

Physiological Host Range of *Trissolcus Mitsukurii*, A Candidate Biological Control Agent of *Halyomorpha Halys*

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Abstract

The invasive stink bug *Halyomorpha halys* is a severe agricultural pest of worldwide importance, and chemical insecticides are largely sprayed for the control of its populations. Negative impacts and several failures in chemical pest management led to consider classical biological control as one of the most promising methods in a long-term perspective. The Asian egg parasitoid *Trissolcus japonicus* is the main candidate biocontrol agent of *H. halys*, but more recently a second Asian egg parasitoid, *Trissolcus mitsukurii*, is getting attention after adventive populations were found on *H. halys* egg masses in Europe. Before recommending the use of *T. mitsukurii* for biological control of *H. halys*, a risk analysis is necessary and therefore here we present the first study on the fundamental physiological host range of this parasitoid in Europe. Tests conducted with *T. mitsukurii* on different hemipterans, using three different experimental designs, revealed a broad physiological host range, comparable with the host range displayed by *T. japonicus* under similar laboratory conditions. Specifically, in addition to its coevolved host *H. halys*, *T. mitsukurii* successfully parasitized the majority of tested pentatomid species and one scutellerid, although with highly variable emergence rates. Host egg sizes positively affected parasitoid size and female egg load. Further studies, testing more complex systems that involve olfactory cues from host and host plants, will simulate different aspects of the parasitoid host location behavior under field conditions, allowing in-depth evaluation of the possible risks associated with the use of *T. mitsukurii* as a biocontrol agent of *H. halys*.

Key Message

- 1) *Trissolcus mitsukurii* is a potential biological control agent (BCA) of *Halyomorpha halys*
- 2) Recently, adventive populations were found in Italy parasitizing *H. halys*
- 3) A risk analysis is mandatory for the evaluation of biological control programs
- 4) *Trissolcus mitsukurii* displayed in black box tests a host range comparable with that of the main BCA *Trissolcus japonicus*
- 5) More complex experimental designs (e.g., tri-trophic system) are needed for more detailed outputs

Introduction

The Brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), is a polyphagous invasive pest native from Eastern Asia and currently present in several countries of the Americas and Europe, where it causes severe damage to many agricultural crops (Leskey and Nielsen 2018). The low efficacy and high environmental impact of insecticide treatments, combined with the lack of effective native natural enemies, led to consider classical biological control with egg parasitoids a promising method for long-term management of *H. halys* in the invaded areas (Abram et al. 2017). Although recent reviews indicated limitations in the success of classical biological control of stink bugs, research in this field needs to highlight any potential approach to find effective pest control solutions (Abram et al. 2020; Conti et al. 2021). These solutions could emerge from the integration of classical biological control with techniques targeting different instars of the pest (Abram et al. 2020), like plant resistance (Rondoni et al. 2018), exploitation of plant volatiles to improve parasitoid efficacy (Rondoni et al. 2017), manipulation of stink bug behavior and parasitoid conservation (reviewed by Conti et al. 2021).

The introduction of an exotic organism, including a candidate biological control agent, is strictly regulated in order to avoid potential negative and permanent ecological consequences (van Lenteren et al. 2006; EPPO 2014; FAO-IPPC 2017). Therefore, non-target risk studies of the candidate biological control agent are mandatory and precede any further step for the petition and approval of its release in the field. Laboratory investigations are the first step for risk analysis of the exotic natural enemy and allow to explore its fundamental physiological host range. Then, several different testing methods need to be integrated in a multiple step workplan, including behavior and chemical ecology. Such studies are necessary for moving from a physiological host range outcome to an ecological host range perspective, which allows to prevent or minimize the risk of a negative impact caused by a voluntary human intervention in the ecosystem (van Lenteren et al. 2006).

The Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) is considered so far the most valid candidate for biological control of *H. halys* (Zhang et al. 2017; Conti et al. 2021). Its host range, outside the natural one, was investigated in the USA, Europe and New Zealand, confirming its oligophagous habitus but also showing its host preference versus *H. halys* (Hedstrom et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Lara et al. 2019; Haye et al. 2019; Sabbatini Peverieri et al., 2021). Adventive populations of *T. japonicus* were discovered recently in Europe and the USA (Talamas et al. 2015; Sabbatini Peverieri et al. 2018; Stahl et al. 2019). Surprisingly, a second Asian egg parasitoid of *H. halys*, *Trissolcus mitsukurii* (Ashmead) (Hymenoptera, Scelionidae), was also found in Italy, as the first record outside its native range (Sabbatini Peverieri et al. 2018). However, while *T. japonicus* ability to attack *H. halys* is well recognized, little is known so far about the preferences and life traits of *T. mitsukurii* on *H. halys* (Zhang et al. 2017; Arakawa and Namura 2002; Arakawa et al. 2004). This parasitoid species is known mainly from Japan, where it has been recorded parasitizing in the field eggs of about ten species of Pentatomidae (Yasumatsu and Watanabe 1964; Hokyo et al. 1966; Ryu and Hirashima 1984; Arakawa and Namura 2002). Additionally, in laboratory test conducted in Japan, *T. mitsukurii* was also able to parasitize another pentatomid species in the genus *Plautia* (Arakawa and Namura 2002). Therefore, *T. mitsukurii* seems to be an oligophagous egg parasitoid. In Japan it was reported in the past as one of the most active natural enemies of *Nezara viridula* L. (Hemiptera, Pentatomidae) (Kiritani and Hokyo 1962; Hokyo and Kiritani 1963), although more recent data from the same country reported this parasitoid as commonly associated to *H. halys* (Arakawa and Namura

2002; Arakawa et al. 2004). Instead, the high parasitization rate reported in China for *T. mitsukurii* on *H. halys* (Chu et al., 1997), due to an original misidentification should be assigned to *T. japonicus* as stated by Yang et al. (2009).

Field data from Northern Italy showed high rates of parasitism of *H. halys* eggs by *T. mitsukurii*, suggesting that this egg parasitoid could be a valid candidate for biological control of the invasive stink bug in addition to *T. japonicus* (Benvenuto et al. 2020; Scaccini et al. 2020; Zapponi et al. 2020). Because no specific and recent information is available on the host range of *T. mitsukurii*, a risk analysis workplan needs to be initiated before implementation of this egg parasitoid as a biological control agent is considered (van Lenteren et al. 2006). The present work is the first contribution to the exploration of the physiological host range of *T. mitsukurii*, conducted comparing its performance on *H. halys*, the target pest of biological control, and other Hemipteran non-target species that are common in Europe.

Material And Methods

2.1 Field sampling, insect collection and rearing

Halyomorpha halys adults were collected in Northern Italy in 2020, both in overwintering sites and in the field during the growing season, whereas adults of non-target Hemiptera species were collected in *H. halys* habitats and surrounding areas, of North and Central Italy, during the 2020 growing season. Non-target Hemiptera species were chosen based on the potential host range of the parasitoid and the information mined from the literature, and according to the centrifugal phylogenetic approach (Kuhlmann and Mason 2003; Kuhlmann et al. 2006). Beside *H. halys*, the following non-target herbivorous Hemipteran species were investigated: the pentatomids *Acrosternum heegeri* (L.), *Aelia acuminata* (L.), *Ancyrosoma leucogrammes* (Gmelin), *Carpocoris pudicus* (Poda), *Dolycoris baccarum* (L.), *Eurydema ventralis* Kolenati, *Eurydema oleracea* (L.), *Graphosma italicum* (Muller), *N. viridula*, *Palomena prasina* (L.), *Piezodorus lituratus* (F.), and *Sciocoris* sp., the scutellerid *Eurygastermaura* (L.), and the coreid *Gonocerus juniperi* Herrich-Schäffer. The two predator species *Arma custos* (Fabricius) (Hemiptera, Pentatomidae) and *Rhynocoris iracundus* (Poda) (Hemiptera, Reduviidae) were also considered (see Table 1).

