

ATTRACTANT PHEROMONE AND ALLOMONE FROM THE METATHORACIC SCENT GLAND OF A BROAD-HEADED BUG (HEMIPTERA: ALYDIDAE)

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Abstract

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Females of *Alydus eurinus* (Say) release an attractant pheromone from their metathoracic scent gland. Conspecific males and, to a lesser extent, females and nymphs were attracted to blends containing the female-specific essential pheromone components 2-methylbutyl butyrate and (*E*)-2-methyl-2-butenyl butyrate, whereas individuals of *Alydus pilosulus* Herrick-Schaeffer were not attracted. When attacked, however, alydid adults emit chemicals for defense—butyric and hexanoic acids in *A. eurinus*—from the metathoracic scent glands. Mimicry is actually the first line of defense for most broad-headed bugs, including the common North American species studied here, whose nymphs are remarkable ant mimics and whose adults strongly resemble spider wasps (Hymenoptera: Pompilidae). The possibility that disparate heteropterans (Hemiptera) produce sexual pheromones in their metathoracic scent glands must be considered in future pheromone research on heteropterans, especially for species with the specialized lines of defense indicated by aposematism or mimicry.

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Résumé

Les femelles d'*Alydus eurinus* (Say) ont une glande métathoracique qui émet une phéromone d'attraction. Les mâles de l'espèce, et à un degré moindre les femelles et les larves, sont attirés par les mélanges qui contiennent les composantes essentielles de la phéromone de la femelle, le butyrate de 2-méthyl-butyle et le butyrate de (*E*)-2-méthyl-2-butenyle, alors que les individus d'*Alydus pilosulus* Herrick-Schaeffer ne sont pas attirés. Lorsqu'ils sont attaqués, les adultes d'alydides émettent de leurs glandes des substances de défense—acides butyrique et hexanoïque chez *A. eurinus*. Le mimétisme est le principal système de défense des Punaises à grosse tête, y compris les espèces nord-américaines communes étudiées ici, dont les larves sont des imitations remarquables de fourmis et dont les adultes ressemblent fort à des pompiles (Hymenoptera : Pompilidae). Il faut envisager, dans les programmes de recherche sur les phéromones des hétéroptères, la possibilité que des hétéroptères (Hemiptera) de diverses lignées émettent des phéromones sexuelles à partir de leurs glandes métathoraciques odorantes, surtout chez les espèces qui possèdent des systèmes de protection spécialisés tels l'aposématisme et le mimétisme.

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Introduction

Alydids (Hemiptera: Heteroptera) are noteworthy insects, in that their nymphs are often remarkable ant mimics (Yonke and Medler 1968) and their adults “give off an odor reminiscent of someone with a bad case of halitosis” (Borror and DeLong 1971). Members of the most highly evolved subfamily in the group, the Alydinae,

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feed primarily on the seeds of various Leguminosae, often in aggregations of mixed species, but curiously, they have frequently been collected feeding on carrion and feces (Schaefer 1980).

Nearly 30 years ago one of us (JRA) investigated the scent-gland morphology of ant-mimetic alydid nymphs and the chemistry of alydid scent gland secretions (Aldrich and Yonke 1975). Among the bugs investigated were *Alydus eurinus* (Say) (Hemiptera: Alydidae) and *Alydus pilosulus* Herrick-Schaeffer, whose adults, particularly those of *A. eurinus*, are also mimetic as a first line of defense; *A. eurinus* adults have a narrow black body with bright orange under the wings, resembling spider wasps (Hymenoptera: Pompilidae) at rest or in flight (Borror and DeLong 1971). Adults and late-instar nymphs of these two species often abundantly coexist on bush clover, *Lespedeza capitata* Michx. (Leguminosae). Butyric and hexanoic acids were identified as the primary components of the adult metathoracic scent gland secretions of these *Alydus* spp., accounting for their extraordinary repugnance. Behavioral (Numata and Hidaka 1990) and chemical investigations of the soybean pest *Riptortus clavatus* Thunberg (Hemiptera: Alydidae, Alydinae), demonstrated that, although males release an aggregation pheromone from their metathoracic scent gland, adults are still able to emit defensive compounds from their metathoracic scent glands when disturbed (Leal and Kadosawa 1992; Leal *et al.* 1995; Mizutani *et al.* 1997). In *Leptocoris chinensis* (Dallas) (Hemiptera: Alydidae, Leptocorisinae), females attract males with a sex pheromone emitted from their metathoracic scent gland (Leal *et al.* 1996). Therefore, given the opportunity to collect large numbers of *A. eurinus* adults from bush-clover stands at the Beltsville Agricultural Research Center (BARC), we decided to reinvestigate the semiochemistry of this alydid using more modern techniques than employed previously, including capillary gas chromatography with electroantennogram detection (GC-EAD), to identify the pheromone of this insect.

