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ORIGINAL PAPER

Sex pheromone component ratios and mating isolation among three *Lygus* plant bug species of North America

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Abstract The plant bugs *Lygus hesperus*, *Lygus lineolaris*, and *Lygus elisus* (Hemiptera: Miridae) are major pests of many agricultural crops in North America. Previous studies suggested that females release a sex pheromone attractive to males. Other studies showed that males and females contain microgram amounts of (*E*)-4-oxo-2-hexenal, hexyl butyrate, and (*E*)-2-hexenyl butyrate that are emitted as a defense against predators. Using gas chromatography–mass spectrometry, we found that female *L. lineolaris* and *L. elisus* have a 4:10 ratio of hexyl butyrate to (*E*)-2-hexenyl butyrate that is reversed from the 10:1 ratio in female *L. hesperus* (males of the three species have ~10:1 ratio). These reversed ratios among females of the species suggest a behavioral role. Because both sexes have nearly equal amounts of the major volatiles, females should release more to attract males. This expectation was supported because *L. hesperus* females released more hexyl butyrate (mean of 86 ng/h) during the night (1800–0700 hours) than did males (<1 ng/h). We used slow-rotating pairs of traps to test the attraction of species to blends of the volatiles with a subtractive method to detect synergism. Each species' major butyrate ester was released at 3 μg/h, the minor butyrate according to its ratio, and (*E*)-4-oxo-2-hexenal at 2 μg/h. The resulting catches of only *Lygus* males suggest that (*E*)-4-oxo-2-hexenal is an essential sex pheromone component for all three species, (*E*)-2-hexenyl butyrate is essential for *L. elisus* and *L. lineolaris*, and hexyl butyrate is essential for *L. hesperus*. However, all three components are recognized by each species since ratios of the butyrate esters are critical for conspecific attraction and heterospecific avoidance by males and thus play a role in reproductive isolation among the three species. Because *L. hesperus* males and females are known to emit these major volatiles for repelling ant predators, our study links defensive allomones in *Lygus* bugs with an additional use as sex pheromones.

Keywords Pheromone identification · Slow-rotating trap pair · GC-MS analysis · Trap catch · Defensive allomones · Pest management · Heteroptera · Miridae

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Introduction

Plant bugs (Hemiptera: Miridae) of the genus *Lygus* feed on meristematic tissues of plants, causing shedding of buds, blooms, seeds, and fruit (Debolt and Patana 1985). Among several *Lygus* species of economic importance in the northern hemisphere, *Lygus hesperus* Knight is the most abundant in western North America and *Lygus lineolaris* (Palisot de Beauvois) is the most important in eastern North America (Clancy and Pierce 1966; Kelton 1975). In California, *L. hesperus* is probably the dominant species followed by *Lygus elisus* Van Duzee, while *L. lineolaris* is rarely found (Mueller et al. 2003). The three species are present in Arizona with *L. hesperus* being more common in alfalfa (*Medicago sativa* L.) while all three species are found in lesquerella, *Physaria fendleri* (A. Gray), an arid oilseed crop (Blackmer and Byers 2009). All three species are polyphagous pests of many agricultural crops, including cotton, strawberries, alfalfa, apples, and pears (Debolt and Patana 1985; Scott 1977; Young 1986).

Earlier studies have found that males of *L. lineolaris* and *L. hesperus* are attracted to caged virgin females, suggesting the presence of sex pheromones (Scales 1968; Strong et al. 1970; Graham 1987, 1988). However, it has been challenging to

identify the sex pheromone components. Aldrich et al. (1988) found that males and females of *L. lineolaris* and *L. hesperus* contain and release similar amounts of hexyl butyrate and (*E*)-2-hexenyl butyrate. Ho and Millar (2002) found that quantities of hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-2-hexenal, and 1-hexanol were similar in solvent extracts of male and female *L. hesperus* metathoracic glands. In odor collections, virgin females released proportionately more (*E*)-4-oxo-2-hexenal and (*E*)-2-hexenal than males. In the field, they found no attraction of *L. hesperus* to many different ratios of hexyl butyrate and (*E*)-2-hexenyl butyrate or other blends of C4–C8 butyrate esters. Wardle et al. (2003) put 50 male or 50 female *L. lineolaris* crowded together in a bottle causing them to become "agitated" and compared this to ten bugs of each sex that could climb on the metal screen and remain "calm." Both sexes of the agitated treatments released large amounts of several compounds, of which hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal were the major components. They suggested that release of the components serves as an alarm pheromone. However, Byers (2006) presented forceps-pinched bugs releasing the volatiles to within a few millimeters of resting bugs and elicited no reaction in the latter. Instead, a defensive allomone function was proposed because ants that attacked the bugs were repelled by emissions of the three major components contained in relatively large amounts (1–20 μg) in the metathoracic glands of both sexes. The question remains, however, can the components also be used as a sex pheromone if females can regulate their release while males use the compounds only for defense?

In Europe, Innocenzi et al. (2004) showed that both male and female *Lygus rugulipennis* contained the same three components in amounts from 1 to 45 μg. They report the difficulty of releasing the components at constant rates with rubber septa. When using polyethylene capsules, (*E*)-4-oxo-2-hexenal was unstable in certain mixtures and its release rate declined during field trials due to degradation. The release of the three possible binary blends and the tertiary blend did not attract more *L. rugulipennis* than the unbaited control. Innocenzi et al. (2005) then used microcapillaries with the compounds in solvent and showed that *L. rugulipennis* males were significantly attracted to the binary blend of hexyl butyrate and (*E*)-4-oxo-2-hexenal. The tertiary blend, including (*E*)-2-hexenyl butyrate, caught fewer males and was not significantly different from the blank, although the tertiary blend caught more males of *Lygus pratensis*. It seemed that higher release rates from capsules were less attractive than the lower release rates from microcapillaries (0.25–2 μg/h). Frati et al. (2009) reported that female *L. rugulipennis* release about 85 ng/h of hexyl butyrate when resting on host plants compared to 15 ng/h for females without host plants, the latter being similar to releases by males. The three volatiles have also been shown to comprise the female-produced sex pheromone of the sorghum plant bug *Stenotus rubrovittatus* (Matsumura) (Yasuda et al. 2008). Recently, a male-derived antiaphrodisiac sex pheromone, myristyl acetate, was identified for the North American *Lygus* species *L. hesperus*, *L. lineolaris*, and *L. elisus* that is inserted into females during mating and repels males for a few days (Brent and Byers 2011).

