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Original Article

Biocontrol implications of multiparasitism by *Trissolcus mitsukurii* and *Trissolcus japonicus* on the invasive brown marmorated stink bug

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Short title: *Multiparasitism between* Trissolcus *species*

Abstract

The egg parasitoids *Trissolcus japonicus* (Ashmead) and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) are the most effective biocontrol agents of the invasive agricultural pest *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) in its native range (east Asia). *Trissolcus japonicus* and *T. mitsukurii* are sympatric in the native areas. In northern Italy, where *H. halys* is a major pest of fruit orchards, adventive populations of both species are spreading, and *T. japonicus* is artificially released under a classical biocontrol program against *H. halys*. This laboratory study aimed to assess the outcome of competition when both species share the same resource and possible implications for the biocontrol of the invasive stink bug. Egg masses of *H. halys* were offered to each parasitoid after previous parasitization by the other species. Parasitoid behaviour, number of ovipositions, and successfully developed parasitoids were recorded. Additionally, contest behaviour was assessed when both species were released simultaneously on the same egg mass. Results showed that both *T. japonicus* and *T. mitsukurii* were able to parasitize an egg mass already parasitized by the other species. Competition occurred within the host eggs and each species outperformed the other when it was the first to oviposit. Importantly, the overall contribution to *H. halys* mortality was not affected by the interaction between parasitoids, as non-parasitized eggs were 4–6% in the absence of competition and <8% in its presence, respectively. When simultaneously released on the egg mass, *T. mitsukurii* was more aggressive, engaging in chase-off events in 71% of cases compared to 50% of *T. japonicus*.

Abbreviated abstract

Halyomorpha halys (Heteroptera: Pentatomidae) is an invasive pest of crops. Adventive populations of its egg parasitoids *Trissolcus japonicus* and *T. mitsukurii* (Hymenoptera: Scelionidae) are spreading across northern Italy. *Trissolcus japonicus* was selected for an Italian biocontrol program against *H. halys*. Multiparasitism experiments indicate that the order of arrival on the host's eggs is crucial to ensure the most successful parasitization and that competition between the two species did not result in reduced *H. halys* egg mortality in the laboratory.

Graphic for Table of Contents

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Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae), native to Eastern Asia (Lee et al., 2013), is an invasive pest of agricultural crops in North America and Europe (Maistrello et al., 2017, 2018; Leskey & Nielsen, 2018). In its native range, eggs of *H. halys* are attacked by several species of hymenopteran egg parasitoids among which *Trissolcus japonicus* (Ashmead) (Scelionidae) is the dominant species in China, with parasitism ranging from 50 to 90% (Yang et al, 2009; Qiu, 2010; Zhang et al., 2017) whereas *Trissolcus mitsukurii* (Ashmead) is the prominent parasitoid in Japan (Arakawa et al., 2004, 2002). In native Asia, distribution data indicate that populations of *T. japonicus* and *T. mitsukurii* occur in sympatry (Yonow et al., 2021). According to a Climex model, both *H. halys* and the two parasitoid species are expected to expand their range globally, with a high degree of overlap in their predicted distributions (Yonow et al., 2021).

Field surveys over a large area of northern Italy, Switzerland, and Slovenia revealed the widespread presence of both *T. mitsukurii* and *T. japonicus*, with comparable performance in terms of exploitation efficiency and parasitoid impact (Rot et al., 2021; Zapponi et al., 2021), thus indicating their suitability as candidates for *H. halys* biocontrol programs in Europe. However, in European countries the use of exotic biocontrol agents is restricted by the 'Habitats directive' (Council Directive 92/43/EEC, 21/05/1992, on the conservation of natural habitats and of wild fauna and flora).

In 2020, in Italy, following the high negative economic impact caused by *H. halys* on fruit production and the repercussions on the entire agro-food chain, a decree of the Italian Ministry of Environment and of Land and Sea Protection (MATTM, 2020) authorized *T. japonicus* for the biocontrol of *H. halys*, leading to one of the largest biocontrol projects ever attempted in Italy.

The success of biocontrol programs might be affected by the outcome of interactions between biocontrol agents. Parasitoids that share the same ecological niche and the same host resource are likely to encounter hosts that are already parasitized, enhancing the possibility of multiparasitism. Competitive interactions could alter parasitoids' reproductive success and thus have null, antagonistic, or synergistic effects on host mortality (Brodeur & Boivin, 2006; Cusumano et al., 2012; Harvey et al., 2013; Valente et al., 2019; Stahl et al., 2020). Furthermore, multiparasitism might be affected also by extrinsic factors such as host quality and density, parasitoid density, competing taxa, species parasitization sequence, the time interval between parasitization, and the developmental rates of the competing species (Godfray, 1994; Pedata et al., 2002; Sithole & Lohr, 2017).

If more than one female parasitoid exploits a host patch, they can interfere directly through aggressive behaviours which include striking the opponent with antennae/wings/legs, biting, and stinging (Brodeur & Boivin, 2006). Ownership status strongly affects contest outcomes: owners are usually advantaged as they have already inspected the resource before the competitors' arrival and therefore have a more accurate estimation of its value and intruders must be much larger than owners to take over the hosts (Petersen & Hardy, 1996; Mohamad et al., 2015).

This study aimed to investigate the potential for multiparasitism of *H. halys* eggs by *T. japonicus* and *T. mitsukurii* by evaluating the number of ovipositions and the successful parasitoid emergence at various time intervals. Furthermore, observations on contest behaviour were performed by recording the number of chase-offs, ovipositions, and the number and duration of contacts with the host resource and their consequence on parasitoid emergence when the two species followed a different order of introduction in the contest.

