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Attractiveness of Harlequin Bug, *Murgantia histrionica*, Aggregation Pheromone: Field Response to Isomers, Ratios, and Dose

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Abstract A two-component pheromone, (3*S*,6*S*,7*R*,10*S*)- and (3*S*,6*S*,7*R*,10*R*)-10,11-epoxy-1-bisabolen-3-ol (murgantiol), present in emissions from adult male harlequin bugs, *Murgantia histrionica*, is most attractive in field bioassays to adults and nymphs in the naturally occurring ratio of ca. 1.4:1. Each of the two individual synthetic stereoisomers is highly attractive to male and female adults and nymphs, but is more attractive in combination and when deployed with a harlequin bug host plant. Blends of 8 stereoisomers also are highly attractive, suggesting that isomers not found in the natural pheromone are not repellent. Deployment of an inexpensive non-stereospecific synthetic pheromone holds promise for efficient trapping and/or use in trap-crops for this important pest in North America.

Mention of commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

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Keywords Murgantiol · Trap plant · Pheromone trap · (3*S*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol, (3*S*,6*S*,7*R*,10*R*)-10,11-epoxy-1-bisabolen-3-ol · (1*S*,4*S*)-4-((*R*)-4-((*S*)-3,3-dimethyloxiran-2-yl)butan-2-yl)-1-methylcyclohex-2-enol · (1*S*,4*S*)-4-((*R*)-4-((*R*)-3,3-dimethyloxiran-2-yl)butan-2-yl)-1-methylcyclohex-2-enol, Hemiptera, Pentatomidae, insect pest, pest management

Introduction

The harlequin bug, *Murgantia histrionica* (Hahn) (Hemiptera: Pentatomidae), has been a serious pest of cole crops (*Brassica* spp.) in the southern USA for about 150 years, and also infests other plants in the Brassicaceae (mustard family) and Capparidaceae (caper family) (Aldrich et al. 1996; Chittenden 1920; Wallingford et al. 2011). Zahn et al. (2008, 2012) showed that an aggregation pheromone, emitted by adult males feeding on a plant host, was attractive to both sexes of adults in the laboratory, and concluded that the pheromone was a single isomer of 10,11-epoxy-1-bisabolen-3-ol [= epoxyalcohol 4-[3-(3,3-dimethyloxiran-2-yl)-1-methylpropyl]-1-methylcyclohex-2-en-1-ol], dubbed murgantiol.

Khrimian et al. (2014b) constructed a synthetic library of stereoisomers of 10,11-epoxy-1-bisabolen-3-ol to establish the identity of isomers constituting the aggregation pheromone produced by adult male brown marmorated stink bugs, *Halyomorpha halys* (Stål). In a companion paper, expanding on the use of this synthetic stereoisomer library, Khrimian et al. 2014a showed that (3*S*,6*S*,7*R*,10*S*)- and (3*S*,6*S*,7*R*,10*R*)-10,11-epoxy-1-bisabolen-3-ol (SSRS and SSRR, respectively) occur in a 1.4:1 ratio in emissions of adult male harlequin

bugs. In this study, we examined the attractiveness of these and related stereoisomers and their combinations, in a series of trap- and plant-based choice experiments, using field trapping of wild, and field-collected-and-released, harlequin bug adults and nymphs.

Methods and Materials

Insects Harlequin bugs were collected by hand from their host plants on gardens and small farms within 80 km of Beltsville, MD, USA (mostly from mixed vegetables in Leonardtown, St. Mary's County, MD, USA), and held under natural photoperiod and temperature of 20-25 °C with potted collard plants to feed upon, until used in releases within 3 weeks of collection. Other traps caught wild harlequin bugs without nearby releases, as noted.

Plants Collard plants (*Brassica oleracea* L., acephala group, cv. Champion or Vates) were grown in 3.8 L pots and deployed in field bioassays, with or without candidate attractants. At the time of field deployment, plants were 20–30 cm tall and approximately 40 cm in breadth.

Synthetic Attractants Individual isomers were prepared according to the methods of Khrimian et al. (2014a, 2014b). Chemical purities of tested stereoisomers were ≥95 %, and stereoisomeric purities were: SSRS 95 % *dr* (diastereomeric ratio; percentage of main stereoisomer to sum of minor stereoisomers); *SSRR* 95 % *dr*; (3*R*,6*R*,7*R*,10*R*)-10,11-epoxy-1-bisabolen-3-ol (RRRR) 79 % *dr*; (3*R*,6*R*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol (RRRS) 91 % *dr*; (3*S*,6*S*,7*S*,10*R*)-10,11-epoxy-1-bisabolen-3-ol (SSSR) 92 % *dr*. Mixed-isomer preparations, containing both *cis*- and *trans*-10,11-epoxy-1-bisabolen-3-ols, were prepared from (7*R*)-4-(6-methylhept-5-en-2-yl)cyclohex-2-enone (Hagiwara et al. 2002) following Zahn et al. (2008). Mixed isomer lure #1 (MIX1), used in all 2012 trials, had a ratio of *cis*- and *trans* stereoisomers of 3:1, and an approximate 1:1 ratio of SSRS to SSRR). The faster eluting fraction from the Zahn et al. (2008) synthesis, containing about equal amounts of four *cis* stereoisomers, and the slower eluting fraction, containing equal amounts of four *trans* stereoisomers, were isolated by flash chromatography and mixed at a 3:1 ratio. Mixed isomer lure #2 (MIX2), used in the June 2013 two-way choice trial with released adults, was a crude mixture of eight stereoisomers of 10,11-epoxy-1-bisabolen-3-ol with 7*R* configurations, prepared analogously to MIX1, but without further purification. The ratio of *cis*- and *trans*- stereoisomers from the reaction was 1:2. As with MIX1, SSRS and SSRR (which are both *cis* isomers) were in an approximate 1:1 ratio. All lures were gray rubber septa (1-F SS 1888 GRY, West Pharmaceutical Services, Lititz, PA, USA) washed in a Soxhlet apparatus with hexane and dried for 12 h before loading with candidate attractants as described in Khrimian et al. (2008). See supplemental information for additional details on lure preparation.

