



Odor learning improves efficacy of egg parasitoids as biocontrol agents against *Tuta absoluta*

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Abstract

Trichogramma spp. (Hymenoptera: Trichogrammatidae) are generalist egg parasitoids widely used as biological control agents, yet with limited efficacy against *Tuta absoluta* (Meyrick; Lepidoptera: Gelechiidae), a key pest of tomato. Olfactory cues related to the host-plant complex influence the searching behavior in parasitoids. Studies have shown that female *Trichogramma* spp. can learn odor cues affecting their preference. We assessed if learning increases the response to tomato odor and whether it improves the parasitism rate of *Trichogramma achaeae*, *Trichogramma evanescens*, and *Trichogramma dendrolimi*. The parasitoids were either naive, reared for one generation on *T. absoluta* eggs, or conditioned associatively with an oviposition experience on a factitious host in the presence of tomato odor. Subsequently, their arrestment response (searching time) to *T. absoluta*-infested tomato leaves was observed in a static four-chamber olfactometer. Rearing the parasitoids on *T. absoluta* increased the arrestment time of *T. dendrolimi* strongly and that of *T. evanescens* slightly but not that of *T. achaeae*. In contrast, oviposition experience on a factitious host with the tomato odor (leaves or extract) increased the searching time of *T. achaeae* and *T. evanescens* strongly but did not improve the response of *T. dendrolimi*. Concerning parasitism improvement, only *T. achaeae* showed an increased parasitism rate after associative learning. Hence, the learning capacity of *T. achaeae* should be further explored to improve its biological control efficacy against *T. absoluta*.

Keywords Tomato pinworm · Biological control · Odor conditioning · Olfactometer · *Trichogramma* efficiency

Key messages

- We investigated the response of *Trichogramma* parasitoids to tomato leaves in an olfactometer.
- The odor of tomato leaves innately repelled all three species.
- Learning enhanced the arrestment response of all three species in the olfactometer.
- The conditioning process suitable to induce learning differed among species.

- Only for *T. achaeae*, learning translated into a higher parasitism efficacy.

Introduction

Hymenopteran parasitoids are contributing to the reduction of important arthropod pest species worldwide. Parasitoid wasps lay their eggs in or on the body of other arthropods, where the immatures will develop, resulting in the host's death. The efficiency of host searching behavior is determinant for the reproductive success of the female parasitoid wasps. For parasitoids of herbivorous hosts, their searching ability often relies on their response to odor cues from the host and the host plant (Bjorksten and Hoffmann 1998; Takasu and Lewis 2003; Vet and Groenewold 1990). This response may be affected by genetic mechanisms (Prevost and Lewis 1990; Gu and Dorn 2000) or learning (Kruidhof et al. 2019). Learning can occur through different processes and at diverse moments during the life cycle of a parasitoid. When emerging, for instance, a wasp can learn the cues of its

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environment (Kester and Barbosa 1991; Hastings and Godfray 1999). Adult wasps can establish an association between two stimuli or between a stimulus and a response through experience, the so-called associative learning (Kruidhof et al. 2019). Learning behavior can be exploited for manipulating the efficacy of augmentative biological pest control as parasitoids can be conditioned to become more efficient in the different phases involved in host searching and host acceptance (Giunti et al. 2015; Kruidhof et al. 2019).

