Acta Scientiarum
http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v39i4.34943

# Age and density of eggs of *Helicoverpa armigera* influence on *Trichogramma pretiosum* parasitism

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**ABSTRACT.** This study evaluated the parasitism of *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) on eggs of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) at different ages and densities. The rates of parasitism and emergence, the number of parasitoids emerged per egg and sex ratio of offspring were evaluated in both experiments. Eggs of *H. armigera* up to 36 hours provided greater parasitism and emergence of adults compared to eggs up to 60 hours old. The number of parasitoids, which emerged per host egg, was greater than one and the sex ratio remained around 80% of females, regardless of the egg development stage. Females of *T. pretiosum* responded with superior rates of parasitism, emergence and number of parasitoids per egg at the densities of 20 and 25 eggs of *H. armigera*. These results indicate that *T. pretiosum* parasite with superior performance in eggs of up to 36h of age and densities of 20 eggs per female day$^{-1}$ in laboratory conditions. These results will help to establish the intervals between releases of parasitoids, aiming to control this pest, when adjusted with knowledge of the survival of the parasitoids in the field and in pest infestation.

**Keywords:** biological control, exotic pest, egg parasitoid, host-parasitoid interaction, noctuidae.

## Influência da idade e densidade de ovos de *Helicoverpa armigera* no parasitismo de *Trichogramma pretiosum*

**RESUMO.** Este trabalho avaliou o parasitismo de *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) em ovos de *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) em diferentes idades e densidades. As taxas de parasitismo e emergência, número de parasitoides emergidos por ovo e razão sexual dos descendentes foram avaliados em ambos os experimentos. Ovos de *H. armigera* de até 36 horas proporcionaram maior parasitismo e emergência dos adultos em comparação com ovos com até 60 horas de idade. O número de parasitoides, que emergiram por ovo hospedeiro, foi superior a 1 e a razão sexual permaneceu em torno de 80% de fêmeas, independentemente do estágio de desenvolvimento do ovo. Fêmeas de *T. pretiosum* responderam com taxas superiores de parasitismo, emergência e número de parasitoides por ovo nas densidades de 20 e 25 ovos de *H. armigera*. Estes resultados indicam que *T. pretiosum* parasita com desempenho superior em ovos de até 36h de idade e densidades de 20 ovos por fêmea dia$^{-1}$ em condições laboratoriais. Estes resultados ajudarão a estabelecer os intervalos entre as liberações de parasitoides, visando o controle desta praga, quando ajustado com o conhecimento da sobrevivência dos parasitoides no campo e na infestação de pragas.

**Palavras-chave:** controle biológico, praga exótica, parasitoide de ovo, interação hospedeiro-parasitoide, noctuidae.

## Introduction

Recently, the cotton bollworm Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae), which is a cosmopolite and polyphagous pest, has been introduced in Brazil (Czepak, Albernaz, Vivan, Guimarães, & Carvalhais, 2013; Specht, Sosa-Gómez, Paula-Moraes, & Yano, 2013; Gómez et al., 2016). Since then, it has been causing losses in the production of several crops including cotton, corn, soybean, and tomatoes (Bueno, Yamamoto, Carvalho, & Bueno, 2014; Czepak et al., 2013; Mastrangelo et al., 2014; Pratissoli, Lima, Pirovani, & Lima, 2015; Tay et al., 2013).

The main injuries are the defoliation and the attack on the reproductive structure by larvae. In addition, the mechanical damage in the fruit increases the occurrence of pathogens (fungi and bacteria) that can proliferate and deteriorate the quality of the fruit (Kakimoto, Fujisaki, & Miyatake, 2003). In earlier instars, the larvae preferably feed on leaves, remaining exposed to agent controls in this phase (Figueiredo et al., 2006). On the other hand, in later instars, they attack the reproductive structure of plants, which can be located in the lower parts protected by the canopy, or even sheltered inside the fruits becoming partially protected.