Our first objective was to determine the variation in amounts of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal in male and female *L. hesperus*, *L. lineolaris*, and *L. elisus* collected from alfalfa and lesquerella crops. Differences in ratios of any of the volatiles that were species-specific or sex-specific would be candidates for sex pheromone components. Extracts of males and females were also examined for amounts of myristyl acetate that may interact with attractive sex pheromone components. The second objective was to collect volatiles from virgin male and female *L. hesperus* on host plant material to determine any differences between the sexes in emissions of the three components. Ultimately, we wanted to test the three major volatiles and any other indicated compounds for attraction to the *Lygus* spp. in slow-rotating trap pairs in the field (Byers et al. 1990).

Methods and materials

Insects

L. hesperus were collected from the field (University of Arizona farm, Maricopa, AZ, USA) using sweep nets and reared several generations in the laboratory on organically grown green beans (*Phaseolus vulgaris* L.) and synthetic diet (Debolt 1982) at 14:10 L/D and constant 27 °C. Feral individuals of *L. hesperus*, *L. lineolaris*, and *L. elisus* used in solvent extracts were similarly obtained from either alfalfa or lesquerella fields. Feral or reared bugs were separated by sex according to the presence or lack of an ovipositor. To obtain virgins, males and females were separated immediately after adult eclosion (<1 day of age) and maintained as above on green beans and synthetic diet until used at 10 to 16 days of adult age.

Solvent extraction and quantification of identified volatiles in *Lygus* spp.

To determine amounts of the major volatiles that could be used as sex pheromone components, individual male and female *Lygus* adults were extracted and analyzed by gas chromatography–mass spectrometry (GC-MS). Adults of the three *Lygus* species were collected in April 2007 from lesquerella fields and *L. hesperus* and *L. lineolaris* in September 2007 from alfalfa fields. Because (*E*)-4-oxo-2-hexenal was later found to be unstable in extracts, we collected *L. hesperus* and *L. lineolaris* from alfalfa (May–June 2013), and

after each maceration, the supernatant extract was immediately analyzed by GC-MS.

Individual male and female *Lygus* adults were extracted in 100-μl hexane with internal standards of ethyl heptanoate and (+)-carvone (each 1 ng/μl, Sigma-Aldrich, St. Louis, MO, USA). The adults were macerated in hexane in a 100-μl conical vial with a blunt, nickel-plated tapestry needle (Prym-Dritz Corp., Spartanburg, SC, USA). The supernatant from the macerated insect was transferred using a pipette into a glass insert (W.R. Grace, Columbia, MD, USA) in a 2-ml vial to retard degradation of (*E*)-4-oxo-2-hexenal (Byers 2006). Chemical analysis of volatiles was carried out by autosampler injection (Varian 8400, Palo Alto, CA, USA) of 1 μl of each extract into a Varian 3800 gas chromatograph (GC) coupled to a Varian Saturn 2000 mass spectrometer (MS, ion trap at 70 eV). The GC column was a chiral fused-silica capillary (Cyclodex-B, J&W Scientific, Folsom, CA, USA) 60 m long×0.25 mm ID coated with 0.25-μm permethylated β-cyclodextrin. Helium at a constant flow of 1.2 ml/min was used as carrier gas. The injection port was held at 250 °C, and the analysis was performed in the splitless mode for 0.75 min then 60:1 split to 5 min. The GC oven temperature program was 40 °C for 2 min, then 10 °C/min to 60 °C and held 10 min, then 3 °C/min to 150 °C, and then 20 °C/min to 230 °C and held 10 min.

Compounds were identified by comparison of retention times and mass spectra to those of commercial standards (all >98 % purity, Sigma-Aldrich and Bedoukian Research, Danbury, CT, USA) using the NIST08 (National Institute of Standards, USA) spectral reference library. (*E*)-4-Oxo-2-hexenal was synthesized in one step from 2-ethylfurane according to Moreira and Millar (2005) giving a yield of 15 %. After purification, the chemical purity was 91 % with isomeric purity 99 % (RI=963 for *E*-isomer and 942 for *Z*-isomer on 30-m HP-5 column). To quantify chemicals in solvent extracts, the total ion chromatogram areas at the respective retention times were adjusted for quantities and MS response factor sensitivities (Byers 2006) and compared with areas of an internal standard. Selected monitoring of ions 83, 89, or 71 was used to estimate smaller amounts of (*E*)-4-oxo-2-hexenal, hexyl butyrate, and (*E*)-2-hexenyl butyrate, respectively, that co-eluted with volatiles (Byers 2005).

Collection of airborne volatiles from *L. hesperus* and dispensers during the day

To determine whether female *L. hesperus* release sex pheromone components during the day, airborne collections of volatiles from virgin males and females on a host plant (green bean pods) were performed. In the procedure for each experiment, ambient air was pulled using a laboratory vacuum at 400 ml/min through an activated charcoal trap (Alltech Associates, Deerfield, IL, USA) and flowmeter into a 1.9-L wide-mouth glass jar with Teflon-lined lid (VWR, Radnor, PA, USA). The jar contained green beans and virgin male or female *L. hesperus* from the laboratory colony. Volatiles released in the interior of the jar were transferred using Teflon tubing to a 20×2.3-mm ID Teflon tube filled with Porapak Q (80/100 mesh, Alltech Associates). The volatiles from particular experiments were collected at 25 °C for 2 to 13 h for bug emissions and for 30 min in 250-ml jars (VWR) for testing of chemical dispensers for field use. In preliminary work, no breakthrough of the three major *Lygus* compounds was observed with a second Porapak plug after 2, 4, or 13 h. After airborne collection, the Porapak Q filter was removed and 200 μl of hexane with internal standards was passed through the adsorbent into a glass insert for subsequent GC-MS analysis. Porapak extracts of airborne volatiles were analyzed similarly as for the insect extracts.

