grain beetles (Wong et al., 1983; Millar et al., 1985a,b; A.M. Pierce et al., 1985), we found that males produced two or more unsaturated macrocylic lactones which acted synergistically as aggre-

gation pheromones. Moreover, the response of mixed-sex beetles was not inhibited by the presence of the antipodes in the pheromone mixture. The major differences between *C. quadricollis* and the other cucujids studied to date is that the aggregation pheromone appears to be a single component. However, both quadrilure and macrolides are unsaturated esters.

The ee of natural quadrilure is 91%. Whether the *S* isomer of the pheromone acts as a synergist or is inactive has not been clearly established on the basis of the present data. The lower threshold for significant response by females as compared to males (Figure 6) might mean the *S* isomer acts as synergist or the females are just inherently more sensitive to *R* isomer than are the males. Additional bioassays would be required to resolve this question.

The terpene-like compound **4** is a homolog of the pheromone but has no apparent effect on ambulatory beetles in the modified pitfall olfactometer. However, its influence on in-flight beetles has not been tested. Although the geometry of the C-2,3 double bond of **4** is unknown, its skeletal structure is reminiscent of (2*Z*,6*Z*)-7-methyl-3-propyl-2,6-decadien-1-ol, which is produced by the codling moth, *Laspyeria pomonella* L. (Bowlus and Katzenellenbogen, 1973). Availability of the 2*E* and 2*Z* isomers of **4** would permit not only a more thorough investigation of its effects on the behavior of *C. quadicollis* but also confirmation of the exact structure of the natural compound.

The most interesting aspect of semiochemical production by *C. quadricollis* is the appearance of (*R*)-(−)-1-octen-3-ol (**5**) in the volatiles of segregated males and females. That the (*R*)-(−)-**5** is active is consistent with response by *C. quadricollis* to only the *R* isomer of the pheromone. Although 1-octen-3-ol is produced by a wide variety of molds and fungi, there was no visible mold growth in the aeration cultures, and production of this compound is possibly restricted to the beetles. Additional aerations under conditions of low moisture to preclude mold growth on the oats or of the segregated sexes in absence of food would have to be undertaken to confirm that the beetles produce **5.** Being a repellent, compound **5** could function alone as a female-produced epideictic pheromone (Prokopy, 1981) to promote dispersion of females. Because response to the aggregation pheromone is not reduced by a large excess of **5,** aggregation on a food source and mating would not be inhibited.

Acknowledgments—We thank Dr. R.B. Davis for a cutlure of *C. quadricollis*; Dr. A. Mosandl for samples of the chiral 1-octen-3-ols; Dr. G.G.S. King for some chirality determinations; and J. Dodic, A. Javer and E. Merletti for technical assistance.

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LIPID CUES FOR SEED-CARRYING BY ANTS IN *Hepatica americana*

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Abstract—We investigated the chemical basis for ants carrying the seeds of *Hepatica americana*, an ant-dispersed plant of eastern North America. A laboratory bioassay of seed and elaiosome extracts was based on the distance test items were carried by *Pogonomyrmex rugosus*. Ants responded equally to isolated elaiosomes and to the diglyceride fraction. Diolein was a major component of the diglyceride fraction, which is consistent with a finding that 1,2-diolein releases seed-carrying by *Aphaenogaster rudis*. Ants' response to the free fatty acid fraction was less intense. Gas chromatography of the fatty acid fraction indicated that oleic acid was a primary component, and oleic acid is known to elicit necrophoric behavior. No evidence supports an earlier suggestion that ricinoleic acid stimulates seed-carrying behavior. Ants failed to respond to seeds from which elaiosomes were removed.

Key Words—Hymenoptera, Formicidae, *Pogonomyrmex*, *Hepatica americana*, ants, Ranunculaceae, diglyceride, elaiosome, myrmecochory, seed dispersal

INTRODUCTION

The dispersal of seeds by ants is a widespread phenomenon involving many taxa (Beattie, 1985). Many species of plants, especially forest herbs of mesic forests, are dispersed by ants that are not granivorous. In these systems, ants usually are attracted to external appendages on seeds called elaiosomes (Sernander, 1906). Elaiosomes have evolved independently in several plant taxa,

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but generally elaiosomes are rich in lipids and carbohydrates (Bresinsky, 1963). Many species of ants that encounter elaiosomes quickly remove them (and the attached seeds) to nests, where the elaiosomes are fed to larvae. After elaiosomes are removed from seeds, the seeds may be scattered on refuse piles near ant nests or abandoned underground in the nest. The potential benefits to the plant include removal from parents with associated reduction of risks from parent–offspring competition or seed predation by rodents, and deposition in better microsites for germination and establishment (see Beattie, 1985, for a review).

Chemicals mediate the specific behavior of ants toward elaiosomes or any other potential food item (Wilson, 1971). Ants' possible responses to seeds include ignoring it, eating the elaiosome in situ, carrying the seed directly to a nest refuse area, carrying the seed to a nest, and recruiting other workers to the seed depot. Specific chemical releasers are known for each of these reactions, although only a relatively small number of species have been evaluated (Bradshaw and Howse, 1984). The fate of a seed is related to whether the elaiosome elicits feeding, necrophoric behavior, foraging, or recruitment, each of which may have chemical releasers.

The two previous studies of ants' response to elaiosomes disagree about the chemical that stimulates carrying behavior. Bresinsky (1963) identified ricinoleic acid as the primary stimulus for *Lasius fuliginosus* to carry elaiosomes of several plants, including *Viola odorata* (Violaceae). Marshall et al. (1979) found that the ant *Aphaenogaster rudis* did not respond to ricinoleic acid. Instead, they identified 1,2-diolein as the specific and only compound from *Viola odorata* elaiosomes that elicited carrying behavior.

Our study has two purposes. The first is to test whether the elaiosomes of *Hepatica americana* (Ranunculaceae) contain factors to elicit seed-carrying behavior and identify the active components. Second, we have modified the bioassay techniques reported by Marshall et al. (1979) to eliminate the need to define a complex behavioral index and the associated requirement of long periods of direct observation.

METHODS AND MATERIALS

Organisms. Hepatica americana is a spring-flowering, perennial herb, common in wooded habitats from Nova Scotia to northern Florida and west to Manitoba, Iowa, and Missouri. The flowers are without nectar, frequently opening before the last snows of spring, and seeds are produced through autogamy (Motten, 1982). Flowers in an outdoor experimental plot in Ohio produced an average of 9.7 seeds per flower (maximum = 20, $N = 54$). Seeds mature by late May and are dispersed by early June in Ohio and West Virginia.

The seeds are 5 mm long, 2 mm wide, and carry a lipid-rich elaiosome at one end.

Pogonomyrmex rugosus inhabits arid habitats from western Texas to southern Nevada and in central California between elevations of 240 and 1300 m (Snelling and George, 1979). It is a common harvester ant, which is primarily a seed eater, although it may eat insects. Seeds are taken to large nests, and in certain conditions stored seeds may sprout before being consumed (Wheeler, 1910; Creighton, 1950; Davidson, 1977). Consequently, seed harvesting may confer dispersal benefits, as has been documented for *Pogonomyrmex californicus* (Bullock, 1974; O'Dowd and Hay, 1980).

This combination of plant and ant species was chosen to test the generality of previously reported chemical stimulation of seed carrying by ants. Further, *P. rugosus* is readily available through commercial sources, reducing the seasonal problems of obtaining colonies. Ant colonies (Carolina Supply Co.) were maintained in plastic boxes (27 × 35 cm). The boxes had a plaster floor, and the sides were coated with Fluon to prevent the ants from escaping. At one side of each box, a test tube covered with aluminum foil and containing moistened cotton was used as an artificial nest.

Bioassay. The index of attractiveness was the distance a test item was moved by ants. Test items included whole seeds, seeds without elaiosomes, detached elaiosomes, and inert cubes containing elaiosome extract. Each assay was performed with 10 test items by placing them in the nest box at the end farthest from the ant nest. The origins of the test items were marked. After test items were exposed to ants for 60 min, we measured the distance each item was moved. This scoring method was used because the ants moved the majority of items at least 1 cm and because no assumptions were needed about the "intensity" of different ant behavioral categories (Marshall et al., 1979). Distances of movement were compared by analysis of variance with group differences determined by the T method (Sokal and Rohlf, 1981).

Polyporous strips (BioQuip Company) were cut into cubes approximately the size of a *H. americana* seed. The cubes were washed in methanol, then chloroform, and finally diethyl ether, then dried at 50°C. (Kuskis, 1978). Each cube was given a unique number. Both size and texture allowed ants to pick up cubes with their mandibles.

We used three controls: polyporous cubes washed and numbered as above; similar cubes with 5 μl chloroform added (the carrier used for elaiosome extracts); and *H. americana* seeds from which the elaiosomes had been removed. The last control tested for chemical stimulation of seed movement, as compared to ants' response to seed shape or texture.

Extraction and Identification of Attractant. Hepatica americana plants were obtained from Burpee Seed Co. Flowering was induced by maintaining potted

plants at 7°C during the day (8 hr) and 2°C at night (16 hr), then shifting daylength to 12 hr with temperatures of 21°C and 18°C. Harvested seeds were stored under nitrogen gas at −25°C in the presence of BHT to prevent autoxidation and transesterification reactions among unsaturated lipids (Wren and Szczepanowska, 1964).

The extraction process and associated bioassays were done twice. Total lipids were isolated by homogenizing elaiosomes according to Folch et al. (1957) at 0°C with a 2:1 (v/v) chloroform–methanol mixture containing 0.05% BHT. The tissue fraction was diluted to 20-fold the original volume of the tissue sample. After equilibrium at 24°C, the homogenate was filtered through a fatfree paper. The crude extract was washed by adding 0.2 its volume of chloroform–methanol–water (3:48:47) containing 0.02% $CaCl_2$. The extract was centrifuged at 4°C for 10 min at 2400 rpm; the upper phase was removed, and the interface was rewashed. Finally, the lower phase and the remaining upper phase were made into one phase by adding methanol. Nitrogen gas was used to evaporate solvent from the extract, which was then stored at −25°C.

Polar and nonpolar lipids were separated using a silicic acid column (Mallinckrodt silicic acid, 325 mesh) (Hirsh and Ahrens, 1958). The mixture of lipids was dissolved in as small a volume of chloroform as possible and pipetted onto the column. The separation was done with 150 ml chloroform (for nonpolar lipids) and then 150 ml methanol (for polar lipids).

