


# Behavioral responses of the egg parasitoid *Trissolcus japonicus* to volatiles from adults of its stink bug host, *Halyomorpha halys*

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**Abstract** *Halyomorpha halys*, originating from East Asia, has become an important agricultural pest in northern temperate regions since its accidental introduction into North America in the mid-1990s and Europe in 2007. *Trissolcus japonicus* is the predominant egg parasitoid of this pest in its native range. Here, we tested the hypothesis that *T. japonicus* uses volatiles associated with *H. halys* adults during host location. The effects of these volatile compounds on *H. halys* adults were also assessed. Chemical analysis with GC-MS and electrophysiological analysis with GC-EAD revealed the presence of two

bioactive volatile compounds from *H. halys* adults, *n*-tridecane and (*E*)-2-decenal. In the Y-tube assays, female *T. japonicus* were attracted by *n*-tridecane but strongly repelled by (*E*)-2-decenal. Furthermore, host searching time of female *T. japonicus* was significantly reduced when an *H. halys* egg mass was treated with *n*-tridecane and prolonged when treated with (*E*)-2-decenal or a mixture of (*E*)-2-decenal and *n*-tridecane. Male *H. halys* were significantly attracted by *n*-tridecane, whereas females had no response to this compound in the Y-tube assays. (*E*)-2-Decenal strongly repelled male and female *H. halys* in the Y-tube assays. We discuss the multi-functional kairomonal and other possible roles of these semiochemicals at different trophic levels in the context of intra- and inter-specific chemical communication, in the *H. halys*–*T. japonicus* system.

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**Keywords** Brown marmorated stink bug · Pentatomidae · Scelionidae · Host–parasitoid interactions · Kairomones · *n*-Tridecane · (*E*)-2-Decenal

## Key message

- We tested the hypothesis that *Trissolcus japonicus* utilizes volatiles associated with *Halyomorpha halys* adults during host location.
- *n*-Tridecane and (*E*)-2-decenal were the most abundant defensive compounds released from adult *Halyomorpha halys*.
- Functioning as a kairomone, *n*-tridecane significantly attracted *T. japonicus* and reduced its host searching time.
- (*E*)-2-Decenal was confirmed to be a repellent allelomone to its parasitoid *T. japonicus*.

## Introduction

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is a polyphagous stink bug native to China, Japan and Korea (Hoebeke and Carter 2003). This stink bug was accidentally introduced into North America in the mid-1990s (Hoebeke and Carter 2003; Garipey et al. 2014), and Europe in 2007 (Wermelinger et al. 2008). Since then, BMSB has spread rapidly in northern temperate regions (Haye et al. 2015), becoming a major agricultural pest across a wide range of commodities in North America (Rice et al. 2014). Its population increased exponentially during 2004–2011, for example, at a rate of 75% per year in New Jersey (Nielsen et al. 2013). The BMSB feeds on over 120 different plants including many economically important fruits, vegetables and field crops (Leskey et al. 2012; Lee et al. 2013; Northeastern IPM Center 2014). High populations of BMSB severely damage fruit crops, with up to 90% of peaches damaged and an estimated US\$ 37 million losses in apple produce in mid-Atlantic fruit orchards in the USA (Leskey and Hamilton 2010; Leskey et al. 2012). In northern China, BMSB was one of the most serious orchard pests causing 50–80% losses in peach and pear production in the 1980s (Wang and Wang 1988; Qin 1990). However, its population is suppressed by several natural enemies in its native range, particularly hymenopteran egg parasitoids (Yang et al. 2009; 2015; Zhang et al. 2015).

*Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) (syn. *T. halyomorphae* Yang; Talamas et al. 2013) is a solitary endoparasitoid of BMSB eggs. In China, *T. japonicus* is predominant within the egg parasitoid guild (Haye et al. 2015; Talamas et al. 2015; Yang et al. 2015), with parasitism rates up to 70% and an average annual rate of 50% (Yang et al. 2009). Recently, *T. japonicus* was found to be established in the USA, presumably as a result of an accidental introduction (Talamas et al. 2015; Herlihy et al. 2016). Due to its higher parasitism rate than other parasitoids and other favorable reproductive attributes such as short developmental time and female-biased sex ratio, *T. japonicus* is a potentially effective biological control agent of BMSB (Yang et al. 2009).

