Influence of intra- and inter-specific competition between egg parasitoids on the effectiveness of biological control of *Euschistus heros* (Hemiptera: Pentatomidae)

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HIGHLIGHTS

- Parasitoid competition can reduce the parasitism rates on brown stinkbug eggs.
- In primary parasitism, the parasitoid offspring viability was reduced by superparasitism and multiparasitism.
- *Trissolcus basalis* reduced the reproductive success of *Telenomus podisi*.
- *T. basalis* was incapable to discriminate parasitized eggs by conspecifics.
- In sequential parasitism, the pattern of parasitoid emergence curves changed and it can lead to asynchrony of host-parasitoid dynamic.

ARTICLE INFO

ABSTRACT

Telenomus podisi and *Trissolcus basalis* are two important parasitoids used as biological control agents against the brown stink bug *Euschistus heros*. The interaction between these parasitoid species in the field, however, can have different effects on the effectiveness of pest control, depending on the density of the species present and the conditions under which the species find their host's eggs in the field. This study aimed to analyze the effects that may arise when two parasitoid species interact directly or indirectly during foraging at *E. heros* egg sites. Through experimental scenarios, the effects of intra- and inter-specific competition on unparasitized and previously parasitized host eggs (i.e. simultaneous and sequential parasitism, respectively) on the parasitism rates, emergence rates and emergence patterns were evaluated. For simultaneous parasitism, high emergence rates occurred when *T. podisi* was the only parasitoid species present in the system. In the presence of *T. basalis*, the parasitism rates remained high only when *T. podisi* was also present, although the emergence rate was considerably reduced due to multiparasitism. In sequential parasitism, *T. podisi* showed higher parasitism rates and offspring emergence due to its greater ability to discriminate eggs previously parasitized by conspecifics. In this scenario, the offspring of the first parasitoid that interacted with host eggs had greater success; however, the larval competition inside the host eggs led to changes in the species emergence patterns as well as to high offspring mortality. Competition changed the emergence pattern of the parasitoid offspring, which can lead to a decoupling of the host-parasitoid dynamics synchrony, by reducing the chances of encounters between the species and consequently the effectiveness of long-term pest control.

1. Introduction

Cultivation of the soybean *Glycine max* (L. Merrill) grows annually and is one of the most economically important crops in the world. Among the insect pests that cause damage to soybean production, phytophagous stink bugs (Hemiptera: Pentatomidae) stand out as the main group. The brown stink bug - *Euschistus heros* (Fabricius), the small green stink bug - *Piezodorus guildinii* (Westwood) and the green stink bug - *Nezara viridula* (Linnaeus) make up the soybean pest complex of greatest economic importance. Great variations in the distribution and

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abundance of these species have been observed in the different producing regions of this oilseed in Brazil. *Euschistus heros* was relatively rare in Brazil until the 1970 s. Nowadays its status has shifted from secondary pest to key soybean pest, being considered one of the most abundant main pests of the crop in Brazil, notably in the warmer regions, from the north of the state of Paraná to the Brazilian Midwest (Panizzi and Slansky, 1985; Cividanes and Parra, 1994). The change in the status of the pest is attributed to several factors, with the increase in global average temperatures in the soy-producing regions being considered one of the most important.

Several species of natural enemies, such as predators and parasitoids, are found in soybean crops, reducing the populations of stink bugs, and keeping them below the economic injury level (Corrêa-Ferreira and Moscardi, 1995). Among the most frequently exploited natural enemies of stink bugs in biological programs are egg parasitoids, which prevent hatching of the pest eggs and thus prevent damage to crops. About twenty species of microhymenopterans have been reported as potential biological control agents, with *Trissolcus basalis* (Wollaston) and *Telenomus podisi* Ashmead (Hymenoptera, Scelionidae) being the most important (Corrêa-Ferreira and Moscardi, 1995; van Lenteren et al., 2018). Most of these parasitoids attack eggs of several stink bugs and some species show a certain preference, as is the case of *T. podisi* for *E. heros* eggs and *T. basalis* for *N. viridula* eggs (Sujii et al., 2002; Lagôa, et al., 2020).

Climate change and the expansion of soybean cultivation in different regions of Brazil have influenced the population dynamics of stink bug pests, as reflected by the observed reduction of populations of *N. viridula* and the increase in the abundance and distribution of *E. heros*, which has become the key pest in soybean crops today. Consequently, it is expected that the parasitoid community associated with this species will also be influenced by the new distribution patterns of the stink bug species. For example, *T. basalis*, the main parasitoid of *N. viridula*, may have more frequent encounters with the eggs of *E. heros* in soybean crops and parasitize them more frequently. The territorial expansion of *E. heros* populations also allows for the expansion of *T. podisi* in regions where *T. basalis* was predominantly associated with *N. viridula*, leading to a higher frequency of interactions between the two parasitoid species (Corrêa-Ferreira, 1993; Sujii et al., 2002).

It is known that in systems where two parasitoids exploit the same host, competition can exclude one of the species (Hassel, 1986), or lead to resource sharing, facilitating coexistence between species (Godfray, 1994). The mechanisms that explain the coexistence or displacement between parasitoids can be very useful for application in biological control programs (Sorribas et al., 2010). The occurrence of *T. basalis* and *T. podisi* and the releasing of both species as biological control agents of *E. heros* has been reported in soybean crops (Corrêa-Ferreira and Moscardi, 1995). Thus, the study of the competition between parasitoid species for *E. heros* eggs can help elucidate the process of population regulation of the host, contributing to the planning and management of biological control in soybean crops.

This study aimed to evaluate the effects of intra- and inter-specific competition between two parasitoids, *T. podisi* and *T. basalis*, on the parasitism of *E. heros* eggs and on the rates and patterns of successful parasitoid offspring emergence. These effects were assessed in two different experimental scenarios: (i) exposure of either two female wasps of a single parasitoid species or one female wasp of each of the two parasitoid species simultaneously to unparasitized *E. heros* eggs, and (ii) exposure of either one female wasp of a single parasitoid species or one female wasp of each of the two parasitoid species to host eggs previously parasitized by the conspecifics and/or the other parasitoid species.

2. Material and methods

2.1. Insects

The rearing of the stink bugs and parasitoid species in the laboratory

started with insects provided by the Insect Biology Laboratory, Department of Entomology and Acarology, at the University of São Paulo – USP/ESALQ. The rearing of *E. heros* was carried out according to a methodology adapted from Mendoza et al. (2016). The colony was reared in plastic cages (24 × 24 × 10 cm) under laboratory conditions (25 ± 2 °C, 12 h photoperiod, and 60 ± 10% RH). A diet based on beans, peanuts and cottonseed moistened with water was provided for the insects. Four strips of cotton fabric (3 × 10 cm) were placed on the upper part of the plastic cages, which served as substrate for laying eggs. Egg masses were collected daily and transferred to Petri dishes with filter paper moistened with sterile-distilled water. The eggs were used for maintaining the laboratory colony and for experiments.