Neutral glycerides were separated by thin-layer chromatography. Plates (Eastman Kodak Co. Chromagram Sheets, 100-μm-thick silica gel) were activated for 30 min at 115°C, then cooled to room temperature. Samples of 5 μl were dried under nitrogen gas. Neutral lipid separation was carried out at 5°C with two different solvent systems: hexane–diethyl ether–glacial acetic acid (30:70:1, v/v/v) and diethyl ether–isooctane (5:95, v/v; Wren, 1960). After chromatography, the solvent was evaporated from the plate with nitrogen (Skipski et al., 1965).

Lipid spots on the chromatograms were tentatively identified with iodine vapor, using reference compounds for orientation. The technique allowed detection of 0.1 μg of lipid (Simms and Larose, 1962). Lipids were eluted from the silica gel by scraping off the undetected lipid band from one plate at a location corresponding to an iodine-detected band from another plate. The lipids were extracted from the gel with 5 ml chloroform–methanol (4:1, v/v) and centrifuged to settle the gel (Kuskis, 1978). The extract was decanted and the procedure repeated with two further aliquots of diethyl ether (Skipski and Barclay, 1968). The extracts were combined and evaporated under nitrogen to a volume of 1 ml.

The TLC fractions were separated into mono-, di-, and triglycerides using HPLC. A commercially packed Radial Pak-Silica column, with 10-μm spheri-

cal particles (Waters Assoc.) was used. A mobile phase mixture of HPLC-grade hexane–isopropanol (95:5, v/v) was used at a flow rate of 1.0 ml/min, generating 350 psi within the system. The standards used were α-monopalmitin, diolein, and tristearin obtained from Sigma Chemical Company. Standards were dissolved in chloroform at 10 mg/ml and stored at $-25°C$ until used.

For gas chromatographic analysis, fatty acid methyl esters were prepared from the diglyceride fraction of the nonpolar lipids using BF_3–methanol (Metcalfe and Schmitz, 1961). The fatty acid methyl esters were separated on a Hewlett-Packard gas chromatograph-700 series with a 577-A Electrometer and a flame ionization detector. A stainless-steel column measuring 6 ft $\times$ $\frac{1}{8}$ in., packed with 10% SP-2330 on $\frac{100}{120}$ mesh Chromosorb W AW was used at 200°C with nitrogen as a carrier gas at 20 ml/min.

RESULTS

General Bioassay Results. Bioassay results were consistent for replicated extracts (for polar lipids, nonpolar lipids, and total lipids) and where different solvent systems were used for TLC (Table 1). Within treatments, coefficients of variation generally ranged between 10% and 40%.

The bioassay indicated four relatively homogeneous groups of treatments (Table 1). The controls, group I, were moved short distances if they were moved at all. These treatments included seeds with elaiosomes removed and polyporous strips washed with water or chloroform. At the other extreme, group IV treatments stimulated the most carrying behavior by ants. Items in this treatment group were usually carried the maximum distance, which was back to the ant nests. Included in Group IV were intact seeds, isolated elaiosomes, crude extracts of nonpolar lipids, and TLC fractions 3 (hexane–diethyl ether–acetic acid solvent), and 10 (diethyl ether–isooctane solvent). These TLC fractions will be shown below to include diglycerides. Storage did not affect results, as indicated by the similarity between intact seeds and isolated elaiosomes. The intact seeds were assayed within hours after they were harvested, while the isolated elaiosomes were stored at $-25°C$ (with antioxidant) for at least seven days after they were removed from diaspores.

Items in treatment group III were moved modest distances (Table 1), but significantly less than those for group IV. Activity was expected for the first lipid extract (total lipids) because it included nonpolar lipids. TLC fractions 13 and 5 also stimulated some carrying activity; these fractions included free fatty acids, separated by using different solvent systems. Finally, items from the group II treatments were investigated by ants, but not carried back to the nest. This group included polar lipids, mono- and triglycerides, and cholesterol.

TABLE 1. MOVEMENT OF BIOASSAY UNITS BY ANTS INDUCED BY COMPONENTS OF *Hepatica americana* SEEDS[a]

Treatment	Distance moved, cm (95% CI)	Homogeneous groups ($P < 0.05$)	Group
Water-washed polyporous cubes	0.8 (0.17)		I
$CHCl_3$-washed polyporous cubes	0.9 (0.18)		
Seeds without elaiosomes	1.8 (0.51)		
Polar lipid extract B	3.6 (0.67)		II
TLC-ISO fraction 2 (monoglycerides)	4.0 (0.52)		
TLC-ISO fraction 4 (triglycerides)	4.1 (0.47)		
TLC-HEX fraction 4 (triglycerides)	4.3 (0.45)		
TLC-HEX fraction 2 (monoglycerides)	4.4 (0.50)		
TLC-ISO fraction 5 (cholesterol stnd)	4.4 (0.55)		
Polar lipid extract A	6.5 (1.26)		
TLC-ISO fraction 6 (free fatty acids)	10.0 (0.64)		III
TLC-HEX fraction 5 (free fatty acids)	11.8 (0.58)		
Polar + nonpolar lipid extract A	12.7 (1.75)		
Polar + nonpolar lipid extract B	13.6 (0.90)		
TLC-ISO fraction 3 (diglycerides)	16.2 (0.56)		IV
TLC-HEX fraction 3 (diglycerides)	16.3 (0.47)		
Elaiosomes without seeds	16.5 (1.42)		
Nonpolar lipid extract B	17.3 (0.58)		
Intact seeds	17.5 (1.68)		
Nonpolar lipid extract A	17.8 (0.79)		

[a] Average distances (measured in cm) are followed by the 95% confidence interval of the mean. TLC solvent systems used were ether–isooctane (ISO) and hexane–diethyl ether–acetic acid (HEX). Lines connecting treatments indicate statistically indistinguishable distances moved (T method, Sokal and Rohlf 1981). Replicated extracts are indicated by A and B.

Identification of TLC Fractions. Both solvent systems showed two fractions of nonpolar lipids with significant potency to stimulate ant carrying behavior (Figure 1). From the plates run in hexane–diethyl ether–acetic acid (Figure 1A), both standards and HPLC indicated fraction 2 to be monoglycerides, fraction 3 to be diglycerides, and fraction 4 to be triglycerides. Fractions 2, 3, and 5 from the diethyl ether–isooctane solvent system (Figure 1B) corresponded to mono-, di-, and triglycerides. The fraction with the most activity in both solvent systems was diglycerides.

Gas chromatography of the active (diglyceride) fractions from both solvent systems (Figure 2) showed them to have peak retention times identical to oleic acid (18:1). This implicates either 1,2- or 1,3-diolein as the most common

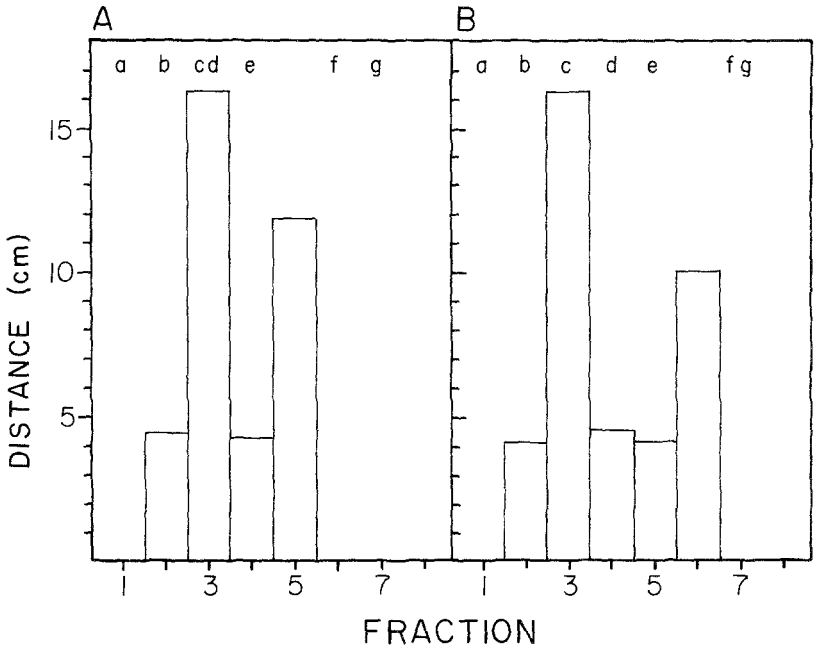

FIG. 1. Bioassay activity of fractions of nonpolar lipid extracts from thin-layer chromatography. The standards used for TLC separation were: α-lecithin (a), α-monopalmitin (b), diolein (c), cholesterol (d), tristearin (e), BHT (f), and squalene (g). The activity for each fraction is expressed as the average distance an inert cube coated with the fraction was moved by *Pogonomyrmex rugosus*. (A) Solvent = hexane–diethyl ether–acetic acid (30:70:1, v/v/v); (B) solvent = 5% diethyl ether in isooctane (v/v).

compound in the bioassays, although other fatty acids were observed, especially from the diethyl ether–isooctane solvent system.

The diglyceride fractions were not the only fractions of nonpolar lipids eliciting significant carrying behavior by ants. Fractions 5 (hexane–diethyl ether–acetic acid solvent) and 6 (diethyl ether–isooctane solvent) induced carrying behavior, but at a lower intensity than diglycerides (Table 1). These fractions had R_fs from the TLC plates that would correspond to hydrocarbons, esters, or fatty acids (Wren, 1960). Gas chromatography of the methyl esters showed that both fractions contain large peaks of 18-carbon, unsaturated fatty acids (Figure 3). The fatty acids represented were oleic (18:1), linoleic (18:2), and linolenic (18:3).

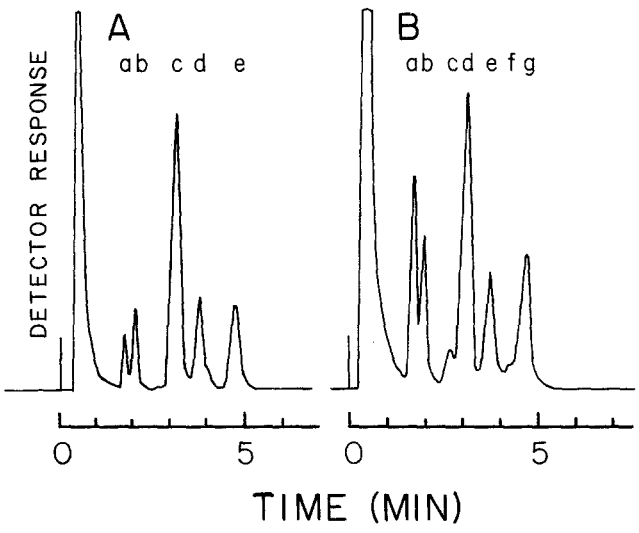

FIG. 2. Gas chromatography of methyl esters of fatty acids from diglycerides that were isolated with TLC. (A) From TLC fraction 3, using hexane–diethyl ether–acetic acid solvent. Identified fatty acids included palmitoleic (a), stearic (b), oleic (c), linoleic (d), and linolenic (e). (B) From TLC fraction 3, using ethyl ether–isooctane solvent. Identified fatty acids included palmitic (a), palmitoleic (b), stearic (c), oleic (d), linoleic (e), arachidonic (f), and linolenic (g).