Materials and Methods

Insects. *Alydus eurinus* late-instar nymphs and adults were collected (39°02'N, 76°55'W) from bush clover (*L. capitata*) in July and August 1999, and maintained in the laboratory at $26 \pm 1^\circ\text{C}$ under 16L:8D on fresh green beans (Leguminosae: *Phaseolus vulgaris* L.), raw sunflower (Compositae: *Helianthus annuus* L.), soybean seeds [Leguminosae: *Glycine max* (L.)], and water. Field-collected male and female adults were maintained separately, and virgin adults were added to the respective cages as field-collected nymphs molted to adults.

Collection of Volatiles. Groups of 10–30 either all male or all female adults were introduced into 1-L 4-necked glass vessels, each containing a fresh green bean and a water bottle. Air was drawn by vacuum (1 L/min) into the vessel through 6- to 14-mesh activated charcoal and out of the vessel through two traps containing about 200 mg of Super QTM (2 cm \times 1.5 cm o.d.; Alltech Associates, Inc., Deerfield, Illinois).² Bugs were aerated for 1–3 d. The adsorbent was eluted with CH₂Cl₂ (4 \times 0.5 mL) and stored at -4°C until used.

Gas Chromatography – Electroantennogram Detector Analysis. The GC-EAD system was as previously described (Zhang *et al.* 1997), except that a 60 m \times 0.25 mm i.d., 0.25- μm film thickness, DB-5TM capillary column was used (50°C for 2 min, to 300°C at 10°C/min, held for 10 min; J&W Scientific, Folsom, California). Antennal

² Mention of commercial products does not constitute an endorsement by the USDA.

recordings were made using a holder constructed from a piece of acrylic plastic (8 cm long \times 0.8 cm wide \times 0.6 cm thick), into which, near its end, two 1.25 mm diameter wells about 3 mm apart were drilled; a notch cut between the two wells allowed air-flow. Each well was filled with 0.9% NaCl solution; the cut end of an antenna was inserted in one well and the uncut tip of the antenna was inserted in the other well. Each well was connected *via* a gold wire to a high-impedance 1:100 amplifier with automatic base-line drift compensation, and the antennal preparation was cooled to about 5°C inside a water-cooled condenser (RTE-100, NESLAB Instruments, Inc., Portsmouth, New Hampshire). The flame ionization and electrophysiological output signals were recorded using Hewlett-Packard ChemStationTM software.

Compound Identification. Samples were analyzed by gas chromatography – mass spectrometry (GC–MS) on an HP 6890 GC coupled to an HP 5973 Mass Selective Detector, using the same type of GC column and conditions as listed above, except that helium was the carrier gas. Compound identifications were verified by coinjection and spectral comparisons with known standards purchased or prepared from precursors from Aldrich Chemical Company (Milwaukee, Wisconsin). The enantiomers of 2-methylbutyl butyrate were resolved on a γ -cyclodextrin trifluoroacetyl capillary column (30 m \times 0.25 mm i.d.; 40°C for 10 min, to 50°C at 1°C/min, held for 2 min; Advanced Separation Technologies, Inc., Whippany, New Jersey).

Field Test. Traps were made of transparent cylindrical containers (20.2 \times 19.7 cm; Tri-State Molded Plastics, Inc., Dixon, Kentucky), by cutting two holes (9 cm diameter) in opposite sides and covering each hole with an inwardly projecting screen funnel. Pheromone solutions were prepared so that a compound in each blend was present at the same concentration per 100 μ L CH₂Cl₂ dose of each blend [*i.e.*, 3.33 mg total active ingredient (AI) per dose for the two-component blend, 5 mg total AI per dose for the three-component blend, and 10 mg total AI per dose for the four-component blend (Fig. 3 and below)]. Traps were baited with gray rubber septa (5 \times 9 mm; Thomas Scientific, Philadelphia, Pennsylvania) loaded with 100 μ L of a pheromone solution. Every 3–5 d, traps were rebaited with fresh pheromone-impregnated septa, and trapped insects were removed.