Laboratory colonies were established and maintained using rearing cages (BugDorm 4F4545, Insect MegaView Science Co. Ltd., Taichung, Taiwan) in rearing rooms at $26 \pm 1^\circ\text{C}$ and 16:8 L:D. For the phytophagous species, a variety of plant sources was used as food: seeds (*Arachis hypogaea*), fresh vegetables (*Daucus carota*, *Phaseolus vulgaris*, *Brassica oleracea*, *Capparis spinosa*), fresh fruits (*Malus domestica*, *Actinidia deliciosa*). Food was purchased from local markets. Plants were self-produced and were used when at least 10cm tall. The predator species were reared on *Acanthoscelides obtectus* Say (Coleoptera, Chrysomelidae) adults, *N. viridula* nymphs and *Tenebrio molitor* L. (Coleoptera, Tenebrionidae) pupae. For all species, food and water (provided with wet cotton) were replenished three times per week. Paper towels were hung inside the rearing cages as oviposition substrates, and eggs were collected daily. As a measure of standard quality of laboratory hemipteran rearing, the viability of eggs was occasionally recorded (Tab. 1). At this scope, batches of egg masses produced by target and non-target species were reared in climatic chamber (HPP750, Memmert GmbH + Co. KG, Schwabach, Germany) at $26 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and 16:8 L:D up to egg hatching.

Trissolcus mitsukurii was obtained from *H. halys* egg masses collected in 2018 and 2019 in infested sites of Northern Italy. These egg masses were reared in the laboratory and the emerged *T. mitsukurii* individuals (several dozens of specimens) were used to start a laboratory colony. Adults of the egg parasitoid were fed with pure honey droplets. Periodically, fresh egg masses (<24h old) of *H. halys* were offered as host for parasitization and juvenile development. The permanent colony of the egg parasitoid was established and maintained in climatic chamber at $26 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and 16:8 L:D; such conditions were considered as standards for the present work and were used for all experiments. Adults of *T. mitsukurii* and parasitized egg masses were housed in glass tubes (15cm length, 2cm diam), closed on both ends by a plastic net (250 μm mesh). Establishment and maintenance of the egg parasitoid colony and all experiments (see below) were conducted under quarantine conditions at CREA facilities. Quarantine laboratories are authorized for studies on exotic beneficial organisms in the framework of *H. halys* biological control program (MiPAAF, DG/DIR/DIR05/0013647-19/04/2018 and SFR Regione Toscana 203304 – 12/04/2018).

2.2 No-choice black box tests

A total of three different experimental conditions were considered for the no-choice black box tests. In the first and second experiment, host egg masses were handled as little as possible to avoid the introduction of external contaminants, and any adjustment of size (egg number per mass) was avoided, as to simulate the natural number of host eggs, per egg batch, that female parasitoids would likely encounter in the field (Hedstrom et al. 2017; Botch and Delfosse 2018). For *G. juniperi*, which lays individual eggs, ten eggs were aligned and glued (using non-toxic clear school Elmer's® glue, Newell Office Brands) on a cardboard (15mm x 80mm), each egg distanced 5 mm from the other. For the tests, one egg mass (or a row of ten single eggs) of a given host species was placed in a glass tube (15cm length, 2cm diam.), and one parasitoid female, 7-d old, was introduced for two different exposure times. Specifically, in the first experiment, females were kept for 24h and a small drop of honey was added on the glass internal surface as food source. In the second experiment, the exposure time was reduced to 2h and no food was provided.

In the third type of black box no-choice experiment, only one egg was provided to the female for a total time of 2h. In this experimental condition, a small glass vial (7cm length, 0.5cm diam.) was used and closed with a cotton ball to prevent the parasitoid from escaping. This assay is an extreme simplified exposure condition that has been previously shown to provide reliable results for no-choice tests with egg parasitoids (Sabbatini Peverieri et al. 2021). Tested eggs were glued (Elmer's® glue) on the cardboard to permit a sufficient stability.

In all no-choice experiments, only *T. mitsukurii* females originating from *H. halys* eggs were tested, and only fresh eggs (< 24h old) of *H. halys* and non-target species were employed. Before use, eggs and egg masses were inspected under a stereo microscope to assess their suitability for the tests. In case of, e.g., non-characteristic color of the eggs, unusual egg arrangement in the batch, unswollen eggs in the mass, or unusual low number of eggs per mass, the egg or the egg mass was discarded. Parasitoids were removed at the end of the allotted time (24h or 2h). Eggs were maintained in the glass vials (single eggs) or tubes (egg masses) and were reared in climatic chamber at standard conditions until nymph hatching or parasitoid emergence. Eggs that did not hatch nor produced a parasitoid after three weeks from tests, were classified as “dead eggs”. These were dissected and inspected under a stereomicroscope, and the content was eventually classified as: dead parasitoid (pupae or pharate adult), dead Hemiptera nymph, or undetermined content.

2.3 Effect of host egg size on parasitoid size and egg load

The effects of host eggs on the parasitoid size and egg load was evaluated using *T. mitsukurii* females emerged from target and non-target hosts of the no-choice black box tests. For this purpose, the volume (mm³) of eggs of host species was calculated by the parabolic barrel formula $V = \pi H(3r^2 + 4Rr + 8R^2)/15$, where “H” is the egg height, “r” is the radius of the operculum and “R” is the radius at the center of the egg (Abram et al. 2016; Botch and Delfosse 2018). The head and thorax width and the length of the hind tibia were measured from females emerged from target and non-target hosts. Additionally, parasitoid females from target and non-target host eggs were reared individually for 7 days at standard conditions in climatic chamber. At the seventh day of rearing, the females were dissected and the egg load in their ovary was counted after staining with 1% toluidine blue (Sabbatini Peverieri et al. 2020).

2.4 Paired choice black-box tests using parasitoids reared on different hosts

In a first paired-choice experiment, seven-days old *T. mitsukurii* females reared on *H. halys* eggs were singly used in dual-choice tests in Petri dishes (9cm diam.). A female was allowed to exploit two fresh egg masses (< 24h), one of *H. halys* and one of a non-target species (n=12). *Arma custos* or *D. baccarum* were chosen as non-target species because of the high parasitization rates that were recorded in the no-choice tests (over 50% of successful parasitization). *Arma custos* was also chosen as this is a key species for the safeguarding approach in risk analysis. The number of eggs of tested egg masses was not adjusted in order to maintain the similar egg numbers that *T. mitsukurii* would encounter in the field (Haye et al. 2019). The two egg masses were placed at the bottom of the Petri dish and at the opposite side of the arena. The position of the egg species was inverted at each replicate. The parasitoid female was placed at the center of the arena and tested for a 2h time span in climatic chamber at standard condition. This reduced time of egg mass exposure was adopted because in the previous no-choice experiment the parasitoid females displayed to parasitize host egg masses within this short time interval, and because this would limit the opportunity for multiple parasitization, which is likely to occur in a 24h exposure test (Haye et al. 2019). At the end of the exposure, the females were removed from the arena and the egg masses were reared in the climatic chamber at standard condition until the eggs hatched or the parasitoids emerged. Eggs that did not hatch nor produced a parasitoid after three weeks from the experiment were dissected to determine the contents as described previously.

A second paired-choice experiment was conducted to evaluate possible effects of the rearing host on host preferences by *T. mitsukurii*. For this purpose, different colonies of *T. mitsukurii* were established and maintained on egg masses of the non-target species, *D. baccarum* or *A. custos*, with the same method used when reared on *H. halys* as host. The same experimental design as described above was applied using *T. mitsukurii* parasitoid females originated from host eggs of either *D. baccarum* or *A. custos*. When females did not make a choice, the replicate was excluded from the analysis.