Field Experiments Two types of field release-recapture experiments were undertaken: 2-way choice squares, and 7-way choice squares. For each release, at least 100 adults and/or nymphs were released from the center of a plot with a few detached collard leaves, to simulate rapid host degradation. Release time was in late afternoon to twilight, to discourage immediate flight of adults. Trap plants were checked, and all the bugs on the plant and within 30 cm of it were collected the next day and at least three times during the week, over which time, numbers collected typically dwindled to near zero, consistent with the attracted insects originating from the initial release. Treatments were re-randomized after each inspection.

Two-way choice squares were undertaken, using trap plants baited or not baited with experimental lures (see Supplementary Fig S1), during September and October 2012, with the exception of two trials, respectively, during June and August-September 2013. In 2012, two small fallow release areas (0.1 ha) on BARC North Farm, Beltsville, MD, USA (39°02′00″N 76°55′54″W) were used with 4 randomized blocks of two treatments each, separated by 13 m, arranged at 12 m spacing on each of the 4 edges (roughly N, S, E, and W) of each release area (Fig. S2). Trials in 2013 were also on BARC North Farm, with the same layout of two-way choice squares, in a 3.5 ha fallow field, 440 m south of the 2012 release area. The top and lure basket of a green Unitrap (Great Lakes IPM, Vestaburg, MI, USA) was positioned immediately over the top of the plant, using a 1 m tall bendable steel green PVC-coated flower stake (Fig. S1). The pot containing the plant was sunk into a hole into which a second empty pot was nested, and field soil covered the lip of the pot to provide unimpeded access by walking bugs to the stem of the test plant. Test bugs were released at the center of each square. The 2-way choice tests undertaken were as follows:

A. SSRS (2 mg) vs. blank, with 100 nymphs and 100 adults released, 17–24 September 2012;
B. SSRR (2 mg) vs. blank, with 231 adults released, 15–22 October 2012;
C. RRRR (2 mg) vs. blank, with 229 adults released, 15–22 October 2012;
D. RRRS (2 mg) vs. blank, with 200 adults and 100 nymphs released, 7–15 October 2012;
E. MIX2 (31 mg loading of a mix that contained 2 mg SSRS and 2 mg SSRR) vs. blank, with 150 adults released, 5–10 June 2013;
F. MIX1 (10.66 mg loading of a mix that contained 2 mg SSRS and 2 mg SSRR) vs. blank, with 223 nymphs released, 10–14 September 2012; and

G. SSSR (2 mg) vs. blank, with 200 adults and 200 nymphs released, 26 August–3 September 2013.

Seven-way choice squares were employed on the South Farm of Beltsville Agricultural Research Center (BARC), College Park, MD, USA at the edges of a single 1 ha field (39°01′00″N 76°56′30″W; planted with squash in 2012 and sweet corn in 2013), deploying 4 randomized blocks of 7 treatments each, corresponding to the four sides of the field, with the release point in the center of the field, with each trap or trap plant separated by 12 m within the block and ca. 18 m between blocks at the corners (Fig. S2).

To test for effect of different ratios of the two identified pheromone isomers, seven-way choice squares were set out, using trap plants baited with rubber septa lures loaded as described above and in the supplemental information, with experimental isomer blends with 4 mg total of SSRS and SSRR on 2–9 July 2013. The treatments were as follows:

a) All SSRS (4 mg SSRS, 0 mg SSRR).
b) 6:1 (3.43 mg SSRS, 0.57 mg SSRR).
c) 3:1 (3 mg SSRS, 1 mg SSRR).
d) Natural ratio of 1.4:1 (2.33 mg SSRS, 1.67 mg SSRR).
e) 1:1 (2 mg SSRS, 2 mg SSRR).
f) 1:3 (1 mg SSRS, 3 mg SSRR).
g) All SSRR (0 mg SSRS, 4 mg SSRR).

Lures and plants were deployed as in the 2-way choice tests (Fig. S1). Six hundred adult and 600 3rd to 5th instar nymphal field-collected harlequin bugs were released at the center of the sweet corn field at approximately 1700 h on 2 July 2013, and collected from plants on 3, 4, 5, 8, and 9 July.