The adults of *T. podisi* and *T. basalis* were kept separate in polyethylene bags (20 × 25 cm). Inside the bags, droplets of pure honey were provided to feed the parasitoids. Newly obtained egg masses from the *E. heros* colony were given to the parasitoids in Petri dishes (15 × 2 cm) inside the bags. After 24 h, the plates containing eggs were removed from the bags and kept in environmental chambers (25 ± 2 °C, 12 h photoperiod, and 60 ± 10% RH) until the emergence of the parasitoids. After emergence, adult parasitoids were released into polyethylene bags to start the new cycle.

2.2. Experimental design

The effects of intra- and inter-specific competition on parasitism rates, emergence rates, emergence patterns and sex ratio of *T. podisi* and *T. basalis* were analyzed in different experimental scenarios that evaluated: (*i*) simultaneous parasitism – exposure of two females of a single parasitoid species or one female of each parasitoid species simultaneously to unparasitized stink bug eggs and (*ii*) sequential parasitism, where one female of a single parasitoid species or one female of each parasitoid species simultaneously were able to choose from eggs previously parasitized by parasitoid species or unparasitized eggs. In both scenarios, the competition could lead to the occurrence of super- or multiparasitism. The objective of these experimental scenarios was to evaluate the potential of the parasitoid species as biological control agents, considering the occurrence of competition between two species or competition between conspecifics after release in the field.

The interactions took place in experimental arenas, represented by glass test tubes (2 × 8 cm) closed with film paper to prevent the escape of parasitoids. *Euschistus heros* egg masses, obtained from the lab colony, were carefully removed from the cotton fabric inside the plastic cages, counted and allocated inside the glass tubes (with the operculum positioned upward). All experiments were carried out between 2:00 pm and 6:00 pm (following an experimental standard) at the Interaction Ecology Laboratory, Department of Ecology and Evolutionary Biology, at the Federal University of São Carlos.

2.2.1. Simultaneous parasitism: Competition effects on unparasitized host eggs

Within each experimental arena, twenty host eggs up to 24 h old were offered to two previously mated parasitoid females aged approximately 48 h. The parasitoid females received honey *ad libitum* until the end of the experiment. The effects of intra- and inter-specific competition for host eggs were quantified through 3 scenarios: (TB *vs.* TB) 2 *T. basalis* females, (TP *vs.* TP) 2 *T. podisi* females and (TB *vs.* TP) a *T. basalis* female and a *T. podisi* female. Fifteen replicates were made for each experimental scenario divided into three blocks, with 4, 6 and 5 replicates each, respectively. The use of blocks was necessary because the replicates were performed on different days. A control group, without parasitoids, was added to the experiment.

After 24 h, the parasitoid females were removed and the eggs were placed in Petri dishes (15 × 2 cm) in a 25 ± 2 °C climatic chamber, 60 ± 10% RH and a 14 h photoperiod until stink-bug nymphs hatched and/or parasitoids emerged. After all parasitoid emergence and/or nymph host hatch had ceased (after approximately 20 to 22 days), the remaining

eggs of *E. heros* were classified as follows: (*i*) parasitized host eggs (parasitoids emerged successfully), (*ii*) parasitized but dead host eggs (parasitoids failed to develop or emerge); (*iii*) unparasitized host eggs (hatched); (*iv*) unparasitized but dead host eggs, and (*v*) host eggs dead from unknown causes (Moreira and Becker, 1986, adapted for *E. heros*).

The parasitism rates (number of parasitized eggs + parasitized but dead host eggs in relation to the total number of eggs provided), the emergence rates (number of parasitoids that emerged in relation to the total number of parasitized + parasitized but dead host eggs) and the sex ratio of the offspring (proportion of emerged females) in each experimental scenario were recorded. The host mortality resulting from the parasitism in this experimental scenario was estimated by the Schneider-Orelli formula, given by: $(b-k)/(1-k)$, where b and k represent the ratio of dead hosts in the treatment and in the control, respectively (Wang et al., 2019).

2.2.1.1. Sequential parasitism: Effects of previously parasitized hosts on adult female parasitism rates. This experiment aimed to determine whether the parasitoid is capable of discriminating against parasitized host eggs in the presence or absence of another parasitoid species. For this, 12 *E. heros* eggs were made available to the parasitoid females for 24 h. The total set of 12 eggs was composed of 3 groups: 4 eggs previously parasitized by *T. podisi*; 4 eggs parasitized by *T. basalis* and 4 eggs that were not parasitized. Female choice in the presence or absence of competitors was assessed in three experimental scenarios: (TB) 1 *T. basalis* female, (TP) 1 *T. podisi* female, and (TB *vs.* TP) 1 *T. basalis* female + 1 *T. podisi* female. Each parasitoid received honey *ad libitum* until the end of the experiment.

The previous parasitism of the eggs occurred 24 h before the experiment in a 25 ± 2 °C climatic chamber, $60 \pm 10\%$ R.H and 14 h photoperiod. This exposure time was chosen based on preliminary observations, assuring 100% parasitism of hosts by the first parasitoid species. For this, test tubes (2×8 cm) closed with film paper were used, containing six unparasitized eggs up to 24 h old and a female having previously mated. The procedure was done for both species. To standardize the age of the egg groups for the females during the experimentation, the unparasitized eggs made available to the females were 48 h old. After 24 h of parasitism, the parasitoid females were removed and the eggs separated and placed in Petri dishes (15×2 cm) in a climatic chamber at 25 ± 2 °C, $60 \pm 10\%$ R.H and 14 h photoperiod until stink-bug nymphs hatched and/or parasitoids emerged. After the offspring emerged, the emergence rates (number of emerging parasitoids), parasitism rates (number of parasitized eggs + parasitized but dead host eggs in relation the total number of eggs provided) and sex ratio of the offspring in each group of eggs were recorded. Ten replicates were made for each experimental scenario. A control group, without parasitoid release, was added to the experiment. Parasitism success was considered to be 100% when there was the emergence of only one parasitoid species from all of the groups of eggs (unparasitized, previously parasitized by *T. podisi* and previously parasitized by *T. basalis*). Parasitoid emergence of 100% (*i.e.*, success in parasitism) suggests that the females were able to identify eggs already parasitized by their conspecific and parasitized only unparasitized eggs and eggs parasitized by another species. In this case, their offspring were able to develop and succeed in competition (multiparasitism) with immature forms of other species present in the eggs. To evaluate whether females avoided eggs already parasitized by conspecifics, the emergence patterns were compared.

2.2.1.2. Emergence patterns. The number of parasitoids emerged per day was counted in all scenarios. From the removal of the females (day 1) until the day of the last emergence of the offspring, after the parasitoids were quantified, they were removed from the experimental arenas. To analyze and quantify the effects of competition on the development time and the offspring emergence rates of the species in the

different competition scenarios, the emergence pattern (*i.e.*, cumulative probability of emergence /day) of the offspring from the parasitized *E. heros* eggs in the interaction scenarios was analyzed. The analysis considered two important factors, reproductive success, given by the number of offspring (*i.e.*, emerged parasitoids) and the period in which the offspring developed inside the eggs until the emergence of adults.