2.5 Statistical analysis

In no-choice tests with host egg masses exposure for 24h and 2h, the percentages of hatched Hemiptera eggs, successfully parasitized eggs (emergence of the parasitoid) and dead eggs were analyzed by Kruskal-Wallis and Dunn’s multiple comparison post-hoc test using *H. halys* as control species. Data were analyzed only for the species showing at least five replicates (although the entire dataset was reported). Pairwise comparison among species in single host egg exposure for 2h was performed using Chi-square test with Yates’ correction. Pooled data on parasitoid female dimensions (head width, thorax width, hind tibia length) were analyzed with Pearson correlation coefficient. The functional relationship between the egg size of host species, emerged parasitoid female size and ovaries egg load were analyzed through linear regression model. In choice-test the percentages of hatched Hemiptera eggs, successfully parasitized eggs (emergence of the parasitoids) and dead eggs were compared using the Mann-Whitney *U*-test. Ovarian egg load between target and non-target species were compared with Kruskal-Wallis and Dunn’s multiple comparison post-hoc test. Statistics were performed using Graphpad Prism 8.

Results

3.1 No-choice black box tests

When Hemiptera egg masses were exposed to *T. mitsukurii* for 24h, most eggs were suitable or partially suitable for parasitoid development, with significantly different emergence rates from the diverse tested species ($\chi^2=112.7$; $P<0.0001$) (Table 2). *Trissolcus mitsukurii* was able to parasitize and develop in 12 pentatomid species out of the 14 that were tested (85.71 % of tested species), and in the only tested scutellerid, *E. maura*, although with highly variable success. Conversely, it was not able to exploit the remaining pentatomids *A. leucogrammes* and *E. ventralis*, the coreid *G. juniperi* and the reduviid *R. iracundus* (Table 2). The emergence rate of *T. mitsukurii* from *H. halys* egg masses was very high, close to 100%, and not significantly

different compared to emergence from the pentatomids *A. heegeri*, *A. custos*, *C. pudicus*, *D. baccarum*, *G. italicus*, *P. prasina*, *P. lituratus*, *Sciocoris* sp. (8 out of 13 tested non-target pentatomid species) and the scutellerid *E. maura* (Table 2; see Table S1 for statistics). Instead, emergences were significantly lower, compared to *H. halys*, when eggs of *Aelia acuminata*, *E. oleracea* and *N. viridula* were tested (Table 2; see Table S1 for statistics). Sex ratios were strongly female-biased in most of the species tested (Table 2).

The percentages of Hemiptera eggs that hatched after exposure to *T. mitsukurii* were significantly different ($\chi^2=80.04$; $P<0.0001$) and partially complementary to parasitoid emergence rates. Almost none of the eggs of *H. halys*, *A. heegeri*, *D. baccarum* and *P. prasina* hatched, while hatching percentages were relevant for the other pentatomid species, although only *A. leucogrammes* and *E. ventralis* showed significantly higher rates compared to *H. halys*. The percentages of dead eggs (no hatching and no parasitoid emergence) were significantly different among the tested species ($\chi^2=73.50$; $P<0.0001$). Dead eggs were present in all Hemiptera species except *H. halys* and *P. prasina* (but only one egg mass of the latter could be tested), and percentages were notably higher in *A. acuminata*, *E. oleracea*, *E. ventralis* and *N. viridula* than in *H. halys* (Table 2; see Table S1 for statistics). Dead specimens of *T. mitsukurii* (pupae and pharate adults) were detected in eggs of suitable and partially suitable pentatomid host species, with the only exceptions of *H. halys*, *D. baccarum* and *P. prasina*. No dead parasitoid specimens were recorded in eggs of the scutellerid *E. maura*, although several eggs died for unknown reason (undetermined content). While no *T. mitsukurii* emerged from *A. leucogrammes* and *E. ventralis*, dissection of dead eggs revealed the presence of high numbers of dead parasitoid specimens (adults and pupae) in both species. Instead, dissections of dead eggs of the coreid *G. juniperi* and the reduviid *R. iracundus* never reported the presence of dead pupae or adults.

When the Hemiptera egg masses were exposed to *T. mitsukurii* for 2h, results were similar to the 24h exposure experiment and the emergence rates were significantly different among the tested species ($\chi^2=77.38$; $P<0.0001$) (Table 3; see Table S1 for statistics). *Trissolcus mitsukurii* was able to parasitize and develop in 6 out of the 9 pentatomid species that were tested (66.67% of tested species) and in the scutellerid *E. maura* (Table 3). Specifically, the emergence rates of the pentatomids *A. heegeri*, *A. custos*, *C. pudicus*, *D. baccarum*, and *G. italicus* (5 out of 8 non-target pentatomid species) and the scutellerid *E. maura* were not significantly different from that of *H. halys* (Table 3; see Table S1 for statistics). Sex ratios were strongly female-biased in most of the species tested (Table 3). Hatching rates of Hemiptera eggs were significantly different ($\chi^2=46.52$; $P<0.0001$) and complementary with parasitoid emergence rates and dead eggs. Percentages of dead eggs were also significantly different ($\chi^2=74.06$; $P<0.0001$) and consistent with previous data. Dead pupae and adults of the parasitoid were observed in most of the tested species, but not in *H. halys*, *A. heegeri* and *D. baccarum*. Specifically, eggs of *A. acuminata* showed lower parasitization success compared to *H. halys*, with significantly lower parasitoid emergence and higher dead eggs containing dead parasitoids. Similarly, eggs of *N. viridula* showed no parasitoid emergence and significantly higher dead eggs containing dead parasitoids. Instead, a high rate of nymphs emerged from *E. ventralis* eggs and no parasitoid emerged, while some dead parasitoids were found in the eggs (Table 3; see Table S1 for statistics).

When a single Hemiptera egg was exposed to *T. mitsukurii* for 2h, six out of seven pentatomid species (85.71% of tested species), and the scutellerid *E. maura* were suitable for *T. mitsukurii* development (Table 4). Percentages of parasitoid emergence from eggs of non-target species were significantly lower compared to those from *H. halys* (Table 4; see Table S2 for statistics), except for *P. prasina* where 100% of emergence success was recorded, like in *H. halys*. No parasitoids emerged from eggs of *N. viridula* and no parasitoid pupae or pharate adults were found in dead eggs.

3.2 Effect of host egg size on parasitoid size and egg load

The three body dimensions measured on *T. mitsukurii* females (head width, thorax width and hind tibia length, see Table S3) revealed to be highly correlated (head width vs. thorax width: $r=0.96$, $P<0.0001$; head width vs. hind tibia length: $r=0.81$, $P<0.0001$; thorax width vs. hind tibia length: $r=0.81$, $P<0.0001$). Within each Hemiptera host species, the female head width was used as main parameter to analyze in the linear regression model as a function of host egg volume. Regression analysis displayed a positive trend between the volume of host eggs and the head width of the emerged parasitoid females ($r^2=0.7673$, $P=0.0002$) (Fig. 1).

The egg loads (see Table S4) of *T. mitsukurii* females originated from the different Hemiptera host species were significantly different ($\chi^2=65.75$; $P<0.0001$). Parasitoid females that developed in *H. halys* exhibited a higher number of eggs in their ovaries compared to other non-target pentatomid species (see Table S4 for statistics), except for *C. pudicus* and *A. heegeri* and the scutellerid *E. maura*. Regression analysis displayed a positive trend between host egg volume and the ovarian egg load ($r^2=0.8053$, $P<0.0001$) (Fig. 2).

