*Journal of Chemical Ecology, Vol. 27, No. 6, 2001*

# CLOSE-RANGE ATTRACTION IN *Lygocoris pabulinus* (L.)

FALKO P. DRIJFHOUT[1,*] and ASTRID T. GROOT[2]

[1]*Laboratory of Organic Chemistry, Phytochemical Section*
*Wageningen University*
*Dreijenplein 8, 6703 HB Wageningen, The Netherlands*

[2]*Plant Research International, Wageningen UR*
*P.O. Box 16, 6700 AA Wageningen, The Netherlands*

(Received May 1, 2000; accepted February 10, 2001)

**Abstract**—Males of the green capsid bug, *Lygocoris pabulinus*, exhibit a specific courtship behavior, i.e., a vibration of the abdomen. When both live and dead females were offered to males, this vibration behavior was elicited in most of the males tested. When females were dissected into separate body parts, heads, wings, and legs elicited equal responses, while thorax plus abdomen elicited a much lower response. When separate body parts were extracted, the leg extracts elicited significantly stronger responses than any other extract. This suggests that female *L. pabulinus* legs are either the source of a close-range sex pheromone or that pheromone is accumulated on the legs due to grooming behavior. The leg extracts contained several hydrocarbons such as *n*-alkenes, *n*-alkanes, and some methylalkanes. Female extracts contained more (*Z*)-9-pentacosene and male extracts contained more (*Z*)-9-heptacosene. Substrates on which females had walked elicited similar responses as female legs, indicating that the pheromone is deposited on the substrate. This enlarges the functional range of low-volatility compounds, which are thought to function only when sexes are in close vicinity or in contact.

**Key Words**—Heteroptera, Miridae, contact sex pheromone, male vibration, (*Z*)-9-pentacosene, (*Z*)-9-heptacosene, cuticular hydrocarbons, close-range pheromone, SPME.

## INTRODUCTION

Sex pheromones are commonly used by insects to locate mates at long range and to stimulate mating at close range (Carlson et al., 1971; Cardé et al., 1975; Muhammed et al., 1975). Long-range sex pheromones were first described and chemically identified in moths (Butenandt et al., 1959) and are now widely used for

*To whom correspondence should be addressed. e-mail: Falko.Drijfhout@bio.oc.wau.nl



0098-0331/01/0600-1133$19.50/0 

monitoring of lepidopterous pests (e.g., Minks and Van Deventer, 1992; Cardé and Minks, 1997). In recent years identification of nonlepidopteran sex pheromones has received growing attention (Hardie and Minks, 1999). In mirids (Heteroptera: Miridae), whose virgin females attract males, long-range sex pheromones have been identified for three species so far (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998; McBrien and Millar, 1999).

Close-range sex pheromones initiate courtship behavior. Such pheromones are usually less volatile than long-range pheromones (Blomquist et al., 1993). Despite their low volatility, close-range pheromones may play an important role in the decision of an insect to land at a certain spot (Carlson et al., 1971). Without the addition of such pheromones, arriving males may not enter a trap (Cardé et al., 1975; Kennedy, 1977). In mirids close-range sex pheromones have not yet been reported. Major focus has been on attractive and alarm compounds from the metathoracic and accessory scent glands (e.g., Carayon, 1971; Staddon, 1979; Aldrich, 1988). Compounds identified from these glands have carbon chain lengths of 2–15 and are most commonly acids, aldehydes, ketones, alcohols, and esters (Staddon, 1979; Aldrich, 1988). Close-range pheromones may have carbon chain lengths of 20–30 or even more (Blomquist et al., 1993). The source of long-range pheromones in mirids has been suggested to be the metathoracic scent gland (Aldrich, 1988), or at least the thoracic region (Millar et al., 1997), although Graham (1988) identified the ovipositor region as source of attraction. Since the chemical nature of close-range pheromones may differ completely from long-range pheromones, their sources probably differ as well.

To identify close-range sex pheromones, a specific arousal or courtship behavior of one of the sexes should be distinguished. Males of the green capsid bug [*Lygocoris pabulinus* (L.), Heteroptera: Miridae] exhibit a characteristic sex-specific courtship behavior, a repeated vibration of the abdomen; only males vibrate in the presence of females and only when they are sexually mature (Groot et al., 1998). We used this vibration behavior of male *L. pabulinus* to determine the source of attraction in females at close range. Additionally, we attempted to identify compounds involved in this close-range attraction.

## METHODS AND MATERIALS

*Insects*. *Lygocoris pabulinus* was reared on potted potato plants, (cultivar Bintje) in wooden cages in a greenhouse at $22 \pm 2^{\circ}$C, $65 \pm 5$ % relative humidity, and 18L : 6D photoregime, following the procedure of Blommers et al. (1997). Every two to three days newly emerged adults were collected from the rearing cages, after which the sexes were isolated in separate rearing cages. In this way, virgin males and females of known age were continuously available for the experiments (Groot et al., 1998).

*Bioassays*. One to two hours before each test, virgin males 6–9 days old were collected from the separate rearing cages and isolated in small glass tubes. Glass Petri dishes 5 cm diam. were cleaned with acetone, and the bottoms were covered with white filter paper disks of the same diameter. The stimuli to be tested were placed in the Petri dishes, after which one male per dish was introduced. Stimuli consisted of one bug equivalent per Petri dish, and originated from bugs that were virgin and 6–9 days old. All males were observed for 15 min. If a male in a dish started to vibrate within this period, that dish was set aside and counted as a positive response. The number of Petri dishes with positive responses was calculated as a fraction of the total number of Petri dishes in which the stimulus had been applied. Different stimuli were tested at the same time, and stimuli were tested on several different days. All experiments were carried out at 19–23°C between 10:00 and 14:00 hr C.E.T.

In a second experiment, a solid-phase microextraction (SPME) needle (100 $\mu$m polydimethylsiloxane coating from Supelco, Bellefonte, Pennsylvania) was positioned on the bottom of the Petri dish through a hole in the side. A metal strip was placed in a V-shape around the needle to reduce the amount of space the bug had, thus increasing the chance of bug–needle contact. One or two bugs were then placed in the Petri dish for 2–3 hr.

