Intensity of intraguild predation of parasitized eggs by mirid predators depends on time since parasitization: a case study with the parasitoid *Trichogramma pretiosum* and the predator *Macrolophus basicornis* attacking *Tuta absoluta* eggs

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Abstract

Concurrent releases of several species of natural enemies for pest control have been studied in various crops with either positive, neutral, or negative results. To control the pest *Tuta absoluta* (Meyrick) (Lepidoptera Gelechiidae), only the egg parasitoid *Trichogramma pretiosum* Riley (Hymenoptera Trichogrammatidae) is applied on many hectares with tomatoes in South America. Use of the mirid predator *Macrolophus basicornis* Stal (Hemiptera Miridae) is considered either alone or in combination with the parasitoid *T. pretiosum*. To determine if intraguild relationships between the two natural enemies negatively affect concurrent releases, unparasitized eggs and eggs parasitized by *T. pretiosum* were exposed to the predator. Knowing which stages of parasitized eggs are consumed or rejected by the predator is important for development of a release strategy resulting in highest pest mortality. *M. basicornis* consumed 1-2 day old parasitized eggs as well as unparasitized eggs, and, consequently, expressed strong intraguild predation (IGP). Five and 9-day old parasitized eggs were often not consumed, and, therefore, exerted the phenomenon of competitive exclusion (CE). Predation rates of old parasitized eggs were very low and similar in no-choice and choice tests, and in experiments with a large (100), medium (50) and very limited (10) number of preys offered, demonstrating a strong CE effect. Interestingly, in choice experiments with unparasitized and old parasitized eggs, predators started to consume unparasitized eggs significantly later than when only unparasitized or young parasitized eggs were offered. This suggests that old, parasitized eggs in some way reduce the foraging activity of *M. basicornis*. We expect that stronger pest reduction by concurrent releases of both natural enemies will only occur at the start of the tomato production season and when the egg parasitoid is introduced seven days before the mirid predator in order to reduce intraguild predation of parasitized eggs by the predator.

Key words: intraguild predation, competitive exclusion, biological control of invasive pest, South American tomato leaf miner, *Phthorimaea absoluta*.

Introduction

The South American tomato leaf miner *Tuta* (= *Phthorimaea*) *absoluta* (Meyrick) (Lepidoptera Gelechiidae) has since long been a problem in tomato crops in Latin America (Guedes and Picanço, 2012). The pest was accidentally introduced into Spain in 2006, then spread over Europe, to northern Africa and Africa South of the Sahara, the Near East, and Asia (Biondi *et al*., 2018), and might, according to climate models, establish in the field in Mexico and southern USA, and in greenhouses in other parts of the USA and Canada (Early *et al*., 2022) as well. Pest populations can be reduced by chemical control, but need frequent treatments (1-4 × week) due to the mining behaviour of the larvae and as a result, *T. absoluta* may develop resistance to pesticides rather fast (Guedes and Picanço, 2012). Control of the pest is also possible with biological control agents, and this method is, among others, applied successfully in Europe and Latin America (Ferracini *et al*., 2019). During the past decades, more than 200 natural enemies have been associated with

T. absoluta, but only five species are currently commercially applied (van Lenteren *et al*., 2021). Combined introductions of two species of natural enemies have been proposed to improve biological control of this pest (Cabello *et al*., 2015), and the issue of releasing more than one natural enemy species has recently surfaced related to biological control of *T. absoluta* in Brazil, as release of egg parasitoids alone is time consuming and expensive (Bueno *et al*., 2023).

In European tomato crops, two natural enemies - the mirid predator *Nesidiocoris tenuis* (Reuter) (Hemiptera Miridae) and the generalist egg parasitoid *Trichogramma achaeae* Nagaraja et Nagarkatti (Hymenoptera Trichogrammatidae) - can be released concurrently for control of the *T. absoluta* (Cabello *et al*., 2015). However, the mirid predators may negatively affect the impact of parasitoids because they frequently feed on eggs earlier parasitized by *T. achaeae* (Calvo *et al*., 2012; Chailleux *et al*., 2013), a process called Intraguild Predations (IGP) (Rosenheim and Harmon, 2006). On the other hand, when the predator avoids to feed on parasitized eggs, releases

of the parasitoid may limit prey availability, development, survival and adult performance of the predator and, thus, have a negative effect on reproduction of the predator, a phenomenon addressed as Competitive Exclusion (CE) (Tilman, 1982). So, both IGP of parasitized prey by the predator and CE of the predator by the parasitoid may play a role in a system with concurrent releases. A study on acceptance of *Heliothis zea* Boddie (Lepidoptera Noctuidae) eggs parasitized by *Trichogramma pretiosum* Riley (Hymenoptera Trichogrammatidae) by the predator *Orius insidiosus* Say (Hemiptera Anthocoridae) shows the role of IGP and CE (Ruberson and Kring, 1991): two- and 5-day old parasitized eggs were encountered as frequent as unparasitized eggs. However, unparasitized eggs were accepted for feeding twice as frequently as the parasitized eggs. Consequently, *O. insidiosus* has fewer prey available when eggs have been parasitized and particularly when pupal formation of the parasitoid had taken place within the egg, illustrating the effect of CE. But also *T. pretiosum* is negatively affected by the predator, because larvae of the parasitoid are killed when attacked within the prey egg, indicating the role of IGP.

The effects of hemipteran predators on *Trichogramma* egg parasitoids and vice versa have been described in a number of recent papers (Calvo *et al*., 2012; Chailleux *et al*., 2013; Cabello *et al*., 2015; Varshney and Ballal, 2018; Mirhosseini *et al*., 2019) and usually show a similar pattern: predators will consume unparasitized prey eggs and often also easily feed on eggs that have been parasitized recently, which contain young stages of the parasitoid. However, they hardly feed on eggs with the older pupal stages of the parasitoid. Studies describing the effect of combined releases of hemipteran predators and *Trichogramma* egg parasitoids reveal conflicting results: some mention a better control effect after concurrent release (Cabello *et al*., 2015), others do not show significant differences between concurrent and single species releases or state that only one of the two species - usually the predator - alone does control the pest (Calvo *et al*., 2012), and still others point at a negative effect of one species on the control effect of the other species (Guven *et al*., 2017; Mirhosseini *et al*., 2019).