3.3 Paired choice black-box tests using parasitoids reared on different hosts

In the *H. halys* vs. *D. baccarum* choice tests, *T. mitsukurii* females when reared on *H. halys*, always performed a choice for parasitization. In *H. halys* vs. *A. custos* choice-tests, *T. mitsukurii* females failed to interact with host eggs in 1 out of total 12 egg masses when the parasitoid females originated from *H. halys*, and in 2 out of total 12 egg masses when the females originated from *A. custos*.

When reared on *H. halys*, emergence of the progeny of *T. mitsukurii* females showed no significant differences between *H. halys* and each of the two non-target host species (*H. halys* vs. *D. baccarum*, $U=48$, $P=0.1398$; *H. halys* vs. *A. custos*, $U=38.50$, $P=0.1339$). Percentages of dead eggs were not significantly different neither (*H. halys* vs. *D. baccarum*, $U=58$, $P=0.3811$; *H. halys* vs. *A. custos*, $U=45.50$, $P=0.3342$) (Fig. 3). A lower percentage of nymphs hatched from *H. halys* compared to *A. custos* eggs ($U=30$, $P=0.0434$), while similar percentages emerged from *H. halys* and *D. baccarum* eggs ($U=65$, $P=0.6924$).

When reared on a non-target host (*D. baccharum* or *A. custos*), the emergence of the progeny of *T. mitsukurii* females showed no significant differences in the case of *H. halys* vs. *D. baccharum* ($U=72$, $P>0.9999$) and in that of *H. halys* vs. *A. custos* ($U=30.50$, $P=0.1214$) (Fig. 4). Additionally, no differences were observed in the percentage of dead eggs (*H. halys* vs. *D. baccharum*, $U=57$, $P=0.8444$; *H. halys* vs. *A. custos*, $U=28$, $P=0.0927$) nor in the percentage of hatched nymphs (*H. halys* vs. *D. baccharum*, $U=65$, $P=0.6924$; *H. halys* vs. *A. custos*, $U=37.50$, $P=0.3541$).

Discussion

The egg parasitoid *T. mitsukurii* has been poorly investigated till now as a candidate biological control agent of *H. halys*, especially when compared to other egg parasitoid species like *T. japonicus* and the non-coevolved *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae), on which a large literature is available from China, Europe, USA and New Zealand. Field data from Japan indicate for *T. mitsukurii* an oligophagous habitus (Yasumatsu and Watanabe 1964; Hokyo et al., 1966; Ryu and Hirashima, 1984; Arakawa and Namura 2002), and data from the invaded areas in Europe (Northern Italy) evidenced the ability of this parasitoid to locate and successfully parasitize *H. halys* egg masses in the field (Sabbatini Peverieri et al. 2018; Benvenuto et al. 2020; Scaccini et al. 2020; Zapponi et al. 2020). Furthermore, laboratory studies confirm that the reproductive biology of this parasitoid on *H. halys* is similar to that of *T. japonicus* on the same host (Sabbatini Peverieri et al. 2020).

In our experiments under laboratory conditions and 24 hours of egg mass exposure, *T. mitsukurii* displayed the ability to parasitize and develop successfully in 8 (61.54%) out of 13 tested non-target pentatomid species, with no significant differences compared to the coevolved host *H. halys*, while additional 3 species were partially suitable although at a significantly lower level compared to *H. halys*. These results were largely confirmed when *H. halys* and non-target species were exposed to the parasitoid only for 2 hours, as *T. mitsukurii* successfully parasitized 5 (62.50%) out of 8 non-target pentatomid species with similar rates than when parasitizing *H. halys*. Additionally, the scutellerid *E. maura* was successfully parasitized in both the 24h and 2h tests, whereas the coreid *G. juniperi* and the reduviid *R. iracundus* were not. This can be at least partially explained if we consider that scutellerids belong to the same superfamily of pentatomids, i.e., Pentatomoidea. In previous laboratory investigations, *E. maura* was revealed to be physiologically suitable also for *T. japonicus* (Haye et al. 2019). However, because scutellerids were never found as hosts of neither *T. mitsukurii* nor *T. japonicus* in the field, it is possible to hypothesize that other ecological factors, e.g., habitat preferences, interspecific competition or oviposition periods, may limit the access to this host. Laboratory host range of *T. mitsukurii* can be considered similar to that of *T. japonicus*, which showed comparable results in previous host specificity tests on European non-target species, as 13 pentatomids were suitable for parasitoid development (Haye et al. 2019; Sabbatini Peverieri et al. 2021).

The highly variable rates of *T. mitsukurii* emergence from non-target eggs, both in the 24h and 2h exposure experiments, could be discussed in terms of host egg recognition/acceptance and suitability, based on data on parasitoid emergence, egg eclosion, egg death and the presence of dead parasitoids in eggs. Thus, *H. halys*, *A. heegeri* and *D. baccharum* appear to be easily recognized by *T. mitsukurii* and highly suitable hosts for this parasitoid under laboratory conditions, as emergence rates were always very high, egg hatching low, and almost no dead eggs were recorded. Similar results were also observed in *P. prasina*, although this species was tested with only one egg mass due to the poor rearing success in the laboratory. Most of the other non-target species seemed to be less suitable, although statistically not different from *H. halys*, because of the high presence of dead eggs, often containing a dead parasitoid pupa or a pharate adult (*A. custos*, *C. pudicus*, *G. italicum*, *P. lituratus*, *Sciocoris* sp.). Still, rates of successful parasitism of this species were rather high. High rates of parasitization successes under laboratory conditions were observed also for *T. japonicus* in China (Zhang et al., 2017), while other studies in the USA and New Zealand reported lower rates both on the target, *H. halys*, and no targets, probably due to the different physiological status of tested females (Hedstrom et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Lara et al. 2019). In our study, a few non-target pentatomid hosts showed significantly lower *T. mitsukurii* emergence rates compared to emergence from *H. halys*. This was due either to low recognition and parasitization, as indicated by high egg hatching rates (*A. leucogrammes*), or to low suitability to parasitoid development, as indicated by the high rates of dead eggs (*A. acuminata*, *E. oleracea*, *N. viridula*), or to both reasons (*E. ventralis*). The occurrence of dead *T. mitsukurii* pupae or adults in several Hemiptera host eggs further reflects a low physiological suitability. Remarkably, dead parasitoids were found also in the eggs of pentatomid species that did not allow any parasitoid adult emergence, i.e., *E. ventralis* and *A. leucogrammes*. In the field, such cases might act as an evolutionary trap, as was stated for *T. japonicus* in similar cases, and unsuitable biochemical contentment of hemipteran eggs can here play a key role (Abram et al. 2014; Haye et al. 2019).

Surprisingly, as discussed above, the eggs of *N. viridula* showed a very low suitability rate for *T. mitsukurii*. This is a rather unexpected result because, although not coevolved due to the different geographical origin, *N. viridula* was addressed in the past as a main host for *T. mitsukurii* in its area of origin (Kiritani and Hokyo 1962; Hokyo and Kiritani 1963; Hokyo et al. 1966; Arakawa and Namura 2002; Arakawa et al. 2004). In our experiment, a very low parasitoid emergence rate and a high rate of dead eggs was detected, especially if compared with the high hatching rate observed in unexposed *N. viridula* egg masses. Moreover, in only about one tenth of the dead eggs it was possible to find dead parasitoid pupae or adults, whereas the egg content could not be determined in the other cases. We can hypothesize that parasitization occurred also in these cases, but the parasitoid failed to survive to late instars, further suggesting that *N. viridula* eggs are not suitable for larval development of *T. mitsukurii*. A similar result was observed in *T. japonicus*, as the parasitizing females caused 100% mortality in *N. viridula* eggs, but no emergence of adults were observed, nor parasitoid presence could be clearly identified from egg dissection (Haye et al. 2019).