Materials and methods

Insect colonies

Overwintering *H. halys* specimens were collected during autumn 2019 from various locations in Modena and Reggio Emilia (Emilia-Romagna region, northern Italy) and were stored inside wooden boxes in a dark room at 10 °C for 9 weeks, as described in Taylor et al. (2017). The surviving individuals were relocated to cubic cages (15 cm side) (BugDorm; NHBS, Bonn, Germany). About 25 males and 25 females per cage were placed in climatic chambers at 25 °C and L16: D8. The bugs were provided with fresh organic seasonal vegetables and peanuts twice a week and with pieces of paper and a small plant of *Peperomia* sp. (Piperaceae) as egg-laying substrates. The egg masses used to maintain the parasitoid colony were collected daily and underwent cold storage at 9 °C according to Wong et al. (2020). Freshly laid egg masses (<24 h old) were utilized in the experiment. *Trissolcus japonicus* and *T. mitsukurii* emerged from field-collected *H. halys* egg masses. They were reared in BugDorms (125 mm side) inside climatic chambers at 25 °C and L16: D8, and were provided with drops of a honey-water solution (70:30 ratio), directly placed on the mesh of the BugDorm. Cold-stored egg masses were offered to the parasitoids (one egg mass per female), and after 3 days each egg mass was moved to a single empty vial, where the parasitoids emerged.

Experimental procedure

Freshly laid *H. halys* egg masses (<24 h old) consisting of 28 eggs were gently split in two portions by means of entomological forceps and pins. Newly emerged parasitoids of each species were mated daily (one female and one male) and placed for 1 week in vials (Falcon 50 ml, whose lid was replaced by a piece of pantyhose secured with an elastic band) and provided with drops of honey-water solution (70:30 ratio). All trials were conducted in a climatic chamber at 25 °C and L16: D8.

Parasitization in absence of interaction (ab_in). Mated females (n = 20) of each species were individually transferred into Petri dishes (9 cm diameter) with a single mass of 14 eggs and their behaviour was video-recorded for 3 h.

Multiple parasitism at various time intervals. Already parasitized masses of 14 eggs (n = 120) were offered to a newly mated female belonging to the other species. The second parasitization took place after 0, 2, or 5 days. The second parasitization was video-recorded for 3 h. Each treatment was replicated 20× for each parasitoid species.

Contest behaviour during real-time interactions. Masses of 14 eggs were offered in a Petri dish (9 cm diameter) to a single mated 1-week old female of each parasitoid species. As soon as this female came into contact with the egg mass, a second female belonging to the other species was released into the same Petri dish and the number of ovipositions and their behaviour was recorded for 3 h for both species. The first introduced female was considered to have contacted the egg mass as soon as she touched an egg with the first pair of legs. For each parasitoid species, 15 replicates were performed.

Data collection

Video-recording was performed in all cases using a wireless camera (HC-V380; Panasonic, Kadoma, Japan). In the first two set of experiments the following behaviours were recorded:

Acceptance latency: period of time elapsed between the beginning of the experiment and the first oviposition;

No. ovipositions (including marking): Probing the host, inserting the ovipositor, and making head-pumping movements and body vibrations associated with egg-release. Partially exerting the ovipositor and sweeping it over the surface of the host egg in ∞-shaped movements as described by Field (1998).

For the contest behaviour experiments, for each parasitoid we recorded:

No. ovipositions, as described above;

No. chase-off events: running directly at the other female, sometimes lunging with wings raised, making contact and biting as described by Field (1998). Chase-events began when one female attacked the other, which then moved away rapidly, without retaliation (Peterson & Hardy, 1996);

No. contacts with the egg mass: examination of host egg surface by drumming it with the antennae;

Duration of each contact (in s);

No. markings;

No. injuries during combats.

After each video-recording period, the parasitoids were removed and the parasitized egg masses were kept at 25 °C and L16: D8 for 30 days, observing them daily to record the number of host nymphs and the number of parasitoids emerged. After 30 days, all unhatched eggs were dissected to determine the number and species of fully developed parasitoid adults that had failed to emerge. The parasitoids were identified based on the taxonomic keys reported in Talamas et al. (2015).

The behaviour and oviposition activity were analysed with the software 'Boris' (Friard & Gamba, 2016). To ensure the repeatability of the observations, all videos were analysed by the same author (D Iotti) who had previously been trained on pre-recorded materials in order to correctly recognise and interpret the various behaviours described above.

Data analysis

The number of successfully developed parasitoids (SDP) was calculated by adding the number of emerged parasitoids to the number of parasitoids that had failed to emerge, as recorded during the egg dissection. All statistical analyses were performed using R v.3.6.0 (R Core Team, 2020). For each test, $\alpha = 0.05$.

Considering the parasitization of egg masses at different intervals of time, differences in oviposition latency period, number of ovipositions, and number of SDP were evaluated. A linear model was used to compare oviposition latencies between *T. japonicus* and *T. mitsukurii* for each treatment. One-way ANOVA followed by Tukey's honestly significant difference (HSD) test was used to compare oviposition latencies among treatments (ab_in; 0, 2, and 5 days) within parasitoid species. Log (oviposition latency + 0.5) transformation was used in this analysis to normalize the data. Comparison in number of ovipositions and SDP between *T. japonicus* and *T. mitsukurii* for each treatment were performed using a generalized

linear Poisson model. Kruskal-Wallis test followed by Dunn's post hoc test was used to assess differences in number of ovipositions and SDP among treatments (ab_in; 0, 2, 5 days) for each parasitoid species. Furthermore, when the egg masses were offered to both parasitoid species, species rate (%) was calculated as (number of SDP / no. eggs parasitized) × 100%, also considering the fraction of parasitoids that did not emerge. Lastly, χ^2 test was used to compare number of SDP between *T. japonicus* and *T. mitsukurii* within treatment (0, 2, 5 days) when they both are either the first or the second species to parasitize the egg masses.

