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IDENTIFICATION AND SYNTHESIS OF MALE-PRODUCED SEX PHEROMONE COMPONENTS OF THE STINK BUGS *Chlorochroa ligata* AND *Chlorochroa uhleri*

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Abstract—The reproductive behaviors of the stink bugs *Chlorochroa ligata* and *C. uhleri* were studied in the laboratory. Adults of both species became sexually mature about 12–14 days after the final molt, and both sexes mated multiple times during their lifetimes. The mean duration of copulation was 54 ± 24 min for virgin bugs and 46 ± 33 min for experienced bugs for *C. ligata* and 78 ± 55 min for field-collected *C. uhleri* of unknown mating status. Male *C. ligata* were found to transfer a significant fraction of their body mass (19%) to females during mating. Sexually mature *C. uhleri* males produced three sex-specific compounds, methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate, methyl (2*E*,6*E*)-farnesoate, and methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, in a ratio of 100:0.9:0.6. These three compounds were also produced by sexually mature male *C. ligata* in a ratio of 100:0.5:0.4. Identifications of the compounds were confirmed by synthesis. Production of the male-specific compounds peaked in late afternoon to early evening, coincident with the peak period of reproductive activity. Laboratory and field bioassays demonstrated that female bugs were attracted to odors from live males and to reconstructed blends of the male-specific compounds.

Key Words—*Chlorochroa ligata*, *Chlorochroa uhleri*, Pentatomidae, pheromone, methyl (*E*)-6-2,3-dihydrofarnesoate, methyl (2*E*,6*E*)-farnesoate, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, reproductive behavior.

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INTRODUCTION

Phytophagous pentatomid bugs are pests of a broad spectrum of agriculturally important crops including fruits, nuts, row crops, and vegetables (McPherson and McPherson, 2000). These insects are characterized by piercing and sucking mouth parts, which both immatures and adults use to inject saliva into plants, liquefying plant tissues. This feeding damage causes premature abortion or deformation of seeds and fruiting bodies and also transmits disease. The adult insects are strong fliers and can migrate from field to field as hosts are harvested or senesce (McPherson and McPherson, 2000). Current monitoring methods include beating tray or sweep-net sampling (McPherson and McPherson, 2000), both of which are labor intensive and time consuming. There is an urgent need for better monitoring methods, and traps baited with attractant pheromones may provide such methods. However, sex specific compounds have been identified for only a few stink bugs, including *Nezara viridula* (L.) (Aldrich et al., 1987; Baker et al., 1987; Brézot et al., 1994), *Euschistus spp*. (Aldrich et al., 1991, 1994; Borges et al., 1998a), *Plautia stali* Scott (Sugie et al., 1996), *Thyanta pallidovirens* Stål (Millar, 1997), *Piezodorus hybneri* Gmelin (Leal et al., 1998), *P. guildinii* (Westwood) (Borges et al., 1998b), and *Biprorulus bibax* Breddin (James et al., 1994). The complexities involved in the identification and development of applications of stink bug pheromones have been summarized by McBrien and Millar (1999) and are further complicated by the growing body of evidence that stink bug reproductive behaviors are mediated by both chemical and substrate-borne vibrational signals (e.g., Ota and Çokl, 1991; Çokl et al., 2001).

The conchuela stink bug, *Chlorochroa ligata* (Say), is found in the western regions of the United States and Canada, and it has been reported as far east as Missouri, Kansas, and South Dakota (Buxton et al., 1983; Henry and Froeschner, 1988). *C. ligata* adults are usually large grayish black bugs but the species exhibits a wide variation in color. Overall, the species is generally dark in color, with the lateral margins of the thorax, the basal third to two thirds of the costal margins of the wing corium, and the tip of the scutellum varying in color from pale yellow to bright crimson (Morrill, 1910; Buxton et al., 1983). The strain infesting apple and pear orchards in California is commonly black with reddish marginal borders and a reddish spot in the middle of the back (Ohlendorf, 1999), whereas *C. ligata* found in blackberry fields in Washington State were black with pale yellow borders (Fish and Alcock, 1973).

A congeneric species, *Chlorochroa uhleri* (Stål), commonly known as Uhler's stink bug, is distributed through the western United States, where it damages wheat (Caffrey and Barber, 1919), tomato (Ohlendorf, 1990), cotton (Morrill, 1910), and alfalfa (Russell, 1952). In California, it can have several generations per year, and details of its life history have been reported by Morrill (1910) and Russell (1952). The color of *C. uhleri* is green above and paler and yellower ventrally. The

callosities at the base of the scutellum are moderate to small. *C. uhleri* is morphologically similar to another congener, *Chlorochroa sayi* Stål, and the two species frequently have been confused (McPherson and McPherson, 2000), particularly as both *C. sayi* and *C. uhleri* can be found on the same host plants. The most useful character for separating the two species is the purple flecks on the membrane of the hemelytra of *C. sayi*, which are absent in *C. uhleri* (Buxton et al., 1983).

We recently reported the identification of pheromones from *C. sayi* (Ho and Millar, 2001a). Here, we report the results of investigations of the reproductive behavior and semiochemistry of *C. uhleri* and *C. ligata.* Our specific objectives were: (1) to describe the reproductive behaviors of *C. uhleri* and *C. ligata*; (2) to identify volatile chemicals produced by each species, focusing particularly on sex-specific compounds that might be sex pheromone components; and (3) to test reconstructed blends of sex-specific compounds as stink bug attractants in laboratory and field bioassays.

METHODS AND MATERIALS

Insects. A colony of *C. uhleri* was started from egg masses obtained from Paul da Silva, Kearney Agricultural Station, Parlier, California. Additional bugs were collected from Russian thistle (*Salsola iberica* Sennen) in Riverside County in the summers of 1998 and 1999. A colony of *C. ligata* was started from adult bugs provided by Dr. Les Ehler, University of California, Davis, California. A second colony of *C. ligata* was started in the summer of 1999 from specimens provided by Christian Krupke, Washington State University Tree Fruit Research and Extension Center, Wenatchee, Washington. Both species were reared on green-bean pods grown without pesticides, raw shelled peanuts and sunflower seeds, and bouquets of alfalfa and seasonal weed species, exactly as previously described for *C. sayi* (Ho and Millar, 2001a). Bugs were kept under a 16L:8D cycle, at 23 $\pm$ 2°C, and $>$50% relative humidity.

Observations of Reproductive Behavior. Mating behaviors of sexually mature adult *C. ligata* and *C. uhleri* were video-taped in time lapse mode as described for *C. sayi* (Ho and Millar, 2001a), using individual pairs of bugs in plastic tubes, with green-beans provided for food. Observations of mating behaviors were carried out using virgin, laboratory-reared *C. ligata.* For *C. uhleri*, due to the low number of bugs in the laboratory colony at the time the experiments were conducted, field collected insects of unknown age and mating status were used, after first segregating the bugs by sex for 4 days. A total of 27 matings were observed from 13 pairs of *C. ligata* in 14 days, and 28 matings from 12 pairs of *C. uhleri* over eight days. The time each copulation started, duration of each copulation, and the total numbers of copulations per pair were recorded. The numbers of matings initiated per 2 hr time period were plotted.

Weight Change during Mating. The experimental setup to examine weight change during mating was as previously described for *C. sayi* (Ho and Millar, 2001a). For *C. ligata*, 122 virgin pairs and 52 experienced pairs were observed between March 27 to April 10, 2000. Copulations were observed among a total of 13 virgin pairs and 11 experienced pairs. Because there were not enough *C. uhleri* in the laboratory colony, weight changes during mating experiments were not recorded.

Collection and Analysis of Insect-Produced Compounds. Volatile chemicals were collected and analyzed as previously described for *C. sayi* (Ho and Millar, 2001a). Briefly, volatiles were collected on activated charcoal traps from groups of sexed virgin adult bugs held in glass aeration chambers with green-beans. Volatiles from green-beans were collected as a control. Compounds were eluted from the traps with pentane and ether, and analyzed by splitless gas chromatography on a DB-17 column (30 m $\times$ 0.25 mm, J&W Scientific, Folsom, California), with a temperature program of 50°C for 1 min, 10°C/min to 250°C. Injector and detector temperatures were 250°C and 280°C, respectively, with helium carrier gas. Extracts were also analyzed by splitless coupled gas chromatography-mass spectrometry (GC-MS) with a Hewlett-Packard 5890 GC fitted with a DB5-MS column (20 m $\times$ 0.2 mm id.), and interfaced to an H-P 5970B mass selective detector (electron impact ionization, 70 eV). The GC was programmed from 40°C/1 min, 10°C/min to 250°C, with injector and transfer line temperatures of 250 and 280°C, respectively. Compounds were tentatively identified from interpretation of the mass spectra, and from matches with the NBS-NIH mass spectral data base. Identifications were confirmed by comparison of retention times and spectra with those of authentic standards.

Bioassays Using Odors from Live Male Bugs, Extracts of Bug Volatiles, or Synthetic Chemicals as Test Stimulants. Laboratory bioassays were carried out with a vertical glass Y-tube olfactometer, exactly as described previously (Ho and Millar, 2001a), using sexually mature virgin adults. Bugs were used only once. Not all cohorts of virgin male *C. uhleri* produced male-specific compounds, as determined by GC analysis of collected bug odors. Consequently, only live males from *C. uhleri* cohorts that had been demonstrated to be producing male-specific compounds were used as sources of attractant in bioassays, with female responders.

Field Bioassays. The complete blend of male-specific compounds from each species (see below) was tested in field bioassays as described by Ho and Millar (2001a). Lures consisted of solutions of 20 mg of the racemic major compound, methyl (*E*)-6-2,3-dihydrofarnesoate, together with the minor components, methyl (2*E*,6*E*)-farnesoate (0.12 mg) and racemic methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate (0.18 mg) absorbed onto 10 cm lengths of natural latex rubber tubing. Controls consisted of sections of tubing treated with CH_2Cl_2. Lures and controls were hung in Russian thistle plants, with the number of bugs on each plant counted daily during the late afternoon. Lures and controls were rerandomized

among plants after each count. In the first sets of trials (see below), responding bugs were collected, counted, and released, whereas in later trials, all responding bugs were collected and taken back to the laboratory to augment the laboratory colonies.

