

Olfactory responses of *Trissolcus mitsukurii* to plants attacked by target and non-target stink bugs suggest low risk for biological control

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Abstract

Field release of classical biocontrol agents requires prior risk assessment to ensure that non-target effects are averted. No-choice and choice bioassays can provide basic information on the physiological host range of the candidate agent. However, other experimental methods, like olfactometer bioassays of host-plant complexes, can depict more realistically the likelihood that the released biocontrol agent localizes non-targets in the field. *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) is native to Asia and invasive in North America and Europe, where it represents a major threat for crops. The Asiatic egg parasitoid *Trissolcus mitsukurii* (Ashmead) is adventive in Italy and exhibits high parasitization ability on *H. halys*. In the light of evaluating *T. mitsukurii* for *H. halys* biocontrol, its physiological host range has been already partially explored. Here, we investigated the ability of the parasitoid to exploit odours associated with *H. halys* or with the following non-targets: *Arma custos* F., *Dolycoris baccarum* L., *Eurydema ventralis* Kolenati, and *Nezara viridula* L. (all Hemiptera: Pentatomidae). Specifically, using a Y-tube olfactometer, we tested the response of parasitoid females to plants bearing naturally laid eggs, to physogastric females or eggs of the different species. We observed that *T. mitsukurii* is only attracted by plants challenged by eggs of *H. halys* or *N. viridula*, while it is not attracted by physogastric females or eggs alone. Remarkably, *T. mitsukurii* was almost repelled by plants bearing eggs of the beneficial *A. custos*. Our results usefully contribute to a more valuable assessment of the potential non-target risks in case of parasitoid release.

Key Message

- *Trissolcus mitsukurii* is an oligophagous candidate biocontrol agent of *Halyomorpha halys*
- *T. mitsukurii* ability to exploit long-range odours associated with plant-host complexes was unknown
- *T. mitsukurii* was attracted by plants bearing eggs of *H. halys* or *Nezara viridula*
- Conversely, the parasitoid was repelled by plants bearing eggs of the predator *Arma custos*
- Olfactometer bioassays can provide reliable data on host location likelihood in field

Introduction

Management of invasive arthropod species in agricultural settings encompasses multiple strategies, including the introduction and release of exotic natural enemies (Kenis et al. 2017). The release of a biocontrol agent that is coevolved with the target herbivore allows the restoration of ecological processes, with positive implications for pest suppression (Hoddle 2004). On the other hand, biocontrol agents can have negative effects on the community of native natural enemies, with consequences for local biodiversity that can hardly be foreseen (Van Lenteren et al. 2006; de Clercq et al. 2011; Roy et al. 2016). Therefore, selection of biological control agents is a crucial aspect for maximizing the benefits of pest control and, at the same time, minimizing ecological risks (Loomans 2021). Recent national and

international regulations require that applications for licensing candidate biocontrol agents are supported by thorough risk assessment documentation (Mason et al. 2017). Simple evaluation of the physiological host range provides a base ground and a first understanding of whether a natural enemy can be suitable to become a biocontrol agent of a given target pest (Abram et al. 2021; Sabbatini-Peverieri et al. 2021). Such protocols typically encompass no-choice and choice laboratory bioassays in small settings, i.e., Petri dish arenas, or larger insect cage tests (Haye et al. 2020). Remarkably, effective interactions of the introduced natural enemy in the field and the general impact on local community depend on many ecological factors. Among these, the ability of the natural enemy to exploit odours from challenged plants (synomones) and/or host instars (kairomones) at a long-range distance (Hilker and Meiners 2008; Hilker and Fatouros 2016). In the hierarchical process of host/prey location, these odours can help the natural enemy to efficiently locate the host (Conti and Colazza 2012; Meiners and Peri 2013). However, when dealing with exotic species, the reliability of host-associated cues could be hardly predictable as a consequence of the lack of coevolutionary history between members of the plant-host-parasitoid tritrophic system (Conti et al. 2004; Desurmont et al. 2014; Martorana et al. 2017). Therefore, incorporating olfactometer bioassays in pre-release risk assessment can be crucial for determining the likelihood of the species interactions in open field conditions (Conti et al. 2004; Ferracini et al. 2015; Avila et al. 2016; Bertoldi et al. 2019). Here, we explored this concept on an exotic egg parasitoid as a candidate biocontrol agent of an invasive stink bug and conducted olfactometer bioassays to investigate behavioural responses towards odours associated with target and non-target species.

