

Interspecific competition between *Trissolcus japonicus* and *Trissolcus mitsukurii*, two promising candidates for biocontrol of *Halyomorpha halys*

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HIGHLIGHTS

- *Trissolcus japonicus* and *T. mitsukurii* are valid potential biological control agents of *H. halys*.
- Interspecific competition among BCAs can affect pest control success.
- Multiparasitism caused a lower reproductive potential offspring production and wasted searching time.

GRAPHICAL ABSTRACT



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ABSTRACT

The invasive stink bug *Halyomorpha halys*, native to east Asia, is a severe agricultural pest of worldwide importance and biocontrol is a promising method for its long-term management. The two Asian egg parasitoids *Trissolcus japonicus* and *Trissolcus mitsukurii* are considered to be effective candidates as biological control agents. These species can co-occur in the same habitats and interspecific competition for hosts could therefore have an important role in natural control. We assessed the foraging behavior and the progeny production of both species under laboratory conditions with three different interspecific competition scenarios. With indirect competition, *H. halys* egg masses previously parasitized by the female of one species were offered to the competitor females after 0, 3, 5, or 7 days. With direct extrinsic competition, females of each species were tested in arenas either when introduced simultaneously (synchronous release) or when half of the host egg mass had already been parasitized by the other species (asynchronous release). Both *Trissolcus* species were able to parasitize host eggs already parasitized by the other species, although the progeny production always favored the species that arrived first on the host egg mass. However, in the synchronous releases scenario, *T. mitsukurii* displayed more aggressive behavior and spent more time defending the host egg mass than *T. japonicus*, resulting in a higher progeny production. Our results showed that multiparasitism among *T. japonicus* and *T. mitsukurii*, at least under

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laboratory conditions, resulted in a general waste of searching time, energy, eggs laid, and a lower reproductive potential by the second-arriving species on the host egg mass.

1. Introduction

The invasive Asian stink bug *Halyomorpha halys* Stål (Hemiptera, Pentatomidae) is a severe agricultural pest introduced accidentally in recent decades into several countries of the Americas, in Europe and in the Caucasus region (Leskey and Nielsen, 2018). Biological control is seen as one of the most effective long-term management approaches (Roversi et al., 2016; Abram et al., 2017). The solitary Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) is the main antagonist in the area of origin and is considered the most promising candidate for classical biological control in invaded regions (Zhang et al., 2017). This parasitoid has been extensively investigated as a biocontrol agent in the USA, Europe and New Zealand, and is the biocontrol agent targeted for release in the field after petition approvals by local authorities (Hedstrom et al., 2017; Botch and Delfosse, 2018; Haye et al., 2019; Sabbatini Peverieri et al., 2021). In Europe, Italy was the first country to initiate a large-scale release program of laboratory-reared *T. japonicus* (MiPAAF, 2020). Additionally, adventive populations of *T. japonicus* have also been recently detected in Europe and in the USA (Talamas et al., 2015; Sabbatini Peverieri et al., 2018; Stahl et al., 2019; Dieckhof et al., 2021). Moreover, adventive populations of *Trissolcus mitsukurii* (Ashmead) (Hymenoptera, Scelionidae), a less-studied egg parasitoid of eastern Asia origin, was recently found attacking *H. halys* in Italy and in neighboring countries of Slovenia and France, highlighting its role in the parasitoid guild of *H. halys* and leading researchers to consider it as a second candidate for biocontrol in the pest's invaded regions (Sabbatini Peverieri et al., 2018; Benvenuto et al., 2020; Rot et al., 2021; Yonow et al., 2021; Bout et al., 2021). While *T. japonicus* has been the subject of an extensive body of scientific literature, especially concerning risk analysis of its introduction into new areas, such studies about *T. mitsukurii* are currently in an embryonic stage (Giovannini et al., 2021a; Rondoni et al., 2022). Little is known about its lifecycle and its impact on the host species (Arakawa and Namura, 2002; Arakawa et al., 2004; Sabbatini Peverieri et al., 2020; Scaccini et al., 2020).

Trissolcus japonicus and *T. mitsukurii* co-occur in part of the Asian range of *H. halys*, but while *T. japonicus* is reported widely throughout this range, *T. mitsukurii* has been reported in the scientific literature mostly from Japan (Zhang et al., 2017; Yonow et al., 2021; Arakawa and Namura, 2002; Arakawa et al., 2004; Hokyo and Kiritani, 1963; Hokyo and Kiritani, 1963, 1966; Kiritani, 1964; Hokyo et al., 1966; Ryu and Hirashima, 1984; Yang et al., 2009; Nishimoto et al., 2015). Recent discoveries during an extensive monitoring program of both species in the same areas in Northern Italy, indicated that these species are sympatric there as well (Zapponi et al., 2021). Moreover, it is expected that the adventive populations of *T. japonicus* and *T. mitsukurii* reported in this area will naturally spread into neighboring countries and/or new introductions will occur elsewhere (Rot et al., 2021; Bout et al., 2021; Dieckhoff et al., 2021).

Previous studies on interspecific competition between *T. japonicus* and native European and Asian egg parasitoids [*Anastatus bifasciatus* (Geoffroy), *Anastatus japonicus* Ashmead (Hymenoptera, Eupelmidae), and *Trissolcus cultratus* (Mayr) (Hymenoptera, Scelionidae)] found strong aggressive behavior by *T. japonicus* females, but also by *T. mitsukurii* against the latter, as recently reported by Costi et al., 2022. However, successful competition is also affected by the time of arrival to the host eggs (Konopka et al., 2017a; Haye et al., 2021; Mi et al., 2021). Similarly, studies on interspecific competition involving *T. mitsukurii* within the parasitoid guild of *Nezara viridula* L. (Hemiptera, Pentatomidae) [*Trissolcus basalis* (Wollaston) and *Telenomus nakagawai* Watanabe (Hymenoptera, Scelionidae)] documented its aggressive behavior

in host patch defense (Hokyo and Kiritani, 1966; Hokyo et al., 1966; Field and Calbert, 1999; Nishimoto et al., 2015).

Interspecific competition in egg parasitoids can be “direct” or “indirect”. Direct (extrinsic) interspecific competition occurs when females of different species simultaneously attack the same host egg mass, or their juvenile stages are both present in the host egg (direct intrinsic competition) (Boivin and Brodeur, 2006; Cusumano et al., 2012). Indirect competition occurs when a female encounters a host egg mass already parasitized by a different species and reacts by adapting its foraging behavior (e.g., deciding to multiparasitize, or not). In direct competition, female interactions can be resolved in different ways: leaving the host patch, co-exploiting the patch, or engaging in aggressive physical interactions (e.g., fights using the mandibles). The resulting interactions may be negative for both competitors, by wasting searching time, loss of energy and risk of severe injury (Hokyo and Kiritani, 1966; Field et al., 1998).

Scelionid female behavior during host egg parasitization is typically stereotyped (Hokyo and Kiritani, 1966; Field, 1998) and can be summarized as follows: 1) host egg inspection by antennation; 2) egg acceptance with ovipositor drilling of the chorion; 3) oviposition; 4) marking of the parasitized host egg. Marking is adaptive by enabling recognition of already parasitized eggs to avoid self-superparasitism (Okuda and Yeorgan, 1988) and is a clear sign of successful oviposition in scelionid species (Hokyo and Kiritani, 1966; Field, 1998; Wilson, 1961; Van Baaren et al., 1994; Weber et al., 1996). Marked eggs are also recognizable by conspecifics (thus avoiding superparasitism), while in interspecific competition, in most of cases studied, the marked egg recognition appears uncommon (Boivin and Brodeur, 2006; Okuda and Yeorgan, 1988). During the post-ovipositional period, females may exhibit additional behaviors, such as continuing to inspect the egg mass, guarding, resting or grooming (Field, 1998).