For trial 1, August 3–16, 1999, lures with the three-component blend of *C. uhleri* together with solvent-treated controls were deployed at test sites in Ontario, Moreno Valley, Redlands, and Riverside, California, with three replicates of each treatment in each block. Bioassays were evaluated as described above, with the counted bugs being released.

For trial 2, August 19–24, 1999, six experimental blocks were set up in Russian thistle patches in Moreno Valley and Riverside, testing the three-component blend of *C. uhleri*, and a solvent control. Each treatment and control was replicated three times at each site. Bioassays were evaluated each evening from ~17:00 till dusk, collecting all responding bugs.

For both trials, where appropriate, the numbers of responding bugs were transformed, using the square root $(x + 0.5)$ transformation, and the transformed data were subjected to two-way ANOVA, with differences between treatments and controls determined by Dunnett's method. For those data sets that did not satisfy the assumptions of ANOVA, differences between treatments and controls were determined with the nonparametric multiresponse permutation procedure (Biondini et al., 1985; McCune and Mefford, 1999).

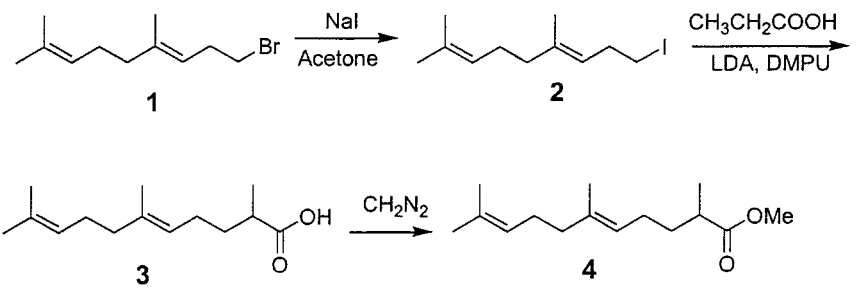
Chemicals. (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-hexenyl acetate, undecane, dodecane, tridecane, tetradecane, and pentadecane were purchased from Aldrich Chemical (Milwaukee Wisconsin), and (*E*)-2-octenyl acetate was obtained from Bedoukian Research (Danbury, Connecticut). (*Z*)-2-Octenyl acetate was synthesized by acetylation of (*Z*)-2-octenol with acetyl chloride and triethylamine in ether. Racemic methyl (*E*)-6-2,3-dihydrofarnesoate was synthesized as previously described (Ho and Millar, 2001a). (*E*)-4-Oxo-2-hexenal was synthesized as described by Ho and Millar (2001b).

Synthesized Compounds. Flash chromatography was carried out with 0.04 to 0.063-mm silica gel (Aldrich Chemical Co., Milwaukee, Wisconsin). ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 and 75.48 MHz, respectively, using a General Electric QE 300 instrument. GC-MS analyses (electron impact ionization, 70 eV) were performed on a Hewlett-Packard 5890 gas chromatograph interfaced to an HP 5970B mass selective detector (Palo Alto, California). A DB-5MS column was used (20 m $\times$ 0.2 mm, 0.25 μm; J&W Scientific, Folsom, California). Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl immediately before use. Routine work-up of reactions included drying solutions over anhydrous Na_2SO_4 and concentrating by rotary evaporation under partial vacuum (~80 mm Hg). All reactions were carried out under argon atmosphere in oven-dried glassware unless otherwise specified.

Synthesis of Methyl (2E,6E)-Farnesoate. A solution of trimethylphosphonoacetate (2.18 g, 12 mmol; Lancaster Synthesis, Windham, New Hampshire)

in THF (20 ml) was cooled to −30°C under Ar, and *n*-butyllithium (4.8 ml of 2.5 M in hexane, 12 mmol) was added dropwise. The mixture was warmed to 0°C, and geranyl acetone (1.94 g, 10 mmol) was added dropwise. The mixture was warmed to room temperature, stirred 2 hr at room temperature, during which time considerable precipitate formed, then heated to reflux for 1 hr. The mixture was then cooled, poured into 1 M aq. $NaHCO_3$, and extracted three times with hexane. The combined hexane extracts were backwashed with brine, dried over anhydrous Na_2SO_4, and concentrated, giving a crude yield of 3 g of a ∼2:1 mixture of (2*E*,6*E*)- and (2*Z*,6*E*)-methyl farnesoates. The crude mixture was purified by flash chromatography on silica gel (5 × 25 cm, eluting with 1 liter each of 1%, 1.5%, and 2% ether in hexane). The combined fractions containing each component were concentrated and Kugelrohr distilled (oven temperature 120°C, 0.2 mm Hg vacuum), yielding 1.2 g and 0.3 g of pure (2*E*,6*E*)- and (2*Z*,6*E*)-methyl farnesoates, respectively. Methyl (2*E*,6*E*)-farnesoate: ^{1}H NMR: δ 5.68 (br. s, 1H, H2), 5.09 (br. t, 2H, $J = \sim 8$ Hz, H6,10), 3.69 (s, 3H, OCH_3), ∼2.18 (m, 4H, H4, H5), 2.18 (d, 3H, $J = 1.23$ Hz, H3′), 2.12–1.95 (m, 4H, H8, H9), 1.69 (br. s, 3H, H12), 1.61 (br. s, 6H, H7, H12′). ^{13}C NMR (CDCl3, 75.48 MHz): δ 167.27, 160.19, 136.16, 131.41, 124.20, 122.85, 115.20, 50.78, 40.94, 39.67, 26.66, 25.69, 25.94, 18.84, 17.69, 16.02. EI-MS: 250 (M^+, 1), 207 (2), 136 (4), 121 (13), 114 (24), 81 (22), 69 (100), 53 (18), 41 (85). Methyl (2*Z*, 6*E*)-farnesoate: ^{1}H NMR: δ 5.68 (br. s, 1H, H2), 5.18 (br. t, 1H, J = 6.2 Hz, H6), 5.10 (br. tt, 1H, $J = 6.7$, 1.1 Hz, H10), 3.69 (s, 3H, OMe), 2.65 (t, 2H, $J = 7.8$ Hz, H4), 2.18 (overlapped dt, 2H, $J \sim 7.8$, 7.7 Hz, H5), 2.12–1.95 (m, 4H, H8, H9), 1.90 (d, 3H, $J = 0.8$ Hz, H3′), 1.68 (br. s, 3H, H12), 1.62 (br. s, 3H, H12′), 1.59 (s, 3H, H7′). EI-MS: 250 (M^+, 7), 235 (1), 218 (1), 207 (12), 175 (5), 149 (17), 137 (10), 121 (36), 114 (28), 109 (22), 81 (31), 69 (100), 53 (19), 41 (55). The ^{1}H NMR and mass spectral data for both compounds matched literature data [NMR: Savu and Katzenellenbogen (1981); MS: Wainwright et al. (1996)].

Synthesis of Racemic Methyl (E)-5-2,6,10-Trimethyl-5,9-Undecadienoate (Scheme 1). For homogeranyl iodide (**2**), NaI (2.3 g, 15 mmol) was dried in a Kugelrohr distillation apparatus at 100°C under vacuum for 2 hr. After cooling,

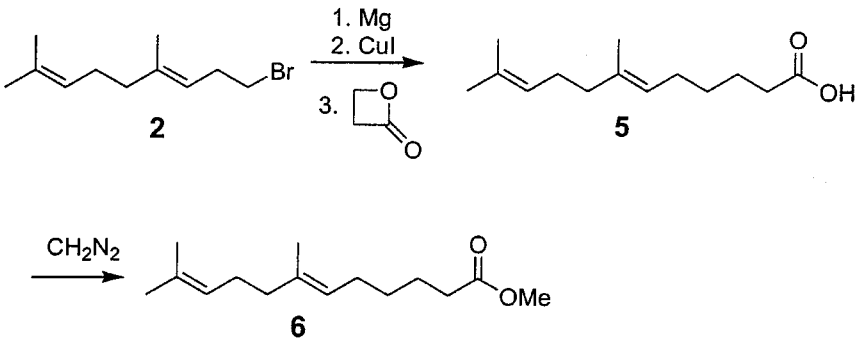


SCHEME 1.

25 ml acetone, homogeranyl bromide **1** (0.7 g, 3 mmol, prepared as described by Ho and Millar, 2001a), and 10 mg of Na_2CO_3 were added, and the mixture was stirred under Ar at room temp, monitoring the reaction by GC. When the reaction was complete (~4 hr), the mixture was poured into 150 ml water and extracted with hexane. The hexane layer was washed with aq. sodium bisulfite and brine, dried, and concentrated. The crude product was stored in the freezer until used, with a piece of copper wire added to retard degradation. EI-MS *m/z*: 278 (M^+, 0.7), 263 (1.3), 235 (9), 151 (2.7), 123 (12), 95 (150) 81 (16), 69 (100), 41 (74).