Considering the recent occurrence of the pest in Brazil, management practices adapted to the Brazilian agricultural are still scarce, mainly because there is a diversity of host crops in multiple cycles coupled with a favorable climate. There are no insecticides registered for *H. armigera* in Brazil (Vivan, Torres, & Fernandes, 2017). However, strategies of biological control have been reported in Brazil as promising options for control of the cotton bollworm, such as: strains of virus HzSNPV (Ardisson-Araújo, Sosa-Gómez, Melo, Báo, & Ribeiro, 2015), Cry toxins and entomopathogenic fungi (*Metarhizium anisopliae*, *Metarhizium rileyi* and *Nomuraea rileyi*) (Costa et al., 2015; Vivan, Torres, & Fernandes, 2017), larval parasitoid (Guerra, Guerra, Ribas, Gonçalves, & Mastrangelo, 2014), and egg parasitoid (Carvalho et al., 2017; Zuim, Rodrigues, Pratissoli, & Torres, 2017).

Egg parasitoids are an important strategy for Integrated Pest Management (IPM) because they promote the reduction of pest population before it can damage the crops (Bale, Lenteren, & Bigler 2008). In addition, this technology is used in pest control in other countries with the same occurrence of the pest, which in few years proved to be satisfactory (Öztemiz, 2008; Arain et al., 2014; Wang, He, Zhang, Lu, & Babendreier, 2014). Among the parasitoids, the *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) is the most studied and used throughout the world for its easiness and efficiency of propagation in the laboratory for later release in the field, especially in the control of the Lepidoptera pests (Davies, Pufke, & Zalucki, 2009; Davies & Zalucki, 2008). However, the biological changes of the pest may result in different answers regarding the use of the parasitoid.

The performance of a parasitoid in IPM depends in part on its host selection behaviors. Especially, host-egg ages can affect host selection by the parasitoid. In the natural environment, host-searching female *Trichogramma* can encounter host eggs at different developmental stages, from freshly laid eggs to eggs close to hatching, containing fully developed neonates. Usually, freshly deposited eggs are preferred because old eggs adversely affect the parasitism rate of parasitoid (Rocha, Kolberg, Mendonça Jr., & Redaelli, 2006). However, the numbers of parasitized host-egg by parasitoid tends to decrease with the increasing age of the host (Pizzol, Desneux, Wajnberg, & Thiéry, 2012). The parasitism efficiency can also be strongly influenced by the density of target species (Kalyebi et al., 2005). Understanding the relationship between host density and parasitism rate would help predict parasitoid potential for pest control.

Therefore, it is fundamental to know the relationship suitable between number of parasitoids to be released in relation to the density of host eggs presented in the agroecosystem, and the host-egg age more favorable to parasitism, in order to determine the release intervals of the parasitoid in the field (Ko et al., 2014; Polanczyk, Pratissoli, Holtz, Pereira, & Furtado, 2007). Thus, the aim of this study was to evaluate the effects of *H. armigera* egg age and density on biological characteristics of *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae).

## Material and methods

### I - Obtainment and rearing of *Helicoverpa armigera*

The collection of *H. armigera* larvae had been done in tomato crops in the city of Alegre (latitude -20.760258, longitude -41.455440), Espírito Santo state, and then taken to laboratory within the infested tomato fruit; which later were individually identified in plastic pots (10 cm of diameter x 8 cm of height). These pots were filled up to ¼ with sterile sand (4 hours in a drying oven at 170°C) to allow the larvae to build the shelter and pass to the pupal stage. The pupae were then transferred to acrylic cages (45 cm high x 40 cm wide x 50 cm depth) until the emergence of the adults. After four days, time needed for sexual maturation, the adults were collected and inserted into Poly-vinyl chloride tubes (20 cm of diameter x 25 cm of height) coated internally with kraft paper, being the top end closed with paper towel and voil fabric, and the bottom end supported in Styrofoam covered with kraft paper. The adults were daily fed with 10 % honey solution. The *H. armigera* eggs deposited on the inner wall of the tubes and on the towel paper have been collected and packed in plastic containers. After hatching, the larvae were individualized in glass tubes (8.5 x 2.5 cm) filled in up to 1/3 of its volume with an artificial diet based on beans, wheat germ and soybean meal (Giolo et al., 2006). The larvae were kept in these containers until the pupal stage. The pupae were treated with chlorine hypochlorite solution to 10% and washed in deionized water. All developmental stages of the pest were in climatic room (25 ± 1°C, RH 70 ± 10% and photoperiod of 14 hours).