In the first experiment, four sets of 20 virgin males and four sets of 20 virgin females were placed inside 1.9-L glass jars with six green beans, and odors were collected as described above. A jar with only green beans served as a control for each set. Volatiles were collected during the day in 1- or 2-h periods except during the night when 12-h collections were performed. In a second set of experiments, new jars were employed for each 2- or 12-h (overnight) collection period, with the green beans and insects replaced at each sampling interval (N=43 for each sex; 7–23 May 2008). In a third set of experiments, volatiles from the same individual virgin males or females were collected during 24 h in periods of 2 h throughout the day and for a 12-h period overnight (N=9 each sex, 16–21 June 2008). To relate the amounts released by individuals in the three experiments to amounts released during defense, an experiment was conducted in which tips of female abdomens were sharply squeezed with forceps for 1 s inside a jar to stimulate the expulsion of their metathoracic gland contents. The jar was then immediately closed, and volatiles were collected for 12 h using two Porapak filters in series to determine if breakthrough of compounds occurred through the first filter.

Response of *Lygus* spp. to potential sex pheromone components released from slow-rotating trap pairs

The three major volatiles of the *Lygus* species, (*E*)-4-oxo-2-hexenal, hexyl butyrate, and (*E*)-2-hexenyl butyrate, were tested in slow-rotating pairs of traps in the field with a subtractive method to evaluate synergism among components (Byers et al. 1990; Byers 1992). However, we suspected that ratios of hexyl butyrate to (*E*)-2-hexenyl butyrate could also be critical to attraction because insect extractions and airborne collections showed that females of *L. hesperus* had a substantially different ratio (10:1) than females of *L. lineolaris* and *L. elisus* (4:10). Baits with the desired ratios of hexyl butyrate to (*E*)-2-hexenyl butyrate were achieved using glass tubes of

4.4 mm ID×50 mm long each filled at the bottom with 100 μl of 1:10 dilutions (*v*/*v* in canola oil) of one of four stock solutions: 50 % hexyl butyrate, 5 % (*E*)-2-hexenyl butyrate (for *L. hesperus*), 20 % hexyl butyrate, and 50 % (*E*)-2-hexenyl butyrate (for *L. lineolaris* and *L. elisus*). The target release rate of ~3 μg/h at 25 °C for the 50 % hexyl butyrate and 50 % (*E*)-2-hexenyl butyrate solutions and proportional release rates for the other mixtures were achieved by the dilution method (Byers 1988) as determined by 30-min volatile collections of dispensers in 250-ml jars. Because (*E*)-4-oxo-2-hexenal was unstable during synthesis and purification, it was immediately dissolved as 10 % *w*/*w* in polyethylene glycol (400 MW, Sigma-Aldrich) with 1 % butylated hydroxytoluene (BHT) and stored at −20 °C. In initial experiments, however, we found that this compound degraded in the field as shown by GC-MS analysis of volatile collections of dispensers. Volatile collections showed that (*E*)-4-oxo-2-hexenal was released at a rate of ~3 μg/h initially and ~1 μg/h after 18 h in the field. This necessitated that solutions of (*E*)-4-oxo-2-hexenal be renewed daily by placing 25 μl of fresh 10 % solution in the bottom of a 2-ml glass vial (6 mm ID×31 mm long).

The slow-rotating trap pair system allows blends of chemicals to be compared in many different positions during a few hours, thus moderating the usual variation in captures on fixed-position traps due to spatial and temporal effects (Byers et al. 1990). The slow-rotating trap pair was constructed with metal arms that suspended two white delta traps (triangular opening 22 cm long×18 cm wide×14 cm high, sticky floor 18×17 cm, Alpha Scents, West Linn, OR, USA) 4.4 m apart about 30 cm above the crop canopy. A 12-V lead–acid battery powered a 0.5-rpm gear motor and gearbox to rotate the trap pair once every 20 min. Six rotating trap pairs were placed in a line with a 14-m spacing between rotors on the edge of a lesquerella field (April–May 2013). Three rotating trap pairs were moved and placed similarly in the middle of an alfalfa field for additional tests (June–September). The dispensers of the three components were held in a metal screen (5-mm mesh) cage hung centrally inside traps to protect against direct sun and jostling from wind. The traps were placed on the rotors at about 1900 hours, rotated throughout the night, and then removed at 1100 hours the next morning since flight of the three *Lygus* species was expected in the evening (2000–2200 hours) and to a lesser extent in the morning (Mueller and Stern 1973; Rancourt et al. 2000).

The three-component blend for each species was compared to a blank trap or to the same blend where one of the components was missing (subtracted). In one test, the *L. hesperus* blend was compared to the *L. lineolaris*–*L. elisus* blend in the same rotor. In other tests, the two blends at the standard dosage described above (1×) and at one tenth (0.1×) and tenfold higher release rates (10×) for the butyrates were tested for all species. However, the (*E*)-4-oxo-2-hexenal was released at 0.1× (1 % in PEG) in the low dosage test and at 1× rates in tests of the 1× and 10× rates of the butyrates. The 1× blend was tested against both the lower and higher doses in two rotors. The effect of addition of a 1:10 dilution of 1 % (*E*)-2-hexenal added to the full three-component *L. hesperus* blend was also tested. In addition, traps with ten virgin female *L. hesperus* on four green beans were tested against traps with green beans only in a rotor at the same time as the synthetic compounds were tested. The females and beans were held inside clear plastic tube sections (5.5 cm diameter×8 cm long) covered on their ends with fine cloth mesh and suspended centrally inside the delta traps.