For (*E*)-5-2,6,10-*trimethyl-5,9-undecadienoic acid* (**3**), the procedure was adapted from that of Prashad et al. (1993). Lithium diisopropylamide (LDA) (1.5 M, 7.5 ml, 11.23 mmol) was added to 20 ml freshly distilled THF in an oven dried flask in an ice bath. Propionic acid (0.36 ml, 5 mmol) then was added dropwise, followed by 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU) (0.7 ml, 5.7 mmol) as cosolvent. The mixture was stirred at room temperature for 20 min, then cooled to 0°C, and crude homogeranyl iodide **2** (0.7 g, 2.5 mmol) in 2 ml THF was added over 10 min. The mixture was stirred at room temperature, monitoring by GC. When there was no further change in the product ratio, the mixture was poured into 100 ml 1 M HCl and extracted with hexane (1 × 100 ml, 2 × 50 ml). The combined hexane layers were extracted with 1 M NaOH (1 × 100 ml, 2 × 50 ml). The combined aq. NaOH layers were back-extracted with ether, then acidified with 100 ml 3 M HCl and extracted with ether (1 × 100 ml, 2 × 50 ml). The combined ether layers were backwashed with brine, dried, and concentrated, yielding 0.4 g of acid **3** (purity 95% by GC, 71% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.1 (m, 2H, H5 and H9), 2.5 (sextet, 1 H, $J = 7$ Hz, H-2), 2.06 (m, 7H, H2, H4, H7, and H8), 1.8 (m, 1H, H3), 1.69 (s, 3H, H11), 1.61 (2 × s, 2 × 3H, H6′ and H11′), 1.45 (m, 1H, H3′), 1.19 (d, 3H, J = 7 Hz, H2′). ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 182.99, 136.03, 131.41, 124.27, 123.33, 39.71, 38.75, 33.50, 26.62, 25.71, 25.48, 17.70, 16.86, 15.99. EI-MS *m/z*: 224 (M^+, 1), 209 (1.6), 181 (34), 163 (7.5), 137 (5.4), 109 (43), 81 (19), 69 (100), 41 (96). The spectra matched those reported by Coates and Freidinger (1970).

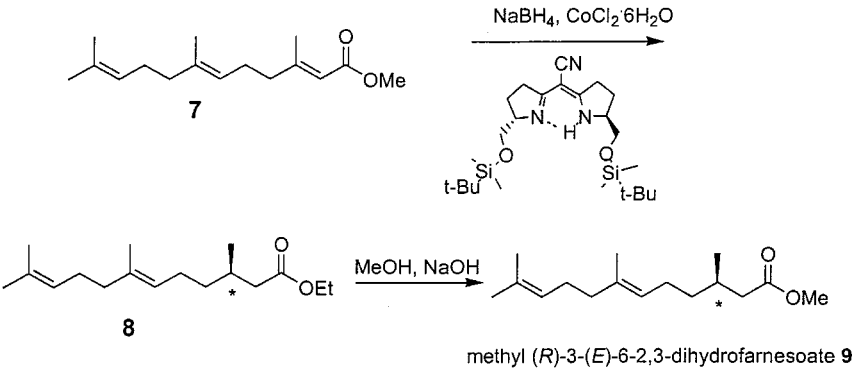
For *methyl* (*E*)-*5-2,6,10-trimethyl-5,9-undecadienoate* (**4**), acid **3** was dissolved in ~5 ml ether, cooled in an ice bath, and a freshly prepared solution of diazomethane in ether (prepared according to Aldrich Tech. Information Bulletin AL-180, Aldrich Chem. Co., Milwaukee, Wisconsin) was added until the free acid was consumed (monitoring by persistence of the yellow color of excess diazomethane). Nitrogen was bubbled vigorously through the solution to discharge traces of excess diazomethane; then the mixture was concentrated and Kugelrohr distilled at 0.1 mm Hg, oven temperature 70°C, yielding 0.34 g of ester **4** (58% yield, 92% purity by GC). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.09 (t, 2H, $J = 7$ Hz, H5 and H9), 3.67 (s, 3H, OCH_3), 2.46 (sextet, 1H, $J = 6.9$ Hz, H2), 1.88–2.15 (m, 7H, H2, H4, H7 and H8), 1.6–1.8 (m, 1H, H3), 1.68 (s, 3H, H11), 1.60 and 1.58



SCHEME 2.

(2 × s, 2 × 3H, H11′ and H6′), 1.35–1.5 (m, 1H, H3′), 1.15 (d, 3H, $J = 6.9$ Hz, H2′). ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 177.33, 135.82, 131.37, 124.28, 123.50, 51.48. 39.73, 38.89, 33.79, 26.65, 25.71, 25.57, 17.69, 17.09, 15.95. EI-MS *m/z*: 238 (M^+, 0.7), 223 (3.3), 195 (19), 163 (10), 123 (7.4), 109 (75), 88 (22), 69 (84), 55 (20), 41 (100).

Synthesis of Racemic Methyl (*E*)*-6-7,11-Dimethyl-6,10-Dodecadienoate (Scheme 2).* For (*E*)-6-7,11-dimethyl-6,10-dodecadienoic acid (**5**), the procedure was adapted from those described by Fujisawa et al. (1980) and Sato et al. (1980). Homogeranyl bromide **2** (0.9 g, 3.9 mmol, purity 82%) was dissolved in ~10 ml of freshly distilled THF in a dropping funnel. One milliliter of the solution was added to a flask containing Mg turnings (0.2 g, 8 mmol), with several small crystals of iodine and 5 drops of dibromoethane. The mixture was stirred until the iodine color disappeared, and then the rest of the solution was added dropwise over ~2 hr, warming gently to 30–40°C with an oil bath. When the addition was complete, the solution was heated at 40°C for 1 hr, then cooled to room temperature The resulting solution of Grignard reagent was transferred by syringe into a solution of β-propiolactone (0.25 g/0.22 ml, 4 mmol; Aldrich Chemical) and powdered CuI (0.04 g, 0.2 mmol) in 5 ml dry THF at ~−10°C (ice-salt bath) in a 100-ml flask under Ar over 30 min. The mixture was warmed to 0°C and stirred until the reaction was complete. The reaction mixture was poured into 100 ml 1 M HCl and extracted with hexane (1 × 100 ml, 2 × 50 ml). The combined organic layers were extracted with 1 M NaOH (1 × 100 ml, 2 × 50 ml), and the combined aq. NaOH extracts were then back-extracted once with ether. The aq. NaOH extract was then acidified with 100 ml 3 M HCl, and extracted with ether (1 × 100 ml, 2 × 50 ml), the combined ether extracts were washed with brine, dried, and concentrated, yielding 0.4 g of acid **5**. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.75 (br s, 1 H, COOH), 5.11 (m, 2H, H6 and H10), 2.36 (t, 2H, $J = 7.4$ Hz, H2), 1.9–2.1 (m, 6H, H5, H8, H9), 1.68 (s, 3H, H12), 1.60 (s, 6H, H7′ and H12′), 1.5–1.7 (m, 2H, H3), 1.3–1.5



SCHEME 3.

(m, 2H, H4). ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 180.52, 135.49, 131.38, 124.36, 123.99, 39.78, 34.11, 29.25, 27.52, 26.73, 25.74, 24.32, 17.73, 16.05. EI-MS *m/z*: 224 (M^+, 0.5), 209 (1.8), 181 (28), 137 (5), 123 (15), 109 (12), 95 (36), 69 (100), 41 (77).

For *methyl* (*E*)*-6-7,11-dimethyl-6,10-dodecadienoate* (**6**), a portion of (*E*)-6-7,11-dimethyl-6,10-dodecadienoic acid **5** was esterified with diazomethane as described for 4. EI-MS *m/z*: 238 (M^+, 0.5), 223 (0.6), 195 (18), 163 (6), 137 (9), 123 (11), 109 (21), 95 (49), 69 (82), 41 (100).

Synthesis of Methyl (R)-3-(E)-6-2,3-Dihydrofarnesoate (Scheme 3). The reaction was carried out exactly as described by Pfaltz (1989) and Leutenegger et al. (1989). A successful reaction required rigorous degassing of solvents and starting materials, and exclusion of oxygen. Thus, methyl (2*E*,6*E*)-farnesoate **7** (0.25 g, 1 mmol) in a 25-ml three-necked round-bottom flask equipped with a magnetic stir bar, with the necks fitted with a stop-cock and two new rubber septa, was degassed by several cycles of pumping under vacuum and refilling the flask with N_2. All solvents and solutions were degassed by sonicating while pumping under house vacuum, then refilling with N_2; this was repeated several times. Then 0.5 ml ethanol were added to the flask by syringe, followed by 0.25 ml of a solution of $CoCl_2 \bullet 6H_2O$ (100 mg, 0.42 mmol) in ethanol (5.5 ml), a solution of (1*S*,9*S*)-1,9-bis[(*t*-butyldimethylsiloxy)-methyl]-5-cyanosemicorrin (10 mg, 0.022 mmol; Fluka #14556) in 0.5 ml ethanol (mixture turns deep blue), and a solution of $NaBH_4$ [85 mg (2.2 mmol) in 0.8 ml dimethylformamide] (mixture turns brown, cloudy). The mixture was degassed again by several freeze–pump–thaw cycles, using a Dry Ice–acetone bath. The flask was then sealed tightly and the mixture was stirred for 45 hr at room temperature. A sample taken indicated that the reaction was complete, with no trace of starting material, but that the product had been 60% transesterified to the ethyl ester. The mixture was worked up by

pouring into 100 ml water and extracting with 3 × 50 ml CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with water and brine, dried, and concentrated. The crude ethyl ester **8** was then transesterified by stirring with methanol (10 ml) and one pellet of NaOH (∼250 mg) overnight at room temperature. The mixture was poured into 50 ml water and extracted with hexane, washing the hexane solution with brine. The solution was dried, concentrated, and Kugelrohr distilled (oven temperature 110°C, 0.3 mm Hg), yielding 229 mg of methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate **9**, >98.5% pure by GC. The enantiomeric excess was determined to be 89.6% by hydrolysis of the acid followed by formation of the diastereomeric amides by reaction with (*S*)-(–)-α-methylbenzylamine and GC analysis (see below).