DISCUSSION

Our bioassay for chemical inducement of carrying is simpler than previous measures. Marshall et al. (1979) used an index based on specific behaviors of the ants as they responded to test substances. Others (Gordon, 1983; Wilson et al., 1958; Howard and Tschinkel, 1976) have used the frequency of movement of test items. Using distances has several advantages. During tests, ant colonies do not have to be directly observed for hours, allowing more tests to be conducted simultaneously. Our bioassay yields ratio data that are more easily interpreted and replicated than indices based on behavioral categories. Distances also provide more information on the degree of ant reaction than scoring test items as moved or not moved.

Interpreting any bioassay using ant behavior requires consideration of ant social behavior. The social context of an ant colony can influence workers' responses to chemicals. For example, the activity patterns of *Pogonomyrmex badius* colonies influenced the deposition sites of pieces of filter paper covered with oleic acid (Gordon, 1983). Variation added by different social situations

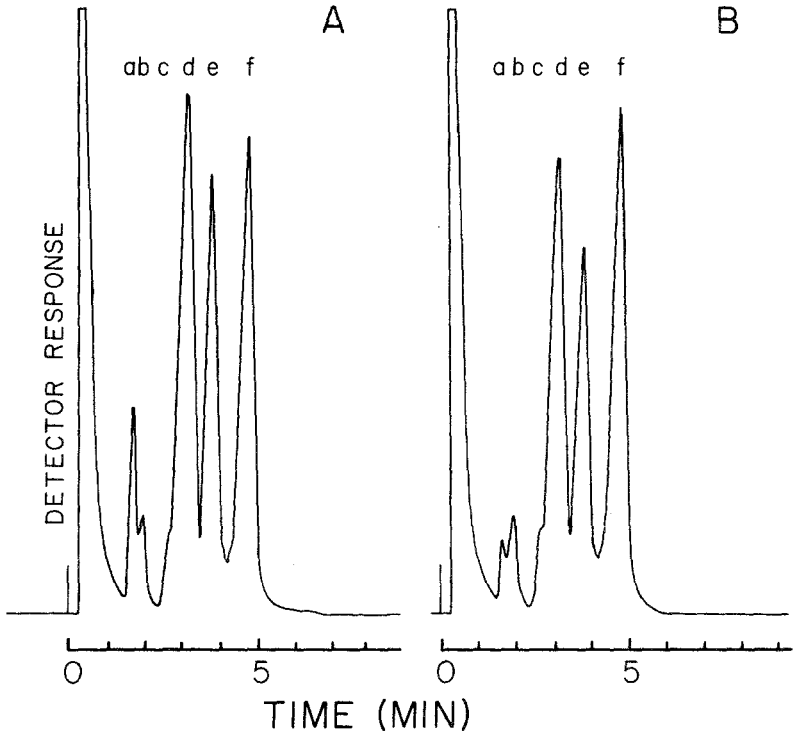

FIG. 3. Gas chromatography of methyl esters of free fatty acids isolated with TLC. (A) From TLC fraction 5, using hexane–diethyl ether–acetic acid solvent. (B) From TLC fraction 6, using diethyl ether–isooctane solvent. Identified peaks from both A and B are: palmitic (a), palmitoleic (b), stearic (c), oleic (d), linoleic (e), and linolenic (f) acids.

should be minimal in our bioassay for the following reasons. First, all ants were workers so there were no differences among colonies or through time as a result of a developing brood. Second, the ants were maintained in artificial nests, minimizing nest repair behavior. Finally, knowing the specific deposition site (nest vs. refuse pile or in between) is not critical for determining components of seeds that elicit carrying behavior. Consequently the simple measurement of distances is adequate for this purpose and has the advantages mentioned above.

Diglycerides on *Hepatica americana* seeds are responsible for their attractiveness to *Pogonomyrmex*. Given the predominance of oleic acid in the gas chromatographic analysis of the diglyceride fraction, diolein was the most common diglyceride isolated for *Hepatica americana* elaiosomes. This parallels the

findings of Marshall et al. (1979) that the primary active portion of *Viola odorata* elaiosomes is 1,2-diolein. They also report that 1,3-diolein does not elicit seed-carrying by *Aphaenogaster rudis*. Although the free fatty acid fraction also stimulated some carrying, this was only about 60% as effective as the isolated diglycerides. Intact seeds, isolated elaiosomes, and diglycerides showed the same level of activity in the bioassay, so free fatty acids do not add measurably to ants' response to fresh *Hepatica* seeds. In the absence of diglycerides, however, free fatty acids should induce some seed carrying by ants.

Previous studies of the chemical nature of ant–seed interactions give conflicting results. Bresinsky's (1963) original work demonstrated the importance of lipids to stimulation of seed-carrying behavior. Using bioassays with standards, he identified the free fatty acid ricinoleic acid as a stimulant but did not mention diglycerides. Amounts of ricinoleic acid sufficient to stimulate seed carrying by *Lasius fuliginosus* were isolated from two *Luzula* spp., *Melica nutans*, *Veronica hederifolia*, and four *Viola* spp. Marshall et al. (1979) observed no response of *Aphaenogaster rudis* to ricinoleic acid, but these ants responded strongly and specifically to the diglyceride 1,2-diolein. In their study, ricinoleic acid was clearly not an active compound in elaiosomes of *Viola odorata* (a species studied by Bresinsky). Ours is the third study of this phenomenon, and our results for *Hepatica americana* and *Pogonomyrmex* point to 1,2-diolein (or at least a similar diglyceride) as the primary attractant; although free fatty acids from *H. americana* attracted ants, we could not identify a significantly large amount of ricinoleic acid in *H. americana* elaiosomes. A conservative conclusion is that the basis for seed carrying varies with species of plant, ant, or both. Alternately, Bresinsky (1963) may have missed the major source of natural attraction in the species he studied because diglycerides were not included in that research program.

The stimulation of seed-carrying behavior is different from mechanisms promoting other kinds of ant feeding and carrying behavior. Feeding behavior in *Solenopsis* is released by a variety of compounds, but neither ricinoleic acid nor diolein are phagostimulants (Vinson et al., 1967; Vinson, 1972). In terms of dispersal, direct stimulation of feeding would potentially inhibit the carrying of seeds back to nests. Corpse-carrying behavior is stimulated by oleic acid for *Solenopsis* spp. (Wilson et al., 1958; Blum, 1970; Howard and Tschinkel, 1976), *Myrmica vindex* (Blum, 1970), and *Pogonomyrmex badius* (Gordon, 1983). Oleic acid did not stimulate seed-carrying behavior for *Aphaenogaster rudis* (Marshall et al., 1979). *Pogonomyrmex rugosus*, in this study, may have showed a weak response to the fatty acid fraction, perhaps because it contained oleic acid. In nature, responding to seeds as "corpses" would probably result in movement of seeds to refuse piles rather than into ant nests. The nutritional benefit of elaiosomes to larvae would then be lost.

One proposal to explain why ants carry elaiosome-bearing seeds to nests

is that the chemical stimulants of carrying in elaiosomes mimic (or are the same as) compounds that trigger ant responses to arthropod prey (Carroll and Janzen, 1973; Horvitz and Beattie, 1980). Alternatively, brood-tending behavior may be related to seed carrying. Bigley and Vinson (1975) concluded that triolein induces *Solenopsis invicta* to carry objects to brood chambers and to tend inert paper as they would treat sexual brood. In their experiments, diolein evoked similar behavior, but at a lower intensity. There are insufficient data to test hypotheses about why ants as taxonomically different as *Pogonomyrmex rugosus* and *Aphaenogaster rudis* should show similar responses to diolein. Many other ant species pick up elaiosomes and seeds (Beattie, 1985), so this chemical mechanism (or mechanisms) may be widespread.

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CHEMOTAXONOMIC IMPLICATIONS OF THE VENOM CHEMISTRY OF SOME *Monomorium "antarcticum"* POPULATIONS

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Abstract—A comparative analysis of the venom alkaloids produced by ants in the genus *Monomorium* (=*Chelaner*) collected on North Island and South Island, New Zealand, has been undertaken. All of the ants produce *trans*-2,5-dialkylpyrrolidines along with 3,5-dialkylpyrrolizidines. The structures and sterochemistry of the novel alkaloids *trans*-2-butyl-5-(8-nonenyl)pyrrolidine, (5*E*,8*Z*)-3,5-di(5-hexenyl)pyrrolizidine, and (5*Z*,8*E*)-3-methyl-5-(8-nonenyl)pyrrolizidine were established by unambiguous synthesis. The geographic distribution and the chemotaxonomic significance of the alkaloids produced by these ants are discussed.

Key Words—Ants, *Monomorium*, Hymenoptera, Formicidae, 2,5-dialkylpyrrolidines, 3,5-dialkylpyrrolizidines, ant venom alkaloids, chemotaxonomy.

INTRODUCTION

A large variety of saturated nitrogen heterocycles has been identified in the venoms of ant species in the myrmicine genera *Monomorium* and *Solenopsis* (Jones et al., 1982b). 2,6-Dialkylpiperidines and 2,5-dialkylpyrrolidines are most commonly found, and (5*Z*,9*Z*)-3-alkyl-5-methylindolizidines have also been reported from both genera (Ritter and Persoons, 1975; Jones et al., 1984). On the other hand, except for the occurrence of (5*Z*,8*E*)-3-heptyl-5-methylpyrrolizidine in a *Solenopsis* sp. (Jones et al., 1980b), the 3,5-dialkylpyrrolizidine system has not been detected in the alkaloids produced by any other formicid species.

Recently we isolated and identified (5*E*,8*Z*)-3-(8-nonenyl)-5-(*E*,1-propenyl)pyrrolizidine (**5**) as the major alkaloidal component in the venom of *Monomorium* (=*Chelaner*) *antarcticum* (Jones et al., 1986; Bolton, 1987). In order to determine whether these results were typical of other *Monomorium* populations, we have analyzed the alkaloidal extracts of single-nest collections from 28 locations on North and South Island, New Zealand (Figure 1). The results show that there are major differences in the alkaloids produced by different populations. A variety of pyrrolidines and pyrrolizidines have been detected, and the structures and sterochemistry of one novel pyrrolidine and two novel pyrrolizidines from these ants have been established. These results further indicate that the venom alkaloids of these ants may provide useful taxonomic characters.