The first pheromone blend (blend I), tested from 23 to 30 August 1999, was a 1:1:1 blend by volume of butyl butyrate, (*S*)-(–)-2-methylbutyl butyrate, and (*E*)-2-methyl-2-butenyl butyrate, reflecting the GC peak areas of the three most EAD-active compounds in extracts from *A. eurinus* females. Four traps baited with this treatment were deployed along with four unbaited control traps. Traps were hung about 1 m from the ground and at least 15 m apart along a chain-link fence bordering a field of bush clover at BARC-West (39°02'N, 76°55'W) in which *A. eurinus* and *A. pilosulus* had been collected by sweep-netting. On 9 September, the supply of blend I was exhausted and this treatment was discontinued.

Additional testing was conducted at the original site and at a similar site nearby from 30 August through 7 November 1999, as follows. Three other pheromone blends (blends II, III, and IV) were prepared with combinations of butyl butyrate, (*S*)-(±)-2-methylbutyl butyrate, (*E*)-2-methyl-2-butenyl butyrate, and hexyl butyrate (Fig. 3): blend II was the same as blend I, except racemic 2-methylbutyl butyrate was used; blend III contained only the two most EAD-active components; and blend IV contained hexyl butyrate at four times the concentration of the other esters, reflecting the greater abundance of this compound in natural *A. eurinus* extracts. Two traps per treatment were deployed and serviced as described above in a randomized block design at each site (a total of four traps per treatment), including a set of four replicates of blend I,

until the supply of this mixture was consumed (9 September). Two unbaited control traps were also deployed at each site.

Statistical Analysis. Analysis of variance (ANOVA) was performed with the MIXED procedure (SAS Institute Inc. 1997). Data were $\log(x + 1)$ transformed to satisfy ANOVA assumptions before least squares (LS) means were calculated. Treatments labeled with the same letter are not significantly different at $\alpha = 0.05$.

Results

Butyric and hexanoic acids (peaks 5 and 6) are the major volatile compounds obtained from *A. eurinus* adults, plus hexyl butyrate (peak 4) (Fig. 1). GC-EAD analysis revealed that the antennae of both male (Fig. 1) and female (not shown) *A. eurinus* are relatively insensitive to these compounds compared with antennal responses to the much less abundant esters butyl butyrate (peak 1) [m/z (%): 41 (34), 43 (50), 49 (16), 56 (40), 71 (100), 84 (14), 101 (4)], 2-methylbutyl butyrate (peak 2) [m/z (%): 41 (24), 43 (50), 49 (9), 55 (16), 70 (54), 71 (100), 84 (9), 89 (8), 100 (4), 101 (4)], and (*E*)-2-methyl-2-butenyl butyrate (peak 3) [m/z (%): 41 (67), 43 (78), 53 (19), 67 (53), 68 (67), 69 (43), 71 (100), 85 (12), 86 (30), 89 (13), 113 (1), 156 (M^+ , 7)]. In addition, no trace of the latter two butyrates (peaks 2 and 3) could be detected in aeration samples of male *A. eurinus*, and esters 1 and 4 appeared to be more abundant in samples from females than in samples from males (Fig. 2). Chiral GC analysis showed that female *A. eurinus* produce predominantly, if not solely, (*S*)-(-)-2-methylbutyl butyrate.

In the initial field test of the three-component blend that included (*S*)-(-)-2-methylbutyl butyrate, 53 male and one female adult *A. eurinus* were caught in pheromone-baited traps, along with one fourth- and two fifth-instar nymphs (not analyzed statistically). No aldydids were caught in control traps and no *A. pilosulus* adults were captured, although individuals of this species were present at the field site.

The first part of the second field tests (30 August through 9 September), comparing pheromone made with (*S*)-(-)-2-methylbutyl butyrate (blend I) with pheromone made with racemic 2-methylbutyl butyrate (blend II) and unbaited control traps, showed a significant treatment effect ($F_{2,20} = 17.82$; $P = 0.0001$). Paired comparisons of LS means (*t* tests) showed that blends I and II were each highly significantly different from controls ($P < 0.001$), and indicated that blend II was marginally more attractive than blend I at $\alpha = 0.05$ (transformed LS means \pm SE = 0.61 ± 0.089 and 0.40 ± 0.089 , respectively; $P = 0.0524$).