Determination of Absolute Configuration of Insect-Produced Methyl (E)-6-2,3-Dihydrofarnesoate. This procedure was adapted from a method reported by Huffer and Schreier (1990), scaling down the apparatus and quantities of reagents used. Thus, 10 μg of synthetic racemic or methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate was hydrolyzed in 20 μl of EtOH and 2 μl of aq. 4 M NaOH in a Keele vial (Wheaton Scientific Products, Millville, New Jersey), heating in a 50°C water bath for 2 hr. After cooling, the EtOH was evaporated under a gentle stream of N_2. Then 20 μl of 1 M HCl were added to the dry vial, and the mixture was extracted with pentane (3 × 100 μl) to recover the free (*E*)-6-2,3-dihydrofarnesoic acid. The pentane solution was dried by filtering through a Pasteur pipet containing a 0.5-cm layer of anhydrous $MgSO_4$, then transferred to a 250-μl conical glass vial insert inside a 4-ml glass vial. The solution was evaporated just to dryness under a gentle stream of N_2 and 20 μl of $CHCl_3$ were added, followed by 2 μl of a $CHCl_3$ solution of (*S*)-(–)-α-methylbenzylamine (10 μg/μl; Aldrich Chemical) and 4 μl of a solution of dicyclohexylcarbodiimide (DCC) (6 μg/μl in $CHCl_3$). The vial was sealed tightly with a Teflon-lined screw-cap, and the vial was heated in a 50°C water bath for 30 min. The mixture was then analyzed by GC and GC-MS on DB-5 and DB5-MS columns (20 m × 0.32 or 0.2 mm, respectively, J&W Scientific, Folsom, California) in splitless mode. GC and GC-MS conditions were: 150°C/0 min, 4°C/min to 275°C, hold at 275°C for 10 min, injector temp 300°C, detector or transfer line temp 280°C.

A pooled aeration extract from mature male *C. uhleri* containing approximately 1.2 mg of the major defensive compound, tridecane, and 22 μg of the major male-specific compound, methyl (*E*)-6-2,3-dihydrofarnesoate was fractionated on a silica gel solid-phase extraction cartridge as previously described (Ho and Millar, 2001a). The fraction containing methyl (*E*)-6-2,3-dihydrofarnesoate was hydrolyzed, and the resulting (*E*)-6-2,3-dihydrofarnesoic acid was converted to the diastereomeric methylbenzylamide derivative and analyzed as described above. This procedure was repeated with pooled aeration extracts from *C. ligata* males containing approximately 64 μg of tridecane and 11 μg of the major male-specific compound, methyl (*E*)-6-2,3-dihydrofarnesoate.

RESULTS

Basic Reproductive Behaviors. The reproductive behaviors of *C. uhleri* and *C. ligata* were similar. For both species, the male bug began close-range courtship with antennation of the female bug, followed by head butting the posterior end of the female bug. If the female was receptive, she stopped walking, lowered her head, and raised her abdomen. Then, the male turned around, coupled his genitalia with those of the female, and copulated in an end-to-end position. During mating, both female and male bugs were in a 'head-stand' position, shaped as an inverted 'V', with the abdomen higher than the head. When copulation was complete, one of the bugs swung from side to side to disengage from the other. If the female was not receptive, she walked away. In some cases, even though the female bug stopped and raised her abdomen in the receptive position, the male would still head butt for some time before he turned around and mated with her. In cases where the female assumed the receptive position with her abdomen raised, and the male failed to couple with her genitalia, the male resumed head butting and started over again. In some cases, the male had to pursue the female with periodic bouts of head butting for hours, before the female acquiesced and copulated with the male. In other cases, the female mated with the male after a relatively brief courtship of only a few minutes of head butting.

Weight Change after Mating. Experiments with 122 virgin pairs and 52 experienced pairs of *C. ligata* resulted in a total of only 13 observations of copulation between virgin pairs and 11 copulations of experienced pairs. For virgin insects, females gained $28 \pm 1\,0$ mg in weight (mean $\pm$ SD, $11 \pm 4\%$ of body weight), and males lost 42 ± 11 mg ($19 \pm 5\%$ of body weight) during copulation (Figure 1a). There was negligible weight change in the control group, with females losing 3 ± 3 mg, and males losing 1 ± 2 mg ($N = 16$). There was a trend towards increasing weight change in both sexes with increasing duration of mating [Figure 1a, $R^2 = 0.1527$ (females) and 0.4926 (males)].

For experienced pairs, weight change was not correlated with duration of mating for either sex (Figure 1b, experienced pairs, females, $P = 0.72$ and males, $P = 0.42$). Experienced males lost an average of 42 ± 7 mg ($19 \pm 3\%$ of body weight) during mating, and females gained 24 ± 10 mg ($11 \pm 5\%$ of body weight). Overall, these results indicate that virgin and experienced males transfer material equivalent to a significant fraction of their body mass to females during mating. For both virgin and experienced pairs, there was a small overall net weight loss, probably due to defecation; drops of feces were frequently seen on the walls of the cages at the termination of the experiments.

We were not able to carry out analogous experiments with *C. uhleri* because of insufficient numbers of bugs in the laboratory colonies.

Diurnal Patterns in Mating Behavior. The diurnal rhythm of mating behavior of adult *C. ligata* is shown in Figure 2a. Of 23 sexually mature, lab-reared pairs

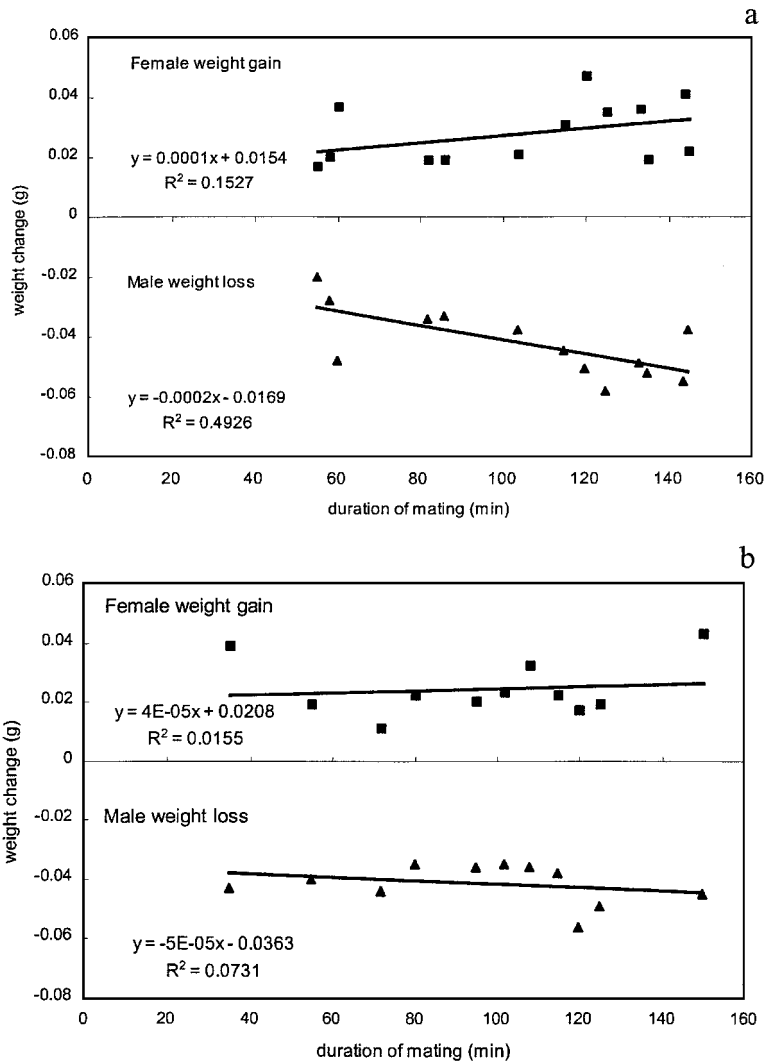


FIG. 1. Weight change of *C. ligata* after mating. (a) Virgin insects: females gained 0.028 ± 0.010 g (mean $\pm$ SD) (*t* test versus controls, $P = 6 \times 10^{-8}$, and males lost 0.042 ± 0.011 g in weight during mating (*t* test versus controls, $P = 1.8 \times 10^{-8}$) ($N = 13$). Linear regression was carried out on the weight change versus duration of mating for each sex. Female weight gain was not correlated with duration of mating ($P = 0.1868$), whereas male weight loss was correlated with mating duration ($P = 0.0075$). (b) Experienced insects: females gained 0.024 ± 0.010 g (mean $\pm$ SD) in weight (*t* test versus controls, $P = 1.3 \times 10^{-6}$), and males lost 0.042 ± 0.007 g with every mating (*t* test versus controls, $P = 1 \times 10^{-9}$) ($N = 11$). Linear regression was carried out on the weight change versus duration of mating for each sex. Neither female weight gain ($P = 0.7154$) nor male weight loss ($P = 0.4212$) was correlated with mating duration.