The sympatry of *T. japonicus* and *T. mitsukurii* as reported by recent field data both in Italy (Zapponi et al., 2021) and Europe in general (see Bout et al., 2021; Dieckhoff et al., 2021; Rot et al., 2021), and the potential opportunity to use these species in biological control programs against *H. halys*, stimulated a number of studies on their interactions with each other or with different species of the parasitoid guild, in order to assess possible pest control outcomes, as firstly evidenced by Konopka et al. (2017a, 2017b), Haye et al. (2021), Mi et al. (2021) and successively by Costi et al. (2022). Indeed, interspecific competition can potentially disrupt biological control programs, or cause synergism, or it may have no influence at all (Boivin and Brodeur, 2006; Casula et al., 2006; Mills, 2006; Ode et al., 2022).

In laboratory experiments, we investigated the mechanisms and behaviors displayed during interspecific competition between *T. japonicus* and *T. mitsukurii* and the way these species exploit common resources. The outcomes focused on three different experimental scenarios which are potentially predictors for field occurrence: i) when a female of one species encountered a host egg mass previously parasitized by a female of the other species which had already departed (indirect competition); ii) when females of each species were released simultaneously in an arena containing a host egg mass (direct competition with synchronous release); iii) when a female of one species encountered a host egg mass already parasitized but still attended by a female of the other species (direct competition with asynchronous release). We then quantified and analyzed principal behavior patterns exhibited by *Trissolcus* females of each species under these competitive scenarios in order to estimate the success in host egg parasitization, the progeny production of each species, and the potential impact on host egg survival.

In view of the considerable interest in egg parasitoids of *H. halys*,

similar studies on this subject were conducted concurrently by several research groups. Although the results obtained from such studies are similar to ours (e.g., see Costi et al., 2022), the different approaches used for the experimental designs (e.g., use of unmanipulated and naturally-sized egg masses, different timing in direct and indirect competition, synchronous and asynchronous release of females under direct competition, different ethological traits investigated, different origin/haplotypes of the parasitoid populations) provide additional and complementary information that may help to describe potential scenarios for biological control programs.

2. Material and methods

2.1. Insect origins and rearing

Halyomorpha halys colony was established from individuals collected at infested sites in Northern Italy in 2020. Collected specimens were maintained in the lab in insect rearing cages (BugDorm® 4F4545, MegaView Science, Taiwan, China) and fed with fresh fruits (apples, kiwi), vegetables (carrots, green beans), potted plants (soybean), and seeds (peanuts and soybean); water was provided with wet cotton. *Trissolcus mitsukurii* females were obtained from laboratory colonies originally established with *H. halys* egg masses collected from infested sites in Northeastern Italy (*T. mitsukurii* strain H5). *Trissolcus japonicus* females were obtained from permanent lab-reared colonies of populations originally collected in Beijing (China) (*T. japonicus* strain H7). *Trissolcus mitsukurii* was selected due to the recent attention resulting from its occurrence in North Italy as a newly-reported member of the parasitoid guild of *H. halys*, while *T. japonicus* of Beijing origin was selected because this population is the most-investigated worldwide for classical biological control actions and has been mass-reared for field release. Colonies of both egg parasitoids were maintained on fresh *H. halys* egg masses (parasitized at age < 24 h); adult parasitoids were fed with pure honey droplets replenished twice a week. Adults and parasitized host eggs were maintained in glass tubes in climatic chambers under standard conditions of 26 °C, 60 %RH, and 16:8 L:D. A total of 156 *H. halys* egg masses were used in the tests (mean of 27.53 ± 0.19 S.E. eggs/ mass).

2.2. Indirect competition

To evaluate the parasitization behavior and the progeny production by *T. japonicus* and *T. mitsukurii* females when one species was provided with a *H. halys* egg mass already completely parasitized by the other species, a fresh *H. halys* egg mass was placed in a plastic Petri dish (30 mm in diameter) and exposed to a single naïve female of *T. japonicus* or *T. mitsukurii* (7 d old post-eclosion for optimal accumulation of ovarian eggs, see Sabbatini Peverieri et al., 2020). Its foraging behavior in the arena was video-recorded (Panasonic WV-BP330/GE, Suzhou Matsushita Co. Ltd., Suzhou, China) for 2.5 h starting from the first contact with the host egg mass in the arena. Preliminary analysis showed that 2.5 h was a sufficient time-lapse to permit complete egg mass parasitization under non-competitive conditions for both egg parasitoids. Behavioral tests were conducted under laboratory conditions (25 °C; 55 % RH) and a constant cold light source.

For tests with *T. japonicus* as the first-arriving species, the egg mass was then removed from the arena and from the *T. japonicus* female and transferred to a climatic chamber for juvenile development as needed for the successive interspecific competition test. These egg masses were offered to *T. mitsukurii* females at four different intervals: immediately (T0), after 3 days (T3), 5 days (T5) and 7 days (T7). The development stage of parasitoid juvenile present at these intervals included: egg, developing larva (LI-LII), mature larva (including prepupa) and pupa stadium (Giovannini et al., 2021). At each scheduled time interval, a female *T. mitsukurii* (naïve and 7d in age) was introduced into the arena containing the *H. halys* egg mass previously parasitized by *T. japonicus*;

its behavior was videorecorded for 2.5 h. Each treatment was replicated 12 times.

The same experimental design was used when *T. mitsukurii* was given host eggs to parasitize first, then followed by *T. japonicus*. Following the experimental exposures to parasitoid females, the egg masses used were maintained in the climatic chamber until emergence of progeny. Additionally, twelve *H. halys* egg masses were offered to females of each species for parasitization as no-competition controls to compare progeny production and parasitization behavior. Offspring were identified to species and sexed; female development times were also recorded.

The following behaviors and parameters were recorded:

- time spent by the female inspecting the egg mass before beginning parasitization (acceptance time);
- percentage of host eggs parasitized successfully (oviposition and subsequent host egg marking);
- percentage of host eggs parasitized successfully before the first abandonment of the host egg mass;
- percentage of host eggs producing parasitoid progeny;
- percentage of *H. halys* eggs hatched and dead.

Due to the complexity of the behavioral patterns shown by *Trissolcus* during the parasitization process, data on additional behavioral parameters are reported in [Supplementary material S1](#). These data are relevant to the scope of our research and add ethological information for the studied species.

2.3. Direct competition with synchronous release of females

In this experiment, we simulated simultaneous arrival of two parasitoid females at the same *H. halys* egg mass. To evaluate parasitization behavior, species interactions and progeny production by the two parasitoids, a fresh *H. halys* egg mass was placed in a plastic Petri dish (30 mm in diameter) and one naïve female (7 days old) of each *Trissolcus* species was introduced simultaneously into the arena. To distinguish *T. mitsukurii* from *T. japonicus* females during behavioral analysis, the mesoscutum of each female was marked with a different color of acrylic tempera (Zenacolor®, BMS International, Grand Bay, Mauritius) using a fine brush (series 7n. 000, Winsor and Newton®, London, UK) before being used in the tests. Preliminary observations showed that the marking on the mesoscutum did not affect mobility, parasitization behavior, longevity or fecundity. The behavior of both females in the arena was videorecorded for 2.5 h and started when the females were introduced in the arena. At the end of the exposure the females were removed from the arena and the egg mass transferred to a climatic chamber and reared under standard conditions. The egg mass was checked daily until eggs hatched or parasitoids emerged. Offspring were identified to species and sexed, and the female development time was recorded. The experiment was replicated 12 times.