This paper deals with the intraguild interactions between the Neotropical mirid predator *Macrolophus basicornis* (Stal) (Hemiptera Miridae) and the egg parasitoid *T. pretiosum* when released together to reduce populations of *T. absoluta*. *M. basicornis* is able to kill large numbers of *T. absoluta* eggs and larvae (van Lenteren *et al*., 2017; 2018) and shows a pest kill rate that is considerably larger than the innate population growth rate of the pest (van Lenteren *et al*., 2021), indicating that the predator can kill more prey eggs per unit of time than *T. absoluta* can produce. Thus, this predator may need only to be released at the start of the tomato production season in order to provide permanent control, similar to the current practice in Europe with *N. tenuis* (Calvo *et al*., 2012). *T. pretiosum* kills fewer prey eggs per unit of time than *T. absoluta* can deposit (van Lenteren *et al*., 2021) and needs to be released weekly in very large numbers to sufficiently reduce *T. absoluta* populations (Bueno *et al*., 2023).

The aims of this study were to answer the questions (1) whether IGP of parasitized eggs occurs by the mirid predator, and, (2) if CE by the parasitoid takes place by making parasitized prey eggs unsuitable for predation. We hypothesize that younger prey eggs containing eggs or larvae of the parasitoid will be easily consumed by the predator, but that older eggs containing pupal stages of the parasitoid can no longer be penetrated by the mouthparts of the mirid predator or are rejected for consumption.

Materials and methods

Plants and insects

Tomato plants *Solanum lycopersicum* (L.) cv. Santa Clara were reared in pots and used in pest insect rearing after they reached a height of 30 cm. Adults of the pest insect *T. absoluta* were collected from tomato in Sao Paulo State, Brazil, and maintained in mesh cages (90 × 70 × 70 cm) with tomato plants in the laboratory according to Pratissoli and Parra (2001). New tomato plants were regularly placed into the cages to keep a stock rearing of *T. absoluta* at 25 ± 2 °C, RH 70 ± 10% and a photoperiod of 14:10 (L:D). Newly-emerged adults from this rearing were collected and allowed to lay eggs for use in experiments.

The predator *M. basicornis* was collected on tobacco *Nicotiana tabacum* (L.) near Lavras, Minas Gerais, Brazil and reared as previously described (Bueno *et al*. 2013; Bueno *et al*., 2018), using tobacco plants as oviposition substrate and with UV-irradiated *Ephestia kuehniella* Zeller (Lepidoptera Pyralidae) eggs as prey in climate rooms at 25 ± 2 °C, 70 ± 10% RH and a photoperiod 14:10 (L:D). Adult female *M. basicornis* predators of up to seven days old were used in the experiments and had been starved for 24 hours, but had access to water. The parasitoid *T. pretiosum* was obtained from Koppert Biological Systems Brazil, and then reared on UV-irradiated eggs of *E. kuehniella* in climate rooms at 25 ± 1 °C, 70 ± 5% RH and a photoperiod of 16:8 (L:D) according to Parra (1997). Adult parasitoids used in the experiments were less than 24 hours old. All rearings and the predation experiments were done in the Laboratory of Biology of Insects, part of the Department of Entomology and Acarology at the College of Agriculture "Luis de Queiroz" (ESALQ), University of São Paulo (USP), Piracicaba, Brazil.

General procedures

Shortly before the start of a test, the eggs of *T. absoluta*, whether unparasitized or parasitized, were transferred with a fine paint brush to a tomato leaflet on which they would be exposed to the predator. Mortality due to transfer of *T. absoluta* eggs was not determined in these experiments. We have been using the same method to transfer eggs from one leaflet to another one with the tip of a fine paint brush for many years. In earlier experiments the mortality of 12,680 unparasitized prey eggs was measured after transfer, and the natural egg mortality together with egg manipulation mortality ranged from 0% to 1.12% (van Lenteren *et al*., 2016). These low percentages of mortality made us decide not to correct our data for death due to handling eggs.
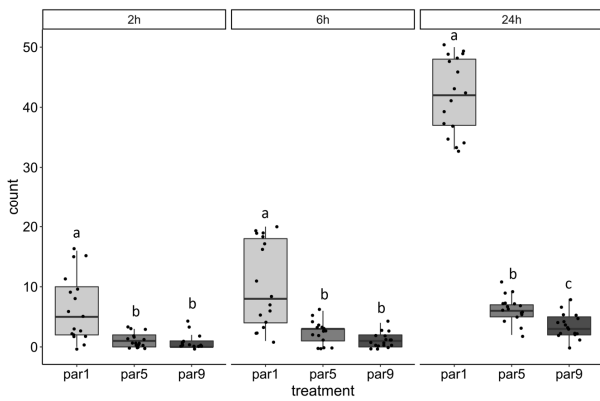
Figure 1. No-choice predation experiment with *M. basicornis* and *T. absoluta* eggs parasitized by *T. pretiosum*. Eggs were parasitized 1 (par1), 5 (par5) and 9-days (par9) before exposure. Cumulative average number of eggs consumed per predator - for each time point - after 2, 6, and 24 hours exposure to *M. basicornis*. Bold horizontal lines show medians, boxes contain the 25th-50th percentiles, whiskers show the upper and lower quartiles and points represent individual replicates. Different letters indicate statistically significant difference (P < 0.05, GLM).

Although *T. absoluta* egg development takes up to 4 days at 25 °C (Silva *et al.*, 2015), only unparasitized eggs of maximally 24 hours old were used in all tests. Hence, we tested whether older unparasitized *T. absoluta* eggs were equally attractive for mirid consumption by offering 25 maximally 24 hours eggs together with 25 3-4 day old eggs in a choice experiment on tomato leaflets. The numbers of eggs eaten was counted after 6 and 14 hours. The number of replicates was 17.

Prey of five different categories were offered to female *M. basicornis* predators: unparasitized 1-day old *T. absoluta* eggs, and *T. pretiosum* parasitized *T. absoluta* eggs of 1-, 2-, 5- and/or 9 days after being parasitized. The immature parasitoid is in the egg-larval stage in the 1- and 2-day old parasitized *T. absoluta* eggs, in the early pupal stage in 5-day old parasitized prey eggs, and in the late pupal stage in the 9-day old parasitized prey eggs at 25 ± 1 °C, 70 ± 5% RH and a photoperiod of 16:8 (L:D) (Consoli *et al.*, 1999). The eggs were placed with a fine paint brush on a tomato leaflet kept inside a small Petri dish (4.5 cm diameter). The petioles of the leaves were kept in a 2 ml Eppendorf tube with water to maintain the leaf in good condition during the 24 hours experimental period.