Considering the contest behaviour during real-time interactions, a qualitative analysis of the data was performed when both species engaged in chase-off events. Parasitoid emergence (%) and differences in chase-off events were calculated when *T. japonicus* was introduced first and when *T. mitsukurii* was introduced first. Parasitoid emergence was obtained as a fraction of the number of SDP of the species introduced first and total number of eggs offered to the parasitoids, whereas differences between chase-off events were calculated as the difference between the number of chase off-events of the species introduced second and the number of chase-off events of the species introduced first. Moreover, a generalized Poisson model was performed to assess differences in the number of contacts, marking, and chase-off events whereas a linear regression model was used to compare the length (in s) of each parasitoid contact with the egg masses between the two species. Log (contact's duration + 0.5) transformation was used in this analysis to normalize the data.

Results

Multiple parasitism at various time intervals

Both *T. japonicus* and *T. mitsukurii* oviposited in egg masses already parasitized by the other species with a range of accepted egg masses from 80 up to 100% (Table 1). The number of ovipositions did not differ among treatments for *T. japonicus* (Kruskal-Wallis test: P = 0.067) whereas the number was higher for *T. mitsukurii* when the second parasitization occurred after 5 days (treatments ab_in vs. 5 days; Kruskal-Wallis followed by Dunn's test: P = 0.004). Differences between species within each treatment were not found (Generalized Poisson model: P>0.05).

Comparing the two species, oviposition latency differed within the treatments ab_in and 2 days, with *T. mitsukurii* having a significantly longer latency period (Table 1). No differences were detected in treatments 0 and 5 days. Considering each species seperately, no differences were detected among treatments for *T. mitsukurii* (one-way ANOVA: $F_{3,73}$ = 0.642, P = 0.59), where the first oviposition was usually recorded after 5–9 min (Figure 1).

For *T. japonicus*, significant differences were detected (one-way ANOVA: $F_{3,74} = 4.552$, P = 0.006), with a longer oviposition latency in treatment 0 days (>18 min) compared to the other treatments (ranging from <1 to 9 min; Figure 1).

Considering the number of SDP, if the egg masses were first parasitized by *T. mitsukurii*, the number of emerged *T. mitsukurii* was always higher than that of *T. japonicus* (generalised Poisson model: P<0.001; Figure 2A, Table 2). Similarly, if parasitization by *T. japonicus* occurred first, the number of successfully emerged *T. japonicus* was always higher than that of *T. mitsukurii* (generalised Poisson model: P<0.001; Figure 2B, Table 2).

If *T. mitsukurii* was the species introduced first, the percentage of non-parasitized eggs ranged between 2.5 (0 days) and 6.8% (2 days), and if *T. japonicus* was introduced first it ranged between 2.9 (5 days) and 7.5% (5 days) (Table 3). Considering parasitized eggs, values of *T. japonicus* and *T. mitsukurii* success for the trial where *T. japonicus* and *T. mitsukurii* were introduced first, resulted significantly different at 0 and 2 days (Tm vs. Tj, 0 days: 81.7 vs. 67.7%; 2 days: 90.0 vs. 79.5%; χ^2 test: both P = 0.001; Table 4). In treatment 5 days, 100% of the eggs were parasitized by the first species introduced, regardless the species (Table 4).

Contest behaviour during real-time interactions

During real-time interactions, if *T. japonicus* was the first to approach the egg mass, it was always the first species to oviposit, and chase-off events occurred in 53.3% of the trials. If *T. mitsukurii* was the first to approach the egg mass, in 60% of the replicates it was also the first species to oviposit, and chase-off events occurred in 80% of the trials. If *T. japonicus* was introduced first, both species marked the same eggs in four replicates out of 15 (26.7%; 12 eggs/egg masses on average) whereas if *T. mitsukurii* was introduced first this happened in seven replicates out of 15 (46.7%; seven eggs/egg masses on average). Charging and chasing behaviours by one individual causing the retreat of the other were common but injury almost never occurred. In one case where *T. mitsukurii* was introduced first, after wrestling one antenna of *T. japonicus* was bitten off. In all cases where both parasitoid species did not exhibit contest behaviour, *T. japonicus* had an emergence rate of 100% regardless of the order of their first contact with the egg mass. The qualitative analysis (Figure 3) was developed considering only the replicates in which at least one of the two species performed chase-off events. Considering the trials where *T. japonicus* was introduced first (Figure 3A), *T. mitsukurii* and *T. japonicus* rarely exhibited contest behaviour (six chase-off events in total), and when *T. mitsukurii* chased *T. japonicus* (differences in chase-off events >0), the

emergence rate of *T. japonicus* was reduced from 100 to 50% (Figure 3A).