The elapsed time and numbers of the following behaviors and events were recorded for both species:

- time from release of each female in the arena to first contact with the host egg mass (discovery of egg mass);
- time spent by each female on the egg mass for parasitization (including egg inspection, antennation, drilling, egg laying and marking);
- time spent by each female standing motionless on the egg mass or moving across host eggs without further inspection (guarding);
- time spent by each female in aggressive interactions with each other (biting or wrestling);
- time spent by the female away from the egg mass after first contact;
- number of successful ovipositions in host eggs followed by marking;
- number of attempted ovipositions interrupted due to interaction with the competitor (not resulting in a complete oviposition with marking);
- number of times a female chased the other female away from the

host egg mass (attacks);

- i) number of times a female won a physical contest;
- l) number of offspring produced by each of the two competing females;
- m) percentage of *H. halys* eggs hatched and dead.

2.4. Direct competition with asynchronous release of females

Direct (extrinsic) interspecific competition was also investigated with sequential release of the two species together in an arena containing an *H. halys* host egg mass. The same procedures used in experiment 2.3 were applied except that the second female was released into the arena after a short delay, permitting the first female introduced (hereafter the “resident” female) to attack a portion, but not all, of the host eggs in the mass before the second female was introduced. Previous analysis of parasitization behavior showed that *T. japonicus* takes less time to oviposit compared to *T. mitsukurii*, thus the females of *T. japonicus* and *T. mitsukurii* were allowed to parasitize the *H. halys* egg mass for 60 min and 75 min, respectively. Preliminary observations showed that these intervals were sufficient to permit the parasitization of approximately half of the host egg mass. The second female was then introduced into the arena (hereafter the “intruder” female). The delayed arrival of the second female to the same egg mass produced an asymmetry in resource investment due to the partial parasitization of the mass by the first arriving species (Field and Calbert, 1999). Their direct competition was videorecorded for 90 min to analyze the behavior of both contestants. Twelve replicates of each succession combination (*japonicus* followed by *mitsukurii* and the reverse) were conducted.

The times and number of events of the following parameters were recorded for both species, as described for experiment 2.3:

- a) time spent for parasitization;
- b) time spent for guarding;
- c) time spent for fighting;
- d) time spent away from the host egg mass;
- e) number of attacks;
- f) number of times a female won a physical contest;
- g) total progeny produced;
- h) percentage of *H. halys* eggs hatched and dead.

2.5. Data analysis and statistics

The behavior of *Trissolcus* females during parasitization and direct extrinsic and indirect interactions was recorded with BORIS software (Friard and Gamba, 2016). Data obtained from videorecording and from rearing the parasitized *H. halys* host egg masses were used to build the data sets in all three experiments.

Simple generalized linear models for binomial distribution with a logistic link function were adopted to evaluate the effect of the different exposure times on the behavioral responses recorded in indirect competition (including also the behaviors described in S1) and a Kruskal-Wallis test followed by Dunn’s post hoc test ($p < 0.05$). The dead and hatched *H. halys* eggs in absence of competition were analyzed by Mann-Whitney’s test and pooled where no significant differences were detected. Release experiments, concerning both direct competition with synchronous and asynchronous release of females, were analyzed by a generalized linear model and a Kruskal-Wallis test followed by Dunn’s post hoc test ($p < 0.05$). The $\ln(x + 0.5)$ transformation was used in this analysis to normalize the data in order to meet normality assumptions/criteria and stabilize the variance of the population. In the test of asynchronous release of females, Mann-Whitney’s test was used to verify the homogeneity of the first arriving females parasitization. All statistics were performed using SPSS 20.0 software.

3. Results

3.1. Indirect competition

The total number of eggs parasitized was similar in the absence of competition and also in most of the competitive scenarios, regardless of the order of species succession and the time intervals between the two sequential parasitization exposures (Table 1); significant differences in parasitization behavior occurred only in treatment T7, where the second female attacked fewer *H. halys*, regardless of species order ($F_{17,198} = 5.810, p < 0.001; H = 45.140, p < 0.001$). The mean range of parasitized eggs per egg mass before the first female departed the egg mass was variable between 34 % and 100 % and no differences were detected among most of the combinations; also in this case, the only significant differences occurred in treatment T7 regardless of species successions ($F_{17,198} = 8.102, p < 0.001; H = 66.620, p < 0.001$). The mean acceptance time of host eggs by females in the different combinations was variable and ranged from 42 and 109 s ($F_{17,198} = 4.345, p < 0.001; H = 65.210, p < 0.001$). *Trissolcus mitsukurii* females had a longer acceptance time than *T. japonicus* in absence of competition, but no differences were detected among competitive scenarios. The percentages of dead eggs in absence of competition were extremely low both in *T. japonicus* and *T. mitsukurii* (zero and 1.49 %, respectively) and not different ($U = 48,000, p = 0.093$); such data were pooled and compared with the treatments. Under the competitive interactions, higher numbers of dead eggs were recorded in all other combinations than in absence of competition ($F_{17,198} = 12.370, p < 0.001; H = 68.350, p < 0.001$). *Halyomorpha halys* eggs that hatched were observed at an extremely low rate (<0.1 % of all the exposed host eggs). No hatched eggs were recorded in absence of competition in both species, similarly to results observed among the competitive scenarios (Table 1; $F_{9,110} = 0.818, p = 0.601; H = 1.39, p = 0.592$).

Both *T. japonicus* and *T. mitsukurii* had similar high levels of progeny production in non-competitive control replicates, reaching a mean of 98.20 % and 97.31 % respectively ($F_{17,198} = 103.700, p < 0.001; H = 183.400, p < 0.001$). The second arriving females of both species produced a lower number of progeny than females in absence of a competition scenario. No significant difference in the number of progeny was recorded for the first arriving females except in the T0 treatment, where the progeny production was significantly lower than in the absence of competition (Fig. 1).

3.2. Direct competition with synchronous release of females

Trissolcus japonicus was the first to discover the egg mass in seven of the 12 replicates. *Trissolcus japonicus* and *T. mitsukurii* females both encountered the host egg mass within a few minutes of starting the test, on average 3.16 min and 4.27 min ($F_{1,22} = 0.009, p = 0.923; H = 0.12, p = 0.729$), respectively. *Trissolcus japonicus* spent significantly less time (31.79 % of the total time observed) inspecting and parasitizing the egg mass than *T. mitsukurii* females (59.10 %) ($F_{1,22} = 3.102, p = 0.092; H = 4.201, p = 0.040$) (Fig. 2). Duration of guarding behavior also differed between the two species ($F_{1,22} = 20.420, p < 0.001; H = 12.610, p < 0.001$). *Trissolcus mitsukurii* spent more time guarding the egg mass (28.03 %) than *T. japonicus* (0.76 %). The two species also differed in time spent away from the egg mass ($F_{1,22} = 26.330, p < 0.001; H = 14.080, p < 0.001$). *Trissolcus japonicus* was more often away from the host patch compared to *T. mitsukurii* (64.70 % vs 9.30 % of the total available time). The total time spent in aggressive interactions (i.e., fighting), occupied less than a minute (0.59 %) of the total time.

