

# Behavioral responses of predaceous minute pirate bugs to tridecane, a volatile emitted by the brown marmorated stink bug

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**Abstract** Since its introduction from Asia, the brown marmorated stink bug, *Halyomorpha halys* (Stål), has become a pest of many agricultural crops in the USA. Insect predators utilize *H. halys* as prey; however, the chemical cues used by them in prey location are largely unknown. Here, we hypothesized that tridecane, an *H. halys*-associated volatile, acts as a kairomone for the predaceous minute pirate bug, *Orius insidiosus* (Say). We conducted laboratory, greenhouse, and field experiments to: (1) assess the attraction of adult *O. insidiosus* to *H. halys*-damaged bean pods and tridecane in cage studies, and its effect on egg predation; (2) video record the behavioral response of *O. insidiosus* males and females to tridecane in a four-arm olfactometer; and (3) test whether *Orius* spp. are attracted to tridecane-baited sticky traps in peach, blueberry, and sunflower fields, and its effect on egg predation. In the greenhouse, *O. insidiosus* was attracted to *H. halys*-damaged pods and artificial plants baited with tridecane in the presence of prey (sentinel eggs); however, this attraction rarely led to increased egg predation. Although both *O.*

*insidiosus* males and females spent more time in the arm of the olfactometer with tridecane, males were more responsive at a lower concentration than females. Similarly, *Orius* spp. were more attracted to tridecane-baited than unbaited traps in the field; however, there was no effect on egg predation. Altogether, while *O. insidiosus* is attracted to tridecane, this attraction did not always lead to higher predation, suggesting that this kairomone likely acts as an arrestant.

**Keywords** *Orius insidiosus* · *Halyomorpha halys* · BMSB · Conservative biological control · Invasive pests · Kairomones

## Key message

- We tested the hypothesis that the predator *Orius insidiosus* utilizes volatiles associated with *Halyomorpha halys* during prey location.
- *Orius insidiosus* spent more time foraging on bean plants damaged by *H. halys* in the presence of prey.
- Tridecane was a major compound emitted by *H. halys* and *H. halys*-damaged plants.
- Laboratory, greenhouse, and field experiments showed attraction of *O. insidiosus* to tridecane.
- An increased attraction to tridecane by *O. insidiosus* did not always enhance predation.
- Tridecane arrested *O. insidiosus* foraging behavior.

## Introduction

As proposed by the enemy release hypothesis, invasive pests usually impose a major challenge to integrated pest management (IPM) programs because they often arrive to a

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new territory free from their natural enemies (Keane and Crawley 2002; Colautti et al. 2004; Pimentel et al. 2005). Natural enemies in the invaded territories are rarely adapted to utilize these novel food items; therefore, it may take several years until they are able to regulate the pests' populations (e.g., Chabaane et al. 2015). A possible reason is that native natural enemies do not usually recognize the chemicals emitted from these invasive pests (i.e., kairomones) and are consequently unable to locate them (Desurmont et al. 2014). Thus, identifying and understanding the response of natural enemies to volatiles emitted by invasive pests is critical for their conservation and augmentative releases in agro-ecosystems.

Natural enemies of herbivorous insects, i.e., predators and parasitoids, are known to use chemical cues to find their host/prey (Turlings et al. 1990). Such cues involve volatiles either emitted by plants in response to insect damage or emitted by the herbivores themselves (Ninkovic et al. 2001; De Boer and Dicke 2004; Van Den Boom et al. 2004; Zhu and Park 2005). In fact, natural enemies can be attracted to specific volatile compounds, or even to specific ratios of volatiles within blends, produced by their host (De Moraes et al. 1998; Reddy et al. 2002; Colazza et al. 2014). These volatiles, once identified, can be used to attract natural enemies into agro-ecosystems (Khan et al. 2008; Rodriguez-Saona et al. 2012; Kelly et al. 2014). For example, methyl salicylate (MeSA), a volatile commonly emitted by plants after herbivore damage (also known as herbivore-induced plant volatiles, HIPVs), has been reported to elicit behavioral responses in several groups of insect predators of agricultural pests (James and Price 2004; Rodriguez-Saona et al. 2011; Gadino et al. 2012; Kelly et al. 2014).

*Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), commonly referred to as brown marmorated stink bug (BMSB), is an invasive pest introduced into the USA (USA) from Asia (Leskey et al. 2012a; Xu et al. 2014). It was originally detected in Allentown, Pennsylvania (USA), in 1996 (Hoebeke and Carter 2003), and since then it has been reported and intercepted in 42 states (Northeastern IPM Center 2016). *Halyomorpha halys* has a large host range (Nielsen and Hamilton 2009; Northeastern IPM Center 2016) and, since its introduction in the USA, it has caused more than \$37 million in losses to apples (Leskey et al. 2012b), as well as substantial economic losses to other crops such as ornamentals, vegetables, and field crops (Kuhar et al. 2012). Due to *H. halys*, insecticide use has increased in many cropping systems (Leskey et al. 2012a, b). Alternative management strategies such as conservation biological control have not yet been implemented, although studies with native natural enemies of *H. halys* have been initiated in the USA (Rice et al. 2014; Morrison et al. 2016). Insect predators can play an

important role in *H. halys* control in vegetables, ornamentals, tree fruit, and field and organic crops (Leskey et al. 2012a). Leskey et al. (2012a) reported generalist predators in the Chrysopidae, Reduviidae, Lygidae, and Anthocoridae families using *H. halys* as prey. However, the chemical cues used by natural enemies to locate *H. halys* remain unknown.