To obtain 1-day old parasitized eggs, *T. absoluta* eggs laid during the previous 24 hours were exposed to *T. pretiosum* parasitoids during 24 hours. After 24 hours the parasitoid adults were removed and the 24-48 hours old *T. absoluta* eggs, which thus contain parasitoid eggs of 0-24 hours, were offered to the predator *M. basicornis*. Reasoning in the same way, in the 2-day test, the parasitoids in the *T. absoluta* eggs were 24-48 hours old, in the 5-day test, the parasitoids were 96-120 hours old and in the 9-day test, the parasitoids were 192-216 hours old. In 1- and 2-day old parasitized eggs, it is not possible to see whether the *T. absoluta* eggs have all been parasitized. To determine what the average percentage parasitism of these eggs is, 18 batches of 100 eggs were exposed to *T. pretiosum* for 24 hours and the result of parasitism was determined after 5 days when the parasitized eggs had turned black. The average percentage parasitism of the 18 replicates was 91.00% (SE ± 1.43). Thus, in the experiments with 1- and 2-day old parasitized prey eggs, on average less than 10% of the eggs were unparasitized. In the tests with 5- and 9-day old parasitized eggs, the percentage parasitism was always 100%, as only dark coloured parasitized eggs were transferred to a tomato leaflet for exposure to the predator.

Predators were provided with unparasitized and/or parasitized prey eggs either in a no-choice or two-choice test in so-called black box predation experiments, i.e. we did not observe the predation behaviour. In the first experiment, we offered a total of 50 prey eggs. As the predator *M. basicornis* is able to consume on average 100 eggs per 24 hours (van Lenteren *et al.*, 2016), the limited number of eggs offered in the first experiment may have influenced the predation rate of parasitized hosts. Therefore, we offered a total of 100 prey eggs in the second experiment. Next, in the third experiment, we only provided 10 prey eggs to test if predation rates towards unparasitized and parasitized eggs changed under very limited provision of prey. Unparasitized and parasitized eggs of different ages were offered, and egg consumption was determined after various time intervals. The experiments are described in detail below.

Black box predation experiments
Experiment 1 - No-choice and choice predation experiment with unparasitized and 1-, 5-, and 9-day old parasitized eggs, 50 eggs offered

In the no-choice experiment, 50 unparasitized *T. absoluta* eggs or 50 eggs parasitized by *T. pretiosum* were distributed over a tomato leaflet randomly, and in the choice experiment, 25 unparasitized + 25 parasitized eggs were used. Parasitized eggs of three different ages - 1-, 5- or 9 day old at the start of the experiment - were tested. After 2, 6 and 24 hours exposure to the predator *M. basicornis*, eggs were counted and those being empty and with a ruptured chorion were considered consumed by the predator.

Experiment 2 - No-choice and choice predation experiment with unparasitized and 1-, 2-, and 5-day old parasitized eggs, 100 eggs offered

In the no-choice experiment, 100 unparasitized *T. absoluta* eggs or 100 eggs parasitized by *T. pretiosum* were distributed over a tomato leaflet randomly, and in the choice experiment, 50 unparasitized + 50 parasitized eggs were used. Parasitized prey eggs of 1-, 2- and 5-day old were used in this experiment. After the exposure period of 24 hours, the numbers of eggs eaten by the predator were determined.

Experiment 3 - Choice predation experiment with limited numbers of unparasitized and 1-, 5-, and 9-day old parasitized eggs, 10 eggs offered

In this third predation experiment, a very limited number of preys were offered. The number of prey eggs consumed was counted after 2, 6 and 24 hours. In total 10 eggs were offered, five of each type of prey.

Statistics

Generalised linear models (GLMs) with Poisson error distribution and log link function were used to analyse count response variables - i.e. numbers of egg consumed - fitting the treatments (unparasitized eggs, eggs parasitized at different time points) as categorical fixed factor. When over dispersion was detected, we corrected this by fitting a quasi-Poisson distribution in the models. Significance of the explanatory variables were tested with likelihood-ratio tests (LRTs) (Crawley, 2012). GLMs with gamma error distribution and reciprocal link function were used to analyse time-to-event data (i.e., time before the first feeding occurs), fitting the treatment (unparasitized eggs, eggs parasitized at different time points) as fixed factor. Significance of the explanatory variables was obtained with F- tests (Crawley, 2012).

If the GLMs detected significant differences amongst factor levels, we proceeded to pairwise comparisons to determine which differed using the *glht* function found in the *multcomp* package of the R software (Bretz *et al.*, 2010). Model fit was assessed with residual plots. All statistical analyses were performed using R statistical software version 3.6.2 (R-Core-Team, 2018).

The raw data of all experiments are provided in the supplemental material.

Results

Consumption of young and old *T. absoluta* eggs by *M. basicornis*

In the black box predation experiments, we only provided unparasitized *T. absoluta* eggs of maximally 24 hours. To be sure that older, unparasitized are not of inferior quality, we performed a choice test offering young and old unparasitized prey eggs. *T. absoluta* eggs of 3-4 days old were consumed as frequently as eggs of

maximally 1-day old. The average numbers of eggs consumed after 6 hours were 9.88 and 10.53, for 1-day old and 3-4 day old eggs respectively, and after 24 hours these numbers were 23.41 and 23.71. No statistical differences were found regardless of the time point (GLM 2 hours: z = 0.59, n = 15, P = 0.555; 6 hours: z = 0.28, n = 16, P = 0.778; 24 hours: z = 0.17, n = 16, P = 0.860).
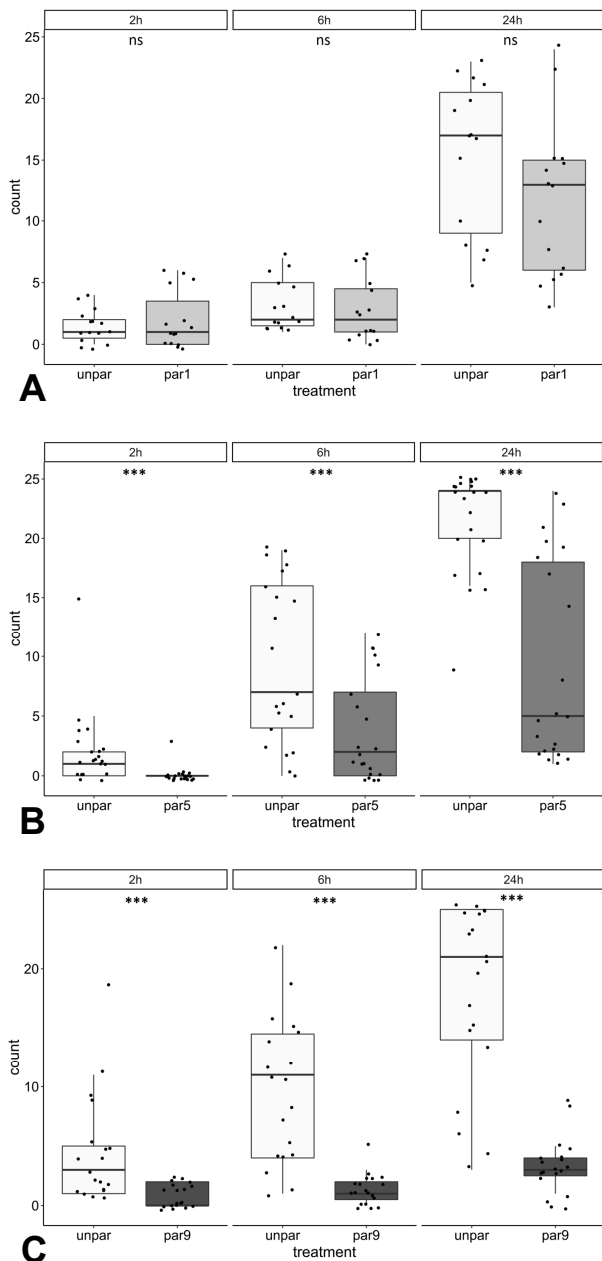
Black box predation experiments