The extremely simplified experiment conducted using a single host egg, exposed to *T. mitsukurii* for 2h, showed that among the seven pentatomid and one scutellerid species tested, most of them were detected as physiologically suitable, failing only in the case of *N. viridula*, which confirms its extremely low physiological suitability for *T. mitsukurii*. Remarkably, in such kind of test, only *H. halys* and *P. prasina* allowed 100% parasitism success

by *T. mitsukurii*, while parasitoid emergence from all other non-target host species was significantly lower. Comparing the three methods of host egg exposure to females of *T. mitsukurii* adopted in the present study (exposure of egg masses for 24h and for 2h and exposure of single eggs for 2h) and considering only the Hemiptera species that were tested in all three experiments, the 2 h test with single egg exposure failed to detect host physiological suitability in one case, the less suitable host species *N. viridula*. Additionally, a high number of hatched eggs was observed in all species except *H. halys* and *P. prasina*. These results indicate that a single host egg was not easily recognized and probed by the parasitoid. Therefore, by exposing a single egg, the degree of host acceptance and physiological suitability might be more difficult to define since the effect of clustered eggs is not taken into account. However, the use of single host egg exposure was already adopted for host specificity tests with *T. japonicus* and the output of physiological host suitability range was comparable with the results from experiments conducted using egg mass exposed for 24h using similar host species (Haye et al. 2019; Sabbatini Peverieri et al. 2021). This simple method of exposure might permit to filter a first list of suitable non-target species to investigate successively in more complex systems, optimizing resources and time.

In future tests, the time of egg exposure to parasitoids can be reduced (depending on the size of arena and on complexity of tests) as a few hours are adequate rather than a 24h experimental design. Here, considering outputs of the present work, three hours might act as optimal exposure time. The reduction of time might be a relevant factor in both choice and no-choice tests since this would increase parasitoid selectivity by reducing the number of encounters with egg masses, thus preventing host acceptance as a consequence of multiple contacts (Haye et al., 2019). Under field conditions, a first rejection of a potential host species might turn out in the parasitoid searching for more suitable hosts, while this is not permitted in the constrained environments under laboratory studies (Botch and Delfosse 2018; Boyle et al. 2020).

In our experiments we also aimed at comparing the host preference of *T. mitsukurii* in black-box choice tests under 2h exposure and the effect of rearing host, using *H. halys* vs. either one of two suitable non-target species, *D. baccarum* and *A. custos*. No significant evidence of host preference by *T. mitsukurii* females was observed. Additionally, the host species used to rear *T. mitsukurii* females did not appear to play a significant role in further host selection. Conversely, in two-choice tests, *T. japonicus* was shown to prefer *H. halys* more frequently when tests were conducted in small arenas, but only partially in more complex systems using large cages and plants as ovipositing substrates (Hedstrom et al. 2017; Botch and Delfosse 2018; Haye et al. 2019).

The last experiment was conducted to evaluate the effect of host egg size on the size and egg load of emerged *T. mitsukurii*. The results indicated that host egg volume significantly affected the size of emerging adult females and the number of the eggs in their ovaries, i.e., larger host eggs produced larger females with higher egg loads, confirming previous findings by Arakawa and Namura (2004). Since many non-target host species are smaller in size than *H. halys*, it can be assumed that a population of *T. mitsukurii* originating from non-target species results in females with lower reproductive ability. This was already speculated for *T. japonicus* by Botch and Delfosse (2018) and observed successively in laboratory tests (Sabbatini Peverieri et al. 2021).

Conclusions

Trissolcus mitsukurii displayed in laboratory tests no host specialization, showing capability to successfully parasitize most of tested Pentatomoidea species, including *A. custos*, an Asopinae predator, as was previously assessed also for *T. japonicus*. However, while in our laboratory experiments *T. mitsukurii* showed similar host preference toward more than one pentatomid species, *T. japonicus* preferred its coevolved host *H. halys* both in laboratory and field conditions (Milnes and Beers 2019; Botch and Delfosse 2018). It is well known that the testing conditions in the laboratory simplified environment can be misleading in non-target risk analysis and the results must be carefully evaluated (Haye et al. 2019). Thus, more complex systems are needed to better simulate field environmental factors, and experiments should adopt multidisciplinary and integrated approaches. These were considered in the case of *T. japonicus* and the large literature on host range tests in the laboratory evidenced, on a global scale, that the physiological host range of this parasitoid embraces more than two dozens of species among tested pentatomids (Zhang et al. 2017; Hedstrom et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Haye et al. 2019; Lara et al. 2019). Meanwhile, by means of olfactometer bioassays and testing volatiles from hosts and host-induced plant volatiles, things appear to be different and a higher level of species selection can be put in evidence, as these experiments allow to predict what cues from which host species the parasitoids are able to exploit in the field during host location (Bertoldi et al. 2019).

Considering the topical interest of classical biological control of *H. halys*, new field data on host preference by *T. mitsukurii* in Europe are expected to appear soon in the literature. Combined with the information we provide here, this will help understand the actual parasitoid host range of this exotic parasitoid in the novel area of its establishment.

Declarations

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Tables

Table 1 List of the European native and exotic Hemiptera tested for *Trissolcus mitsukurii* physiological host suitability and relative egg hatching rates in the rearing facility (n.a. = not assessed)

Target and non-target species	Family, subfamily, tribe	Egg mass hatching rate in the rearing facility
<i>Acrosternum heegeri</i> Fieber	Pentatomidae, Pentatominae, Pentatomini	n.a. (94.0%*)
<i>Aelia acuminata</i> (L.)	Pentatomidae, Pentatominae, Aelini	n.a.
<i>Ancyrosoma leucogrammes</i> (Gmelin)	Pentatomidae, Podopinae, Graphosomatini	n.a.
<i>Arma custos</i> (F.)	Pentatomidae, Asopinae, Asopini	77.67%
<i>Carpocoris pudicus</i> (Poda)	Pentatomidae, Pentatominae, Carpororini	95.7% (76.61%*)
<i>Dolycoris baccarum</i> (L.)	Pentatomidae, Pentatominae, Carpororini	88.6% (88.0%*)
<i>Eurydema oleracea</i> (L.)	Pentatomidae, Pentatominae, Strachiini	n.a.
<i>Eurydema ventralis</i> Kolenati	Pentatomidae, Pentatominae, Strachiini	93.1% (96.67%*)
<i>Graphosoma italicum</i> (Muller)	Pentatomidae, Pentatominae, Graphosomatini	83.33%
<i>Halyomorpha halys</i> (Stål)	Pentatomidae, Pentatominae, Cappaeiini	93.35% (90.39%*)
<i>Nezara viridula</i> L.	Pentatomidae, Pentatominae, Pentatomini	92.34% (85.58%*)
<i>Palomena prasina</i> (L.)	Pentatomidae, Pentatominae, Carpororini	n.a. (97.56%*)
<i>Piezodorus lituratus</i> (F.)	Pentatomidae, Pentatominae, Piezodorini	n.a. (88.0%*)
<i>Sciocoris</i> sp. Wollaston	Pentatomidae, Pentatominae, Sciocorini	n.a.
<i>Eurygaster maura</i>	Scutelleridae, Eurygastrinae, Eurygastrini	90.15%
<i>Gonocerus juniperi</i> Herrich-Schäffer	Coreidae, Coreinae, Gonocerini	n.a. (100%*)
<i>Rhynocoris iracundus</i> (Poda)	Reduviidae, Harpactorinae, Harpactorini	n.a. (96.67%*)

* Data from Sabbatini Peverieri et al., (2020) using the same rearing condition are reported within parentheses