In the sequential parasitism, the analysis of the emergence pattern allowed evaluation of the species' ability to avoid superparasitism and/or multiparasitism in two situations: when females forage alone or in the presence of another competing species. The control group was used as a reference for the time of offspring emergence in the absence of interspecific competition inside of the eggs for the comparison of the emergence period of the first and second parasitism events, since it is expected that, in previously parasitized eggs (48 h before onset of the experiment), the offspring emergence occurs in a shorter period, based on the emergence period observed in the control group.

2.3. Statistical analysis

In the simultaneous parasitism analysis, a multinomial model was fitted to the proportions of parasitized eggs, parasitized but dead host eggs, unparasitized but dead host eggs and host eggs dead from unknown causes, considering the effects of scenario (TB *vs.* TB, TP *vs.* TP and TB *vs.* TP) and block on the linear predictor. The significance of the effects was assessed using likelihood ratio tests. The host mortalities in the different scenarios and blocks were compared using one-way ANOVA.

Quasi-binomial generalized linear models were fitted to the proportion data (parasitism rates, emergence rates and sex ratio), considering the effects of scenario (TB *vs.* TB, TP *vs.* TP and TB *vs.* TP for the simultaneous parasitism experiment; TB, TP or TB *vs.* TP for the sequential parasitism experiment), block and the interaction between scenario and block in the linear predictor. The significance of the effects was assessed using F tests, and multiple comparisons were performed by obtaining 95% confidence intervals for the linear predictors. Goodness-of-fit was assessed using half-normal plots with simulated envelopes (Moral et al., 2017).

Weibull cure rate models were fitted to the time of emergence data (Ramirez et al. (2020)), implemented through GAMLSS (Rigby and Stasinopoulos, 2005). In the model, the "cured" fraction represents the insects that never emerged in the 24-day observation period (*i.e.*, fraction of non-emergence). We included the effect of block and scenario on the linear predictor for the mean (modelled with a log link), and scenario on the linear predictors for the variance (modelled with a log link) and the fraction of non-emergence (modelled with a logit link). We then performed backwards model selection starting from the linear predictor for the mean, then variance given mean, and finally non-emergence rate given mean and variance. Using the final selected model, we produced plots of the cumulative distribution functions for each scenario, which represent the probability of emergence over time. To assess goodness-of-fit we used half-normal plots with a simulated envelope (Moral et al., 2017) and worm-plots for the randomized quantile residuals (Rigby and Stasinopoulos, 2005). All analyses were performed using R (Core Team, 2020). All data and R scripts are available at: https://github.com/rafamoral/tbtpcompetition.

3. Results

3.1. Simultaneous Parasitism: Competition effects on unparasitized host eggs

At the end of parasitism, in each experimental scenario, most eggs were parasitized and offspring emergence was high (Fig. 1). The proportions of parasitized eggs, parasitized but dead host eggs and unparasitized but dead host eggs were different for each scenario ($LR = 967.35$, $df = 12$, p < 0.05). Unparasitized host eggs were observed only
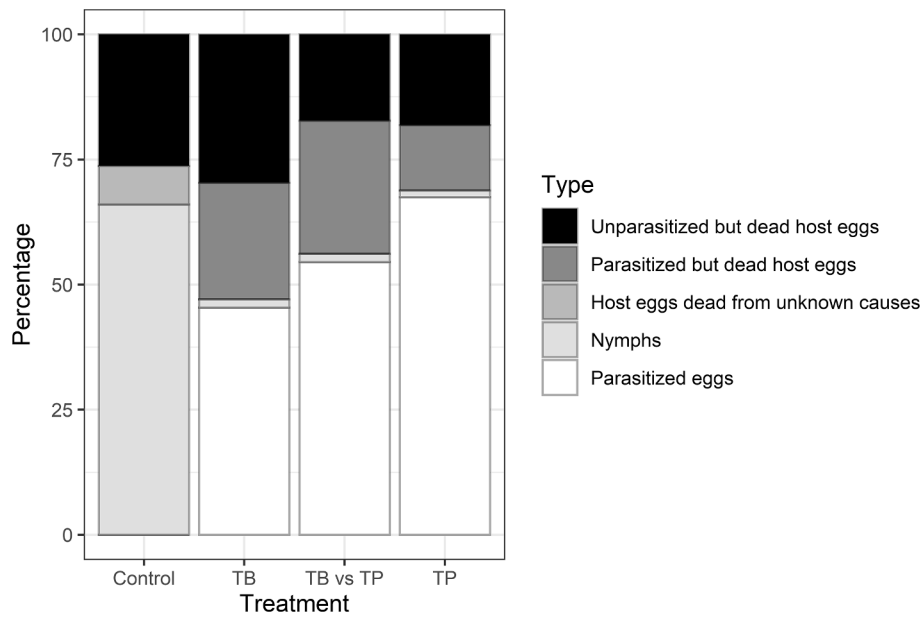
Fig. 1. Percentage of parasitized eggs, parasitized but dead host eggs (parasitoids failing to develop or emerge), unparasitized but dead host eggs, host eggs dead from unknown causes and hatched nymphs of *E. heros* in the experimental scenarios for interspecific (TB *vs.* TB; TP *vs.* TP) and interspecific (TB *vs.* TP) competition and control group. TP and TB represent, respectively, the parasitoid species *T. podisi* and *T. basalis*.

in the control group, in which the parasitoids were absent. The hatching of host nymphs was high in the control group, indicating that the mortality rates of *E. heros* observed in the experimental scenarios studied were mainly due to the action of the parasitoids (Fig. 1). The host mortality was marginally different among the competition scenarios (experimental scenario: $F_{2,40} = 3.07$, p = 0.057; block: $F_{2,40} = 7.04$p < 0.05). The host mortality was higher when the host eggs were exposed to both parasitoid species simultaneously, followed by the scenarios in which the host eggs were exposed to *T. podisi* or *T. basalis* alone (71.9%, 70.6% and 51.7%, respectively).