Generalist egg parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are common natural enemies of many Lepidoptera species. They are relatively easy to mass-rear and are successfully used in inundative and inoculative biological control programs worldwide (van Lenteren 2000). It has been shown that *Trichogramma* spp. can learn volatile plant compounds (Bjorksten and Hoffmann 1995; Pashalidou et al. 2010; Wilson and Woods 2016). Recent attempts have been made to use *Trichogramma* spp. against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in Europe (Urbaneja et al. 2012; Zappalà et al. 2013). This key pest of tomato has become a significant threat to tomato production in Europe and Africa (Biondi et al. 2018; Mansour et al. 2018) and has reached China in 2017, the worldwide biggest tomato producer (Zhang et al. 2020a). Populations of *T. absoluta* established first in the province Xinjiang (Zhang et al. 2020a), followed by Yunnan (Zhang et al. 2020b), Guizhou, Sichuan, Hunan, Guangxi, and Chongqing, causing severe damage in Xinjiang (yield losses up to 86%) and Yunnan (Zhang et al. 2021). To control the pest, Chinese tomato growers currently rely on insecticides, including Emamectin benzoate, Avermectin-chlorobenzoyl, Methoxy fenozide, and Chlorantraniliprole (Amire et al. 2020), as well as on the microbial pesticide *Bacillus thuringiensis* (Bt-G033A) (Zhang et al. 2020c). However, *T. absoluta* has developed high levels of resistance to several of the most popular insecticides, thus implying that the invading individuals may already bear or will develop resistance to those insecticides (Han et al. 2018; Guedes et al. 2019; Desneux et al. 2021). Therefore, new tools that are safe for human health and the environment are highly needed for managing this invasive pest. The egg parasitoids *Trichogramma* spp. are one possible option. Unfortunately, in Europe, several factors limit the success of *Trichogramma* spp. in augmentative biological control against *T. absoluta*, such as the low attraction to infested tomato plants (Milonas et al. 2019) and the fact that *T. absoluta* eggs are not laid as egg patches (Tropea et al. 2012). Our study aimed to evaluate if learning can improve the success of *Trichogramma* spp. against *T. absoluta*.

We focused on three commercially available species, namely *Trichogramma achaeae* Nagaraja & Nagarkatti, *Trichogramma evanescens* Westwood, and *Trichogramma dendrolimi* Matsumura. We selected *T. achaeae* since this

species is currently used for biological control of *T. absoluta* in Europe (Desneux et al. 2021) and *T. evanescens* and *T. dendrolimi*, as both are commonly occurring in China (Zang et al. 2021). In China, *T. dendrolimi* is mass-reared and widely used in maize fields against the Asian corn borer (ACB) *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae) and to a lesser extent on rice fields against *Chilo suppressalis* Walker (Lepidoptera: Crambidae) (Zang et al. 2021).

The aims were to explore different mechanisms by which experience increases parasitoid attraction toward tomato plants and whether this differs between *Trichogramma* species. To address this, three questions were investigated: (1) What is the innate response of the parasitoids to volatile cues of the tomato plant? (2) Does experience of cues from host remains and tomato plants at emergence improve this response? (3) Does an oviposition experience in the presence of tomato plant cues result in associative learning and increased preference to the plant-host complex? Finally, we explored whether the behavior modified by learning in the three *Trichogramma* species translates into differences in parasitism efficacy.

Materials and methods

Plants and insects

Tomato plants, *Solanum lycopersicon* cv. Rentita, were grown in greenhouses at 25 ± 5 °C and $60 \pm 10\%$ RH. Plants were grown in pots (10 cm dia.) with long-term fertilizer mixed with the soil (3 kg/m³, Manna Cote 4 M, Hauert Düngerwerke GmbH, Germany). Eggs of *T. absoluta* were provided by Andermatt Biocontrol (Switzerland) and a colony was established in a climate chamber at 25 ± 1 °C, $70 \pm 10\%$ RH and a 16:8 h L:D photoperiod). Adults were kept in mesh cages (50×50×50 cm) (bug dorm, MegaView Science Co., Ltd., Taiwan) and provided cotton soaked in honey-water (10% v/v) placed on the top of the cage and tomato plants for egg laying. After seven days, plants with eggs and young larvae were moved to a another cage to start a new colony. Moth eggs used in the experiments were between 4 and 16 h old.

The commercially available egg parasitoids, *T. evanescens*, *T. achaeae* and *T. dendrolimi* were obtained from AGROLINE Bioprotect (Switzerland), Bioline AgroSciences (France), and AMW Nützlinge (Germany), respectively. Colonies of parasitoids were established on UV-irradiated eggs of *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae) from AGROLINE Bioprotect (Switzerland) in climate chambers (22 ± 1 °C, $70 \pm 10\%$ RH, 16:8 h L:D). Rearing was established in large acrylic glass tubes (10 cm dia.; 30 cm length), and the parasitoids were fed on honey-agar

(water:agar:honey, 1 l: 8 g: 0.1 l). Two to three times per week, food and a post-it® with pasted fresh host eggs were added to the rearing and parasitized eggs were removed and placed in incubation containers (5 cm dia.; 10 cm length). After about 10 days, newly emerged adults from these containers were transferred back into the rearing tube. The parasitoid species were maintained for at least 50 generations on *E. kuehniella* eggs before experimentation.