Experiment 1 - No-choice and choice predation experiment with unparasitized and 1-, 5-, and 9-day old parasitized eggs, 50 eggs offered

In the no-choice test, a significant effect of the treatment was found on the numbers of eggs consumed regardless of the time point (GLM 2 hours: χ^2 = 120.03, df = 2, P < 0.001; 6 hours: χ^2 = 176.35, df = 2, P < 0.001; 24 hours: χ^2 = 863.47, df = 2, P < 0.001). After 2 hours, the numbers of 1-day old parasitized eggs eaten was significantly higher than 5-day and 9-day old parasitized eggs, whereas no differences were found between 5- and 9-day old parasitized *T. absoluta* eggs (figure 1). The same effect was found when predators were exposed for 6 hours, while consumption levels differ significantly among all three categories after 24 hours of exposition (figure 1). The average number of eggs preyed per individual per 24 hours when 50 prey eggs were offered was 41.9, 6.3, 3.5 for 1-, 5- or 9-day old parasitized *T. absoluta* eggs, respectively. These results show that the number of parasitized eggs eaten is decreasing with the interval between parasitism and exposure to the predator. Further, the percentage of predators not eating during the first two hours is strongly increasing with the age of the parasitized eggs offered (table 1). The effect is less strong, but still present in the second interval, and almost disappeared during the third interval. The percentage of all available prey eggs eaten is highest in the experiment where 1-day old parasitized eggs were offered, is considerably lower when 5-day old parasitized eggs were given, and is lowest when 9-day old parasitized eggs were presented (table 1).

The results of the choice test show no significant differences, in terms of numbers of eggs consumed, between unparasitized *T. absoluta* eggs and 1-day old parasitized eggs only at 24 hours but not at 2 hours and 6 hours (GLM 2 hours: z = 0.96, n = 11, P = 0.338; 6 hours: z = 0.53,

Table 1. No-choice predation experiment with *M. basicornis* and *T. absoluta* eggs parasitized by *T. pretiosum.* Number/percentage of predators not preying and percentage of total number of offered prey eggs consumed; 50 prey eggs were offered to the predators; *T. absoluta* eggs were parasitized 1 (par1), 5 (par5) and 9-days (par9) before exposure.

	Predation period			
Prey	0-2 hours	0-6 hours	0-24 hours	N
	Number / percentage of predators not preying			
par1	2 /12	0 / 0	0 / 0	17
par5	7 / 41	5 / 29	0 / 0	17
par9	11 / 65	6 / 35	1 / 6	17
	Percentage of total number of offered prey eggs consumed			
par1	13	21	84	17
par5	2	5	13	17
par9	1	2	7	17

Figure 2. Choice predation experiment with *M. basicornis* and unparasitized *T. absoluta* eggs or eggs parasitized by *T. pretiosum.* Eggs were parasitized 1 (par1) (**A**), 5 (par5) (**B**) and 9-days (par9) (**C**) before exposure. Cumulative average number of eggs consumed per predator - for each time point - after 2, 6, and 24 hours exposure to *M. basicornis*. Bold horizontal lines show medians, boxes contain the 25th-50th percentiles, whiskers show the upper and lower quartiles and points represent individual replicates (*** P < 0.001, ns = not significant).

n = 14, $P = 0.596$; 24 hours: z = 2.82, n = 14, $P = 0.005$). Overall, results showed that parasitized eggs are increasingly less consumed with an increasing interval after parasitism (figure 2). Significant effects were found in the combination of unparasitized with 5-day old parasitized eggs at 2 hours (z = 4.54, n = 14, $P < 0.001$), 6 hours (z = 6.91, n = 18, $P < 0.001$) and 24 hours (z = 9.72, n = 20,

$P < 0.001$) (figure 2). A similar effect was observed when comparing unparasitized versus 9-day old parasitized eggs regardless of the time point (2 hours: z = 6.24, n = 18, $P < 0.001$; 6 hours: z = 9.34, n = 18, $P < 0.001$; 24 hours: z = 12.23, n = 18, $P < 0.001$) (figure 2).

The numbers of unparasitized eggs that were consumed across the three choice combinations was significantly different after 24 hours ($\chi^2 = 17.97$, df = 2, $P = 0.015$). Also, the numbers of eggs consumed differed significantly among 1-day, 5-day and 9-day old parasitized eggs ($\chi^2 = 90.929$, df = 2, $P < 0.001$), as the predator consumed fewer 9-day old eggs compared with the two other treatments.

The percentage of unparasitized eggs consumed after 2, 6 and 24 hours is increasing over time and highest in the choice tests combined with 5- and 9-day old parasitized eggs (table 2). The percentage of 1-day old parasitized eggs consumed after 2, 6 and 24 hours shows a similar trend as that of the unparasitized eggs, but the increase is slower with 5-day old parasitized eggs and still slower with 9-day old parasitized eggs (table 2).
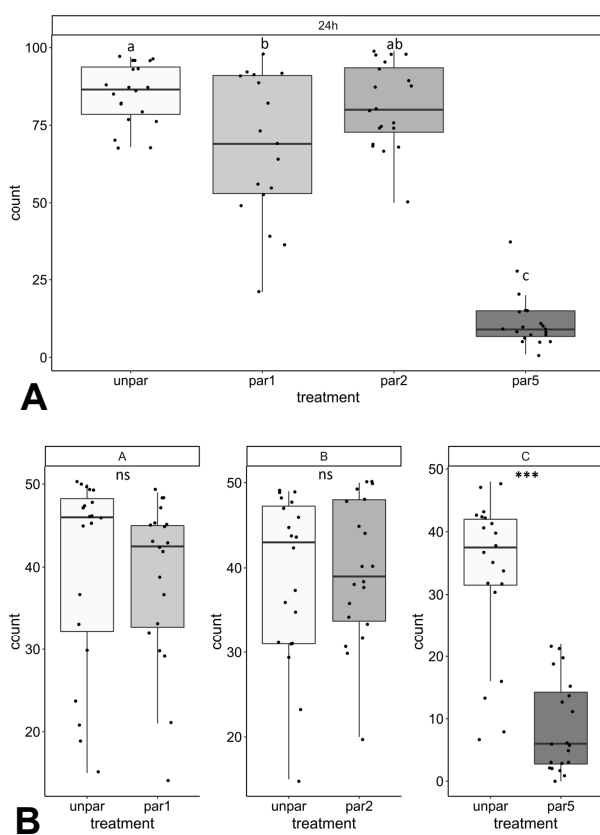
Experiment 2 - No-choice and choice predation experiment with unparasitized and 1-, 2-, and 5-day old parasitized eggs, 100 eggs offered