The scenario (TP *vs.* TP; TB *vs.* TB or TP *vs.* TB) and the experimental blocks influenced the parasitism rates of *E. heros* eggs ($F_{2,40} = 3.45$, p < 0.05 and $F_{2,42} = 10.15$, p < 0.05, respectively). In general, the odds of obtaining parasitized eggs in the experimental arenas with only *T. podisi* (TP *vs.* TP) or *T. podisi* and *T. basalis* females together (TB *vs.* TP) competing for eggs were 1.92 and 2.26 times higher in relation to the arena with only *T. basalis* females (TB *vs.* TB) (Table 1.). The parasitoid emergence rates in *E. heros* eggs also varied according to the scenario and the different experimental blocks ($F_{2,40} = 7.67$, p < 0.05 and $F_{2,42} =$
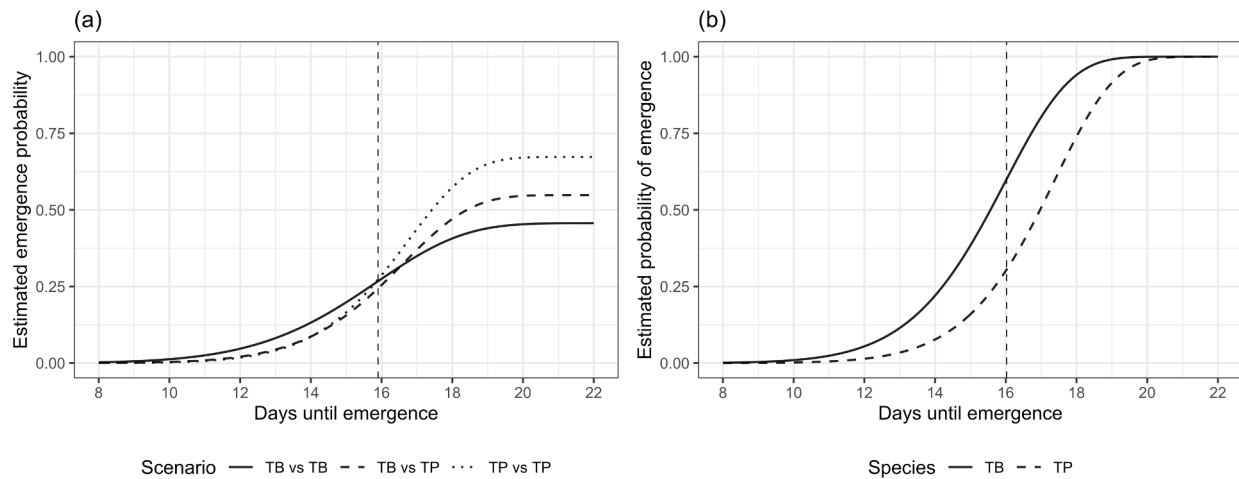
3.77, p < 0.05, respectively), with the odds of emergence being 2.83 higher in the experimental arenas in which there was only *T. podisi* (TP *vs.* TP) when compared to the other two setups (Table 1.).

Aiming to compare the effects of intra- and inter-specific competition on parasitoid species, the emergence rates in the arenas with only one of the species (TB *vs.* TB or TP *vs.* TP) and in the presence of both species (TB *vs.* TP) have been compared. The presence of another species during the foraging of host eggs by parasitoid females and the experimental blocks influenced the emergence rates of *T. basalis* (another spp: $F_{1,26} = 18.77$, p < 0.05; block: $F_{2,27} = 16.81$, p < 0.05) and *T. podisi* (another spp: $F_{1,26} = 88.85$, p < 0.05; block: $F_{2,27} = 3.65$p < 0.05; another spp × block: $F_{1,26} = 11.52$, p < 0.05). The offspring emergence rates were significantly higher for species in competition with conspecifics than for those facing interspecific competition (Table 1). The emergence rates of *T. basalis* and *T. podisi* also were compared within the interspecific scenario (TB *vs.* TP). In this scenario, the effect of the experimental blocks on the parasitism rates of each species was significant ($F_{2,12} = 19.48$, p < 0.05). Among the parasitized eggs in these experimental arenas, 61% corresponded to *T. basalis* and 39% to *T. podisi*.

The type of competition scenario and the experimental blocks did not influence the sex ratio (number of females/number of males) of the offspring of the parasitoids ($F_{2,39} = 0.55$, p = 0.55 and $F_{2,41} = 0.58$p = 0.56, respectively). The respective mean percentages of females in the offspring were 88.8% (±1.86), 92.6% (±1.98) and 93.1% (±1.90) in the TB *vs.* TP, TP *vs.* TP and TB *vs.* TB interaction arenas.

3.1.1. Emergence pattern

In general, the period for the emergence of parasitoids in simultaneous parasitism occurred within 20 days after the parasitoid release; however, the analysis indicated that the pattern of emergence over time varied according to species in different interaction scenarios (mean: LR = 18.82, df = 2, p < 0.05; variance: LR = 60.84, *df* = 2, p < 0.05; fraction of non-emergence: LR = 14.55, df = 2, p < 0.05). In the scenario where only *T. basalis* was present (TB *vs.* TB - intraspecific competition), the emergence period was shorter compared to the others (Fig. 2a). In the second week after parasitism (15th day), more than 50% of the offspring had already emerged, indicating a shorter development time of the immature phase of the species. The emergence curves of the parasitoids in the TP *vs.* TP and TP *vs.* TB scenarios were similar in the second

Table 1
Mean (±SE) percentage of *E. heros* eggs parasitized and mean (±SE) percentage of the parasitoid *T. basalis* (TB) or *T. podisi* (TP) offspring having successfully emerged in the presence of intra- or interspecific competition.

Scenarios	Parasitism rate	Emergence rate	Emergence rate (within TB *vs.* TP scenario)
TP *vs.* TP	80% (±0.03) a	83.9% (±0.02) a A	TP 28% (±0.07) B
TB *vs.* TB	69% (±0.06) b	59% (±0.07) b A	TB 36% (±0.07) B
TB *vs.* TP	83% (±0.05) a	66.2% (±0.05) b	

Means within columns followed by different small letters are significantly different in comparison among parasitism and emergence rates in competition scenarios. Means within rows followed by different capital letters are significantly different in comparison between TB and TP emergence rates in intra and interspecific competition scenarios (P < 0.05).

Fig. 2. Emergence patterns (cumulative probability) of the offspring of *T. basalis* and *T. podisi* as a function of time (days), for: (a) scenarios of intraspecific competition: *T. basalis* (TB *vs.* TB- continuous line) and *T. podisi* (TP *vs.* TP- long dashes) and interspecific competition *T. basalis vs. T. podisi* (TB *vs.* TB – short dashes) and (b) for interspecific competition scenarios: between *T. basalis* species (TB - continuous line) and *T. podisi* (TP – dashed line).

week after parasitism (8th to 15th day). However, unlike the scenario where there were only TB females, most parasitoids emerged in the third week after parasitism (16th to 22nd day). From the third week, in the scenario where there were only *T. podisi* females (TP *vs.* TP), the offspring number was larger, and the emergence of parasitoids occurred more quickly than in the TB *vs.* TP scenario, whose emergence curve presented an intermediate offspring number and emergence time when compared to the other scenarios, TB *vs.* TB and TP *vs.* TP.

The curve for the TB *vs.* TP scenario (interspecific competition) considered the total number of emerged parasitoids, with no distinction between species. Therefore, to assess the period of the offspring emergence of the two species after interspecific competition, an analysis was also made considering only the quantity of viable parasitized eggs in this scenario. The analysis within the scenario in which the females of the two species competed for eggs (TB *vs.* TP) showed that the offspring emergence time varied between species (mean: LR = 192.75, *df* = 2, p < 0.05; variance: LR = 1.42, *df* = 2, p = 0.49; fraction of non-emergence: LR = 14.55, *df* = 2, p < 0.05). The *T. basalis* offspring continued to have a shorter emergence period and lower viability than *T. podisi* offspring. The interspecific competition between parental females did not influence the emergence time of the surviving offspring (Fig. 2b).