Parasitoids use volatile compounds from their hosts in the process of locating and recognizing a host (Vinson 1984; Conti and Colazza 2012). Each host developmental stage is a potential source of kairomones for parasitoids. Previous studies showed that scelionid parasitoids frequently use several types of semiochemicals that originate directly from stink bugs eggs (Tognon et al. 2016), or indirectly from non-host stages of stink bugs, such as defensive compounds released from the metathoracic glands (Laumann et al. 2009). For example, *Telenomus*

*podisi* Ashmead (Hymenoptera: Scelionidae) was attracted to volatiles from egg masses of *Euschistus heros* (F.) (Hemiptera: Pentatomidae) (Michereff et al. 2016) and crude gland extracts of *E. heros* (Laumann et al. 2009). For *Euschistus conspersus* Uhler, Tognon et al. (2016) showed that the main attractive egg surface volatile was the male-produced aggregation pheromone component, methyl (2*E*,4*Z*)-2,4-decadienoate, that attracts *Telenomus podisi* and *Trissolcus erugatus* Johnson females. *Trissolcus basalis* (Wollaston) was attracted to the eggs of the *Nezara viridula* (L.) (Hemiptera: Pentatomidae) via a recognition kairomone from the adhesive coating of host eggs (Bin et al. 1993) and by the metathoracic gland secretion of host adults (Laumann et al. 2009). Furthermore, female *T. basalis* used *n*-nonadecane, a cuticular hydrocarbon from *N. viridula* adult males, to discriminate male and female hosts (Colazza et al. 2007). However, the native North American egg parasitoids, *Trissolcus erugatus* and *Telenomus podisi*, were repelled by extracts of BMSB eggs and a blend of the synthetic C<sub>16,18,20</sub> aldehydes identified from BMSB eggs (Tognon et al. 2016). Our previous study also showed that *T. japonicus* females had a higher number of trichodea sensilla than males, and papillary sensilla were only found in females. These sensilla might be involved in host recognition and acceptance during the drumming behavior, a host selection step observed in *T. japonicus* (Yang et al. 2016).

In the present study, we tested the hypothesis that *T. japonicus* uses volatiles associated with BMSB adults during host location. Therefore, we recorded electrophysiological and behavioral responses of *T. japonicus* to volatile extracts of BMSB adults and to individual antennally active compounds, respectively. Moreover, we assessed the effects of these volatile compounds to BMSB adults in order to evaluate the functions of these semiochemicals.

## Materials and methods

### Maintenance of BMSB and parasitoid colonies

A laboratory colony of BMSB was established from wild individuals collected from a peach orchard in Beijing, China (E116°12'41"; N40°02'06") in June 2016 and used for the experiments after two generations of rearings. Adults were continuously reared on a diet of pods of organic green beans (*Phaseolus vulgaris* L.) and cobs of corn (*Zea mays* L.) in rearing cages (60 × 60 × 60 cm) at 25 ± 1 °C, 65 ± 5% RH and 16 L: 8 D photoperiod. Each cage contained about 50 paired adults. Food was replaced every 2 days. Eggs were collected daily and maintained in

separate nymphal rearing cages (same size as above) under the same conditions. Newly developed adults were regularly removed from the nymphal rearing cages and evenly distributed among the adult rearing cages.

*Trissolcus japonicus* was originally obtained from egg masses of *H. halys* collected from the same peach orchard in Beijing in June 2016 and used for the experiments after four generations of rearings in the laboratory. Laboratory colonies were maintained in transparent acrylic rearing cages (25 × 25 × 25 cm) and fed daily with a cotton wick saturated with 10% honey solution under laboratory condition of 25 ± 1 °C, 65 ± 5% RH and 16 L: 8 D photoperiod. To maintain the laboratory colony, egg masses of *H. halys* from the laboratory colony were provided to *T. japonicus* in the parasitoid rearing cage. Parasitized eggs of *H. halys* were then transferred to a separate parasitoid rearing cage, and the newly emerged adult wasps were collected to use in experiments.

### Semiochemical extraction and analysis

Two BMSB females or males were randomly selected from the laboratory colony and immersed in 2 mL of hexane for 15 min in a 20-mL glass vial, which contained 200 µg *n*-nonanal as an internal standard. The solvent with volatile extracts was then transferred by a Pasteur pipette and collected with a 0.45-µm micropore filter (4 mm diameter, PFTE-4-4 Iso-Disc™, Supelco, USA) to another clean glass vial. Samples were kept at –20 °C until analyses.