In the second experiment with no-choice and choice tests, the results are in line with those of the first experiment. However, the total numbers of unparasitized and 1-day old parasitized prey eggs consumed are significantly higher in this experiment, where 100 instead of 50 eggs were offered in the no-choice test, and 50 + 50 eggs instead of 25 + 25 eggs in the choice test. Here, in the no-choice test, on average 85 unparasitized and 68 1-day old parasitized were consumed (figure 3A), while in the first experiment on average 42 1-day old parasitized were consumed (no data for predation of unparasitized eggs for the no-choice situation were collected in the first experiment). In the current choice test, on average 47 eggs of the unparasitized and on average 43 eggs of the 1-day old parasitized eggs were consumed (figure 3B), whereas in the first experiment these numbers were 15 for unparasitized and 12 for 1-day old parasitized eggs. This indicates that the numbers of eggs offered in the first experiment with no-choice and choice tests (in total 50 eggs) were insufficient to meet the maximum predation capacity of *M. basicornis*.

In no-choice conditions, the parasitism status of the prey significantly affected the numbers of eggs consumed after 24 hours ($\chi^2 = 1512.6$, df = 3, $P < 0.001$) with 5-day old parasitized eggs being consumed significantly less than the other three categories. When the numbers of unparasitized and parasitized prey in the choice tests are considered, we found that the numbers of eggs consumed are not different between unparasitized and 1-day old parasitized eggs (z = 0.51, n = 19, $P = 0.614$) as well as between unparasitized and 2-day old parasitized eggs (z = 0.38, n = 19, $P = 0.705$) (figure 3B). Yet the predator consumed significantly more unparasitized eggs when offered together with 5-day old parasitized eggs (z = 15.83, n = 19, $P < 0.001$). The total number of eggs consumed (i.e. "parasitized + unparasitized eggs" within a

Table 2. Two-choice predation experiment with *M. basicornis* and *T. absoluta* eggs either unparasitized (unpar) or parasitized (par) by *T. pretiosum*. Percentage of total number of prey eggs consumed. Eggs were parasitized 1, 5 and 9-days before exposure; 25 unparasitized and 25 parasitized eggs were simultaneously offered to the predators.

| Prey	Predation period			
	0-2 hours	0-6 hours	0-24 hours	N
	Percentage of total number of offered prey eggs consumed			
unpar	6	13	62	15
par1	6	11	47	15
unpar	9	38	86	21
par5	1	15	37	21
unpar	18	29	71	19
par9	3	2	13	19

Figure 3. Predation experiment with *M. basicornis* and *T. absoluta* eggs either unparasitized (unpar) or parasitized (par) by *T. pretiosum*. (**A**) No-choice bioassays, average number of eggs consumed per predator after 24 hours with 100 eggs offered to *M. basicornis*. Different letters indicate statistically significant difference (P < 0.05, GLM). (**B**) Two-choice bioassays, average number of eggs consumed after 24 hours for the three combinations tested (*** P < 0.001, ns = not significant). Eggs were parasitized 1 (par1), 2 (par2), and 5-days (par5) before exposure. Bold horizontal lines show medians, boxes contain the 25th-50th percentiles, whiskers show the upper and lower quartiles and points represent individual replicates.

choice) differed among the treatments ($\chi^2 = 289.41$, df = 2, $P < 0.001$) with significantly lower numbers of eggs consumed in the combination unparasitized + 5-day old parasitized eggs. The number of unparasitized eggs that were consumed across the three choice combinations was not significantly different ($\chi^2 = 13.06$, df = 2, $P = 0.159$). However, the number of parasitized eggs consumed differed significantly ($\chi^2 = 524.13$, df = 2, $P < 0.001$), as the predator consumed fewer 5-day old eggs compared with 1-day and 2-day old parasitized eggs.

Experiment 3 - Choice predation experiment with limited numbers of unparasitized and 1-, 5-, and 9-day old parasitized eggs, 10 eggs offered