Table 2 Physiological suitability of Hemiptera eggs for *Trissolcus mitsukurii* in 24h black box tests. Asterisks within each column indicate significant differences among the non-target species and *Halyomorpha halys* (Kruskall-Wallis followed by Dunn's Multiple Comparison test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.a. = not assessed) (see Table S1 for statistics)

Species (replicates)	n. eggs/egg mass (mean \pm SE)	% of eggs with parasitoid emergence/egg mass (mean \pm SE)	% of females (mean \pm SE)	% of hatched eggs / egg mass (mean \pm SE)	% of dead eggs (mean \pm SE)	n. of parasitized egg masses /with parasitoid emergence/total n. of eggs exposed	total n. of dead eggs		
							with undetermined content	with dead hemipteran nymphs	with dead parasitoid
<i>Halyomorpha halys</i> (12)	27.42 \pm 0.29	99.40 \pm 0.60	90.22 \pm 3.29	0.60 \pm 0.60	0 \pm 0.00	12 / 12 / 329	0	0	0
<i>Acrosternum heegeri</i> (12)	13.25 \pm 0.46	98.32 \pm 1.24	89.78 \pm 1.37	0 \pm 0.00	0.49 \pm 0.49	12 / 12 / 159	0	0	1 (adult)
<i>Aelia acuminata</i> (12)	11.83 \pm 0.24	7.20 \pm 3.57***	72.00 \pm 12.64	25.00 \pm 13.06	67.80 \pm 12.26***	7 / 4 / 142	93	0	13 (all adults)
<i>Ancyrosoma leucogrammes</i> (10)	11.50 \pm 0.45	0 \pm 0.00***	-	90.00 \pm 10.00***	10.00 \pm 10.00	1 / 0 / 115	3	0	9 (all adults)
<i>Arma custos</i> (12)	14.33 \pm 0.63	66.32 \pm 12.51	90.65 \pm 1.54	25.00 \pm 13.06	8.67 \pm 5.05	9 / 9 / 172	0	0	14 (all adults)
<i>Carpocoris pudicus</i> (12)	14.08 \pm 0.87	75.00 \pm 13.06	92.22 \pm 0.60	9.22 \pm 7.37	15.78 \pm 9.95	12 / 9 / 169	0	0	29 (28 pupae, 1 adult)
<i>Dolycoris baccarum</i> (12)	13.92 \pm 0.34	98.29 \pm 0.89	92.08 \pm \pm 1.02	0.55 \pm 0.55	0.59 \pm 0.59	12 / 12 / 167	1	0	0
<i>Eurydema oleracea</i> (10)	10.10 \pm 0.38	21.38 \pm 11.09**	82.22 \pm 5.35	25.00 \pm 11.33	55.21 \pm 11.50***	4 / 4 / 101	42	0	12 (all adults)
<i>Eurydema ventralis</i> (12)	11.83 \pm 0.32	0 \pm 0.00***	-	63.81 \pm 11.49**	36.19 \pm 11.49*	5 / 0 / 142	12	0	41 (6 pupae, 35 adults)
<i>Graphosma italicum</i> (12)	14.58 \pm 0.45	78.57 \pm 8.05	84.52 \pm \pm 2.85	15.16 \pm 8.07	6.28 \pm 2.74	11 / 11 / 175	1	0	10 (all adults)
<i>Nezara viridula</i> (12)	28.25 \pm 0.97	1.90 \pm 1.57***	100	29.04 \pm 10.45	68.36 \pm 10.89***	4 / 2 / 339	207	0	22 (all adults)
<i>Palomena prasina</i> (1)	12	100 n.a.	75 n.a.	0 n.a.	0 n.a.	1 / 1 / 12	0	0	0
<i>Piezodorus lituratus</i> (5)	10.60 \pm 1.29*	68.24 \pm 18.39	68.37 \pm 7.29	22.97 \pm 19.33	8.79 \pm 5.77	4 / 4 / 53	0	0	6 (all adults)
<i>Sciocoris</i> sp. (8)	10.38 \pm 0.57	81.60 \pm 12.25	83.07 \pm 2.72	10.94 \pm 9.28	6.07 \pm 3.29	8 / 7 / 83	1	0	5 (all adults)
<i>Eurygaster maura</i> (12)	11.67 \pm 0.53	87.36 \pm 8.32	90.74 \pm \pm 1.34	9.03 \pm 8.30	3.61 \pm 2.51	11 / 11 / 140	6	0	0
<i>Gonocerus juniperi</i> (12)	10.00 \pm 0.00	0 \pm 0.00***	-	97.50 \pm 1.31***	2.51 \pm 1.30	0 / 0 / 100	3	0	0
<i>Rhynocoris iracundus</i> (3)	32.22 \pm 0.88	0 \pm 0.00 n.a.	-	74.33 \pm 21.16 n.a.	25.67 \pm 21.16 n.a.	0 / 0 / 97	26	0	0

Table 3 Physiological suitability of Hemiptera eggs for *Trissolcus mitsukurii* in 2h black box tests. Asterisks within each column indicate significant differences among the non-target species and *Halyomorpha halys* (Kruskall-Wallis followed by Dunn's Multiple Comparison test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) (see Table S1 for statistics)

Species (replicates)	n. eggs/egg mass (mean \pm SE)	% of eggs with parasitoid emergence/egg mass (mean \pm SE)	% of females (mean \pm SE)	% of hatched eggs / egg mass (mean \pm SE)	% of dead eggs (mean \pm SE)	n. of parasitized egg masses /with parasitoid emergence/total n. of eggs exposed	total n. of dead eggs		
							with undetermined content	with dead hemipteran nymphs	with dead parasitoid
<i>Halyomorpha halys</i> (12)	27.58 \pm 0.74	81.69 \pm 3.41	94.76 \pm 0.44	18.31 \pm 3.41	0.00	12/12/331	0	0	0
<i>Acrosternum heegeri</i> (12)	14.50 \pm 0.68	91.11 \pm 8.30	89.68 \pm 1.13	8.89 \pm 8.30	0.00	11/11/174	0	0	0
<i>Aelia acuminata</i> (12)	10.92 \pm 0.34	3.19 \pm 2.53*	66.67 \pm 13.61	23.41 \pm 11.78	73.39 \pm 11.46***	6/1/131	82	0	18 (all adults)
<i>Arma custos</i> (12)	15.33 \pm 0.49	40.52 \pm 14.47	92.84 \pm 0.11	58.33 \pm 14.87	1.15 \pm 0.77	5/5/184	0	0	2 (all adults)
<i>Carpocoris pudicus</i> (12)	12.33 \pm 0.33	88.46 \pm 8.65	88.37 \pm 1.09	8.97 \pm 8.30	2.56 \pm 2.56	11/11/160	0	0	4 (all adults)
<i>Dolycoris baccarum</i> (12)	14.00 \pm 0.24	83.28 \pm 4.41	85.85 \pm 3.44	5.40 \pm 3.53	11.40 \pm 4.26	10/10/175	11	0	0
<i>Eurydema ventralis</i> (12)	11.67 \pm 0.19	0 \pm 0.00**	-	90.66 \pm 3.78	9.28 \pm 3.77	1/0/140	9	0	4 (all adults)
<i>Graphosoma italicum</i> (12)	14.75 \pm 0.31	77.12 \pm 5.84	86.85 \pm 2.30	18.87 \pm 6.36	4.00 \pm 2.72	12/12/177	0	0	7 (all adults)
<i>Nezara viridula</i> (12)	34.25 \pm 1.16	0 \pm 0.00**	-	22.85 \pm 10.62	77.15 \pm 9.38***	5/0/411	260	0	27 (all adults)
<i>Eurygaster maura</i> (12)	13.58 \pm 0.26	88.64 \pm 8.13	92.48 \pm 0.93	8.33 \pm 8.33	3.02 \pm 1.08	11/11/163	0	0	5 (all adults)