### II – Alternative host creation *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)

The creation was established in laboratory (25 ±

1°C, RH 70 ± 10% and photoperiod of 14 hours) according to the methodology developed at the Center for Scientific and Technological Development in Phytosanitary Management of Pests and Diseases (NUDEMAFI), using homogenized diet based on whole-wheat flour (60%), cornmeal (37%) and yeast (3%). The diet was distributed in plastic boxes (30 x 25 x 10 cm) with corrugated cardboard (25 x 2 cm) on the bottom, being distributed about 0.3 g of eggs per box on the surface of the diet. The *E. kuehniella* adults were collected daily using an adapted vacuum cleaner, and transferring to PVC cages (150 mm of diameter x 25 cm of height) containing zigzag-folded nylon screen inside, which served as an oviposition substrate. The *E. kuehniella* eggs have been daily collected, for five days, and subsequently stored and kept in the refrigerator at 4 ± 1°C, for up to 20 days (Milanez, Pratissoli, Polanczyk, Bueno, & Tufik, 2009).

### III - Obtainment and multiplication of *Trichogramma pretiosum*

This study was carried out using the commercial strain known as Tricho-Strip-P of Koppert Biological Systems Ltda (Piracicaba, São Paulo State) acquired and maintained in NUDEMAFI laboratory at 25 ± 1°C, RH 70 ± 10% and photoperiod of 14 hours. The multiplication of these parasitoids was done in eggs of *E. kuehniella,* an alternative host. Using arabic glue (10% p $v^{-1}$), the alternative host eggs were fixed in a light blue card (8.0 x 2.0 cm), blocked with a germicidal lamp during 50 min. (Pratissoli et al., 2010). These cards were then inserted in glass tubes (8.5 x 2.5 cm) with female parasitoids recently emerged. After 24 hours, the cards were transferred to a clean tube and stored until the emergence of the next generation.

### IV – Influence of age of *Helicoverpa armigera* eggs on parasitism by *Trichogramma pretiosum*

The *T. pretiosum* female recently emerged were individualized in microtubes (2 mL), with a honey droplet deposited on the inner wall. The *H. armigera* eggs of different ages, 0-12, 24-36 and 48-60 hours, were removed from the creation and exposed to parasitism by female of *T. pretiosum* for 24 hours. Thirty eggs were fixed with gum arabic (10% p $v^{-1}$) in a light blue card (0.5 x 2.5 cm), with the help of a brush. After 24 hours of parasitism, the females were removed and the cards with parasitized eggs were kept in the microtubes until the emergence of the new parasitoids. Daily inspections were performed on the microtubes for removing the neonates larvae once they can damage the parasitized eggs. When parasitoids emerged, the average number of parasitized eggs, the percentage of emergence, the sexual rate [Nº. female/ (Nº. females + Nº. of males)] and the numbers of parasitoids emerged per egg were estimated.

The experiments were conducted in a completely randomized design, with three treatments represented by age range of eggs and 20 repetitions (female). The premises of ANOVA were tested using “PROC UNIVARIATE” and the data were transformed when necessary. Results were subjected to analysis of variance by PROC ANOVA and Tukey test (HSD) ($p < 0.05$) for the separation of the means using the statistical program SAS, version 9.0 (SAS Institute 2001).

### V - Influence of the density of *Helicoverpa armigera* eggs on *Trichogramma pretiosum* parasitism

*Trichogramma pretiosum* female recently emerged were individualized in microtubes (2 mL), with a honey droplet deposited on the inner wall. The *H. armigera* eggs of 24 -36 hours of age were removed from the creation and fixed with gum arabic (10% p $v^{-1}$), in different densities (15, 20, 25 and 30) in a light blue card (0.5 x 2.5 cm), with the help of a brush. The cards were offered to female parasitism during 24h in climate chambers (25 ± 1°C, 70 ± 10% RH and photoperiod of 14 hours). After this period, the females were removed and the cards with parasitized eggs were kept in the microtubes until the emergence of the new parasitoids. Daily inspections were performed for removing neonates larvae. The parasitized eggs were used to calculate: parasitism (%), emergence (%) and sexual rate [Nº. female/ (Nº. females + Nº. of males)], as well as to estimate the numbers of parasitoids emerged per egg.