Considering the trial where *T. mitsukurii* was introduced first (Figure 3B), the number of its chase-off events (n = 10) and its emergence rate increased (differences in chase-off events <0). Both, when *T. japonicus* was introduced first and when *T. mitsukurii* was introduced first, when *T. japonicus* chased *T. mitsukurii* off (Figure 3A: differences in chase-off events <0; Figure 3B: differences in chase-off events >0) its emergence rate was 100%. The number of contacts was higher for *T. japonicus* when it was introduced first and their duration was significantly greater. No differences were detected when *T. mitsukurii* was introduced first; in fact, within this treatment *T. mitsukurii* increased the number and duration of its contacts with the egg mass. Even though the number of chase-off events was significantly higher in *T. mitsukurii* than in *T. japonicus*, regardless of the order of introduction, the number of markings was higher for *T. japonicus* within the treatment with *T. japonicus* introduced first (Table 5).

Discussion

Considering that adventive populations of *T. japonicus* and *T. mitsukurii* are currently co-occurring and successfully spreading in northern Italy (Zapponi et al., 2021), this study evaluated multiparasitism, i.e., how each parasitoid species would react in presence of *H. halys* egg masses already parasitized by the other species, further assessing the behaviour when both species were on the same egg mass.

According to our findings, both species oviposited on eggs that had already been parasitized by the other species. *Trissolcus mitsukurii* showed a longer oviposition latency (about 5–9 min) compared to *T. japonicus* (usually around 1 min), except in the case of freshly parasitized eggs (treatment 0 days), when the first oviposition by *T. japonicus* occurred after >18 min. Thus, the eggs freshly parasitized by the other species have cues that temporarily delay the acceptance only in the case of *T. japonicus*, although this did not prevent the second female from parasitizing all the available eggs. This might indicate that, compared to *T. mitsukurii*, *T. japonicus* has a greater ability to detect these cues, which could be semiochemicals and/or visual or tactile cues. Interspecific discrimination has evolved in some parasitoid guilds and can be mediated at long-range distance by semiochemicals, as in the case of *Cotesia marginiventris* (Cresson) whose females avoid host larvae with body odours of the superior larval competitor *Campoletis sonorensis* (Cameron) (Tamò et al., 2006; Cusumano et al., 2012). In any case, as this study did not take into account superparasitism, i.e., subsequent parasitism by the same species, it is not possible to state whether either one of

the species has a higher sensitivity in discriminating already occupied host eggs. Further studies are currently underway to define whether and how the females of both species are able to discriminate between the host eggs already parasitized by their own species vs. those parasitized by other species, both in laboratory and semi-field conditions.

Our results indicate that even though the number of recorded ovipositions was similar under different treatments, the number of successfully developed parasitoids (SDP) was related to the order of arrival on the egg mass. In other words, the first parasitoid species that finds and exploits an egg mass is also the one with the highest reproductive success. Similar results occurred also when *T. japonicus* interacted with the European native species *Trissolcus cultratus* (Mayr) (Konopka et al., 2017). These findings seem to indicate a competition between the two species occurring inside the egg, where the substances injected by the first female during oviposition might play a role by suppressing or interfering with the development of the species that comes second, as suggested by Konopka et al. (2017). Moreover, being the first species to exploit the host is usually advantageous because the offspring can start ingesting limited resources sooner than competitors (Cusumano et al., 2012). However, in other studies, the results show the opposite response with the first female's egg clutch destroyed by a second conspecific or allospecific female (Pérez-Lachaud et al., 2002; Hardy et al., 2013). In addition, when both species approached the egg mass first and when both species came second, the number of successfully developed parasitoids was higher in *T. mitsukurii* than in *T. japonicus*. Considering that in both species there were no differences in the number of ovipositions, these results might indicate a greater success of *T. mitsukurii* in larval competition.

Our data showed that, in absence of interactions under laboratory conditions, *T. japonicus* and *T. mitsukurii* have a very high parasitization efficiency, with only 4 and 6% of non-parasitized eggs, respectively, thus confirming previous studies (Scaccini et al., 2020; Zapponi et al., 2021). If the species were competing for the same egg mass, the parasitism due to each of the species changed according to the order of arrival on the egg. However, taking into account the parasitization of both species, the percentage of non-parasitized eggs was always <8%. Therefore, the overall contribution to *H. halys* mortality is essentially unchanged. A similar absence of adverse effects on the overall efficacy of parasitization was observed when the competition between *T. japonicus* and *Anastatus japonicus* Ashmead was investigated (Zhang et al., 2017).

When the females of the two species interacted directly on the same egg mass, if *T. japonicus* arrived first, in 50% of the cases they did not engage in contest behaviour.

However, if *T. mitsukurii* arrived first, chase-off events increased to 71%, thus showing stronger aggressive behaviour compared to *T. japonicus*, especially if it was the first species to approach the egg mass. *Trissolcus mitsukurii* behaviour could be explained by the 'bourgeois' strategy, in which an individual could behave aggressively only when in the role of the owner (Maynard Smith & Parker, 1976; Kokko et al., 2006). These observations confirmed the importance of ownership status in the outcome of the wasp competition whereby females that arrive first on a patch have an advantage against subsequent intruders (Field & Calbert, 1998; Keeler & Chew, 2008). Another explanation for the more aggressive behaviour of *T. mitsukurii* compared to *T. japonicus* is that, based on our results, in the absence of chase-off events, *T. mitsukurii* had a lower possibility to increase its chance to produce offspring. In fact, in absence of chase-off events, *T. japonicus* parasitized the entire egg mass, regardless the order of arrival on the egg mass. Furthermore, it is also possible that the experimental setup with a smaller egg clutch compared to the 28 eggs usually laid by *H. halys*, may have influenced the aggressiveness of the two species, the general outcome of the competition and contest behaviour by modifying the perceived host value (Humphries et al., 2006; Stockermans & Hardy, 2013).