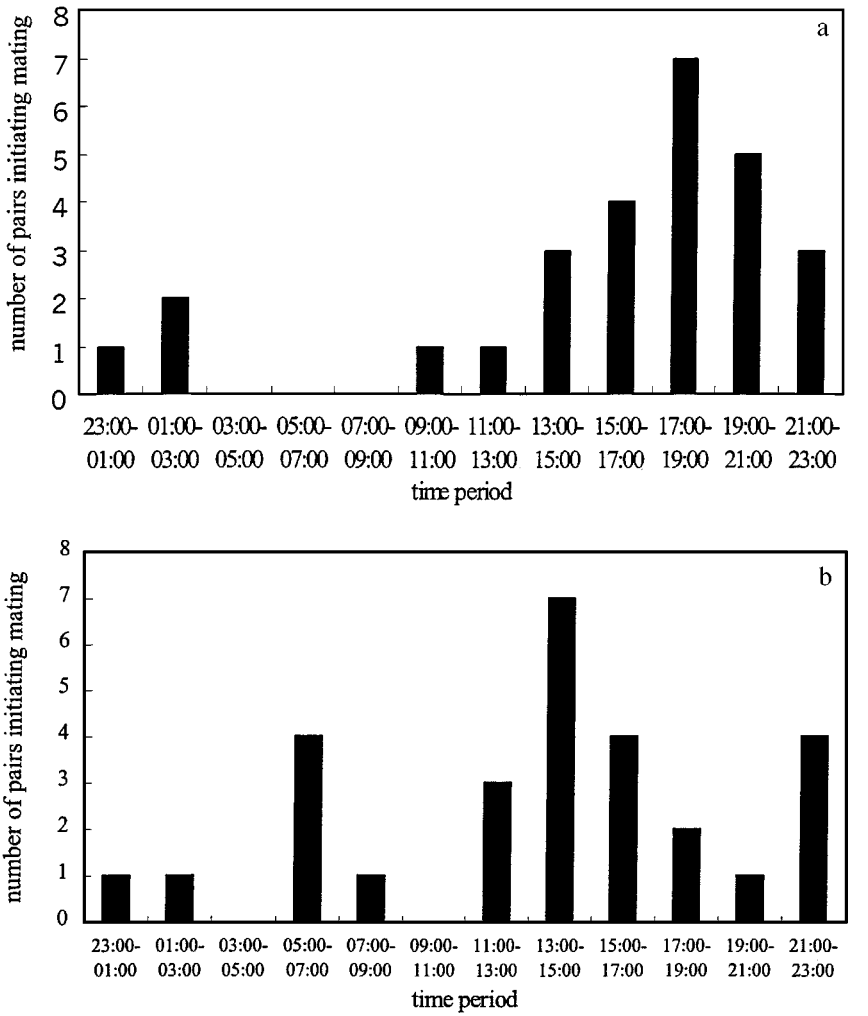


FIG. 2. Diurnal pattern of mating behavior of *C. ligata* (a) and *C. uhleri* (b), showing number of pairs that initiated copulation during successive 2-hr time periods. Lights were on from 06:00 to 20:00 hr each day. The *C. ligata* trial was run with 13 pairs of insects for 14 consecutive days, with 27 matings observed. The *C. uhleri* trial was run with 12 individual pairs of insects for 8 consecutive days, with 28 matings observed.

observed over a 14 day period, 13 pairs mated a total of 27 times. No mating was observed for the other 10 pairs because of the death of one or both bugs of the pair. For pairs that mated more than once, the mean latency between copulations was 50 ± 20 hr (range 22–92 hr). The duration of mating for virgin females

paired with virgin males (54 ± 24 min; $N = 12$), and for experienced females and males (46 ± 33 min; $N = 15$) was not different (two tailed t test, $df = 25$, $P = 0.44$). The largest percentage of copulations (>25%) were initiated between 17:00–19:00 hr (Figure 2a). The remainder of copulations generally were initiated in the afternoon and evening hours, from 13:00 to 23:00 hr, indicating that this species is sexually active primarily during the latter half of the photophase.

Twelve pairs of field-collected *C. uhleri* adults of unknown age and mating status, held in sexed groups for 4 days before beginning observations, mated a total of 28 times during the 8 day observation period. The overall pattern of reproductive activity is shown in Figure 2b. Mating duration ranged from 8 to 193 min, (mean ± SD, 78 ± 55 min). Eight of the 12 pairs mated more than once, one pair did not mate at all, and three pairs mated only once due to the death of one of the pair. Of the eight multiply-mating pairs, two pairs mated twice the same day, and then every one to two days. Another three pairs mated every other day. The interval between mating ranged from 10 to 51 hr (mean 26 ± 14 hr). Females usually laid eggs between copulations. Mating was initiated mostly in the afternoon, with 25% of the 28 matings observed being initiated between 13:00–15:00.

Identification of Compounds from Odors Produced by Adult C. uhleri and C. ligata. Typical gas chromatograms of aeration extracts from groups of 5–10 sexually mature, virgin adult female and male *C. uhleri* are shown in Figure 3. Several compounds appeared in both female and male aeration extracts, including (*E*)-2-hexenal, (*E*)-2-octenal, dodecane, (*E*)-2-octenyl acetate, tridecane, tetradecane, and pentadecane. Female extracts also contained the known bug defensive compound (*E*)-4-oxo-2-hexenal (Aldrich, 1995) and a small amount of undecane. All compounds except (*E*)-4-oxo-2-hexenal were tentatively identified by comparison of their mass spectra with those from the National Bureau of Standards GC-MS database. (*E*)-4-Oxo-2-hexenal was tentatively identified by comparison of the mass spectrum with a reference spectrum obtained from J. R. Aldrich. Identifications then were confirmed by comparison of retention times and mass spectra of the insect-produced compounds with those of authentic standards. The C_{11}-C_{15} hydrocarbons, (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-octenyl acetate, and (*E*)-4-oxo-2-hexenal are probably defensive compounds from the metathoracic glands (Ho and Millar, 2001b). Aeration extracts from green-bean controls were found to contain trace amounts of the common plant volatiles hexanol, (*E*)-2-hexenol, (*E*)-2-hexenal, and linalool.

Three male-specific compounds (Figure 3, peaks A, B, and C) in a ratio of 0.9:100:0.6, were first detected in aeration extracts 12 days after the final molt in each of two cohorts of virgin males. The mass spectrum of the most abundant of the three compounds, peak B, suggested a molecular mass of 252 with major fragment ions of *m/z* 69, 109, 123, 177, and 209. The compound was tentatively identified as methyl (*E*)-6-2,3-dihydrofarnesoate by comparison of its mass spectrum with the

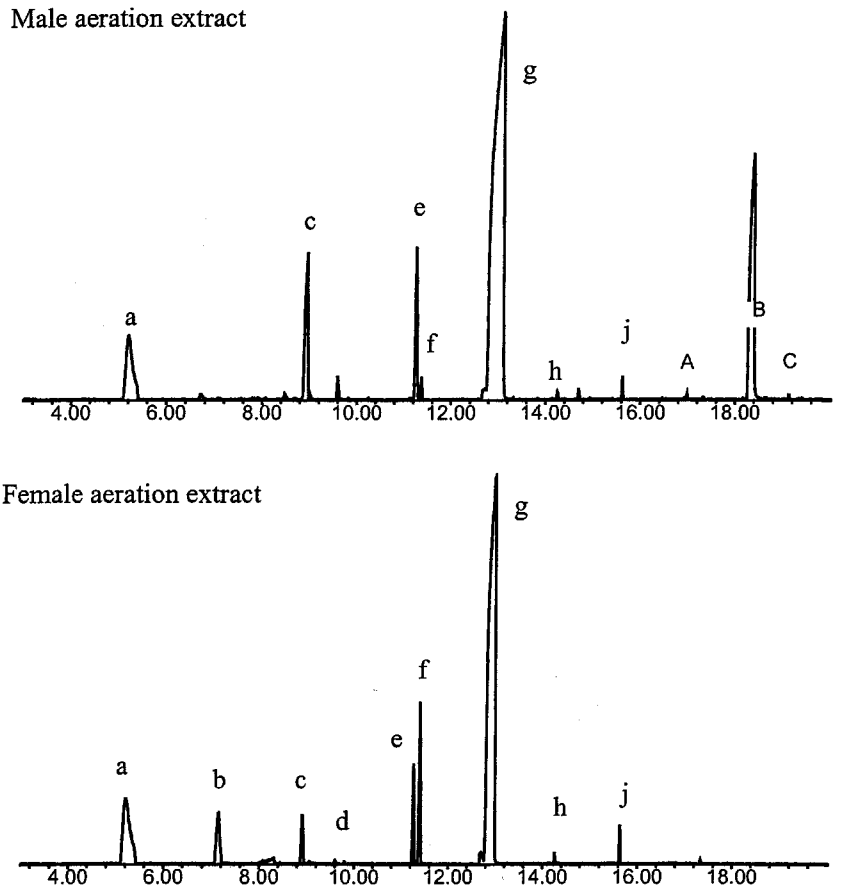


FIG. 3. Gas chromatograms of aeration extracts of sexually mature male (top trace) and female (bottom trace) *C. uhleri*. GC conditions: column DB5-MS (20 m × 0.2 mm ID), initial temperature 50°C (1 min), 10°C/min to 250°C, 250°C (10 min). Three compounds were found only in male aeration extract: A: methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate; B: methyl (*E*)-6-2,3-dihydrofarnesoate; C: methyl (2*E*,6*E*)-farnesoate. Other peaks: a: (*E*)-2-hexenal; b: (*E*)-4-oxo-2-hexenal; c: (*E*)-2-octenal; d: undecane; e: dodecane; f: (*E*)-2-octenyl acetate; g: tridecane; h: tetradecane; j: pentadecane.

mass spectral database. The identification was confirmed by synthesis of methyl (*E*)-6-2,3-dihydrofarnesoate, and match-up of mass spectra and GC retention times on two columns of different polarity (DB-5MS and DB-17).

Peak C was tentatively identified as methyl (2*E*,6*E*)-farnesoate based on its molecular ion at *m/z* 250 (corresponding to a possible molecular formula of

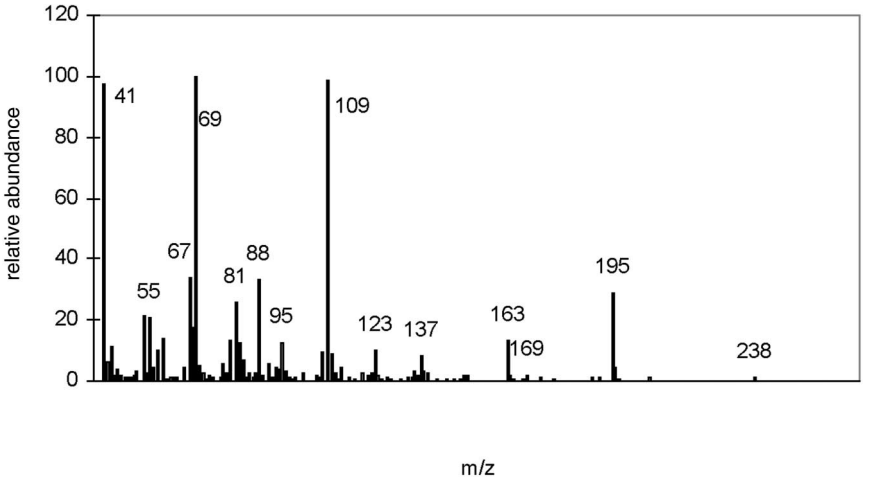


FIG. 4. Mass spectrum of (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate.