To compare the responses of harlequin bug to doses of mixed preparations containing pheromone, with and without host plants, we undertook three trapping experiments. Each of them used MIX1, described above. First, large black pyramid traps, described previously for brown marmorated stink bugs (Leskey et al. 2012), were used for field trials on North Farm, Beltsville, MD, with 5 treatments in 4 blocks, from 5 June through 3 July 2012. These traps were spaced ca. 50 m apart along a field edge with unmanaged forest habitat, with the following loadings on rubber septa: 0 mg (blank), 0.1 mg, 1.0 mg, 10 mg, and 100 mg (the 100 mg treatment consisted of 10×10 mg septa hung together by threading them with wire to hang in the trap). Hercon Vaportape II (Hercon Environmental, Emigsville, PA, USA) was added as a killing agent to prevent escape from traps. No harlequin bugs were released; captures were all wild bugs. All adults and nymphs, on or in any part of a trap, were recorded and collected. Traps were collected twice weekly, and re-randomized at each collection, with lures being replaced once after 2 weeks.

Large black pyramid traps also were used in test dose–response tests, with and without the host plant. Four treatments were randomized in each of four blocks: traps were spaced ca. 50 m apart along a field edge with unmanaged forest and hedgerow habitat on North Farm, Beltsville, MD, 21–28 October 2012. The four treatments were 10 mg and 100 mg of MIX1 on rubber septa (the higher dose consisting of 10×10 mg septa hung together as above), with or without a single potted collard plant placed by the base of the trap. No harlequin bugs were released nearby; hence captures were wild bugs. All bugs on or in any part of the trap or plant were recorded and collected. Traps were set up on 21 October, and collected on 24, 25, 26, and 28 October, and re-randomized at each collection.

To determine harlequin bug dose responses with and without plants, mini-pyramid traps (Fig. S1, Great Lakes IPM, Vestaburg, MI, USA) were employed in a 7-way choice square (Fig. S2) on the South Farm of Beltsville Agricultural Research Center (BARC), College Park, Maryland, at the edges of a single 1 ha field planted with squash as described above, from 27 June through 11 July 2012. Tops were modified to connect the provided funnel with a 500 ml Nalgene® translucent polyethylene container, and fitted with fine mesh screen on two sides, and with an opening at the top, into which the Unitrap that held the candidate lure snugly fit. For the appropriate treatments, a single potted collard plant was placed at the foot of the trap (Fig. S1). Treatments used the MIX1 lure described above, as follows:

A) Trap only, blank lure.
B) Trap with collard plant, blank lure.
C) Trap with 3 mg lure.
D) Trap with 10 mg lure.
E) Trap with collard plant and 10 mg lure.
F) Trap with 3×10 mg lures (30 mg total, hung as described above).
G) Trap with 10×10 mg lures (100 mg total, hung as described above).

Field-collected adult harlequin bugs were released at 2100 h on 28 June (800 bugs) and at approximately 1630 h on 9 July (an additional 170 adults). All bugs on or in any part of the trap or plant were recorded and collected. Lures were placed out on 27 June at 1800 h. Traps were collected before the first release on 28 June, on 29 June, and on 2, 5, 9, and 11 July, and were re-randomized at each collection.

Statistical Analysis For two-way choice tests, results were analyzed using binomial statistics (statistical significance with null hypothesis of no preference, i.e., P =0.5, and ratio of captures with 90 % 2-sided confidence intervals; Zar 2009; Pezzullo 2014; Lowry 2014) on totals for each treatment over the entire approximately 1-week sampling period. For the 7-way blend test, we employed an overall χ^2 goodness-of-fit test, followed by six pre-planned orthogonal χ^2 goodness-of-

fit tests contrasting individual treatments and sets of treatments, as follows, based on the null hypothesis of equal numbers captured by each treatment (SAS Institute 1998; Pezzullo 2014; Lowry 2014):

1) Blends (treatments b-f above) vs. pure isomers (treatments a and g).
2) Natural ratio of 1.4:1 (d) vs. off-ratio blends (b, c, e, and f).
3) SSRS-biased blends (b and c) vs. SSRR-biased blends (e and f).
4) Within SSRS-biased blends, more-biased (6:1 ratio, b) vs. less-biased (3:1 ratio, c).
5) Within SSRR-biased blends, more-biased (1:1 ratio, b) vs. less-biased (3:1 ratio, c).
6) Within pure isomers, SSRS (a) vs. SSRR (g).

If any goodness-of-fit test indicated significant (P <0.05) deviation from equal numbers per treatment, we constructed a 90 % binomial confidence interval of the ratio of the more attractive treatment (or group of treatments), based again on the null hypothesis expectation of equal numbers captured by each treatment. Overall patterns in captures of adults vs. nymphs, and captures of males vs. females, were tested for differences using 2×7 χ^2 contingency tests, and, if the overall test were significant (P <0.05), with 6 subsequent 2×2 Fisher's exact tests for group-specific differences, using the same pre-planned contrasts as listed above.

We also performed regression analysis (SAS Institute 1998) for similarity to the natural blend using an index of similarity as follows: similarity for lure x, S_x=[min($SSRS_x$, $SSRS_{natural}$)+min($SSRR_x$, $SSRR_{natural}$)]/4, using the lure loading in mg. Thus, the natural ratio lure had a similarity value of 1.0, and, for instance, the 1:3 SSRS:SSRR lure had a similarity value of $S_{1:3}$=[min(1, 2.33)+min(3, 1.67)]/4=2.67/4=0.67. For dose–response experiments, we employed regression analysis to test for significant y-intercept, linear and nonlinear responses, and differences in slope between treatments, with and without added host plants.