Trissolcus mitsukurii females were significantly more likely to exhibit aggressive behavior, initiating a greater number of attacks than *T. japonicus* ($F_{1,22} = 20.070, p < 0.001; H = 11.020, p < 0.001$), and were more likely to win the physical contests ($F_{1,22} = 20.000, p < 0.001; H = 10.830, p < 0.001$) (Fig. 3). The presence of a competitor on the egg mass sometimes induced females to interrupt oviposition, although such

Table 1

Behavioral parameters and egg survival (mean \pm S.E.) of *Trissolcus japonicus* and *Trissolcus mitsukurii* at different time intervals, between successive parasitization exposures to *Halymorpha halys* egg masses.

	Interval between initial parasitization and 2nd species introduction	% host eggs parasitized (host eggs marked)	% eggs parasitized during first encounter with egg mass	Host time acceptance (s)	% <i>H. halys</i> dead eggs	% <i>H. halys</i> hatched eggs
No competition	Tj	100 \pm 0.00	100 \pm 0.00	43.83 \pm 10.05	0.00 \pm 0.00	0.00 \pm 0.00
	Tm	98.51 \pm 0.69	83.63 \pm 7.57*	85.67 \pm 11.95**	1.49 \pm 0.67	0.00 \pm 0.00
<i>T. japonicus</i> first – <i>T. mitsukurii</i> second	T0 Tj	96.07 \pm 2.99	96.07 \pm 2.99	41.75 \pm 5.93	4.24 \pm 1.58*	0.08 \pm 0.08
	T0 Tm	89.04 \pm 4.82	76.48 \pm 6.44	109.08 \pm 18.15		
	T3 Tj	100 \pm 0.00	97.88 \pm 1.44	42.42 \pm 10.88	5.28 \pm 2.23**	0.00 \pm 0.00
	T3 Tm	91.37 \pm 5.81	88.11 \pm 6.26	80.25 \pm 6.87		
	T5 Tj	100 \pm 0.00	100 \pm 0.00	45.50 \pm 6.23	4.53 \pm 1.29**	0.08 \pm 0.08
	T5 Tm	83.43 \pm 8.48	97.06 \pm 1.80	79.17 \pm 10.80		
	T7 Tj	98.40 \pm 1.60	98.08 \pm 1.92	64.17 \pm 16.01	9.41 \pm 3.94**	0.17 \pm 0.17
	T7 Tm	58.30 \pm 8.85***	34.74 \pm 10.19**	69.58 \pm 9.44		
<i>T. mitsukurii</i> first – <i>T. japonicus</i> second	T0 Tm	95.78 \pm 2.12	78.31 \pm 5.75	85.75 \pm 11.57	3.05 \pm 0.89*	0.08 \pm 0.08
	T0 Tj	100 \pm 0.00	97.22 \pm 3.13	54.17 \pm 6.76		
	T3 Tm	98.85 \pm 0.49	86.70 \pm 6.79	83.92 \pm 8.52	9.70 \pm 2.99***	0.00 \pm 0.00
	T3 Tj	86.83 \pm 5.41	86.55 \pm 5.57	50.25 \pm 6.86		
	T5 Tm	97.90 \pm 0.69	78.97 \pm 8.09	101.00 \pm 11.96	7.31 \pm 2.05***	0.17 \pm 0.11
	T5 Tj	96.68 \pm 2.32	90.12 \pm 7.96	107.50 \pm 46.45		
	T7 Tm	99.39 \pm 1.11	87.13 \pm 7.81	92.92 \pm 13.70	4.77 \pm 1.78**	0.25 \pm 0.18
	T7 Tj	74.12 \pm 8.51**	37.22 \pm 11.66***	61.33 \pm 19.66		

Sequence day interval of the two successive parasitoid exposures: T0 = immediate successive exposure, T3 = 3 days, T5 = 5 days; T7 = 7 days. Within each column, for each species behaviour, an asterisk indicates a significant difference between the treatment and the absence of competition; the dead and hatched eggs of *H. halys* recorded in each competition sequence treatment are compared with the absence of competition treatments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) (data of dead and hatched eggs were pooled).

interruptions were infrequent and did not differ significantly between the two species ($F_{1,22} = 3.944$, $p = 0.059$; $H = 3.968$, $p = 0.043$). *Trissolcus mitsukurii* successfully completed more ovipositions (including marking of the host egg) (mean of 17.83 ± 2.67 S.E.) than *T. japonicus* (9.67 ± 2.56 S.E.) although this difference was not significant ($F_{1,22} = 1.749$, $p = 0.200$; $H = 3.741$, $p = 0.053$). Nevertheless, *T. mitsukurii* produced significantly more progeny (16.08 ± 2.42 S.E.) than *T. japonicus* (6.67 ± 2.18 S.E.) ($F_{1,22} = 4.861$, $p = 0.038$; $H = 5.070$, $p = 0.024$). No dead or hatched *H. halys* eggs were observed in any of the replicates.

3.3. Direct competition with asynchronous release of females

A mean of 17.00 ± 1.22 (S.E.) and 17.58 ± 0.58 (S.E.) eggs were parasitized by *T. japonicus* and *T. mitsukurii*, respectively, before introducing the female of the other species ($U = 56,500$, $p = 0.384$). Parasitization behavior (including host inspection and oviposition) was not significantly different for the two species when *T. japonicus* arrived first (resident species) and *T. mitsukurii* was introduced as second species (intruder) ($F_{1,22} = 0.162$, $p = 0.691$; $H = 0.120$, $p = 0.729$) (Fig. 4). In the reverse case, when *T. mitsukurii* was the first species introduced and *T. japonicus* was second, differences in parasitization behavior were significant ($F_{1,22} = 11.200$, $p = 0.003$; $H = 6.750$, $p = 0.009$). Similarly, guarding behavior was not significantly different when *T. japonicus* was the first species introduced in the arena ($F_{1,22} = 1.200$, $p = 0.285$; $H = 0.563$, $p = 0.451$), but when *T. mitsukurii* was the first species introduced, the females spent more time in egg mass guarding ($F_{1,22} = 9790.000$, $p < 0.001$; $H = 17.280$, $p < 0.001$). When *T. japonicus* was the resident species, no significant differences were observed in time spent away from the egg mass ($F_{1,22} = 2.092$, $p = 0.162$; $H = 1.688$, $p = 0.193$). In contrast, when *T. mitsukurii* was the resident species, *T. japonicus* spent significantly more time away from the egg mass ($F_{1,22} = 115.700$, $p < 0.001$; $H = 17.280$, $p < 0.001$).

The number of attacks was not significantly different between

resident-intruder combinations when *T. japonicus* female was the resident ($F_{1,22} = 0.466$, $p = 0.502$; $H = 0.120$, $p = 0.729$), whereas when *T. mitsukurii* was the resident female, it had a greater number of attacks versus *T. japonicus* ($F_{1,22} = 145.900$, $p < 0.001$; $H = 17.280$, $p < 0.001$) (Fig. 5). No difference between species was observed in resolution of the aggressive interactions (combination: *T. japonicus* resident species: $F_{1,22} = 0.447$, $p = 0.511$; $H = 0.120$, $p = 0.701$; combination: *T. mitsukurii* resident species: $F_{1,22} = 1.239$, $p = 0.278$; $H = 0.853$, $p = 0.249$). The first-arriving females, regardless of species, produced the greatest number of progeny (combination: *T. japonicus* resident species: $F_{1,22} = 31.450$, $p < 0.001$; $H = 15.870$, $p < 0.001$; combination: *T. mitsukurii* resident species: $F_{1,22} = 124.100$, $p < 0.001$; $H = 17.280$, $p < 0.001$). No dead or hatched *H. halys* eggs were observed in any of the replicates.

