Combining visual cues and pheromone blends for monitoring and management of the tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae)

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Running title: Visual and olfactory cues of *Lygus lineolaris*

Abstract

BACKGROUND: The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) is considered the most damaging pest of cotton (*Gossypium hirsutum* L.) in the mid-southern United States. Previous studies have reported the role of different ratios of volatile metathoracic gland components such as hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal in eliciting low level attraction of *L. lineolaris*. In this study, we tested different visual cues (colored sticky cards) in combination with olfactory cues (pheromone blends) to optimize the attraction and capture of *L. lineolaris* in the field.

RESULTS: Red-colored sticky cards were more attractive to *L. lineolaris* adults than white, blue or yellow cards. Red sticky cards combined with blends of three potential pheromone components attracted significantly more *L. lineolaris* adults than sticky cards without a blend added. Traps baited with a blend of hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal in 4:10:7 ratio, respectively, caught a significantly higher number of *L. lineolaris* than those baited with 10:4:2 or 7:10:4 blends or an unbaited control in the first week of the experiment.

CONCLUSIONS: Combining visual cues (red color) with olfactory cues (pheromone blends) significantly increased the capture of *L. lineolaris* in the field. This device or a future iteration could contribute towards sustainable and environmentally-appropriate early-season monitoring and management of *L. lineolaris* in the field.

Keywords: *Lygus lineolaris*, pheromones, visual cues, sticky cards, hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-4-oxo-2-hexenal

1. INTRODUCTION

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae), is reported to feed on 700 plant species that belong to 55 families in North America, including over 130 economically-important crops such as cotton, soybeans, tomatoes, apples, grapes etc.[1-3] Compared to other *Lygus species* such as *L. hesperus* (Knight) and *L. elisus* (Van Duzee) that are found in agricultural crops in North America, *L. lineolaris* is the most widely distributed and is reported from Mexico to Alaska.[4] Many conducive factors, including a wide host range, polyphagous feeding behavior, favorable overwintering conditions, and a high rate of development of resistance to conventional pesticides have favored establishment of *L. lineolaris* as a significant pest. In the southern United States, it is particularly important as a pest of cotton (*Gossypium hirsutum* L.) with 6.9 million acres of cotton estimated to be infested by *Lygus species* in 2021.[5]

Before the start of the cotton cropping season in early summer, the first one or two generations of *L. lineolaris* are completed on wild, non-crop plants.[6,7] During the early cropping season, overwintered generations move from wild hosts into cotton or other crops.[7] Feeding damage associated with cotton is mainly sap-feeding by both nymphs and adults on the meristematic tissues of cotton buds ("squares") and small bolls, resulting in square abscission, and death of pinhead squares.[8] Preventing damage to the first fruiting position during the pre-bloom period is critically important to maximizing cotton yield.[9] Management of *L. lineolaris* is heavily dependent on synthetic insecticides including organophosphates, pyrethroids, and carbamates, insect growth regulators such as novaluron, and newer insecticides such as sulfoximines and neonicotinoids that have both contact and systemic activity.[10] *Lygus lineolaris*

has developed resistance to many of these insecticide classes with different modes of action which necessitates further research for developing new control strategies.

Considering this pest's wide host range, seasonal distribution and movement among different host plants and wild hosts, and overwintering behaviors, semiochemical based pest management strategies using insect sex pheromones and other behavior-modifying chemicals could be important in developing early season monitoring tools, mass trapping and mating disruption strategies. Both females and males of *L. lineolaris* produce chemical compounds in the metathoracic gland (MTG) including hexyl butyrate (HB), (*E*)-2-hexenyl butyrate (E2HB) and (*E*)-4-oxo-2-hexenal (E4OH).[11-14] Wardle et al.[14] found these compounds and their blends tended to be repellent in static-air laboratory bioassays. Although these compounds elicited behavioral and antennal responses from both sexes of *L. lineolaris,* experiments failed to attract either sex in the field.[15]

Byers et al.[16] reported that the ratios of HB, E2HB and E4OH produced by females of *L. lineolaris*, *L. elisus*, and *L. hesperus* varied among species. These authors[16] also demonstrated the attraction of conspecific males to the corresponding blend in field trapping tests and suggested the blend differences contribute to the prevention of cross mating among these species. Fountain et al.[17] reported that females of four European *Lygus* species also produce specific blends of these three compounds, although there was some cross-attraction in field trapping tests. They also developed dispensers for these compounds, which released them in essentially the same ratios as in the blends loaded.[17] These were used by Parys and Hall[18] to test different blends in combination with white sticky cards under field conditions to attract *L. lineolaris.* A lure loaded with HB, E2HB and E4OH in a ratio of 4:10:7 was most effective in trapping *L. lineolaris*. The number of *L. lineolaris* collected using this blend was only 2.5 insects/week,

82 mainly males, but was similar to the number of *L. lineolaris* caught on traps baited with virgin
83 female insects.

84 Behavioral responses to visual cues are also critical in the host finding and feeding
85 behavior of insects.[19] Identification and optimization of color cues is important in the design and
86 development of efficient traps that combine visual and olfactory cues.[19] Prokopy et al.[20] reported
87 that darker colors were less attractive to *L. lineolaris* compared with non-UV reflecting white,
88 yellow, and clear painted plexiglass rectangles. In another study by Legrand and Los[21], more *L.*
89 *lineolaris* were caught on pink sticky traps than white traps. Trap design,[22,23] height, placement,
90 and time of the day[23,24] also influence *L. lineolaris* trap capture.

91 In this study, we investigated the effects of trap color and addition of lures containing
92 blends of pheromone components on catches of adult *L. lineolaris* on sticky traps, and the
93 interaction of these visual and olfactory cues. Different colored sticky cards were tested in cotton
94 fields to determine their efficacy in attracting *L. lineolaris*. The most attractive color was
95 selected and tested in combination with different pheromone blends under field conditions.
96 Pheromone blend that attracted the most number of *L. lineoalris* was also tested with different
97 trap colors to confirm there was no unexpected interaction of color and olfactory cues, in order to
98 provide an optimised trap and lure for monitoring *L. lineolaris*.

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2. MATERIALS AND METHODS

2.1 Measurement of reflectance from sticky cards

Spectrophotometer readings were carried out in the laboratory following the protocol of Chittka and Kevan,[25] using an Avantes AvaSpec-2048 spectrophotometer and an AvaLight-DH-S-BAL Deuterium-Halogen light source (Avantes, Leatherhead, Surrey, UK), calibrated relative to a white standard (Avantes WS-2). Measurements were taken with a fine probe 155 (FCR7UV200-2-1.5 x 100) inside a light shade box that excluded ambient light and held the probe at 45° angle to the stimulus surface. A minimum of three measurements was taken per sample. Spectra were processed by Avantes AvaSoft 8 software to produce spectra of percentage reflectance of the incident light with 1 nm increments between 300 and 700 nm, the normal visual sensitivity range of insects, and imported into MS Excel for processing.

2.2 Collection and analysis of volatiles from *Lygus lineolaris*

For collection of volatiles, eggs of *L. lineolaris* laid on green beans were sent by courier from the US to the UK where they were reared through to adults on green beans under 12h:12h L:D conditions with temperatures 25 °C and 20 °C, respectively, and 50% relative humidity. Fifth instar nymphs were separated by sex and reared to adults in individual petri dishes (9 cm dia). Using procedures identical to those described in Fountain et al.[17] volatiles were collected from undisturbed, individual, virgin female or male *L. lineolaris* adults at 3-7 d old for 24-h periods under the same conditions as those used for rearing. Collections were analyzed by gas chromatography (GC) with flame ionization detection (FID), GC coupled to mass spectrometry and GC coupled to electroantennographic (EAG) recording from the antenna of a male insect. As

with other *Lygus* species,[17] the main female-specific, EAG-active components were hexyl butyrate (HB), (*E*)-2-hexenyl butyrate (E2HB), and (*E*)-4-oxo-2-hexenal (E4OH). Collections were made from *L. lineolaris* adults reared on green beans from eggs from the Stoneville laboratory culture in May 2016, adults reared from eggs laid by adults collected in the field at Stoneville in July 2016, adults from another batch eggs from the Stoneville laboratory culture in October 2016, and adults from eggs from adults collected in the field at the USDA Arid Land Agricultural Research Center, Maricopa, AZ in May 2017.