The Brown Marmorated Stink Bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), is an economically important invasive pest of major concern worldwide (Leskey and Nielsen 2018). Its aptitude to aggregate and hide in small spaces, e.g., shipping containers, facilitates its worldwide spread (Nixon et al. 2019). Indigenous of East Asia, this species was accidentally introduced in the US and detected firstly in 1996 (Hoebeke and Carter 2003). The feeding on many plant species, including a large number of crops and fruit plants, caused about US \$37 million losses to the apple crop in the US in 2010 (Leskey and Nielsen 2018). In Europe, *H. halys* was first observed in 2004 (Switzerland) and since then discoveries have multiplied in the European countries, including Italy (Haye et al. 2014; Cianferoni et al. 2018; Maistrello et al. 2018). After its establishment in Italy, *H. halys* has damaged field crops and orchards, causing serious economic damage with fruit losses that were estimated at nearly EUR 600 million in 2019 (Bariselli et al. 2016; CSO Italy 2020; Rot et al. 2021).

Current management strategies against *H. halys* include chemical, biotechnological and physical control, while classical and augmentative biological control are promising methods under investigation (Conti et al. 2021; Zapponi et al. 2021). The most effective natural enemy of *H. halys* appears to be the Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), which exhibits high field parasitism rates in its original area (Yang et al. 2009; Abram et al. 2017; Zhang et al. 2017). Adventive populations of *T. japonicus* have been documented in North America and Europe, where this species apparently tracked its host in the invaded areas (Kaser et al. 2018; Abram et al. 2019; Haye et al. 2020). Additionally, feral populations of another Asian egg parasitoid species, *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae), have been recently detected in northern Italy on *H.*

halys egg masses (Sabbatini-Sabbatini-Peverieri et al. 2018; Moraglio et al. 2020; Scaccini et al. 2020). Frequently described as an indigenous parasitoid of *Nezara viridula* L. (Hemiptera: Pentatomidae) in Japan (Hokyo and Kiritani 1963, Arakawa et al. 2004), *T. mitsukurii* parasitizes also *H. halys* and other stink bug species in China and Japan (Ryu and Hirashima 1984; Arakawa and Namura 2002; Chen et al. 2020). In Italy, adventive populations of *T. mitsukurii* have shown high parasitism efficacy on *H. halys*, comparable to that of *T. japonicus* (Scaccini et al. 2020). This considering, *T. mitsukurii* is reckoned as a promising egg parasitoid of *H. halys* in Italy and neighbouring countries (Bout et al. 2021; Rot et al. 2021), and is currently under evaluation for the development of a pre-emptive biological control program of the stink bug in Australia and New Zealand (Caron et al. 2021).

A detailed screening of *T. mitsukurii* host range using choice and no-choice Petri dish experiments was conducted previously and revealed oligophagy of this parasitoid limited to members of the Pentatomoidea superfamily (Giovannini et al. 2021). However, the capability of this parasitoid to exploit odours from the plant-host system for locating stink bug eggs in the field is unknown. Results from previous studies revealed that *Trissolcus* spp. are primarily attracted by oviposition-induced plant synomones (Colazza et al. 2004; Conti et al. 2010; Tognon et al. 2016; Borges and Blassioli-Moraes 2017; Bertoldi et al. 2019). Here we hypothesized that the oligophagous behaviour shown by *T. mitsukurii* during assessment of its physiological host range (Giovannini et al. 2021) would result narrower if the chemical ecology of this parasitoid is investigated. Specifically, only odours associated with those host species that exhibit coevolutionary history with *T. mitsukurii* would elicit behavioural responses of the parasitoid. Therefore, we conducted olfactometer bioassays and tested whether the parasitoid differently responds to odours from plants exposed to oviposition of *H. halys* or non-target native stink bugs, specifically *Arma custos* F., *Dolycoris baccarum* L., *Eurydema ventralis* Kolenati (Hemiptera: Pentatomidae) and *N. viridula*. Odours from physogastric females and egg masses alone were also tested. Of the different species, *H. halys* and *A. custos* naturally occur in *T. mitsukurii* native area (De Clercq 2000; Hamilton et al. 2018). The other stink bugs evaluated, *D. baccarum*, *E. ventralis* and *N. viridula*, also occur in Asia, where their presence should be considered outside their native range (Panizzi et al. 2000; Rider et al. 2002; Rider 2006; Esquivel et al. 2018).

Results of this investigation would help understanding the possible ecological impact of the exotic parasitoid *T. mitsukurii* in novel ecosystems. Additionally, they would provide base ground data for the preparation of a risk-assessment document, in support of a petition for releasing the parasitoid in areas that are seriously infested by *H. halys*.

Materials And Methods

Origin of insects and rearing

Stink bug colonies were established from adults collected in spring and summer 2020 in Northern and Central Italy, during entomological field trips in fruit orchards, herbaceous crops and uncultivated areas. Adult stink bugs were collected by sweep netting or visual handpicking on grasses, bushes and trees.

Field collected adults were transferred to the laboratory and reared in insect cages (BugDorm 4F4545, Insect MegaView Science Co. Ltd., Taichung, Taiwan) under environmental controlled conditions ($25 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and 16:8 h L:D). Phytophagous stink bugs were maintained with a mixed diet based on fruits, vegetables and seeds, whereas the predatory *A. custos* was fed with *Tenebrio molitor* L. pupae (Coleoptera: Tenebrionidae). Food was replaced three times per week. A daily wetted cotton piece placed inside an opened Petri dish was used for water provision. About 10 paper towels (20 cm \times 20 cm) were added inside each rearing cage to provide an oviposition substrate.