In the third experiment a very limited number of prey eggs was offered. The trends in the numbers and percentages consumption of the different types of prey is similar as in the previous experiments: unparasitized and 1-day old parasitized eggs are consumed well, 5-day old ones less and 9-day old ones are least eaten (figure 4). When considering the combination between unparasitized and 1-day old parasitized eggs, an effect of the exposition time was found: in fact, significant differences were observed only when predators were allowed to feed for 6 hours ($z = 1.999$, n = 34, $P = 0.045$) and 24 hours ($z = 2.67$, n = 35, $P = 0.011$) but not for 2 hours ($z = 1.63$, n = 22, $P = 0.101$) (figure 4). In all the combinations between unparasitized and 5-day old parasitized eggs, a significant effect was found regardless of the time point (2 hours: z= 4.50, n = 13, $P < 0.001$; 6 hours: z =5.91, n = 24, $P < 0.001$; 24 hours: z = 5.93, n = 27, $P < 0.001$) (figure 4). Similar results were also found for comparisons between unparasitized and 9-day old parasitized eggs (2 hours: z = 6.24, n = 18, $P < 0.001$; 6 hours: z = 9.34, n = 18, $P < 0.001$; 24 hours: z = 12.23, n = 18, $P < 0.001$) (figure 4). The total number of eggs consumed (i.e. "parasitized + unparasitized eggs" within a choice) differed among all the treatments ($\chi^2 = 30.54$, df = 2, $P < 0.001$). During the first experiment, predators started to feed later when exposed to 5- and 9-day old parasitized egg in the no-choice test (table 1). In this experiment we observed the same effect even in a choice test (table 3): about 50% of the predators did not feed during the first two hours, and even during first 6 hours 10-30% of the predators did not consume eggs.
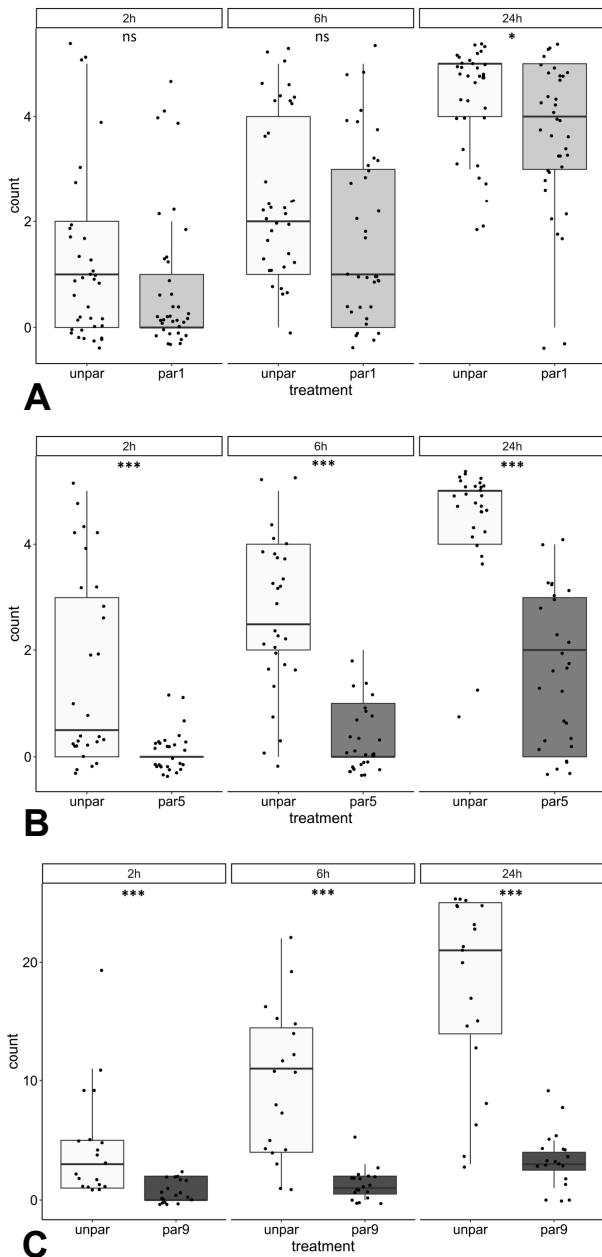
Figure 4. Average cumulative number of eggs consumed per predator after 2, 6 and 24 hours exposure to *M. basicornis* in choice experiment with 5 unpar and 5 par eggs offered. Eggs were parasitized 1, 5 and 9-days before exposure. (**A**) unpar + par1 day; (**B**) unpar + par5 day; (**C**) unpar + par9 day. Bold horizontal lines show medians, boxes contain the 25th-50th percentiles, whiskers show the upper and lower quartiles and points represent individual replicates (* P < 0.05, *** P < 0.001, ns = not significant).

Discussion and conclusions

The results of the black box predation experiments showed that, both in no-choice and choice tests:
- 1- or 2-day old parasitized eggs are consumed at the same rate as unparasitized eggs,
- 5- or 9-day old parasitized eggs are consumed at a much lower rate than unparasitized or 1- and 2-day old parasitized eggs,

- predators start to feed significantly later when provided with 5- and 9-day old parasitized eggs than when given unparasitized or 1-day old parasitized eggs.

These results support our hypothesis saying that prey eggs containing a parasitoid egg or larva are easily consumed by the predator, but not the older eggs with the pre-pupal or pupal stage of the parasitoid. Interestingly, the results were similar in no-choice and choice tests, and in experiments with a large (100/24 hours), medium (50/24 hours) and a very low (10/24 hours) number of preys offered (50-100 eggs per 24 hours), which demonstrates a strong effect of CE. The predator *M. basicornis* is able to consume an average of 101 prey eggs per 24 hours (van Lenteren *et al.*, 2016). Thus, in experiment 1, where 50 eggs were offered, and in experiment 3 with 10 eggs, the amount of food provided was far below the predation capacity of the predator. Still, in these experiments the number of old, parasitized eggs consumed was less than 10 out of 50 (1st experiment) and less than 3 out of 10 (3rd experiment) and similar to the numbers consumed in the 2nd experiment where ample preys were provided. All this information demonstrates that the low consumption rates of old, parasitized prey eggs are very consistent and suggests that (1) these old eggs can no longer be penetrated by the mouth parts of the predator, (2) these eggs are rejected for feeding after having been penetrated, or (3) the predator rejects the old parasitized eggs before attempting to feed.

The currently held opinion about prey searching behaviour of mirid predators is that they do not search by vision or smell, but encounter prey randomly (Wheeler, 2001) and reject old parasitized eggs after contact because they can no longer be penetrated by the stylets of the predators. Eggs of many Lepidoptera parasitized by *Trichogramma* spp. become dark due to the deposition of melanin to the inner surface of the host egg chorion at the end of the larval stages and the start of prepupa formation of the parasitoid (Clausen, 1940; Metcalfe and Breniere, 1969; Alrouechdi and Voegelé, 1981; Knutson, 1998; Pintureau *et al.*, 1999). In general, melanin protects the insect egg against desiccation, UV light and natural enemies (Pintureau *et al.*, 1999; Hilker *et al.*, 2023). No detailed data are available for development of *T. pretiosum* in *T. absoluta*, but the total immature development time at 25 °C of 10.3 days in *T. absoluta* (Pratissoli and Parra, 2000) is very similar to that of 10.4 days in *E. kuehniella* (Consoli *et al.*, 1999). So, in our experiments, *M. basicornis* was exposed to *T. absoluta* eggs with the parasitoid in the egg-larval stage (1- and 2-day old parasitized prey eggs) and to the early and late pupal stages of the parasitoid in the prey egg (5- and 9-day old parasitized eggs). In behavioural-observation studies conducted simultaneously with these predation experiments, we saw that *M. basicornis* can penetrate old parasitized eggs, and that these eggs - in the rare cases that they are contacted - are accepted for consumption at the same rate as unparasitized eggs (van Lenteren *et al.*, 2023). Thus, suppositions 1 (predator cannot penetrate old eggs) and 2 (eggs are rejected for consumption after being penetrated) are not correct for *M. basicornis*.

Is there any evidence supporting supposition 3 (i.e. the predator rejects old parasitized eggs before attempting to

Table 3. Two-choice predation experiment with *M. basicornis* and *T. absoluta* eggs either unparasitized (unpar) or parasitized (par) by *T. pretiosum*. Percentage of total number of offered prey eggs consumed and number/percentage of predators not preying. Eggs were parasitized 1, 5 and 5-days before exposure, 5 unpar + 5 par eggs were offered to the predators.