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic analyses were performed on a Gow-Mac model 750P using a 2-m × 2-mm-ID glass column packed with 5% SP-1000 on 100–120 mesh Supelcoport. This instrument was programmed from 40°C to 200°C at 10°C/min as soon as the solvent had eluted. Retention times and temperatures were found to be reproducible within one degree on a given day. Preparative gas chromatography was performed on a Varian model 920 using a 2-m × 5-mm-ID aluminum column packed with either 10% SP-2100 or 10% SP-1000 on 100–120 mesh Supelcoport. Infrared spectra were obtained from neat liquid films with a Perkin-Elmer 1320 grating infrared spectrophotometer. [^{1}H]NMR spectra were obtained from $CDCl_3$ solutions at 80 MHz using a Varian FT-80 spectrometer and at 360 MHz using a Nicolet 360-MHz spectrometer. Chemical shifts (δ) are reported downfield from tetramethylsilane in parts per million (ppm). Mass spectra were obtained using an LKB-9000 GC-MS at an ionizing voltage of 70 eV and fitted with a 2-m × 2-mm-ID glass column packed with 1% SP-1000. High-resolution mass spectra were obtained

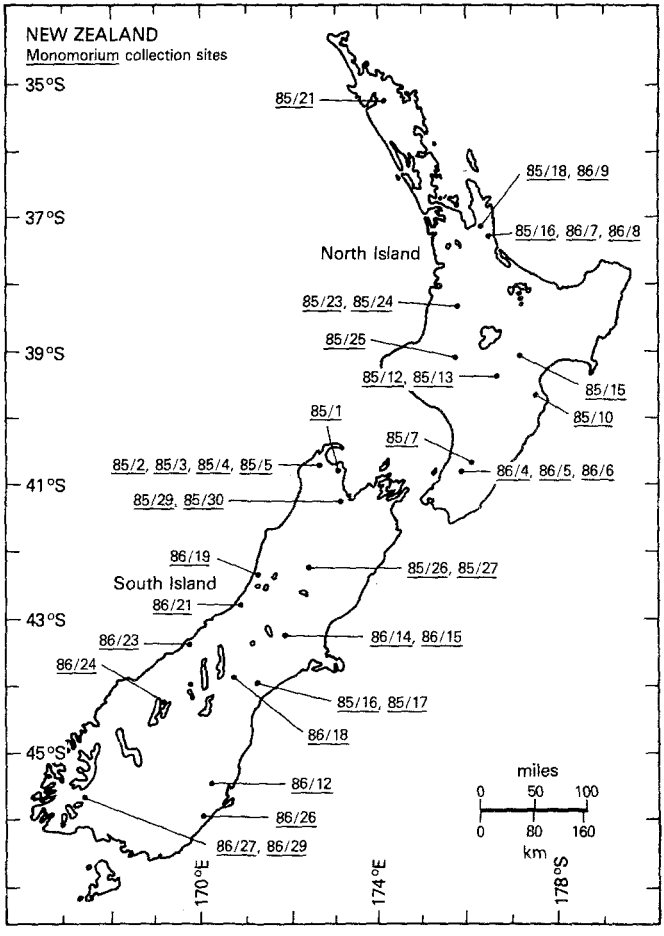


FIG. 1. Map of New Zealand showing the *Monomorium* collection sites.

using a VG-7070F in the EI mode at an ionizing voltage of 70 eV. Melting points are uncorrected.

Ants. The ants were collected during the summer months (December–February) of 1984–1985 and 1985–1986 at the sites indicated on the map. Each collection (Table 1) of ca. 100–200 workers of *Monomorium* sp. was made from a separate nest and immediately placed in a vial containing 1–2 ml of methylene chloride. In addition, five to nine individual queens from nests 85/24, 85/25, 85/26, 85/27, and 86/17 were also collected and stored separately. For analyses, these mixtures were reduced to ca. 0.2 ml with a slow stream of nitrogen.

TABLE 1. ALKALOIDS FOUND IN NEW ZEALAND *Monomorium* POPULATIONS

Collection number	Site	Alkaloids[a]										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
85/1	Motupipi, N.W. Nelson South Island, N.Z.	–	–	–	–	*	–	–	–	–	–	–
85/2	Aorere Valley, N.W. Nelson South Island, N.Z.	0	–	–	+	–	–	*	0	–	–	–
85/3	Aorere Valley, N.W. Nelson South Island, N.Z.	–	–	–	–	*	–	–	–	–	–	–
85/4	Aorere Valley, N.W. Nelson South Island, N.Z.	+	–	–	+	–	–	*	+	–	–	–
85/5	Aorere Valley, N.W. Nelson South Island, N.Z.	+	–	–	–	*	0	–	–	–	–	–
85/7	Tararua Forest Park South Island, N.Z.	+	–	–	–	*	0	–	–	–	–	–
85/10	Japeka Orchard, Hastings North Island, N.Z.	–	–	–	+	–	–	*	–	–	–	–
85/12	W. of Taruarau Hill Summit North Island, N.Z.	–	–	–	+	–	–	*	–	–	–	–
85/13	8 km E. of Taruarau Hill North Island, N.Z.	+	–	–	–	*	0	–	–	–	–	–
85/15	Summit of Napier-Taupo Hwy North Island N.Z.	+	–	–	–	*	0	–	–	–	–	–
85/16	10 km N. of Waihi North Island, N.Z.	0	–	–	–	*	0	–	–	–	–	–
85/18	Kauaeranga Valley North Island, N.Z.	0	–	–	–	*	0	–	–	–	–	–
85/21	16 km N. of Okaihau North Island, N.Z.	–	0	–	+	+	–	–	–	*	+	–
85/23	26 km S. of Te Kuiti North Island, N.Z.	–	–	–	–	*	–	–	–	–	–	–
85/24	30 m from 85/23	–	–	–	–	–	–	*	0	–	–	–
85/24	Queens		–	–	0	–	–	*	–	0	–	–
85/25	8 km N. of National Park North Island, N.Z.	–	–	–	–	*	–	–	–	–	–	–
85/25	Queens		0	–	–	*	0	–	–	0	–	–
85/26	Maruia Springs Bridge South Island, N.Z.	0	0	*	–	–	–	+	–	–	–	+
85/26	Queens	–	–	*	–	–	–	*	–	–	–	0
85/27	Maruia Springs Bridge South Island, N.Z.	+	–	0	–	–	–	*	0	–	–	–
85/27	Queens	–	–	+	–	–	–	*	–	–	–	–
85/29	Motueka R. near Tapawera South Island, N.Z.	0	–	–	–	*	0	–	–	–	–	–
85/30	Motueka R. near Tapawera South Island, N.Z.	–	–	–	–	–	–	–	–	–	–	–
86/4	Tararua Forest Park North Island, N.Z.	–	–	–	–	*	–	–	–	–	–	–

TABLE 1. Continued

Collection number	Site	Alkaloids[a] 1	2	3	4	5	6	7	8	9	10	11
86/5	Tararua Forest Park North Island, N.Z.	–	–	–	–	–	–	*	–	–	–	–
86/6	Tararua Forest Park North Island, N.Z.	–	–	–	–	*	–	–	–	–	–	–
86/7	10 km N. of Waihi North Island, N.Z.	–	–	–	–	*	0	–	–	–	–	–
86/8	10 km of Waihi North Island, N.Z.	–	–	–	–	–	–	*	–	–	–	–
86/9	Kauaeranga Valley North Island, N.Z.	–	–	–	–	*	0	–	–	–	–	–
86/12	14 km E. of Middlemarch South Island, N.Z.	+	–	+	0	*	–	–	–	–	–	–
86/12	Males	–	–	–	–	–	–	–	–	–	–	–
86/14	Porter's Pass, 15 km W. of Springfield South Island, N.Z.	–	–	–	+	*	–	–	–	–	–	–
86/15	Porter's Pass, 15 km W. of Springfield South Island, N.Z.	+	–	0	0	*	0	–	–	–	–	–
86/16	Peel Forest South Island, N.Z.	+	–	0	0	*	0	–	–	–	–	–
86/16	Queens	–	–	–	–	*	0	–	–	–	–	–
86/17	Peel Forest South Island, N.Z.	+	–	–	–	*	0	–	–	–	–	–
86/17	Queens	–	–	–	–	*	0	–	–	–	–	–
86/18	Burke's Pass 16 km E. of Tekapo South Island, N.Z.	+	–	–	–	*	0	–	–	–	–	–
86/19	North Beach, Cobden Greymouth, South Island, N.Z.	0	–	–	–	*	0	–	–	–	–	–
86/21	Lake Kanieri South Island, N.Z.	–	–	–	–	*	0	–	–	–	–	–
86/23	17 km N. of Karangarua River South Island, N.Z.	0	–	–	–	*	0	–	–	–	–	–
86/24	24 km N. of Lake Hawae South Island, N.Z.	0	–	–	–	*	0	–	–	–	–	–
86/26	Waipori Gorge 19 km from Outram, South Island, N.Z.	0	–	–	–	*	0	–	–	–	–	–
86/27	Lake Monowai South Island, N.Z.	–	–	+	–	–	–	*	0	–	–	+
86/29	1 km from Lake Monawai South Island, N.Z.	0	–	–	0	–	–	*	–	–	–	–

[a] * = major component; + = minor component (10–15%); 0 = trace; – = not detected.

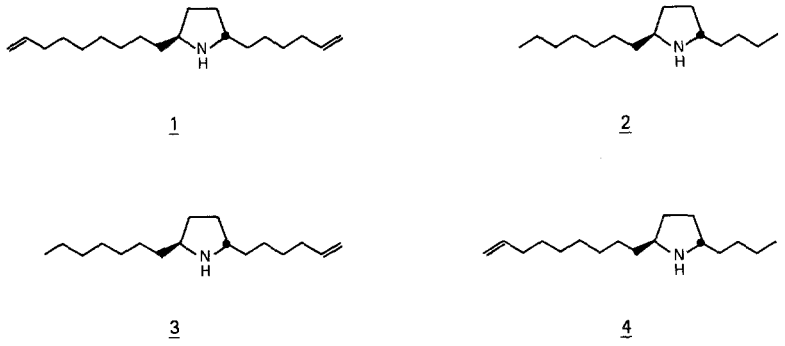


FIG. 2. Structures of alkaloids 1–4.