Table 4 Physiological suitability of Hemiptera eggs for *Trissolcus mitsukurii* in 2h exposure of a single host egg. Among brackets number of females tested; asterisks within each column indicate significant differences among the non-target species and *Halyomorpha halys* (Chi-square test with Yates' correction, *** $P < 0.001$) (see Table S2 for statistics)

species	% of emerged parasitoids	% of hatched eggs	% of dead eggs	
			with undetermined content or a dead hemipteran nymph	containing pupae or adults
<i>Halyomorpha halys</i> (31)	100	0	0	0
<i>Acrosternum heegeri</i> (30)	46.67***	53.33	0	0
<i>Arma custos</i> (43)	30.23***	55.81	9.31	4.65
<i>Dolycoris baccarum</i> (30)	20.00***	63.33	16.67	0
<i>Graphosoma italicum</i> (30)	26.67***	16.67	0	56.67
<i>Nezara viridula</i> (30)	0***	56.67	43.33	0
<i>Palomena prasina</i> (26)	100	0	0	0
<i>Eurygaster maura</i> (34)	2.94***	26.47	61.77	8.82

Figures

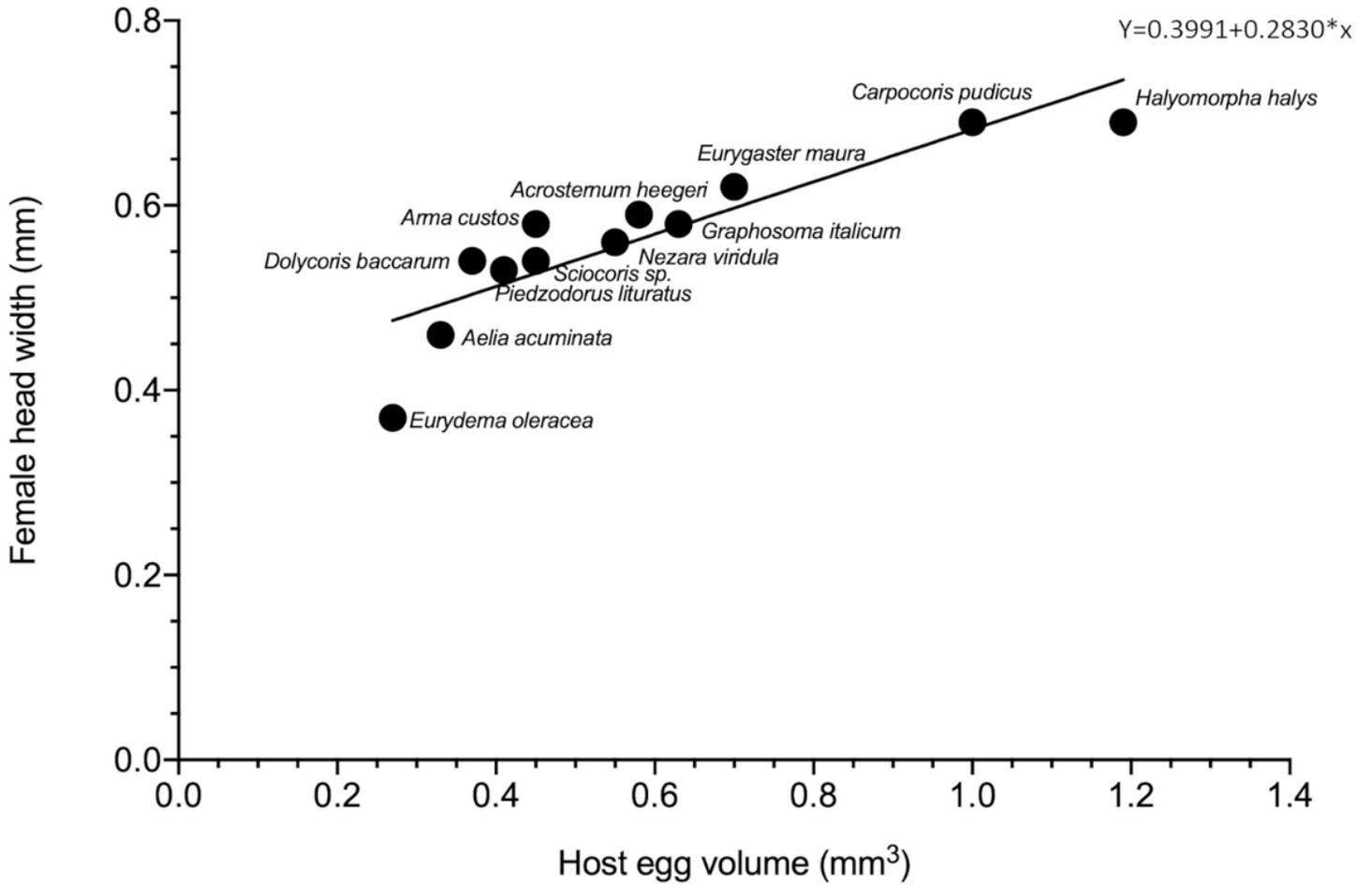


Figure 1

Regression analysis of *Trissolcus mitsukurii* females head width as a function of egg volume of Hemiptera host species