Statistics

Differences between means of chemical amounts between the sexes of a species for each experiment were analyzed by *t* tests (MicroSoft Excel) with *P* values adjusted by the Bonferroni–Holm method for multiple comparisons (Westfall et al. 1999) at $\alpha = 0.05$. In comparisons of full three-component pheromone blends with a blank trap or an alternate two-component blend in the rotating trap pairs, insect counts from the alternate or blank trap were subtracted from the paired capture from the full blend and this difference was $\log_{10}$-transformed. Means and 95 % confidence intervals of the transformed differences were calculated using the MEANS procedure of SAS (SAS Institute 2010). Where the confidence interval did not include zero, a significant difference was declared between the paired trap treatments. Where the trap with the alternative lure captured more insects than the paired trap with the full pheromone blend in at least one replication, the log difference could not be calculated because the difference was negative. In those cases, captures for the two treatments were compared by an exact goodness of fit test (the EXACT statement of PROC FREQ; SAS Institute 2010). Insect captures for all traps baited with a full three-component blend were summarized to compare catch frequencies of the three species in relation to blend ratios using the Fisher exact test (EXACT option of PROC FREQ).

Results

Solvent extraction and quantification of identified volatiles in *Lygus* spp.

Individual *Lygus* bugs taken from alfalfa contained relatively large amounts of (*E*)-4-oxo-2-hexenal (means from 1.5 to 3.2 μg), hexyl butyrate (6 to 13 μg), and (*E*)-2-hexenyl butyrate (0.4 to 20 μg) (Table 1). Individuals of *L. hesperus* and *L. lineolaris* that were sampled from alfalfa in September 2007 (Table 1) appeared to contain more of the major volatiles than those feeding in lesquerella in April 2007 (Fig. 1). The ratio of hexyl butyrate to (*E*)-2-hexenyl butyrate was similar in *L. hesperus* males and females (31:1 and 35:1, respectively) in alfalfa (September 2007, Table 1), but the ratio was lower at

Table 1 Amounts (ng/individual, ±SE) of volatiles in males and females of *L. hesperus* and *L. lineolaris* taken from alfalfa in 19–26 September 2007

Extracted material	(*E*)-2-Hexenal	(*E*)-4-Oxo-2-hexenal	Hexyl butyrate	(*E*)-2-Hexenyl butyrate	Myristyl acetate
L. hesperus ♂ (*N*=10)	14±4	1,536±241	11,570±1,316	370±129	20±6
L. hesperus ♀ (*N*=10)	53±10[a]	1,644±464	12,706±1,630	364±103	1±1[a]
L. lineolaris ♂ (*N*=10)	18±6	2,876±359	13,024±1,337	1,521±333	18±3
L. lineolaris ♀ (*N*=10)	96±30[a]	3,202±572	5,893±639[a]	19,827±2,498[a]	0[a]

[a] Male and female mean amounts within a species were significantly different (*t* test, adjusted $P<0.05$)

15:1 in males and 9:1 in females from lesquerella (April 2007, Fig. 1). The ratio of these two butyrate components in *L. lineolaris* males was 8.6:1 (Table 1) and 4.7:1 (Fig. 1) in alfalfa and lesquerella, respectively. Male *L. elisus* from lesquerella had a similar ratio of 5.9:1 (Fig. 1). Thus, males of all three species have predominantly hexyl butyrate, as do the females of *L. hesperus*. However, the ratio of hexyl butyrate to (*E*)-2-hexenyl butyrate is reversed in females of *L. lineolaris* (~1:3.3 in alfalfa and lesquerella) and females of *L. elisus* (1:2.9 in lesquerella, Fig. 1).

Because the major volatiles are from the metathoracic gland and used for defense (Byers 2006), the total amounts of butyrates (hexyl butyrate plus (*E*)-2-hexenyl butyrate) for each sex might be similar. This was the case for *L. hesperus* in September in alfalfa (11.9 and 13.1 μg total butyrates for males and females, respectively, Table 1) and in lesquerella (5.4 and 6.3 μg, Fig. 1). *L. lineolaris* individuals also had large amounts, with males and females having mean totals of 14.5 and 25.7 μg, respectively, when in alfalfa (Table 1) and 7.6 and 13.2 μg, respectively, when in lesquerella (Fig. 1). In lesquerella, *L. elisus* males had 4.9 μg of total butyrates compared to 7.6 μg in females.

Both sexes of all three species in alfalfa or lesquerella contained relatively small amounts of (*E*)-2-hexenal (≤0.10 μg). Males of *L. lineolaris* contained significantly less (*E*)-2-hexenal than females in alfalfa (Table 1) and in lesquerella (males 44±8 ng and females 106±21 ng; adjusted $P=0.007$). *L. elisus* males also appeared to contain less (*E*)-2-hexenal (15±5 ng) than females (43±15 ng) in lesquerella, but this difference was not significant at $\alpha=0.05$ after adjustments for multiplicity (adjusted $P=0.12$). Males of *L. hesperus* had less than females in alfalfa (Table 1) but not in lesquerella (males 65±21 ng and females 70±32 ng; adjusted $P=0.89$). The mean amounts of myristyl acetate per male *L. hesperus* or *L. lineolaris* were 18–20 ng (Table 1). This compound was sometimes found in low amounts in females that probably had recently mated, which gave a small mean amount (Table 1).

Adults of *L. hesperus* and *L. lineolaris* collected from alfalfa in May–June 2013 and analyzed immediately after maceration to minimize degradation also contained large amounts of the major volatiles (Fig. 2). The ratios of (*E*)-4-oxo-2-hexenal/hexyl butyrate/(*E*)-2-hexenyl butyrate were 7:91:2 for male and 5:90:5 for female *L. hesperus* and 6:86:8 for male and 5:25:70 for female *L. lineolaris* (Fig. 2).