Results

Attractive Isomers In 2-way choice field bioassays, using released bugs and lures deployed with collard plants (Fig. 1), adults and nymphs responded strongly to the two synthetic stereoisomers (SSRS and SSRR) present in the male-emitted volatiles. The SSRS lure attracted ca. 36 % of both nymphs, and adults released, 6-fold more than did the unbaited plants. The SSRR lure attracted ca. 9 % of adults released, 21-fold more than did the unbaited plants. The confidence intervals for the ratio of captures for baited to unbaited plants overlapped for these two isomers, and were highly significant (P <0.001) in all cases.

Unattractive Isomers In contrast, the other closely related stereoisomers, RRRR and RRRS, failed to attract more bugs than did plants with blank lures (Fig. 1). Based on overlap of chromatographic peaks of a fifth synthetic isomer, SSSR, with the pheromone component SSRR (Khrimian et al. 2014a), SSSR also was tested vs. a blank lure in 2013. In this test, a total of 147 adults were collected from the SSSR-baited plants, vs. 157 from the blank plants (binomial test non-significant; P =0.303); male and female response was similar (Fisher's exact test P =0.139). Nymphal totals (182 on SSSR-baited plants vs. 222 on blank plants) indicated a slight, but significant, repellency of the lure (binomial test P =0.026; 1.22×; 90 % c.i.: 1.03, 1.45). Adult and nymphal numbers captured were greater than those released, due to the large numbers of harlequin bug populations in and near the release field in late summer 2013.

Mixed Isomers The 2-way choice tests to measure the attractiveness of mixed isomer preparations MIX1 (for nymphs) and MIX2 (for adults) in separate releases, confirmed the strong attraction of the mixed isomer preparations as shown in Fig. 1.

7-way Test of Isomer Blends In the 7-way choice test of SSRS/SSRR binary blends, 286 adults and 63 nymphs (of the 600 adults and 600 nymphs released) were captured on the 28 trap plants. The overall difference among the isomer blends was highly significant (Goodness of fit χ^2=71.54, df =6, P<0.001; see Fig. 2a). The five blends were more attractive than the two single-component lures (3.00× more attractive; 90 % c.i.: 2.27, 4.04; P <0.001). Bugs were more strongly attracted to the natural ratio of isomers (1.4:1 SSRS:SSRR) than to the four off-ratio blends of SSRS and SSRR (1.60× more attractive; 90 % c.i.: 1.29, 1.98; P <0.001). Single-isomer lures (4 mg of SSRS vs. 4 mg of SSRR) differed in numbers of bugs captured; pure SSRS was 2.15× more attractive than pure SSRR (90 % c.i.: 1.19, 4.03; P =0.014). However, among the off-ratio blends, the two SSRS-biased blends (6:1 and 3:1) did not capture more bugs than the SSRR-biased blends (1:1 and 1:3); neither were there differences between the pairs of SSRS- and SSRR-biased blends. The overall trend was that blends similar to the natural blend, using the similarity index described above, had the greatest total captures. The regression of total captures on similarity to natural blend (Fig. 2b) was highly significant (P=0.001; r^2=0.902).

We tested for a life-stage effect on captures, but found that a 7×2 χ^2 comparison of adult vs. nymphal captures showed no difference (χ^2=4.253; P=0.64) between life stages. However, a similar comparison of male vs. female captures revealed a difference between the sexes of adult bugs in their attraction to