Trissolcus mitsukurii populations initiated from *H. halys* parasitized egg masses originally collected in fruit orchards in north-eastern Italy. The parasitoid colony was yearly replenished with new field-collected specimens (Sabbatini-Peverieri et al. 2018). For breeding maintenance, 1-d-old *H. halys* egg masses were exposed to a parasitoid female for 24h. The wasps were held in glass tubes (2 cm diam. \times 15 cm length) sealed on both sides with a plastic mesh. A diet of honey droplets was dispensed on a rectangular cardboard (2 cm large \times 4 cm length), offered to parasitoids and replenished two times per week. Males and females were kept together to permit mating and 7-d-old females were isolated in glass tubes before bioassays. Maintenance of the egg parasitoid colony (at $25 \pm 1^\circ\text{C}$, $60 \pm 5\%$ and 16:8 h L:D) and all olfactometer bioassays (see below) were conducted under officially authorized quarantine conditions at CREA facilities (DG/DISR/DISR05/0013647-19/04/2018).

Plant rearing and exposure for stink bug oviposition

Seeds of soybean, *Glycine max* (L.) Merrill, and cauliflower, *Brassica oleracea* var. *botrytis* L., were sown in plastic pots (6.5 cm height \times 5.5 cm diam. at mid-height) containing a horticultural substrate. Plants were grown and maintained in a rearing room ($24 \pm 2^\circ\text{C}$, $55 \pm 10\%$ RH, 16: 8 h L:D) and irrigated every 2 d. A mixture (1.4 g/L) of fertilizer (5-15-45, NPK, Plantfol, Valagro, Italia) was added one week after plant emergence. Two plants, 2- to 4-weeks old (approx. 15-20 cm tall) depending on plant species, were placed inside an insect cage containing mated females of each colony of the stink bug species tested. In details, soybean plants were used for *H. halys*, *D. baccarum*, *N. viridula*, and *A. custos*, while cauliflower plants were used for *E. ventralis*. Plants were checked twice per day for the presence of egg masses laid on the abaxial leaf surface. Whenever eggs were detected, plants were removed from the cages and tested for their attractiveness within 24 h. If no eggs were found after 3 d of exposure, plants were replaced with new clean plants.

Odour sources tested in the bioassays

Using a Y-tube olfactometer we tested the behavioural responses of *T. mitsukurii* to plant-host odours associated with native and non-native stink bugs. For each of the five stink bug species, i.e., *A. custos*, *D. baccarum*, *E. ventralis*, *H. halys*, and *N. viridula*, the following treatments were evaluated:

a) *Plant + Eggs*: a plant exposed to stink bug feeding and bearing 1 naturally laid egg mass (< 24 h old);

b) *Females*: a batch of four females in the ovipositional phase, i.e., with physogastric abdomen (Polajnar et al. 2016; Rondoni et al. 2017a);

c) *Eggs*: about 100 eggs of < 24 h old. Only egg masses laid on paper towels were tested.

Controls consisted of clean air (AIR) for treatments with females and eggs, or of an insect-unexposed soybean or cauliflower plant (CLEAN PLANT) for treatments with plants.

Olfactometer bioassays

The female behavioural responses to the different odour stimuli were investigated in a Y-tube olfactometer (common stem: 90 mm length; arms: 80 mm length each at 100° angle between arms and equally distant from the common stem, internal section: 15 mm × 10 mm), carved in a plexiglass plate (200 mm × 190 mm × 10 mm thick) and sandwiched between two glass sheaths (each plate: 200 mm × 150 mm × 5 mm thick), which provided the upper and lower closure of the olfactometer. Two identical Y-tube olfactometers were simultaneously used, allowing to observe the behaviour of two parasitoids at the same time. A stream of environmental air was inflated by a diaphragm pump (KNF Italia S.r.l., Milan, Italy) through an activated charcoal filter (260 mm length × 40 mm internal diam.). The airflow was humidified through a Dreschel bottle (250 mL volume) containing distilled water and split into two identical routes, each consisting of a flowmeter that regulated the airflow at 0.8 L/min and a glass chamber (30 cm height × 10 cm internal diam., ~1.9 L volume) containing the odour source (treatment or control cue). The glass chamber was sealed at the base to a Teflon disk (7 mm height, 14 cm diam.) using Parafilm M[®] sealing film (Heathrow Scientific, Vernon Hills, IL, USA). The airflow was conveyed using tubes in Silicone (6 mm internal diam.). Tubes were connected to the chamber by means of plastic opened screw caps (Kartell Spa, Noviglio, Italy). The airflow coming out from the glass chamber was eventually split again into two and conveyed to one of the two arms of both olfactometers. A digital flowmeter (mod. GFM17, Aalborg, New York, USA) was used to measure the flow rate entering each olfactometer arm (~200 mL/min). The olfactometer device was surrounded by a black fabric curtain to minimize external cues from the room and was illuminated by two 36 W cool white fluorescent tubes located above the device. About 30 min before the bioassays, parasitoids, stink bugs and plants were moved to the bioassay room, maintained at 25 °C, to acclimatize. Bioassays were conducted from 09:00 to 16:00. In detail, a parasitoid female was introduced into the central stem of the Y-tube and the behaviour was recorded. After 4 bioassays the position of the tubes entering the Y-tube arms was switched, to avoid possible bias. Additionally, the glass plates were cleaned with a laboratory detergent, rinsed with tap water and acetone, whereas the plexiglass part of the olfactometer was cleaned with detergent, rinsed with tap water, and finally rinsed with distilled water. Each *T. mitsukurii* female was observed once for 10 min. The insect residence time, i.e., the time spent in each olfactometer arm and in the common stem was visually recorded with JWatcher 1.0 (Blumstein et al. 2006; Rondoni et al. 2021). For each treatment, depending on insect and egg masses availability, 3-4 repetitions of the same odour stimulus (plant bearing an egg mass, stink bug female, or eggs) were tested. Fifty-six to 74 parasitoids