Where they were major or minor components, the structures of alkaloids **1–9** (Fig. 2 and 3) were assigned by comparison of their mass spectra and gas chromatographic retention times with those of authentic samples. Compounds **1, 2, 5,** and **6** were available from previous studies (Jones et al., 1980a, 1986). The mass spectrum observed for (5*E*,8*Z*)-3-(8-nonenyl)-5-(*E*,1-propenyl)pyrrolizidine (**5**) was as follows: *m/z* (rel intensity) 275(2, M+), 274(2), 271(1), 260(1), 246(1), 244(1), 234(1), 160(2), 151(11), 150(100), 122(6), 110(1), 109(2), 108(3), 107(3), 106(1), 105(1), 95(1), 94(1), 91(1), 82(5), 81(4), 80(4), 80(4), 79(8), 70(2), 69(3), 68(9), 67(7), 56(3), and 55(12). The

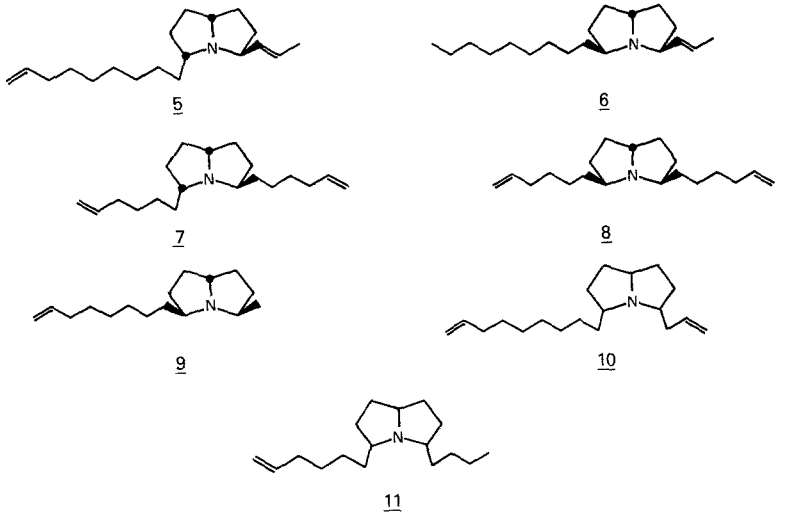


FIG. 3. Structures of alkaloids 5–11.

preparations of **3, 4, 7, 8,** and **9** are described herein. A small amount (ca. 0.1 mg) of the major alkaloidal component from collection 85/4 was obtained by preparative gas chromatography. The 360-MHz ^{1}H spectrum showed only the signals at δ = 5.7 (d of d of t, J = 18, 10, and 6 Hz), 5.0 (br d, J = 18 Hz), and 4.9 (br d, J = 10 Hz) in the olefinic region of the spectrum.

In collection 85/21, the structure of a minor component, 3-allyl-5-(8-nonenyl)pyrrolizidine (**10**), was suggested by its mass spectrum: *m/z* (rel intensity) 275(2, M+), 274(1), 271(0.5), 234(100), 160(0.5), and 150(30). In collections 85/26 and 86/27 a minor component, 3-butyl-5-(5-hexenyl)pyrrolizidine (**11**), was also suggested by its mass spectrum: *m/z* (rel intensity) 249(6, M+), 248(3), 245(6), 202(18), 193(12), 192(76), 176(17), 167(10), 166(100), and 124(5).

1-Heptadecen-7,10-dione(12a). A solution containing 2.9 g of 1-dec-3-one (19 mmol), 2.1 g of 6-heptenal, and 0.5 g of 5-(2′-hydroxyethyl)-4-methyl-3-benzylthiazolium chloride in 3 ml of triethylamine was heated to reflux overnight under a nitrogen atmosphere. After the usual work up, Kugelrohr distillation (0.01 mm Hg) gave 3.0 g (60%) of solid diketone **12a:** mp 48–49°C; NMR δ = 5.8 (1H, d of d of t, J = 18, 10, and 6 Hz), 5.0 (1H, br d, J = 18 Hz), 4.9 (1H, br d, J = 10 Hz), 2.67 (4H, s), 2.45 (4H, t, J = 6.8 Hz), 2.05 (2H, m), 1.6–1.26 (14H, m), and 0.87 (3H, br t); MS *m/z* (rel. intensity) 266(3, M+), 211(15), 198(33), 183(22), 182(6), 167(5), 155(36), 154(20), 140(13), 139(20), 136(10), 127(74), 114(38), 111(37), 107(22), 95(48), 83(49). 81(19), 71(50), 69(43), 68(20), 67(33), 57(65), 55(81), 43(94), and 41(100). Calculated for $C_{17}H_{30}O_2$, 266.2246; observed, 266.2226.

2-Heptyl-5-(5-hexenyl)pyrrolidine (Monomorine IV) (3). A solution containing 1.0 g (3.8 mmol) of diketone **12a,** 0.3 g of ammonium acetate, 0.3 g of sodium cyanoborohydride, and 0.05 g of potassium hydroxide in 15 ml of methanol was stirred overnight under a nitrogen atmosphere. The usual work up provided 0.7 g of an oily product that was greater than 90% pure by gas chromatographic analysis. Gas chromatography on a 5% SP-1000 column showed two isomeric components having retention times of 3.0 and 3.2 min under isothermal conditions (200°C), and having identical mass spectra. MS *m/z* (rel intensity) 251(5, M+), 250(2), 194(8), 181(5), 178(7), 169(10), 168(90), 153(12), 152(100), 96(7), 86(4), 84(8), 83(8), 82(19), 69(9), 67(10), 55(12), and 41(13). Calculated for $C_{17}H_{23}N$, 251.2613; observed, 251.26099.

16-Heptadecen-5,8-dione(12b). A mixture containing 0.9 g (8 mmol) of 1-hepten-3-one, 1.2 g of 9-decenal, and 0.26 g of 5-(2′-hydroxyethyl)-4-methyl 3-benzylthiazolium chloride in 2.5 ml of triethylamine was heated to reflux overnight under a nitrogen atmosphere. After the usual work up, 2.0 g of crude (ca. 85% pure by GLC analysis) diketone **12b** was isolated. Preparative GLC provided a sample with the following physical characteristics: mp 49–50°C; δ = 5.8 (1H, d of d of t, J = 18, 10, and 6 Hz), 5.0 (1H, br, d, J = 18 Hz),

4.9 (1H, br d, J = 10 Hz), 2.66 (4H, s), 2.44 (4H, br t), 2.02 (2H, br m), 1.28 (14H, br m), 0.89 (3H, br t); MS *m/z* (rel intensity) 266(3, M±), 224(7), 209(12), 181(11), 169(3), 156(58), 153(8), 141(68), 127(30), 114(46), 113(40), 95(36), 85(89), 83(85), 81(30), 71(46), 69(58), 67(34), 57(89), 55(96), 43(32), and 41(100). Calculated for $C_{17}H_{30}O_2$, 266.2246; observed, 266.22309.

2-Butyl-5-(8-nonenyl)pyrrolidine(4). A mixture containing 1.0 g of diketone **12b,** 0.3 g of ammonium acetate, and 0.25 g of sodium cyanoborohydride in 15 ml of anhydrous methanol was stirred overnight under a nitrogen atmosphere. After the usual work up, 0.8 g of an oily product was obtained that was ca. 85% pure by GLC analysis. Gas chromatographic analysis (5% SP-1000 column at 200°C) showed two components having retention times of 3.2 and 3.6 min, respectively, with identical mass spectra: MS *m/z* (rel intensity) 251(1, M+), 250(2), 220(1), 208(2), 207(2), 206(2), 195(12), 194(77), 180(1), 166(2), 152(6), 127(12), 126(100), 96(4), 83(6), 82(18), 69(5), 68(5), 67(6), 55(13), and 41(12). Calculated for $C_{17}H_{33}N$, 251.2613; observed, 251.26025.

1,18-Nonadecadien-7,10,13-trione (13). A solution containing 10 g of 6-heptenal (89 mmol), 3.7 g of divinyl ketone (45 mmol), and 2.5 g of 5-(2′-hydroxyethyl)-4-methyl-3-benzylthiazolium chloride in 12 ml of triethylamine was mechanically stirred and heated under a nitrogen atmosphere to 70°C overnight. After the work up, the crude triketone was recrystallized from methanol to give 7.6 g of white crystals: mp 72–74°C; NMR δ = 5.8 (2H, d of d of t, J = 8, 10 and 6 Hz), 5.0 (2H, br d, J = 18 Hz), 4.9 (2H, br d, J = 10 Hz), 2.70 (8H, s), 2.45 (4H, br t, J = 7 Hz), 2.01 (4H, M), 1.5 (8H, m); MS *m/z* (rel intensity) 306(6, M+), 288(8), 238(26), 223(12), 220(18), 210(6), 195(30), 177(10), 167(12), 163(16), 139(30), 127(48), 121(12), 111(90), 107(46), 99(18), 83(50), 71(10), 69(24), 68(16), 67(28), 55(100), and 41(50). Calculated for $C_{19}H_{30}O_3$, 306.2194; observed, 306.2175.

Reductive Amination of 13. A solution containing 1.0 g of triketone **13** (3.3 mmol), 0.25 g of ammonium acetate, 0.2 g of sodium cyanoborohydride, and 0.05 g of potassium hydroxide in 50 ml of methanol was stirred overnight under a nitrogen atmosphere. After the usual work up, 0.6 g of an oily residue was obtained which was nearly 90% pure by GLC analysis. Gas chromatographic analysis (2-m × 2-mm column packed with 5% SP-1000 on Supelcoport) at 200°C showed three components, **14, 8,** and **7** in the ratio of 1:9:3, which had retention times of 2.8, 4.0, and 10.4 min, respectively. The three components had almost identical mass spectra: MS *m/z* (rel intensity) 275(4, M+), 271(2), 234(5), 202(5), 193(15), 192(100), 164(3), 122(4), 109(4), 108(5), 82(5), 81(6), 69(4), 68(8), 67(8), 67(16), 55(14), and 41(25). Calculated for $C_{19}H_{33}N$, 275.2613; observed, 275.2607.

The three isomers, eluting in the order **14, 8,** and **7,** were separated by preparative GLC (2-m × 5-mm column packed with 10% SP-1000 on Supelcoport) and [^{1}H]NMR spectra were obtained for each. All three showed signals

at δ = 5.8(2H, d of d of t, J = 18, 10, and 6 Hz), 5.0 (2H, br d, J = 18 Hz), and 4.9 (2H, br d, J = 10 Hz). In addition to a broad multiplet from δ = 2.3–1.1 (24H), the three isomers showed the following signals in the δ = 4.0–2.5 region: **14,** none; **8,** δ = 3.6 (1H, br m), 2.6 (2H, br m); **7,** δ = 3.6 (1H, m), 2.94 (2H, br m).