Collection of airborne volatiles from *L. hesperus* and dispensers during the day

In the first experiment using groups of 20 virgin males or females inside 1.9-L glass jars, inconsistent results were

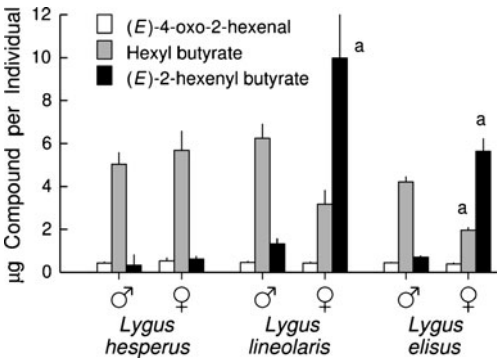

Fig. 1 Amounts of three major volatiles extracted from males and females of *L. hesperus*, *L. lineolaris*, and *L. elisus* taken from lesquerella fields (10–25 April 2007). The *letter* "a" denotes a significant difference in mean amounts of the indicated compound between males and females of the same species (*t* test, adjusted $P<0.05$)

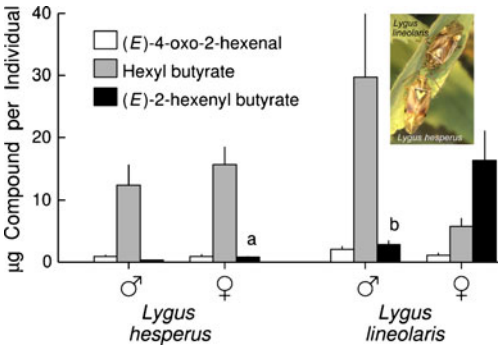

Fig. 2 Amounts of three major volatiles of males and females of *L. hesperus* and *L. lineolaris* taken from alfalfa (30 May–6 June 2013) and analyzed by GC-MS immediately after solvent extraction of macerated individuals. The *letter* "a" denotes a significant difference in mean amounts of (*E*)-2-hexenyl butyrate between females of each species, while the *letter* "b" denotes a significant difference in means for the same volatile between males and females of *L. lineolaris* (*t* test, adjusted $P<0.05$)

obtained except that none of the major *Lygus* volatiles were collected from green beans in the control jars. These inconsistencies could have occurred because variable numbers of bugs in each jar expelled the contents of their defensive glands. When volatiles were collected from individual bugs replaced each 2- or 12-h (overnight) sampling period, a mean of 9.9± 2.8 ng/h (±SE, $N=32$) of hexyl butyrate was collected per female in periods during the daylight, while during the night 61.6±27.9 ng/h per female ($N=11$) was emitted ($P=0.004$, t test). Three of the 32 individual males released high levels of hexyl butyrate (750, 2,550, and 2,750 ng/h) due to expulsion of their defensive glands. These quantities were comparable to amounts released by forceps-pinched females. Without the three male outliers, males released a mean of only 3.5± 1.1 ng/h during the day and 0.9±0.2 ng/h during the night.

We wanted to avoid disturbing individuals during their introduction to the collection jar; therefore, the same individuals remained for 24 h in a jar. Using this procedure, very low levels of released compounds were found from males (Fig. 3). Also, females did not release high levels in the first three periods of collection spanning 1000 to 1800 hours. The onset of darkness may have triggered the release of hexyl butyrate in females (mean 86.5 ng/h) that was considerably higher than that from males (0.014 ng/h) (Fig. 3). This difference was not significant due to variable releases and adjustment for multiple comparisons (Bonferroni–Holm); however, the overall mean release rate from males (0.8 ng/h) was significantly less than that from females (28.8 ng/h, $P=0.004$). The release of (*E*)-4-oxo-2-hexenal ranged from about 6 to 11 % that of hexyl butyrate, while that of (*E*)-2-hexenyl butyrate was about 4 to 8 % that of hexyl butyrate in the females where these compounds could be readily quantified (data not shown).

Response of *Lygus* spp. to potential sex pheromone components released from slow-rotating trap pairs

In all three species, (*E*)-4-oxo-2-hexenal is likely a pheromone synergist since its subtraction from the three-component blend of each species caused attraction to cease (Fig. 4). Hexyl butyrate for *L. hesperus* and (*E*)-2-hexenyl butyrate for both *L. lineolaris* and *L. elisus* are essential sex pheromone components (Fig. 4). Although baits of both *L. hesperus* and *L. lineolaris–L. elisus* had the same three components released in similar microgram per hour rates, *L. hesperus* was attracted only to its conspecific ratio of components, while *L. lineolaris* and *L. elisus* were attracted only to the ratio representing these two species (Fig. 4). Only males of the three *Lygus* species were caught on any traps, indicating the sex-specific pheromone activity of the component blends. In cases where trapping was begun at 1900 hours, checked at 0600 hours, and terminated at 1100 hours (June–September), 96 % of *L. hesperus* and 69 % of *L. lineolaris* males were caught in the evenings ($N=100$ and $N=99$, respectively). Captures were not observed between 0900 and 1800 hours. Both species were caught only in relatively calm wind speeds (<3 m/s).

Males of *L. hesperus* likely avoided the component ratio of the heterospecifics, while *L. lineolaris* and *L. elisus* likely avoided the *L. hesperus* ratio. This conclusion is supported by respective 2×2 Fisher exact tables where the catch of *L. hesperus* and *L. lineolaris* and the catch of *L. hesperus* and *L. elisus* in response to two ratios (10:1 and 4:10) of hexyl butyrate and (*E*)-2-hexenyl butyrate are compared. The comparisons show that the catch ratios for each pair of species are

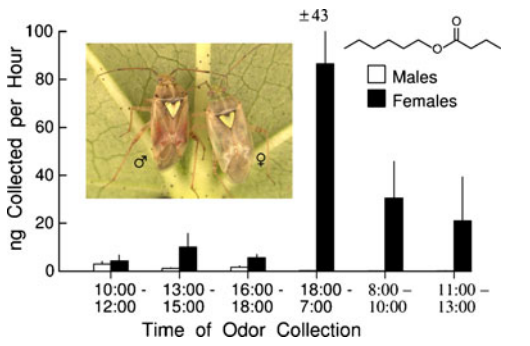

Fig. 3 Airborne amounts (ng ± SE) of hexyl butyrate collected per hour during the day from individual male and female *L. hesperus* inside glass chambers containing green beans. *Photo insert* shows male and female *L. hesperus* on the underside of a cotton leaf