were eventually evaluated for each treatment with a plant bearing an egg mass, 44 to 70 parasitoids for stink bug female tests, and 32 to 77 insects for the stink bug eggs tests.

Statistical analyses

The walking behaviour of the parasitoid in the olfactometer was described by the residence time, i.e., the time spent by the female in each olfactometer arm, and by the first choice, i.e. first entrance in either olfactometer arm. Females that did not exhibit any choice or only entered in either olfactometer arm for a limited time (< 30 s) were considered not responding and were discarded from the analysis (similar to Peri et al. 2011). For the analysis, the logarithmic transformation of the ratio between the residence time in the treatment arm versus the residence time in the control arm was calculated. This transformation (log-ratio) ensured that only one measure per insect was later analysed (Rondoni et al. 2017b). Generalized linear models (GLMs) with Gaussian error distribution (for residence time data) or with binomial error distribution (for first choice data) were fitted to test differences of treatment versus control within each odour source. Analyses were conducted under R statistical environment (R Core Team 2020).

Results

Behavioural responses to odours from plants bearing an egg mass

Trissolcus mitsukurii females positively responded to odours associated with the target host (soybean plant with an egg mass of *H. halys*) as its residence time in the treatment arm was higher compared to that in the control arm (contrast result for Gaussian GLM: $P = 0.012$) (Figure 1, Table S1). Similarly, *T. mitsukurii* was attracted (higher residence time compared to control) to odours associated with *N. viridula* (soybean plant with an egg mass) ($P = 0.029$). Noteworthy, parasitoids appeared to be almost repelled by odours associated to *A. custos* (soybean with an egg mass), displaying a lower residence time in treatment vs. control arm ($P = 0.028$). In contrast, *T. mitsukurii* did not respond to odours associated with *D. baccarum* (soybean plant with an egg mass) or *E. ventralis* (cabbage plant with an egg mass), as residence time was similar between control and treatments ($P \geq 0.26$ for both comparisons). First choice data confirmed the preference of female egg parasitoids for plants carrying *H. halys* eggs (contrast results for binomial GLM: $P = 0.0062$) (Figure 1, Table S2). Conversely, first choices were similar between control and treatments for all the other tested species ($P \geq 0.17$).

Behavioural responses to odours from stink bug females

Trissolcus mitsukurii females did not prefer *H. halys* ($P = 0.95$) nor those of the indigenous species, *A. custos*, *D. baccarum*, *E. ventralis* and *N. viridula* females as residence time was similar in the treatment and control arms ($P \geq 0.34$ for all the comparisons) (Figure 2, Table S3). Correspondingly, first choice data did not differ between control and treatments ($P \geq 0.40$ for all comparisons) (Figure 2, Table S4).

Behavioural responses to odours from stink bug eggs

Females of *T. mitsukurii* did not exhibit any attractiveness towards *H. halys* ($P = 0.85$), or of all other indigenous stink bugs, as residence times in treatment and control did not differ ($P \geq 0.11$ for all the comparisons) (Figure 3, Table S5). First choice analysis confirmed the absence of a significant attractivity of the eggs of all stink bug species tested ($P \geq 0.13$ for all comparisons) (Figure 3, Table S6).

