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Influence of *Grapholita molesta* semiochemicals on chemotaxis, parasitism, and learning ability of *Trichogramma pretiosum*

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Abstract

Trichogramma pretiosum Riley (Hymenoptera Trichogrammatidae) is known to use a wide range of chemical cues to locate its hosts. Its presence in areas where mating disruption control has been used for *Grapholita molesta* (Busck) (Lepidoptera Tortricidae) has led to questions about the effect of these semiochemicals on the behaviour of this parasitoid. Therefore, we evaluated the chemotactic responses and parasitism of *T. pretiosum* at different ages to *G. molesta* sex pheromone, as well as the ability of this parasitoid to learn and to recognize *G. molesta* eggs volatiles. Chemotaxis of 24, 48, 72, and 96 h-old *T. pretiosum* mated males and females was recorded using an olfactometer, contrasting the synthetic sex pheromone blend (*Z*-8-dodecenyl acetate, *E*-8-dodecenyl acetate, and *Z*-8-dodecenol - 10 µl at 0.001%) versus hexane. We also observed the response of parasitoids (females with and without experience on the host eggs) to *G. molesta* rinsed and unrinsed eggs (olfactometry and parasitism). Our results evidenced that *T. pretiosum* females are able to recognize *G. molesta* sex pheromone and also that learning process on *G. molesta* eggs increases their chemotactic response and parasitism in unrinsed eggs. Understanding the interactions between *T. pretiosum* and *G. molesta* is the first step to design a rational combination among different sustainable pest control techniques.

Key words: pheromone, oriental fruit moth, kairomone, olfactometry, egg parasitoid.

Introduction

Trichogramma pretiosum Riley (Hymenoptera Trichogrammatidae) is an important parasitoid species of Lepidoptera eggs (Pratissoli *et al*., 2005) widely distributed in the Americas (Pinto and Stouthamer, 1994; Zucchi *et al*., 2010). It can parasitize the oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera Tortricidae) (Poltronieri *et al*., 2008; Tortosa *et al*., 2014), which is considered the most important pest of peach and apple orchards in Brazil (Botton *et al*., 2011).

Mating disruption is also a valuable tool to control *G. molesta* and consists of releasing a large volume of synthetic sex pheromone (*Z*-8-dodecenyl acetate, *E*-8-dodecenyl acetate and *Z*-8-dodecenol) in the field, which hinders the moth from finding mates, thereby decreasing the oviposition of viable eggs (Arioli *et al*., 2013). This pheromone is also used for monitoring moth populations (Botton *et al*., 2011; Arioli *et al*., 2013). Nevertheless, synthetic commercial pheromones can also act as a kairomonal stimulant, interfering in host localization by parasitoids, and consequently impacting biological pest control (Bayoumy *et al*., 2011).

The task of finding a suitable host is challenging for female parasitoids, because the hosts tend to remain inconspicuous to their natural enemies (Hoedjes *et al*., 2010). To mitigate this problem, oophagous parasitoids can use a variety of strategies in their search behaviour (Huigens *et al*., 2010), and for trichogrammatids, chemical cues are the most important ones (Nordlund, 1994; Schmidt, 1994; Hoedjes *et al*., 2010; Gontijo *et al.,* 2019). These cues include volatile organic compounds (VOC) emitted by plants under herbivore attack (Vet and Dicke, 1992; Schweiger *et al*., 2014), synomones induced by deposition of host eggs (Gontijo *et al*., 2019; Ali and Wright, 2021; Nascimento *et al*., 2021), and kairomones produced by the phytophagous insects (Xu *et al*., 2014; Gontijo *et al*., 2019).

Studies have found that the host sex pheromone can provide long-range cues for the parasitoids (Fatouros *et al*., 2008; Colazza *et al*., 2010; Gontijo *et al*., 2019). *T. pretiosum* showed innate attractiveness to sex pheromone of its hosts, Noctuidae species, such as *Helicoverpa zea* (Boddie) and *Mamestra brassicae* (L.) (Noldus *et al*., 1991). These authors also observed that the sex pheromone of the female moth *M. brassicae* adsorbed onto the leaf surface of Brussels sprout plants attracts conspecific male moths and *Trichogramma evanescens* (Westwood) (Hymenoptera Trichogrammatidae) females. In addition, *Trichogramma* spp. have the ability to attach themselves to a host (phoresy), probably guided by pheromones, suggesting that hitchhiking is a strategy used by these wasps to gain access to freshly laid moth eggs (Fatouros *et al*., 2007; Huigens *et al*., 2010; Fatouros and Huigens, 2012; Xu *et al*., 2014).