Minute pirate bugs (*Orius* spp.) (Hemiptera: Anthocoridae) have been observed feeding on *H. halys* egg masses (Biddinger et al. 2012; Morrison et al. 2016). One of its members, *Orius insidiosus* (Say), is widespread throughout the USA (Shipp and Ramakers 2004) and is a well-known predator of thrips, aphids, mites, and eggs and young larvae of various agricultural pests (Iglinsky and Rainwater 1950; Hansen et al. 2003; Osekre et al. 2008; Harwood et al. 2009). *Orius* spp. and other anthocorids frequently exploit host-plant and prey-associated odors, also known as kairomones, to guide them during foraging (Reid and Lampman 1989; Lattin 1990; Aldrich et al. 2007). For example, Teerling et al. (1993) demonstrated that *Orius tristicolor* (White) searches more intensively in the presence of the alarm pheromone (decyl and dodecyl acetates) of its prey the western flower thrips, *Frankliniella occidentalis* (Pergande). Additionally, *O. tristicolor* and *Orius similis* Zheng are attracted to sticky traps baited with MeSA in hop yards (James 2005) and cotton fields (Yu et al. 2008).

In the present study, we tested the hypothesis that *O. insidiosus* utilizes volatiles associated with *H. halys* during prey location. Specifically, we conducted a series of laboratory, greenhouse, and field experiments to: (1) determine the behavioral response of *O. insidiosus* to beans damaged by *H. halys* in greenhouse cages, and evaluate the effects of this response on egg predation; (2) isolate and identify the volatiles emitted from *H. halys* nymphs and adults, and from *H. halys*-bean associations; (3) test the behavioral response of *O. insidiosus* to the major volatile emitted from *H. halys* (tridecane) in cage studies and under field conditions (blueberries, peaches, and sunflower); and (4) examine in more detail, using an olfactometer and video recordings, the effects of tridecane on *O. insidiosus* foraging behavior.

## Materials and methods

### Insects

A laboratory colony of *H. halys* was sourced from a colony maintained at the Rutgers Agricultural Research and Extension Center (RAREC; Bridgeton, NJ, USA) and from adults collected in May 2014 from peach orchard in Bridgeton, NJ. The colony was kept in insect rearing cages

(30 × 30 × 30 cm; Bugdorm Insect Cage; MegaView Science Co Ltd, Taichung, Taiwan). *Halymorpha halys* was fed with organic carrots, apples, broad bean pods, and sunflower seeds; food was changed every two days, and water was provided with soaked cotton wool in small containers (50 ml). Paper towels were placed inside each cage as oviposition substrate. *Orius insidiosus* adults for olfactometer and greenhouse experiments were obtained from Rincon-Vitova Insectaries (Ventura, CA, USA) and sexed as described by Herring (1966). Both insect colonies were maintained in an environmental chamber kept at 25 ± 1 °C, 50 ± 10% RH, and 14L:10D.

For greenhouse experiments and volatile collections, *H. halys* adults and nymphs were separated from the colony and starved for 24 h prior to being placed on experimental plants. For cage and olfactometer experiments, individual *O. insidiosus* adults were placed in smaller plastic containers and deprived of food overnight until the start of the bioassays. In order to assess predation in the greenhouse and field experiments, egg masses of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), were used due to their commercial availability (Rincon-Vitova Insectaries) and also because they are commonly used by *O. insidiosus* as prey in nature (Andow 1990; Musser and Shelton 2003).

## Plants

For volatile collections and to assess *O. insidiosus* attraction to *H. halys*-damaged plants, common bean plants (*Phaseolus vulgaris* L.) (Burpee; Warminster, PA, USA) were used due to their fast growth and because bean pods are a suitable food source for *H. halys*. Seeds of beans were sown in pots filled with standard potting mix with new plants being sowed at 2-week intervals. Plants were kept in a greenhouse at the Rutgers P.E. Marucci Center (Chatsworth, NJ, USA), at 26 ± 1 °C, 50 ± 10% RH, and 14L:10D. Plants were used when pods were fully developed (approximately 4–5 weeks after seeding), watered three times a week or more often if needed, and fertilized biweekly with PRO-SOL 20-20-20N-P-K All Purpose Plant Food (Pro Sol Inc; Ozark, AL, USA) (each plant received approximately 100 ml of a 2 g of fertilizer per liter of water solution).

## Behavioral response of predators to infested plants

We conducted an experiment in the greenhouse to investigate the attraction of *O. insidiosus* to volatiles emitted from bean pods damaged by *H. halys* and whether this attraction leads to increased predation of *O. nubilalis* eggs. Our treatments consisted of: (1) *H. halys*-damaged plants (pods) with prey (i.e., eggs); (2) *H. halys*-damaged plants

with no prey; (3) undamaged plants with prey; and (4) undamaged plants with no prey. For the damage treatments, five *H. halys* adults were kept with bean pods inside closed white organza bags (Uline; Pleasant Prairie, WI, USA) for 72 h. Bags and insects were removed prior to the choice tests (see below). For the treatments with prey, four pieces of wax paper each containing an individual *O. nubilalis* egg mass (each egg mass had approximately 25 eggs) were glued to a green plastic card; the card was then pinned to the underside of one of the fully matured leaves—one card per plant.

For each choice test, four plants (two of each treatment) were placed in cages (60 × 60 × 60 cm; Bugdorm Insect Cage; MegaView Science Co Ltd), equidistant from each other, and with plants treated in the same manner placed diagonal from each other. Ten *O. insidiosus* adults (mixed sexes) were released in the morning (10:00 h) inside each cage (center), and their position was recorded every hour for 6 h (i.e., total of six times per day from 10:00 to 14:00 h) for 2 days. To measure egg predation, the numbers of *O. nubilalis* eggs per mass were counted before and after each experiment; eggs were examined under the microscope for signs of predation by piercing and sucking. Each choice test was replicated four times.

## Volatile collection and analysis

We collected volatile organic compounds (VOCs) emitted from *H. halys*-damaged bean pods, *H. halys* nymphs and adults, and mechanically damaged bean pods in the greenhouse (26 ± 1 °C, 50 ± 10% RH, and 14L:10D), using a pull system (Tholl and Röse 2006). Individual fully developed bean pods were covered with organza bags. Treatments consisted of bean pods with 5 *H. halys* adults or nymphs (3rd–5th instar) inside the bags; 5 *H. halys* nymphs only; 5 *H. halys* adults only; mechanically damaged bean pods; bean pods only; and empty bags (blank controls). Insects were allowed to feed on the pods for 2–3 days and then removed prior to volatile collections. Mechanical damage consisted of 20 sequential pokes (10 pokes per day for 2 days) on each pod using sharpened forceps. Each treatment was repeated 3–6 times.