$C_{16}H_{26}O_2$); significant fragment ions of *m/z* 41, 69, 114, 136, and 235; and an excellent match with a spectrum of the authentic compound in the mass spectral database. The identification was confirmed by matching the mass spectrum, and retention times on DB-5MS and DB-17 GC columns, with those of a synthesized standard of methyl (2*E*,6*E*)-farnesoate.

The mass spectrum of the small peak A showed a possible molecular weight of 238 amu (Figure 4). The molecular weights of peak A and the major component, methyl (*E*)-6-2,3-dihydrofarnesoate, were 14 mass units different, and both gave similar mass spectral fragmentation patterns in the lower mass ranges. This suggested that the unknown might be a homolog of methyl (*E*)-6-2,3-dihydrofarnesoate, with one methylene group less. There were two obvious possibilities. First, the compound might be derived from chain shortening of methyl (*E*)-6-2,3-dihydrofarnesoate by one carbon, that is, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate. Alternatively, the structure might be methyl (*E*)-6-7,11-dimethyl-6,10-dodecadienoate, from removal of the methyl group on carbon 3 of methyl (*E*)-6-2,3-dihydrofarnesoate. Other possibilities, such as removal of a methyl group further along the chain, seemed unlikely because of the similarities between the mass spectra of this compound and methyl (*E*)-6-2,3-dihydrofarnesoate, whereas removal of an allylic methyl from carbons 7 or 11 of methyl (*E*)-6-2,3-dihydrofarnesoate would have shifted the masses of most of the major fragments by 14 mass units. To confirm the structure of the unknown, the two most likely compounds were synthesized. By comparison of the retention times and mass spectrum of the unknown with those of the synthetic compounds, compound A was identified

as methyl (*E*)-5-2,6,10-trimethyl- 5,9-undecadienoate. A diagnostic mass spectral fragment with *m/z* 88 (Figure 4), arising from McLafferty rearrangement of the methyl ester (Silverstein and Webster, 1998), aided the conclusive identification. This fragment was not seen in the spectrum of methyl (*E*)-6-7,11-dimethyl-6,10-dodecadienoate, and the retention time of this compound was different from that of the insect-produced compound.

The absolute configuration of the insect-produced methyl (*E*)-6-2,3-dihydrofarnesoate was determined by hydrolysis of the ester to the free acid, followed by derivatization with (*S*)-(–)-α-methylbenzylamine. GC analysis of the resulting amide diastereomer determined that its retention time matched that of the corresponding derivative prepared from synthetic methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate. The pair of derivatives from derivatization of racemic (*E*)-6-2,3-dihydrofarnesoic acid were resolved to baseline on a DB-5MS column [(*R*)-3- enantiomer, 22.76 min; (*S*)-3 enantiomer, 22.94 min], unequivocally indicating that the bugs produce methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate.

There was also a chiral center in the minor component, (*E*)-5-methyl-2,6,10-trimethyl-5,9-undecadienoate. This compound was present in trace amounts in the fraction containing methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate, but after the two-step hydrolysis and derivatization procedure, a peak corresponding to this compound could not be located in the chromatograms.

Typical gas chromatograms of aeration extracts from female and male *C. ligata* are shown in Figure 5. The compounds shared by both females and males included (*E*)-2-octenal, dodecane, tridecane, and pentadecane. Extracts from females also contained (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, undecane, and tetradecane. Sexually mature males also produced the same three male-specific compounds as *C. uhleri*, in a similar ratio [methyl (*E*)-6-2,3-dihydrofarnesoate, methyl (2*E*,6*E*)-farnesoate, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, 100:0.4:0.5]. Furthermore, GC analysis of the diastereomeric derivative formed from the *C. ligata*-produced methyl (*E*)-6-2,3-dihydrofarnesoate determined that it too had the (*R*)-3 configuration. As with *C. uhleri*, the amount of the minor component methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate was too small to be able to determine its absolute configuration by the derivatization method. Thus, the male-specific blends of compounds from *C. uhleri* and *C. ligata* appear to be remarkably similar, even to the extent of the major, chiral component having the same absolute configuration.

Dynamics of Production of Male-Specific Compounds. Less than half the cohorts of virgin male *C. uhleri* produced male-specific compounds. For example, between November 1997 and November 1998, of 28 cohorts of virgin males $>$10 days old that were aerated continuously for at least 8 days, only 13 cohorts produced detectable amounts of male-specific compounds. In a second set of aerations conducted between February 6 and March 24, 1999, only three of 17 cohorts

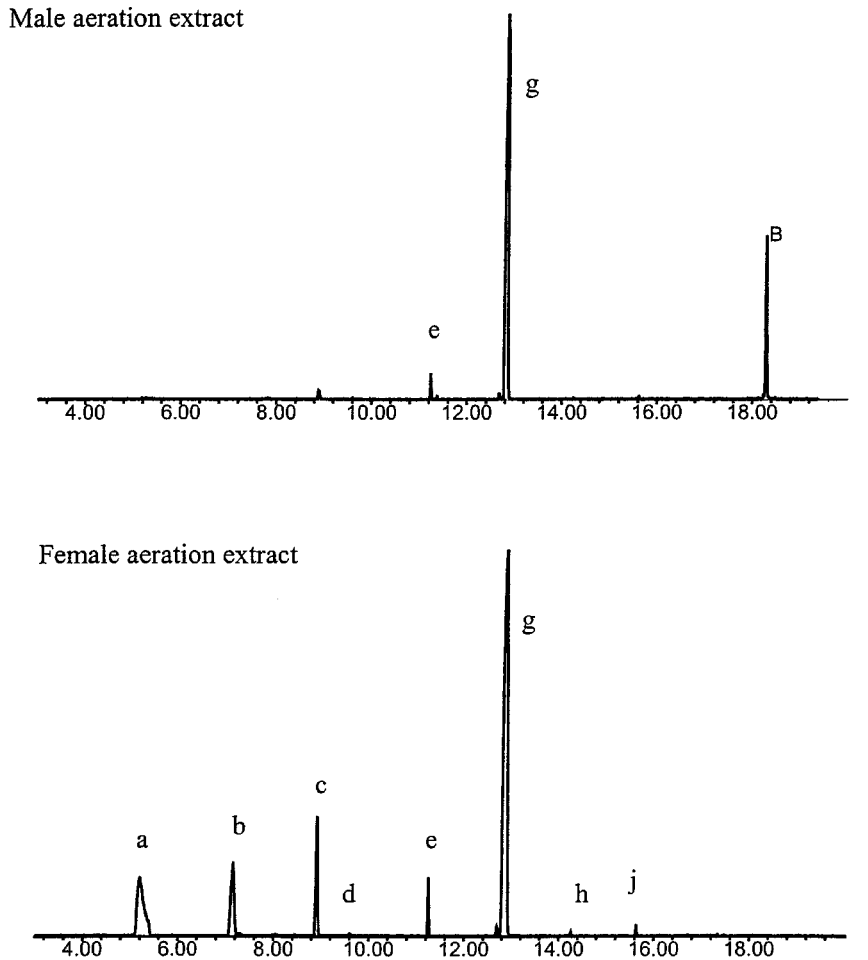


FIG. 5. Gas chromatograms of male (top trace) and female (bottom trace) *C. ligata* aeration extracts. GC conditions: column DB5-MS (20 m × 0.2 mm ID), oven temperature: 40°C (1 min), 10°C/min to 250°C, 250°C (10 min). Peak B: methyl (*E*)-6-2,3-dihydrofarnesoate. Other peaks: a: (*E*)-2-hexenal; b: (*E*)-4-oxo-2-hexenal; c: (*E*)-2-octenal; d: undecane; e: dodecane; f: (*E*)-2-octenyl acetate; g: tridecane; h: tetradecane; j: pentadecane.

of virgin males >12 days old aerated for periods of one week produced male-specific compounds. This second set of aerations was conducted with individuals reared from eggs laid by bugs collected in late fall of 1998.

For *C. ligata*, male-specific compounds were first detected from three cohorts of virgin males 13, 14, and 14 days, respectively, after the final molt. Unlike

C. uhleri, all cohorts (>20 total) of males that were aerated produced male-specific compounds.

Diurnal Rhythm of Production of Male-Specific Compounds. Aeration data from seven cohorts of male *C. ligata* were pooled and analyzed by 2-way ANOVA to determine the diurnal rhythm of production (Figure 6a). Maximum production of male-specific compounds occurred from 19:00 to 21:00 hr, and the quantities produced were significantly greater than for the next most productive periods between 17:00 and 19:00 and 21:00 and 23:00 hr. During peak production, males produced about 35 ng/bug/hr of the major male-specific compound.

For cohorts of male *C. uhleri* that did produce male-specific compounds, production followed a diurnal cycle, increasing from midday and peaking in the late afternoon at 17:00–19:00 (Figure 6b). The mean amount of the most abundant compound, methyl (*E*)-6-2,3-dihydrofarnesoate, produced during the peak production period was 8.8 ± 4.7 ng/bug/hr. Lower levels of this compound were detected during the entire 24 hr cycle.