Host egg's kairomones can also act as a cue that is detectable at short distances or by contact (Kaiser *et al*., 1989; Colazza *et al*., 2010). Response to volatiles of lepidopteran egg masses has been reported for the trichogrammatids *T. pretiosum* (Gross *et al*., 1981; Nordlund *et al*., 1987; Vet *et al*., 1995) and *Trichogramma brassicae* (Bezdenko) (Renou *et al*., 1992).

Although parasitoids display an innate behaviour when searching for hosts (Papaj and Lewis, 1993), it can be modified through experience, which may result in a learning process associated with the acquisition of information that is beneficial to parasitoids fitness (Meiners and Peri, 2013). *Trichogramma* adults can learn to recognize compounds present on or inside the host chorion (Le Rec and Wajnberg, 1990; Vinson, 1998; Nurindah *et al*., 1999). It can reduce the time spent in host selection (Beserra and Parra, 2003) or change parasitism preference (Vargas *et al*., 2017). Besides, age can also influence the response of *Trichogramma*, what might be associate with hormones involved in age-dependent behavioural sensitivity (Pak *et al*., 1986; Garcia *et al*., 2001; Ya and Vaghina, 2007). Thus, in this study, we assessed the influence of *G. molesta* sex pheromone, learning ability, as well as age on chemotactic responses and parasitism of *T. pretiosum* under laboratory conditions.

Materials and methods

Insects

T. pretiosum was obtained from specimens collected from eggs of *H. zea*, in a corn crop, located at the Experimental Station SEAPDR/Floresta (29°41'24"S 53°48'42"W), in Santa Maria, Rio Grande do Sul, Brazil. The wasps were identified by the Laboratory of Insects Biology at the University of São Paulo through multivariate morphometrics by Dr. Jaci Mendes. The parasitoids were multiplied and maintained (62 generations) in *Ephestia kuehniella* Zeller (Lepidoptera Pyralidae) eggs, following the methodology of Parra (1997) under controlled environmental conditions (25 ± 1 °C, 60 ± 10% RH, 12L:12D photoperiod).

The colony of *G. molesta* was maintained in the laboratory of Biology, Ecology, and Biological Control (Bioecolab) of Federal University of Rio Grande do Sul under controlled environmental conditions (25 ± 1 °C, 60 ± 5% RH, 16L:8D photoperiod). The adults were maintained in cages of 2 L made of polyethylene terephthalate (PET) bottles, which also served as oviposition sites, and fed with a solution of honey and water (15%). Every three days eggs masses were collected and transferred to recipients containing artificial diet for the larval phase. The diet was based on dried apple, beer yeast, corn meal, and wheat germ (Ivaldi-Sender, 1974).

Pheromone

A commercial blend of *G. molesta* synthetic sex pheromone containing the components *Z*-8-dodecenyl acetate, *E*-8-dodecenyl acetate, and *Z*-8-dodecenol in the ratio 93:6:1 (Bedoukian®) was used for the bioassays, diluted in hexane at 0.001%. This concentration was chosen based on preliminary olfactometry tests. It was the lowest concentration at which the parasitoids initiated their response.

Olfactory response of *T. pretiosum*

The chemotactic responses bioassays were conducted in a dual-choice glass Y-tube olfactometer, consisted of a bifurcated glass tube (1 cm internal diameter, 12 cm stem length, 5 cm arm's length and 50° angle) positioned horizontally on the surface. The odours sources were placed inside translucent plastic tubes (4 cm high, 1 cm diameter) connected to the extremities of the olfactometer. An air flow, previously filtered with active carbon, was adjusted to 0.3 L/min (0.15 L/min per arm) using a calibrated flowmeter connected to the air pump. To avoid spatial bias the olfactometer was rotated on its horizontal axis (180° rotation) after every three replicates, and was replaced by another previously washed and sterilized (oven-dried at 120 °C) after every six replicates. At each replacement of the olfactometer, the odours sources were changed for new ones. Bioassays were carried out under fluorescent light (60 W, luminance of 290 lux) situated behind the odour sources at the olfactometer room (25 ± 1 °C, 60 ± 5% RH).