Volatiles from inside the bags were collected on 30 mg Super-Q adsorbent traps (Alltech; Deerfield, IL, USA) by pulling air at a rate of 300 ml min<sup>-1</sup> with the aid of micro-diaphragm air pumps (Sensidyne; Saint Petersburg, FL, USA). Prior to each collection, traps were cleaned by rinsing them three times with 5 ml of dichloromethane (99% purity; Sigma-Aldrich; St. Louis, MO, USA). VOCs were collected for 48 h, starting at 10:00 h. After collection, Super-Q traps were eluted with 150 µl dichloromethane; 5 µl of 400 ng of *n*-octane (Sigma-Aldrich) was added to each sample as an internal standard (IS).

Samples were analyzed on a Hewlett Packard 6890 Series Gas Chromatograph (GC) equipped with a flame ionization detector (FID) (Agilent HP-1 column; Santa Clara, CA, USA): 10 m × 0.53 mm × 2.65 μm, He as carrier gas: constant flow rate = 5 ml min<sup>-1</sup>, velocity = 39 cm s<sup>-1</sup>. The program for separation and quantification was set up at 40 °C initial temperature (1 min), followed by a temperature ramp of 14 °C min<sup>-1</sup> to 180 °C (2 min), then 40 °C min<sup>-1</sup> to 200 °C, then 200 °C (2 min). Compounds (ng h<sup>-1</sup>) were quantified based on comparison of peak areas with that of the IS (*n*-octane). Identification of compounds (for at least one sample per treatment) was performed on a Varian 3400 GC coupled to a Finnigan MAT 8230 Mass Spectrometer (MS) (Supelco MDN-5S column; Bellefonte, PA, USA): 30 m × 0.32 mm × 0.25 μm. The program was 35 °C initial temperature (1 min), followed by a temperature ramp of 4 °C min<sup>-1</sup> to 170 °C, then 15 °C min<sup>-1</sup> to 280 °C. The MS data were acquired and processed in a Finnigan MAT SS300 data system, and compounds were identified by GC retention index, and comparison of their retention times to those of commercially available compounds and their spectral data to those from the NIST library (Rodriguez-Saona et al. 2009, 2011).

### Behavioral response of predators to tridecane

We identified tridecane (C13) as the major compound emitted by *H. halys*-damaged bean pods and from *H. halys* nymphs and adults (see Results). Likewise, Solomon et al. (2013) and Harris et al. (2015) identified tridecane as the dominant defensive volatile compound secreted from the scent glands of *H. halys* when disturbed. Thus, we conducted experiments to test the attraction of *O. insidiosus* adults to tridecane (≥99% purity; Sigma-Aldrich). Choice tests consisted of 1, 10, or 100 ng/μl of tridecane (diluted in hexane; Sigma-Aldrich) versus a control (hexane without tridecane). These concentrations were used to mimic a range of amounts (peaks) emitted from *H. halys* detected by GC (see above). Instead of bean plants, we used artificial plants of similar size (30 cm tall) and color (green) to avoid any additional volatile cues. The artificial plants had eight leaves and were placed individually in plastic containers (250 ml) filled with sand. Four artificial plants were placed inside cages (Bugdorm Insect Cage), as described above for natural plants. Two of the plants had a septum (Precision Seal<sup>®</sup>; Sigma-Aldrich) containing 20 μl of one of the tridecane concentrations, while the other two had a septum without tridecane. All plants received three *O. nubilalis* egg masses as described above. Fifteen *O. insidiosus* adults (mixed sexes) were released at 10:00 h in the center of the cage, and their location (numbers on plants) was recorded every hour for 6 h day<sup>-1</sup> and for a

total of 3 days. Egg predation was measured as described above. Each choice test was replicated 15 times.

Release rates from tridecane-treated septa ( $N = 3$  per concentration) were calculated by placing individual septum in 6-ml glass vials (Fisher Scientific Inc, Pittsburg, PA, USA), collecting headspace volatiles from vials as described above for 24 h, and calculating release rates based on a linear regression with known quantities of tridecane; releases rates (mean ± SE) for 1, 10, and 100 ng/μl concentrations were: 1.12 ± 0.21, 1.79 ± 0.27, and 3.78 ± 0.95 ng day<sup>-1</sup>, respectively.

### Olfactometer assays

The response of *O. insidiosus* to tridecane was further investigated in a four-arm olfactometer (Volatile Assay Systems; Rensselaer, NY, USA). Three concentrations were tested (1, 10, and 100 ng/μl). The arena consisted of an acrylic chamber (10 × 10 cm) that was divided into four zones. A removable plastic chamber (2 × 2 cm) was installed at the end of each arm, closed by a cork with a hole covered with gauze for air flow. The top of the arena was covered with a glass plate to prevent insects from escaping. The experiment was conducted in a dark room at 25 ± 1 °C; a light bulb was positioned on the top of the arena such that the amount of light (~2500 lx) was similar for all arms. Each treatment was tested by pipetting 20 μl of one of the tridecane concentrations onto a piece of Whatman No. 1 filter paper (2.5 cm<sup>2</sup>; Sigma-Aldrich). The tridecane-treated filter paper was then placed inside the chamber of one of the arms (randomly selected) of the olfactometer; the other arms had filter papers with 20 μl of hexane (controls). A single adult *O. insidiosus* (male or female) was released in the center of the arena, and the time that it spent in each arm of the arena was tracked and recorded for 10 min using a video camera (model: acA1300-60gm GigE; Basler, Ahrensburg, Germany) and analyzed with the software Ethovision XT (Noldus Information Technology, Wageningen, Netherlands). Treatments in the olfactometer were replaced, and their position changed, every five insects. The entire arena and the odor chambers were cleaned using soap water and hexane and then dried. Each treatment was replicated 30 times for each gender.