2.3 Preparation of lures and measurement of longevity under laboratory conditions

Three candidate pheromone blends were formulated in pipette-tip dispensers, which were reported to provide sustained release of these compounds in a ratio similar to those loaded in the dispenser.[17] Two of these were based on results obtained above from adult female *L. lineolaris* of Stoneville origin containing HB, E2HB, and E4OH in 100 : 40 : 20 ratio, respectively, and those of Maricopa origin in 70 : 100 : 40 ratio. Based at Maricopa, Byers et al.[16] reported an optimum lure for *L. lineolaris* releasing HB, E2HB, and E4OH at 1.2, 3.0 and 2.0 µg/h, respectively, and a third blend was prepared containing these compounds in 40 : 100 : 70 ratio, respectively.

HB and E2HB were obtained from Sigma-Aldrich (Gillingham, Dorset, UK) and were >99% pure. E4OH was prepared at the Natural Resources Institute as described by Fountain et al.[17] The blends were formulated in sunflower oil with the major component at 10%. 4-Methyl-2,6-di-*tert*-butylphenol (BHT; 10% of major component) and Waxoline Black (1% of major component) were added as antioxidant and UV screener, respectively. The blend (100 µl) was then formulated on cigarette filters in polypropylene disposable pipette tips (1 mL; Fisher

145 Scientific, Loughborough, UK) which were sealed with a crimp seal and wrapped in duct tape to
146 exclude light, leaving the small end of the pipette open. Lures were packed in sealed aluminum
147 foil bags and stored in a refrigerator (4 ºC) before use.

148 Release rates from the three blends were measured as described by Fountain et al. [17]
149 Dispensers were maintained in a laboratory wind tunnel at 27 °C and 8 km/h windspeed. At
150 intervals, volatiles were collected by passing charcoal-filtered air (2 L min^{-1}) over single lures
151 held in a glass vessel (10 cm x 3 cm dia) and trapping volatiles on filters containing Porapak Q
152 (200 mg; 50-80 mesh; SigmaAldrich). Trapped volatiles were eluted from the filters with
153 dichloromethane (1 mL) and analysed by GC-FID as for collections from live insects against an
154 internal standard of decyl acetate (2 µg). Collections were carried out for 3 h at 27 °C and results
155 are the means of two replicates.

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157 **2.4 General field experiment procedures**

158 Four field experiments were performed using different colored sticky cards and pheromone
159 blends during 2021 and 2022 to study the visual and olfactory cues used by *L. lineolaris*. The
160 experimental site was in the United States Department of Agriculture (USDA) research farm,
161 Southern Insect Management Research Unit, located in Stoneville, MS. The cotton field (5 acres)
162 was planted with different cotton varieties, and our experiments were performed during the
163 flowering and squaring stage of Bollguard II cotton (DeltaPine 1646 B2XF).[1] This cotton variety
164 is one of the most planted cotton varieties in the mid-southern United States. Sixteen rows of
165 cotton (285 m long and 60 cm between rows) were used for the experiments. Insecticidal

applications were avoided in order to have a high population of *L. lineolaris* in the experimental plots.

Sticky cards were tied to a 30 cm x 2 mm-thick vinyl coated cable (Lowe's Inc. Mooresville, NC, USA) attached to a 104 or 160 cm steel-painted metal traditional shepherd's hook (LG Sourcing, Inc., North Wilkesboro, NC, USA) using gorilla black duct tape (Gorilla Glue Company, Cincinnati, OH), dependent on the plant's height (Fig. 1A). The shepherd's hook was used to keep the sticky cards 15 cm above the plant canopy and prevented contact with the plant. This setup allowed easy visibility and free movement of the sticky cards in the wind and prevented the cards from damage under windy and rainy conditions. Where used, pheromone lures were attached horizontally to the center of the double-sided red sticky card using a small metal wire, with the tip pointing away from the card (Fig. 1B).

The species and sex of the captured *L. lineolaris* were determined based on the morphological characteristics of the abdomen. The female has a groove that begins at the bottom and rises to the middle of her abdomen as the ovipositor lies in the center, almost hidden. This groove is absent for males, and the abdomen is tapered at the end.[26-28] Cotton plants were sampled for *L. lineolaris* before and during the experiments. Forty plants (10 plants/row) were randomly sampled by visual inspection of fruiting structures for *Lygus* adults and nymphs in four rows of the experiment plots.

2.5 Comparison of catches of *Lygus lineolaris* on different colored sticky cards

A previous study[18] reported the attraction of *L. lineolaris* to pheromone blends using non-UV white sticky cards. This experiment compared the attraction to red, blue, white, and yellow sticky

cards. The double-sided sticky cards (25 cm x 11.25 cm) were purchased from commercial vendors: red (Pherocon SWD trap; Trécé Inc., Adair, OK), non-UV white (Great Lakes IPM, Inc., Vestaburg, MI), blue and yellow (Alpha Scents Inc., West Linn, OR). All these sticky cards have a hot melt glue and are used in monitoring and trapping of different insects. The sticky cards were arranged 30 m apart in the same row and 2.4 m apart between rows in a randomized complete block design (1 July 2021). Each treatment was replicated six times. On day 7, each sticky card was collected and placed inside a labeled Ziploc bag, and the number of *L. lineolaris* was counted and recorded in the laboratory. The male: female ratio of the insects was determined for all the *L. lineolaris* trapped on three out of six replications of each card color used. The plants were visually inspected on day 3 during the 7-day experiment. Data were analyzed by ANOVA followed by Tukey's HSD for means separation using the JMP statistical program (SAS, Cary, NC, USA).

2.6 Comparison of catches of *Lygus lineolaris* on red sticky cards with candidate pheromone lures in paired test

As the red sticky card alone was highly attractive to *L. lineolaris* in the previous field experiment, the synergistic effect of attaching pheromone lures (olfactory cue) to the red sticky cards (visual cue) in increasing *L. lineolaris* capture in the field was evaluated. The experiment was performed as paired test in which each baited card was paired with an unbaited sticky card control with an empty 1 mL pipette tip placed 90 cm away. The treatment pairs (Byers blend vs. control, Stoneville blend vs. control, and Maricopa blend vs. control) were arranged 30 m apart in a completely randomized design in the cotton field, with six replications for each treatment.

After deployment (15 August 2021), *L. lineolaris* trapped on the paired sticky card treatments were counted at 2, 5, and 7 days and the male: female ratio was recorded. Visual sampling of the cotton plants was performed on day 3 of the experiment. Data were analyzed by paired *t*-test using JMP statistical program (SAS, Cary, NC, USA).

2.7 Comparison of catches of *Lygus lineolaris* on red sticky cards baited with candidate pheromone blends

The paired experiment showed a significantly higher attraction of *L. lineolaris* towards all three different pheromone blends than the paired, unbaited controls. Using a randomized complete block design experiment, we evaluated the attraction of *Lygus* to these lures during the peak *L. lineolaris* activity in cotton to identify which blend ratio attracted the highest number of *L. lineolaris* and to investigate the activity of these lures under field conditions. Treatments were the three pheromone blends (Byers, Stoneville, and Maricopa) attached to the red sticky cards and the unbaited, red sticky card control. The treatments were arranged 30 m apart in the same row and were replicated six times in four rows in a randomized complete block design. Starting 28 August 2021, the number of *L. lineolaris* captured on the sticky cards was monitored weekly for six weeks. The red sticky cards were replaced every week, and the same attractant lure was used for 6 weeks to study the residual activity of these lures. The numbers of *L. lineolaris* caught on the sticky cards were counted, and a subsample was stored in the refrigerator before determining the male: female ratios. Visual sampling of the cotton plants was performed weekly during the 6-week duration of the experiment. Overall mean differences in *L. lineolaris* catch between treatments were analyzed by ANOVA followed by Tukey's HSD, using JMP statistical program (SAS, Cary, NC, USA).