Discussion And Conclusions

Among the different stink bug-associated odours tested in the olfactometer, only those from plants bearing an egg mass of *H. halys* or *N. viridula* elicited positive attraction in *T. mitsukurii* females. These results validated our hypothesis that a more in-depth evaluation of the parasitoid host specificity, through chemical ecology investigation, would further restrict the parasitoid host range that was depicted by physiological host range assays. Indeed, several non-coevolved hosts, although accepted under laboratory simplified conditions (i.e., no-choice and paired choice black-box tests), would be hardly located in the field due to lack of suitable host-associated odours. This interpretation conceptually agrees with results from prior studies on *T. japonicus*, another *H. halys* exotic biocontrol agent that is currently released in Italy (Zapponi et al. 2021). In fact, *T. japonicus* displayed a relatively wide physiological host range in laboratory choice and no-choice assays (Zhang et al. 2017; Haye et al. 2020; Sabbatini-Peverieri et al. 2021), but a more restricted oligophagy in olfactometer or in field conditions (Bertoldi et al. 2019; Milnes and Beers 2019).

Our results also indicate that soybean plants challenged by *H. halys* can emit oviposition-induced plant volatiles that are detected by the coevolved parasitoid *T. mitsukurii*. This response is consistent among residence time and first choice data. Volatile emission induced by host oviposition represents an indirect defence for the plants, as already demonstrated in different systems involving *Trissolcus* egg parasitoids (Colazza et al. 2004; Conti et al. 2010; Bertoldi et al. 2019), and is an exceptionally reliable signal of the presence of target hosts in the canopy (Hilker and Meiners 2008; Conti and Colazza 2012). Induced plant volatiles act on long-distance range and are easily detected by the egg parasitoids, allowing them to rapidly locate the host that is suitable only for a short period of time (Vet and Dicke 1992; Colazza et al. 2004). The ability of *T. mitsukurii* to exploit oviposition-induced plant volatiles for locating *H. halys*, although it is investigated here for the first time, was recently hypothesized following results of open field surveys in northeast Italy or France (Zapponi et al. 2020; Bout et al. 2021). For instance, higher parasitization of *H. halys* by *T. mitsukurii* was detected in naturally-laid eggs compared to sentinel eggs (Zapponi et al. 2020). In other two studies, *T. mitsukurii* exhibited remarkable discovery efficiency of naturally laid eggs on plant tissues, with 27.3 to 46.5 % of parasitised egg masses (Scaccini et al. 2020; Bout et al. 2021).

Noteworthy is also the positive attraction of *T. mitsukurii* towards plants bearing eggs of non-coevolved *N. viridula*. Our result, together with the fact that *T. mitsukurii* positively responds to tracks of *N. viridula* females (Scala et al. 2021), would suggest high ability to locate eggs in the field. Indeed, the parasitoid is considered a main enemy of *N. viridula* in Japanese areas where the stink bug has established (Hoyko et al. 1996; Arakawa et al. 2004). Surprisingly, despite this remarked discovery ability, the emergence rate

displayed by *T. mitsukurii* was null or very low, in the case of the adventive Italian population (Scaccini et al. 2020; Giovannini et al. 2021), and moderate (e.g., ~40%) for Japanese strains (Kiritani and Hôkyo 1962; Hokyo et al. 1966). This mismatch between the positive response of *T. mitsukurii* to cues associated with *N. viridula* and the low suitability of this host for parasitoid development is partially consistent with lack of coevolution, as *N. viridula* is of Ethiopian-South Mediterranean origin (Vinson 1975; Esquivel et al. 2018; Konopka et al. 2018). However, this does not explain why the parasitoid responds to cues associated with the novel host. A hypothetical explanation for this could be that *N. viridula* induces in soybean a defensive response that might be similar to that induced by other herbivores, like *Nezara antennata* Scott, native to Eastern Asia (Panizzi et al. 2000) and listed as host of *T. mitsukurii* (Ryu and Hirashima 1984). It is known that *N. viridula* and *N. antennata* share common volatile compounds (Borges and Blassioli-Moraes 2017), however, whether they also induce similar plant responses is unknown.

Previous choice and no-choice bioassays underlined high acceptance and suitability of *D. baccarum* for *T. mitsukurii* (Giovannini et al. 2021). Surprisingly, our data did not reveal any behavioural response of this parasitoid to odours from soybean plants bearing egg masses of *D. baccarum*. *Dolycoris baccarum* has a wide distribution in the Palearctic region (Panizzi et al. 2000), occurring also in Asia where it is a pest of several crops including soybean (Kobayashi 1981; Nakamura and Numata 2006). Although *D. baccarum* is listed in the host range of *T. mitsukurii* (Ryu and Hirashima 1984), there are no quantitative data related to the prevalence rate of this parasitoid. On the other hand, naturally-laid and sentinel egg masses of *D. baccarum* are highly parasitized in Chinese orchards by other parasitoids, i.e., *T. japonicus* (Zhang et al. 2017). Similarly, in Korea and Japan, *Trissolcus nigripedius* Nakagawa and *Telenomus gifuensis* Ashmead (both Hymenoptera: Scelionidae) are commonly found parasitizing *D. baccarum* eggs laid in crop field, including soybean (Mahmoud and Lim 2008), sometimes demonstrating high prevalence (Lim et al. 2007). Therefore, while some parasitoid species seem to effectively track host eggs of *D. baccarum* in the field, the same cannot be demonstrated for *T. mitsukurii*, suggesting that parasitization by this species might be occasional.