The responses of *T. pretiosum* inexperienced males and females (24, 48, 72, and 96 h-old) to *G. molesta* synthetic sex pheromone were evaluated. For this bioassay, at the end of one olfactometer arm, a filter paper (80 g/m^2/1 × 3 cm) folded in a bagpipe shape, containing 10 µl of the synthetic pheromone (0.001% concentration), was added and then tested against the same volume of hexane solvent (control) in the other arm.

Also, the responses of *T. pretiosum* females (24, 48, 72, and 96 h-old) inexperienced or experienced on *G. molesta* eggs were evaluated contrasting rinsed and unrinsed eggs. To acquire experience, these parasitoids (± 200) were exposed to a PET irregular bottle pieces containing 300 *G. molesta* eggs (up to 24 h-old), for five hours. A plastic piece containing 50 unrinsed eggs (up to 24 h-old) was placed in one arm of the olfactometer, and in the other, 50 rinsed eggs. The eggs were rinsed according to the methodology of Tognon *et al*. (2018), which consists in immersing eggs in solvent hexane (99%) for five minutes.

Insects then were positioned individually at the beginning of the central arm of the Y-tube and observed for 10 minutes. Parasitoids that moved at least 3 cm into one arm and remained there for at least 60 seconds were considered responsive. If no choice was made within 10 minutes, the wasp was recorded as non-responsive, and was excluded from statistical analysis. At least 40 parasitoids (replicates) were tested per treatment (contrasts). Each parasitoid was used only a single time to prevent learning.

Parasitism of *T. pretiosum* in *G. molesta* eggs

The parasitism of *T. pretiosum* females (24, 48, 72, and 96 h-old) inexperienced and experienced in *G. molesta* eggs were evaluated on rinsed and unrinsed eggs in a two-choice bioassay. The experience acquisition and the eggs rinsing followed the same method described above on the olfactometer bioassay.

Females were released individually into glass tubes (3 × 7 cm) containing two irregular PET bottle pieces with 50 eggs (up to 24 h-old) of each type (rinsed and unrinsed). The tubes were sealed with Parafilm® (Bemis Flexible Packaging) and females were exposed for 3 hours. After this exposure, the eggs were transferred to new glass tubes, where they were separated according to the type (rinsed and unrinsed), and were kept in climatic chambers (25 ± 1 °C, 60 ± 5% RH, 16L:8D photoperiod). Each replicate consisted of one tube (one parasitoid and 100 eggs), in a total of 20 replicates per treatment. Thirteen days after exposure (approximately), the number of parasitized eggs was recorded.

Statistical analysis

All data were analysed with generalized linear models (GLMs) in the statistical software R 4.0.0 (RStudio Team, 2020). A binomial distribution was used for the parasitoid choice frequency in the olfactometer bioassays. Also, the proportion of non-responsive parasitoids within each age, inexperienced and experienced on *G. molesta* eggs was analysed with a binomial distribution. The negative binomial distribution was used for parasitism data contrasting rinsed and unrinsed eggs within each age of the parasitoid. The goodness-of-fit of the model was confirmed with a half-normal plot (hnp package) (Moral *et al*., 2017).

Results

Chemotactic response of *T. pretiosum* (inexperienced) to *G. molesta* sex pheromone

T. pretiosum mated males of any age did not distinguish between the odours of pheromone blend or solvent hexane (deviance analysis with binomial model; 24 h-old: $\chi^2 = 1.8$, df = 1, $P = 0.179$; 48 h-old: $\chi^2 = 0.8$, df = 1, $P = 0.371$; 72 h-old: $\chi^2 = 3.2$, df = 1, $P = 0.072$); 96 h-old: $\chi^2 = 3.2$, df = 1, $P = 0.072$) (figure 1A). Mated females (24, 48 and 72 h-old) were more attracted to the synthetic pheromone blend of *G. molesta* than hexane (deviance analysis with binomial model; 24 h-old: $\chi^2 = 16.8$, df = 1, $P < 0.001$; 48 h-old: $\chi^2 = 5.1$, df = 1, $P = 0.025$; 72 h-old: $\chi^2 = 58.9$, df = 1, $P < 0.001$) (figure 1B). However, when females were 96 h-old, they preferred the solvent over the pheromone blend (deviance analysis with binomial model; $\chi^2 = 10.0$; df = 1; $P = 0.002$) (figure 1B).