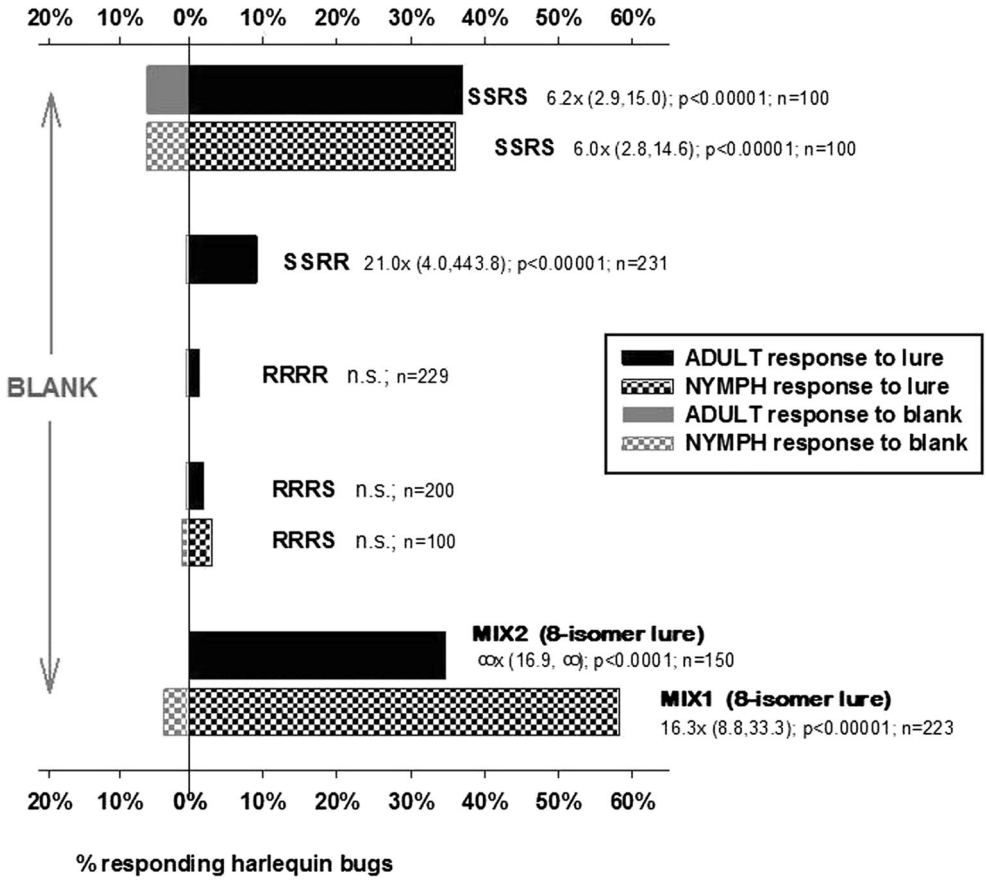

Fig. 1 Responses of adult and nymph harlequin bugs to lures, in 2-way field bioassays with collard plants, containing 2 mg each of individual isomers of 10,11-epoxy-1-bisabolen-3-ol, as noted by stereospecificity at carbons 3,6,7, and 10, respectively, and mixed lures MIX1 and MIX2 (described in text), compared in each case to blank lures. Preferred lure is noted with magnitude of effect, 90 % confidence interval, 1-way binomial *P*-value and *N*= number of bugs released in trial. September-October 2012 (except for June 2013 for adult trial with MIX2 lure), North Farm, Beltsville Agricultural Research Center, Beltsville, MD, USA, using single collard trap plants in 2-way choice arrangement

the seven lures (χ^2=23.69, *df* =6, *P* <0.001). Of the six Fisher's exact tests for blend comparisons, males and females differed only for two: natural vs. biased blends (*P* =0.025), and with pure lures, SSRS vs. SSRR lure (*P* =0.049). In each case, captures of males showed no difference from the null hypothesis (natural=biased blends and SSRS=SSRR), whereas the females showed a preference for natural over biased blends (natural 2.00× more attractive than mean of biased blends; 90 % c.i. 1.45, 2.75; *P* <0.001) and for pure SSRS over pure SSRR (4.50× more attractive; 90 % c.i. 1.71, 14.48; *P* =0.002).

Dose Response to Mixed Isomers±Plant In the dose–response tests, the mixed-isomer preparation attracted both adults in the field, as well as released nymphs and adults. In each case, the dose–response relationship was significant and linear, with a y-intercept not different from zero and, therefore, not included in the model. Harlequin bugs showed a linear dose–response relationship to the mixed isomer lure in field tests with released wild bugs (using mini-pyramid traps, Fig. 3) and wild populations (using large pyramid traps, Fig. 3). For full-size pyramids without the presence of the plants (Fig. 3a), zero bugs were caught in treatments of 0, 0.1, and 1 mg. Treatments with added potted collard plants (Fig. 3b, c) were more attractive than treatments using traps only.

Discussion

The two attractive isomers (SSRS and SSRR) in the 2-way choice tests with single isomers vs. blanks were the same as those identified by Khrimian et al. (2014a) in male emissions. Isomers tested that were not present in the male emissions (RRRR, RRRS, and SSSR) were not attractive. Nymphal and adult responses to SSRS and RRRS were similar when adults and nymphs were released simultaneously. Although 2-way tests for the two mixed isomer preparations (MIX1 and MIX2) were separate tests, and also not conducted simultaneously with other choice tests, the magnitude of preference, as indicated by the confidence interval of the ratio of captures for baited to unbaited trap plants, was very high. This indicates comparable attractiveness to the single isomers tested, and suggests that non-active components of this synthetic mixture were unlikely to be repellent. Mixed isomer preparations are much easier and less expensive to synthesize than are single isomers, providing a strong incentive for their use in the practice of pest monitoring and management.