The bioassay was completely randomized, with four treatments (densities) and 15 repetitions (females). The condition of normality and the homogeneity of variance of the data were tested. Results were subjected to analysis of variance (ANOVA) and the means grouping by Scott-Knott test ($p < 0.05$) using the program R (R Development Core Team, 2013).

## Results

### I - Influence of age of *Helicoverpa armigera* eggs in parasitism by *Trichogramma pretiosum*

The average number of parasitized eggs by *T. pretiosum* varied depending on the eggs embryonic development stage of *H. armigera* ($F_{2, 42} = 39.27$; $p < 0.0001$) (Table 1).

**Table 1.** Parasitism characteristic of eggs of *Helicoverpa armigera* from different ages by *Trichogramma pretiosum* at a constant density of 30 eggs per female (24 hours of exposure) (25 ± 1°C, 70 ± 10% RH and photophase of 14 hours).

| Eggs' age (h) | Number of parasitizes eggs/female [a] | Emergency [a] (%) | Number of emerged individuals/egg host [a] | Sexual Ratio[a] |
|---|---|---|---|---|
| 0-12 | 21.0 ± 0.46 a | 85.8 ± 1.96 a | 1.5 ± 0.07 a | 0.80 ± 0.02 a |
| 24-36 | 21.0 ± 0.34 a | 87.5 ± 1.65 a | 1.3 ± 0.15 a | 0.83 ± 0.01 a |
| 48-60 | 14.8 ± 0.80 b | 77.6 ± 2.39 b | 1.1 ± 0.12 a | 0.79 ± 0.03 a |
| *F* | 39.27 | 6.81 | 2.98 | 0.93 |
| *P* | <0.0001 | 0.0027 | 0.0619 | 0.4006 |

[a] Means (± SE) followed by the same letter, in column, do not differ significantly by Tukey test (p ≤ 0.05).

Eggs of 0-12 and 24-36 hours provided the largest number of parasitized eggs, regarding the eggs of 48-60 hours. The percentage of parasitoids emergence also took similar effect to the number of parasitized eggs, where the eggs of 24-36 hours provided the best emergency rate. The eggs of 48-60 h provided a lower emergence rate ($F_{2,\ 42}$ = 6.41; P = 0.0027), but kept above 70% (Table 1). The number of emerged individuals per parasitized egg ($F_{2,\ 42}$ = 2.98; P = 0.0619) did not differ regardless the age of the eggs of *H. armigera* (Table 1) and neither the sexual rate ($F_{2,\ 42}$ = 0.93; P = 0.4006) which ranged from 79 to 83% of females.

### II - Influence of *Helicoverpa armigera* eggs density on parasitism by *Trichogramma pretiosum*

The percentage of eggs parasitized by *T. pretiosum* varied depending on the density of the eggs of *H. armigera* offered (Table 2).

**Table 2**. Parasitism characteristics of *Helicoverpa armigera* eggs with 24-36 hours old by *Trichogramma pretiosum* in function of different densities of eggs (25 ± 1°C, 70 ± 10% RH and photoperiod of 14 hours).

| Densities | Parasitism[a] (%) | Emergency[a] (%) | No. of emerged individuals/egg[a] | Sexual Ratio[a] |
|---|---|---|---|---|
| 15 | 63.9 ± 1.93 c | 85.7 ± 2.89 a | 1.59 ± 0.05 b | 0.79 ± 0.01 a |
| 20 | 78.3 ± 1.80 a | 89.5 ± 2.08 a | 1.81 ± 0.04 a | 0.79 ± 0.02 a |
| 25 | 72.8 ± 2.38 b | 89.6 ± 2.55 a | 1.68 ± 0.07 a | 0.77 ± 0.02 a |
| 30 | 71.3 ± 1.97 b | 77.1 ± 3.05 b | 1.57 ± 0.04 b | 0.80 ± 0.01 a |
| *F* | 8.43 | 4.78 | 3.87 | 0.76 |
| *P* | 0.0001 | 0.0049 | 0.0138 | 0.5214 |

[a] Means (± SE) followed by the same letter, in column, do not differ significantly by Scott-Knott test (p ≤ 0.05).