Volatile extracts of BMSB adults were concentrated to 10 µL under a gentle nitrogen stream and analyzed by gas chromatography–mass spectrometry (GC-MS) using an Agilent 5973 N mass selective detector coupled with an Agilent 6890 N network gas chromatograph (GC) system equipped with a HP-5 low polar capillary column (30 m × 0.25 mm ID × 0.25 µm, J&W Scientific, Folsom, CA, USA). Each concentrated sample (1 µL) was injected into the GC-MS in splitless mode. Oven temperature was programmed at 45 °C for 1 min, then increased to 280 °C at 10 °C/min and held at that temperature for 5 min. Helium was used as the carrier gas at a constant flow rate of 1 mL/min.

Identification of chemical compounds was by comparing the mass spectra of the chromatographically resolved compounds with entries in the NIST08 database using the data acquisition software MSD Chemstation G1701EA E.01.00.237 (Agilent Technologies, Santa Clara, CA, USA). All volatile compounds showing mass spectra with match factors ≥ 90% were put on a “positive list” of tentatively identified substances, which were then compared against mass spectra of commercial standards. Finally, confirmation of a compound identity was verified

by comparison of the retention time to its authentic standard.

All synthetic chemical standards used in identification, electrophysiological analyses and bioassays were purchased commercially as follows: (*E*)-2-hexenal (97%, J&K, Beijing, China), (*E*)-2-octenal (97%, J&K, Beijing, China), *n*-nonanal (95%, J&K, Beijing, China), *n*-dodecane (99.1%, J&K, Beijing, China), *n*-tridecane (99%, Aldrich, St. Louis, USA), (*E*)-2-decenal (95%, Aldrich, St. Louis, USA) and (*E*)-2-decenyl acetate (95%, PharmaTech, Wuxi, China).

### Coupled gas chromatography–electroantennographic detection (GC-EAD)

GC-EAD was used to identify electrophysiologically active compounds of BMSB volatile extracts from *H. halys* adults. The column and temperature program of the GC was identical to that for the GC-MS analysis described above. Each antenna was prepared following standard procedures by cutting the tip and base of the antenna and immediately mounting the excised antenna between two glass capillary Ag/AgCl electrodes filled with Kaissling saline (Tang et al. 2016). The electrode at the distal end of the antenna was connected via an interface box to a signal acquisition interface board (IDAC; Syntech, Netherlands). EAD signals and flame ionization detector (FID) responses from the GC were simultaneously recorded using Syntech AutoSpike software. Three antennae were tested for each species and/or sex. Bioactive chemicals were identified by crosschecking with GC-MS data.

### Y-tube olfactometer assays

Behavioral responses of *H. halys* adults and *T. japonicus* females to synthetic chemical compounds were tested using glass Y-tube olfactometers (BMSB: stem length 20 cm, diameter 3 cm; arm length 15 cm, diameter 2.5 cm, arm angle 90°; *T. japonicus*: stem length 15 cm, diameter 1 cm; arm length 10 cm, diameter 1 cm, arm angle 90°). Unidirectional airflow was maintained downwind at 0.5 L/min for *H. halys* and 0.1 L/min for *T. japonicus* by connecting the olfactometer stem to a vacuum pump. Ambient air was charcoal-filtered and moistened with distilled water before being pulled through each arm. (*E*)-2-Decenal and *n*-tridecane were prepared in hexane and diluted to 10, 1, 0.1, 0.01 and 0.001 µg/µL solutions. A standard aliquot (4 µL) of the selected concentration was pipetted onto a piece of filter paper, exposed 20 s in the air to allow solvent evaporation, and then placed in one arm of the olfactometer. A piece of filter paper loaded with 4 µL of hexane (control) was placed inside the other arm. In each trial, a single adult *H. halys* (female or male) or *T. japonicus* female was introduced into the base of the

olfactometer stem and allowed to choose between the arm with the synthetic compounds and the hexane control arm. Bioassays with *T. japonicus* were conducted during photophase under natural light indoors without direct sun in the laboratory ( $25 \pm 2$  °C, 40–60% RH), while *H. halys* were tested during scotophase under infrared light in a dark room ( $25 \pm 2$  °C, 40–60% RH). As we observed in pretest, *H. halys* were more active during scotophase in the dark room.

Each insect was allowed 5 min to choose one of the olfactometer arms. A choice was scored positive when an insect entered the arm and either remained adjacent to the filter paper or walked within 5 cm of the filter paper. If an insect stayed in the main stem and did not make a choice within 5 min, it was recorded as no choice. At least 30 individuals were tested for each treatment, and individuals were used only once in these tests. Y-tubes were cleaned with acetone and hexane and then oven-dried at 100 °C for at least 2 h before each trial. The two arms were altered every ten tests to reduce possible position influences. Preliminary Y-tube tests showed no difference in the responses of insects when choices were two filter papers with only hexane (controls).