3.2. Sequential parasitism: Effects of already parasitized hosts on adult female parasitism rates

The analysis showed that the experimental scenario (TB; TP; TB *vs.* TP) influenced the emergence rates of the parasitoids (χ^2 = 123.51; *df* = 9, p < 0.05). Within each scenario, all pairwise comparisons between groups of eggs showed significant differences (Fig. 3a). In the control group, the eggs parasitized by *T. podisi* had higher emergence rates compared to the viability of *T. basalis*. Furthermore, the observed occurrence of failed eggs influenced the emergence rates in the control group (Table 2.). In the single-species release scenarios (TB and TP), the presence of the second parasitoid species decreased the emergence rates of heterospecifics and increased the emergence rates of conspecifics, compared to the emergence rates of the control group, which considered only first parasitism without the presence of second parasitoid females. In the TB scenario produced the highest parasitoid mortality rates (parasitized but dead host eggs). The results indicated the occurrence of multiparasitism and superparasitism (Table 2., Fig. 3a). In the scenario in which the two species compete for eggs (TB *vs.* TP), the emergence rates of new parasitoids were higher compared to the other scenarios. *Telenomus podisi* was the species that exhibited the highest emergence
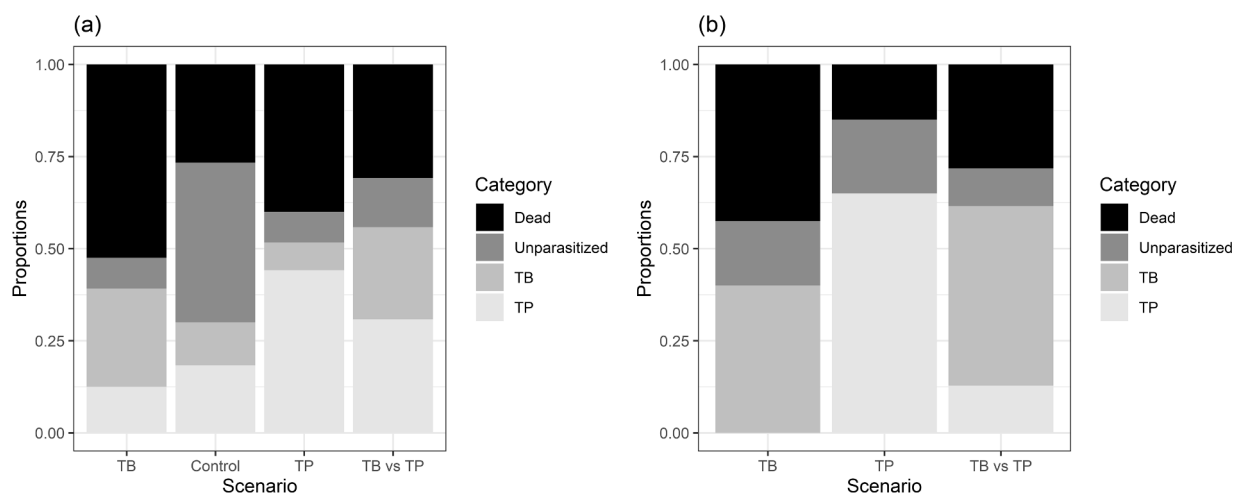
Fig. 3. Average percentage of reproductive success (emergence of offspring) of each parasitoid species, mortality of parasitoids (parasitized eggs without emergence of parasitoids) and failed eggs in the experimental scenarios with second release of: one female of *T. basalis* (TP), one female of *T. podisi* (TP), one female of each parasitoid species (TB *vs.* TP) and Control group (no second release of parasitoids). In (a) experimental scenarios considering three types of host eggs: previously parasitized by TP, TB and unparasitized eggs; and (b) scenarios considering only unparasitized eggs (*i.e.*, without previous parasitism).

Table 2

Mean (±SE) percentages of emerged *T. basalis, T. podisi*, parasitized but dead host eggs and unparasitized but dead host eggs at the end of parasitism in each group of eggs (TB: eggs with previous parasitism of *T. basalis*; TP: eggs with previous parasitism of *T. podisi*; np: non-parasitized eggs) in the experimental scenarios TB, TP, TB *vs.* TP and Control (without the presence of parasitoid females). Dear Dr. Desneux,

Scenarios	Group of eggs	Response				
		T. basalis	*T. podisi*	Parasitized but dead host eggs	Unparasitized but dead host eggs	Total
	tb	0.9 (±0.99)	0.2 (±0.42)	2.8 (±0.91)	0.1 (±0)	4
	tp	0	2.5 (±1.35)	1.4 (±1.42)	0.1* (±0)	4
TP	np	0	2.6 (±1.64)	0.6 (±0.84)	0.8 (±0.91)	4
	% success (Response)	7.5%	44.16%	40%	8.33%	
	tb	1.5 (±0.70)	0	2.2 (±0.78)	0.3 (±0.48)	4
	tp	0.1* (±0)	1.5 (±1.35)	2.4 (±1.42)	0	4
TB	np	1.6 (±1.17)	0	1.7 (±0.94)	0.7 (±0.94)	4
	% success (Response)	26.6%	12.5%	52.5%	8.33%	
	tb	1.1 (±1.05)	0.2*(±0)	1.9 (±1.37)	0.8 (±0.63)	4
	tp	0	2.9 (±1.19)	0.7 (±0.67)	0.3* (±0)	4
TB *vs* TP	np	1.9 (±1.19)	0.6 (±0.96)	1.1 (±0.87)	0.4 (±0.51)	4
	% success (Response)	25%	30.83%	30.83%	12.5%	
Control	tb	1.4 (±1.07)	0	1.7 (±1.05)	0.9 (±0.87)	4
	tp	0	2.2 (±1.54)	1.5 (±1.50)	0.3 (±0.67)	4
	np	0	0	0	Nymphs3.8 (±0.63) 31.66%	4
	% success (Response)	11.66%	18.33%	26.66%	10%	

* Occurrence of an event in only one sample.

rate. The emergence of *T. podisi* from eggs previously parasitized by *T. basalis* was also observed (Table 2.).