Tests of the blends II, III, and IV that were run from 30 August through 7 November (Fig. 3) showed that males were significantly more attracted to all blends than females (Figs. 3 and 4). For *A. eurinus* males, field testing of these blends showed significant treatment effects ($F_{3,171} = 48.11$; $P = 0.0001$). Paired LS mean comparisons showed that blends II, III, and IV were each highly significantly different from controls ($P < 0.001$). Addition of hexyl butyrate (blend III) to the three-component blend (blend II) did not significantly alter attraction (transformed LS means \pm SE = 1.42 ± 0.248 and 1.44 ± 0.248 , respectively; $P = 0.8889$), whereas the omission of butyl butyrate (blend IV; LS mean = 1.02 ± 0.248) did significantly reduce the attraction of males relative to blends II and III ($P < 0.01$ for each comparison). For *A. eurinus* females, the field tests of blends II, III, and IV also showed significant treatment effects ($F_{3,171} = 11.84$; $P = 0.0001$). Paired LS mean comparisons showed that blends II, III, and IV were each highly significantly different from controls ($P < 0.001$), but that blends II, III, and IV were equally attractive to female *A. eurinus* (transformed LS means \pm SE = 0.24 ± 0.077 , 0.22 ± 0.077 , and 0.16 ± 0.077 , respectively; $P < 0.01$ for each comparison).

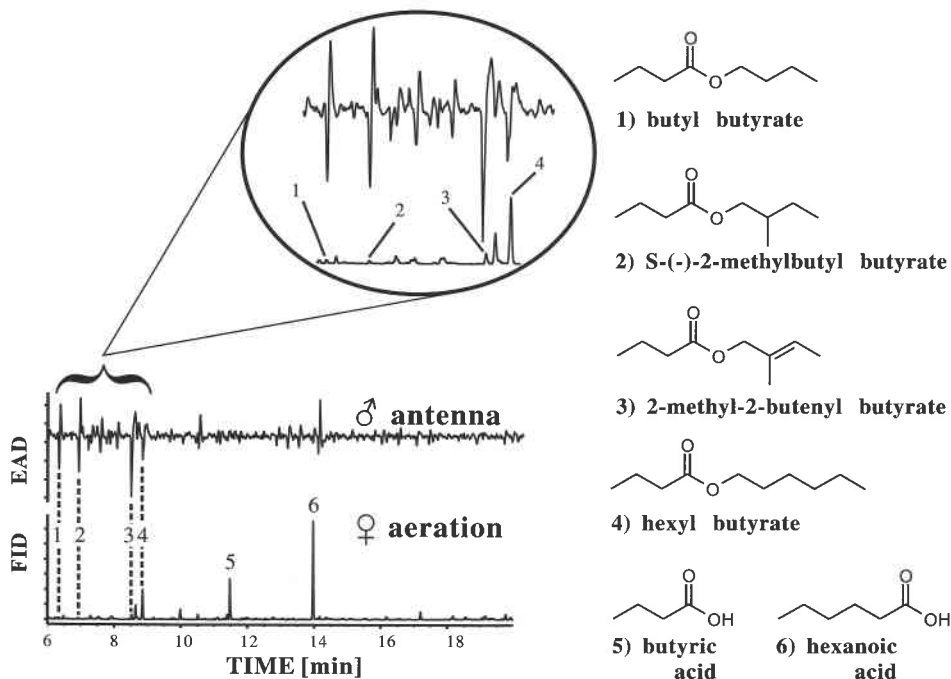


FIGURE 1. Gas chromatogram – electroantennogram detector traces for the metathoracic scent gland extract of *Alydus eurinus*, showing a close-up of the pheromone region and the identified pheromone (1–4) and allomone (5 and 6) compounds.