#### Behavioral responses of *T. japonicus* to *n*-tridecane and (*E*)-2-decenal

A fresh BMSB egg mass (12–24 h old) was placed in the center of a plastic Petri dish (diameter 9 cm). (*E*)-2-Decenal (4 µL; 100 ng/µL), *n*-tridecane (100 ng/µL) or mixture of both was sprayed on the surface of the egg mass using a glass syringe and allowed to dry for 30 s. Then, an individual 3-day-old mated *T. japonicus* female was introduced into the edge of the petri dish. The behavioral response of the *T. japonicus* female was observed for up to 30 min, and the time spent until successful oviposition attack was recorded. Fifteen *T. japonicus* females were used for each treatment, and individuals were used only once. Hexane was applied as a control with the same procedure as described above. The bioassays were conducted in the laboratory at  $25 \pm 2$  °C and 40–60% RH.

#### Statistical analyses

The proportion between the selected choices in the Y-tube experiments were arcsine-transformed before analysis. Binomial test was then used to compare the choice between treatment and control based on the null hypothesis that tested insects showed no preference for each olfactometer arm with treatment or control. Values for searching times (the time period from introduction of the parasitoid until successful oviposition attack in the petri dish) of female

parasitoids were compared with *t* tests for paired samples. If the parasitoid spent more than 30 min to locate the host eggs, its' searching time was recorded as 30 min for statistical analysis. All analyses were performed with the program SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

## Results

#### Bioactive volatile compounds released by *H. halys*

Seven compounds including (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, *n*-dodecane, (*E*)-2-decenal, *n*-tridecane and (*E*)-2-decenyl acetate were identified from BMSB adult extracts by comparison of retention time and mass spectra to those of chemical standards, except 4-oxo-(*E*)-2-hexenal was not yet confirmed with its chemical standard but based on the findings of Moreira and Millar (2005). No difference of volatile compounds was detected between male and female extracts (Fig. 1A, B). However, out of six volatile compounds identified from BMSB adults, only *n*-tridecane and (*E*)-2-decenal elicited electrophysiological antennal responses by female *T. japonicus* and male *H. halys* (Fig. 2A, B). Antennae of female *H. halys* only responded to (*E*)-2-decenal (Fig. 2C).