In no-choice black box experiments, *E. ventralis* eggs represented a poorly suitable host for *T. mitsukurii*, as parasitoids failed to develop inside its eggs (Giovannini et al. 2021). The lack of response towards odours from plants bearing an egg mass of *E. ventralis* in our experiments is consistent with the very low suitability of this species (Giovannini et al. 2021). The stink bug is of Palearctic origin and is only marginally present in the native area of *T. mitsukurii* (Rider et al. 2002; Rider 2006; Giovannini et al. 2021). Therefore, the risk that under field conditions *T. mitsukurii* would parasitize *E. ventralis* appears quite low.

One of the most desired aspects of risk assessment is that the candidate biocontrol agent has no or limited negative effect on beneficials. The fact that plants with *A. custos* eggs repelled *T. mitsukurii* females reduces the risk of non-target parasitisation. In fact, we may expect that in case of intentional release of *T. mitsukurii*, the existence of such an ecological barrier would limit encounters with *A. custos* eggs in the field. The response of *T. mitsukurii* to *A. custos* appears at least partly similar to that of *T. japonicus*, which in no-choice tests parasitized successfully this stink bug predator, but in large cage

tests, using plant bearing egg masses, was less preferred compared to *H. halys* (Haye et al. 2020; Giovannini et al. 2021). Similarly to herbivorous species, zoophytophagous Heteroptera, including *A. custos*, can feed on plant tissues to acquire water and nutrients (Gillespie and Mcgregor 2000). It was demonstrated that some of these species can induce the activation of defensive signalling pathways in plants, with consequent release of volatile organic compounds, which can inform natural enemies of the ongoing attack (Naselli et al. 2016; Bouagga et al. 2018; Martorana et al. 2019). Eventually, behavioural responses of natural enemies to such odour sources can vary in different systems. For instance, oviposition by the zoophytophagous *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) induces the emission of plant volatiles which attract its coevolved parasitoid *Telenomus podisi* (Ashmead) (Hymenoptera: Scelionidae) (Martorana et al. 2019). In another trophic system, oviposition by the predatory *P. maculiventris* on tomato plants did not attract *T. japonicus*, although the parasitoid is able to successfully parasitize *P. maculiventris* eggs and develop inside the host (Bertoldi et al. 2019; Lara et al. 2019). The lack of a coevolutionary history between the two species may explain the inconsistent behaviour of *T. japonicus* (Bertoldi et al. 2019). *Arma custos* (junior synonym *Arma chinensis* Fallou, after Zhao et al. 2018) is historically present in *T. mitsukurii* native area (Zou et al. 2012), hence we can speculate that the observed repellence can be interpreted as a coevolutionary adaptation among the tritrophic system, that might prevent the parasitoid to exploit the predator. Although *A. custos* eggs were highly suitable for *T. mitsukurii* in no-choice black box tests (Giovannini et al. 2021), development of the parasitoid larva is highly risky due to possible predatory (cannibalistic) behaviour of newly hatched *A. custos* nymphs (Zou et al. 2012). Hence, in the first step of the hierarchical process of habitat assessment, the parasitoid could use plant odours to avoid such risky host.

The exploitation of adults-related chemical odours is quite common in egg parasitoids (Afsheen et al. 2008; Conti and Colazza 2012; Rondoni et al. 2017a), however in the present study we noticed lack of *T. mitsukurii* response to females of *H. halys*. Although it is known that volatile and non-volatile cues from physogastric females may represent reliable information for some parasitoids belonging to Scelionidae (Borges and Blassioli-Moraes 2017; Bertoldi et al. 2021), kairomones from stink bug females elicit parasitoid responses mainly at short distance (Colazza et al. 2007; Borges and Blassioli-Moraes 2017). For instance, a previous investigation demonstrated that *T. japonicus* responds to *H. halys* females only in a “close-distance” olfactometer and not in a “long-distance” olfactometer (as the one we have used here) (Bertoldi et al. 2019). In open arena, *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae) responded to *Murgantia histrionica* (Hahn) (Hemiptera: Pentatomidae) physogastric females but not to males (Conti et al. 2003). Concerning *T. mitsukurii*, female extracts of *H. halys* seem to elicit a behavioural response of the parasitoid in Petri dish arena, thus under close-distance environment (Scala et al. 2021).

We did not detect any attraction of *T. mitsukurii* towards odours from eggs of the tested stink bugs. Kairomones from host eggs are typically present in small amounts, hence their role in host location is mainly expected at short distance (reviewed by Vinson 1998; Fatouros et al. 2008; Conti and Colazza 2012). In olfactometers, even though few species of egg parasitoids (e.g., *T. podisi*) were demonstrated to respond to odours directly emitted by eggs (Frenoy et al. 1992; Michereff et al. 2016), so far, investigated

Trissolcus species did not respond (Bertoldi et al. 2019) except when a high number of eggs was placed very close to the air hole at the end of the olfactometer arm (Conti et al. 2003). Hence, we can hypothesize that such poorly detectable odours would only permit host location at proximity, as shown for *T. brochymenae* using short-range bioassays in open arenas (Conti et al. 2003).