### Field experiment

In June–August 2014, we evaluated the response of *Orius* spp. to tridecane in peach ( $N = 4$ ), blueberry ( $N = 4$ ), and sunflower ( $N = 2$ ) fields. Both peach (latitude 39°31'36"N, longitude 75°12'16"W, 37 m altitude) and sunflower (latitude 39°31'41"N, longitude 75°12'08"W, 35 m altitude) fields were located at RAREC (Bridgeton, NJ, USA). The

blueberry fields were located on an organic farm (latitude 39°39'59"N, longitude 74°45'13"W, 18 m altitude) in Hammonton, NJ (USA). Peaches, sunflowers, and blueberries are suitable hosts for *H. halys* feeding and reproduction (Nielsen and Hamilton 2009; Leskey et al. 2012a; Soergel et al. 2015). In each field, the following six treatments were tested in a randomized complete block design: 1, 2, and 3 ml of tridecane, and 3 and 5 ml of MeSA (>99% purity; Sigma-Aldrich), and a control. Yellow sticky cards (23 cm × 28 cm) (ISCA Technologies; Riverside, CA, USA) were baited with each of the treatments in 3 ml (all except for the 5 ml MeSA treatment) or 15 ml (for the 5 ml MeSA treatment) low-density polyethylene vials (Wheaton Science Products; Millville, NJ, USA). One set of traps ( $N = 6$ ) was placed in each of the eight fields (total of 60 traps). Each field was considered a replicate. Traps were placed on plants ~1–1.5 m from the ground and at least 10 m apart, and their position rotated clockwise weekly. Traps were replaced every week for a total of five weeks and were examined in the laboratory under a microscope for *Orius* spp. Release rates were obtained by weighing the vials before and after placing them in the field: release rates (mean ± SE) =  $0.11 \pm 0.01$ ,  $0.26 \pm 0.02$ ,  $0.27 \pm 0.04$ ,  $0.25 \pm 0.05$ ,  $0.49 \pm 0.04$  g wk<sup>-1</sup> for the 1, 2, and 3 ml tridecane, and 3 and 5 ml MeSA treatments, respectively.

In a separate experiment, we evaluated the effects of each of the treatments on egg predation. The experimental design was similar to that described above for sticky traps. The same 10 fields were used in this experiment. Egg masses ( $N = 4$ ) of *O. nubilalis* were glued to a card, as described above for greenhouse experiments, and placed near a vial containing one of the above-mentioned treatments. The cards were then pinned to the underside of leaves, within 20 cm from the vials. Eggs remained in the field for 24 h, after which they were removed from plants, brought to the laboratory, and checked for egg predation under the microscope. Egg predation was evaluated in the same weeks as it was done for the sticky traps.

### Statistical analyses

All statistical analyses were conducted using the software SPSS® Statistics 22 (SPPS Inc; Chicago, IL, USA). Data on the number of *O. insidiosus* adults on plants in greenhouse cages were analyzed by general linear model (GLM), followed by Tukey's HSD tests ( $\alpha = 0.05$ ). Greenhouse data for multiple observations were summed for each treatment prior to analysis. The same GLM analysis was used to compare tridecane emissions among treatments. An independent-sample *t* test was used to compare egg predation and attraction of *O. insidiosus* to tridecane-baited artificial plants versus unbaited plants. The same test was used to compare the time spent by males and females in the

arms containing different tridecane concentrations in the olfactometer bioassays. Total number of *Orius* spp. on traps and percentage of egg predation on blueberry, sunflower, and peach fields were analyzed by repeated-measures analysis of variance (ANOVA), with treatment (fixed effect), crop (fixed effect), and time of sampling (week) (random effect), as well as all possible interactions, as independent variables. A significant ANOVA was followed by Tukey's HSD test ( $\alpha = 0.05$ ). To establish normality, mean number of *Orius* spp. on traps were  $\ln(x + 0.5)$ -transformed prior to analysis. Percent data were arcsine square-root-transformed prior to analysis. The results presented correspond to the original (untransformed) data.

## Results

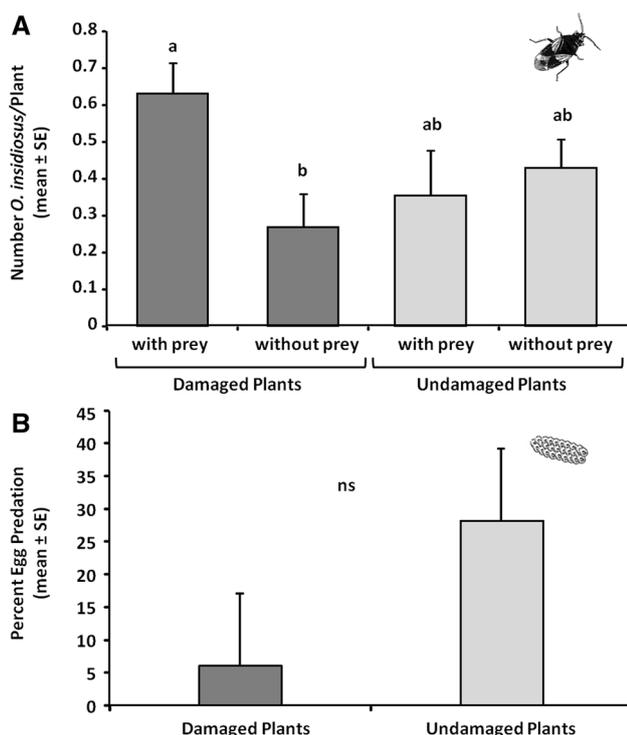
### Behavioral response of predators to infested plants

The presence of *H. halys* feeding damage and prey (sentinel eggs) on bean pods affected *O. insidiosus* attraction (significant damage × prey interaction;  $F = 5.52$ ;  $df = 3, 8$ ;  $P = 0.047$ ), indicating that the combination of both *H. halys* feeding damage and presence of prey attracted more *O. insidiosus*. In fact, there were significantly more *O. insidiosus* adults on bean plants damaged by *H. halys* in the presence of eggs than on damaged plants without prey ( $P < 0.05$ ) (Fig. 1a). There was, however, no effect of *H. halys* feeding damage alone ( $F = 0.394$ ;  $df = 1, 8$ ;  $P = 0.548$ ) or an effect of prey alone ( $F = 2.433$ ;  $df = 1, 8$ ;  $P = 0.157$ ) on *O. insidiosus* attraction.