For the experiments, only female wasps were used. Females and males were distinguished under a stereomicroscope by their dimorphic antennae. In females, the last flagellomeres are usually prominently enlarged, creating an apical club. In males, the antennomere number is often higher and the shape filiform (see Colazza et al. 2009). Females were between 6 and 12 h old and mated. In *Trichogramma* spp., mating takes place just after the female emerges (Waage and Ming 1984). One hour before the start of the experiment, females were isolated in small glass tubes. All experiments took place in climate chambers (22 ± 1 °C, $70 \pm 10\%$ RH, 16:8 h L:D).

Parasitism capacity of *Trichogramma* spp.

We first characterized the parasitism capacity of the three *Trichogramma* species. The experiments were conducted in glass vials, where single females received approximately 400

eggs of *E. kuehniella* daily for 9 days. Parasitism, fertility and sex ratio of offspring were recorded. A detailed description of the experiments and the results can be found in the supplementary electronic material.

Conditioning procedures

The *Trichogramma* spp. females used in the experiments were allowed to gain experience with tomato and host cues. Different conditioning procedures were implemented (Fig. 1).

Emergence learning (EL)

Parasitoids were reared in containers covered with a fine mesh (5 cm dia.; 10 cm length) for one generation on *T. absoluta* eggs on tomato leaflets (5 ± 2 cm length, freshly cut from 3 weeks old plants). The eggs had been laid by *T. absoluta* onto the tomato leaves within 24 h before the assay. To assure the presence of cues from freshly infested tomato plants during the entire parasitoid development, the containers were placed inside cages with *T. absoluta*-infested tomato plants until parasitoid emergence. After emergence, the parasitoids remained in the cage for 6 to 12 h until used in the experiments.

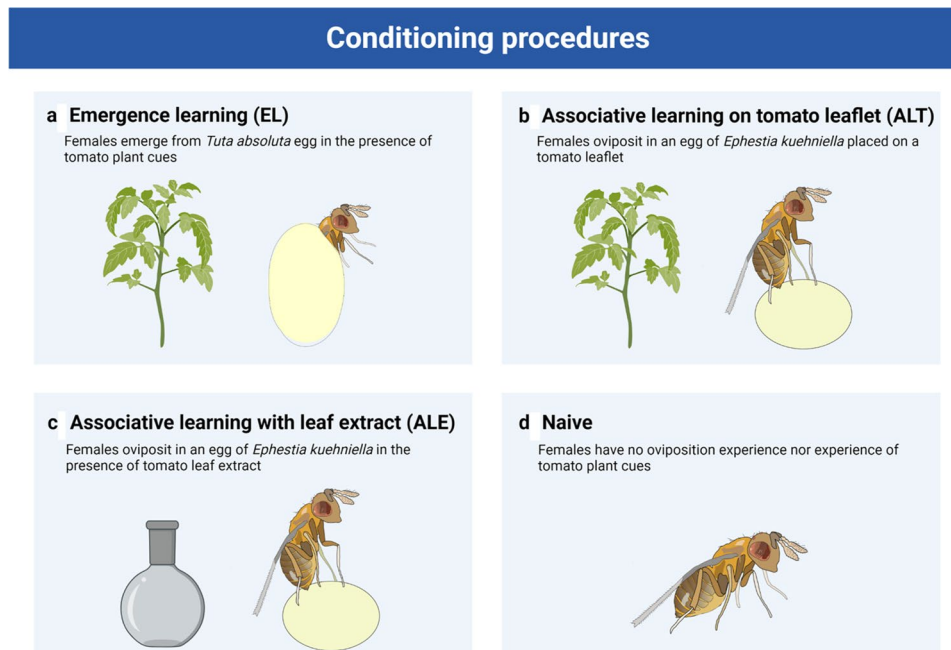


Fig. 1 The *Trichogramma* spp. females were allowed to gain experience with tomato and host cues before the experiments. Different conditioning procedures were used. **a** Emergence learning (EL): parasitoids were reared for one generation on *T. absoluta* eggs on tomato leaflets. **b** Associative learning on tomato leaflet (ALT): female wasps experienced oviposition on *E. kuehniella* eggs placed on a tomato