*Stimuli Tested*. First, a series of live females, dead females, live males, and dead males were tested. Bugs were anesthetized with $CO_2$, after which the heads were clipped off. In a following series freshly anesthetized females were dissected into heads, wings, legs, and thorax plus abdomen. Thorax and abdomen were not subdivided, since clipping would mean cutting through several organs and glands that run from thorax to abdomen, which may then release a variety of chemicals. Third, extracts were made of the different body parts of females, *i.e.*, heads, wings, legs, and thorax plus abdomen. After anesthetizing fresh females with $CO_2$, the body parts were dissected and placed in 1.8- or 4-ml vials. After dissecting all available females, 15–50 $\mu$l of either dichloromethane, pentane, pentane–ether (2 : 1), or water per female was added, the amount of which was set as one female equivalent of the particular extract. The extracts were stored in a freezer ($-20 \pm 2$°C) until used. All extracts were used 1–14 days after the initial dissections. In a fourth series, one female equivalent of a synthetic mixture (2.5 $\mu$g in total) was tested. The synthetic mixture consisted of 1-hexanol (40 ng), hexyl butyrate (500 ng), (*E*)-2-hexenyl butyrate (25 ng), (*Z*)-9-tricosene (150 ng), (*Z*)-7-pentacosene (200 ng), (*Z*)-9-pentacosene (1000 ng), (*Z*)-9-heptacosene (200 ng), tricosane (80 ng), and pentacosane (80 ng) (Table 1 below).

*Chemical Analysis*. Extracts were analyzed with a dual-column GC (HP 6890) equipped with an apolar DB-1 column (J&W Scientific, Folsom, California; 60 m $\times$ 0.25 mm; 0.25-$\mu$m) and a polar Stabilwax column (Restek, Bellefonte, Pennsylvania; 60 m $\times$ 0.25 mm; 0.25-$\mu$m) and two flame ionization detectors. Oven program: 30°C (2 min hold) to 238°C (25 min hold) at 4°C/min. Hydrogen

TABLE 1. AVERAGE COMPOSITION (%) OF EXTRACTS OF LEGS FROM FEMALE AND MALE *L. pabulinus*

| No.[a] | Compound | Composition(%, mean ± SD) | |
|---|---|---|---|
| | | Females | Males |
| 1 | Hexan-1-ol[b] | 1.7 ± 1.8 | 0.6 ± 0.4 |
| 2 | Hexyl butyrate[b] | 19.2 ± 13.7 | 22.3 ± 23.4 |
| 3 | (*E*)-2-Hexenyl butyrate[b] | 0.8 ± 0.5 | 0.8 ± 0.9 |
| 4 | Tricosane[b] | 6.3 ± 1.9 | 0.4 ± 0.7 |
| 5 | (*Z*)-9-Tricosene[b] | 3.1 ± 1.7 | 0.1 ± 0.1 |
| | (*Z*)-9-Tetracosene | ≤0.2 | ≤0.2 |
| | Tetracosane | ≤0.2 | ≤0.2 |
| 8 | 2-Methyltetracosane | 3.3 ± 1.5 | 1.8 ± 0.2 |
| 9 | Pentacosane[b] | 8.6 ± 2.8 | 10.6 ± 4.9 |
| 10 | (*Z*)-9-Pentacosene[b] | 40.8 ± 9.2 | 3.4 ± 2.3 |
| 11 | (*Z*)-7-Pentacosene[b] | 8.7 ± 1.9 | 20.5 ± 4.8 |
| | 9-Hexacosene | ≤0.2 | ≤0.2 |
| | 2-Methylhexacosane | ≤0.2 | ≤0.2 |
| 14 | (*Z*)-9-Heptacosene[b] | 7.6 ± 4.6 | 39.6 ± 13.2 |
| | (*Z*)-7-Heptacosene | ≤0.2 | ≤0.2 |
| | Heptacosane | ≤0.2 | ≤0.2 |
| | (*Z*)-9-Nonacosene | ≤0.2 | ≤0.2 |

[a] Numbers according to Figures 3 and 4.
[b] Compounds used in the synthetic mixture.

was used as the carrier gas (constant flow of 2.4 ml/min, linear velocity: 48 cm/sec). GC-MS analyses were carried out on Varian 3400 GC connected to a Finnigan MAT95 mass spectrometer. The BP5 column (SGE; 25 m × 0.25 mm; 0.25-$\mu$m) was programmed from 50°C to 270°C (4 min. hold) at 5°C/min. The mass spectrometer was operated in EI mode (at 70 eV) and scanning was done from mass 24 to 500 at 0.7 sec/dec. $^1$H NMR (200 MHz) spectra were recorded on a Bruker AC200 spectrometer. FTIR spectra were recorded on a Perkin-Elmer 1725X spectrometer. Identification of compounds in extracts was carried out with GC-MS and by comparison of retention times of reference compounds with GC. The position of the double bond in the alkenes was determined by derivatization with DMDS according to Carlson et al. (1989).

*Chemicals*. Hexanol, tricosane, pentacosane, 1-bromotetradecane, 1-bromohexadecane, 1-bromooctadecane, triphenylphosphine, nonanal, heptanal, *n*-butyllithium in hexane, DMDS, and urea were all purchased from Acros Organics (Geel, Belgium) and hexyl butyrate from Roth (Karlsruhe, Germany). (*E*)-2-Hexenyl butyrate was synthesized according to Drijfhout et al. (2000). (*Z*)-9-Tricosene, (*Z*)-9-pentacosene, (*Z*)-7-pentacosene, and (*Z*)-9-heptacosene were all synthesized as described below. All the chemicals used were >98% pure. All solvents used were distilled twice before use.

*Synthesis of Alkyltriphenylphosphonium Bromide*. A mixture of 0.3 mmol of the alkylbromide (1-bromotetradecane, 1-bromohexadecane, or 1-bromooctadecane) and 0.3 mmol of triphenylphosphine was heated to 140°C under a nitrogen atmosphere for 5 hr. The reaction mixture formed a solid when cooled down. Dry acetone (5 ml) and dry diethyl ether (12 ml) were added to the solid and cooled to −20°C (overnight). After filtration, the alkyltriphenylphosphonium bromide was obtained as white crystals.

*Synthesis of Alkenes*. A slurry of 5 mmol of powdered alkyltriphenylphosphonium bromide in 10 ml of THF was prepared under nitrogen. The mixture was cooled in an ice bath, 5 ml of DMSO was added after which 5 mmol of *n*-butyllithium in hexane was injected. The butyllithium was added at such a rate that the temperature of the mixture remained at 10–15°C. After 5 min, 5 mmol of the aldehyde (nonanal or heptanal) was injected, and the resulting mixture was stirred for 30 min at ambient temperature. The mixture was diluted with water and extracted with petroleum ether 40/60. The dried ($MgSO_4$) extract was filtered and concentrated to give a 90% yield of the alkene. The alkene was further purified with column chromatography on silica gel and eluted with hexane to give a mixture of *Z* and *E* isomers in a ratio of 85 : 15.