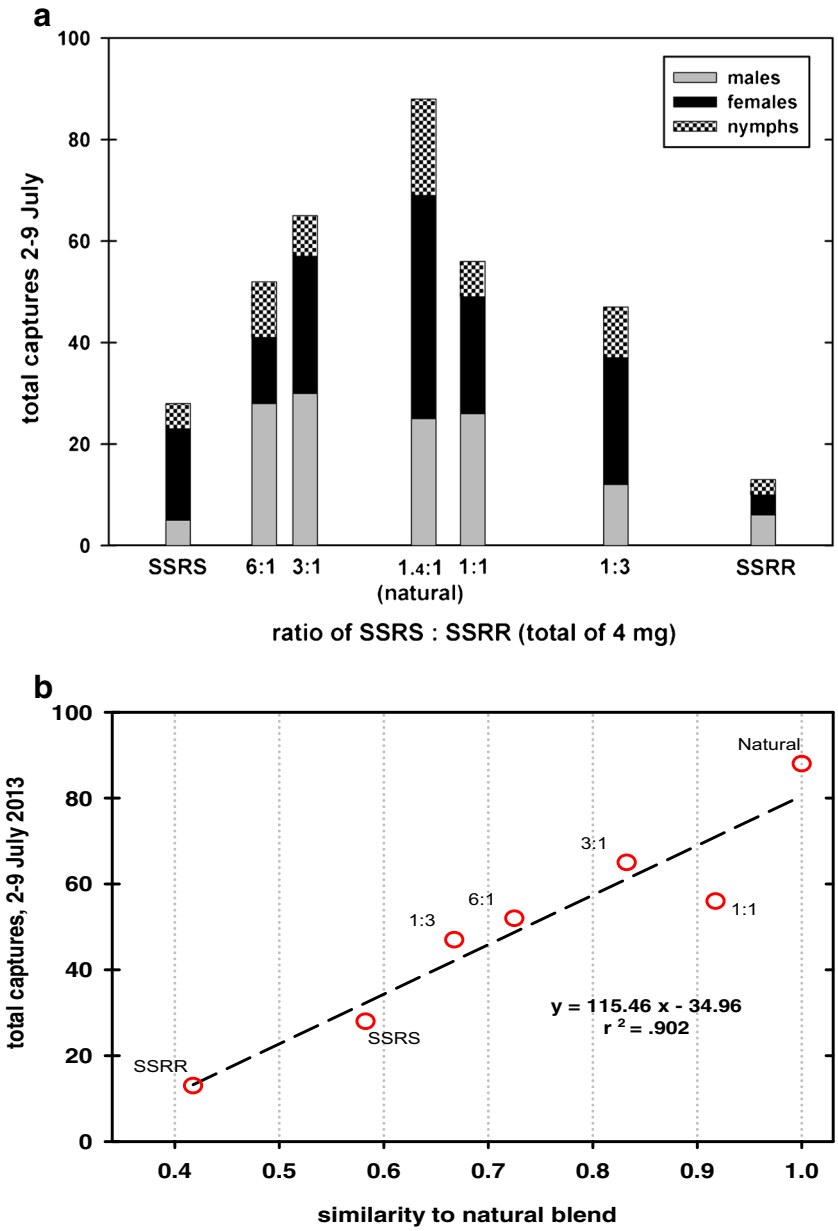

Fig. 2 **a** Responses of adult male and female harlequin bugs and nymphs to lures with 4 mg total of (3*S*,6*S*,7*R*,10*S*)- and (3*S*,6*S*,7*R*,10*R*)-10,11-epoxy-1-bisabolen-3-ol, denoted as SSRS and SSRR respectively. All lures stationed above a single collard plant in 7-way choice arrangement. Totals for all four plants in each treatment, 2–9 July 2013, South Farm, Beltsville Agricultural Research Center, College Park, MD, USA; **b** Total captures (adults plus nymphs) vs. similarity of lure ratio to natural ratio (see text for explanation)

This is the second species of stink bug (Hemiptera: Pentatomidae), after *Halyomorpha halys* (Stål), for which sesquiterpene pheromone components have been identified using chemical and behavioral comparison with synthetic chemical libraries (Khrimian et al. 2014a, 2014b). In combination with analysis of volatiles and appropriate bioassays, use of synthetic chemical libraries in conjunction with appropriate gas chromatographic enantioselective columns is proving a powerful technique for rapid stereochemical characterization of stink bug aggregation pheromones.

Male-produced pheromones of stink bugs are known in some instances to attract only females (sex pheromones) and in other cases to attract both sexes (aggregation pheromones) (Millar 2005). Attractiveness of the pheromone to both sexes does not preclude different responses of the two sexes to different pheromone blends, or to combinations with other volatiles, such as those released by host plants, which Wallingford (2012) found more attractive to male harlequin bugs. In the case of harlequin bug aggregation pheromone, different ratios of the two aggregation pheromone components may evoke a different response in males and females. For females, we found that the natural 1.4:1 ratio of SSRS:SSRR isomers attracted more than the mean of the four other SSRS:SSRR blends; but this was not the case for males. However, our female bugs were of unknown age and probably all mated as they were released en masse with males. Since we did not test for effect of age or mating status, which may affect the response to pheromone components and other olfactory cues,