16-Heptadecen-2,5,8-trione(15). A solution containing 1.54 g (10 mmol) of 9-decenal, 1.35 g of 6-hepten-3,5-dione, and 0.27 g of 5-(2′-hydroxyethyl)-4-methyl-3-benzylthiazolium chloride in 1.4 g of triethylamine was heated to reflux overnight under a nitrogen atmosphere. After the usual work up, 3.2 g of crude triketone **15** were obtained. A sample purified by sublimation had mp 59–62°C; NMR δ = 5.75 (1H, d of d of t, J = 18, 10, and 6 Hz), 5.0 (1H, br d, J = 18 Hz), 4.9 (1H, br d, J = 10 Hz), 2.65 (8H, s), 2.4 (2H, br t), 2.13 (3H, s), 2.08 (2H, m), and 1.25 (10H, m); MS *m/z* (rel intensity 280(4.M+), 262(2), 237(2), 219(1), 209(4), 181(3), 170(28), 155(12), 153(4), 152(27), 135(10), 127(100), 112(7), 111(8), 109(12), 99(85), 95(27), 94(8), 83(17), 81(12), 71(17), 69(24), 67(16), 55(46), 43(62), and 41(35). Calculated for $C_{17}H_{28}O_3$, 280.2038; observed, 280.2025.

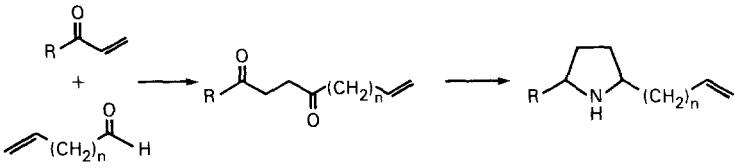
3-Methyl-5-(8-nonenyl)pyrrolizidine (9). A solution containing 1.48 g of triketone **15** (5.3 mmol), 0.51 g of ammonium acetate, 0.67 g of sodium cyanoborohydride, and 0.09 g of potassium hydroxide in 20 ml of methanol was stirred overnight under anhydrous conditions. After the usual work up, GLC analysis (2-m × 2-mm column packed with 5% SP-1000 of 100–120 Supelcoport) showed four components in the ratio 1:40:7:3 (in order of elution) having almost identical mass spectra. MS *m/z* (rel intensity) 249(3,M+), 248(2), 245(0.5), 234(10), 220(2), 180(2), 166(2), 152(2), 138(1), 134(2), 125(11), 124(100), 110(3), 84(5), 81(6), 55(7), and 41(10). Calculated for $C_{17}H_{31}N$, 249.2456; observed, 249.2450.

Samples of the first and second eluting isomers were obtained by preparative GLC (2-m × 5-mm column packed with 10% SP-1000 on 100–120 Suplecoport). The IR spectrum of the first eluting isomer had important bands at 3080, 2915, 2860, 2790, 2700, 2680, 1640, 990, and 905 cm^{-1}. The second eluting, major isomer had NMR δ = 5.8 (1H, d of d of t, J = 18, 10, and 6 HZ), 5.0 (1H, br d, J = 18 Hz), 4.9 (1H, br d, J = 10 Hz), 3.6 (1H, br m), 2.65 (2H, brm), 1.9 (6H, br m), 1.29 (16H, m), and 1.09 (3H, d, J = 5.7 Hz).

RESULTS

The distribution of alkaloids found in the *Monomorium* population samples is shown in Table 1, and the structures of these compounds are depicted in Figures 2 and 3. The pyrrolidines **1–4** are characterized from their mass spectra, which show a pair of intense even mass peaks resulting from α-cleavage of side

chains and a weak molecular ion of odd mass: 2-(5-hexenyl)-5-(8-nonenyl)pyrrolidine (**1**), *m/z* = 277(M+), 194(100), and 152(83); 2-butyl-5-heptylpyrrolidine (**2**), *m/z* = 225(M+), 168(100), and 126(80); 2-heptyl-5-(5-hexenyl)pyrrolidine(**3**), *m/z* = 251(M+), 168(90), and 152(100); 2-butyl-5-(8-nonenyl)pyrrolidine(**4**), *m/z* = 251(M+), 194(77), and 126(100). Pyrrolidines **1** and **2** were available from previous investigations (Jones et al., 1980a, 1982a), and pyrrolidines **3** and **4** were prepared by the previously described methodology based on the reductive amination of the corresponding 1,4-diketones (Scheme 1). The synthetic compounds **1–4** obtained in this manner are nearly



12a; R = C_7H_{15}, n = 4 3; R = C_7H_{15}, n = 4
12b; R = C_4H_9, n = 7 4; R = C_4H_9, n = 7

SCHEME 1.

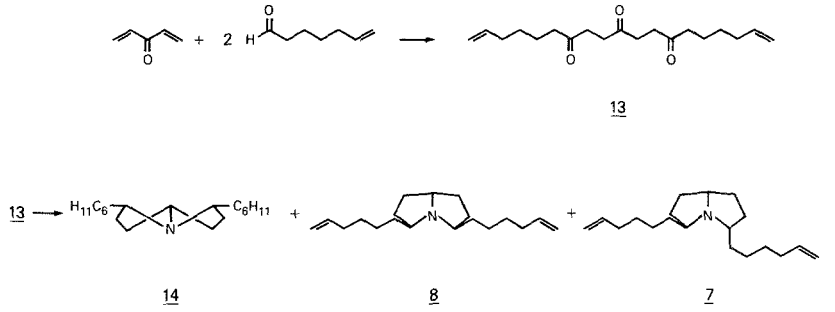
a 1:1 mixture of *cis*- and *trans*-2,5-dialkylpyrrolidine stereoisomers, with the *cis* isomer eluting first from a SP-1000 GC column (Jones et al., 1979; Pedder et al., 1976). In all the ant extracts providing more than trace amounts, direct comparison showed that the natural pyrrolidines **1–4** had gas chromatographic retention times identical with those of the later eluting *trans* isomers.

The 3,5-dialkylpyrrolizidines **5** and **7** are the characteristic major alkaloidal components from all the *Monomorium* collections studied except for collection 85/21 wherein the major alkaloid was pyrrolizidine **9.**

The structure elucidation and synthesis of (5*E*, 8*Z*)-3-(8-nonenyl)-5-(*E*,1-propenyl)pyrrolizidine (**5**) and its 5*Z*,8*E* isomer (**6**) have been described (Jones et al., 1986). They are easily discerned by their mass spectra, *m/z* = 275(M+), and 150(100). All four possible pyrrolizidine stereoisomers are formed in the synthesis of **5** and **6**. Although the major product in that synthesis is the 5*Z*,8*E* isomer **6,** it is only present in trace amounts in the *Monomorium* collections where it was detected. On the other hand, the 5*E*,8*Z* isomer **5,** a minor product from the synthesis, is the major alkaloid in most of the *Monomorium* collections (Table 1).

In nearly one third of the *Monomorium* recollections, the major alkaloidal component appeared to be isomeric with pyrrolizidine **5,** having a mass spectrum [*m/z* = 275(M+), and 192(100)] that indicated only the loss of a C_6H_{11}

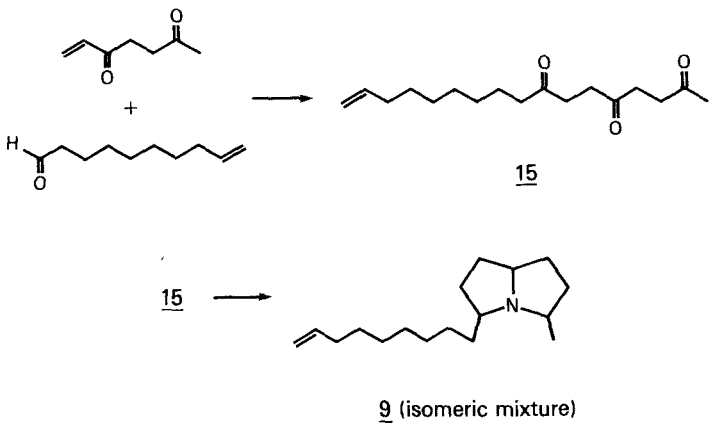
fragment. A very small amount of this compound was obtained from collection 85/4 by preparative gas chromatography. The 360-MHz proton NMR spectrum of this sample showed only the signals characteristic of a terminal double bond in the olefinic region. These results, including the simplicity of the mass spectrum, suggested a symmetrically substituted 3,5-di(5-hexenyl)pyrrolizidine. This assignment was confirmed by the synthesis outlined in Scheme 2. Divinyl



SCHEME 2.

ketone condensed with two equivalents of 6-heptenal in the presence of triethyl amine and a thiazolium salt catalyst (Jones et al., 1980a; Stetter et al., 1977) to form 1,18-nonadecadien-7,10,13-trione (**13**). Reductive amination of triketone **13** with ammonium acetate and sodium cyanoborohydride gave the three possible 3,5-di(5-hexenyl)pyrrolizidines eluting in the order **14, 8,** and **7** (SP-1000 column) in 1:9:3 ratio. These compounds had essentially identical mass spectra, which matched the mass spectra of the natural materials. The stereochemistry of these isomers was readily assigned from their [^{1}H]NMR spectra (Jones et al., 1980b). Pyrrolizidine **14** had no C<u>H</u>-N methine signals below δ = 2.5 ppm, which is indicative of a *trans*-fused pyrrolizidine, whereas **7** and **8** had a multiplet at δ = 3.6 ppm typical of the C_8 proton of *cis*-fused pyrrolizidines. The 5*Z*,8*E* isomer **8** and the 5*E*,8*Z* isomer **7** had two-proton multiplets at δ = 2.6 and 2.94 ppm, respectively, characteristic of their configurations (Jones et al., 1980b). The major 3,5-di(5-hexenyl)pyrrolizidine in these *Monomorium* extracts had a gas chromatographic retention time identical to that of **7,** and traces of **8** were found in some of the collections containing **7** (Table 1).