According to our observations, which were performed for 3 h in Petri dishes in a no-choice setting, all eggs that had previously been parasitized underwent further parasitization. Additional investigations are needed to verify whether this also occurs in the field, when parasitoids have different options. Under natural conditions, *T. japonicus*' seemingly greater ability to detect already parasitized eggs may possibly increase its parasitization success by focusing on finding new, unexploited eggs. Interestingly, this improved egg discrimination ability seems to be confirmed by the results of a large-scale survey on naturally laid *H. halys* egg masses collected in northern Italy, where *T. japonicus* showed greater effectiveness in host discovery compared to *T. mitsukurii* (0.45–53.9% vs. 0.17–20.2%) (Zapponi et al., 2021). Furthermore, it is also possible that under field conditions and high pressure of *H. halys* populations, the two parasitoid species do not interact with each other, largely choosing non-parasitized instead of parasitized eggs, thus favouring an additive effect on the reduction of the target species.

Trissolcus japonicus and *T. mitsukurii* have sympatric populations in various areas of east Asia (Yonow et al., 2021) where they share the same host: *H. halys* eggs. This condition of population sympatry is reconstituting itself also in northern Italy, where the host and both parasitoids have been introduced. It is likely that in conditions of sympatry the two species have developed different strategies to exploit the same host, occupying different niches and/or

exhibiting different behaviours. Besides, it is also possible that, due to the energy cost of competition, the species with a wider host range may adapt to competition by changing host preference, whereas it is likely that specialists with a limited host range are more vulnerable to competition, because they fail to exploit alternative hosts (Nie et al., 2019).

The results of this study seem to indicate a different strategy between these two species when parasitizing the eggs of *H. halys*. *Trissolcus japonicus* has a lower reproductive success if it has to parasitize eggs already occupied by the other species but seems better at discriminating the already parasitized eggs. When both species are released simultaneously on the same egg mass, *T. japonicus* is less prone to engage in contest behaviour and has better parasitization efficiency if *T. mitsukurii* does not intensify its chase-off events. On the contrary, *T. mitsukurii*, besides being more active in contest behaviour, has a greater offspring than *T. japonicus* if it arrives on the egg mass in the first 2 days after the previous parasitization by the other species. Taken individually, each species has a very high parasitization impact. The overall impact considering both species also does not change in case they occur simultaneously on the same egg mass. Although our findings suggest that when the two species share the same egg mass, the overall biocontrol potential will likely be preserved, the species that approaches the egg mass first prevails in intrinsic competition resulting in predominant reproductive success on the other species, with considerable consequences for the desired outcome of biocontrol programs (i.e., diffusion rate and settlement capacity of the released species).

Our results refer to no choice trials conducted under laboratory conditions, therefore differences in host finding and dispersal ability, avoidance of competition through resource and niche partitioning, superparasitism, and the influence of field conditions on the dynamics of competition were not addressed. For this reason, we believe that further experiments are necessary, in particular to investigate the ability of both species to discriminate between parasitized and unparasitized eggs and to avoid intra- and interspecific competition.

Conflicts of Interest

The authors declare no conflict of interest.

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Figure captions

Figure 1 Oviposition latency (s) recorded for *Trissolcus japonicus* (black) and *T. mitsukurii* (grey) when 0, 2, or 5 days elapsed between first and second parasitization, and when interaction was absent (ab_in). Asterisks indicate significant pairwise differences, for *T. japonicus* only (Tukey's HSD test: $*0.01<P<0.05$, $**P<0.01$). 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, and the dots are outliers.

Figure 2 Number of successfully developed parasitoids (SDP) of *Trissolcus japonicus* (black) and *T. mitsukurii* (grey) when 0, 2, or 5 days elapsed between first and second parasitization, in trials where egg masses where first parasitized (A) by *T. mitsukurii* and secondly by *T. japonicus*, or (B) by *T. japonicus* and secondly by *T. mitsukurii.* 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, and the dots are outliers.

Figure 3 Association between differences in chase-off events and emergence rate of (A) *Trissolcus japonicus* (Tj) and (B) *T. mitsukurii* (Tm) when both were the first species to approach the host. On the horizontal axis the number of chase events between the second and the first species introduced, on the vertical axis the number of emerged parasitoids out of the total number of eggs. Differences in chase off events between the two species are positive in A when *T. mitsukurii* chase *T. japonicus*, and in B when *T. japonicus* chase *T. mitsukurii*; differences are negative vice-versa.

Table 1 Median (+ interquartile range; n in parentheses) oviposition latency and number of ovipositions recorded for *Trissolcus japonicus* and *T. mitsukurii* in absence of competition (ab_in) and in presence of competition after 0, 2, or 5 days

Variable	Treatment	*T. japonicus*	*T. mitsukurii*	Estimate[1]	P
Oviposition latency (s)	ab_in	136 [48–272] (19)	552 [122–1649] (19)	1.73	0.007
	0 days	1127 [268–2591] (19)	343.5 [200–1141] (20)	-0.64	0.33
	2 days	66.5 [22–599] (20)	483 [73–1827] (20)	1.69	0.026
	5 days	66 [21–342] (19)	386.5 [106–1295] (16)	1.16	0.17
No. ovipositions	ab_in	14 [14–14] (19)	14 [14–14] (19)	-0.12	0.89
	0 days	14 [14–17] (19)	14 [14–15] (19)	-0.06	0.51
	2 days	15 [14–16] (20)	14 [14–15] (20)	-0.07	0.41
	5 days	17 [14–18] (19)	17.5 [15–19] (16)	-0.09	0.30

[1]Linear regression (oviposition latency) and Poisson regression (no. oviposition) model's estimates and P-values comparing the log (oviposition latency + 0.5) transformed variable and the number of ovipositions between *T. japonicus* and *T. mitsukurii*.