Chemotactic response of *T. pretiosum* (inexperienced or experienced) to rinsed and unrinsed egg volatiles of *G. molesta*

Inexperienced *T. pretiosum* females, at all ages, did not differentiate rinsed and unrinsed *G. molesta* eggs (deviance analysis with binomial model; 24 h-old: $\chi^2 = 4.1$; df = 1; $P = 0.050$; 48 h-old: $\chi^2 = 3.6$, df = 1, $P = 0.056$; 72 h-old: $\chi^2 = 1.6$; df = 1; $P = 0.204$; 96 h-old: $\chi^2 = 0.10$; df = 1; $P = 0.758$) (figure 2A).

Experienced females at all ages were more attracted to unrinsed eggs than rinsed ones (deviance analysis with binomial model; 24 h-old: $\chi^2 = 7.033$, df = 1, $P = 0.008$; 48 h-old: $\chi^2 = 21.78$, df = 1, $P < 0.001$; 72 h-old: $\chi^2 = 4.97$, df = 1, $P = 0.026$; 96 h-old: $\chi^2 = 6.36$, df = 1, $P = 0.012$) (figure 2B).

Parasitism of *T. pretiosum* (inexperienced or experienced) in *G. molesta* eggs

The parasitism of inexperienced *T. pretiosum* on *G. molesta* eggs was only higher in rinsed eggs when the parasitoid was 72 and 96 h-old (table 1). Nevertheless, when *T. pretiosum* was previously exposed to the eggs of its host, parasitism was higher in unrinsed eggs for 72 and 96 h-old parasitoids (table 1).

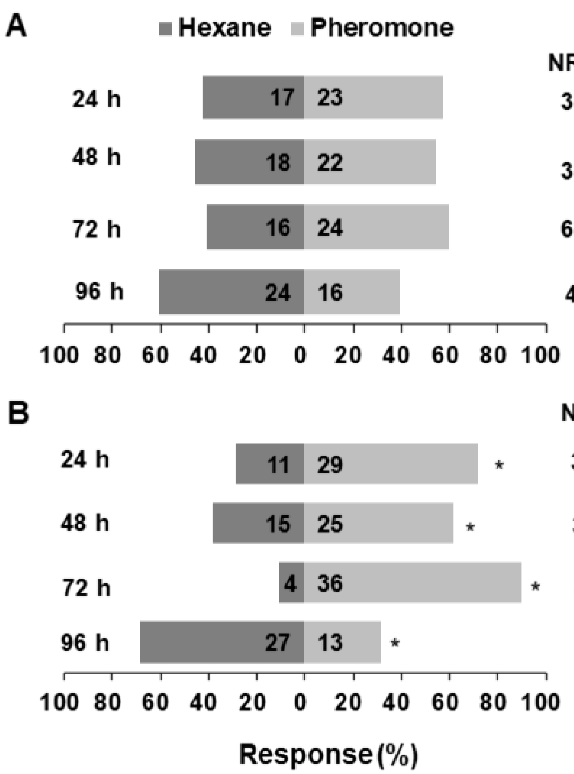

Figure 1. Olfactory response of *T. pretiosum* at different ages to synthetic pheromone blend volatiles of *G. molesta*. **(A)** Response of *T. pretiosum* mated males. **(B)** Response of *T. pretiosum* mated females. The numbers inside the bars are the total numbers of *T. pretiosum* that responded to each treatment. * Significant at 5% according to contrasts of the model (GLM: binomial distribution); NR = non-responsive parasitoids.