*Separation of Z and E Isomers*. The two isomers of the alkenes obtained during the synthesis were separated making use of their different complexation with urea (Leadbetter and Plimmer, 1979). One part of alkene and five parts of urea were dissolved in 20 parts of methanol. This was left to crystallize at room temperature. The white crystals were separated by filtration. The methanol fraction was evaporated to obtain the (*Z*)-isomer in 97% purity. The procedure was repeated to improve the purity of the *Z* isomer. If the alkene could not be dissolved in methanol, isopropanol was added.

*(Z)-9-Tricosene*. $^1$H NMR $\delta$ ($CDCl_3$) 0.83–0.89 (t, 6 H, $-CH_3$), 1.2–1.6 (m, 34 H, $-CH_2CH_2-$), 2.0 (m, 4 H, $=CH-CH_2$), 5.3–5.35 (m, 2 H, CH=CH). MS: $m/z = 322$ ($M^+$); Kovats indices: 2320 on Stabilwax and 2271 on DB-1. IR (film): $\nu_{HC=CH(cis)}$ 722 (m) $cm^{-1}$

*(Z)-7-Pentacosene*. $^1$H NMR $\delta$ ($CDCl_3$) 0.83–0.89 (t, 6 H, $CH_3$), 1.2–1.4, (m, 38 H, $-CH_2CH_2-$), 1.9–2.0 (m, 4 H, $=CH-CH_2$), 5.3, (m, 2 H, CH=CH). MS: $m/z = 350$ ($M^+$); Kovats indices: 2526 on Stabilwax and 2477 on DB-1; IR (film): $\nu_{HC=CH\ (cis)}$ 722 $cm^{-1}$

*(Z)-9-Pentacosene*. $^1$H NMR $\delta$ ($CDCl_3$) 0.83–0.89 (t, 6 H, $-CH_3$), 1.2 (m, 38 H, $-CH_2CH_2-$), 1.9–2.2 (m, 4 H, $=CH-CH_2$), 5.3–5.4 (m, 2 H, CH=CH). MS: $m/z = 350$ ($M^+$); Kovats indices: 2519 on Stabilwax and 2470 on DB-1; IR (film): $\nu_{HC=CH\ (cis)}$ 722 $cm^{-1}$

*(Z)-9-Heptacosene*. $^1$H NMR $\delta$ ($CDCl_3$): 0.9 (t, 6 H, $CH_3$), 1.1–1.5 (m, 40 H, $-CH_2CH_2-$), 2.2 (m, 4 H, $=CH-CH_2$), 5.3 (m, 2 H, CH=CH). MS: $m/z = 378$ ($M^+$); Kovats indices: 2721 on Stabilwax and 2671 on DB-1; IR (film): $\nu_{HC=CH\ (cis)}$ 722 $cm^{-1}$

*Statistical Analysis*. If males responded to a source, differences in responses towards the different sources were statistically analyzed by fitting a logit regression model with overdispersion to the daily observed counts of responses of a test (McCullagh and Nelder, 1989), using the computer program Genstat 5 (release 4.1, PC/Windows NT, 1997). In the model, source was taken as the explanatory variable and the variance was assumed to be proportional to the binomial variance. First, a chi-square test for the residual deviance was conducted to determine overdispersion. The overall effect of treatments was determined by performing an $F$ test for the ratio of the mean deviance for treatment and the mean deviance of the rest. If the overall test was significant ($P < 0.05$), pairwise comparisons by $t$ test between treatment means on the logit scale were conducted.

## RESULTS

Live and dead females elicited similar responses, the fractions of males vibrating being $0.88 \pm 0.08$ and $0.74 \pm 0.09$ (mean $\pm$ SE), respectively (Figure 1A). Live and dead males elicited vibration responses in few males. When the bodies of females were dissected, the head, wings, and legs were equally attractive and as attractive as dead females, while the thorax plus abdomen of females were significantly less attractive (Figure 1B). Responses to freshly dissected wings and heads may be due to grooming, which spreads attractive compounds over the body surface. For confirmation of presence of attractive compounds on the whole body surface, small pieces of filter paper were rubbed over female bodies (after anesthetizing and clipping off heads). When these pieces of paper were offered in clean Petri dishes, almost half of the males tested ($0.41 \pm 0.07$, mean $\pm$ SE) started vibrating ($N = 46$).

After extraction of the separate body parts of females, leg extracts elicited significantly more vibrational response than all other extracts (Figure 1C). Extracts from thorax plus abdomen did not elicit a response from males, which may be due to defensive compounds in the metathoracic gland. Therefore, extracts were also made of female thorax plus abdomen, from which the metathoracic gland was removed by gently cutting the cuticle with two sharp tweezers, trying to destroy as little tissue as possible. Few males responded to this extract (Figure 1C). When differences in responses between freshly dissected body parts and their corresponding extracts were statistically compared, male responses to the leg extracts were not different from responses to freshly dissected legs, while wing and head extracts elicited lower responses ($P < 0.05$) than freshly dissected wings and heads.

Organic solvent extracts stimulated significantly more males to vibrate than water extracts (Figure 2). The graph indicates that "water extracts" caused only minimal vibration in males. Legs from female bugs, used for these "water extracts,"