Although there were ~2 times more *O. insidiosus* adults on damaged plants with prey as compared with undamaged plants with prey, this difference in attraction did not lead to an increase in egg predation ( $F = 1.96$ ;  $df = 1, 4$ ;  $P = 0.24$ ). In fact, numerically, there was ~6× lower percent predation of eggs on damaged compared with undamaged plants (Fig. 1b).

### Volatile collection and analysis

We collected volatiles to understand the mechanisms behind *O. insidiosus* attraction to plant-prey associations. *Halyomorpha halys* nymphs and adults (mixed sexes), as well as bean pods damaged by nymphs and adults of *H. halys*, consistently emitted high amounts of tridecane [retention times of tridecane from our chromatograms deviated from the standard compound by ±0.15 (SD) min, and matched by >95% the library mass spectrum for tridecane] (Fig. 2). However, tridecane emissions varied significantly among treatments ( $F = 13.21$ ;  $df = 6, 17$ ;  $P < 0.001$ ) (Table 1). Bean pods damaged by *H. halys* adults and



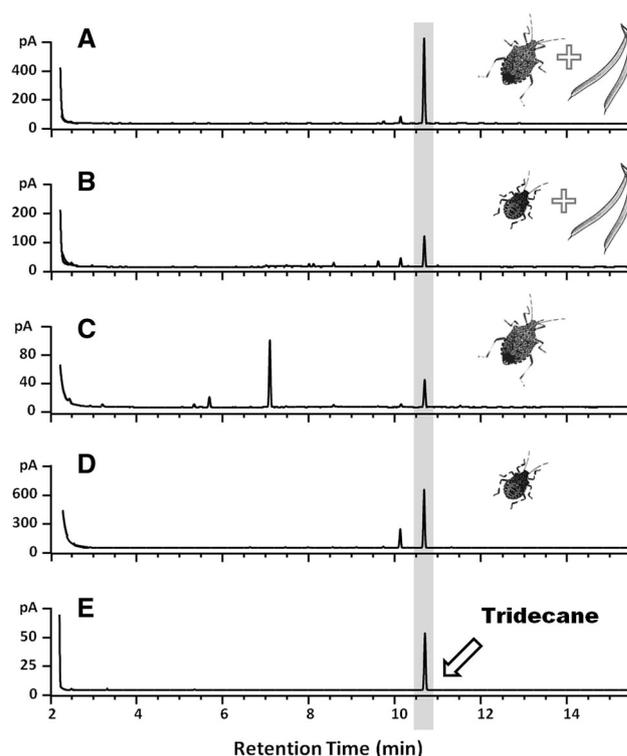
**Fig. 1** Attraction of the minute pirate bug, *Orius insidiosus*, to bean pods previously damaged by *Halyomorpha halys* and undamaged bean pods in the presence or absence of prey (*Ostrinia nubilalis* egg masses) (a). Amount of egg predation on damaged and undamaged bean plants (b). Means with different letters are significantly different (Tukey's HSD test;  $P \leq 0.05$ ); ns no significant differences between treatments

nymphs emitted higher amounts of tridecane compared with undamaged and artificially damaged bean pods (Table 1), indicating that volatile emissions were elevated by insect herbivory. In addition, nymphs produced significantly higher quantities of tridecane than adults (Table 1). Besides tridecane, no notable differences were observed in the emissions of other *H. halys*-bean-associated volatiles (see Fig. 2) and, thus, are not presented here.

### Behavioral response of predators to tridecane

Choice experiments were conducted to investigate the response of *O. insidiosus* to tridecane. *Orius insidiosus* adults were ~60% more attracted to artificial plants baited with tridecane at all concentrations tested as compared with unbaited artificial plants (controls) (1 ng/ $\mu$ l,  $t = 4.47$ ,  $df = 14$ ,  $P = 0.003$ ; 10 ng/ $\mu$ l,  $t = 3.79$ ,  $df = 14$ ,  $P = 0.001$ ; 100 ng/ $\mu$ l,  $t = 6.51$ ,  $df = 14$ ,  $P < 0.001$ ) (Fig. 3a).

Although tridecane at the two lower concentrations (1 and 10 ng/ $\mu$ l) attracted more *O. insidiosus* to artificial plants, we found no differences on egg predation between tridecane-baited and unbaited plants for these concentrations



**Fig. 2** Typical chromatograms of volatiles collected from a bean pods damaged by *Halyomorpha halys* adults, b bean pods damaged by *H. halys* nymphs, c adults only, and d nymphs only. Chromatogram (e) represents the tridecane standard

(1 ng/ $\mu$ l:  $t = 1.50$ ,  $df = 14$ ,  $P = 0.086$ ; 10 ng/ $\mu$ l:  $t = 0.64$ ,  $df = 14$ ,  $P = 0.272$ ) (Fig. 3b). There was, however, 1.8  $\times$  higher predation of eggs in the tridecane treatment at 100 ng/ $\mu$ l as compared with the control ( $t = 2.26$ ,  $df = 14$ ,  $P = 0.027$ ) (Fig. 3b).