To evaluate the species' ability to avoid parasitism of parasitized eggs, we analyzed the parasitism rates of the species in unparasitized eggs present in the experimental scenarios. The analysis of reproductive success showed that the experimental scenario (TP, TB, TB *vs.* TP) and egg host types had an interactive influence on the success of parasitism (mean: LR = 11.19, *df* = 5, p < 0.05; variance: LR = 5.09, *df* = 5, p = 0.63; fraction of non-emergence: LR = 31.41, *df* = 5, p = 0.63). Within each scenario, all pairwise comparisons between groups of eggs showed significant differences. In the TB scenario, it was observed that 42.5% of unparasitized eggs, once parasitized by *T. basalis,* were not viable. Mortality rates for treatments TP and TB *vs.* TP in unparasitized eggs were lower, 15% and 27%, respectively (Fig. 3b). When only one species was present in the experimental arenas, the highest rate of offspring emergence was observed in *T. podisi* in the TP scenario. When females of both species were present in the same experimental arena (interspecific competition – TB *vs.* TP scenario), the species with the most success and emergence of offspring in unparasitized eggs was *T. basalis*. When both species were present, the *T. podisi* offspring emerged in only 12.5% of non-parasitized eggs (Fig. 3b).

The sex ratio of the parasitoid offspring in scenarios where females alone or in interspecific competition foraged in arenas with parasitized and unparasitized eggs was not influenced by the parasitoid species present (TB: F$_{3,28}$ = 0.23p = 0.87 and TP: F$_{3,30}$ = 0.53p = 0.66). In the TB, TP and TB *vs.* TP scenarios, the respective mean percentages of females were 71.5%, 72.2% and 70.4% in the *T. basalis* offspring *vs.* 92%, 82% and 85.7% in those of *T. podisi.*

3.2.0.1. Emergence curves

In the TB scenario, the offspring from eggs previously parasitized by

T. basalis started around the 12th day. The offspring of eggs previously parasitized by *T. podisi* and unparasitized eggs started approximately 2 days later. Thus, in the second week after parasitism (8th to 15th day), most of the parasitoids present (offspring) were constituted by *T. basalis* (first parasitism). In the third week after parasitism (16th to 22nd day) the offspring of *T. podisi* (first parasitism) and *T. basalis* (secondary parasitism - offspring of the females that oviposited in unparasitized eggs) were present. In this scenario, the success of *T. basalis* parasitism in eggs from the first and secondary parasitism was similar at the end of the emergence period of the parasitoids (Fig. 4- TB, solid and dotted lines, respectively).

When only females of the species *T. podisi* were present (TP scenario) in the experimental arena, the offspring from eggs parasitized by first conspecifics began to emerge around the 12th day and extended until the third week after the parasitism started (16th to 22nd day). Offspring from unparasitized eggs and from eggs first parasitized by *T. basalis* started to emerge from the 14th day, but most of the offspring emerged in the third week (16th to 22nd day) after parasitism. In this scenario, the success of the first and second parasitism of *T. podisi* was similar (Fig. 4-TP: dashed and dotted lines, respectively), while the emergence rates of parasitoids in eggs parasitized by *T. basalis* (Fig. 4-TP, solid line) were lower. The diminished emergence of the offspring and the delay in the emergence period of *T. basalis* suggest the occurrence of multi-parasitism and the consequent competition of immature forms inside the eggs, resulting in a delay in the development of the larvae inside the eggs.

In the scenario where females of the species *T. basalis* and *T. podisi* (TB *vs.* TP) competed for the eggs available in the experimental arena, the emergence of offspring from eggs previously parasitized by *T. podisi* and *T. basalis* occurred around the 12th day after the onset of parasitism. The offspring of eggs previously parasitized by *T. podisi* emerged almost
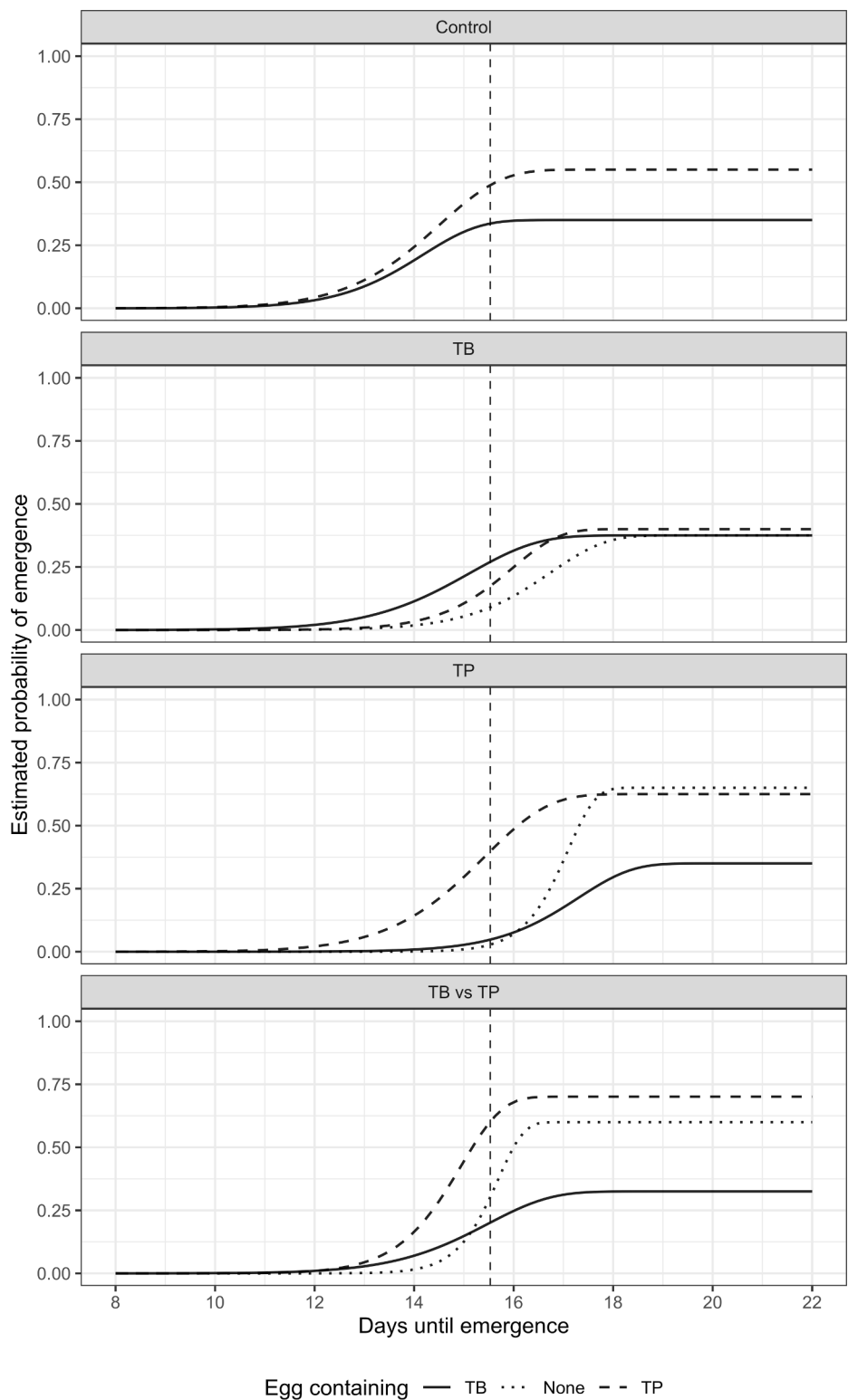
Fig. 4. Emergence pattern (cumulative probability) of offspring of groups of eggs containing TB: previous parasitism by *T. basalis*; TP: previous parasitism by *T. podisi*; None: Unparasitized eggs; of parasitoid species as a function of time (days) in different scenarios: TB- when only the female of the species *T. basalis* was present; TB *vs.* TP- when the females of the species *T.basalis* and *T. podisi* were present simultaneously; TP- when only the female of *T.podisi* species was present in the experimental arena; Control - when only previous parasitism occurred, without the addition of females of the species.