leaflet. **c** Associative learning with a tomato leaf extract (ALE): female wasps experienced oviposition on *E. kuehniella* eggs placed on a filter paper with tomato leaf extract. **d** Naive: control female wasps were reared on *E. kuehniella* eggs without any exposure to *T. absoluta* or tomato cues. This figure was created with BioRender.com

Associative learning on tomato leaflet (ALT)

0.5 to 1 h before the experiment, female wasps experienced oviposition on *E. kuehniella* eggs placed on a tomato leaflet. Approximately 30 eggs of *E. kuehniella* were placed on a freshly cut leaflet from an uninfested tomato plant in a Petri dish (5 cm dia., 1 cm height). Single parasitoid females randomly selected from the rearing were placed on the leaflet and allowed to oviposit. Oviposition (i.e., egg stung with the ovipositor) was confirmed under the stereomicroscope. Immediately after the first oviposition, the females were individually collected with a glass aspirator and transferred into a glass vial until used in the experiments. Females that did not oviposit within 15 min. were discarded (approximately 5%). When testing the memory retention in a separate experiment, experienced females were kept for 24 h before used.

Associative learning with a tomato leaf extract (ALE)

The same procedure as described above was conducted except that, instead of a tomato leaflet, a filter paper (5 cm dia.) with tomato leaf extract was used. The extract was prepared by soaking 3.5 g of tomato leaves for 12 h in 20 ml of solvent (diethyl ether or hexane, Sigma-Aldrich Chemie GmbH, Germany $\geq 95\%$ purity). The solution was filtered through a cotton to remove the leaves and gently heated in a water bath at 60 °C to reduce the volume to 3.5 ml, corresponding to 1 g of tomato leaf per 1 ml extract. The filter paper was placed under the fume hood, 400 μ l of extract were added and the solvent was allowed to evaporate for 5 min before the conditioning. The filter paper was exchanged between each female conditioning process.

Naive

Parasitoids were reared on *E. kuehniella* eggs without any exposure to *T. absoluta* or tomato cues. They served as control in the experiments.

Olfactometer assays

Experimental setup

To test the arrestment response of the *Trichogramma* spp. females to the cues of infested tomato leaves, we used a static four-chamber olfactometer (10 cm dia.), which consisted of a bottom with four chambers, which were covered with a fine mesh providing a walking arena (height 0.5 cm), and a lid made of a glass plate (Collatz et al. 2006). The walking arena was divided in four sectors corresponding to the four olfactometer chambers. The mesh prevented access of the parasitoids to the chambers. The experiments took

place in a climate chamber at 22 °C and 70% RH. A freshly cut tomato leaflet infested with approximately 30 *T. absoluta* eggs (< 24 h old) was placed in one sector of the olfactometer. Factitious leaflets made of green paper of the same size were placed in each of the other sectors. The olfactometer was placed in a cardboard box illuminated from the top to avoid any directional light attraction. One *Trichogramma* spp. female at a time was placed on the walking arena and allowed to settle for 5 s. Subsequently, the time spent on each of the four chambers was recorded for 5 min using the Software the Observer XT11 (Noldus, Netherlands). The positions of the factitious and tomato leaflets were swapped between each replicate to minimize location bias. Every hour, new tomato and factitious leaflets were used. After each series of observation, the chambers were cleaned with 98% Ethanol to remove any remaining volatiles.