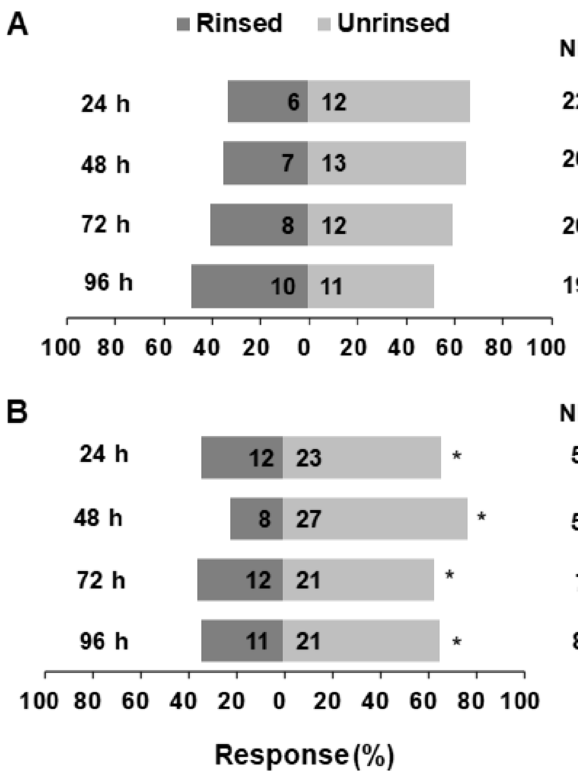

Figure 2. Olfactory response of females of *T. pretiosum* at different ages with and without experience on *G. molesta* eggs to rinsed and unrinsed *G. molesta* eggs. **(A)** Inexperienced parasitoid. **(B)** Experienced parasitoid. The numbers inside the bars are the total numbers of *T. pretiosum* corresponding to that treatment. * Significant at 5% according to contrasts of the model (GLM: binomial distribution); NR = non-responsive parasitoids.

Table 1. Mean number (± SE) of rinsed and unrinsed *G. molesta* eggs parasitized by *T. pretiosum* females, at different ages, with and without experience on its host eggs.

Age in hours	Rinsed eggs	Unrinsed eggs	χ^2 (df)[a]	*P*-value
Without experience				
24	8.7 ± 1.83	7.8 ± 1.90	0.096 (1)	0.757
48	14.9 ± 2.16	7.7 ± 2.30	2.381 (1)	0.123
72	14.9 ± 1.81 *	4.6 ± 1.64	7.300 (1)	0.007
96	16.3 ± 3.29 *	5.5 ± 1.74	5.067 (1)	0.024
With experience				
24	7.6 ± 2.76	11.8 ± 2.42	0.392 (1)	0.531
48	3.6 ± 1.42	6.8 ± 1.27	0.461 (1)	0.497
72	2.1 ± 0.55	12.6 ± 1.16 *	30.431 (1)	< 0.001
96	4.0 ± 1.00	10.2 ± 1.84 *	15.486 (1)	< 0.001

Means for rinsed and unrinsed eggs followed by an asterisk (*) within each age are significantly different by contrasts generated by the model (GLM: negative binomial distribution; $P < 0.05$).
[a] χ^2 value from deviance analysis (degrees of freedom).

Discussion

The synthetic pheromone of *G. molesta* triggered chemotactic responses only in females' wasps and did not interfere in males' responses. Al-Jalely and Xu (2021) observed four types of olfactory sensilla in *T. pretiosum* (chaetica, trichoid, faleate and placoid) with different expression patterns of odorant-binding protein (OBP) genes between male and female, which may be crucial in *T. pretiosum* host-seeking and oviposition behaviours. Thus, the lack of male's response observed in our study, could be related to the absence of OBPs associated with *G. molesta* pheromone, since these chemical cues, probably, do not impact its search behaviour for mating and feeding sites. Males usually emerge before females and remain on egg masses while waiting for their emergence (Pompanon *et al*., 1997; Knutson, 1998), which guarantees copulation before field dispersal (Waage and Ming, 1984). Also, *Trichogramma* males locate their mate essentially with a substrate-borne sex pheromone that is not volatile (Pompanon *et al*., 1997). Thus, it was predictable that males would not respond to the synthetic pheromone of *G. molesta*.

Conversely, *T. pretiosum* females were attracted by *G. molesta* sex pheromone. However, 96 h-old *T. pretiosum* females no longer responded to the sex pheromone of *G. molesta*. The attractiveness to host pheromones by females of different species of the genus *Trichogramma* has already been reported to lepidopteran pests (Boo and Young, 2000; Reddy *et al*., 2002; Xu *et al*., 2014); which share similar pheromone groups of compounds, such as aldehydes and alcohols, that are also part of the sexual pheromone of *G. molesta* (Cardé *et al.*, 1979). The perception of odours is related to the expression of genes responsible for the biosynthesis of OBP in the haemolymph of the antenna, but it usually decreases in older insects (Chang, 2016; Gadenne *et al*., 2016), as observed for females of *T. evanescens* and *T. brassicae* to sex pheromones of *Tuta absoluta* (Meyrick) (Lepidoptera Gelechiidae) (Ahmadi and Poorjavad, 2018). Therefore, our results showed that *T. pretiosum* females are attracted to the synthetic sex pheromone of *G. molesta*, but this attraction can be altered by the age of this parasitoid.