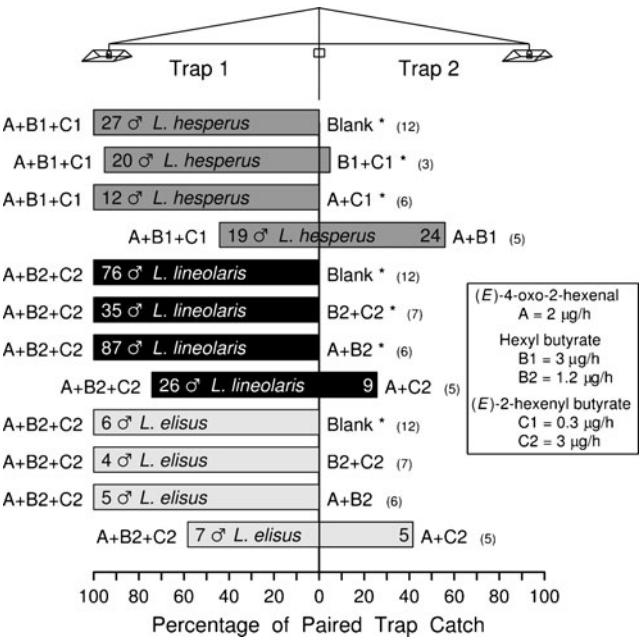

Fig. 4 Total catches of *L. hesperus* in response to blends of (*E*)-4-oxo-2-hexenal (*A*) and a 10:1 ratio of hexyl butyrate (*B1*) and (*E*)-2-hexenyl butyrate (*C1*), and total catches of *L. lineolaris* and *L. elisus* in response to blends of (*E*)-4-oxo-2-hexenal (*A*) and a 4:10 ratio of hexyl butyrate (*B2*) and (*E*)-2-hexenyl butyrate (*C2*) released in slow-rotating trap pairs. *Asterisks* indicate a significant difference between paired trap baits at $P<0.05$ (MEANS procedure of SAS). *Numbers in parentheses at the right* represent the number of trap-days tested per comparison, 30 April–2 September 2013 in lesquerella and alfalfa

Table 2 Summarized catches of male *L. hesperus*, *L. lineolaris*, and *L. elisus* on paired rotating traps releasing 10:1 or 4:10 butyrate ratios representing natural ratios of hexyl butyrate/(*E*)-2-hexenyl butyrate with the same release of (*E*)-4-oxo-2-hexenal (data summarized from field catches on rotating paired traps, Fig. 4)

Species	10:1 ratio of *L. hesperus*	4:10 ratio of *L. lineolaris* and *L. elisus*
L. hesperus[a]	78	0
L. lineolaris	0	224
L. elisus	0	22

[a] Patterns in frequencies of catches corresponding to ratios of butyrate components of *L. hesperus* differ from those of *L. lineolaris* and *L. elisus* (Fisher exact test, $P<0.001$)

significantly different (Table 2) and suggest that *L. hesperus* avoids the heterospecific blend ratio, whereas *L. lineolaris* and *L. elisus* avoid the pheromone blend ratio of *L. hesperus*. Thus, all three components in each of the species are essential for discrimination of sex pheromone blends. The delta trap baited with ten virgin females of *L. hesperus* on green beans caught five male *L. hesperus* compared to none on the blank trap, although this difference was not statistically significant (Fisher exact test, $P=0.17$).

The presence of (*E*)-2-hexenal in larger amounts in female *L. hesperus* (Byers 2006 and Table 1) suggested that this compound should be tested as a pheromone component. However, (*E*)-2-hexenal showed no activity for *L. hesperus* since addition of this compound to the three major components caught 12 male *L. hesperus*, the same number of males caught on the three-component blend without (*E*)-2-hexenal. In alfalfa, the respective *L. hesperus* and *L. lineolaris* three-component blends were compared within the same rotor during the evening and morning (5–6 June 2013). The *L. hesperus* blend caught 12 male *L. hesperus* while the *L. lineolaris* blend caught 30 male *L. lineolaris*, again showing that the two species are very selective for their conspecific blend. Finally, in tests of the standard dosage (1×) against a 0.1× and a 10× dosage for both *L. lineolaris* and *L. hesperus* blends, the 1× versus 10× doses for *L. hesperus* caught 6 males on the 1× only, while the 1× versus 0.1× caught 35 males on the 1× only (26–28 June). No *L. lineolaris* were caught at this time because the alfalfa was mowed and *Lygus* populations did not recover for many days as observed by sweep net sampling.

Discussion

Several factors were critical to successful field-testing of the synthetic sex pheromone components of the three *Lygus* spp. First, the ratios of hexyl butyrate to (*E*)-2-hexenyl butyrate were critical because neither *L. lineolaris* nor *L. hesperus* were attracted to each other's reverse ratio. Secondly, the release rate was important since a 1:10 dilution of the 5 to 50 % concentrations of butyrate components (1×) was the most attractive, while 0.1× and 10× rates were unattractive to *L. hesperus*. The daily renewal of the synergist (*E*)-4-oxo-2-hexenal was also vital since it degraded rapidly in the field, and only traces were detected by airborne collections of dispensers after a few days. Mowing of alfalfa causes adult bugs to leave the fields (Stern and Mueller 1968); thus, tests were generally conducted a week or more after mowing. Finally, because we found that *Lygus* spp. were caught in traps only in relatively calm conditions, we saved limited quantities of (*E*)-4-oxo-2-hexenal by subsequently testing traps for 12 h from 1900 to 1100 hours under mild breezes and temperatures. Further study is needed to improve the synthetic yield of (*E*)-4-oxo-2-hexenal and its stabilization in baits.