Laboratory Bioassays. In vertical Y-tube bioassays, female *C. uhleri* were more attracted to odors from live males than to clean air controls (male odor stimulus, 24 responders, control, 10 responders, 3 nonresponders; χ^2 test, $P = 0.016$). In subsequent bioassays testing different doses of aeration extracts of males (doses of 8–32 ng of the major male specific compound), female bugs showed no preference for the odor extract from males versus the solvent control (extract, 14 responders, control, 18 responders). Because the effects of the pheromone might have been confounded by the presence of defensive chemicals in the crude extracts, no further bioassays were conducted with extracts.

When tested as a single component, 2 μg of the synthesized major male-specific compound, methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate, attracted 26 females, versus 12 females attracted to the solvent control (χ^2 test, $P = 0.023$), with 10 nonresponders. In further bioassays testing the effects of the minor male-specific components, and using racemic (*E*)-6-2,3-dihydrofarnesoate instead of the (*R*)-3 enantiomer, results were unclear. With 4-μg doses of the three-component blend, there were no significant differences in responses of females to the treatment or the control, ($N = 16$; treatment = 6, control = 8, 2 nonresponders). However, a second set of bioassays using 40 μg of the three-component blend resulted in attraction of female bugs to the treatment (treatment = 25, control = 10, 5 nonresponders; $P = 0.011$). In a direct test of the attractiveness of the three-component blend versus the single major component methyl (*E*)-6-2,3-dihydrofarnesoate (40 μg doses), female bugs responded equally to each stimulus (three-component blend = 10, single component = 14, 4 nonresponders; χ^2, $P = 0.414$). This result suggested that the two minor components of the male-specific volatiles are not required for attraction and that they are not pheromone components.

In a final set of laboratory bioassays, mature male *C. uhleri* were repelled by 40 μg of the three-component pheromone blend. Of the 20 bugs tested, 3 went to

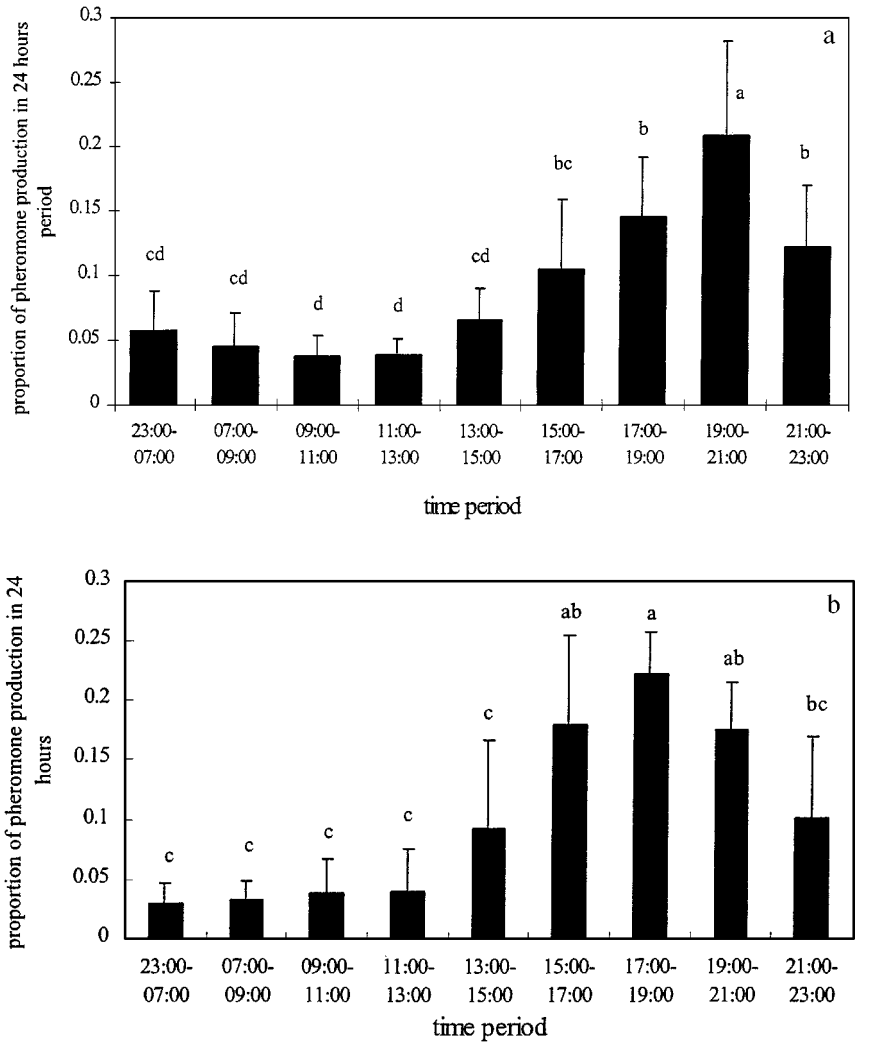


FIG. 6. Diurnal rhythm of production of the major male-specific compound, methyl (*E*)-6-2,3-dihydrofarnesoate, by 7 cohorts of *C. ligata* (a) and 4 cohorts of *C. uhleri* (b) males. *C. ligata*: 2-way ANOVA, for cohort effect, $F = 0.495$, $df = 6, 48$, $P = 0.8086$; for time interval effect $F = 13.326$, $df = 8, 48$, $P < 0.0001$. The maximum amount produced, from 19:00–21:00 hr, was 35.3 ± 32.2 ng/bug/hr. *C. uhleri*: 2-way ANOVA for cohort effect, $F = 0.519$, $df = 3, 24$, $P = 0.6732$; for time interval effect $F = 3.750$, $df = 8, 24$, $P = 0.0056$. The maximum amount, produced from 17:00 to 19:00 hr, was 8.8 ± 4.7 ng/bug/hr. Bars surmounted by the same letter(s) are not significantly different (Student-Newman-Keuls test, $P < 0.05$).

the arm containing the test treatment, 14 went to the control arm, and 3 bugs did not respond (χ^2, $P = 0.008$).

In the vertical Y-tube olfactometer, virgin female *C. ligata* were attracted to odors from live males (male odors versus control, 20 vs. 8 responders, χ^2 test, $P = 0.02$). The results were further categorized into response times of less than or greater than 1 min. This more detailed analysis indicated that when bugs responded more slowly, circling around and taking time to sample the airstreams (i.e., response time >1 min), they chose the arm with the odor of the male bug a large percentage of the time (treatment = 13, control = 2, χ^2, $P = 0.0045$). Furthermore, some of these bugs started up the control arm, then turned back and finally moved all the way to the end of the treatment arm of the olfactometer. In contrast, bugs that made a choice in less than 1 min showed no significant discrimination between the treatment and control arm (seven and six bugs, respectively).

Attempts to demonstrate attraction of sexually mature virgin *C. ligata* females to extracts of male volatiles were not successful, using several different aeration extracts containing varying amounts of the male-specific compounds and the defensive compounds. Possible reasons for this lack of attraction include incorrect dose or interference from the defensive compounds present in the extracts. Consequently, all further laboratory bioassays were conducted with synthetic compounds, in which the blend and dose could be completely controlled.

In the first bioassay of synthetic compounds with female *C. ligata* responders, there were indications that methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate as a single component (2 μg dose) might be attractive to female bugs (treatment = 17, control = 9, nonresponders = 8; χ^2 test, $P = 0.11$). Further bioassays conducted with the two-component blend of methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate with methyl (2*E*,6*E*)-farnesoate (100:1, total dose 2.02 μg) indicated that the two-component blend was more attractive than the control (treatment = 19, control = 6, 6 nonresponders, χ^2 test, $P = 0.009$). In a third series of bioassays, female bugs were more attracted to a three-component blend consisting of racemic methyl (*E*)-6-2,3-dihydrofarnesoate, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate and methyl (2*E*,6*E*)-farnesoate (100:0.8:0.4, total dose 4 μg) than to the control (treatment = 15, control = 1, 4 nonresponders, χ^2 test, $P = 0.0004$). Bioassays directly comparing the attraction of female bugs to the three-component blend versus methyl (*E*)-6-2,3-dihydrofarnesoate as a single component (2 μg doses) were inconclusive due to the limited number of bugs available for bioassays. Of the 11 bugs available, 6 went to the arm containing the single component and 3 went to the arm containing the three-component blend, and 2 did not respond.

In an abbreviated series of bioassays, there was no indication that male *C. ligata* were attracted to or repelled by the three-component blend (treatment = 5, control = 5, nonresponders = 7), but the numbers tested were too low to reach any firm conclusion.

Field Bioassays with C. uhleri *and* C. ligata. In the first field trials conducted August 3–16, 1999, more female *C. uhleri* were attracted to lures containing a three-component blend of the synthesized male-specific components than to solvent treated controls (67 to treatment, 7 to control; two-way ANOVA for treatment effect $F = 7.42$, $df = 2,10$, $P = 0.011$; for block effect $F = 3.48$, $df = 5,10$, $P = 0.043$). Although three times as many males were found on plants with lures versus control plants, the numbers of males attracted were not different between the treatment and control (36 to treatment and 12 to control, multiresponse permutation procedure (MRPP), $P > 0.05$). Interpretation of field test results was complicated by large variations in bug populations among blocks.

A second trial conducted in six blocks from August 19 to 24, 1999, attracted only low numbers of bugs. Significantly more female bugs were collected on baited plants than on control plants (9 vs. 0, MRPP test, $P = 0.01558$). Of the 9 males collected, 7 were on baited plants, and 2 were on control plants.

Because we did not find significant populations of *C. ligata* in the Riverside area in 1999, no specific field experiments were deployed for this species. However, two female *C. ligata* were collected from plants baited with lures for *C. uhleri*. Because male *C. ligata* and *C. uhleri* produce virtually identical blends of male-specific compounds, this was not unexpected. However, it is noteworthy because no other *C. ligata* were seen or collected at that site using visual observations and sweep-netting sampling.