2.8 Comparison of catches of *Lygus lineolaris* on different colored sticky cards baited with the Byers pheromone blend

Previous experiments showed that the Byers blend was the most attractive blend for *L. lineolaris* adults when added to the red colored sticky cards. During the second year, another field experiment (10 August 2022) was done to investigate attraction of *L. lineolaris* towards different colored sticky cards baited with the Byers blend. The red, white, blue and yellow double-sided sticky cards were used as in the first experiment. The different colored sticky cards, with or without Byers blend lures, were arranged 30 m apart in the same row and 6 m apart between rows in a randomized complete block design. Each treatment was replicated five times. Sticky cards were monitored for the number of *L. lineolaris* captured every 7 days after the start of the experiment for four weeks, and the same lure was used throughout the study. Visual sampling of the cotton plants for the number of *L. lineolaris* adults was performed weekly during the 4-week duration of the experiment. Overall mean differences in *L. lineolaris* catch between treatments were analyzed by ANOVA followed by Tukey's HSD, using JMP statistical program (SAS, Cary, NC, USA).

3. RESULTS

3.1 Measurement of reflectance from sticky cards

The percentage reflectances of the incident light between wavelengths of 300 nm and 700 nm for the four different colored traps used in this study were measured in order to define the colors objectively, and are shown in Fig. 2. The white trap reflected across the spectral range while the

peak reflectances of blue (420-500 nm) and yellow (545-700 nm) were at shorter wavelengths than that of the red sticky card (600-700 nm). The total reflectance for each color measured under the same incident light is a measure of the relative brightness of the card, and relative to white (1.00), the brightness of blue, yellow and red cards was 0.41, 0.41 and 0.17, respectively.

3.2 Collection and analysis of volatiles from *Lygus lineolaris*

The relative amounts of hexyl butyrate (HB), (*E*)-2-hexenyl butyrate (E2HB) and (*E*)-4-oxo-2-hexenal (E4OH) in volatiles collected from individual, virgin female *L. lineolaris* adults are shown in Table 1. The blends produced by insects reared from eggs of Stoneville origin, either from a laboratory culture or from the field, were similar with HB as the major component, E2HB at 30-40% and E4OH at 15-28%. However, the blend produced by females reared from eggs from Maricopa was markedly different, with E2HB as major component and a higher relative amount of E4OH. This blend was more similar to that reported for *L. lineolaris* by Byers et al.[16] who were based at Maricopa and reported an optimum lure for *L. lineolaris* releasing HB, E2HB, and E4OH at 1.2, 3.0 and 2.0 µg hr^{-1}. Collections of volatiles from virgin male *L. lineolaris* under the same conditions, using individual, undisturbed adults, did not show detectable amounts of any of the three candidate pheromone components.

3.3 Blends produced by lures and longevity under laboratory conditions

Three blends of the synthetic pheromone components proposed for *L. lineolaris* were prepared based on the results above: a Stoneville blend containing HB, E2HB and E4OH in 100 : 40 : 20 ratio, respectively; a Maricopa blend with the three compounds in 70 : 100 : 40 ratio; and a Byers

277 blend containing the three compounds in 40 : 100 : 70 ratio (Table 1). These were formulated in
278 pipette tip dispensers as 10% solutions in sunflower oil with 10 mg of the major component for
279 use in field tests. The longevity of the lures was determined in a laboratory windtunnel
280 maintained at 27 °C and 8 km h^{-1} windspeed. Results in Supplementary Fig. S1 show that, for all
281 three blends, all three components were released for at least 12 weeks in the low microgram/hr
282 range, although the compositions of the blends released varied over this time (Supplementary
283 Fig. S2). Thus the relative amounts of HB, E2HB and E4OH released (HB = 100) after 1 week
284 and 6 weeks were: Stoneville blend 100 : 37 : 43 and 100 : 37 :16; Maricopa blend 100 : 105 :
285 120 and 100 : 131 : 41; Byers blend 100 : 165 : 223 and 100 : 202 : 110, respectively.

286

287 **3.4 Comparison of catches of *Lygus lineolaris* on different colored sticky cards**

288 Significantly higher numbers of *L. lineolaris* were trapped on red sticky cards compared to
289 yellow, blue, and white sticky cards after 7 days, and numbers caught on the latter three colors
290 were similar ($F_{3,23} = 10.8$; $P < 0.001$, $n = 6$) (Fig. 3). In the visual sampling, the mean number of
291 *L. lineolaris* adults (0.13 $\pm$ 0.04 per plant) and nymphs (0.1 $\pm$ 0.03 per plant) showed the
292 presence of a good population in the cotton field where the experiments were performed. The
293 majority of *L. lineolaris* collected on the sticky cards were males, and the male: female ratio was
294 9:1 for all the insects trapped on the different colored sticky cards. The insects collected on red
295 sticky cards also had a similar 9:1 male: female ratio. Large numbers of dipteran flies were
296 caught on blue and yellow sticky cards, but the numbers on red sticky cards were lower by visual
297 inspection (Fig. 1B).

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3.5 Comparison of catches of *Lygus lineolaris* on red sticky cards with candidate pheromone lures in paired test

Significantly higher numbers of *L. lineolaris* were attracted to red sticky cards baited with pheromone blends, compared with unbaited controls ($P < 0.01$) (Fig. 4). In the paired experiment with the Byers blend, significantly more *L. lineolaris* were trapped on the red sticky card with the lure than on unbaited controls on day 2 ($t = 9.32$, $n = 6$, $P < 0.0001$), day 5 ($t = 16.12$, $P < 0.0001$), and day 7 ($t = 12.04$, $n = 6$, $P < 0.0001$) (Fig. 4A). Similarly, a significantly greater number of *L. lineolaris* were trapped on Stoneville blend on day 2 ($t = 8.97$, $n = 6$, $P < 0.0001$), day 5 ($t = 11.63$, $P < 0.0001$), and day 7 ($t = 12.04$, $n = 6$, $P < 0.001$) (Fig. 4B), and Maricopa blend on day 2 ($t = 12.97$, $n = 6$, $P < 0.0001$), day 5 ($t = 14.32$, $P < 0.0001$), and day 7 ($t = 8.7$, $n = 6$, $P < 0.0001$) (Fig. 4C) than unbaited controls. No significant differences were observed in the number of insects trapped between the different attractant blends ($P = 0.22$, $n = 6$).

The mean numbers of *L. lineolaris* found in the visual sampling of cotton plants for adults (0.23 ± 0.04 per plant) and nymphs (0.3 ± 0.03 per plant) indicated a good field population. The male: female ratio of *L. lineolaris* collected on the sticky cards baited with the Byers blend was 98% males and 2 % females, whereas on the control cards were 94% males and 6% females. The male: female ratio was similar to Byers blend for other blends and their paired controls after 7 days.

3.6 Comparison of catches of *Lygus lineolaris* on red sticky cards baited with candidate pheromone blends

Red sticky cards baited with all three blends attracted more *Lygus* than the unbaited control sticky cards throughout the six weeks of the experiment (Table 2). After the first week, significantly more *L. lineolaris* were trapped on the cards baited with the Byers blend than on those baited with the Maricopa or Stoneville blends, although catches on cards baited with these blends were significantly greater than those on the unbaited red sticky card controls ($F_{3,23}$ = 107.1*;* $P < 0.0001$*,* $n = 6$) (Table 2). No significant differences were observed in the weekly trap catch between the different attractant blends during weeks 2, 4, 5, and 6 (Table 2). During week 3, a significantly higher number of *L. lineolaris* were collected on sticky cards with the Byers and Maricopa blends than with the Stoneville blend (Table 2). Although the numbers of *L. lineolaris* caught on the unbaited cards was similar during each week of the experiment, the numbers caught on the baited cards seemed to decrease markedly after week 4 (Table 2).

The cumulative number of *L. lineolaris* attracted to the baited sticky cards after 6 weeks period was almost 40 times more than the control sticky cards. Overall, the Byers blend (mean per trap ± SE; 317 $\pm$ 18) and Maricopa blend (296 $\pm$ 12) attracted significantly greater number of *Lygus* than Stoneville blend (263 $\pm$ 8) ($F_{2,17}$ = 3.9*;* $P < 0.04$*,* $n = 6$) after 6 weeks. The cumulative number of *Lygus* collected using the Byers blend was significantly higher than the Stoneville blend during all the weeks except week 2. Even though the cumulative numbers were numerically higher for the Byers blend than the Maricopa throughout the experiment, no significant differences were observed.

No significant differences were observed in the male: female ratio of *L. lineolaris* attracted to the different pheromone blends tested. Out of the 1221 *L. lineolaris* adults sampled on the sticky cards from different treatments during the 6-week test period, only 15 were females (1.2%), and the rest, 1206 (98.8%) were males.