One of the advantages of using pheromones as kairomones is to recruit the females near oviposition sites of their hosts, thereby increasing the chance of finding egg masses (Fatouros *et al*., 2008). Another aspect to consider is related to phoresy, i.e., a pheromone detection would allow the parasitoid to directly encounter the host females, to be transported by the herbivore before oviposition, thus not only compensating for the low displacement capacity observed in *Trichogramma* species, but also assisting in the encounter with newly oviposited eggs. This was observed in the laboratory for *T. pretiosum* (Xu *et al*., 2014) and also in the field for *Trichogramma* wasps (Fatouros and Huigens, 2012).

T. pretiosum was able to overcome an innate lack of recognition of volatiles from *G. molesta* eggs by learning as demonstrated in the olfactometer bioassay. The ability to learn is probably a strategy used by generalist parasitoids to address the great variability of chemical information present in the environment (Steidle and van Loon, 2002). Vargas *et al.* (2017) also observed that experienced females (with contact with eggs and eggs extract of *S. frugiperda*) were more attracted to eggs odour from this host than the inexperience ones. In addition, parasitism of inexperienced females was higher in rinsed eggs when wasps were 72 and 96 h-old. In contrast, previously experienced females (72 and 96 h-old) were more responsive to unrinsed eggs.

It is known that oophagous parasitoids usually use kairomones present in eggs and in substances secreted by accessory glands in the process of host search and location (Fatouros *et al*., 2008; Colazza *et al*., 2010). However, these kairomones can act as attractants, repellents or deterrents, preventing parasitism (Colazza *et al.*, 2010). Older experienced parasitoids might have been attracted to these volatiles, due to their previous experience. Moreover, in older inexperienced insects, unrinsed eggs were less parasitized than rinsed ones, which suggests that these volatiles might not have been recognized or could have acted as repellents or deterrents for these wasps. Tognon *et al*. (2017) has already observed that surface compounds of *Halyomorpha halys* (Stal) (Hemiptera Pentatomidae) eggs, from East Asia, prevented parasitism by *Telenomus podisi* Ashmead and *Trissolcus* *erugatus* Johnson (Hymenoptera Scelionidae), both native to North America.

The host acceptance within species is dynamic, varying with physiological state and experience of the parasitoid (Hopper *et al*., 2013). To recognize suitable host eggs, *T. pretiosum* spend more time inspecting them, since this behavior is related to the increase in the viability of the parasitized eggs (Zuim *et al.*, 2017). As older parasitoids had a shorter life expectancy, they might have carefully inspected *G. molesta* eggs in order to recognize and parasitize the most suitable eggs, according to their previous experience. Meanwhile, younger wasps may not have shown preference for either rinsed or unrinsed eggs due to their longer life expectancy, which means that they could lay more eggs and, consequently, they would not have to find the most suitable eggs among *G. molesta* eggs.

Also, in our bioassays, the female wasps were in close contact with the eggs and younger females could have been direct by visual stimuli at first, since both visual and/or volatile stimuli elicit their directional movements (Colazza *et al.*, 2010). Another explanation for the behaviour changes related to age of *T. pretiosum* is probably due to hormones involved in age-dependent behaviour (Pak *et al.*, 1986; Garcia *et al.*, 2001; Ya and Vaghina, 2007). However, to completely understand the relationship among the age of *T. pretisoum*, its previous experience on *G. molesta* eggs, and the oviposition preference of this parasitoid; more studies are needed to verify the components present on the chorion of *G. molesta* egg, and how they are perceived by this parasitoid species.

In summary, our results evidenced that *T. pretiosum* females are able to recognize *G. molesta* sex pheromone and that the learning process on *G. molesta* eggs increases their chemotactic response and parasitism in unrinsed eggs. This is the first study that showed effects of semiochemicals from *G. molesta* on the behaviour of *T. pretiosum*. This ability to learn is probably a strategy used by generalist parasitoids to address the great variability of chemical information present in the environment (Steidle and van Loon, 2002). Consequently, as this species is a generalist parasitoid, learning may play an important role in its efficiency in augmentative biological control programs. Considering that *T. pretiosum* laboratory colonies are maintained in *E. kuehniella* eggs, conditioning olfactory practices with eggs' extracts of the target pest could be done during the parasitoid development, close to the time of its emergence or immediately after it. Also, the presence of synthetic pheromones used for mating disruption control of *G. molesta* could contribute to the attraction of this parasitoid to the area where they are present. However, studies must be done to verify if *T. pretiosum* learning ability will change its innate host-seeking behaviour and its parasitism efficiency on *G. molesta* eggs in the field.

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