### Olfactometer assays

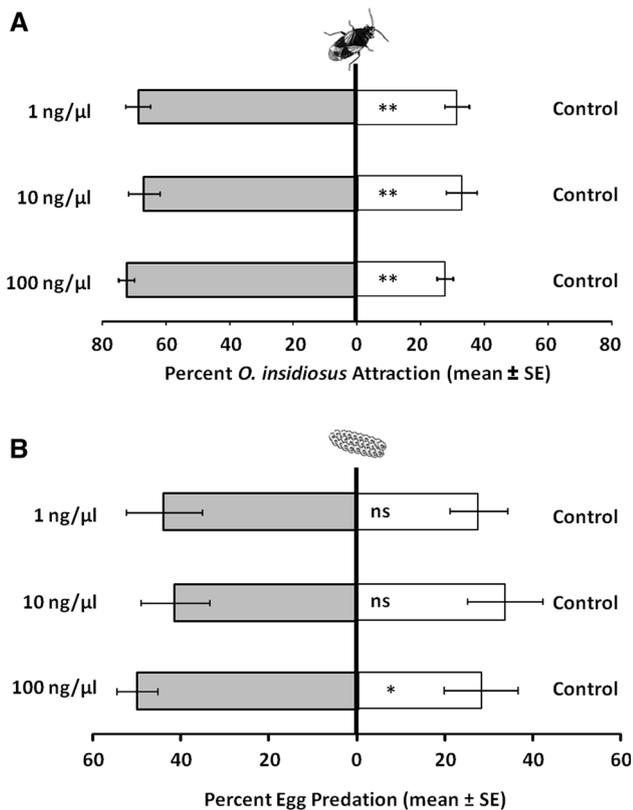
To better understand the behavioral response of *O. insidiosus* toward tridecane, we recorded male and female behaviors toward tridecane in a four-arm olfactometer. *Orius insidiosus* females spent most of their time (>30%) on the tridecane treatment at the highest concentration tested as compared with the control treatments (100 ng/ $\mu$ l,  $t = 2.37$ ,  $df = 1$ ,  $P = 0.021$ ) (Fig. 4a). In contrast, *O. insidiosus* females spent significantly more time on the control treatment than the tridecane treatment at the lowest concentration (1 ng/ $\mu$ l,  $t = -2.310$ ,  $df = 1$ ,  $P = 0.024$ ) (Fig. 4a).

*Orius insidiosus* males spent most of their time (>30%) on both the 10 ng/ $\mu$ l ( $t = 2.502$ ,  $df = 1$ ,  $P = 0.015$ ) and 100 ng/ $\mu$ l ( $t = 2.631$ ,  $df = 1$ ,  $P = 0.011$ ) tridecane treatments (Fig. 4b), demonstrating that both concentrations attracted and arrested males.

**Table 1** Amount of tridecane collected from nymphs and adults of the brown marmorated stink bug, *Halyomorpha halys*, and from *H. halys*-damaged bean pods, mechanically damaged bean pods, bean pods alone, and controls (no insects and plants)

Treatments	N	Tridecane (ng/h) <sup>a</sup> (mean ± SE)	
Feeding damage by 5 adults of <i>H. halys</i>	6	1177.68 ± 780.63	ab
Feeding damage by 5 nymphs of <i>H. halys</i>	3	224.53 ± 68.93	ab
Adults (5) of <i>H. Halys</i>	4	30.93 ± 6.71	bc
Nymphs (5) of <i>H. Halys</i>	4	1579.14 ± 1067.34	a
Mechanical damage	3	6.01 ± 2.23	c
Undamaged bean pods	5	10.18 ± 0.94	bc
Control	3	0.00 ± 0.00	c

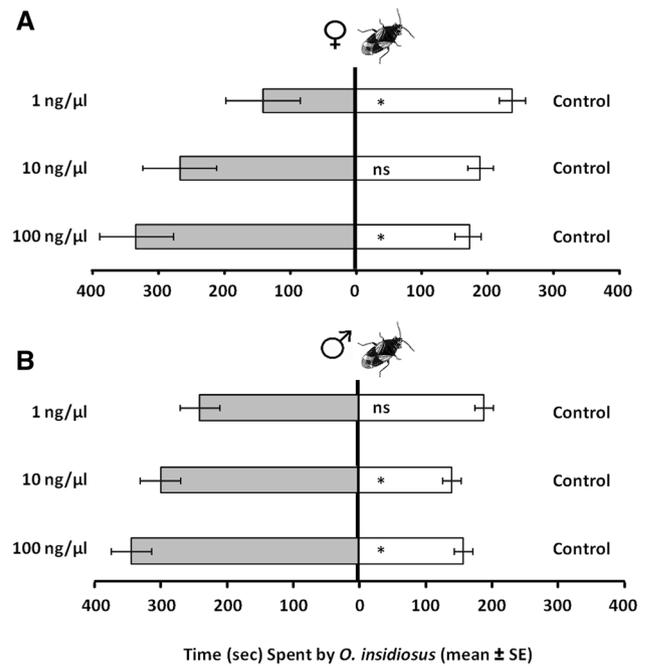
<sup>a</sup> Means with different letters are significantly different (Tukey's HSD test;  $P \leq 0.05$ )



**Fig. 3** Attraction of *Orius insidiosus* to tridecane in choice tests (a). Choices were given between artificial plants baited with one of three different concentrations of tridecane versus unbaited control plants (without tridecane) in greenhouse cage experiments. Amount of egg predation on tridecane-baited and unbaited plants (b). Asterisks indicate significant differences (*t* tests; \*\*  $P \leq 0.01$ , \*  $0.01 < P \leq 0.05$ ); ns no significant differences between treatments.  $N = 15$  replicates per choice test

**Field experiments**

Field experiments were conducted to test whether tridecane affect *O. insidiosus* attraction in three cropping systems: sunflowers, blueberries, and peaches. Tridecane-baited traps attracted *Orius* spp. (significant treatment effect; Table 2A): We found that traps baited with tridecane,



**Fig. 4** Response of females (a) and males (b) of *Orius insidiosus* to tridecane in four-arm olfactometer bioassays. Choice tests consisted of one of three different concentrations of tridecane versus control (without tridecane). Asterisks indicate significant differences (*t* tests; \*  $P < 0.05$ ); ns not significant.  $N = 30$  replicates per choice test

particularly at the dose of 2 ml, attracted  $>2.5\times$  more *Orius* spp. as compared with the unbaited control traps (Fig. 5a). Crop was also significant, with sunflower fields having significantly more *Orius* spp. [mean weekly counts per trap =  $2.92 \pm 0.39$  (SE)] than blueberry ( $0.48 \pm 0.07$ ) and peach ( $0.51 \pm 0.09$ ) fields (Table 2A). However, there were neither treatment-by-crop nor treatment-by-week interaction effects (Table 2A), indicating that the effect of treatment was not influenced by the crop or seasonality. In contrast, MeSA-baited traps did not attract *Orius* spp. in these cropping systems ( $P > 0.05$ ) (Fig. 5a). There were no other significant 2-way or 3-way interaction effects on *Orius* spp. trap captures.