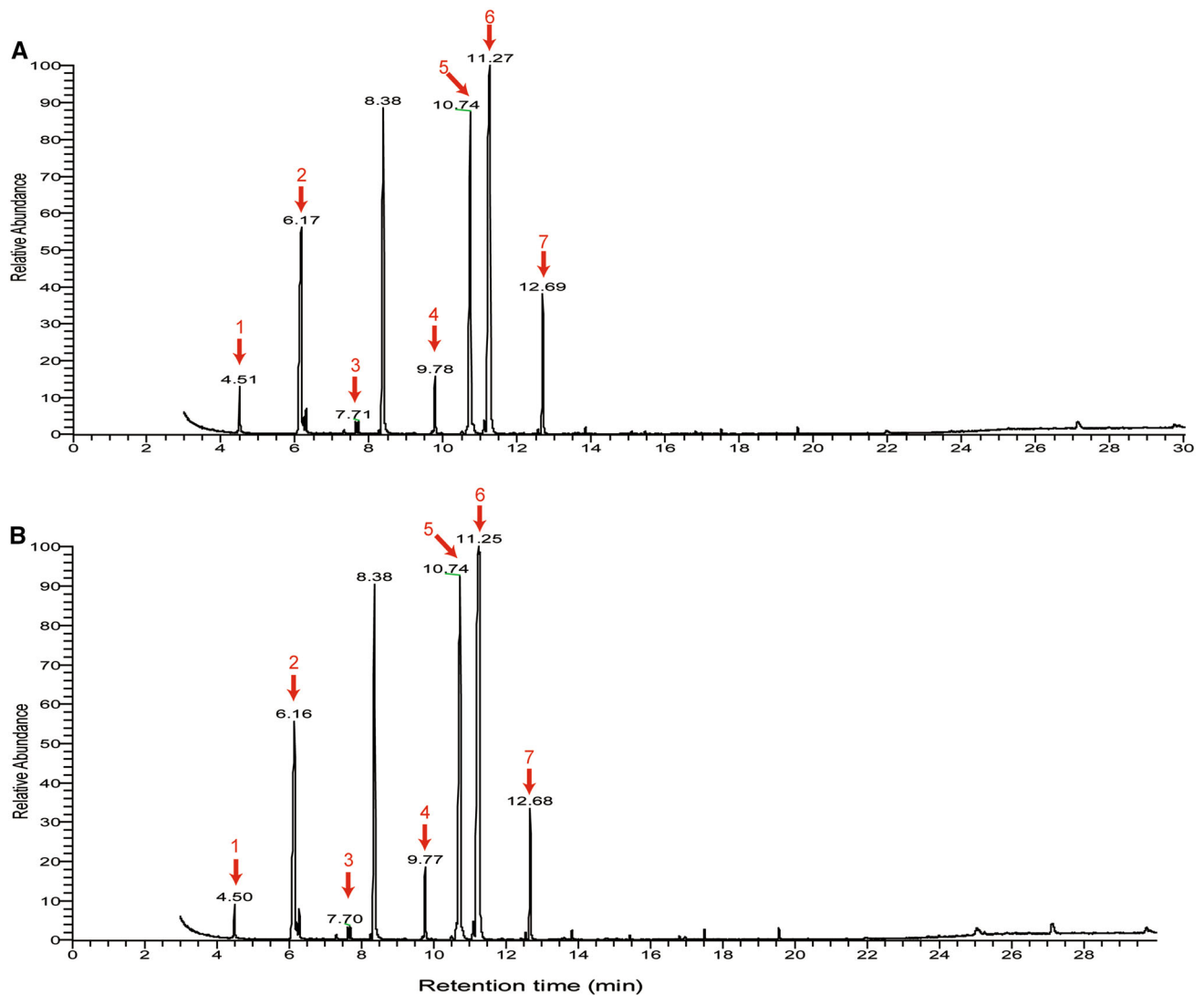
#### Y-tube olfactometer assays

In the Y-tube bioassays, *n*-tridecane significantly attracted female *T. japonicus* at a low dose of 40 ng ( $p = 0.016$ ) (Fig. 3A) and significantly attracted male *H. halys* at high doses (4000 ng,  $p < 0.001$ ; 40,000 ng,  $p = 0.005$ ) (Fig. 3B). However, female *H. halys* had no preference to *n*-tridecane ( $p > 0.05$ , at five treatment concentrations) (Fig. 3C).

In contrast to the results observed with *n*-tridecane, (*E*)-2-decenal significantly repelled female *T. japonicus* from low to high doses of 40 ng ( $p = 0.016$ ), 400 ng ( $p = 0.001$ ), 4000 ng ( $p = 0.005$ ) and 40,000 ng ( $p = 0.043$ ) (Fig. 3D). Similarly, (*E*)-2-decenal at higher dosages significantly repelled both *H. halys* males (4000 ng,  $p = 0.035$ ; 40,000 ng,  $p = 0.043$ ) and females (400 ng,  $p = 0.043$ ; 4000 ng,  $p = 0.050$ ; 40,000 ng,  $p = 0.043$ ) (Fig. 3E, F).

#### Behavioral responses of *T. japonicus* to *n*-tridecane and (*E*)-2-decenal

The host searching time of female *T. japonicus* was significantly reduced when *n*-tridecane was added to the surface of BMSB egg mass ( $166.8 \pm 22.40$  s) in comparison with the control ( $381.6 \pm 38.18$  s;  $t = 3.967$ ,  $df = 14$ ,  $p = 0.0014$ ) (Fig. 4). On the contrary, the searching time



**Fig. 1** Total ion current chromatogram from *Halyomorpha halys* body extracts, **A** female and **B** male ( $N = 3$ ). Peaks of compounds (from left to right) were identified as (1) (*E*)-2-hexenal, (2) 4-oxo-(*E*)-

2-hexenal, (3) (*E*)-2-octenal, (4) *n*-dodecane, (5) (*E*)-2-decenal, (6) *n*-tridecane, and (7) (*E*)-2-decenyl acetate. *n*-Nonanal was used as the internal standard substance with a retention time of 8.38 min