The highest rate of parasitism by *T. pretiosum* ($F_{3,\ 56}$ = 8.43; P = 0.0001) was observed when a density of 20 eggs per female was offered, followed by densities of 25 and 30 eggs and higher than 15 eggs. The offspring production was similar for all densities, being higher than 85.7% except for the density of 30 eggs of *H. armigera*, where the emergence was 77.1% ($F_{3,56}$ = 4.78; P = 0.0049) (Table 2). The offer of 20 and 25 eggs per female provided the largest numbers of parasitoids emerged per egg and proved to be higher than the densities of 15 and 30 eggs per female ($F_{3,\ 56}$ = 3.87; P = 0.0138) (Table 2). The sexual ratio of the offspring, between 77 and 80% of females, was not influenced by the density of eggs offered to females ($F_{3,56}$ = 0.76; P = 0.5214).

## Discussion

Egg-parasitoids have developed strategies to exploit variable host resources, including variation in the quality of a host egg over time (Vinson, 1998). Females *T. pretiosum* were shown to be able to explore a wide range of host egg ages. Results of the present study showed that parasitism ability is substantially affected by age of *H. armigera* host eggs. Although the offspring production occurs at all host ages studied, the average of parasitized eggs was higher for the eggs up to 36 hours old, which demonstrates that eggs until this age provide the best acceptance of this host by *T. pretiosum*. The number of eggs with 48-60 hours of development was reduced, however, an average of 50% (n=15/30) of the egg's density offered was parasitized (Table 1).

The reduction of parasitism of older host eggs by *Trichogramma* match those in previous studies (Bari, Jahan, Islam, & Ali, 2016; Faria, Torres, & Farias, 2000; Moreno, Pérez-Moreno, & Marco, 2009; Pizzol et al., 2012). The *Trichogramma* female are known by their ability to assess the host through its examination with the movements of the antenna and the probing with the ovipositor (Hassan, 1997). With the egg's development, numerous factors are modified. Younger eggs show more attractive nutritional characteristics to parasitoid. However, with the development, nutrient composition is modified into chemically more complex tissues, and the embryonic development makes it more resilient to parasitism causing a reduction in the viability (Vinson, 1997).

The percentage of emergency the *T. pretiosum* adults was over 85.8% in eggs host of 0-12 and 24-36 hours old. A reduction in the emergency was detected in the eggs hots of 48-60 hours old. In these older eggs host, the parasitoids find the embryo in an advanced development stage, with sclerotized cephalic capsule and capable of rotation into all the interior of the egg, thus, being more competitive (Moreno, Pérez-Moreno, & Marco, 2009). In addition, once the hardening of the chorion limits the penetration of the ovipositor, the parasitoids requires a longer manipulation of the host resulting in fewer parasitized eggs (Faria, et al., 2000). The parasitism of older eggs forces the larva of the parasitoid to grow faster (Pizzol et al., 2012), which can generate deformed individuals unable to emerge.

The emergency reduction of the parasitoids due to the embryonic development was noted by several authors (Ko et al., 2014; Pastori, Monteiro, Botton, & Pratissoli, 2010; Poltronieri, Silva, Araujo, Schuber & Pastori, 2008). Among the factors that influence the emergence of parasitoids are: humidity, size, age and the egg nutritional quality. Maintaining the humidity of the egg is important because the dryness of the chorion can make it difficult for the emergence of adults (Nava, Takahashi, & Parra, 2007).