**Table 2** Summary of repeated-measures analysis of variance (ANOVA) for the effects of treatment, crop, and time of sampling (week), and their interactions, on the numbers of *Orius* spp. on semiochemical-baited traps (A) and predation of *Ostrinia nubilalis* egg masses (B)

Variables	df <sup>c</sup>	F	P <sup>d</sup>
<b>A. Trap counts</b>			
Treatments <sup>a</sup>	5	4.92	<b>0.004</b>
Crop <sup>b</sup>	2	40.67	<b>&lt;0.001</b>
Week	4	3.57	0.070
Treatment × crop	10	1.69	0.116
Treatment × week	20	1.11	0.378
Crop × week	8	1.21	0.317
Treatment × crop × week	40	1.44	0.053
<b>B. Predation rate</b>			
Treatments <sup>a</sup>	5	1.17	0.358
Crop <sup>b</sup>	2	1.61	0.258
Week	4	1.40	0.316
Treatment × crop	10	0.47	0.897
Treatment × week	20	1.07	0.412
Crop × week	8	4.00	<b>0.001</b>
Treatment × crop × week	40	1.09	0.339

<sup>a</sup> Treatments consisted of 1, 2, and 3 mL of tridecane, 3 and 5 mL of methyl salicylate (MeSA), and control

<sup>b</sup> Crops were blueberries, sunflowers, and peaches

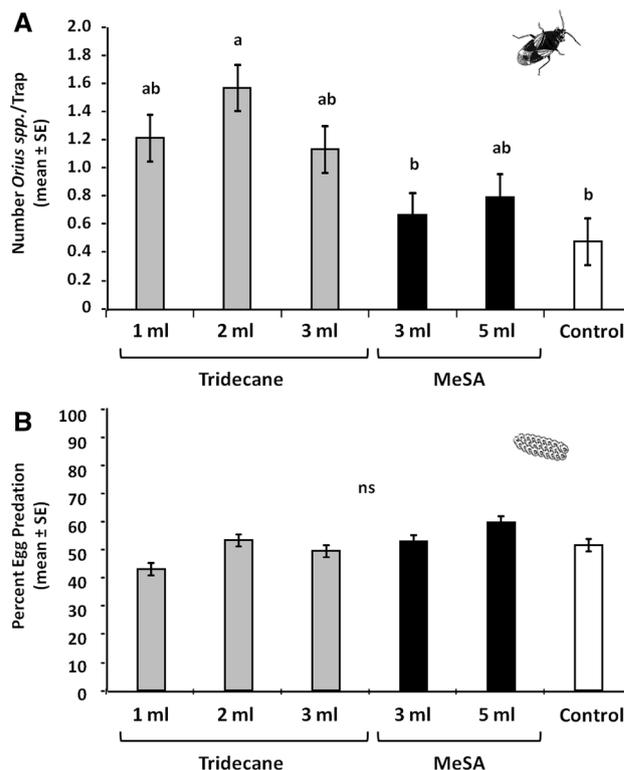
<sup>c</sup> Error  $df = 210$

<sup>d</sup> Numbers in bold are significant at  $\alpha = 0.05$

In addition to *Orius* spp. attraction, we also measured predation of sentinel eggs near baited lures. We found no effect of lure treatment on egg predation (Table 2B; Fig. 5b). We found only a significant crop-by-week interaction (Table 2B); in general, sunflower fields had significantly greater egg predation [mean percent predation =  $60.37\% \pm 4.04$  (SE)] than blueberries ( $45.30\% \pm 2.37$ ), while peaches had intermediate levels of predation ( $54.60\% \pm 3.04$ ), but this effect varied by seasonality. There were no other significant 2-way or 3-way interaction effects on egg predation (Table 2B).

## Discussion

This study shows that: (1) bean plants damaged by *H. halys* are attractive to the predaceous minute pirate bug, *O. insidiosus*, when prey are present; however, this attraction did not lead to higher egg predation; (2) tridecane was a major volatile compound emitted by both nymphs and adults of *H. halys* and by *H. halys*-damaged bean pods; (3) synthetic tridecane attracted *Orius* spp. in greenhouse and field experiments; however, in most instances, it did not affect the predator's consumption of prey eggs; (4) in



**Fig. 5** Attraction of *Orius* spp. to sticky traps baited with three different doses of tridecane, two doses of methyl salicylate (MeSA), and unbaited controls (a). And, amount of predation on *Ostrinia nubilalis* egg masses placed near vials containing these same treatments (b). Means with different letters are significantly different (Tukey's HSD test;  $P \leq 0.05$ ); ns not significant

olfactometer assays, tridecane arrested *O. insidiosus* behavior, which may explain the lack of correlation between attraction and prey consumption. These results confirm our hypothesis that *O. insidiosus* utilizes volatiles associated with *H. halys*, i.e., tridecane, during prey location.

We found attraction of *O. insidiosus* to *H. halys*-damaged plants with prey. This attraction could be due to either volatiles induced by *H. halys* feeding or volatiles emitted by *H. halys* themselves. Herbivore feeding is known to induce volatiles that can attract natural enemies (Turlings et al. 1990; Thaler 1999; James 2003), and *Orius* spp. utilize these HIPVs in prey location (James 2003, 2005). In addition, natural enemies can also use aggregation and alarm pheromones produced by stink bugs (Pentatomidae) during prey location (Aldrich et al. 1984, 1989; Aldrich 1995). For example, the egg parasitoid *Trissolcus basalus* (Wollaston) is attracted to kairomones from *Nezara viridula* (L.) (Colazza et al. 1999). *Orius insidiosus* was, however, less attracted to odors from *H. halys*-damaged beans without prey, indicating that this predator might have learned to associate these odors with the presence of a reward, such as eggs (i.e., associative learning). Although

we expected higher attraction to *H. halys*-damaged plants to lead to greater egg predation, predation of sentinel eggs by *O. insidiosus* was similar on damaged and undamaged plants; see below for a possible explanation. In our studies, we used *O. nubilalis* eggs to assess predation due to their commercial availability and because, as they are generalist predators, we did not expect differences in *O. insidiosus* preference between *O. nubilalis* and *H. halys* eggs as both are acceptable prey items (Musser and Shelton 2003; Morrison et al. 2016); however, this assumption needs to be verified in future studies.