4. Discussion

Under these laboratory experimental conditions, *T. japonicus* and *T. mitsukurii* showed that when in indirect competition for *H. halys* eggs, females of both species seem unable to recognize signs of previous parasitization when encountering egg masses recently parasitized by the other species. This was confirmed by the high percentage of eggs parasitized by the second female arriving in most of the combinations tested. Only when 7 days had elapsed since *H. halys* eggs were first parasitized did late-arriving females change their behavior to attack fewer host eggs than in absence of competition. In this case, the second arriving female was probably able to perceive the presence of the pupa inside the egg (e. g. by detecting chemical cues).

In the field a female may reject a previously parasitized egg mass at any stage in order to search for a more suitable host, but this scenario cannot be investigated with the our experimental design. To obtain information about this potential situation, we evaluated the parasitization ability of the female within the time period from the acceptance of the host and the first abandonment of the egg mass. In this case, we found no differences after five days from initial parasitization compared to the

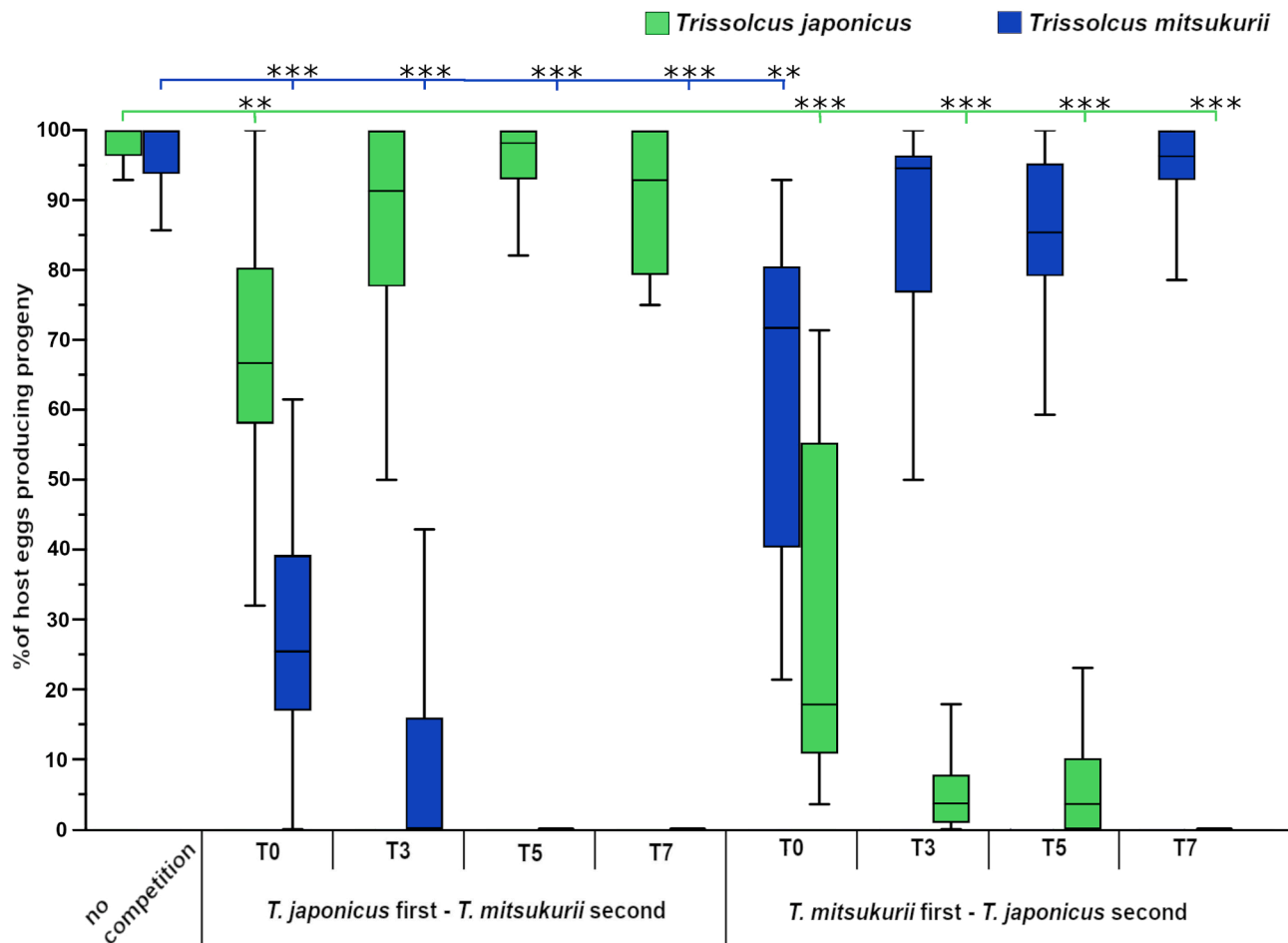


Fig. 1. Progeny production following indirect interspecific competition between *Trissolcus japonicus* and *Trissolcus mitsukurii*: egg masses of *Halyomorpha halys* were first parasitized by one species and then offered to the other species at different time intervals. Interval (days) between successive exposures: T0 = immediate; T3 = 3 days; T5 = 5 days; T7 = 7 days. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) for each treatment with competition absence. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5 \times$ the interquartile range.

absence of competition, but differences emerged in the T7 combination, in which the second arriving female parasitized a lower number of eggs. The ability to multiparasitize was also observed by Haye et al. (2021) with *T. japonicus* when in competition with *T. cultratus*.

In general *T. mitsukurii* females spend more time in evaluating the egg mass for oviposition suitability than *T. japonicus* (host acceptance time). Unfortunately, we cannot compare this parameter from our study with the data obtained by Costi et al. (2022), since their measurement also included the host searching time.

Overall, the competition between the two species did not lead to higher host egg survival, and the number of hatched eggs recorded was extremely low in all the scenarios tested. Instead, the species interactions led to a higher number of dead eggs.

Although females of *T. japonicus* and *T. mitsukurii* in most cases (T0 – T5) parasitized host egg masses at an equal rate independently of the order of species introduction and the interval before discovery of host eggs by the second species, the eventual production of progeny was, on the whole, significantly affected: in most cases progeny produced by the first arriving female was similar to when the competition was absent, with the only exception being when the second species arrived immediately after the first one (T0). In this case, the first arrived female produced significantly fewer progeny. In general, the first arrived species had the highest reproductive success, as was observed by Costi et al. (2022) for the same species and in other *Trissolcus* species (Weber et al., 1996). The outcome of intrinsic competition might be different if the success of the second *Trissolcus* arriving species is subject to a temporal

trophic shift as observed by Haye et al. (2021), where *T. cultratus* was found to be a superior intrinsic competitor of *T. japonicus* when arriving as the second species at zero and three days after initial *T. japonicus* parasitization, but not after 1, 2, 4 or 5 days. Haye et al. (2021) showed that *T. cultratus* is also able to act as a hyperparasitoid (at least under their experimental conditions), but this was not observed in *T. japonicus*. Hyperparasitism was not observed in *T. japonicus* and *T. mitsukurii* also (Costi et al., 2022). In our tests, the second-arriving species was able to produce at least a minimal number of progeny when the elapsed time between initial parasitization and the arrival of the second parasitoid female was short (not more than three days after the primary parasitization) and exceptionally *T. japonicus* also after 5 days. This behavior is explained by Haye et al. (2021) for *T. cultratus* which is also potentially able to exploit a competitor parasitoid larva for its development in addition to the host pentatomid egg.