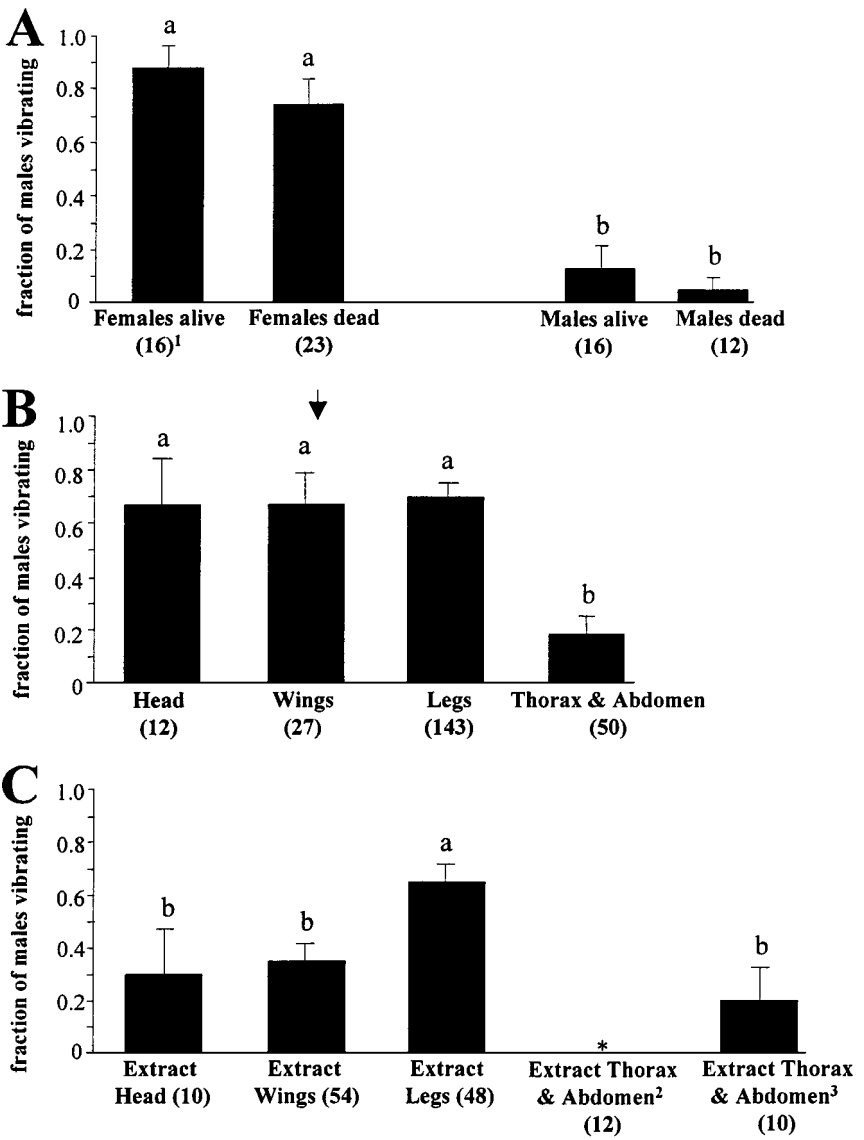


FIG. 1. Male *L. pabulinus* responses (mean ± SE) to different stimuli: (A) whole insects, (B) body parts of females, (C) extracts of female body parts. [1]Total number of males tested; [2]Metathoracic gland left in thorax; [3]Metathoracic gland removed from thorax. Significant differences were determined between sources within one group (A, B, C). Different letters above the bars indicate significant differences in each group at the 5% level. See text for statistical methods used. *Not included in the statistical analyses, as no males had responded.

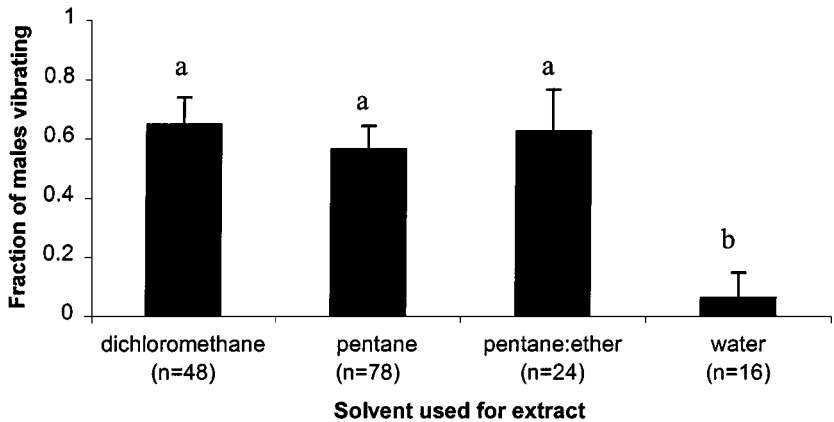


FIG. 2. Response of male *L. pabulinus* to extracts of female legs prepared in different solvents (mean ± SE; n = number of males tested). Different letters above bars indicate significant difference at $P \leq 0.05$ (two-tailed).

still elicited vibratory behavior in males. No significant difference in male vibration occurred using dichloromethane, pentane or the mixture of pentane and ether (Figure 2).

In both male and female leg extracts, the major part of the compounds consisted of hydrocarbons (Figure 3A). These hydrocarbons consisted of alkenes (75%), alkanes (20%), and some monoalkanes (5%). (*Z*)-9-Pentacosene (10) was the most abundant alkene in females, while (*Z*)-9-heptacosene (14) was the most abundant in males. The ratio of (*Z*)-9-pentacosene to (*Z*)-7-pentacosene (11) was opposite in males and females. Furthermore, sometimes the female extracts contained more (*Z*)-9-tricosene than male extracts. (Figures 3B and C). Both male and female extracts contained three oxygen containing compounds: hexyl butyrate (2) and (*E*)-2-hexenyl butyrate (3) and sometimes 1-hexanol (1). Other minor compounds identified in the leg extracts of male and female *L. pabulinus* are listed in Table 1. Table 2 lists some characteristics used to identify the most abundant alkenes present in the extracts.

The vibrational bioassays suggest that female legs are the source of close-range sex pheromone. To determine whether the source of attraction could be defined more precisely, female legs were subdivided into: (A) forelegs, middle legs, and hind legs; or (B) coxae plus femorae, and tibiae plus tarsi. In series A, one pair of forelegs, middle legs, or hind legs of three females was placed in one Petri dish, so that six legs per dish were offered. In series B, the six coxae plus femorae of one female were placed in one Petri dish, and the six tibiae plus tarsi in another. Table 3 shows that all parts of the legs were equally attractive (no significant differences were found between any pair). However, when the overall