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344 **3.7 Comparison of catches of *Lygus lineolaris* on different colored sticky cards baited**
345 **with the Byers pheromone blend**

346 Significantly higher number of *L. lineolaris* were trapped on the red sticky cards baited with
347 lures containing the Byers blend compared to all the other treatments in 4 weeks ($F_{7,39}$ = 202.60*;*
348 $P < 0.001$*,* $n = 5$) (Fig. 5). Both the color and pheromone had a significant effect on trap catch of
349 *L. lineolaris.* In the absence of odor, the red colored traps caught most *L. lineolaris* compared to
350 blue, white and yellow traps, as observed in the first experiment (Figs. 1, 5). Thus addition of the
351 pheromone increased catches eight-fold relative to catches on the unbaited traps for the red and
352 blue traps, seven-fold for the white traps and four-fold for the yellow traps. The mean number of
353 *L. lineolaris* found in the visual sampling of individual cotton plant for adults was 0.22 $\pm$ 0.05
354 per plant, indicating a good sampling population in the field during the study.

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4. DISCUSSION

Colored sticky traps are established tools for early monitoring and mass trapping of pest populations and have been extensively studied in the field and greenhouse for multiple insect orders.[23,29-31] Using color as a visual cue is usually effective from a distance if the trap is large enough to be easily detected by small insect eyes.[32] Previous studies[23,24] showed that *L. lineolaris* were caught on sticky traps during daylight hours, and so it was anticipated that trap color could have an effect on catches. In our first field experiment, we compared catches of *L. lineolaris* adults on four different colored sticky card traps which are commercially-available for monitoring a wide range of insects. The red color was almost sixteen times more attractive than white and nine times more attractive than blue or yellow. This result is not entirely consistent with previous reports. Prokopy et al.[20] reported that darker colors were less attractive to *L. lineolaris* compared with non-UV reflecting white, yellow, and clear painted plexiglass rectangles and Blackmer et al. found no clear differences in numbers of *L. lineolaris* and *L. hesperus* caught on a range of different coloured traps.[23] However, in another study by Legrand and Los[21], more *L. lineolaris* were caught on pink sticky traps than white traps.

Light-emitting diodes (LEDs) have also been used to attract *Lygus* species to traps in the dark, although it is not clear how this compares with attraction to colored cards studied here. Van Tol et al.[33] showed *L. rugulipennis* were attracted to LEDs emitting in the UV-A/violet wavelengths in a laboratory bioassay, whereas more bugs were caught in water traps baited with white LEDs rather than UV-A LEDs in a greenhouse test. Pan et al.[34] reported that green LEDs were most effective in attracting *Apolygus lucorum.*

The colored cards used in our study were defined by their reflectance spectra for objective comparison with other work. These differences in reflectance peaks may explain the

higher orientation of *L. lineolaris* toward red than other colors tested in our experiments. A recent study by Xu et al.[35] found that mirid bugs have long-wavelength sensitive receptors but have lost the medium-wavelength/blue-sensitive ones, indicating that their visual systems are tuned towards detecting longer wavelengths (red, orange, etc.). However, the reflectance spectra of the traps used in our study show that the yellow and white traps have high reflectance at the longer wavelengths as well as the red traps. The overall intensity of reflectance was least for the red traps and the contrast of the "darker" red traps over the bright plant background may be more important than color for their attractiveness. This would suggest the effectiveness of the traps may vary with the crop and this should be investigated before the traps can be used more widely.

Insects use a combination of visual and olfactory cues for host location and mate finding.[34,39] Compared with either olfactory or visual stimuli alone, combining the visual and olfactory cues may augment the accuracy of discriminating between hosts from non-hosts, mates from non-mates and specific parts of host plants, for example. These synergistic responses of insects to host plants and mates using visual, olfactory, and tactile cues have been reported previously.[36-39] In our study, we combined the best visual cue and pheromone blends to increase the attraction and capture of *L. lineolaris*. All the attractant blends trapped significantly more *L. lineolaris* than the paired unbaited red sticky card controls, although it was not clear which blend is most effective for monitoring *L. lineolaris* under field conditions, and which attractant blend has a long residual activity under field conditions. The randomized complete block design experiment comparing the four treatments (three attractant blends and control) clearly showed that Byers blend was more effective in trapping the highest number of *L. lineolaris* than other two blends or the control in the first week, and cumulatively, the Byers blend caught 32% of the total *L. lineolaris* during the first week of the experiment. Also, 75% of all the *L. lineolaris*

403 trapped on the attractant blend sticky cards were collected during the first 3 weeks of the 6-week
404 experiment.

405 The final field experiment comparing catches of *L. lineolaris* on different coloured traps
406 baited with lures containing the Byers pheromone blend confirmed the high attractiveness of the
407 red traps both with and without odor, and that there were no unexpected interactions of color and
408 odor. In this context, it is difficult to define the nature of the interaction as synergistic or not
409 because it is not really possible to measure the number of insects caught by pheromone alone.
410 Rather the interaction is "multiplicative" in that addition of the pheromone increased catches
411 eight-fold relative to catches on the unbaited traps for the red and blue traps, seven-fold for the
412 white traps and four-fold for the yellow traps.

413 With the unbaited traps, catches were overall 90:10 male: female. This male bias has
414 been reported previously for *L. lineolaris*[20,23] and for *L. hesperus*.[23] When the traps were baited
415 with pheromone, this ratio further increased to 98:2 male: female as expected for a female-
416 produced sex pheromone. We did not observe any trends towards a higher female proportion for
417 any of the colors or blends tested.

418 Some studies have reported reduced catch of *L. lineolaris* in traps baited with similar
419 pheromone compounds. Chouinard-Thuly et al.[40] reported the trapping of *L. lineolaris* using
420 sunflower volatiles and pheromone lure on white, non-UV sticky traps in strawberry fields in
421 Quebec, Canada. A significantly lower catch of *L. lineolaris* was observed in traps baited with
422 pheromone + sunflower volatiles than in control traps. Also, *L. lineolaris* responded poorly to
423 sex pheromones in combination with sticky traps. However, neither the composition of the lure
424 nor any release rates of the chemicals used were reported and these are likely to have influenced
425 the results, as are the background colors and odors of strawberries present. The same compounds

426 are involved in defense, mate-finding and aggregation behaviors of mirids, and they play an
427 important role in their intraspecific communication and sexual behavior.[41-43]

428 In this study, dispensers releasing the pheromone components at rates similar to those
429 released from undisturbed, virgin insects were used, and good attraction of male *L. lineolaris*
430 was observed. This confirmed the importance of release rate for these compounds that can have
431 different behavioral effects at different release rates.[17] Nevertheless, it is currently unclear why
432 the composition of the pheromone blend produced by *L. lineolaris* from Stoneville (HB : E2HB :
433 E4OH 100 : 40 : 20) was so different from that produced by *Lygus* of the same species from
434 Maricopa (70 : 100 : 40). The latter agreed more closely with that reported by Byers et al. [16]
435 working at Maricopa (40 : 100 : 70) with E2HB as the major component, whereas HB was the
436 major component in the Stoneville blend.

437 Even more unexpected was the result that in field trapping tests carried out at Stoneville,
438 the Byers and Maricopa blends proved more attractive than the blend produced by *L. lineolaris*
439 from Stoneville. The laboratory release rate studies indicated that the blends released by the
440 pipette-tip dispensers varied with time, although they were broadly consistent with the blends
441 loaded, particularly for the Stoneville blend. The volatiles released from lures loaded with the
442 Byers blend contained higher proportions of E2HB and E4OH relative to HB than those from the
443 other two blends, suggesting this is important for attraction of *L. lineolaris*. Conversely, the
444 proportion of HB in the volatiles released by lures loaded with the Byers blend was lower than
445 that from the other two blends indicating that possibly this component is not necessary.
446 However, in their experiments, Byers et al. [16] found that a two-component lure emitting only
447 E2HB and E4OH was less attractive to *L. lineolaris* than the three-component lure.