The parasitizing behavior of both *Trissolcus* species tested appeared very similar under our test conditions, and indirect interactions among females showed that the first female had a greater success in progeny production and did not suffer severely in competition with the second-arriving species. In contrast, the second-arriving species had less success in progeny production, with a decreasing trend as the elapsed time from first parasitization increased. Moreover, in contrast to the high level of *H. halys* eggs parasitized by the first-arriving females (in which parasitoid eggs were successfully laid), as might be expected, the second-arriving females parasitized host eggs at a rate that decreased slightly over time, although rates of total parasitized host eggs remained

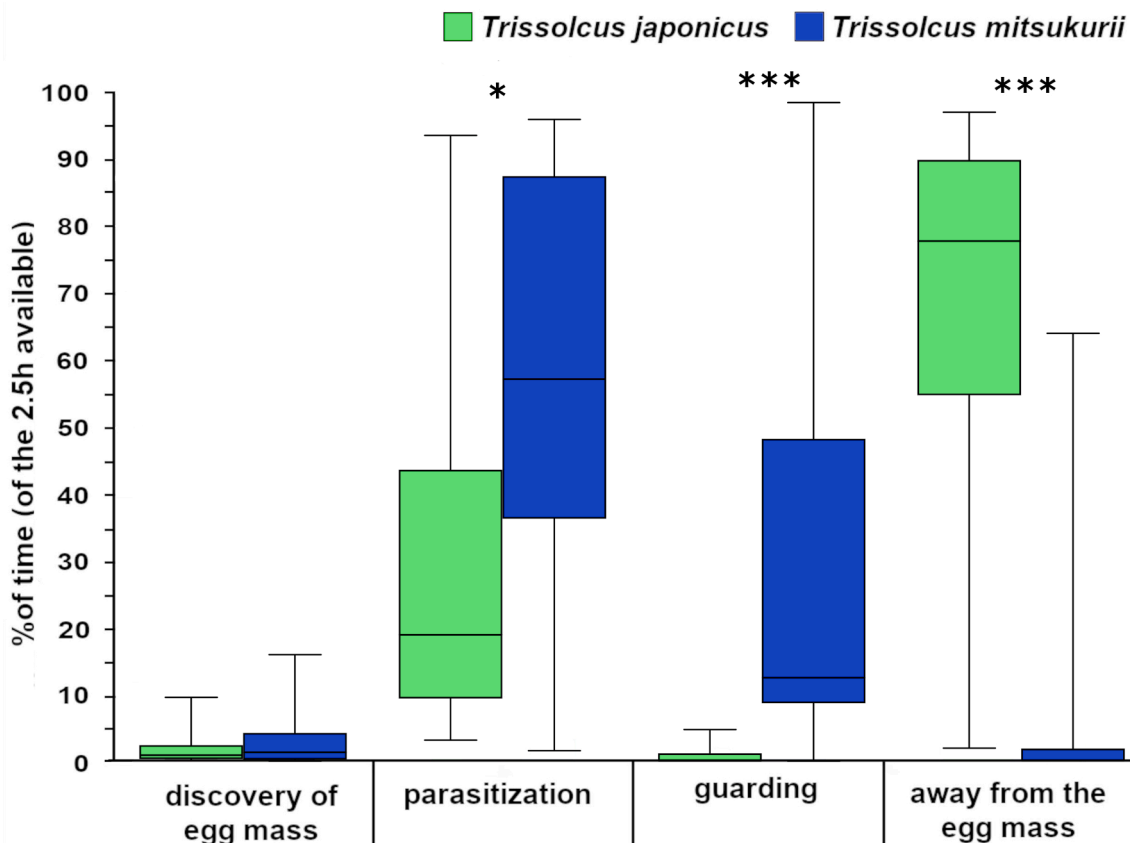


Fig. 2. Behaviors of *Trissolcus japonicus* and *Trissolcus mitsukurii* females released simultaneously in the arena containing an egg mass of *Halyomorpha halys*: time spent in each behavior is reported as a percentage of the total time available (2.5 h per test). Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) within each behaviour trait. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate 1.5 × the interquartile range.

at high levels even at 7 days. The same trend was also observed by Costi et al. (2022) although they concluded that *T. mitsukurii* has a greater intrinsic (larval) competition ability, something that we did not observe. No conclusion can be made about possible differences among haplotypes between our studies since Costi et al. (2022) did not report the haplotype profile of their tested parasitoid populations. The success of first-arriving species is not always the case: when *Trissolcus*-parasitized host eggs are followed by multiparasitism with *Psix* (also Scelionidae) or *Anastatus* (Eupelmidae), in most cases it is the second-arriving species that is most successful in leaving progeny (Weber et al., 1996; Konopka et al., 2017a; Mi et al., 2021). Data collected by Konopka et al. (2017) on competition between *T. japonicus* and *A. bifasciatus* clearly explain the hyperparasitism ability of the tested eupelmid species when it is the second arriving female, which is able to develop successfully in *H. halys* host eggs initially parasitized by *T. japonicus*, at any stage of development of the first arriving parasitoid. Collectively, our data suggests the inability of *T. japonicus* and *T. mitsukurii* to act as hyperparasitoids, while at the same time, both parasitoids are not able to clearly recognize the unsuitability of a previously parasitized host since they will proceed to re-parasitize it.

With respect to biological control of *H. halys*, the experimental laboratory results obtained under our competitive scenarios highlight a potentially important issue when *T. japonicus* and *T. mitsukurii* occur in sympatry: interspecific competition can result in a waste of search time and eggs when the late-arriving species encounters already parasitized host eggs. These results agree with those of Bovin and Brodeur (2006), who described multiparasitism in biological control as having a negative impact if the late-arriving species is intrinsically a weaker species. However, maladaptive behavior of *T. japonicus* described by Haye et al. (2021) (observed in our study by both *T. japonicus* and *T. mitsukurii*)

resulting in a waste of progeny due to the oviposition in an unsuitable developing substrate can be overestimated.