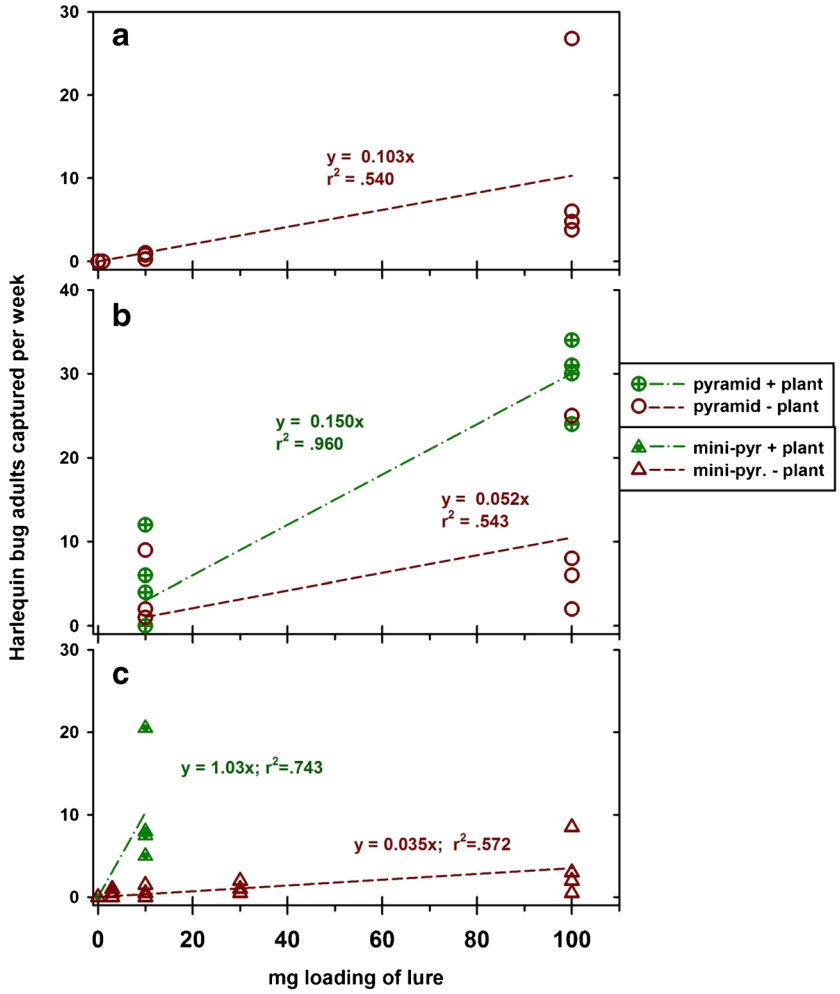

Fig. 3 **a** Dose–response plots of adult harlequin bugs to MIX1 lure using full-size pyramid trap without collard plant at 0 (blank), 0.1, 1, 10, and 100 mg loading. Y-intercept, not significantly different from zero, is excluded from the model. Total counts per week for a total of 4 weeks include those insects in or on traps. North Farm, Beltsville Agricultural Research Center, Beltsville, MD, USA, 5 June through 3 July 2012. **b** Dose–response plots of adult harlequin bugs to MIX1 lure using full-size pyramid trap with and without collard plant. Total counts include those insects in or on trap and plant and pot. Y-intercept, not significantly different from zero, is excluded from the model. 21–28 October 2012, North Farm, Beltsville Agricultural Research Center, Beltsville, MD. **c** Dose–response plots of adult harlequin bugs to MIX1 lure in mini-pyramid trap with and without collard plant. Total counts per week for a total of 2 weeks include those insects in or on trap and plant and pot. Y-intercept, not significantly different from zero, is excluded from the model. 27 June through 11 July 2012, South Farm, Beltsville Agricultural Research Center, College Park, MD, using trap and plant in 7-way choice arrangement

we cannot conclude without further experiments, if indeed male and female harlequin bugs respond differently to these stimuli.

We found that nymphs behaved comparably to adults in attraction to pheromone components. Nymphal attraction also has been demonstrated in response to aggregation pheromone of brown marmorated stink bug, *Halyomorpha halys* (Weber et al. 2014), the predatory stink bug, *Podisus maculiventris* (Sant'Ana et al. 1997), at least three species of *Euschistus* (Aldrich et al. 1991), as well as the alydid bug *Riptortus pedestris* (=*clavatus*) (Leal et al. 1995). Presumably, starved nymphs use the pheromone to assist location of host plants. Late-instar nymphs also find potential mates at plants occupied by conspecifics, as is the case, for instance in immature male mealybug response to female sex pheromone (Mendel et al. 2012). The nymphal numbers recovered in the blend trial precluded examination of the subtleties of their response to isomer ratios. From a pest management perspective, movement of nymphs may be less important than that of adults; however, we have observed mass movement of nymphs in the field following depletion of host plant patches, migrations which could be significant over short distances in agroecosystems.

Trap plants have been used by several stink bug researchers, including Krupke et al. (2001, 2006), who found that the stink bug *Euschistus conspersus* was attracted in greater adult numbers to herbaceous host plants *Verbascum thapsus* (adjacent to tree fruit orchards in Washington state,

USA) baited with its aggregation pheromone vs. similar unbaited plants. However, bugs would not enter traps containing these lures. James et al. (1996) had similar results with *Biprorulus bibax* in Australian citrus orchards. Various explanations may account for the reluctance of bugs to enter baited traps, although neither we nor those authors examined trap designs in detail. However, our results for harlequin bug and the pyramid traps used do not support any sort of repellency of high pheromone doses based on concentrations in the trap [hypothesized by James et al. (1996) for *B. bibax*], since our highest doses consistently lured the most bugs to traps with, or without, accompanying host plants.

Presence of a single potted collard plant exerted a strong positive effect on trap/plant captures. For mini-pyramid traps, the response was 29.4× stronger with the plant than without (ratio of slopes). For full-size pyramids, which present a ca. 5× larger silhouette, the plant effect was still significant, although weaker (2.8x ratio of slopes). This distinction in plant effect with size of trap probably reflects the stronger visual stimulus presented by the large pyramid trap, and is consistent with the importance of both olfactory and visual stimuli provided by potential host plants. Other factors, not included in our study, such as the post-colonization communication by substrate vibration, which this species is known to undertake (Čokl et al. 2004), or the complexities of stink bug mating behavior and diel periodicity of movement (Krupke et al. 2006, 2011), could affect the responses and trap catches of bugs. Furthermore, although our released bugs had a known history after initial capture, we did not know their prior feeding history, age, or other attributes, nor did we know these for the wild bugs caught in trials. Wild hosts are important in stink bug life history, including harlequin bug (Panizzi 1997; Wallingford et al. 2011). Harlequin bugs have distinct preferences among their plant hosts (Chittenden 1920; Ludwig and Kok 1998; Wallingford et al. 2013; and others), and individual bug feeding histories can influence their subsequent behavior (Helmey-Hartman and Miller 2014).