In conclusion, while previous host-acceptance investigations revealed a relatively large level of oligophagy for *T. mitsukurii* in Europe (Giovannini et al. 2021), present results suggest that host location at long distance would likely favour parasitization over *H. halys* (or *N. viridula*), rather than the other stink bugs tested here, in particular the beneficial *A. custos*. Definitely, the presence of such ecological filter would have positive implications for preventing undesired impact on non-targets in case of intentional release of the parasitoid in biological control programs. On the other hand, there are no physiological impediments for *T. mitsukurii* to develop in some non-targets (e.g., *A. custos*), hence when such species co-occur in the field with *H. halys*, they would likely be more exposed to parasitization, still occurring as opportunistic with occasional encountering during foraging. Hence, the bioassays conducted here with *T. mitsukurii* following the host range studies (Giovannini et al. 2021), highlight the need of a multidisciplinary approach in pre-release risk assessment, where results from each step are part of the puzzle that will allow a reliable field scenario prediction. Dedicated field surveys of native and exotic stink bug eggs in those areas where *T. mitsukurii* have fortuitously established would likely help in estimating the relevance of such host-parasitoid interactions and provide better support for the definition of a risk assessment document, necessary for licensing parasitoid releases.

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Figures

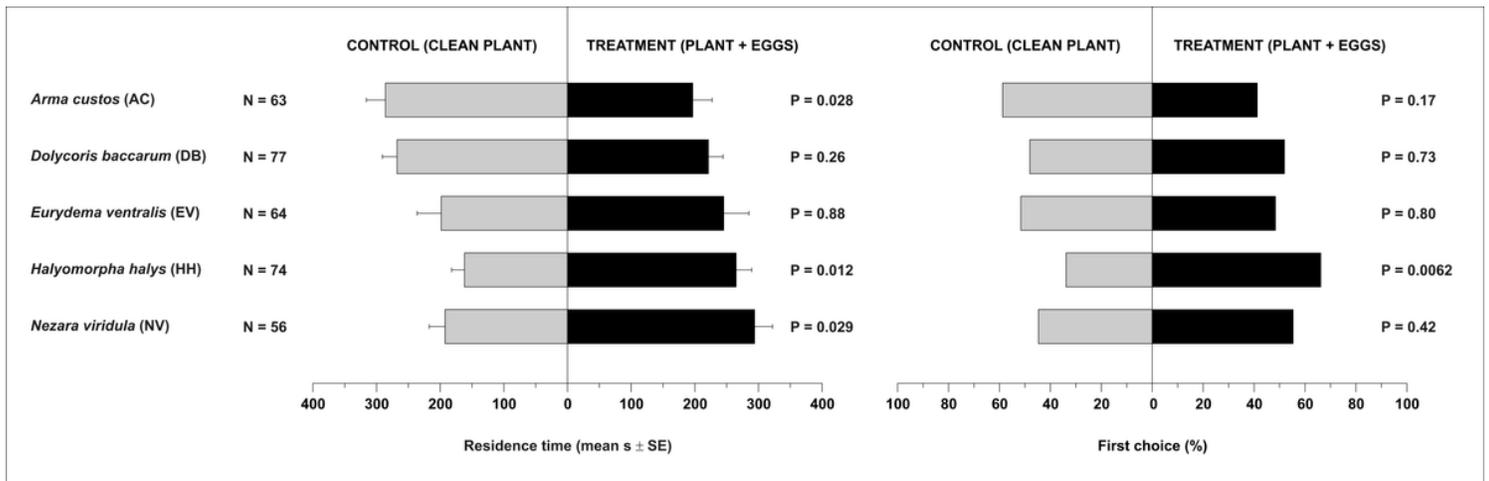


Figure 1

Residence time (mean $s \pm SE$) and first choice (%) of *Trissolcus mitsukurii* females in Y-tube olfactometer exposed to volatiles from soybean plants bearing an egg mass of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Halyomorpha halys* (HH), or *Nezara viridula* (NV), or volatiles from cauliflower plants bearing an egg mass of *Eurydema ventralis* (EV). Control consisted of a clean soybean or cauliflower plant. Planned comparisons were tested within GLM with Gaussian error distribution (residence time) or with binomial error distribution (first choice).