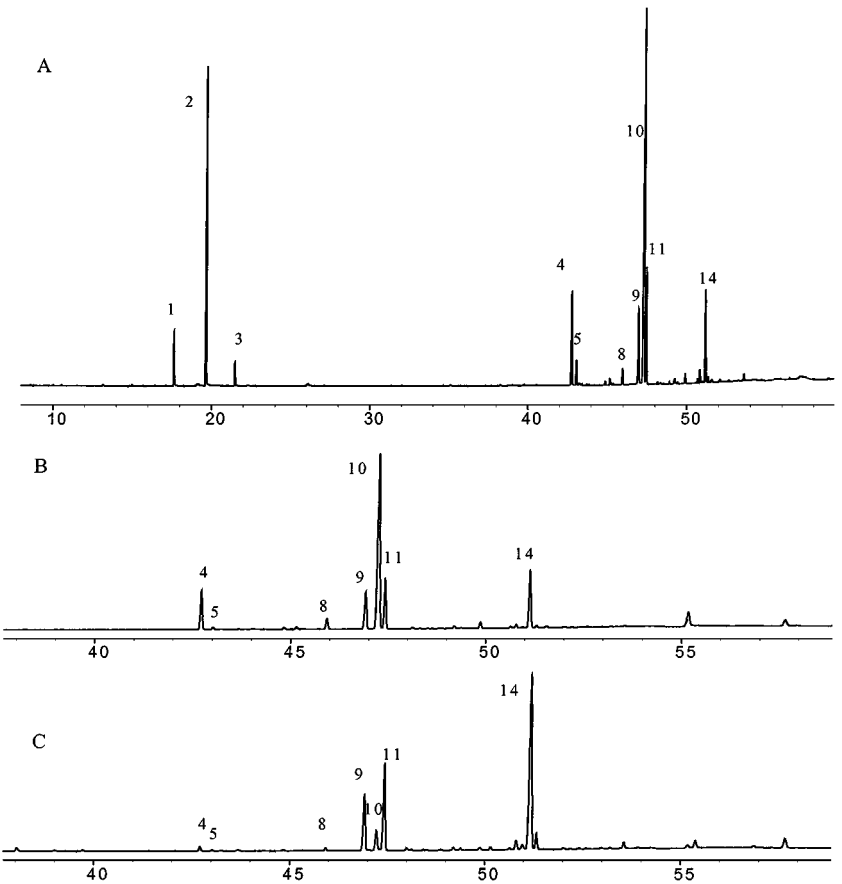


FIG. 3. Gas chromatograms of *L. pabulinus* leg extracts: (A) female leg extract; enlargement of the cuticular hydrocarbon part of female (B) and male (C) extracts. DB-Wax column; FID detector. For explanation of the numbers see Table 1.

response to fore-, middle and hind legs was compared to the overall response to coxae plus femorae and tibiae plus tarsi, responses to entire legs were significantly stronger than to parts.

When legs contain attractive compounds, these compounds may be deposited on the substrate on which female *L. pabulinus* walk. To determine possible deposition of attractive compounds, we tested three different substrates: a piece of potato leaf (cultivar Bintje), a piece of green bean leaf (*Phaseolus vulgaris*, cultivar Miracle), and the glass of an empty Petri dish. One *L. pabulinus* female was allowed to walk in each dish for 75–140 min. As a control, we tested pieces of

TABLE 2. CHARACTERISTICS USED IN IDENTIFICATION OF FOUR MAJOR ALKENES PRESENT IN LEG EXTRACTS OF *L. pabulinus*

| | Kovats indices | | MS characteristics | |
|---|---|---|---|---|
| Compound | DBI | Stabilwax | $M^+$ | $m/z$ after DMDS derivatization |
| 5 | 2271 | 2319 | 322 | 173 ($C_1$–$C_9$), 243 ($C_{10}$–$C_{23}$), 416 ($M^+$) |
| 10 | 2470 | 2518 | 350 | 173 ($C_1$–$C_9$), 271 ($C_{10}$–$C_{25}$), 444 ($M^+$) |
| 11 | 2477 | 2526 | 350 | 145 ($C_1$–$C_7$), 299 ($C_8$–$C_{25}$), 444 ($M^+$) |
| 14 | 2670 | 2719 | 378 | 173 ($C_1$–$C_9$), 299 ($C_{10}$–$C_{27}$), 472 ($M^+$) |

potato leaf or empty dishes on which males had walked for 60–120 min, as well as pieces of potato leaves on which no bug had walked. Table 4 shows that males responded to substrates on which females had walked, while no males showed vibration behavior in any of the control dishes. During the 75–140 min that females walked around in the dishes, a characteristic pheromone-laying behavior was not observed. In addition, desorption of the SPME needle (250°C) revealed the same hydrocarbon profile as found in the leg extracts of the concerning sex. From Figure 4 it is clear that when females walked on or near the needle, a high concentration of (*Z*)-9-pentacosene (10) was deposited on the needle. The volatile compounds 1-hexanol (1), hexyl butyrate (2), and (*E*)-2-hexenyl butyrate (3) were not always present.

When the synthetic mixture, containing compounds derived from female leg extracts, was tested in the vibration bioassay, no males started to vibrate. A few males showed vibratory behavior when male legs, loaded with 5 $\mu$g (*Z*)-9-pentacosene (instead of 1 $\mu$g), were offered (Table 5). Adding (*Z*)-9-tricosene to these legs did not result in more males vibrating. As no biological active compound was found, the possibility of (*Z*)-9-heptacosene acting as a repellent was investigated. Female legs were therefore loaded with a high dose of

TABLE 3. MALE VIBRATION RESPONSE TO DIFFERENT PARTS OF FEMALE LEGS

| Source | Fraction of males responding (±SE) | *N* | | |
|---|---|---|---|---|
| Female legs | 0.70 ± 0.05 | 143 | a[a] | A[b] |
| Forelegs | 0.76 ± 0.14 | 17 | a | |
| Middle legs | 0.71 ± 0.15 | 17 | a | A |
| Hind legs | 0.88 ± 0.10 | 17 | a | |
| Coxae + femorae | 0.45 ± 0.14 | 22 | a | |
| | | | | B |
| Tibiae + tarsi | 0.41 ± 0.14 | 22 | a | |

[a] Different letters indicate signifant differences between pairs ($P < 0.05$).
[b] Different letters indicate significant differences between groups ($P < 0.05$).
See text for statistical methods used.

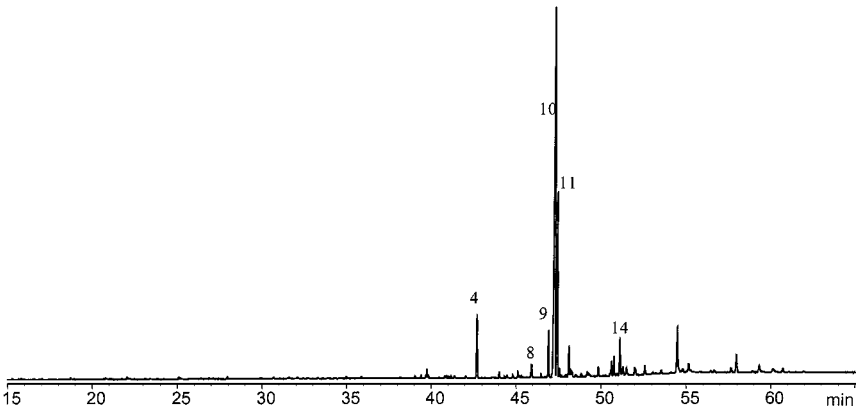


FIG. 4. Gas chromatographic analysis of an SPME needle on which a female bug had walked. DB-Wax column; FID detector. For explanation of the numbers see Table 1.

(*Z*)-9-heptacosene (5–8 $\mu$g). Yet, a similar amount of males showed vibration behavior to these legs as when untreated female legs were offered (Table 5).