Under laboratory conditions, the lures were still releasing significant quantities of all three pheromone components after 12 weeks. In the field, catches of *L. lineolaris* on baited traps were significantly higher than those on unbaited traps for at least six weeks confirming that the lures remained attractive during this time. However, after four weeks catches on the baited traps seemed to be markedly lower than those in previous weeks. Catches on the unbaited traps remained relatively constant during the experiment, suggesting populations of *L. lineolaris* were reasonably stable, so it is probable that the lures were becoming less attractive. This may have been due to the higher temperatures in the field which can reach 36 °C, compared with 27 °C in the laboratory measurements, which would have caused the changes in the composition of the blends released to be more rapid. More detailed comparisons of catches with fresh lures are required to confirm this and determine the useful lifetime of the lures under field conditions.

Further studies are required to understand the extent of geographical and temporal effects on attractiveness of the different blends, and the possible influence of the presence of different species mixes, in order to optimize the pheromone blend for use in monitoring and potentially control of *L. lineolaris.* It would also be useful to increase catches of female bugs on the traps by addition of host-plant volatiles, for example, as has been done for other species of *Lygus*.[33,44,45,46]

5. CONCLUSION

Our experiment results showed that a combination of red sticky cards and the pheromone blends are a useful tool for monitoring and trapping *L. lineolaris* under field conditions. The Byers blend on red sticky cards attracted the highest number of *L. lineolaris* compared to other blends under field conditions. A combination of the red sticky cards with pheromones may have

practical applications to identify and target populations of *L. lineolaris* in early-season natural and cultivated hosts. Future experiments will focus on optimizing the blend ratio that attracted the highest number of *L. lineolaris* in our field experiments. Also, experiments will be designed to test these blends in multistate trials in different cotton-growing areas in the United States. This device or a future iteration based on the visual and olfactory cues can be used for monitoring the early *Lygus* field populations to optimize the more judicious application of insecticides, thereby developing more sustainable *Lygus* management strategies.

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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616
617
618

Tables

Table 1. Relative amounts of hexyl butyrate (HB), (*E*)-2-hexenyl butyrate (E2HB) and (*E*)-4-oxo-2-hexenal (E4OH) in volatiles collected from individual, virgin female *Lygus lineolaris* adults, and relative amounts used in lures.

Source	N	Relative amount (± SE)		
		HB	E2HB	E4OH
Collections from female *Lygus lineolaris*				
Stoneville laboratory (May 2016)	13	100	37.7 ± 1.5	14.8 ± 2.1
Stoneville laboratory (October 2016)	13	100	39.0 ± 1.6	25.5 ± 6.5
Stoneville field (July 2016)	5	100	30.9 ± 2.6	28.3 ± 5.9
Maricopa field (July 2017)	6	100	143.9 ± 19.1	57.7 ± 10.6
Lures				
Stoneville blend		100	40	20
Maricopa blend		70	100	40
Byers blend		40	100	70

Table 2. Mean (± SEM, n=6) number of adult *Lygus lineolaris* collected weekly on red sticky cards containing different pheromone lures (28 August 2021). Means labelled with different letters are significantly different by Tukey's HSD after a significant ANOVA ($\alpha = 0.05$).

Weeks after deployment	Pheromone blends				*F*-ratio	*P*-value
	Byers	Stoneville	Maricopa	Control		
Week 1	101 ± 7^{a}	71 ± 4^{b}	82 ± 4^{b}	1 ± 1^{c}	107.1	<0.0001
Week 2	67 ± 7^{a}	65 ± 2^{a}	63 ± 7^{a}	1 ± 1^{b}	38.1	<0.0001
Week 3	74 ± 5^{a}	51 ± 3^{b}	68 ± 4^{a}	1 ± 1^{c}	104.4	<0.0001
Week 4	49 ± 4^{a}	46 ± 3^{a}	47 ± 3^{a}	3 ± 2^{b}	48.8	<0.0001
Week 5	22 ± 2^{a}	23 ± 2^{a}	26 ± 3^{a}	1 ± 1^{b}	26.1	<0.0001
Week 6	9 ± 2^{a}	6 ± 1^{a}	6 ± 1^{a}	1 ± 1^{b}	8.8	<0.0001

633 **Figure legends**

634 **Figure 1.** Images of the field deployment of red sticky card with pheromone lures. (A) Set up of
635 the sticky card deployment in the field. Pheromone blends in different ratios were dispensed
636 from pipette tips attached horizontally to the center of the red sticky card. (B) *Lygus lineolaris*
637 adults that were attracted and trapped on the red sticky card with pheromone lures. Green dots
638 indicate *L. lineolaris* adults.

639 **Figure 2.** The reflectance spectra of the different colored sticky cards were measured using a
640 spectrophotometer. Figure shows the percentage reflectance measured from the sticky surface of
641 blue, white, red and yellow cards used in the field experiment.

642 **Figure 3.** Mean (SEM, $n = 6$) number of *Lygus lineolaris* adults trapped on different colored
643 (red, yellow, blue, and white) sticky cards in a week under field conditions (01 July 2021).
644 Means labeled with different letters are significantly different by Tukey's HSD after a significant
645 ANOVA ($\alpha = 0.05$).

646 **Figure 4.** Mean (SEM, $n = 6$) number of *Lygus lineolaris* adults captured on red sticky cards
647 with or without the pheromone lures; A) Byers blend, B) Stoneville blend, C) Maricopa blend, 2,
648 5, and 7 days after deployment (15 August 2021). Means labeled with asterisks are significantly
649 greater than their paired control sticky cards by paired t test ($\alpha = 0.05$, $n = 6$).

650 **Figure 5.** Total number (mean $\pm$ SE) of *Lygus lineolaris* adults captured during four weeks on
651 different colored sticky cards with or without the Byers blend pheromone lure (10 August 2022).
652 Means labeled with different letters are significantly different by Tukey's HSD after a significant
653 ANOVA ($\alpha = 0.05$).
654

Fig. 1

Fig. 2

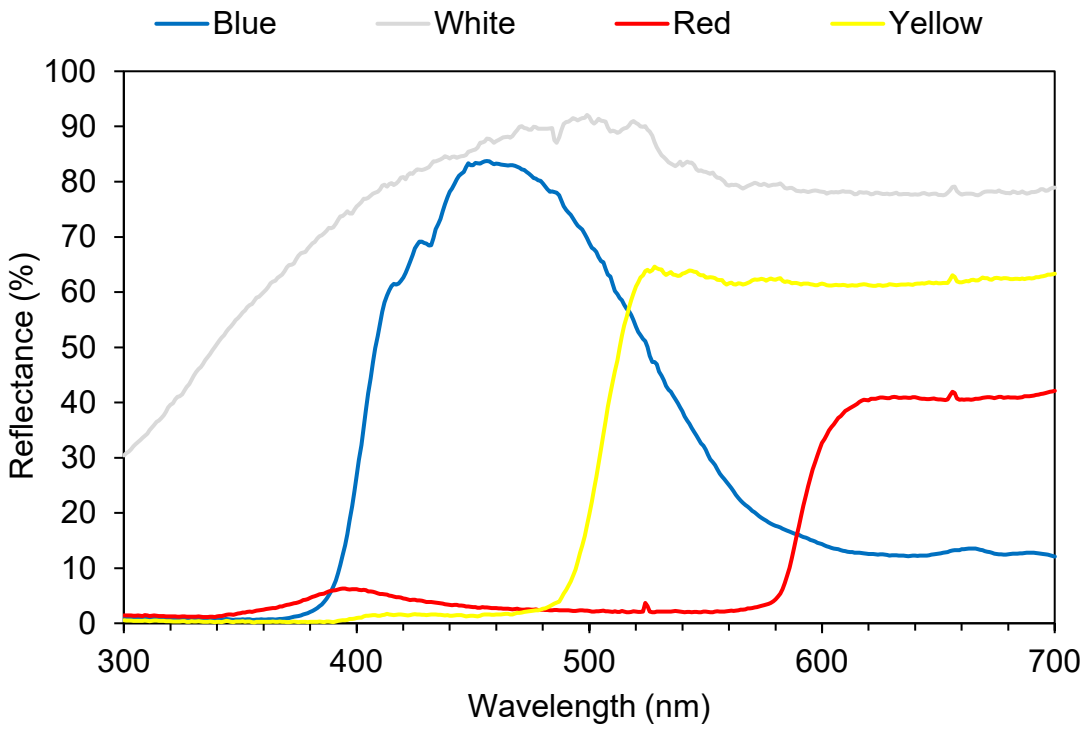

Blue
White
Red
Yellow
Reflectance (%)
100
90
80
70
60
50
40
30
20
10
0
300
400
500
600
700
Wavelength (nm)