Table 2 Median (+ interquartile range; n = 20) number of successfully developed parasitoids (SDP) for *Trissolcus japonicus* and *T. mitsukurii* after selecting trials in which either species was the first to parasitize *Halyomorpha halys* egg masses under a particular treatment (0, 2, or 5 days since the second parasitization)

Treatment	Order of introduction	*T. japonicus*	*T. mitsukurii*	Estimate[1]	P
0 days	*T. mitsukurii* first	1 [0–4]	13 [10–14]	1.50	<0.001
	T. japonicus first	10 [8–11]	3 [2–6]	-0.74	<0.001
2 days	*T. mitsukurii* first	0.5 [0–1]	13 [10–13]	2.20	<0.001
	T. japonicus first	11 [7–13]	1 [0–5]	-1.36	<0.001
5 days	*T. mitsukurii* first	0 [0–0]	13.5 [13–14]	25.90	<0.001
	T. japonicus first	14 [14–14]	0 [0–0]	-24.90	<0.001

[1]Poisson regression model's estimates and P-values comparing SDP between *T. japonicus* and *T. mitsukurii*

Table 3 Total number of *Halyomorpha halys* egg masses (Num), number of eggs exposed to various treatments (parasitization by *Trissolcus mitsukurii* and *T. japonicus*, where ab_in = absence of interaction, and 0, 2, or 5 days indicate the time since second parasitization), related number of non-parasitized eggs (np), and np as percentage of no. eggs exposed [np (%)].

Order of introduction	Treatment	Num	No. eggs	np	np (%)
T. mitsukurii first	ab_in	19	266	16	6.02
	0 days	20	280	7	2.50
	2 days	20	280	19	6.79
	5 days	20	280	15	5.36
T. japonicus first	ab_in	17	238	9	3.78
	0 days	20	280	17	6.07
	2 days	20	280	21	7.50
	5 days	20	280	8	2.86

Table 4 Total number of *Halyomorpha halys* eggs parasitized (out of n = 20 egg masses of 14 eggs each) within various treatments (parasitization by *Trissolcus mitsukurii* and *T. japonicus* at 0, 2, or 5 days since second parasitization, after selecting trials in which either species was the first to parasitize and the emerged species was either *T. mitsukurii* or *T. japonicus*), the number of successfully developed parasitoids (SDP), and species rate (%) indicating SPD as percentage of the no. parasitized eggs

Treatment	Order of introduction	Emerged species	No. parasitized eggs	SDP	Species rate (%)	P
0 days	*T. mitsukurii* first	*T. mitsukurii*	273	223	81.68	< 0.001
		T. japonicus	273	50	18.32	
	T. japonicus first	*T. mitsukurii*	263	85	32.32	
		T. japonicus	263	178	67.68	
2 days	*T. mitsukurii* first	*T. mitsukurii*	261	235	90.04	0.001
		T. japonicus	261	26	9.96	
	T. japonicus first	*T. mitsukurii*	259	53	20.46	
		T. japonicus	259	206	79.54	
5 days	*T. mitsukurii* first	*T. mitsukurii*	265	265	100	–
		T. japonicus	265	0	0	
	T. japonicus first	*T. mitsukurii*	272	0	0	
		T. japonicus	272	272	100	

*P-value obtained using χ^2 test.

Table 5 Median (+ interquartile range) number of contacts with *Halyomorpha halys* egg masses and their duration (in s), number of chase-off events, and markings for *Trissolcus japonicus* and *T. mitsukurii* after either *T. japonicus* or *T. mitsukurii* was the first to approach an egg mass

Behaviour	Treatment	*T. japonicus*	*T. mitsukurii*	Estimate[1]	P
Contacts	*T. japonicus* first	9.0 [3.5–11.5]	0.0 [0.0–11.0]	0.03	0.85
	T. mitsukurii first	6.0 [3.0–6.0]	9.0 [0.5–22.5]	0.80	<0.001
Duration of contact	*T. japonicus* first	1061.1 [604.1–2179.0]	0.0 [0.0–66.9]	-6.25	<0.001
	T. mitsukurii first	101.7 [55.4–2076.6]	259.3 [103.0–465.8]	-1.66	0.10
Chase-events	*T. japonicus* first	0.0 [0.0–2.0]	0.0 [0.0–4.0]	1.20	<0.001
	T. mitsukurii first	0.0 [0.0–1.0]	9.0 [0.0–17.0]	2.54	<0.001
Markings	*T. japonicus* first	14.0 [13.5–14.0]	0.0 [0.0–6.5]	-1.29	<0.001
	T. mitsukurii first	13.0 [1.5–14.0]	14.0 [0–14]	0.13	0.29