## DISCUSSION

The legs of *L. pabulinus* females most consistently elicited vibration behavior in males, suggesting that legs are the source of close-range attraction in this species. Legs have been recognized as the site of sex pheromone release in the aphid *Megoura viciae* (Marsh, 1972), the mosquito *Culiseta inornata* (Lang, 1977), the tsetse fly *Glossina morsitans morsitans* (Carlson et al., 1978), the housefly *Musca domestica* (Schlein et al., 1980), and the parasitoid braconid *Ascogaster reticulatus* (Kainoh and Oishi, 1993). In some species, specific glands in the legs have been

TABLE 4. MALE VIBRATION RESPONSE TO SUBSTRATE ON WHICH FEMALES HAD WALKED

| Source | Fraction of males responding (±SE) | *N* | |
|---|---|---|---|
| Potato leaf on which female walked for 75–140 min | 0.68 ± 0.10 | 39 | a[a] |
| Bean leaf on which female walked for 75–140 min | 0.64 ± 0.13 | 22 | a |
| Empty Petri dish on which female walked for 75–140 min | 0.37 ± 0.09 | 46 | a |
| Potato leaf on which male walked for 60–120 min | 0 | 15 | *b* |
| Empty Petri dish on which male walked for 60–120 min | 0 | 10 | *b* |
| Potato leaf | 0 | 10 | *b* |

[a]Different letters indicate signifant differences ($P < 0.05$). See text for statistical methods used.
[b]Not statistically analysed, as no male had responded.

TABLE 5. MALE VIBRATORY RESPONSE TO FRESHLY DISSECTED LEGS WITH OR WITHOUT ALKENE ADDED

| Source | Fractions of males responding (±SE) | Males ($N$) | |
|---|---|---|---|
| Synthetic mixture | 0 | 32 | a[a] |
| Male legs | 0 | 5 | a |
| Male legs + 5 $\mu$g ($Z$)-9-pentacosene | 0.19 ± 0.11 | 29 | a |
| Male legs + 5 $\mu$g ($Z$)-9-pentacosene + 1 $\mu$g ($Z$)-9-tricosene | 0.05 ± 0.06 | 10 | a |
| Female legs + 5–8 $\mu$g ($Z$)-9-heptacosene | 0.76 ± 0.13 | 21 | b |
| Female legs | 0.70 ± 0.06 | 143 | b |

[a] Different letters indicate significant difference at $P \leq 0.05$ (two-tailed).

identified as the site of sex pheromone excretion (Marsh, 1972; Schlein et al., 1980). In *L. pabulinus*, response to fore-, middle, and hind legs was similarly strong. The lower response to parts of the legs compared to entire legs may be due to the lower amount of leg biomass per Petri dish in the latter group. From these experiments no specific site of possible glands in legs became apparent.

Specific glands in the legs may not synthesize contact sex pheromones. Cuticular hydrocarbons are probably synthesized by oenocytes, large cells that are rich in smooth endoplasmatic reticulum and mitochondria, which appear to be restricted to epidermal tissue in the thorax and abdomen (Gu et al., 1995; Schal et al., 1998). After synthesis, attractive hydrocarbons may be deposited at specific target sites, as in the German cockroach *Blattella germanica*, where the wings accumulate large amounts of pheromone (Gu et al., 1995). The cuticle of the legs may thus be the specific target deposition site of the attractive compounds.

The presence of a close-range sex pheromone on the legs may also be due to grooming. Grooming may either accumulate pheromone from other body parts on the legs (Howard and Blomquist, 1982), or it may spread the pheromone from leg glands over the whole body surface, as in polistine wasps (Beani and Calloni, 1991), whose territorial marking pheromones from leg glands function as sex attractants as well. *L. pabulinus* males and females groom frequently (Groot et al., 1998) and the attractive compounds are not only present on female legs, but also on other body parts as demonstrated by the male response to female wings and heads and to pieces of filter paper rubbed over female bodies. In short, the site of sex pheromone production does not have to be the site of pheromone release, specific glands are not necessarily involved, and grooming may enhance chemical dispersion or accumulation at specific sites.

In mirids, close-range or contact pheromones have not yet been studied, and to our knowledge this is the first study on cuticular hydrocarbons of a mirid species. In *L. pabulinus* the major part of the hydrocarbons consisted of alkenes, followed by alkanes and some methyl alkanes. All compounds were $C_{23}$–$C_{29}$

hydrocarbons. This is also the first time that a clear-cut difference was found in *L. pabulinus* between male- and female-derived compounds, i.e., females produce a high amount of (*Z*)-9-pentacosene, whereas males produce high amounts of (*Z*)-9-heptacosene. Because of the low amount of (*Z*)-9-pentacosene present in males, the ratio of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene is opposite in males and females. To our knowledge, there are only two other studies on cuticular hydrocarbons in heteropteran species, the milkweed bug, *Oncopeltus fasciatus* (Lygaeidae) (Jackson, 1983), and *Triatoma infestans* and *T. mazzotti* (Reduvidae) (Juárez and Blomquist, 1993). In all these species mostly *n*-alkanes, branched monoalkanes, and dimethylalkanes were found. Furthermore hydrocarbons of up to 41–43 carbon atoms were found. More importantly, male and female *O. fasciatus*, *T. infestans*, and *T. mazzotti* had similar profiles, whereas *L. pabulinus* males and females produce a different blend of hydrocarbons.

The hydrocarbons of *L. pabulinus* females appear to elicit vibratory behavior in males, as extracts in water (in which no hydrocarbons are present) were not active. Furthermore, female legs used for water extraction remained active, indicating that the compounds were still present on the legs. Fractionation of the extracts on silica into two fractions (hydrocarbons and oxygenated compounds) suggested that both fractions were needed to elicit vibration behavior in males. The composition of the oxygenated compound fraction of males and females did not show any difference. The similarity of this fraction between males and females was supported by the finding that this fraction from males combined with the hydrocarbon fraction of females did cause vibratory behavior in males (unpublished results).

In various species cuticular hydrocarbons have been identified as contact pheromones (e.g., Muhammed et al., 1975; Carlson et al., 1978; Bolton et al., 1980; Dillwith et al., 1981; Blomquist et al., 1993; Gu et al., 1995; Fukaya et al., 1996; Doi et al., 1997). Alkenes seem to be involved in sexual communication between sexes (Howard and Blomquist, 1982). More precisely, (*Z*)-9-pentacosene is often the main hydrocarbon present in beetles (Baker et al., 1979), ants (Morgan et al., 1992), and bees (Paulmier et al., 1999). In *L. pabulinus*, although (*Z*)-9-pentacosene is the only compound obviously present in much lower amounts in males than in females, the activity of this compound on its own could not be proven in bioassays. Given that the synthetic mixture did not elicit vibration behavior in males, one cannot rule out the possibility of other compounds being part of the contact pheromone. The most abundant male alkene, (*Z*)-9-heptacosene, did not act as a repellent, because female legs sprayed with a high dose of this alkene still elicited vibratory behavior in males (Table 5). More experiments are needed to determine if there are other compounds present in the extracts that act as a vibration elicitor.