Fig. 3

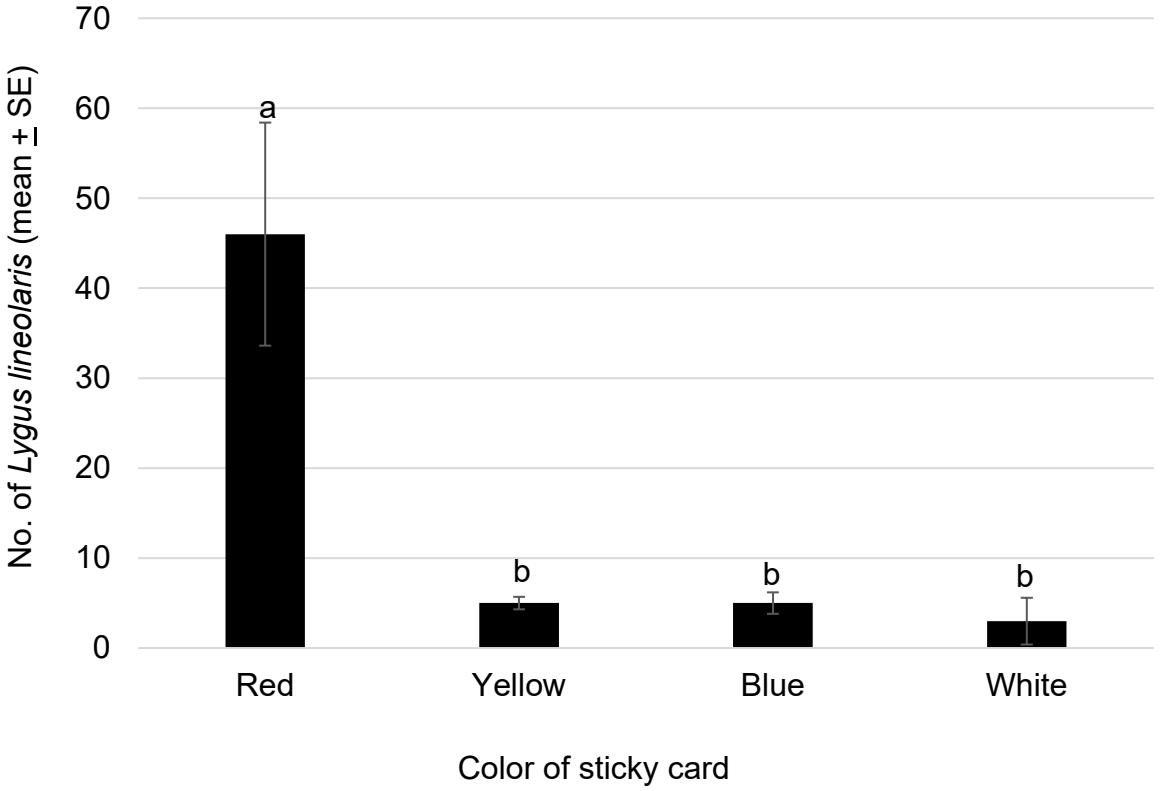

70
60
50
40
30
20
10
0
No. of Lygus lineolaris (mean ± SE)
a
b
b
b
Red
Yellow
Blue
White
Color of sticky card

Fig. 4

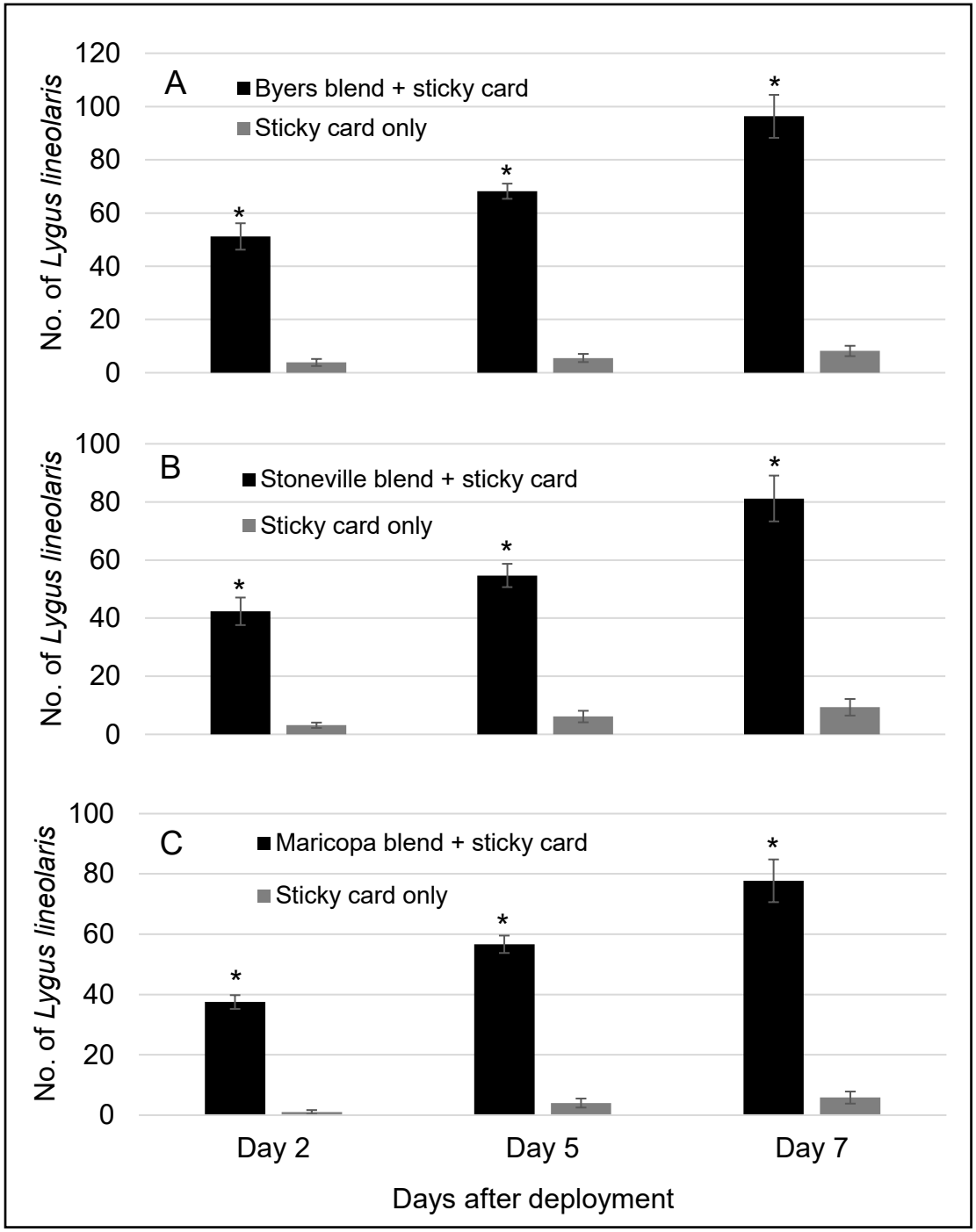

A
Byers blend + sticky card
Sticky card only
No. of Lygus lineolaris
120
100
80
60
40
20
0
*
B
Stoneville blend + sticky card
Sticky card only
C
Maricopa blend + sticky card
Sticky card only
Day 2
Day 5
Day 7
Days after deployment