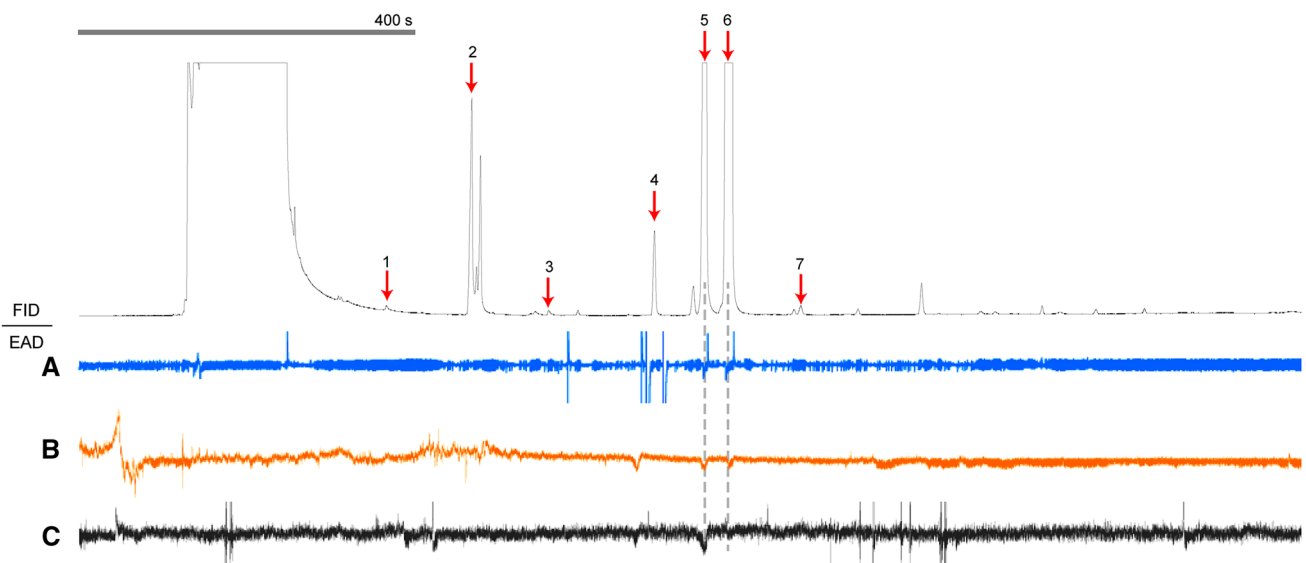
of female *T. japonicus* was significantly prolonged by (*E*)-2-decenal alone ( $1533.3 \pm 119.69$  s;  $t = 9.372$ ,  $df = 14$ ,  $p < 0.0001$ ) and mixture of (*E*)-2-decenal and *n*-tridecane ( $1315.4 \pm 339.63$  s;  $t = 5.137$ ,  $df = 14$ ,  $p = 0.0002$ ) (Fig. 4). Only 4 and 5 *T. japonicus* females (out of 15 in total) successfully located the BMSB eggs within 30 min when (*E*)-2-decenal was added alone or mixed with *n*-tridecane.

## Discussion

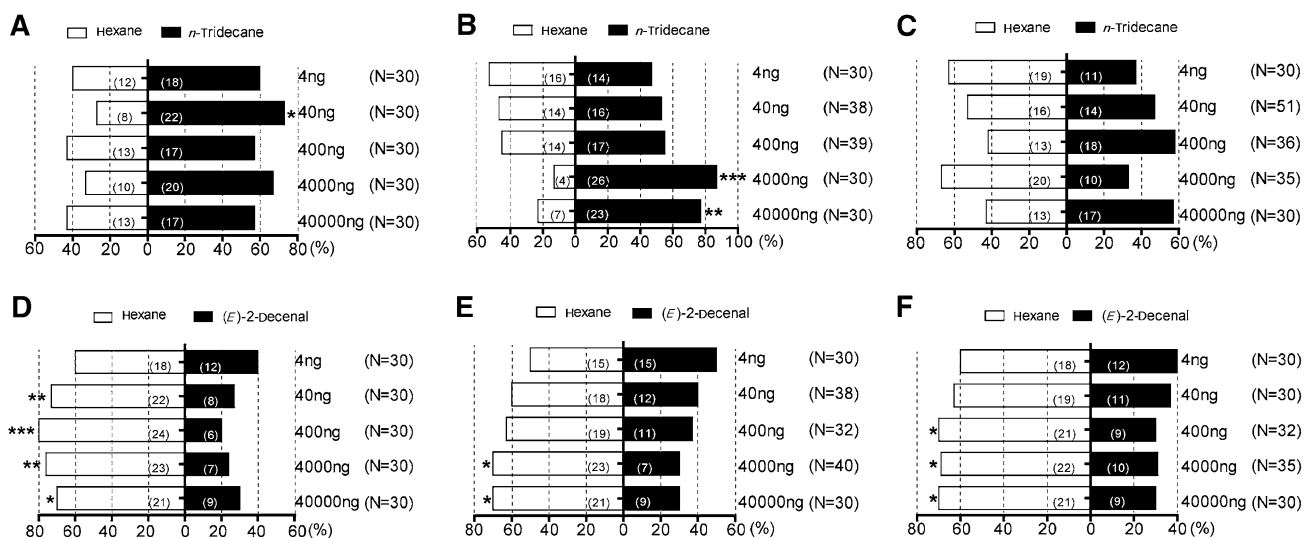
Heteroptera species are known for producing defensive compounds that serve the dual purpose of defense against natural enemies and as alarm pheromones (Aldrich 1988; Kou et al. 1989; Borges and Aldrich 1992; Krall et al.