The lack of *L. hesperus* attraction to baits intended for *L. lineolaris–L. elisus* and the converse lack of *L. lineolaris–L. elisus* attraction to baits for *L. hesperus* are due in part to a strong attraction of the respective species for conspecific sex pheromone. However, it is likely that males of each species avoid entering traps with the heterospecific lure. First, individuals cannot use (*E*)-4-oxo-2-hexenal to avoid heterospecific blends because this compound was released at the same rate for all three species. Furthermore, based on analysis of many dose–response curves of pheromones, the dose of an attractive bait must be altered by five- to tenfold to observe any significant difference in catch (Byers 2013). However, if 9.6 parts of (*E*)-2-hexenyl butyrate are added to the *L. hesperus* bait, then this bait is transformed into the 4:10 bait of *L. lineolaris–L. elisus*, which was unattractive to *L. hesperus*. This is a classic "inhibition" test whereby an attractive lure of *L. hesperus* captures fewer insects when an inhibitor is added. In bark beetles, inhibition of response to attractive pheromone by heterospecific pheromone components has been demonstrated for several species (Byers et al. 2013). The reduced response may be a repellency since bark beetles responding to a trap releasing aggregation pheromone and inhibitors were seen to veer away as they approached within ~1 m of the trap (Byers et al. 2004). In *Phytocoris difficilis* Knight (Miridae), a blend of female-produced sex pheromone components attractive to males was made unattractive by addition of hexyl butyrate and (*E*)-2-hexenyl butyrate that are not found in females (Zhang and Aldrich 2003). Therefore, the catch of the respective *Lygus* species only on their conspecific bait ratio and not on the heterospecific ratio is due not only to attraction but also a likely repellency.

This mechanism of using specific component ratios of butyrates to discriminate and find the appropriate species for mating has not been suggested for species of bugs (Hemiptera). The importance of component ratios in identifying appropriate mates was shown first by Klun et al. (1973) for the European corn borer (*Ostrinia nubilalis* [Hübner], Pyralidae), where different races of the moth are attracted to

specific ratios of geometrical isomers of (*E*)- and (*Z*)-11-tetradecenyl acetate. Löfstedt et al. (1991) showed that three species of *Yponomeuta* ermine moths feeding on the same host plant maintain reproductive isolation even when using the same three compounds for their sex pheromones. Two of these components were produced in particular nonoverlapping ratios of (*E*)- and (*Z*)-11-tetradecenyl acetate that were attractive only to the conspecifics. The case of the three *Lygus* species appears similar but involves two molecules with or without a double bond, rather than two geometrical isomers of a double bond.

Ho and Millar (2002) collected odors for 24 h from individual virgin males and females of *L. hesperus* and found that females began releasing hexyl butyrate at 4 days of age. Females of 8 days of age released 85 ng/h per female compared to only 11 ng/h per male. These release rates were similar to those observed in our airborne collection experiments in which females released hexyl butyrate at 86 ng/h during the night, which was significantly more than that from males (Fig. 3). These release rates for *L. hesperus* are also similar to the amounts collected from mated *L. rugulipennis* females (86 ng/h) and males (8 ng/h) on a host plant (Frati et al. 2009). Mated females and males of *L. rugulipennis* appear to reduce their emissions to <8 ng/h when not on host plants. Although females of *L. hesperus* continuously have the attractive sex pheromone components in their metathoracic gland after mating, nearby males that antennate mated females avoid them for several days while the antiaphrodisiac myristyl acetate remains in the female's gonopore (Brent and Byers 2011).

The release rates of sex pheromone from individual females imply that dosages in the field should not be too high because males are responding to individual females. For example, when synthetic sex pheromone is released at considerably higher rates than natural rates, male moths cease upwind orientation when the concentration exceeds that of a single female (Baker and Roelofs 1981). Innocenzi et al. (2005) indicated that attractive release rates in the field for *L. rugulipennis* occurred at from 0.25 to 2 μg/h for various components. In our field tests in which insects were caught, we released 1–3 μg/h of the same components that attracted either *L. hesperus* or *L. lineolaris* and *L. elisus* depending on the ratio of hexyl butyrate to (*E*)-2-hexenyl butyrate. When release rates of the butyrate components were greatly reduced or increased but with the same release of (*E*)-4-oxo-2-hexenal, no *L. hesperus* were attracted. This represents an exceptionally narrow range of attractive release rates, which usually span three or more orders of magnitude in insects (Byers 2013). Thus, further tests are needed to confirm this result.

The results of field tests with sex pheromone components and the ratios of hexyl butyrate to (*E*)-2-hexenyl butyrate explain how *L. hesperus* (10:1 ratio) maintains reproductive isolation from *L. lineolaris* and *L. elisus* (4:10 ratios) and vice versa. Because *L. hesperus* and *L. elisus* are sympatric in California and Pacific States as well as Arizona, there appears to be strong selection pressure to avoid heterospecific sex pheromone using ratios of the butyrates. Similarly, *L. lineolaris*, found mainly in eastern North America, and *L. hesperus* in western North America nonetheless overlap in many areas of the southern and southwestern USA where the plant bugs would increase their fitness by avoiding heterospecific sex pheromone and detrimental mating. The apparently same pheromone used by the morphologically distinct species, *L. lineolaris* and *L. elisus*, is problematic. However, these two species are largely parapatric over most of their geographic ranges. Further investigation will be necessary to elucidate the mechanisms ensuring reproductive isolation of these species.