Fig. 5

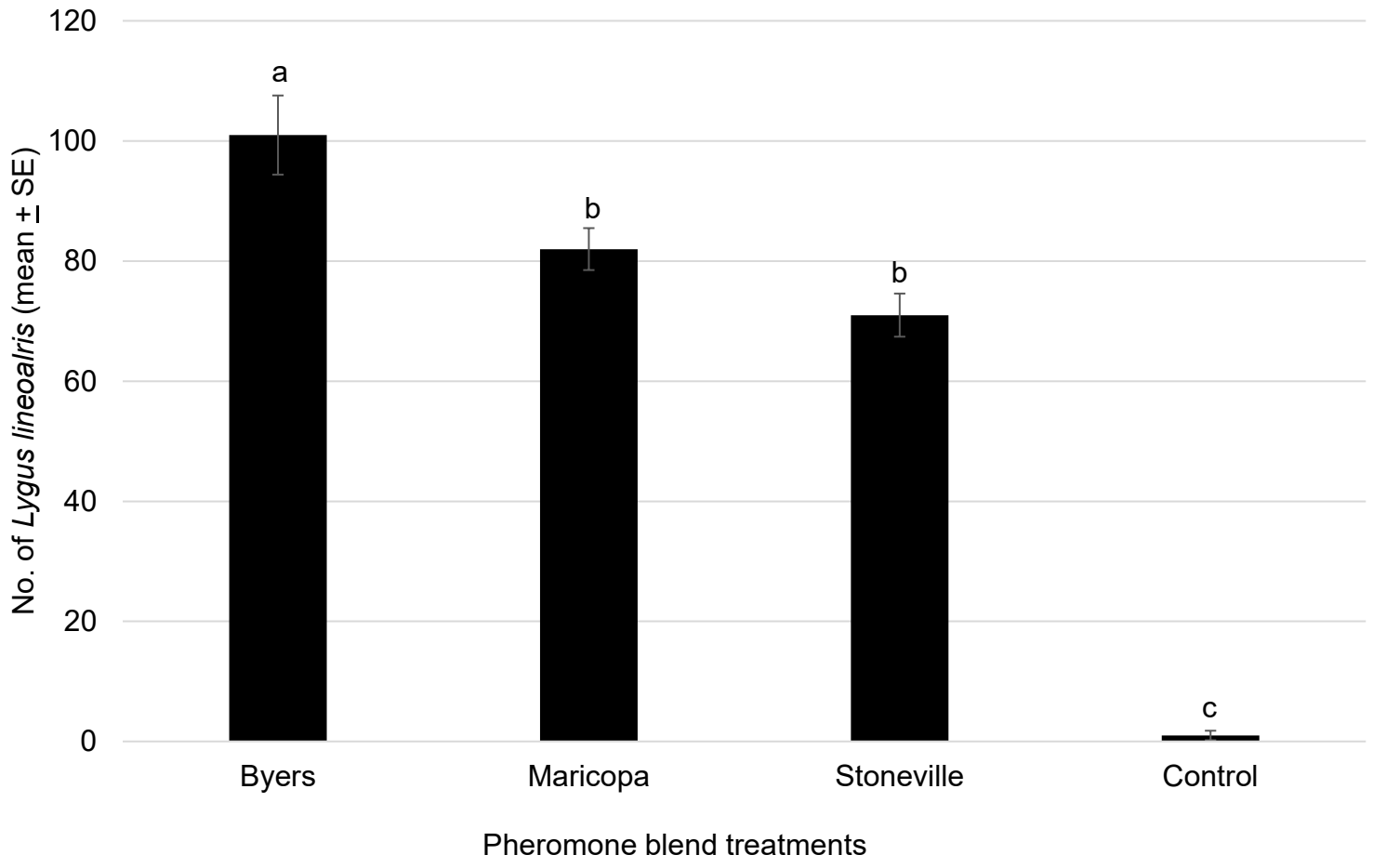
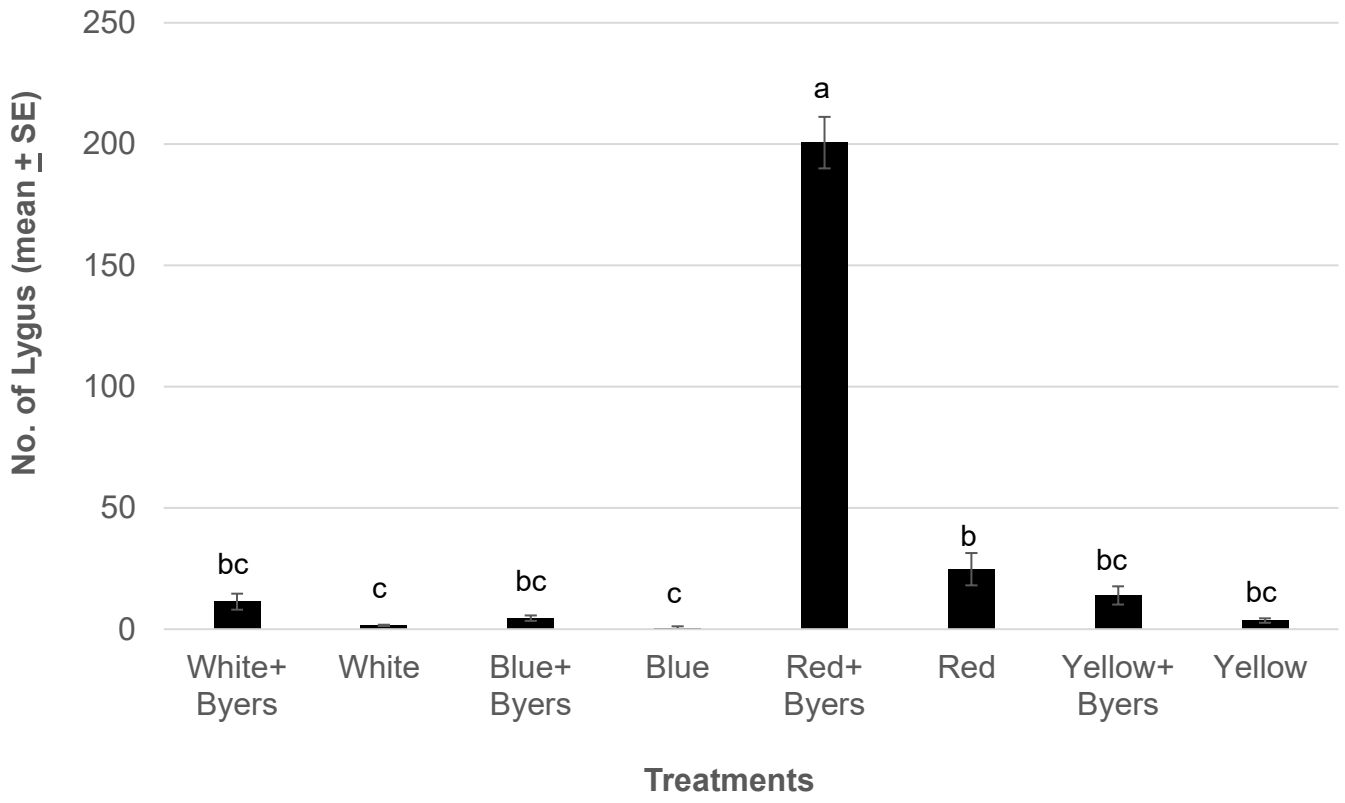
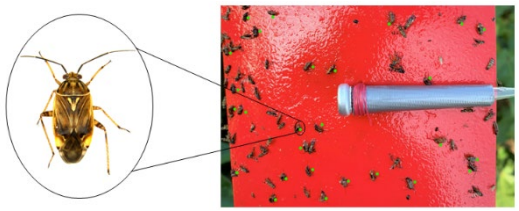

120
100
80
60
40
20
0
No. of Lygus lineoalris (mean ± SE)
a
b
b
c
Byers
Maricopa
Stoneville
Control
Pheromone blend treatments

Fig. 6


250
200
150
100
50
0
No. of Lygus (mean ± SE)
a
b
bc
bc
c
bc
c
bc
White+ Byers
White
Blue+ Byers
Blue
Red+ Byers
Red
Yellow+ Byers
Yellow
Treatments



Supplementary Material

Combining visual cues and pheromone blends for monitoring and management of the tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae)