The attractive compounds on female legs may be deposited on the substrate. Male *L. pabulinus* showed strong responses to substrates on which females had

walked. The fraction of males responding to potato leaves was even similar to responses to female legs. When females were allowed to walk on a SPME needle, a similar chemical profile was found as in attractive female leg extracts. These results support the hypothesis that these hydrocarbons are deposited on the substrate. As a characteristic pheromone-laying behavior was not observed, we suspect that deposition on the substrate occurs passively or that pheromone is adsorbed to the substrate. Adsorption or deposition of attractive compounds on the substrate increases the probability of sex encounters, as it elicits intensive search by males in these areas (Colwell et al., 1978; Fauvergue et al., 1995). Depending on their volatility, these pheromones are active at some distance, as in *M. viciae* (Pickett et al., 1992), or they elicit response at close range or upon contact, as in the other species mentioned. Adsorption of pheromone to a substrate also increases the surface area from which pheromone evaporates, thereby increasing both the rate of volatilization and the possible communication distance (Colwell et al., 1978). Males may follow a gradient of intensity, created by the decay of the compounds over time, to orient their movements towards females (Fauvergue et al., 1995). In this way, the functional range of low-volatility cuticular hydrocarbons would be greatly enlarged.

Identification of the sex pheromones of mirid species is a challenging task. This study indicates that the sex-specific cuticular hydrocarbons, probably in addition to esters, may play a role in the sexual communication of these bugs. Evidence is accumulating that such hydrocarbons not only function as contact pheromones, but are also involved in attraction at a (short) distance. Uebel et al. (1978) demonstrated that field catches of male *Fannia canicularis* and *F. pusio* increased slightly when alkenes were loaded on the lures. Connor et al. (1980) reported that the pheromone of the arctiid moth *Utetheisa ornatrix* (*Z,Z,Z*-3,6,9-heneicosatriene plus a small quantity of an unidentified $C_{21}$ tetraene), perhaps serves as a close-range orientation cue for locating the female. Recently, Schiestl et al. (1999, 2000) reported that flowers of *Ophrys* orchids also mimic the odor profile of bees by using this class of alkenes in order to attract these bees. Successful pheromone trapping of mirids might also have to take these close-range cues into account.

*Acknowledgments*—We thank Anneke Heijboer for rearing *L. pabulinus*, Dr. M. A. Posthumus for carrying out all the GS-MS experiments, and Pieter Vereijken for conducting the statistical analyses. Marcel Dicke, Teris van Beek, and Hans Visser are greatly acknowledged for critical comments on earlier versions of the manuscript. This study was funded by the Dutch Technology Foundation (WBI44.3383).

## REFERENCES

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol*. 33:211–238.

BAKER, J. E., NELSON, D. R., and FATLAND, C. L. 1979. Developmental changes in cuticular lipids of the black carpet beetle, *Attagenus megatoma. Insect Biochem*. 9:335–339.

BEANI, L., and CALLONI, C. 1991. Leg tegumental glands and male rubbing behavior at leks in *Polistes dominilus* (Hymenoptera: Vespidae). *J. Insect Behav*. 4:449–461.

BLOMMERS, L. H. M., VAAL, F. W. N. M., and HELSEN, H. H. M. 1997. Life history, seasonal adaptations, and monitoring of common green capsid *Lygocoris pabulinus* (L.) (Hemiptera: Miridae). *J. Appl. Entomol*. 121:389–398.

BLOMQUIST, G. J., TILLMAN-WALL, J. A., GUO, L., QUILICI, D. R., GU, P., and SCHAL, C. 1993. Hydrocarbon and hydrocarbon derived sex pheromones in insects: Biochemistry and endocrine regulation, pp. 317–351, *in* D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry & Biology. University of Nebraska Press, Lincoln, Nebraska.

BOLTON, H. T., BUTLER, J. F., and CARLSON, D. A. 1980. A mating stimulant pheromone of the horn fly, *Haematobia irritans* (L.): demonstration of biological activity in separated cuticular components. *J. Chem. Ecol.* 6:951–964.

BUTENANDT, A., BECKMANN, R., STAMM, D., and HECKER, E. 1959. Über den Sexuallockstof des Seidenspinner, *Bombyx mori*. Reindarstellung und Kostitution. *Z. Naturforsch*. 14B:283–284.

CARAYON, J. 1971. Notes et documents sur l'appareil odorant métathoraciques des Hémiptères. *Ann. Soc. Entomol. Fr.* 7:737–770.

CARDÉ, R. T., and MINKS, A. K. 1997. Insect Pheromone Research: New Directions. Chapman and Hall, New York, 684 pp.

CARDÉ, R. T., BAKER, T. C., and ROELOFS, W. L. 1975. Behavioral role of individual components of a multichemical attractant system in the Oriental fruit moth. *Nature* 253:348–349.

CARLSON, D. A., MAYER, M. S., SILHACEK, D. L., JAMES, J. D., BEROZA, M., and BIERL, B. A. 1971. Sex attraction pheromone of the house fly: Isolation, identification and synthesis. *Science* 174:76–77.

CARLSON, D. A., LANGLEY, P. A., and HUYTON, P. 1978. Sex pheromone of the tsetse fly: Isolation, identification, and synthesis of contact aphrodisiacs. *Science* 201:750–753.

CARLSON, D. A., ROAN, C., YOST, R. A., and HECTOR, J. 1989. Dimethyl disulfide derivatives of long chain alkenes, alkadienes and alkatrienes for gas chromatography/mass spectrometry. *Anal. Chem.* 61:1564–1571.

COLWELL, A. E., SHOREY, H. H., BAUMER, P., and VAN VORHIS KEY, S. E. 1978. Sex pheromone scent marking by females of *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *J. Chem. Ecol.* 4:717–721.

CONNOR, W. E., EISNER, T., VANDER MEER, R. K., GUERRO, A., GHIRINGELLI, D., and MEINWALD, J. 1980. Sex attractant of an arctiid moth (*Utetheisa orntrix*): A pulse chemical signal. *Behav. Ecol. Sociobiol*. 7:35–63.

DILLWITH, J. W., BLOMQUIST, G. J., and NELSON, D. R. 1981. Biosynthesis of the hydrocarbon components of the sex pheromone of the housefly, *Musca domestica* L. *Insect Biochem*. 11:247–253.

DOI, M., NEMOTO, T., NAKANISHI, H., KUWAHARA, Y., and OGUMA, Y. 1997. Behavioral response of males to major sex pheromone component, *(Z,Z)*-5,25-hentriacontadiene, of *Drosophila ananassae* females. *J. Chem. Ecol.* 23:2067–2078.

DRIJFHOUT, F. P., VAN BEEK, T. A., VISSER, J. H., and DE GROOT, Æ. 2000. On-line thermal desorption-gas chromatography of intact living insects for pheromone analysis. *J. Chem. Ecol.* 26:1383–1392.