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References

Aldrich JR, Lusby WR, Kochansky JP, Hoffmann MP, Wilson LT, Zalom FG (1988) *Lygus* bug pheromones, vis-à-vis stink bugs. Proceedings Beltwide Cotton Production Research Conferences, pp. 213–216

Baker TC, Roelofs WL (1981) Initiation and termination of oriental fruit moth male response to pheromone concentrations in the field. Environ Entomol 10:211–218

Blackmer JL, Byers JA (2009) *Lygus* spp. (Heteroptera: Miridae) host–plant interactions with *Lesquerella fendleri* (Brassicaceae), a new crop in the arid southwest. Environ Entomol 38:159–167

Brent CS, Byers JA (2011) Female attractiveness modulated by a male-derived antiaphrodisiac pheromone in a plant bug. Anim Behav 82: 937–943

Byers JA (1988) Novel diffusion–dilution method for release of semiochemicals: testing pheromone component ratios on western pine beetle. J Chem Ecol 14:199–212

Byers JA (1992) Optimal fractionation and bioassay plans for isolation of synergistic chemicals: The subtractive–combination method. J Chem Ecol 18:1603–1621

Byers JA (2005) A cost of alarm pheromone production in cotton aphids, *Aphis gossypii*. Naturwissenschaften 92:69–72

Byers JA (2006) Production and predator-induced release of volatile chemicals by the plant bug *Lygus hesperus*. J Chem Ecol 32: 2205–2218

Byers JA (2013) Modeling and regression analysis of semiochemical dose–response curves of insect antennal reception and behavior. J Chem Ecol 39:1081–1089

Byers JA, Schlyter F, Birgersson G, Francke W (1990) *E*-myrcenol in *Ips duplicatus*: An aggregation pheromone component new for bark beetles. Experientia 46:1209–1211

Byers JA, Zhang QH, Birgersson G (2004) Avoidance of nonhost plants by a bark beetle, *Pityogenes bidentatus*, in a forest of odors. Naturwissenschaften 91:215–219

Byers JA, Birgersson G, Francke W (2013) Aggregation pheromones of bark beetles, *Pityogenes quadridens* and *P. bidentatus*, colonizing Scotch pine: olfactory avoidance of interspecific mating and competition. Chemoecology in press

Clancy DW, Pierce HD (1966) Natural enemies of some *Lygus* bugs. J Econ Entomol 59:853–858

Debolt JW (1982) Meridic diet for rearing successive generations of *Lygus hesperus*. Ann Entomol Soc Am 75:119–122

Debolt JW, Patana R (1985) *Lygus hesperus*. In: Singh P, Moore RF (eds) Handbook of insect rearing, vol 1. Elsevier, New York, pp 329–338

Frati F, Chamberlain K, Birkett M, Dufour S, Mayon P, Woodcock C, Wadhams L, Pickett J, Salerno G, Conti E, Bin F (2009) *Vicia faba–Lygus rugulipennis* interactions: induced plant volatiles and sex pheromone enhancement. J Chem Ecol 35:201–208

Graham HM (1987) Attraction of *Lygus* spp. males by conspecific and congeneric females. Southwest Entomol 12:147–154

Graham HM (1988) Sexual attraction of *Lygus hesperus* Knight. Southwest Entomol 13:31–37

Ho HY, Millar JG (2002) Identification, electroantennogram screening, and field bioassays of volatile chemicals from *Lygus hesperus* Knight (Heteroptera: Miridae). Zool Studies 41:311–320

Innocenzi PJ, Hall DR, Cross JV, Masuh H, Phythian SJ, Chittamaru S, Guarino S (2004) Investigation of the European tarnished plant bug, *Lygus rugulipennis*: Chemical, electrophysiological, and field studies. J Chem Ecol 30:1509–1529

Innocenzi PJ, Hall D, Cross JV, Hesketh H (2005) Attraction of male European tarnished plant bug, *Lygus rugulipennis* to components of the female sex pheromone in the field. J Chem Ecol 31:1401–1413

Kelton LA (1975) The *Lygus* bugs (genus *Lygus* Hahn) of North America (Heteroptera: Miridae). Mem Entomol Soc Can 95:1–101

Klun JA, Chapman OL, Mattes KC, Wojtkowski PW, Beroza M, Sonnet PE (1973) Insect sex pheromones: minor amount of opposite geometrical isomer critical to attraction. Science 181:661–663

Löfstedt C, Herrebout WM, Menken BJ (1991) Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). Chemoecology 2:20–28

Moreira JA, Millar JC (2005) Short and simple syntheses of 4-oxo-(E)-2-hexenal and homologs: Pheromone components and defensive compounds of Hemiptera. J Chem Ecol 31:965–968

Mueller AJ, Stern VM (1973) *Lygus* flight and dispersal behavior. Environ Entomol 2:361–364

Mueller SC, Summers CG, Goodell PB (2003) A field key to the most common *Lygus* species found in agronomic crops of the central San Joaquin Valley of California. Univ Calif Agric Nat Res Pub 8104:1–12

Rancourt B, Vincent C, De Oliveira D (2000) Circadian activity of *Lygus lineolaris* (Hemiptera: Miridae) and effectiveness of sampling techniques in strawberry fields. J Econ Entomol 93:1160–1166

Scales AL (1968) Female tarnished plant bugs attract males. J Econ Entomol 61:1466–1467

Scott DR (1977) An annotated listing of host plants of *Lygus hesperus* Knight. Bull Entomol Soc Am 23:19–22

Stern VM, Mueller A (1968) Techniques of marking insects with micronized fluorescent dust with especial emphasis on marking millions of *Lygus hesperus* for dispersal studies. J Econ Entomol 61:1232–1237

Strong FE, Sheldahl JA, Hughes PR, Hussein EMK (1970) Reproductive biology of *Lygus hesperus* Knight. Hilgardia 40:105–147

Wardle AR, Borden JH, Pierce HD Jr, Gries R (2003) Volatile compounds released by disturbed and calm adults of the tarnished plant bug, *Lygus lineolaris*. J Chem Ecol 29:931–944

Westfall PH, Tobias RD, Rom D, Wolfinger RD, Hochberg Y (1999) Multiple comparisons and multiple tests using SAS. SAS Institute, Cary

Yasuda T, Shigehisa S, Yuasa K, Okutani-Akamatsu Y, Teramoto N, Watanabe T, Mochizuki F (2008) Sex attractant pheromone of the sorghum plant bug *Stenotus rubrovittatus* (Matsumura) (Heteroptera: Miridae). Appl Entomol Zool 43:219–226

Young OP (1986) Host plants of the tarnished plant bug, *Lygus lineolaris* (Heteroptera: Miridae). Ann Entomol Soc Am 79:747–762

Zhang QH, Aldrich JR (2003) Male-produced anti-sex pheromone in a plant bug. Naturwissenschaften 90:505–508