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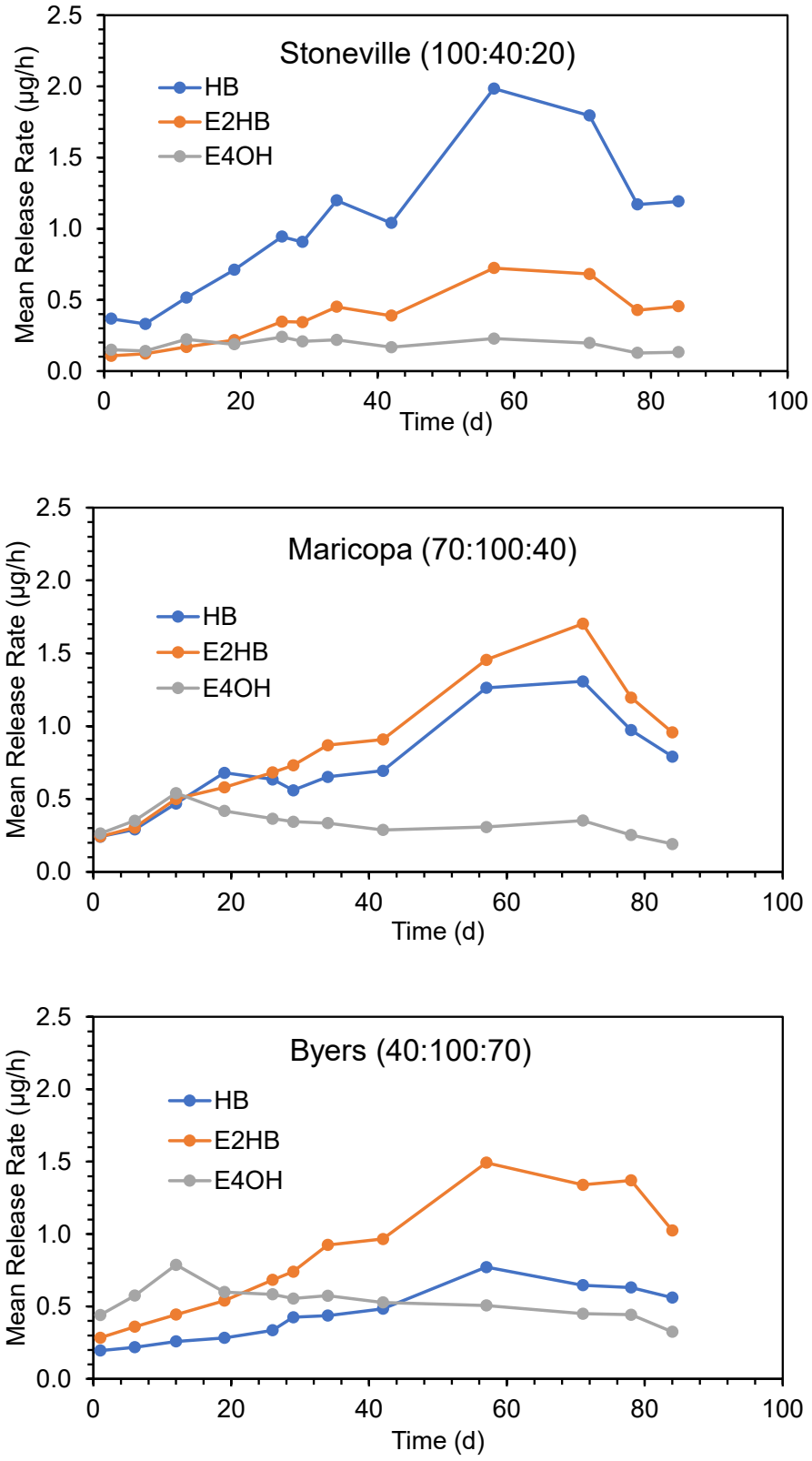
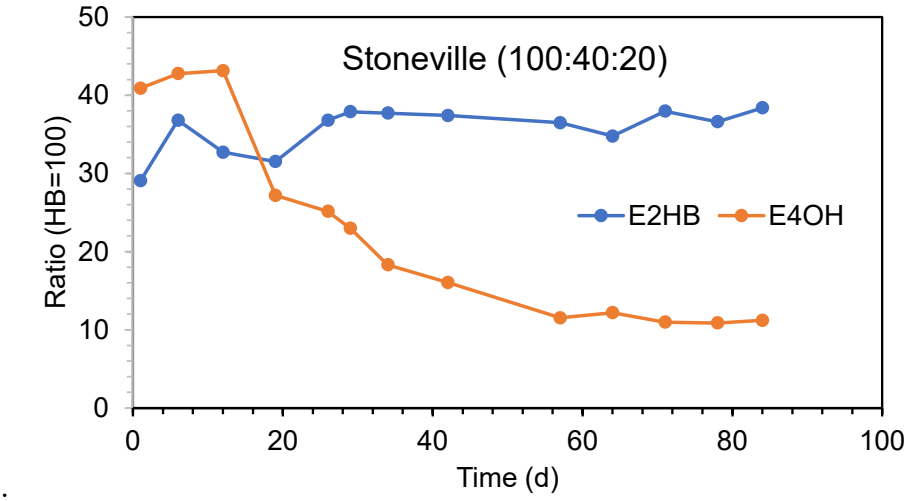


Figure S1. Release rates of hexyl butyrate (HB), (*E*)-2-hexenyl butyrate (E2HB) and (*E*)-4-oxo-2-hexenal (E4OH) from pipette tip dispensers maintained in a laboratory windtunnel at 27 °C and 8 km h^{-1} windspeed (results are mean of two replicates)



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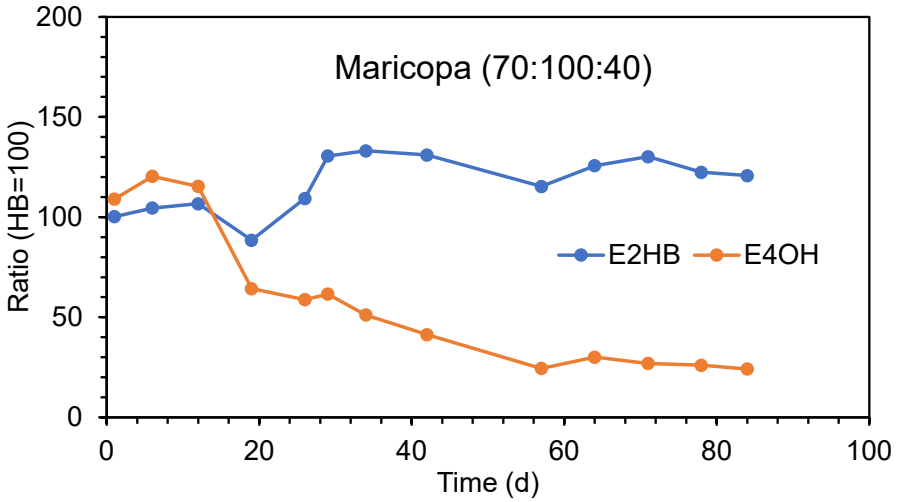

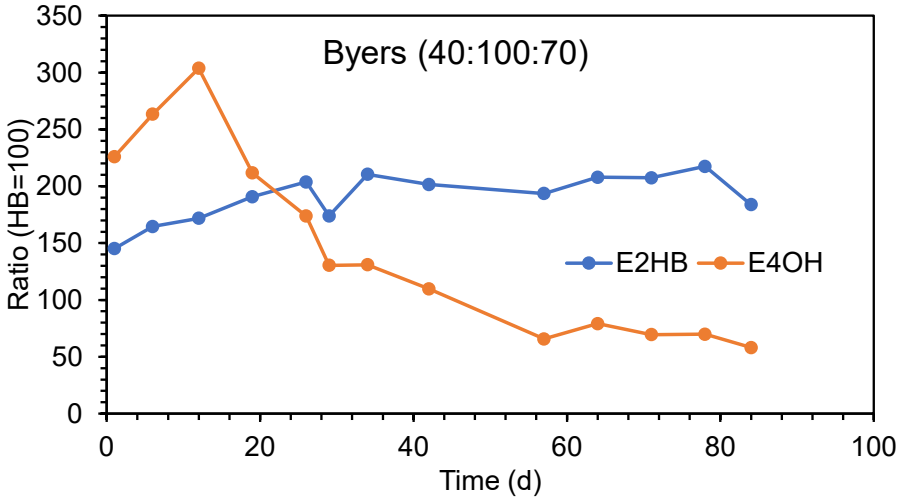

Figure S2. Release rates of (*E*)-2-hexenyl butyrate (E2HB) and (*E*)-4-oxo-2-hexenal (E4OH) relative to hexyl butyrate (HB) = 100 from pipette tip dispensers maintained in a laboratory windtunnel at 27 °C and 8 km h^{-1} windspeed (results are mean of two replicates)