FAUVERGUE, X., HOPPER, K. R., and ANTOLIN, M. F. 1995. Mate finding via a trail sex pheromone by a parasitoid wasp. *Proc. Natl. Acad. Sci. U.S.A.* 92:900–904.

FUKAYA, M., YASUDA, T., WAKAMURA, S., and HONDA, H. 1996. Reproductive biology of the yellow-spotted longicorn beetle, *Psacotheca hilaris* (Pascoe) (Coleoptera: Cerambycidae). III. Identification of contact sex pheromone on female body surface. *J. Chem. Ecol.* 22:259–270.

GRAHAM, H. M. 1988. Sexual attraction of *Lygus hesperus* Knight. *Southwest. Entomol*. 13:31–37.

GROOT, A. T., VAN DER WAL, A. J., SCHUURMAN, A., VISSER, J. H., BLOMMERS, L. H. M., and VAN BEEK, T. A. 1998. Copulation behavior of *Lygocoris pabulinus* under laboratory conditions. *Entomol. Exp. Appl.* 88:219–228.

GU, X., QUILICI, D., JUÁREZ, P., BLOMQUIST, G. J., and SCHAL, C. 1995. Biosynthesis of hydrocarbons and contact sex pheromone and their transport by lipophorin in females of the German cockroach (*Blattella germanica*). *J. Insect Physiol.* 41:257–267.

HARDIE, J., and MINKS, A. K. (eds.). 1999. Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, Oxon, UK, 466 pp.

HOWARD, R. W., and BLOMQUIST, G. J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol*. 27:149–172.

JACKSON, L. J. 1983. Cuticular hydrocarbons of the milkweed bug *Oncopeltus fasciatus* by age and sex. *Insect Biochem*. 13:19–25.

JUÁREZ, P., and BLOMQUIST, G. J. 1993. Cuticular hydroacorbons of *Triatoma infestans* and *T. mazzotti*. *Comp. Biochem. Physiol*. 106B:667–674.

KAINOH, Y., and OISHI, Y. 1993. Source of sex pheromone of the egg-larval parasitoid *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *J. Chem. Ecol*. 19:963–969.

KENNEDY, J. S. 1977. Behaviorally discriminating assays of attractants and repellents, pp. 215–229, *in* H. H. Shorey and J. J. McKelvey, Jr. (eds.). Chemical Control of Insect Behavior, Theory and Application. John Wiley & Sons, New York.

LANG, J. T. 1977. Contact sex pheromone in the mosquito *Culiseta inornata* (Diptera: Culicidae). *J. Med. Entomol*. 14:448–454.

LEADBETTER, G., and PLIMMER, J. R. 1979. An improved preparation of some insect sex attractants: Synthesis and separation of geometrical isomers by formation of urea complexes. *J. Chem. Ecol*. 5:101–108.

MARSH, D. 1972. Sex pheromone in the aphid *Megoura viciae. Nature* 238:31–32.

MCBRIEN, H. L., and MILLAR, J. C. 1999. Phytophagous bugs, pp. 277–304, *in* J. Hardie and A.K. Minks (eds.). Pheromones of Non-Lepidopterous Insects Associated with Agricultural Plants. CABI Publishing, Oxon, UK.

MCCULLAGH, P., and NELDER, J. A. 1989. Generalized Linear Models, 2nd ed. Chapman and Hall, London.

MILLAR, J. G., and RICE, R. E. 1998. Sex pheromone of the plant bug *Phytocoris californicus* Knight (Heteroptera: Hemiptera: Miridae). *J. Econ. Entomol.* 91:132–137.

MILLAR, J. G., RICE, R. E., and WANG, Q. 1997. Sex pheromone of the mirid bug *Phytocoris relativus* Knight. *J. Chem. Ecol.* 23:1743–1754.

MINKS, A. K., and VAN DEVENTER, P. 1992. Practical applications: The European scene, pp. 9–18, *in* R. L. Ridgway, M. Inscoe, and H. Arn (eds.). Insect Pheromones and Other Behavior-Modifying Chemicals. BCPC Monograph No 51. Farnham, British Crop Protection Council.

MORGAN, E. D., HÖLLDOBLER, B., VAISAR, T., and JACKSON, B. D. 1992. Contents of poison apparatus and their relation to trail-following in the ant *Deceton atmigerum. J. Chem. Ecol.* 18:2161–2168.

MUHAMMED, S., BUTLER, J. F., and CARLSON, D. A. 1975. Stable fly sex attractant and mating pheromones found in female body hydrocarbons. *J. Chem. Ecol.* 1:399–412.

PAULMIER, I., BAGNÈRES, A., AFONSO, C. M. M., DUSTICIER, G., RIVIÈRE, G., and CLÉMENT, J. 1999. Alkenes as a sexual pheromone in the leaf-cutter bee *Megachile rotundata. J. Chem. Ecol.* 25:471–490.

PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–90.

SCHAL, C., SEVALA, V. L., YOUNG, H. P., and BACHMANN, J. A. S. 1998. Sites of synthesis and transport pathways of insect hydrocarbons: Cuticle and ovary as target tissues. *Am. Zool.* 38:382–393.

SCHIESTL, F. P., AYASSE, M., PAULUS, H. F., LÖFSTEDT, C., HANSSON, B. S., IBARRA, F., and FRANCKE, W. 1999. Orchid pollination by sexual swindle. *Nature* 399:421–422.

SCHIESTL, F. P., AYASSE, M., PAULUS, H. F., LÖFSTEDT, C., HANSSON, B. S., IBARRA, F., and FRANCKE, W. 2000. Sex pheromone mimicry in the early spider orchid (*Ophrys sphegodes*): Patterns of

hydrocarbons as the key mechanism for pollination by sexual deception. *J. Comp. Physiol. A* 186:567–574.

SCHLEIN, Y., GALUN, R., and BEN-ELIAHU, M. N. 1980. The legs of *Musca domestica* and *Glossina morsitans* females as the site of sex pheromone release. *Experientia* 38:1174–1176.

SMITH, R. F., PIERCE, H. D., and BORDEN, J. H. 1991. Sex pheromone of the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *J. Chem. Ecol*. 17:1437–1447.

STADDON, B. W. 1979. The scent glands of Heteroptera. *Adv. Insect Physiol*. 14:351–418.

UEBEL, E. C., SCHWARS, M., SONNET, P. E., MILLER, R. W., and MENZER, R. E. 1978. Evaluation of the mating stimulant pheromone of *Fannia canicularis*, *F. pusio*, and *F. femoralis* as attractants. *Fla. Entomol*. 61:139–143.