Regarding the number of parasitoids developed per egg, the host-egg age did not play changes, being the number of parasitoids emerged per egg always more than one parasitoid per parasitized egg. This might be explained by the size of the *H. armigera* egg, between 0.42-0.60 mm (Ali et al., 2009), considered big when compared to the eggs of the alternate host *A. kuehniella,* which are around 0.3 mm used for mass creation. These results indicate a larger amount of nutrients in the egg, capable of supporting the development of more than one parasitoid. However, when more than one parasitoid develops in the same egg, there may be competition for food resulting in smaller individuals and lower nutritional quality, reflected in the reduction of the number of parasitized eggs per female (Moreira, Santos, Beserra, Torres, & Almeida, 2009).

Regarding the density of host eggs offered by female parasitoid, the number of parasitized eggs by *T. pretiosum* increased proportionately with the density of *H. armigera* eggs, reaching a mean of 21.7 eggs, when offered the maximum density of 30 eggs. The amount of parasitized eggs tends to increase and stabilize when the parasitoid reach the maximum capacity of parasitism (Pratissoli, Vianna, Reis, Andrade, & Silva, 2005). For example, Pratissoli et al. (2005) observed the maximum parasitism of *T. pretiosum*, when 17.31 eggs were parasitized at the density of 25 eggs of *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), and Faria et al. (2000) registered 31.1 eggs of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) parasitized when was offered up to 60 eggs per female of *T. pretiosum* per day.

The increase in the number of parasitized eggs may be grounded on the decrease in time for each step of the parasitism: egg external recognition penetration of the ovipositor and internal examination, after the parasitism of the first quality egg. Therefore, there is a successive shortening of parasitism behavior, connected to the learning of the female when parasitized the first egg (Moreira, Santos, Beserra, Torres, & Almeida, 2009). However, the learning of females in relation to the number of parasitized eggs depends on the physicochemical characteristics of the host's egg, since they exhibit a seeking behavior characterized by chemical signals (Colazza, Peri, Salerno, & Conti, 2010). When successive generations are kept in the same host, the parasitoid acquires a pre-imaginary conditioning which delays the acceptance of new hosts (Siqueira, Bueno, Bueno, & Vieira, 2012). Nevertheless, the genetic variability of *Trichogramma* results in aggressive females middling with greater acceptance and ability of parasitism in postures with different physical barriers (Beserra & Parra, 2004).

The emergency of adult parasitoids was around 90% when females received 20 or 25 host eggs. These densities were responsible for the higher production of individuals. This proposes that *T. pretiosum* showed greatest performance on *H. armigera* eggs in densities between 20 and 25 host eggs. However, this ability to parasitize can be altered according to the temperature conditions and this abiotic factor must be considered for the biological control efficiency with *T. pretiosum* (Zuim et al., 2017).

In both experiments, the female's offspring sexual rate ranged between 79 and 83% and no statistical difference was observed, this is, neither the host-egg age nor egg density change the sex ratio. The same was observed in other studies (Oliveira, Santana, Bellon, & Oliveira, 2014; Pastori et al., 2010; Poltronieri et al., 2008). The sex ratio of the offspring seems to be influenced by temperature, host egg quality and the symbiont load the female possesses, especially, *Wolbachia* bacteria (Gonçalves et al., 2006; Moiroux, Brodeur, & Boivin, 2014; Vinson, 1997). Factors such as temperature, humidity, and age of the parasitoid females were constant in our bioassays, keeping the sex ratio unchanged.

## Conclusion

Our data showed that older *H. armigera* eggs are less suitable than younger ones for reproduction of *T. pretiosum*, and this is important for planning the release of the parasitoid that must be synchronized with younger eggs of the pest, and thus, does not compromise the efficiency of the parasitoid.

Each *T. pretiosum* female was able to parasitize 70% of the pest eggs, highlighting the potential of this natural enemy for biological control of *H. armigera*.

## Acknowledgements

The authors hereby thank the CAPES for the scholarship given to the first author. The authors are also grateful to the PPGEA/UFRPE and the NUDEMAFI-CCA/UFES by permitting the development of this research; to CNPq and FAPES for the financial support; and we would like to express our appreciation to Dr. Alexandre Specht from EMBRAPA, Cerrado, for the identification of specimen.

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*Received on January 27, 2017.*
*Accepted on July 31, 2017.*