entirely in the second week after parasitism (8th to 15th day) while the period of offspring emergence from eggs previously parasitized by *T. basalis* and unparasitized eggs extended until the third week after parasitism (16th to 22nd day). In this scenario, the success of the offspring from eggs previously parasitized by *T. podisi* and unparasitized eggs (Fig. 4- TB *vs.* TP: dashed and dotted lines, respectively) was greater when compared to the offspring of eggs previously parasitized by *T. basalis* (Fig. 4- TB *vs.* TP solid line). The delay in the *T. basalis* offspring

emergence period (previous parasitized eggs) and the lower offspring emergence rate suggest the occurrence of superparasitism and/or multiparasitism, with consequent mortality of the immature forms inside the egg. The reduction in the *T. podisi* emergence period is also an indication that adult females of the species oviposited in eggs previously parasitized during parasitism, which affected the time of development of the immature forms inside the eggs and consequently the species emergence pattern.

To assess the ability of females of the species to discriminate against previously parasitized eggs in the three scenarios studied (TB, TP, and TB *vs.* TP), the emergence patterns were analyzed considering the total parasitoid offspring having emerged from previously unparasitized eggs. The analysis of reproductive success showed that the parasitoid species influenced the parasitism rates of healthy eggs (mean: LR = 0.2922, *df* = 1, p = 0.2922; variance: LR = 0.34, *df* = 1, p = 0.56). In general, *T. basalis* showed shorter development time and, consequently, shorter emergence time in this host egg type, compared to *T. podisi*, similarly to previous results obtained in unparasitized eggs from simultaneous parasitism experiments (Fig. 5).

4. Discussion

Evaluating effects of the competitive interactions that arise in systems in which the same host can be attacked by different parasitoid species requires effective application of biological control, since competition between species can result in the change of hosts or parasitoid population decrease, thus reducing the efficiency of biological control of the target pest (Murdoch et al., 1998; van Lenteren et al., 2018). In this context, studies that focus on foraging behavior, competitive ability and discrimination capacity of hosts between different parasitoid species can assist in the evaluation and planning of the use of these biological agents.

In the experimental scenarios for simultaneous parasitism, in which only conspecific females competed for *E. heros* eggs not yet parasitized, *T. podisi* exhibited higher rates of parasitism and offspring emergence compared to *T. basalis* populations. This difference in parasitism potential between species had already been reported in previous studies, which showed that *T. basalis* has greater reproductive success and preference for eggs from the host *N. viridula*, while the species *T. podisi* presents strong interaction, high reproductive capacity, and excellent development in eggs of the brown stink bug *E. heros* (Corrêa-Ferreira and Moscardi, 1995; Sujii et al., 2002; Peres and Corrêa-Ferreira 2004; Queiroz et al., 2018). When the host eggs were exposed to females of the two parasitoid species simultaneously, the parasitism rates were higher than 80%. In this case, the joint parasitism of the species showed better results when compared to the situation in which only *T. basalis* was present. In this scenario of interspecific competition, *T. basalis* parasitized more eggs, indicating that females of the species have greater ability to find hosts compared to *T. podisi*. However, the low emergence rates of both parasitoid species indicate the occurrence of multiparasitism, resulting in a low survival rate of the larvae of the species in the competition inside host eggs.

The presence of females of another species during foraging can intensify parasitism, increase the oviposition frequency of the species and, consequently, lead to multiparasitism (Vankosky and Hoddle,

2017). In this context, competition between two species can result in no winner when, for example, the species that shows the better performance in finding the host has a low capacity to exploit it (Cabello et al., 2011). Studies show that females of the genus *Trissolcus* typically exhibit aggressive behavior against conspecifics or females of other species when they encounter host eggs susceptible to parasitism (Field (1998); Cusumano et al. (2010)). Although not evaluated, the report of this aggressive behavior may explain the reduction in the rates of egg parasitism by *T. podisi* in the arenas where *T. basalis* was present.

The higher rates of *T. basalis* parasitism of *E. heros* eggs in the presence of *T. podisi*, however, did not guarantee greater reproductive success for the species. The low emergence rates of *T. basalis* offspring in *E. heros* eggs, in both the presence and absence of *T. podisi*, suggest that the offspring of the species are inefficient in exploiting *E. heros* eggs. The occurrence of parasitized but dead host eggs for the species was high even when there were only females of the species in the experimental arenas, indicating that the effects of multiparasitism, superparasitism and poor adaptation to the host may also have influenced the reproductive success of the species, reducing the offspring emergence rates. The occurrence of superparasitism in *T. basalis* due to the pressure of parasitism had already been reported under laboratory conditions (Thomas Jr. (1972)). Thus, superparasitism may also have contributed to the high mortality observed in the offspring of *T. basalis*, since females lose the ability to discriminate eggs already parasitized by conspecifics when there is a simultaneous attack of more than one female of *T. basalis* in the same oviposition event (Thomas Jr. (1972)).

Multiparasitism is the main factor of offspring mortality when there is competition for hosts between different species. Studies that report the occurrence of multiparasitism in interspecific interactions involving *T. basalis* and/or *T. podisi* report a low rate of species offspring emergence (Cusumano et al. (2010); Cingolani et al., 2013). The occurrence of multiparasitism is dependent on the species' ability to discriminate marks left by other species after parasitism. Thus, not only the presence of other species at the site, but also the host's recognition and selection behavior, which precede oviposition, can influence parasitism rates (Vinson, 1984; Vinson, 1985, Bin et al., 1993). In general, in all experimental scenarios that considered secondary parasitism (i.e., sequential parasitism), the occurrence of parasitized but dead host eggs showed a large increase in the mortality of previously parasitized eggs.

In sequential parasitism, an experimental scenario in which only one *T. basalis* female was present had the lowest rate of offspring emergence, namely only 39% of the parasitized eggs. The results also indicate the occurrence of superparasitism, since the number of eggs previously parasitized by *T. basalis* was reduced. On the other hand, when only one *T. podisi* female was present, the final emergence rate of parasitoids was higher. The final number of *T. podisi* individuals exceeded the number of eggs previously parasitized by their conspecifics, due to the successful parasitism in unparasitized eggs present in experimental arenas, which indicates that the species was able to discriminate conspecific markings in parasitized eggs, thus avoiding superparasitism and population losses of the species. Mortality of eggs previously parasitized by *T. basalis* may be due to the occurrence of multiparasitism; however, the emergence of *T. podisi* in eggs previously parasitized by *T. basalis* suggests greater competitive capacity of the immature species in the acquisition of resources from the hosts.