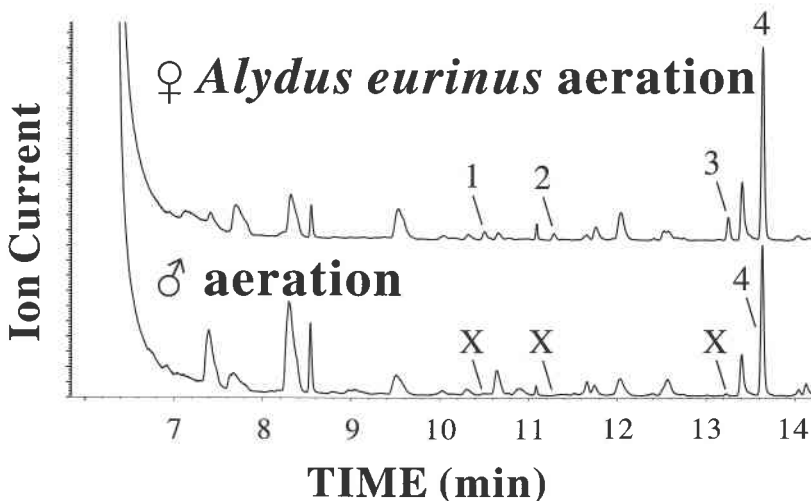
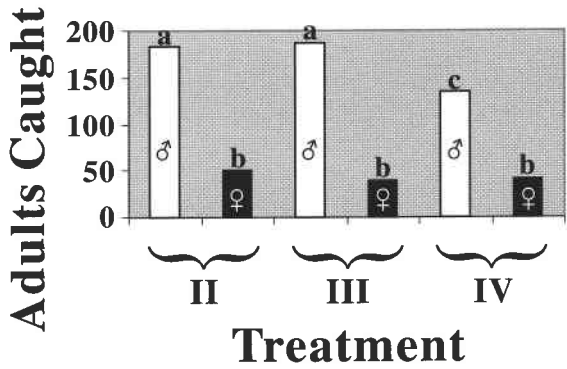


FIGURE 2. The pheromone-ester region of the reconstructed ion chromatograms for female and male *Alydus eurinus* aeration extracts. Compounds are numbered as in Fig. 1, and X indicates the absence of mass spectra matching the corresponding ester identified in the female extract.

From 30 August through 14 September, a total of 15 fifth-instar and four fourth-instar nymphal *Alydus* were captured; these nymphs are assumed to have been *A. eurinus*, because no adult *A. pilosulus* were caught, except for one female in a control trap on 2 September. No individual *A. eurinus* were caught in control traps.



Compound	Blend Ratio (volume)		
	II	III	IV
butyl butyrate	1	1	-
(±)-2-methylbutyl butyrate	1	1	1
2-methyl-2-butenyl butyrate	1	1	1
hexyl butyrate	-	4	-

FIGURE 3. Number of adult *Alydus eurinus* caught in traps baited with three pheromone blends containing racemic 2-methylbutyl butyrate and (*E*)-2-methyl-2-butenyl butyrate (blend IV) with or without butyl butyrate and hexyl butyrate (blends III and II, respectively) from 2 September through 7 November 1999. Bars with different letters are significantly different from each other (ANOVA, *P* < 0.05).

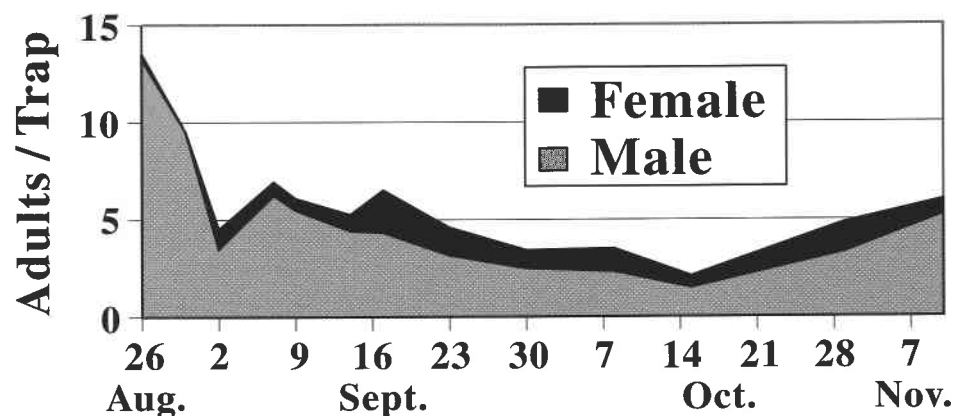


FIGURE 4. Mean trap captures of male and female *Alydus eurinus* from 26 August through 7 November 1999 (blend I during August and blends II–IV during September–November; see text for details).