When the two *Trissolcus* egg parasitoid females were introduced simultaneously in the arena with a single *H. halys* host egg mass (extrinsic competition), both species proved able to oviposit and produce progeny, though with differing success. We observed no significant differences between *T. japonicus* and *T. mitsukurii* in locating the host egg mass in the arena after their release, which occurred within a few minutes. As soon as both females came into contact with each other in the presence of the host egg mass, guarding behavior and mutual aggression rapidly occurred. Whenever a female first arrived on the egg mass, parasitization started quickly after a short egg inspection. In the presence of the competitor, the foraging female's behavioral pattern changed: parasitization was suspended and guarding behavior of the host egg mass was adopted, followed by attempts to repel the competitor when it approached. This sometimes escalated to a physical contest with fighting if the intruder female did not move away from the egg mass, as observed by Field et al. (1998). In our experiments, *T. mitsukurii* was found to be the dominant species of the two, spending a large part of the time guarding the egg mass. It was also more aggressive, initiating a significantly greater number of attacks and winning fights than *T. japonicus* females. Costi et al. (2022) observed a rare exhibition of contest behavior when *T. japonicus* was the first introduced female in the arena, while a greater number of contests was observed when *T. mitsukurii* was introduced as first female. The aggressivity of *T. mitsukurii* appears to be unrelated to the egg mass size (as suspected by Costi et al. 2022). Our results are in line with Costi et al. (2022), although they used an artificially reduced host egg mass size (14 eggs/mass vs the standard egg mass size of 28 eggs, as also used by Haye et al., 2022). A similar case was observed when *T. mitsukurii* was faced with *T. basalis* or

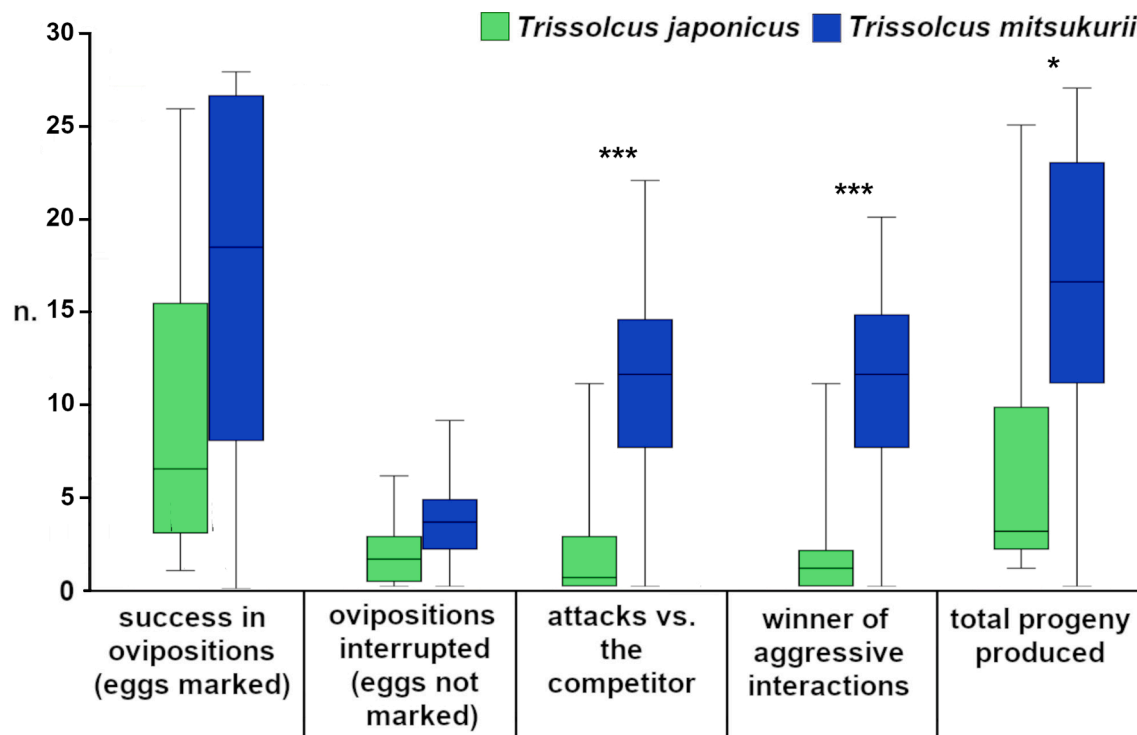


Fig. 3. Behaviors of *Trissolcus japonicus* and *Trissolcus mitsukurii* females simultaneously released in the arena containing an egg mass of *Halyomorpha halys* and numbers of progeny produced. Each category is expressed as the mean number of events recorded during test (2.5 h). Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) within each behaviour trait. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5 \times$ the interquartile range.

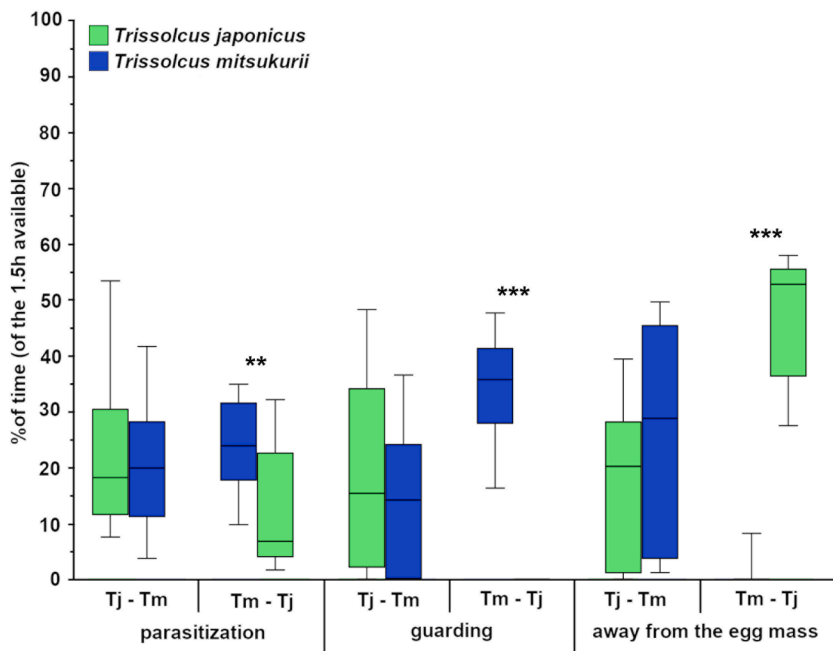


Fig. 4. Behaviors of *Trissolcus japonicus* and *Trissolcus mitsukurii* in direct competition: first access to the *Halyomorpha halys* egg parasitization was given, alternatively, to one species followed by the other. Time spent in each behavior is shown as a percentage of the total time elapsed in the test (1.5 h). Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) within each behaviour trait between the two species. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5 \times$ the interquartile range.

with *T. nakagawai* (Hokyo and Kiritani, 1966; Nishimoto et al., 2015). The fighting behavior between *T. japonicus* and *T. mitsukurii* lasted for a very short time (just a few seconds per event), and always resulted in a retreat from the egg mass by the beaten female. After retreating, the loser moved some distance away from the host egg mass, waiting for an opportunity to return. Of course, in a confined experimental arena the competition between contestants may be exaggerated, engaging several times in a “waiting-game” described by Field et al. (1998), where the

dominant female on the egg mass aggressively defends the host resource, and the intruder female waits for an opportunity to have access to the host. Similar strategies for host exploitation of an already guarded egg mass were observed by Haye et al. (2021) during interactions of *T. cultratus* with *T. japonicus*, where the “stalking” strategy (slow movements) of the first and the “ambush” strategy (fast moves toward the guarding female) of the second species may produce different output in aggressive interactions and progeny production. In our tests we never