The dynamics of immigration, emigration, feeding, and reproduction, for different population cohorts, as well as the interplay between plant and insect-based stimuli, will be important to consider in the use of aggregation pheromones for management of the harlequin bug. With the availability of an efficient synthesis for pheromone isomer mixtures, as well as the ability to deploy individual isomers, the prospects are improved for pest management by manipulation of harlequin bug behavior.

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Conflicts of Interests Authors declare no conflicts of interest.

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Supplementary Information

Preparation and lure loading of precise stereoisomer ratios

For preparation of the treatment, for instance, containing 2.33 mg SSRS and 1.67 mg SSRR per septum, we weighed 46.6 mg of SSRS and 33.4 mg SSRR (both neat). We transferred these materials with the help of 10 ml hexane into a round-bottom Morton flask containing 20 rubber septa pre-washed with hexane in a Soxhlet apparatus. The flask was rotated on a rotary evaporator (without applying vacuum) until the liquid is completely absorbed. Therefore, each septum contained 46.6/20 = 2.33 mg SSRS and 33.4:20 = 1.67 mg SSRR. We applied that same technique for making the other lures.

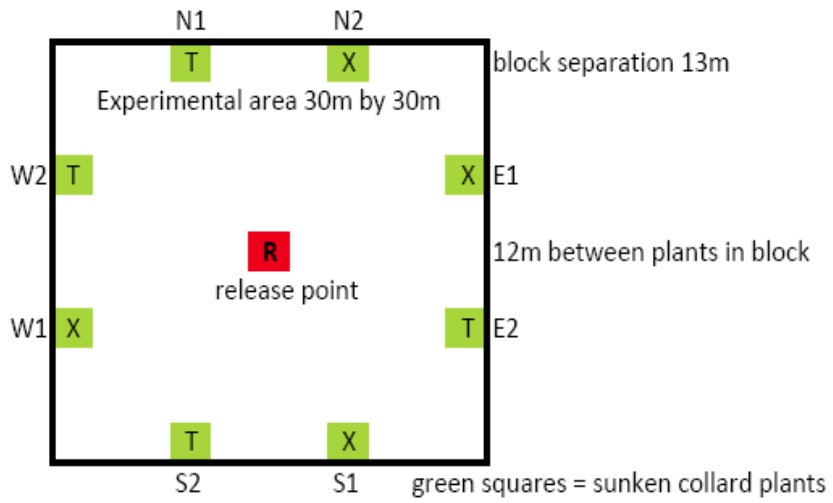
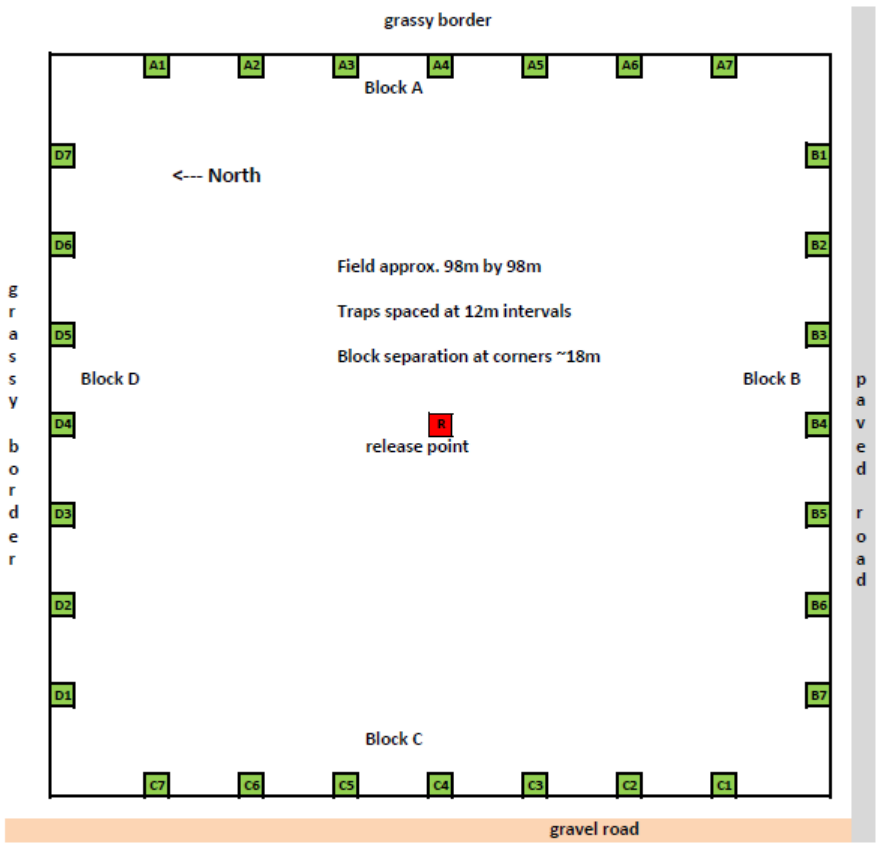
Supplemental Figures

Figure S1 (A) Collard plant with pheromone lure in place, showing Unitrap top with basket containing rubber septum lure underneath the hat.

Figure S1 (B) Minipyramid trap with collard plant.

Figure S2. Experimental release area and trap arrangements:

(A) for 2-way bioassays



(B) for 7-way bioassays.