In the sequential parasitism scenario both species competed for different groups of eggs, resulting in a total egg mortality rate of 30%. Unlike the scenario in which the females of the species competed for unparasitized eggs, the occurrence of competition after a previous attack of parasitoids promoted greater reproductive success of *T. podisi*. These results also suggest a greater ability of the species to avoid ovipositing in eggs already parasitized by conspecifics and a greater competitive ability in the exploitation of hosts. However, neither species was able to discriminate parasitized eggs by the other species. Interspecific discrimination of parasitized hosts is quite uncommon and observed only in closely related parasitoid species (Pijls et al. (2010)). The
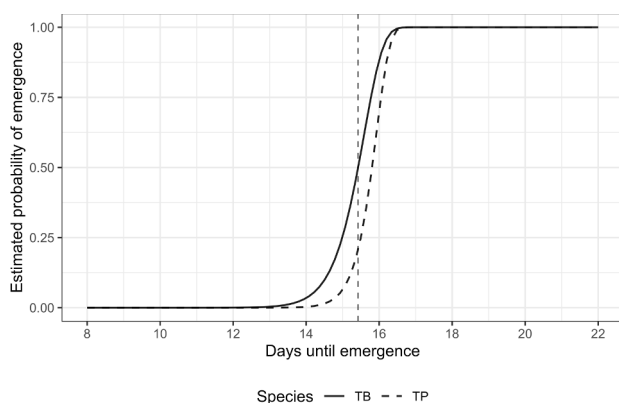

Fig. 5. Emergence pattern (cumulative probability) of the offspring from unparasitized eggs of the species *T. basalis* (TB) and *T. podisi* (TP) as a function of time (All TB, TP and TB *vs.* TP treatments were considered).

parasitized host markings function mainly at a specific level, allowing the females to differentiate chemical marks and signs left by conspecifics, avoiding superparasitism (Okuda and Yeargan, 1988; Van Baaren et al., 1994; Agboka et al., 2002).

In addition to the inability to distinguish interspecific markings, the low density of available hosts, that is, when encounters with unparasitized hosts are infrequent, can also lead to multiparasitism (Godfray, 1994). In this case, ovipositing in already parasitized eggs involves a risk for the parasitoid, since their offspring are forced to compete for resources in the larval stage, which can be lethal (Cingolani et al., 2013). Thus, the time between ovipositions becomes an important factor for the success of a species in multiparasitism (Cusumano et al. (2010)). Generally, parasitizing first provides parasitoids a competitive advantage, which gives older larvae the advantage in a physical confrontation (Irvin et al., 2006; Magdaraog et al., 2012), although larval competition can also be mediated by mechanisms of physiological suppression (Mackauer, 1990; Godfray, 1994; Cusumano et al. (2010)). Either mechanism can cause the death of one or both species, and can affect the development of the larvae, which can reflect in changes in the emergence period of the offspring.

The results suggest a possible confrontation between larvae in multiparasitism that provokes high mortality rates, but also changes in the emergence period of the species. In general, the offspring generated by *T. basalis* emerged in a shorter period compared to the offspring of *T. podisi* when females had only unparasitized eggs available to parasitize. Based on these emergence patterns, it was expected that previously parasitized eggs (which contained approximately 48-hour-old larvae) would show patterns close to those observed in these scenarios, because in the competition these larvae had advantages due to being older. However, in the presence of different groups of eggs, the occurrence of intra-egg larval competition altered the emergence pattern of *T. basalis* with the second parasitism, especially in the presence of *T. podisi* In other words, the occurrence of multiparasitism may have delayed the emergence of parasitoids, suggesting effects of competition on the physiology and development of the immature forms. In a study by Cusumano et al. (2015), the development of *T. basalis* was significantly longer when individuals survived in interspecific competition. Although minor, superparasitism also led to a delay in the emergence time (TB scenario). Changes in the *T. podisi* emergence pattern were less pronounced. The greatest change was observed when only *T. basalis* females (TB scenario) were present when the onset of offspring emergence was delayed.

For the application of biological control, variables such as climate, temperature and phenology of the plant are considered, in addition to carrying out a previous monitoring of the population dynamics of the host's eggs, to analyze the best strategies and periods so that the release of natural enemies coincides with the beginning of the pest infestation (Smith et al., 1986; Parra, 1993). Knowledge of the period for offspring emergence of the biological agents can help to reduce the frequency of parasitoid release in the field. The synchrony between the emergence of parasitoids and the susceptible stages of the hosts is crucial for the success of biological control, as it reduces the survival of the host's offspring. By reducing this "escape" in the host population, new population outbreaks in the following generations can be prevented, keeping the population below the action threshold for a longer period. The results found in this study indicate that interspecific interactions may have an important effect on maintaining populations of parasitoid species in the field. The effectiveness of the joint release of parasitoid species has been discussed by many studies. While some argue that the more species present, the greater the effect of reducing the density of insect pests (De Bach, 1966), other studies report that the presence of multiple species can reduce the effectiveness of biological control when competition or facultative hyperparasitism occurs (Murdoch et al., 1998; Denoth et al., 2002). In fact, the joint use of parasitoid species brings benefits when the niches of the species do not overlap, or partially overlap, for example, when the species attack different stages of the host's life or when the

species are able to discriminate hosts already parasitized by other species (Cusumano et al. (2010)).

The combination of *T. podisi* and *T. basalis* has been utilized to control phytophagous stink bugs such as *N. viridula* and *E. heros* (Côrrea-Ferreira, 2002). The results of the current study show that the rates of parasitism remained high, above 80% when both species were present. Thus, the results suggest that the simultaneous use of the species can impair the long-term persistence of parasitoids in the field, since the viability of the offspring is reduced, which may lead to the need for a higher frequency of releases to achieve pest control throughout the plant cycle. However, the use of simultaneous releases of the species can be effective for the immediate flooding (applied) control, aiming at the control of the *E. heros* population in a short period.

The temporal and spatial co-occurrence of *T. podisi* and *T. basalis* adults in soybean crops can also be common when the release of *T. podisi* occurs in a place where *T. basalis* or even *T. podisi* are already present. Research on naturally parasitized *E. heros* eggs in central Brazil has shown that the coexistence of *Trissolcus* spp. and *T. podisi* can occur in the field (Corrêa-Ferreira and Moscardi, 1995). In these areas, the release of species can lead to more complex interaction scenarios due to the occurrence of sequential parasitism, which can lead to decoupling of the parasitoid host interaction and "refuge" of the pest population, and reducing the efficiency of biological control.

Our results suggest that in areas where *E. heros* is the predominant species, *T. podisi* should be the species chosen for biological control programs. However, further research is needed to elucidate the consequences of *T. podisi* and *T. basalis* interactions in soybean growth regions, considering that the occurrence of other pentatomids and other natural enemy species can affect, directly or indirectly, the competition interaction between these species.

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