Prey	0-2 hours	0-6 hours	0-24 hours	N
		Predation period		
	Percentage of total number of offered prey eggs consumed			
unpar	24	50	86	36
par1	16	36	72	36
unpar	16	52	90	28
par5	1	8	32	28
unpar	16	43	75	33
par9	8	15	23	33
	Number and percentage of predators not preying			
Unpar + par1	12/33.3	1/2.8	0/0	36
Unpar + par5	4/50	3/10.7	0/0	28
Unpar + par9	16/48.9	8/30.3	0/0	33

feed)? Although old parasitized eggs can be penetrated and are consumed by the predator, the numbers of old parasitized eggs consumed is much lower than that of young parasitized eggs both in choice and no-choice experiments in the black box experiments presented in this paper. Behavioural-observation experiments showed that old parasitized eggs are encountered significantly less frequent than young parasitized and unparasitized eggs (van Lenteren *et al*., 2023), suggesting that predators avoid to contact these old eggs. Interestingly, during the first experiment described in this paper, predators started to feed later when exposed to 5- and 9-day old parasitized eggs in the no-choice test than when exposed to 1-day old parasitized eggs (table 1). In the third experiment we observed the same effect even in a choice test (table 3): about 50% of the predators did not feed during the first two hours, and even during first 6 hours 10-30% of the predators did not consume eggs. These observations made us speculate that old, parasitized eggs are not only contacted less frequently than young eggs, but in some way reduce the foraging activity of *M. basicornis*. Results of olfactometer experiments revealed that *M. basicornis* prefers volatiles of tomato leaflets with unparasitized or 1-day old parasitized eggs over volatiles of 5-day old parasitized eggs (van Lenteren *et al*., 2023). Apparently, the predator uses volatile information produced by the combination 'old parasitized eggs-tomato leaflet' to avoid contacting these old eggs, and that these volatiles also reduce the foraging activity of the predator. The results of the black box predation experiments presented in this paper and those of the olfactometer and behavioural-observation experiments shown in van Lenteren *et al.,* (2023) indicate that the current belief that mirids search unsystematically, discover and reject prey only after having physically encountered them (Wheeler, 2001) has to be modified for *M. basicornis*, as this predator does not search randomly and often rejects the old parasitized eggs before contacting them.

Other heteropteran predators also do not distinguish between unparasitized eggs and eggs containing parasitoids early in their development, but reject old parasitized eggs

(Rosenheim and Harmon, 2006). *Macrolophus pygmaeus* Rambur (Hemiptera Miridae) preferentially preys on unparasitized or recently *T. absoluta* eggs parasitized by *T. achaeae* when the eggs are still yellow, but hardly preys on black parasitized eggs (Chailleux *et al*., 2013). The mirid *N. tenuis* consumed significantly more unparasitized eggs than eggs parasitized by *T. achaeae*, and significantly more < 4-day old parasitized eggs than > 4-day old parasitized eggs (Cabello *et al*., 2015).

Concerning the issue whether IGP and/or CE occur when both natural enemies are released for control of *T. absoluta*, we might conclude the following:
- when both natural enemies are released at the same day, large amounts of eggs parasitized by *T. pretiosum* will be consumed by the predator *M. basicornis* during the following days and IGP will be strong;
- when the predator is first released and the parasitoid later, the same will happen and IGP will be strong;
- when parasitoids are first released and the predators after five or more days, old, parasitized eggs will not be consumed at high rates, so in this situation, CE plays an important role. However, this reasoning will only hold at the start of the tomato production season, because the predator will remain permanently in the crop and will consume young, parasitized prey eggs.

The practical implications of our results with regard of using these natural enemies for control of *T. absoluta* in tomatoes in the field or greenhouse need to be tested under realistic tomato production conditions. We expect that combined use of both natural enemies at low pest densities will not result in better control of the pest and the role of the egg parasitoid will be very limited due to IGP. At high densities, combined releases may result in better control at the start of the tomato season and when the parasitoids are introduced a week before the predators, so old parasitized eggs escape predation as the effect of CE. However, later in the season the parasitoids are not expected to add to control, because the predators will remain permanently in the crop and will consume the young, parasitized prey eggs.

Acknowledgements

References

ALROUECHDI K., VOEGELE J., 1981.- Predation des Trichogrammes par les Chrysopides.- *Agronomie*, 1: 187-189.

BIONDI A., GUEDES R. N. C., WAN F. H., DESNEUX N., 2018.- Ecology, worldwide spread, and management of the invasive South American tomato pinworm, *Tuta absoluta*: past, present, and future.- *Annual Review of Entomology*, 63: 239-258.

BRETZ F., HOTHORN T., WESTFALL P. H., 2010.- *Multiple comparisons using R*.- Chapman and Hall/CRC Press, Boca Raton, USA.

BUENO V. H. P., VAN LENTEREN J. C., LINS JR J. C., CALIXTO A. M., MONTES F. C., SILVA D. B., SANTIAGO L. D., PÉREZ L. M., 2013.- New records of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) predation by Brazilian Hemipteran predatory bugs.- *Journal of Applied Entomology*, 137: 29-34.

BUENO V. H. P., MONTES F. C., SAMPAIO M. V., CALIXTO A. M., VAN LENTEREN J. C., 2018.- Performance of immatures of three Neotropical Miridae at five temperatures when reared on *Ephestia kuehniella* eggs on tobacco plants.- *Bulletin of Insectology*, 71: 77-87.

BUENO V. H. P., MONTES F. C., COSTAZ T., DE JONG P. W., PARRA J. R. P., VAN LENTEREN J. C., 2023.- Do releases of the mirid predator *Macrolophus basicornis* (Hemiptera: Miridae) together with the egg parasitoid *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) improve biological control of *Tuta absoluta* (Lepidoptera: Gelechidae) in tomato?- *Journal of Economic Entomology*, 116: 733-741.

CABELLO T., BONFIL F., GALLEGO J. R., FERNANDEZ F. J., GAMEZ M., GARAY J., 2015.- Can interactions between an omnivorous hemipteran and an egg parasitoid limit the level of biological control for the tomato pinworm?- *Environmental Entomology*, 44 (1): 12-26.

CALVO F. J., LORENTE M. J., STANSLY P. A., BELDA J. E., 2012.- Preplant release of *Nesidiocoris tenuis* and supplementary tactics for control of *Tuta absoluta* and *Bemisa tabaci* in greenhouse tomato.- *Entomologia Experimentalis et Applicata*, 143 (2): 111-119.

CHAILLEUX A., BIONDI A., HAN P., TABONE E., DESNEUX N., 2013.- Suitability of the pest-plant system *Tuta absoluta* (Lepidoptera: Gelechiidae)-tomato for *Trichogramma* (Hymenoptera: Trichogrammatidae) parasitoids and insights for biological control.- *Journal of Economic Entomology*, 106 (6): 2310-2321.

CLAUSEN C. P., 1940.- *Entomophagous insects*.- McGraw-Hill, New York, USA.

CONSOLI F. L., ROSSI M. M., PARRA J. R. P., 1999.- Developmental time and characteristics of the immature stages of *Trichogramma galloi* and *T. pretiosum* (Hymenoptera, Trichogrammatidae).- *Revista Brasileira de Entomologia*, 45: 271-275.