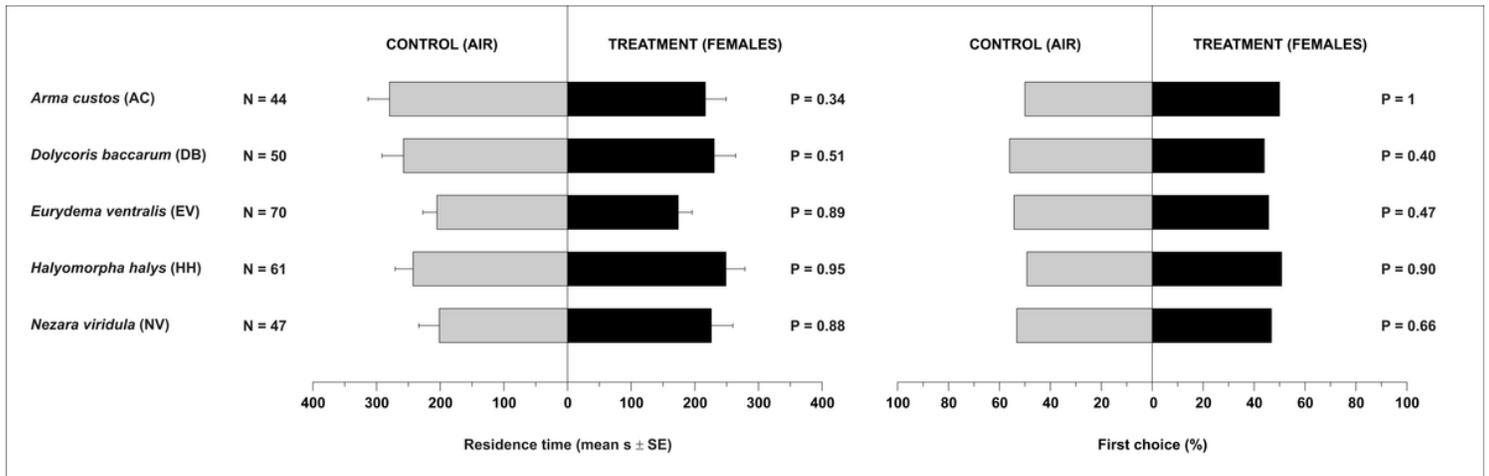


Figure 2

Residence time (mean $s \pm SE$) and first choice (%) of *Trissolcus mitsukurii* females in Y-tube olfactometer exposed to volatiles from females of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Control consisted of clean air. Planned comparisons were tested within GLM with Gaussian error distribution (residence time) or with binomial error distribution (first choice).

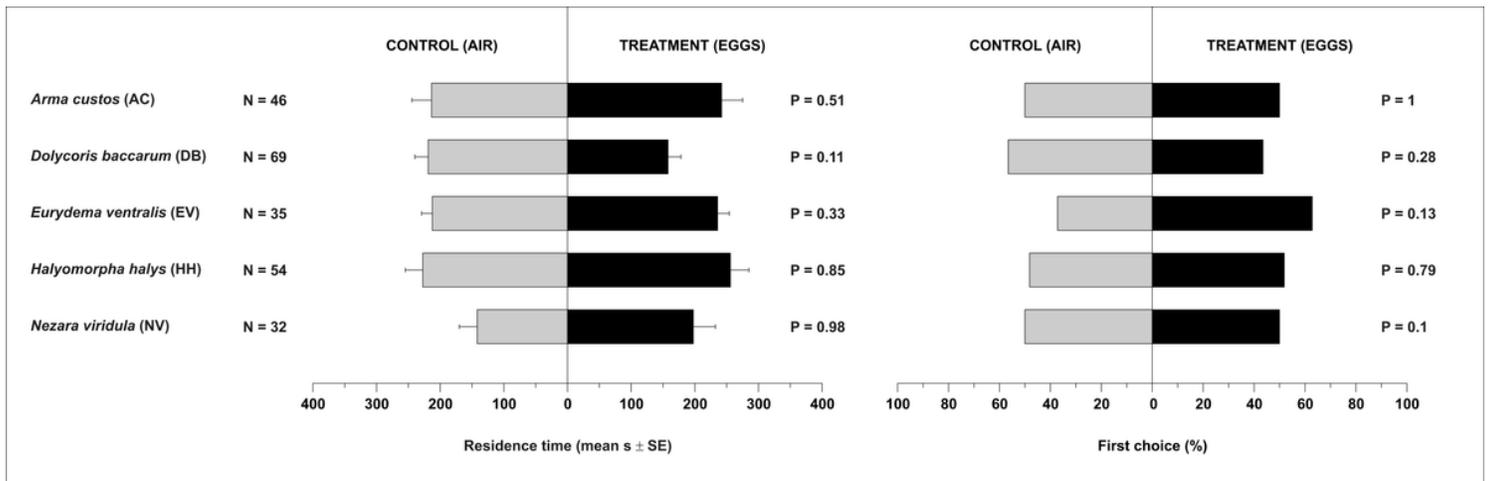


Figure 3

Residence time (mean $s \pm SE$) and first choice (%) of *Trissolcus mitsukurii* females in the control and treatment arm of a Y-tube olfactometer. Control consisted of clean air. Treatments were volatiles from eggs of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Planned comparisons were tested within GLM with Gaussian error distribution (residence time) or with binomial error distribution (first choice).

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