The mass spectrum of the major alkaloid in the extracts from collection 85/21 had a parent ion at *m/z* = 249 and fragments at *m/z* 234(10) and 124(100), indicating the loss of CH_3 and C_9H_{17}, respectively. It was otherwise quite similar to the mass spectrum of the known 3-heptyl-5-methyl pyrrolizidine (Jones et al., 1980b). Its structure and stereochemistry were confirmed as (5*Z*,8*E*)-3-



SCHEME 3.

methyl-5-(8-nonenyl)pyrrolizidine (**9**) by the synthesis outlined in Scheme 3. The appropriate triketone, 16-heptadecen-2,5,8-trione (**15**) was formed in good yield by the condensation of 6-hepten-3,5-dione (Stetter and Landscheidt, 1979) and 9-decenal (Jones et al., 1982a) in the presence of triethylamine and a thiazolium salt catalyst. Reductive amination of **15** produces all four pyrrolizidine isomers in a 1:40:7:3 ratio. The infrared spectrum of the first eluting isomer was typical of a *trans*-fused pyrrolizidine, with Bohlmann C–H bands from 2860 to 2680 cm^{-1}, similar to those of the *trans*-fused isomer of 3-heptyl-5-methylpyrrolizidine (Jones et al., 1980b). The second eluting, major synthetic isomer had a mass spectrum and gas chromatographic retention time identical with those of the major alkaloid in collection 85/21. The proton NMR spectrum of this compound had a one-proton multiplet at $\delta = 3.6$ ppm and a two-proton multiplet at $\delta = 2.6$ ppm, which verify its assignment as the *cis*-fused (5*Z*,8*E*)-3-methyl-5-(8-nonen-1-yl) pyrrolizidine (**9**).

Collection 85/21 also contained small amounts of the *trans*-pyrrolidines **2** and **4** as well as the pyrrolizidine **5.** In addition, a minor component was detected whose mass spectrum [m/z = 275(M+), 234(100), and 150] suggests that it is 3-allyl-5-(8-nonenyl)pyrrolizidine (**10**), and isomer of **5.** The facile loss of 41 ($M-C_3H_5$) would be expected for structure **10,** where it would be an α-cleavage (to nitrogen) as well as cleavage allylic to a double bond. Since **10** is only a minor component, its structure could not be confirmed by other means, and its sterochemistry remains undetermined.

In collections 85/26 and 85/27, a significant component in the alkaloidal extracts is *trans*-2-heptyl-5-(5-hexenyl)pyrrolidine (**3**) (Monomorine IV), which has been reported as a component of the alkaloidal venom of pharoh's ant (*Monomorium pharaonis*) (Ritter et al., 1975). Additionally, a minor component in

collection 85/26 has a mass spectrum [*m/z* 249(M+), 192, and 166] suggesting that it is 3-butyl-5-(5-hexenyl)pyrrolizidine (**11**). As with pyrrolizidine **10,** because of the very small amount of material available, the structure of **11** could not be confirmed by other means, and its stereochemistry is undetermined.

DISCUSSION

The predominant components of the alkaloidal venoms of nearly all the *Monomorium* populations examined in this study are the (5*E*,8*Z*)pyrrolizidines **5** and **7,** which are accompanied in many cases by small amounts of the *trans*-pyrrolidine **1.** The unbranched 19-carbon skeleton of these pyrrolizidines, along with the presence of **1,** is reminiscent of the pyrrolidine mixtures found in many New World *Monomorium* species (Jones et al., 1982a). The pyrrolizidines **5** and **7** can be viewed as bicyclic analogs of **1,** in which the second ring would be formed from the six-carbon side chain to produce **5,** or from the nine-carbon side chain to produce **7.**

The stereochemistry of **5** and **7** is a configuration where one of the alkyl groups is oriented into the cavity of the *cis*-fused pyrrolizidine ring system. Because of the strain and hindrance of this arrangement, the 5*E*,8*Z* configuration is not the most favored, as is reflected in its formation as only a minor product when these compounds are prepared by the reductive amination of the appropriate triketone. In the cases of both **5** and **7,** however, gas chromatographic retention times (SP-1000) of the natural isomers indicate that they are the most polar of the possible pyrrolizidine isomers, which may be related to their biological roles.

The alkaloids from the ants in collection 85/21 are quite distinct from those of the other *Monomorium* collections, being characterized by the 17-carbon (5*Z*,8*E*)pyrrolizidine **9** and its monocyclic homolog **4.** In contrast to **5** and **7,** the stereochemistry of **9,** like that of (5*Z*,8*E*)-3-heptyl-5-methylpyrrolizidine (Jones et al., 1980b), is the least strained or hindered configuration, with both alkyl groups oriented away from the cavity of the *cis*-fused pyrrolizidine system. Furthermore, because of the *trans* substitution patterns of all the five-membered rings in both compounds, **9** is the direct bicyclic homolog of **4.**

The association of pyrrolidine **1** with pyrrolizidines **5** and **7** as well as that of pyrrolidine **4** with pyrrolizidine **9,** suggests a common biological precursor for pyrrolidines and pyrrolizidines with the same carbon skeleton.

Although the alkaloidal mixtures of collections 85/26, 85/27, and 86/27 contain substantial amounts of pyrrolizidine **7,** they are also unique in containing more than traces of the *trans*-pyrrolidine **3,** which has been detected in small quantities in *Monomorium pharaonis* and was designated monomorine IV (Ritter et al., 1975).

The structures of the trace pyrrolizidines **10** and **11** are strongly suggested by their mass spectra and their concomitants. In the first place, the gross fragmentation pattern in the mass spectra of both compounds shows an odd-mass parent with the loss of the side chains to give intense even-mass fragments, where the parent minus the masses of the side chains equals 109 ($C_7H_{11}N-$ the pyrrolizidine nucleus). This phenomenon is characteristic of the mass spectra of all the 3,5-dialkylpyrrolizidines. The loss of C_3H_7 (m/z = 234) in the spectrum of **10,** in contrast with the mass spectrum of **5,** would certainly be expected as the result of allylic cleavage alpha to nitrogen. Secondly, an ion resulting from double-dehydrogenation (M-4, 5%) is observed in the EI mass spectra of **5–9** as well as **10** and **11.** Although this is quite likely an instrumental artifact (Fales et al., 1980), this ion and weak $M-(R-CH_2)$ ions resulting from allylic cleavage of either side chain from a partially aromatized pyrrolizidine nucleus are also observed for **5–9** as well as **10** and **11.** Finally, *trans*-2-heptyl-5-(5-hexenyl)pyrrolidine **3,** the monocyclic homolog of **11,** is an important component of the alkaloidal mixture containing **11.** The concomitance of homologous pyrrolidines and pyrrolizidines is a notable feature in the *Monomorium* alkaloid mixtures predominated by pyrrolizidines **5, 7,** and **9.**

From this study, it is apparent that the 3,5-dialkylpyrrolizidines that characterize the venoms of these *Monomorium* populations are produced stereoselectively by the ants. Similar stereoselection has been observed for the formation of 3-alkyl-5-methylindolizidines in other *Monomorium* as well as *Solenopsis* populations (Jones et al., 1984), although only (5*Z*,9*Z*)-indolizidine isomers have been detected in the venoms of myrmicine ants. On the other hand, New Zealand *Monomorium* populations produced either the (5*E*,8*Z*)- or the (5*Z*,8*E*)-pyrrolizidine stereoisomers, depending on the particular collection examined.

Based on the product ratios from the reductive alkylation of the appropriate triketones, there is relatively little difference in strain and hindrance between the possible 3-alkyl-5-methylindolizidine isomers, whereas there is a much greater difference between the corresponding 3,5-dialkylpyrrolizidine isomers. In the former case, all of the isomers are formed in nearly equal amounts, and in the latter one, the (5*Z*,8*E*)-pyrrolizidine isomer predominates over the others by at least an order of magnitude (Jones et al., 1980b, 1984, 1986). Remarkably, the most common 3,5-dialkylpyrrolizidines found in these *Monomorium* populations are the hindered and strained 5*E*,8*Z* isomers **5** and **7.** Yet, it should be noted that the single 3-methyl-5-alkylpyrrolizidine, **9,** found in *Monomorium* collection 85/21 has the same unstrained 5*Z*,8*E* configuration as an analogous 3-methyl-5-alkylpyrrolizidine isolated from a thief ant (*Solenopsis* sp.) (Jones et al., 1980b).

Although many different species of myrmicine ants are known to produce the same alkaloids in their venoms, there is no precedent for the formation of such structurally different alkaloids as **5, 7,** and **9** by different populations of

the same species (Jones et al., 1982b; MacConnell et al., 1976). From a chemotaxonomic standpoint, the results of this study seem to indicate the presence of perhaps four *Monomorium* "species" in Table 1. Thus the different "species," based on the major alkaloids produced, seem to be: (1) those producing predominantly pyrrolizidine **5,** (2) those producing predominantly pyrrolizidine **7,** (3) those producing predominantly pyrrolizidine **9** (collection 85/21, at northern tip of North Island), and finally (4) those producing the *trans*-pyrrolidine **3** (collections 85/26, 85/27, and 86/27 along with pyrrolizidine **7.** However, what appear to be consistent size and color differences between North Island specimens with **5** or **7** as the major component and their South Island counterparts indicate convergence in the selection of the major alkaloid by allopatric populations and suggest the possibility of two further "species" within the so-called *M. antarcticum* complex. In addition, although collection 85/27 shares **7** as the major component with some northwestern South Island populations, the ants representing these two groups appear markedly dissimilar in worker size and color. Differences in minor components also support this separation. Overall, it is apparent that elucidation of the *M. antarcticum* complex in New Zealand must await much more precise morphological studies in conjunction with further collections and analyses of the venom alkaloids.

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Letter to the Editor

CYANOGENIC GLUCOSIDES AS DEFENSE COMPOUNDS
A Review of the Evidence

The presence of cyanogenic glucosides in plants and their toxic nature has been recognized since antiquity, when traitorous Egyptian priests of Memphis and Thebes were poisoned with the pits of peaches (Bruijn, 1971). The function of cyanogenic glucosides in plants, which, upon tissue damage, hydrolyze to release hydrogen cyanide gas, has been a point of speculation ever since. As with many other plant compounds whose physiological function is not understood, cyanogenic glucosides were first considered to be storage products for nitrogen or waste products (Eyjolfsson, 1970). Today cyanogenic glucosides are considered as a textbook example of defensive plant secondary compounds. Harborne (1982) in a recent text on biochemical ecology states that "There is good evidence that cyanogenesis provides partial, if not complete, protection from predation by a wide spectrum of animal species." Despite the now accepted view of cyanogenic glucosides as antiherbivore compounds, the experimental studies upon which the protective function hypothesis rests have not been critically reviewed. Little attention has been paid to the negative results of many of the studies or to the host specificity of the herbivores involved.