CRAWLEY M. J., 2012.- Generalized linear models, pp. 557-578. In: *The R book* (CRAWLEY M.J., Ed.).- John Wiley & Sons, New Jersey, USA.

EARLY R., RWOMUSHANA I., CHIPABIKA G., DAY R., 2022.- Comparing, evaluating and combining statistical species distribution models and CLIMEX to forecast the distributions of emerging crop pests.- *Pest Management Science*, 78: 671-683.

FERRACINI C., BUENO V. H. P., DINDO M. L., INGEGNO B. L., LUNA M. G., SALAS GERVASSIO N. G., SANCHEZ N. E., SISCARO G., VAN LENTEREN J. C., ZAPPALÀ L., TAVELLA L., 2019.- Natural enemies of *Tuta absoluta* in the Mediterranean basin, Europe and South America.- *Biocontrol Science and Technology*, 29: 578-609.

GUEDES R. N. C., PICANÇO C. M., 2012.- The tomato borer *Tuta absoluta* in South America: pest status, management and insecticide resistance.- *Bulletin OEPP/EPPO*, 42: 211-216.

GUVEN B., KILIC T., MIHCI B., SAHIN C., UYSAL D., 2017.- Investigations on the biological control of *Tuta absoluta* (Lep.: Gelechiidae) in the Aegean region of Turkey.- *Biological Control Society of Turkey*, 8: 59-70.

HILKER M., SALEM H., FATOUROS N. E., 2023.- Adaptive plasticity of insect eggs.- *Annual Review of Entomology*, 68: 451-469.

KNUTSON A., 1998.- *The Trichogramma manual: a guide to the use of Trichogramma for biological control with special reference to augmentative releases for control of bollworm and budworm in cotton*.- Texas Agricultural Extension Service, Texas A&M University, Texas, USA.

METCALFE J. R., BRENIERE J., 1969.- Egg parasites (*Trichogramma* spp.) for control of sugar cane moth borers, pp. 91-115. In: *Pests of sugar cane* (WILLIAMS J. R. M., MATHES R., Eds).- Elsevier, Amsterdam, Netherlands.

MIRHOSSEINI M. A., FATHIPOUR Y., HOLST N., SOUFBAF M., MICHAUD J. P., 2019.- An egg parasitoid interferes with biological control of tomato leafminer by augmentation of *Nesidiocoris tenuis* (Hemiptera: Miridae).- *Biological Control*, 133: 34-40.

PARRA J. R. P., 1997.- Técnicas de criação de *Anagasta kuehniella*, hospedeiro alternativo para produçaõ de *Trichogramma*, pp. 121-150. In: *Trichogramma e o controle biológico aplicado* (PARRA J. R. P., ZUCCHI R., Eds).- FEALQ, Piracicaba, Brazil.

PINTUREAU B., PETINON S., NARDON C., 1999.- Rôle possible des substances excrétées par les Trichogrammes et assombrissant leur hôtes.- *Bulletin de la Société Zoologique de France*, 124: 261-269.

PRATISSOLI D., PARRA J. R. P., 2001.- Seleção de linhagens de *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) para o controle das traças *Tuta absoluta* (Meyrick) e *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae).- *Neotropical Entomology*, 30: 277-282.

R-CORE-TEAM., 2018.- *R: a language and environment for statistical computing*.- R Foundation for Statistical Computing, Vienna, Austria.

ROSENHEIM J. A., HARMON J. P., 2006.- The influence of intraguild predation on the suppression of a shared prey population: an empirical reassessment, pp. 1-20. In: *Trophic and guild interactions in biological control* (BRODEUR J., BOIVIN G., Eds).- Springer, Dordrecht, The Netherlands.

RUBERSON J. R., KRING J. T., 1991.- Predation of *Trichogramma pretiosum* by the anthocorid *Orius insidiosus*, pp. 41-43. In: *Trichogramma and other egg parasitoids, Les Colloques de l'INRA*, 56, Paris, France.

SILVA D. B., BUENO V. H. P., LINS JR J. C., VAN LENTEREN J. C., 2015.- Life history data and population growth of *Tuta absoluta* at constant and alternating temperatures on two tomato lines.- *Bulletin of Insectology*, 68: 223-232.

TILMAN D., 1982.- *Resources competition and community structure*.- Princeton University Press, Princeton, USA.

VAN LENTEREN J. C., HEMERIK L, LINS JR J. C., BUENO V. H. P., 2016.- Functional responses of three Neotropical mirid predators to eggs of *Tuta absoluta* on tomato.- *Insects*, 7 (3): 34.

VAN LENTEREN J. C., BUENO V. H. P., SMIT J., SOARES M. A., CALIXTO A. M., MONTES F. C., DE JONG P., 2017.- Predation of *Tuta absoluta* eggs during the nymphal stages of three Neotropical mirid predators on tomato.- *Bulletin of Insectology*, 70: 69-74.

VAN LENTEREN J. C., BUENO V. H. P., MONTES F. C., HEMERIK L., DE JONG P. W., 2018.- Adult lifetime predation of *Tuta absoluta* eggs by three Neotropical mirid predators on tomato.- *Bulletin of Insectology*, 71 (2): 179-188.

VAN LENTEREN J. C., LANZONI A., HEMERIK L., BUENO V. H. P., BAJONERO-CUERVO J. G., BIONDI A., BURGIO G., CALVO F. J., DE JONG P. W., LÓPEZ S. N., LUNA M. G., MONTES F. C., NIEVES E. L., AIGBEDION-ATALOR P. O., RIQUELME VIRGALA M. B., SÁNCHEZ N. E., URBANEJA A., 2021.- The pest kill rate of thirteen natural enemies as aggregate evaluation criterion of their biological control potential of *Tuta absoluta*.- *Scientific Reports*, 11 (1): 10756.

VAN LENTEREN J. C, BUENO V. H. P., SANTOS-SILVA M., MONTES F. C., CUSUMANO A., FATOUROS N. E., 2023.- The Neotropical mirid predator *Macrolophus basicornis* u ss volatile cues to avoid contacting old, *Trichogramma pretiosum* parasitized eggs of *Tuta absoluta*.- *Bulletin of Insectology*, 76 (2): 229-237.

VARSHNEY R., BALLAL C. R., 2018.- Intraguild predation on *Trichogramma chilonis* Ishii (Hymenoptera: Trichogrammatidae) by the generalist predator *Geocoris ochropterus* Fieber (Hemiptera: Geocoridae).- *Egyptian Journal of Biological Pest Control*, 28: 5.

WHEELER A. G. 2001.- *Biology of the plant bugs (Hemiptera: Miridae): pests, predators, opportunists*.- Cornell University Press, Ithaca, NY, USA.

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