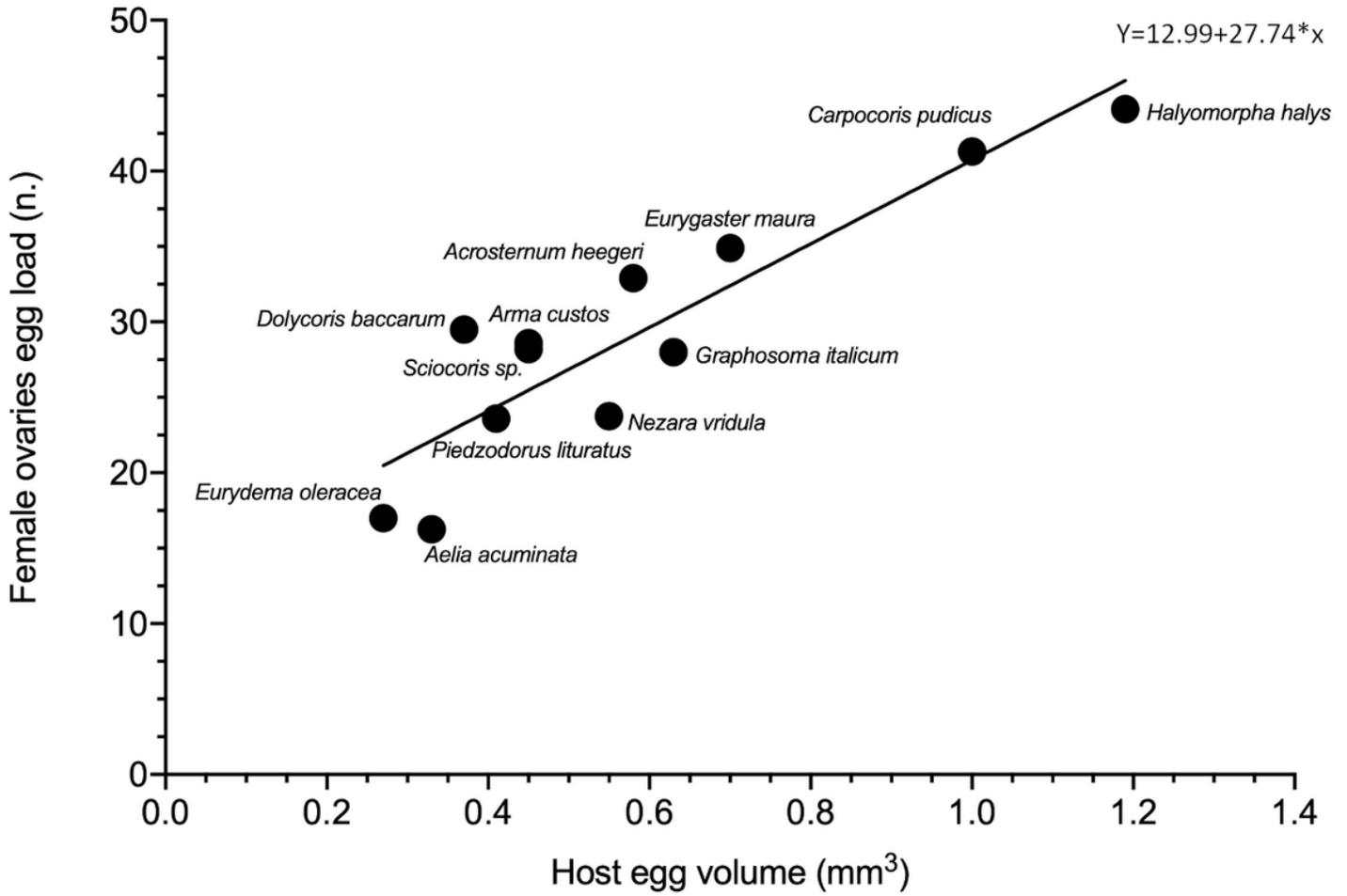


Figure 2
Regression analysis of *Trissolcus mitsukurii* female ovaries egg load (at 7 days in age) as a function of egg volume of Hemiptera host species

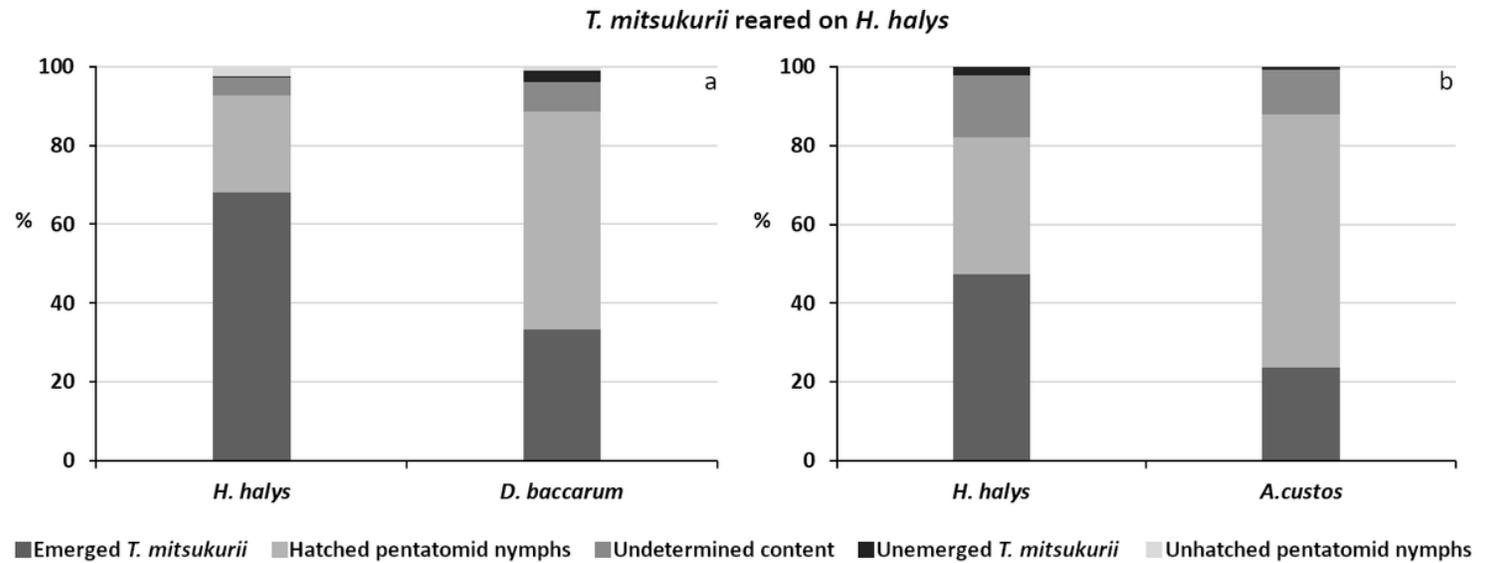


Figure 3
Outputs of choice tests using *Trissolcus mitsukurii* females, originated from *Halyomorpha halys*, that were exposed for 2 h to egg masses of target and non-target species in pairwise comparisons (A = *H. halys* vs. *Dolycoris baccarum*; B = *H. halys* vs. *Arma custos*)

T. mitsukurii reared on non-target species

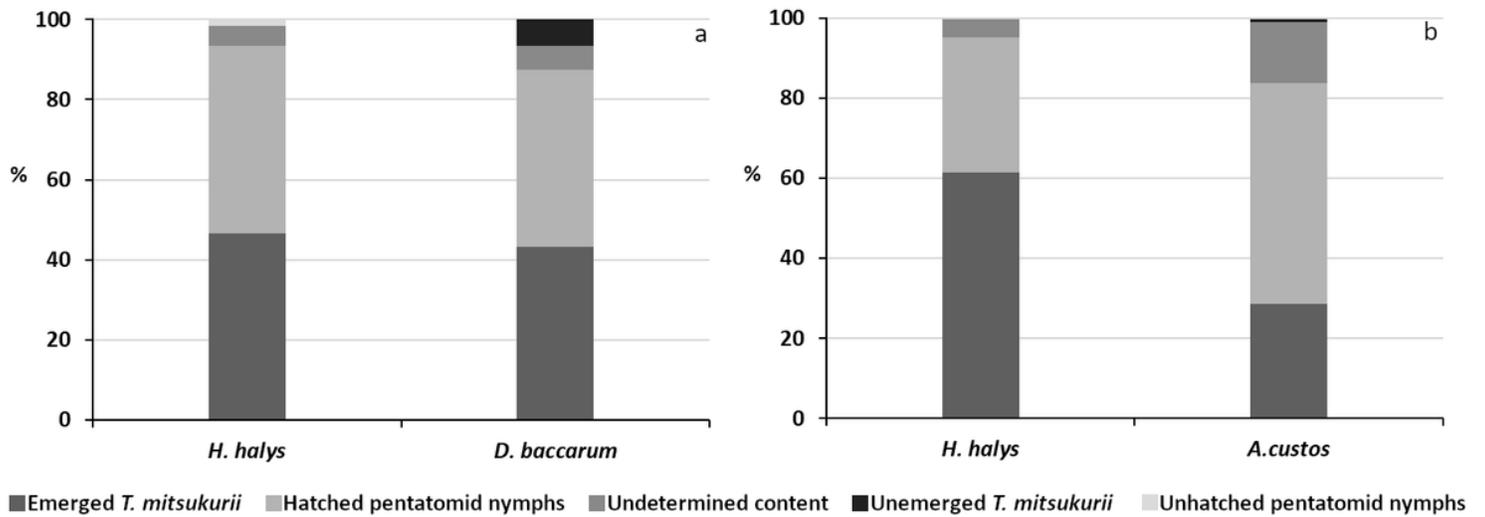


Figure 4

Outputs of choice tests using *Trissolcus mitsukurii* females, originated from non-target species *Dolycoris baccarum* or *Arma custos*, that were exposed for 2 h to egg masses of target and non-target species in pairwise comparisons (A = vs. *Dolycoris baccarum*; B = vs. *Arma custos*)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementarymaterialTablesS1S2S3S4.docx](#)