1999; Zarbin et al. 2000; Pareja et al. 2007; Noge et al. 2012; Solomon et al. 2013). Our present study showed that seven defensive compounds, (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, *n*-dodecane, (*E*)-2-decenal, *n*-tridecane and (*E*)-2-decenyl acetate, were extracted from *H. halys* males and females, although only *n*-tridecane and (*E*)-2-decenal elicited electrophysiological antennal responses from BMSB adults and *T. japonicus* females. Further behavioral bioassays showed that female *T. japonicus* and male *H. halys* were attracted by *n*-tridecane but strongly repelled by (*E*)-2-decenal, indicating the multifunctionality of defensive chemicals at different trophic levels of the *H. halys*–*T. japonicus* system. These results also confirm our hypothesis that *T. japonicus* uses volatiles associated with BMSB adults, i.e., *n*-tridecane, during host location.





**Fig. 2** GC-EAD recordings of *Trissolcus japonicus* female **A** and *Halyomorpha halys* male **B** and female **C** to volatile extracts of *H. halys* adults ( $N = 3$ ). GC peaks were identified as (1) (*E*)-2-hexenal, (2) 4-oxo-(*E*)-2-hexenal, (3) (*E*)-2-octenal, (4) *n*-dodecane, (5) (*E*)-2-decenal, (6) *n*-tridecane, and (7) (*E*)-2-decenyl acetate

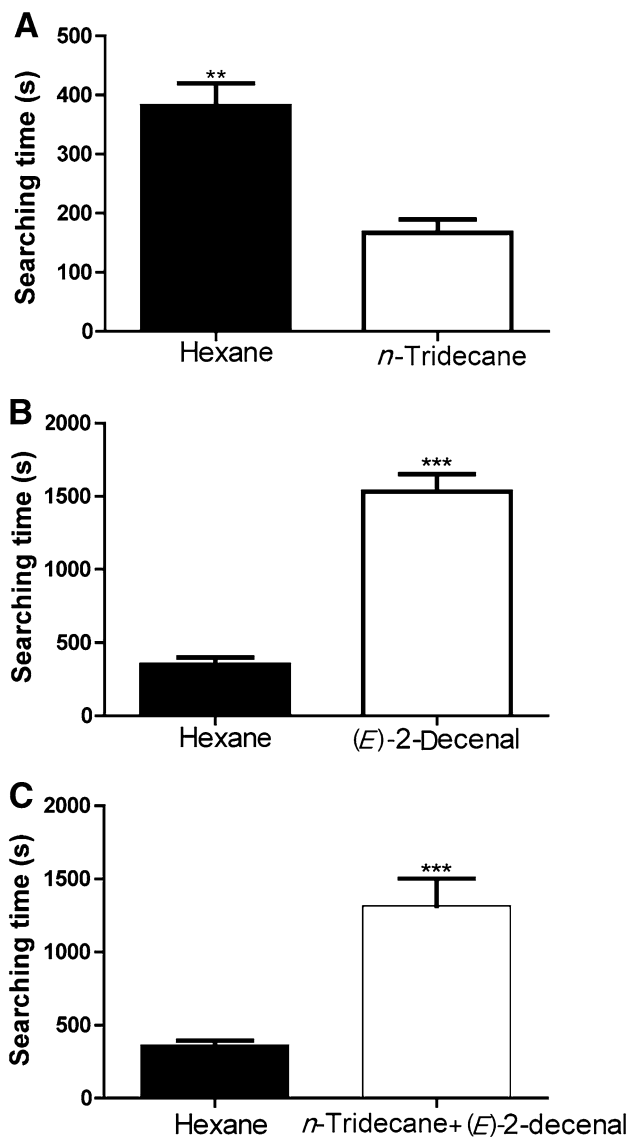


**Fig. 3** Olfactory responses of *Trissolcus japonicus* and *Halyomorpha halys* to *n*-tridecane (**A**, *T. japonicus* female; **B**, *H. halys* male; **C**, *H. halys* female) and (*E*)-2-decenal (**D**, *T. japonicus* female; **E**, *H. halys*

male; **F**, *H. halys* female) in Y-tube olfactometer assays. Binomial test; \*  $0.01 < p \leq 0.05$ ; \*\*  $0.001 < p \leq 0.01$ ; \*\*\*  $p \leq 0.001$