[1]Linear regression (for period of contact) and Poisson regression (for the other behaviours) model's estimates and P-values comparing the log (period of contact + 0.5)-transformed variable and the number of contacts, chase-events, and markings between *T. japonicus* and *T. mitsukurii*.
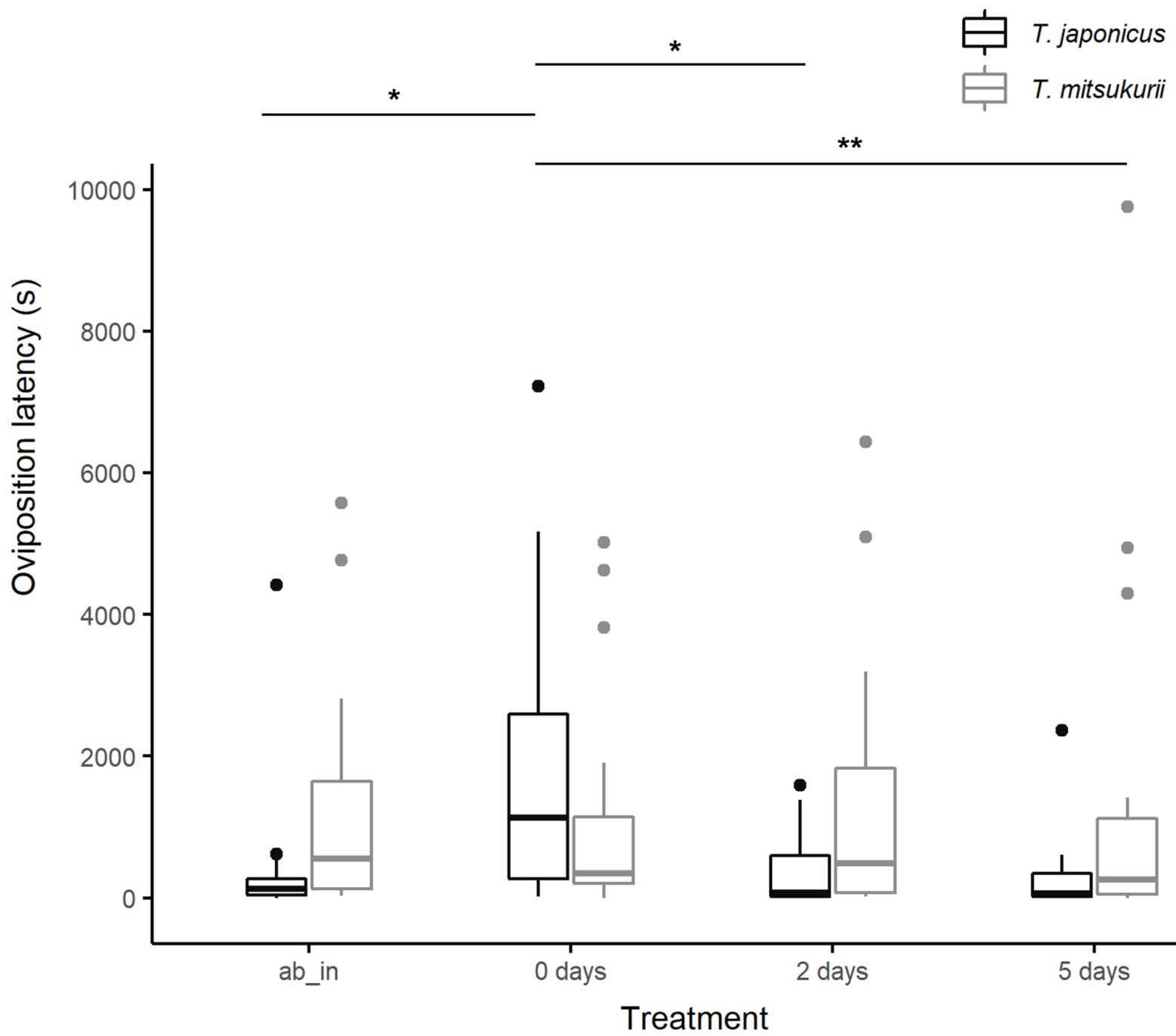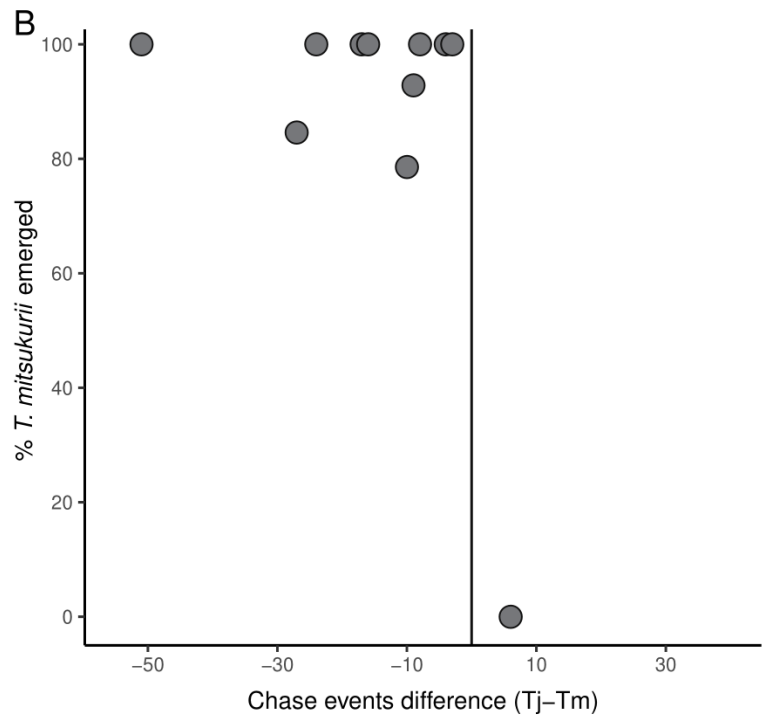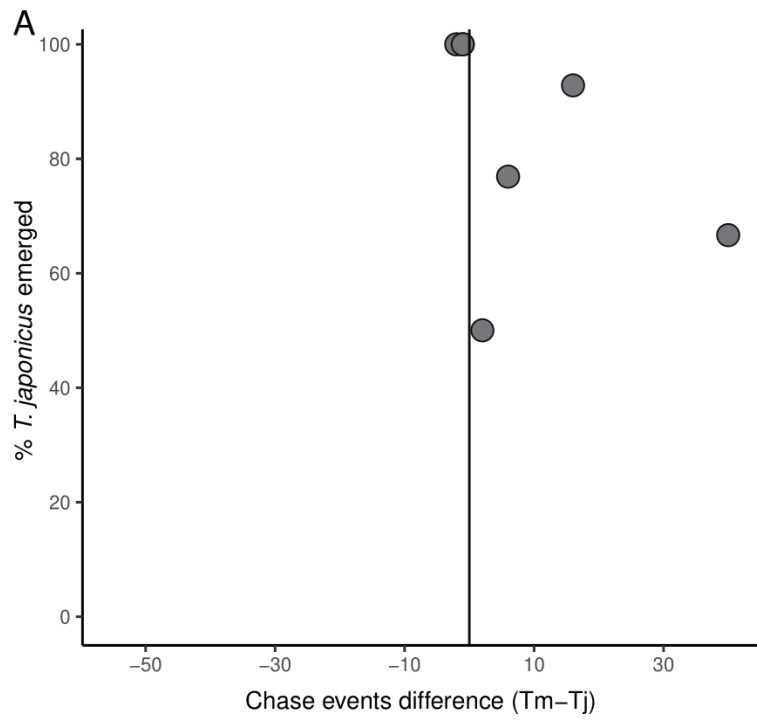
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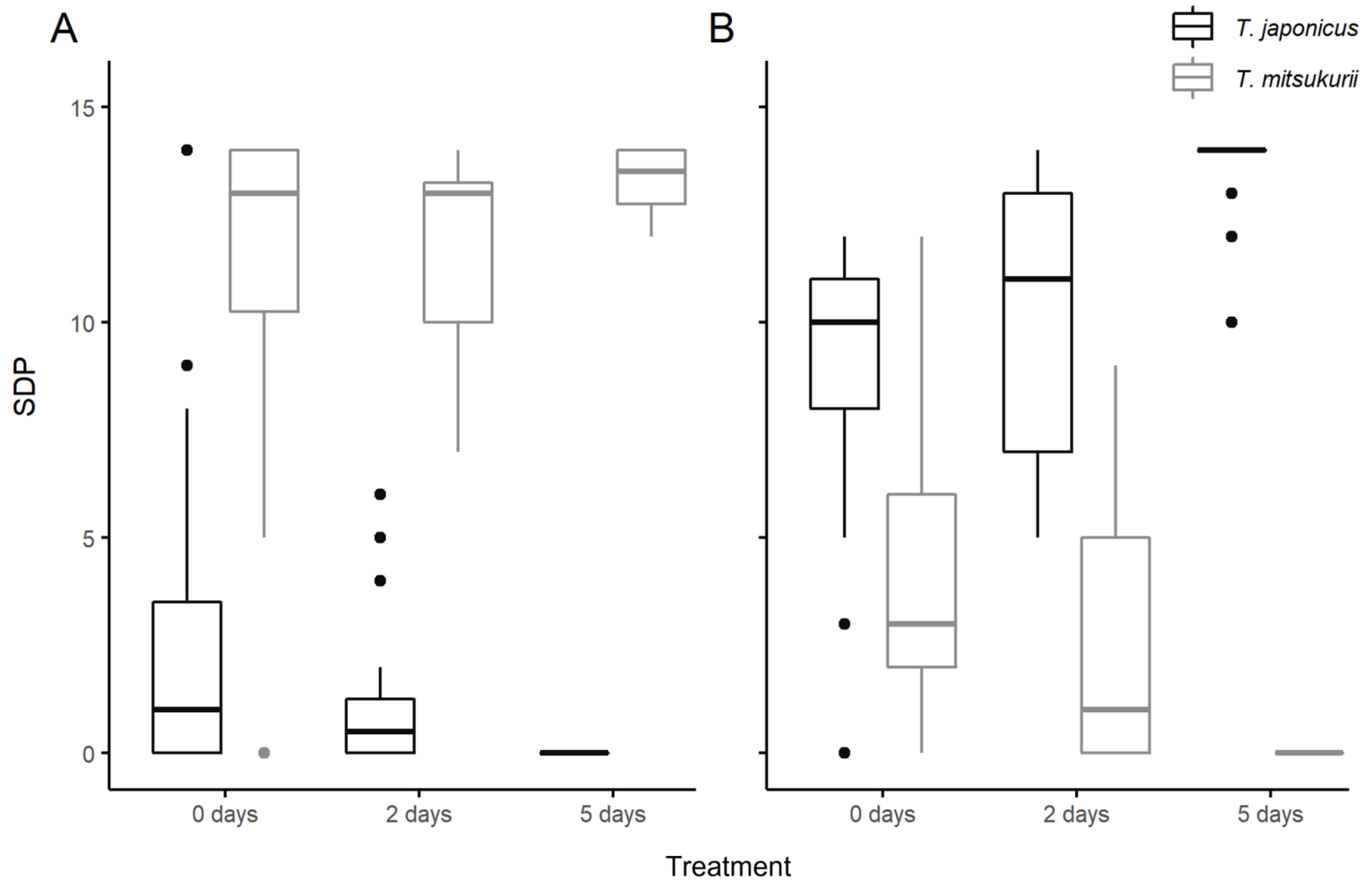
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