Identifying the volatile cues used by natural enemies in host location is critical for potential manipulation of their behaviors in agro-ecosystems (e.g., Khan et al. 2008). We found that *H. halys* and *H. halys*-bean associations emit high quantities of the alkane hydrocarbon tridecane; however, the origin of tridecane in *H. halys*-bean associations is unknown. *Halyomorpha halys* is known to produce tridecane (Solomon et al. 2013; Harris et al. 2015); however, Colazza et al. (2004) reported induced emissions of tridecane from bean plants after feeding and oviposition damage by *N. viridula*. Here, we found that feeding by *H. halys* might actively induce tridecane production and emissions because damaged pods emitted more tridecane than mechanically damaged and undamaged pods, suggesting the presence of an insect oral elicitor (Peiffer and Felton 2014). Alternatively, exposed pods might have passively absorbed and released the tridecane emitted from *H. halys*. Moreover, *H. halys* nymphs produced more tridecane than adults. In contrast, Harris et al. (2015) reported 10- and 100-fold higher emissions of tridecane from groups of *H. halys* males as compared with groups of nymphs and females, respectively; we used mixed sexes, which might have been biased toward one of the sexes and may explain these discrepancies. Regardless, our and previous studies (Harris et al. 2015) identified tridecane as the major component of the *H. halys* glandular secretions.

Tridecane attracted *O. insidiosus* in greenhouse and field studies. In addition to *H. halys*, this volatile is produced by several native North American stink bugs species, including *Chlorochroa uhleri* Say, *N. viridula*, *Erthesina fullo* Thunberg, and *Thyanta pallidivirens* Stål (Aldrich 1988; Kou et al. 1989; Ho and Millar 2001; Fucarino et al. 2004). Thus, it is likely that *O. insidiosus* uses a volatile (kairomone) emitted by *H. halys* that is also common to other stink bug species, such as tridecane, to find its prey. In fact, eggs and nymphs of stink bugs, i.e., *N. viridula* and *Piezodorus guildinii* (Westwood) (Tillman 2011; Tillman et al. 2015), including *H. halys* (Morrison et al. 2016), are suitable food items for *O. insidiosus*. Interestingly, both *O. insidiosus* males and females produce pheromones for sex recognition and tridecane is a major male-specific compound in *O. insidiosus* (Aldrich et al. 2007). Although

virgin females are attracted and arrested by volatiles emitted from males, the role of tridecane in *O. insidiosus* sexual behaviors remains unknown. It is also unclear why females spent less time in the tridecane arm of the olfactometer at very low concentrations, unless there is a threshold that indicate the presence of prey at which females respond positively to tridecane; otherwise they may avoid areas emitting low levels of tridecane that are not indicative of prey presence. *Orius* spp. were not attracted to MeSA in blueberry, sunflower, and peach fields. In a meta-analysis, Rodriguez-Saona et al. (2011) showed that anthocorids as a group respond positively to MeSA. However, Rodriguez-Saona et al. (2011) and Woods et al. (2011) found no behavioral effects of MeSA on *Orius* spp. in cranberries and hop yards, respectively, indicating that attraction of these predators to MeSA might be crop dependent.

Similar to our earlier findings with plant material, attraction of *O. insidiosus* to synthetic tridecane did not lead to an increase in egg predation when tested in the greenhouse (at the lower concentrations) or in the field, which led us to speculate that *O. insidiosus* was being arrested by tridecane. This speculation was confirmed in olfactometer studies. Although many studies have documented attraction of natural enemies to plant-prey-associated volatiles, fewer studies have observed their behavior after attraction. For example, it is widely known that aphid products, such as sugar-rich honeydew (e.g., Leroy et al. 2014), and aphid alarm pheromones ((*E*)- $\beta$ -farnesene) (e.g., Beale et al. 2006) attract and arrest their natural enemies. In our study, we showed that *O. insidiosus* spends more time near the tridecane odor source, i.e., an arrestant response; particularly males that were more responsive to lower tridecane concentrations than females. These results suggest that tridecane might have distracted this predatory insect in both greenhouse and field experiments by increasing its foraging time in high tridecane-emitting areas and, as a result, limited its ability to consume more prey.

In conclusion, our study highlights the role of a prey (*H. halys*)-related volatile (tridecane) on predator (*O. insidiosus*) attraction and egg predation. Volatiles from plant-prey associations, such as HIPVs, could be used in augmentative and conservation biological control (Khan et al. 2008). Still, the chemical cues used by natural enemies of *H. halys* in prey location are largely unknown. Tridecane may serve a dual function for *O. insidiosus*, both as a kairomone in prey location and as a pheromone in mate/conspecific location. Unless the goal is to simply retain the predator in specific areas (i.e., provide a refugium), the applicability of tridecane to attract *O. insidiosus* in agricultural systems might be limited at this point because, based on our findings, attraction to tridecane does not necessarily lead to

increased predation. In addition to tridecane, *H. halys* secretes (*E*)-2-decenal—an alarm compound (Harris et al. 2015). Stink bugs, including *H. halys* (Khrimian et al. 2014; Harris et al. 2015), also produce aggregation pheromones (Aldrich 1988). Further studies are needed to investigate whether these pheromones (i.e., aggregation and alarm pheromones) alone or in combination with HIPVs can be used to manipulate natural enemy behaviors and improve biological control of *H. halys*.

## Author contributions

CR-S, JP, and DFF conceived and designed experiments. DFF and JP conducted experiments and analyzed data. DFF and CR-S wrote the manuscript. GCH and ALN provided insects and field sites. CR-S, GCH, ALN, and ACB provided financial support. All authors read and approved the manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain studies with humans participants performed by any of the authors.

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