Both (*E*)-2-decenal and *n*-tridecane were also detected in other previous studies with *H. halys* (Solomon et al. 2013; Harris et al. 2015; Fraga et al. 2016). They were released from all nymphal instars and adult BMSB. Males and nymphs produced similar amounts of *n*-tridecane, but females produced less. Conversely, nymphs produced more (*E*)-2-decenal than males or females (Harris et al. 2015). The differences in the amounts of these two chemicals released by the different BMSB life stages and sexes may be related to their different functions at different life stages of BMSB. Female BMSB showed no olfactory response to

*n*-tridecane in both the GC-EAD analysis and the Y-tube assays, while BMSB males were attracted by *n*-tridecane in the Y-tube assays. Harris et al. (2015) suggested that *n*-tridecane could reduce the amount of aggregation pheromone produced by male BMSB. We deduced that *n*-tridecane might be involved in mediating chemical communications among BMSB males to regulate aggregation or mating location behavior. Furthermore, *n*-tridecane has also been reported in other pentatomids such as *Euschistus bififormis* Stål (Noge et al. 2012) and *E. conspersus* (Tognon et al. 2016).



**Fig. 4** Searching time (mean  $\pm$  SE) of *Trissolcus japonicus* in locating host eggs in a Petri dish when *n*-tridecane **A** (*E*)-2-decenal **B** or their mixture **C** was added, and hexane as control ( $N = 15$ ). Paired *t*-tests; \*\*  $0.001 < p \leq 0.01$ ; \*\*\*  $p \leq 0.001$

(*E*)-2-Decenal was previously reported as a major component of the defensive compounds in the pentatomids *Acrosternum aseedum* Rolston, *Chinavia impicticornis* (= *Acrosternum impicticorne*) (Stål), *Chinavia ubica* (= *Acrosternum ubicum*) (Rolston), *Cosmopepla bimaculata* (Thomas), *E. tristigmus* (Say) and *N. viridula* (Aldrich et al. 1984; Borges and Aldrich 1992; Mattiacci et al. 1993; Pavis et al. 1994; Krall et al. 1999; Pareja et al. 2007; Fávoro et al. 2011). This compound functions as an allomone against some animals, such as the Chinese praying mantid, *Tenodera aridifolia sinensis* Saussure (Mantodea: Mantidae) (Noge et al. 2012), birds (Aldrich, 1988) and green lizards (Gregorovičová and

Černíková 2015). We confirmed that (*E*)-2-decenal is a repellent to *T. japonicus* that significantly extends the host searching time of this parasitoid. However, (*E*)-2-decenal attracted *T. basalis* and stimulated searching behavior (Mattiacci et al. 1993; Laumann et al. 2009). As the aldehyde associated with alarm pheromone detected from the male metathoracic scent gland of the pentatomid *Erthesina fullo* (Thunberg) (Kou et al. 1989), (*E*)-2-decenal would also function as an alarm pheromone for *H. halys* and repelled both male and female *H. halys* at higher dosages. However, the exact biological function of (*E*)-2-decenal still needs further study.

Here, we report for the first time that *n*-tridecane is a kairomone used by *T. japonicus* to locate BMSB eggs and searching efficiency was significantly improved in laboratory bioassays. Interestingly, *n*-tridecane was also released by BMSB-damaged bean pods and found to attract the predator bug *Orius insidiosus* (Say) (Hemiptera: Anthoridae) in greenhouse and field situations although there was no effect on BMSB egg predation (Fraga et al. 2016). The semiochemistry of *H. halys* and *Trissolcus* and *Telenomus* spp. egg parasitoids has recently been studied in more detail, showing that volatiles found on the surface of eggs are critical for host recognition (Tognon et al. 2016). Further research is still required to understand the complexity of these semiochemicals in the context of tritrophic interactions of host plants–BMSB–*Trissolcus japonicus* in order to develop more effective biological control program for sustainable management of *H. halys* in agroecosystems.

#### Author contributions

YZZ, JPZ, GHC and FZ conceived and designed the experiments. YZZ, JPZ, and HXZ conducted experiments. YZZ, LLR and RT analyzed the data. JPZ, GHC and FZ wrote the manuscript. All authors took part in discussing, reading and approving 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** This article does not contain any studies with human participants or vertebrate performed by any of the authors.

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