I examined 23 papers that experimentally tested the hypothesis that cyanogenic glucosides have a defensive function or that report nonexperimental observations pertaining to that hypothesis (Angseesing and Angseesing, 1973; Angseesing, 1974; Bernays et al., 1977; Bishop and Korn, 1969; Cooper-Driver and Swain, 1976; Corkill, 1952; Crawford-Sidebotham, 1972; Dirzo and Harper, 1982a, b; Dritschilo et al., 1979; Ellis et al., 1977a, b; Fisk, 1980; Jones, 1962, 1966; Keymer and Ellis, 1978; Lane, 1962; Miller et al., 1975; Nayar and Fraenkel, 1963; Scriber, 1978; Whitman, 1973; Woodhead and Bernays, 1977; Woodhead et al., 1980). I noted the following information from each paper: (1) the number of trials conducted (some papers report more than one trial); (2) the type of trial, e.g., anecdotal observations, correlations, controlled laboratory experiments, or controlled field experiments; (3) the plant species used; (4) the herbivore species studied; (5) the response variable measured; (6) the data analysis; and (7) the results. I tabulated the information for all trials but retained only trials where the data from the experiments were presented and analyzed with appropriate statistical tests and where statistical tests supported

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the claimed results. Using this criterion, I was able to retain only 51 trials using 41 herbivore species of the original 120 trials using 74 species of herbivores.

The results from the trials that I examined are summarized in Table 1. Of the 800 plant species known to be cyanogenic (Eyjolfsson, 1970), just six species have been examined in plant–herbivore studies and only two species (*Trifolium repens* and *Lotus corniculatus*) accounted for 75% of the trials. Twenty-nine herbivore species have been tested but just two species (*Arion ater* and *Agriolimax reticulatus*), both mollusks, were used in 22% of all trials.

Some of the classic papers upon which the role of cyanogenic glucosides as defensive compounds rests deserve close reexamination. Among the papers that lack adequate statistical analyzes are three of the four most-referenced works used to support the defensive function hypothesis. The two most-referenced works (Jones, 1962, 1966) present graphs of a negative relationship between cyanogenic glucoside score and the amount of eating, without calculating either the correlation coefficient or the significance level of the relationship. The graphs include many nonindependent observations of the same feeding individuals tested on successive nights. The fourth most-referenced paper, (Whitman, 1973) does present a proper statistical analysis of the data on feeding choice. The results, however, are not statistically significant. In spite of this, the trend of Whitman's results (selective eating of acyanogenic morphs of *Trifolium repens*) is cited in other works without qualification (Ellis et al., 1977a, b).

Only 53% of the herbivore species tested demonstrated preference for acyanogenic plants. Despite the relatively high percentage of "negative" results, the notion that cyanogenic glucosides are protective compounds against herbivores has become firmly entrenched in the literature and textbooks. Drit-

TABLE 1. RESULTS OF LITERATURE REVIEW OF PLANT CYANOGENESIS AND HERBIVORY STUDIES[a]

Herbivore/ pathogen	Results				Total species (*N*)
	No preference	Acyanogenic preference	Cyanogenic preference	Conflicting results	
Fungi	2(1)	0(0)	1(1)	0(0)	2
Mollusca	6(5)	28(14)	0(0)	2(2)	16
Insecta	3(3)	4(4)	0(0)	4(3)	12
Vertebrata	0(0)	0(0)	0(0)	1(1)	1
Totals	11(8)	32(18)	1(1)	7(7)	31
Percent of totals					
Trials	21.6	62.7	2.0	13.7	
Species	23.5	52.9	2.9	20.6	

[a] Number of trials (number of species tested).

schilo et al. (1979) observed 38 insect species colonizing cyanogenic and acyanogenic morphs of *Trifolium repens*. Fourteen of the species had higher abundance on the acyanogenic morph, eight species were more abundant on the cyanogenic morph, and 16 species showed no preference. There was also no difference in visible feeding damage to leaves of cyanogenic and acyanogenic plants. Yet the conclusion of the paper is "selection by some insect species against the acyanogenic phenotype may be added to a growing list of selective agents involved in the cyanogenesis polymorphisms." Jones (1966) also ignored negative results. He found selective eating for the acyanogenic morphs of *Lotus corniculatus* in only one of three years of field studies. Yet, in the summary of his paper, he reports only "In 1960 a significant excess of *Lotus corniculatus* L. plants growing in wild populations which were found to have been eaten were acyanogenic." Furthermore, many negative results never get published as a large proportion of studies on cyanogenesis and herbivory are abandoned due to discouraging results (D. Root, personal communication).

Virtually all of the studies on plant cyanogenesis and herbivory have ignored the feeding specificity of the herbivore. The relationship of the herbivore to the plant being tested is important to conclusions drawn about the defensive function of cyanogenic glucosides. The glucosides do not provide protection against all herbivores, for some have the ability to sequester or detoxify the compounds. Herbivores that are specialists on cyanogenic plants are not expected to have the same response as generalist feeders.

Although cyanogenic glucosides inhibit enzyme functioning and are toxic at high levels, none of the studies reported complete avoidance of cyanogenic plants. No cases of herbivore death were attributed to the glucosides. The lack of a strong avoidance of cyanogenic plants, along with the high percentage of negative results (nearly 25%) suggests that the cost of cyanogenic glucosides to many herbivores is not high.

The many negative results deserve greater attention. If cyanogenic glucosides are defensive compounds to all herbivores, why do only 65% of the published trials support the hypothesis? How do so many herbivores escape or overcome the compounds? Scriber (1978) found that a polyphagous herbivore, the southern armyworm (*Spodoptera cidania*), showed no growth, energy budget, or nitrogen assimilation efficiency response to cyanogenic morphs of *Lotus corniculatus*. He attributes the lack of any metabolic cost to feeding on cyanogenic morphs to the armyworm's system of inducible mixed function oxidases (MFOs) that can detoxify a wide range of compounds. The role of MFOs in the survival of polyphagous herbivores has been questioned by Gould (1984).

The relationship between plant cyanogenesis and herbivores is complex. Care must be taken to avoid overstating the evidence and oversimplifying the results. Cyanogenic glucosides are widely accepted as defensive plant secondary compounds. A review of the literature showed that only 53% of the species

tested showed preference for acyanogenic morphs. Reevaluation of some of the classic papers that are widely cited as evidence in support of the defensive compound hypothesis showed that later citations sometimes overstate the results of the original work. Herbivore specificity must be taken into account in conducting and interpreting the results of experiments with plants containing cyanogenic glucosides. Further work with a fresh approach and of a critical nature may begin to reveal the details of the fascinating relationship between cyanogenesis and herbivory.

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Letter to the Editor

WHAT IS CHEMICAL ECOLOGY? A Reply

In a recent letter to this journal, Clive G. Jones (1988) suggests that the mix of articles in the *Journal of Chemical Ecology* is weighted too heavily toward chemistry and not enough toward ecology. He suggests the society consider rewriting its statement of purpose to more explicitly emphasize the ecological significance of the chemical interactions reported. I will not address this latter suggestion, nor is it the intent of this letter to dispute the former.

In Table 1 of his letter, Jones provides a survey of papers published in this journal in the years 1985–1986. He reports "<1" paper of a general review nature; yet I know offhand of two (Rittschoff and Bonaventura, 1986; Bakus et al., 1986) which are clearly review papers dealing with the chemical ecology of marine systems. In a brief survey I also found at least three papers (Roelofs et al., 1985; Lofstedt and van der Pers, 1985; Saelinger, 1985) that directly address the significance of pheromones to reproductive isolation, a topic with important evolutionary and community ecology consequences. I suspect that given more time I would find even further reason to question Jones' tabulation of papers. However, even if we agree with him, in general if not in detail, that an imbalance of approaches or areas of study exists, I feel additional comments are in order.

Science is of its nature analytical. Even ecologists, whose ultimate interests lie at higher orders of complexity, by and large spend their time teasing apart, via analytical techniques (experimental field manipulations, laboratory studies, multivariate analyses), the separate aspects of ecological interactions. Food web dynamics and systems analysis are two exceptions to the rule.

If one surveys recent articles in the *Journal of Cell Biology* or in *Cell*, one finds a predominance of papers dealing with molecular structure and function, molecular biology, and molecular genetics rather than processes at the integrated cellular level. Are molecular biology and genetics what cellular biology is about? In a way they are, and in a way they aren't. Is the characterization of significant semiochemicals what chemical ecology is about? In a way it is, and in a way it isn't. Perhaps expecting a higher proportion of papers in this journal to be "general chemical ecology" is like expecting a higher proportion of papers

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in *Cell* to be about function of the integrated cell—an altogether reasonable but perhaps unrealistic expectation.

Furthermore, perhaps even more so than cellular biology, chemical ecology is still in its youth. Chemical ecology is still primarily a descriptive science, reporting chemical-mediated interactions that have not before been noted, and identifying the chemicals involved. As more such interactions are found, and the molecules identified, it is a natural consequence that review papers and papers emphasizing higher-order ecological aspects will appear. To accelerate this process, I suggest that, as in both *Cell* and the *Journal of Cell Biology*, minireviews be made a regular feature in this journal. Such minireviews, appearing several times a year, by authorities in the field, could summarize recent advances in a particular topic. Methodologies such as field traps, release devices, GLC or HPLC advances, egg parasitism attractants, pheromone-related control of mammalian estrus, or receptor response mechanisms are just a few areas in which a number of papers have recently appeared in this journal. It might be suggested that those minireviews not specifically dealing with methodologies emphasize the ecological significance of the semiochemicals. Solicitation, review, and publication of such minireviews on a regular basis is no small undertaking. Yet they are likely, once references to them find their way into the wider ecological literature, to find a much broader readership for this journal. And that in itself may be the single most important step toward bringing into these pages papers of the type Jones prefers.

But papers that do no more than report the observation of semiochemical-mediated interactions or the characterization of the compounds involved are of themselves important. Such research may not be publishable in other journals; yet it is crucial that these studies reach the literature. In this respect the *Journal of Chemical Ecology* already performs a critical service.

I would suggest, then, that although in general we may agree with Jones, things are not so bad as they may appear. There may be more effective ways of improving matters than changing the stated purpose of the society.

Ken Parejko
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Announcement

EVOLUTIONARY ASPECTS—MAIN THEME AT THE SIXTH INTERNATIONAL MEETING OF ISCE

The 6th International Meeting of ISCE will be held at the University of Göteborg on the west coast of Sweden, August 7–11, 1989. During this meeting, evolutionary aspects of chemical ecology will be emphasized. Our meeting will follow directly after the IUPAC Congress in Stockholm. At that Congress, there will be a Section devoted to Chemical Communication. Transportation by train on Monday, August 7 from Stockholm to Göteborg will be arranged. Welcome to the 1989 Meeting. Please write to Dr. Gunnar Bergström regarding any aspect of the meeting.

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