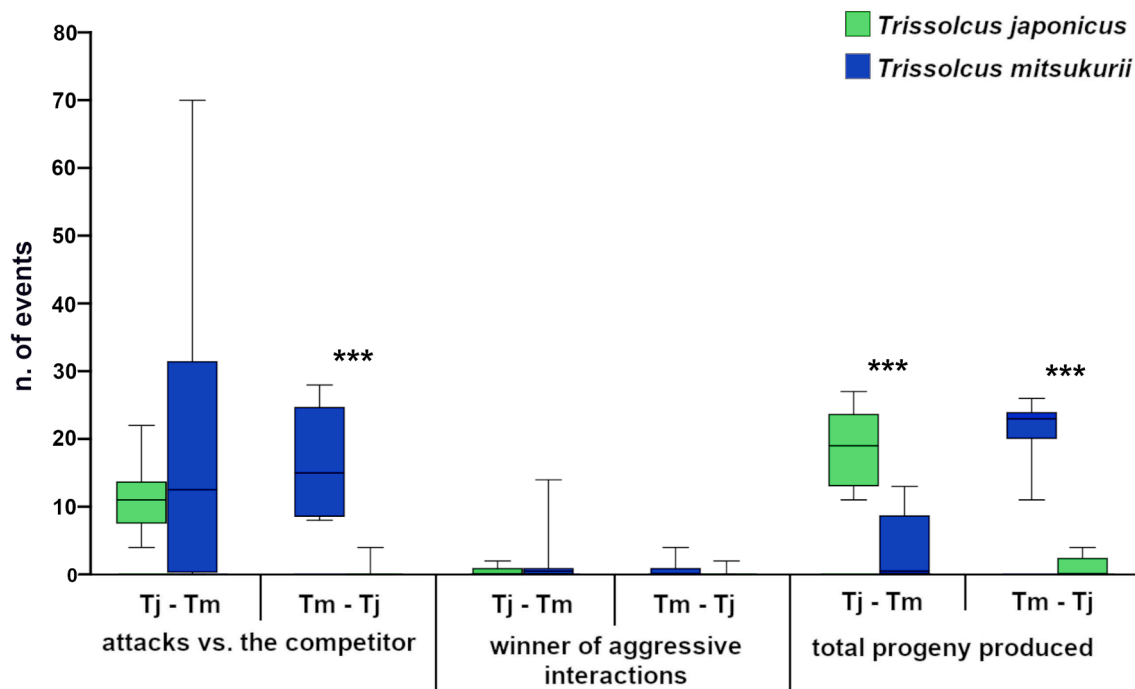


Fig. 5. Aggression behavior of *Trissolcus japonicus* and *Trissolcus mitsukurii* by progeny production in direct competition with first access, alternatively for the two parasitoid species, to the *Halyomorpha halys* egg parasitization. During the test time (1.5 h), the number of events were recorded with Tj-Tm (*T. japonicus* is the first species in the arena (resident), *T. mitsukurii* the species introduced (intruder)) and Tm (resident)-Tj (intruder) sequences. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) within each behaviour trait between the two species. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5 \times$ the interquartile range.

observed signs of body injuries after aggressive interactions. Similarly, in *T. basalis* interspecific competition, body injuries were rare (Field et al., 1998). Guarding and fighting are risky and expensive in terms of time for parasitization, as well as being costly in energy; however, these behaviors permit the most aggressive females to defend the host patch from a competitor and assure successful progeny production (Field et al., 1998). Results of our work and that of Costi et al. (2022) suggest *T. mitsukurii* to be a superior extrinsic competitor, but despite displaying less aggressive behavior *T. japonicus* females were able to exploit opportunities for parasitization; these events generally occurred when *T. mitsukurii* females were engaged in oviposition. In female competition, *T. mitsukurii* spent nearly twice as much time in parasitization activity than *T. japonicus*, and produced on the whole a higher number of progeny, which partially contrasts with Costi et al (2021). However, *T. japonicus* displayed superior competitive ability and aggression in host patch defense when in contests with congeneric (*T. cultratus*) and non-congeneric (*A. bifasciatus* and *A. japonicus*) species that attack *H. halys* (Konopka et al., 2017a, 2017b; Haye et al., 2021; Mi et al., 2021). Strong aggressive host patch defense was observed also in other Scelionidae, *T. basalis* and *T. nigripedius* (Wilson 1961; Cumber, 1964; Mahmoud and Lim, 2008). Interactions could also be less aggressive in some scelionids, for example *Gryon* sp. nr. *gonikopalense* Sharma and *Trissolcus hyalinipennis* Rajmohana & Narendran, where both species appear to be more tolerant of the presence of interspecific foraging females (Hougardy and Hogg, 2021).

In our tests of extrinsic competition with asynchronously released *Trissolcus* females, parasitizations in progress by the resident female abruptly stopped upon arrival of a second female to the host egg mass, and the resident behavior changed from foraging to guarding and defense. In this situation, the time spent in parasitization by both females during the contest was not significantly different. Female *T. mitsukurii* were also able to access and dominate the host egg mass when it arrived first, and in some cases also when it arrived second. However, guarding behavior was strongly influenced by species arrival sequence, and

T. mitsukurii spent the most time guarding eggs when it was the resident species, while no difference was detected between *T. japonicus* as the resident species and *T. mitsukurii* as the intruder. When *T. japonicus* was the intruder, it spent most of the time away from the egg mass. Guarding behavior in both species frequently led to an attack on the competitor, and *T. japonicus* was less successful when it was the intruder. No differences in number of attacks were detected when *T. japonicus* was the resident species and *T. mitsukurii* the intruder. However, contests with asynchronous arrival on the host egg mass by the two *H. halys* parasitoids resulted in greater success of the first-arriving species in term of total progeny produced. This was quite clear since there was less success in emergence when multiparasitism occurred and more of the half of egg mass was already parasitized by the first-arriving species. Similar results were observed by Field and Calbert (1999) during intraspecific competition by *T. basalis* with differences in arrival time of greater than 30 min.

5. Conclusions

Indirect competition between females of the two *Trissolcus* species led to more progeny produced by the first-arriving species, even though in most cases neither species recognized previously parasitized host eggs, resulting in multiple parasitism.

Although interspecific competition resulted in the death of the host close to 100 %, both in direct and indirect competition, multiparasitism within *T. japonicus* and *T. mitsukurii* resulted in significant waste of eggs and potential progeny of the second-arriving species. When simultaneously present, *T. mitsukurii* was more aggressive than *T. japonicus*, dominating the host resources and producing more progeny. When the arrival of females of the two species was asynchronous, although *T. mitsukurii* was also aggressive as the intruder, success in progeny production was biased in favor of the resident female. The interactions among the two species, despite the simplified arenas which cannot account for various environmental factors, may suggest that the reduced

progeny production by the second arriving female could have negative impact on its population dynamics. However, conditions in the field and factors such as environment adaptations, host searching abilities and interaction with other species, are important factors that need to be further evaluated (Denno and Finke, 2006; Ode et al., 2022). While considering that data obtained in this study derive from simplified laboratory experimental conditions, results obtained may nevertheless be useful in designing plans for field releases within biological control programs, and may contribute to understanding evolution of competition in the field (Boivin and Brodeur, 2006; Collier et al., 2002; Denno and Finke, 2006). Indeed, outcomes of laboratory studies in simplified experimental microcosms need to be carefully evaluated since, as shown by Haye et al. (2021), increased oviposition pressure in confining test arenas can affect parasitization behavior and successful emergence of progeny, leading to an overestimation of effects that occur in the field. Thus, studies using more complex microcosms should be adopted for further studies of competition, and supported where possible by actual field data where the species of interest occur in sympatry.

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Data availability statement

Raw data are available by author upon request by third parties.

CRediT authorship contribution statement

Lucrezia Giovannini: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Giuseppino Sabbatini-Peverieri:** Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Sauro Simoni:** Formal analysis, Writing – review & editing. **Rita Cervo:** Supervision, Writing – review & editing. **Kim Alan Hoelmer:** Supervision, Writing – review & editing. **Pio Federico Roversi:** Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocontrol.2022.105068>.

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