Discussion

Despite earlier predictions to the contrary (Aldrich 1988*b*), various male or female true bugs produce ester-dominated attractant pheromones in their metathoracic scent gland in addition to defensive compounds, for example, Lygaeinae (Aldrich *et al.* 1997, 1999), Miridae (Smith *et al.* 1991; Millar *et al.* 1997; Millar and Rice 1998), and

Alydidae (Leal *et al.* 1995, 1996). In the case of *A. eurinus*, females release a blend of butyrate esters that are attractive specifically to conspecific males and, to a lesser extent, females and nymphs. Hexyl and butyl butyrates were identified from the metathoracic scent gland secretion of *A. eurinus* in the original investigation (Aldrich and Yonke 1975), and hexyl butyrate is, in fact, produced in the lateral glands (JR Aldrich, unpublished data). It is probable that the female-specific esters, 2-methylbutyl butyrate and (*E*)-2-methyl-2-butenyl butyrate, are also synthesized in the lateral glands of the metathoracic scent gland complex, but this requires verification. Although predominantly (*S*)-(-)-2-methylbutyl butyrate was detected in the chiral-column analysis of an aeration extract of female *A. eurinus*, the possibility exists that (*R*)-(+)-2-methylbutyl butyrate is also produced and plays a role in attraction, because pheromone made with the racemic compound was marginally more attractive than pheromone made with the (*S*)-(-)-enantiomer. Discovery that the *A. eurinus* pheromone attracts only conspecifics, although individual *A. pilosulus* were present in the bush-clover field sites, indicates that pheromones are involved in the isolation of these congeners. In addition, the fact that pheromones for species in the genus *Alydus* consist of butyrate esters may explain why these and related bugs are routinely collected feeding opportunistically on carrion and feces. It is likely that butyrate esters serve as kairomones for the bugs after forming spontaneously from rancid acids in these food sources (Worden *et al.* 1989).

A recent report on the bean bug, *Riptortus linearis* (F.), indicates that males produce an aggregation pheromone attractive to both sexes and second-instar nymphs, whereas females appear to release a pheromone attractive only to conspecific males (Higuchi and Nakamori 1999). Furthermore, *Riptortus* spp. are subject to egg parasitism by encyrtid wasps that use attractant pheromones of their hosts as kairomones to locate areas likely to have eggs (Leal *et al.* 1995; Mizutani *et al.* 1997). Thus far, we have seen no evidence that parasitoids use the *A. eurinus* pheromone as a kairomone, and there are no records of parasitoids from North American Alydidae (Arnaud 1978; Yonke and Medler 1968). An unusual feature of chemical communication in the genus *Riptortus* is the discovery that only two of the three components of the *R. clavatus* pheromone (Leal *et al.* 1995) are biosynthesized in the lateral glands of the metathoracic scent gland [(*E*)-2-hexenyl (*E*)-2-hexenoate and (*E*)-2-hexenyl (*Z*)-3-hexenoate]; the third essential component (myristyl isobutyrate) is secreted from cells on the abdominal sternum of males (Walter Leal, personal communication).

Another level of semiochemical complexity is exhibited by some species of Alydidae and related families, in which males possess a ventral abdominal gland, the secretion of which exudes onto the surface of the genitalia during courtship and copulation (Aldrich 1995). In *Mirperus scutellaris* Dallas (Hemiptera: Alydidae), the ventral abdominal secretion is almost purely (*E*)-2-octenol but, in this species, males also accumulate secretion containing (*E*)-2-octenol [plus (*E*)-2-hexenyl butyrate] in the lateral glands of the metathoracic scent gland complex (Aldrich *et al.* 1993). For *Leptoglossus clypealis* Heidemann (Hemiptera: Coreidae), laboratory bioassays and observations of mating behavior suggest that the ventral abdominal gland secretion serves as both an attractant and an aphrodisiac (Wang and Millar 2000). Males of the genera *Riptortus* and *Alydus* do not possess ventral abdominal glands (Aldrich 1995), but species in each of these genera communicate at close range by stridulation (Schaefer and Pupedis 1981; Numata *et al.* 1989).

The semiochemical duality seen in bugs producing sexual pheromones and allomones in the same gland is most likely a function of the morphologically elaborate metathoracic scent gland apparatus in Heteroptera (Hepburn and Yonke 1971). Normally the gland consists of a median reservoir containing aldehydic and acidic defensive compounds (Lygaeidae and Alydidae) or concentrated esters (some Miridae) and a pair of lateral accessory glands in which esters are commonly produced (Aldrich

1988a). Earlier evidence indicated that certain compounds stored in the metathoracic-gland reservoir were enzymatically derived from lateral-gland esters (Aldrich *et al.* 1978); however, in those bugs in which attractant pheromones are also liberated from the metathoracic scent gland, it is obvious that the calling insects are able to selectively release pheromone components. The possibility that disparate heteropterans produce sexual pheromones in their metathoracic scent glands must be considered in future pheromone research on this group of insects, especially for those species with the specialized lines of defense indicated by aposematism or mimicry.

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