DISCUSSION

Reproductive Behavior. The close-range courtship of phytophagous stink bugs follows characteristic steps, including males approaching females (over short distances), followed by antennation and head-butting of females by males, and end-to-end copulation (Fish and Alcock, 1973; Borges et al., 1987; Wang and Millar, 1997; Ho and Millar, 2001a). The daily rhythms of reproductive activity also appear similar among species, with courtship and copulation being initiated primarily in the afternoon and evening [e.g., *Chlorochroa ligata* (Fish and Alcock, 1973), *Nezara viridula* (Harris and Todd, 1980), *Euschistus heros* (Borges et al., 1998a), *Thyanta pallidovirens* (Wang and Millar, 1997), *Acrosternum hilare* and *T. pallidovirens* (H. McBrien and J. Millar, unpublished data)]. Thus, late afternoon and evening were chosen as the optimum time to conduct both laboratory and field bioassays.

The patterns of *C. uhleri* and *C. ligata* reproductive activity coincided with the maximum production of male-specific compounds. However, male-specific compounds were detected throughout the 24-hr monitoring periods, suggesting that the compounds were produced continuously with only the production rate varying. This pattern may have developed for several possible reasons. First, parasitoids use stink bug pheromones as kairomones (Aldrich, 1995), and timing peak production to parts of the day with waning light levels may reduce the risk of

parasitization (Harris and Todd, 1980). Second, because of the long copulation times, pheromone production in late afternoon may be timed so that males and females find each other around dusk and then continue copulation under the cover of darkness. Third, mating activities may be timed for optimum temperature and humidity conditions. Reproductive activities in the heat of the day increase the risk of desiccation, whereas cooler nighttime temperatures may hinder movement and activity.

Male *C. ligata* transferred substantial weights of material to females during mating, with body weights of both sexes changing significantly after copulation. Weight changes occurred with both virgin and experienced insects, and mean duration of copulation was similar for virgin and experienced insects. Similar weight changes after mating occur with *T. pallidovirens* (Wang and Millar, 1997), and it has been documented that pentatomid females obtain nutrients from males, often in the form of large, nonfertilizing sperm (Schrader, 1960; Mitchell and Mau, 1969; Mau and Mitchell, 1978; Kasule, 1986). For *T. pallidovirens*, these materials resulted in enhanced egg production by females, increasing the reproductive output of both sexes (Wang and Millar, 1997). This increase in reproductive output should in turn encourage multiple copulations by both sexes, which is indeed a characteristic of pentatomid reproduction.

There are several possible explanations for male stink bugs being the pheromone-producing sex. For example, adult stink bugs are polyphagous and mobile, and they move into and out of habitats quickly (McPherson and McPherson, 2000). However, the details of the dynamics of these migrations are unknown, and it is possible that males are the pioneering sex in colonizing new habitats. Under such a scenario, males, upon finding a good habitat, might produce pheromones to attract females, with oviposition commencing within a day or so after copulation to the mutual benefit of both sexes. Alternatively, because parasitoids use bug semiochemicals as kairomones to locate their hosts (Aldrich, 1995), selective pressures may have favored males as the pheromone-producing gender.

Male Specific Compounds. The male-specific compounds produced by *C. uhleri* and *C. ligata* included two known terpenoid compounds, methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate and methyl (2*E*,6*E*)-farnesoate, and a new terpenoid natural product, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate. The blends of male-specific compounds produced by the two species were remarkably similar, even to the extent that the absolute configuration (3*R*) of the major compound, methyl (*E*)-6-2,3-dihydrofarnesoate, was the same. In contrast, the two species are quite different morphologically, with *C. ligata* adults being dark brown or black with a band of orange or yellow outlining the body, whereas *C. uhleri* are green in color. Although detailed distribution records and habitat preferences are not available, these two species appear to share or at least overlap in ranges and host plants, and we have caught bugs of both species in the same field plots on the same day. The fact that the two species are sympatric, coupled with the apparently identical

pheromone blends, suggests that communication with pheromones is only part of the mate location system. Research on these (McBrien and Millar, unpublished data) and other species (Harris et al., 1982; Çokl, 1985; Kon et al., 1988; Ota and Çokl, 1991; Ryan and Walter, 1992; Jeraj and Walter, 1998; Miklas et al., 1999, Çokl et al., 2001) suggests that over shorter distances, mate location by phytophagous pentatomids involves species-specific and possibly even strain-specific substrate-borne vibrational signals (Ryan et al., 1996; Jeraj and Walter, 1998).

The blend of male-specific compounds produced by the congener *C. sayi*, which is morphologically very similar to and is frequently confused with *C. uhleri* (McPherson and McPherson, 2000), is entirely different from that produced by *C. uhleri* and *C. ligata.* The *C. sayi* blend is composed primarily of the monoterpenoid methyl geranate, with traces of methyl citronellate and methyl (*E*)-6-2,3-dihydrofarnesoate (Ho and Millar, 2001a), the latter being the only shared compound between *C. sayi* and the other two species. To our knowledge, these are the only reports of methyl (*E*)-6-2,3-dihydrofarnesoate being found in nature. It was first synthesized for treating liver disease (Yamatsu et al. 1981), and because of its structural similarity to insect juvenile hormones, it also has been used in the study of juvenile hormones (Campbell et al., 1998). The homolog, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, has never been reported from natural sources. Methyl farnesoate has been found to function as a juvenile hormone in crustaceans (Homola and Chang, 1997), but to our knowledge, it has never before been reported as an insect pheromone component.

In studying the dynamics of production of the male-specific compounds, it is unclear why they were detected in all of the *C. ligata* cohorts aerated but less than half of the *C. uhleri* cohorts. One possible explanation is that reproductive diapause, and the cessation of pheromone production, might be triggered easily in *C. uhleri*, despite the fact that the temperature, humidity, diet, and the length of the photophase (16L:8D) in the rearing room were constant. Even cohorts that failed to produce detectable levels of pheromone were not in complete reproductive diapause because mating did occur in the laboratory colonies. However, the close confines and relatively crowded conditions under which the bugs were reared may have precluded any need for the use of pheromones.

Laboratory Bioassays. Laboratory and field bioassays were complicated by the fact that females did not respond strongly to odors from conspecific males, to extracts from males, or to reconstructed blends of male specific compounds. Analogous problems have been reported during attempts to bioassay possible pheromone components for other phytophagous bug species. For example, blends of two male-specific compounds, *trans*- and *cis*-(*Z*)-bisabolene epoxide, were weakly attractive to female *Nezara viridula* in laboratory bioassays (Brézot et al., 1994). Despite the importance of this bug as a worldwide agricultural pest (Panizzi, 1997), there has been only a single short report of field bioassays of the pheromone, in which low numbers of bugs were attracted (Aldrich et al., 1993). Male-specific compounds

also have been identified from a number of *Euschistus* spp. (Aldrich et al., 1991), but field bioassays resulted in collection of less than one bug per trap-day (Aldrich et al., 1991; Borges et al., 1998b). Laboratory bioassays with *Euschistus heros* (Borges et al., 1998a), *E. obscurus* (Borges and Aldrich, 1994), and *Piezodorus hybneri* (Leal et al., 1998) also demonstrated that attraction of female bugs to male volatiles or synthetic pheromone components was relatively weak, with less than 50% of the individuals tested being attracted to the test treatments.

Field Bioassays. Several reports indicated that commonly used insect trap designs were not effective for phytophagous stink bugs and that bugs were frequently attracted to the vicinity of traps but not right into traps (Aldrich et al., 1991; Sugie et al., 1996). In perhaps the most extreme example, James et al. (1996) caught no spined citrus bugs (*Biprorulus bibax*) in pheromone-baited traps placed in citrus trees, but found that large numbers of bugs were attracted to trees containing traps. Consequently, instead of using traps of unknown efficacy, we decided to use a "trap plant" concept, in which the numbers of bugs on plants baited with pheromone lures or solvent controls were counted. Before deploying bioassays, in a pilot experiment run to determine the optimum time to conduct bug counts, female bugs were not seen close to lures in the early morning when temperatures were still cool, nor were they seen in the upper half of the plants close to the lures during the heat of the day. The optimal period for attraction, in agreement with the laboratory studies and with previously reported data from other species, appeared to be late afternoon through to darkness. Consequently, field tests were evaluated from about 17:00 hr until dark.

Field test results followed the same general pattern as that seen in the laboratory, with weak attraction of adult female bugs to lures. Several other points emerged. First, there were indications that lures also attracted male bugs; the number of males on baited plants was always higher than the number near controls, in all tests run. However, there may be an alternative explanation for this apparent attraction of males to pheromone lures. Males foraging for females may be attracted to or arrested on plants with females in response to cues associated with females, such as the shorter range substrate-borne vibrational cues described above. Further studies will be required to determine whether males are indeed attracted to the synthetic male pheromone or whether the apparent attraction is actually arrestment or attraction in response to female-produced cues. There was no indication that the lures were attractive to immature bugs, as has been reported for at least one other stink bug species (Aldrich et al., 1987).

It must be mentioned that the trap plant concept has limitations. First, bugs that were attracted to lure-baited plants in the late afternoon had disappeared by the next morning, indicating that the attraction is transient. Thus, if used as a bug monitoring strategy, trap plants would need to be checked consistently during late afternoon each day. Second, because bugs are free to move away from the lure-baited plants, counting the bugs on a trap plant provides a snapshot of one point

in time, rather than a cumulative count of all the bugs that have been attracted. Consequently, a trap plant strategy may be less sensitive than a method that retains most or all of the bugs attracted.

In total, the laboratory and field bioassay data provide strong evidence that one or more of the *C. uhleri* and *C. ligata* male-specific compounds are sex pheromone components. Because of the weak attraction to the male-produced compounds, and despite laboratory and field bioassay attempts extending over several years, it remains to be determined whether the male-produced pheromones consist of the single component methyl (*E*)-6-2,3-dihydrofarnesoate, or a blend of this compound with minor male-specific components. Efforts to address this question are continuing, in parallel with trap development and study of stink bug substrate-borne vibrational signals.

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