Experimental design

In the first experiment, we tested the arrestment response of female *T. achaeae* after the following conditioning procedures: (a) EL; (b) ALT; (c) ALT after 24 h. We evaluated in parallel if ALT females discriminate between infested and uninfested leaves. We arranged a choice test by placing an infested tomato leaflet into the test chamber and an uninfested leaflet in the control chamber of the olfactometer. In the second experiment, we tested the response of female *T. achaeae* after the following conditioning procedures: (a) ALE with diethyl ether; (b) ALE with hexane. Finally, in the last experiment, we tested the response of female *T. evanescens* and *T. dendrolimi* after the following conditioning procedures: (a) EL (b) ALT. For each assay, naive wasps were tested as control.

Just after being used in the experiment, all naive and EL females were offered *E. kuehniella* eggs for a maximum of 15 min. Females that did not oviposit were discarded (approximately 5% across groups). This was done to minimize the bias in the associative learning treatments by selecting only females motivated to oviposit during the first 15 min conditioning period before the experiment—the final sample sizes range from 16 to 28 individuals across groups.

Efficacy assays in tubes

Experimental setup

Experiments were conducted with all three *Trichogramma* species to test whether the conditioning leading to an increased arrestment response in the olfactometer also increases parasitism efficacy compared to naive females. Mated parasitoid females of the conditioning treatments that resulted in arrestment response in the olfactometer were placed individually in glass tubes (3 cm dia., 12 cm height)

closed with a lid ventilated with a fine mesh and containing a single tomato leaflet with ten *T. absoluta* eggs. The eggs were collected from infested leaves and transposed at an equal distance (approximately 5–10 mm) on the abaxial side of a fresh tomato leaflet directly by hand using a fine wet pencil. The leaflet stem was placed into a solution of agar water (8 g:1 l) to provide moisture.

Experimental design

Based on the results from the olfactometer experiments, we tested ALT females for *T. achaeae* and *T. evanescens* and EL females for *T. dendrolimi*. *Trichogramma achaeae* females were allowed for 2 h to parasitize the offered *T. absoluta* eggs, whereas, for *T. evanescens* and *T. dendrolimi*, the period was extended to 6 h since they required more time to start parasitization. In the end, the females were removed, and the tubes were incubated. After five days, the eggs parasitized (visible as blackened eggs) were counted. Between 25 and 30 replicates were conducted per parasitoid species and treatment. The experiments took place in a climate chamber at 22 °C, 70% RH, and artificial light. For each assay, naive wasps were tested as control.

Efficacy assays in mesh cages

Experimental setup

Mesh cage assays were conducted with *T. achaeae* to assess whether the conditioning leading to increased parasitism in the tube also increased the efficacy at a larger scale. Ten mated ALT females were placed together in a mesh cage (15 cm dia.; 30 cm height) containing a tomato compound leaf with seven leaflets. Twenty *T. absoluta* eggs were transposed on each leaflet (2 to 3 eggs per leaflet at an equal distance) on the abaxial side directly by hand using a fine wet pencil. The leaf stem was placed into a solution of agar water to provide moisture.

Experimental design

T. achaeae females were allowed four days to parasitize the eggs. The eggs parasitized were counted four days later. Twenty replicates each were conducted for ALT females and naive females as control. The experiments took place in a climate chamber at 22 °C, 70% RH and 16:8 h L:D.

Statistical analysis

For statistical analysis, the computer software NCSS (2020) (NCSS, LLC, US) was used. Data were tested for normal distribution using the Shapiro–Wilk test, and visual inspections of the data were made using Q–Q-plots. Since data

were not normally distributed, we used a Wilcoxon signed-rank test to analyze the arrestment response of parasitoids to infested tomato leaves, i.e., to compare the time spent above the chamber with the infested leaflet to the time spent above the opposite chamber of the four-chamber olfactometer. For the efficacy tests, data were normally distributed, and variance was homogenous (Modified-Levene equal variance test). We considered the females laying zero eggs during the time allowed as not responding. A chi-square test of independence was performed to examine the relation between the treatment and the number of females not responding during the time allowed. For females that had oviposited, a two-sample unpaired *t* test of equal variance was used to compare the mean number of eggs parasitized between the treatments. For all analyses, we considered a *p* value lower than 0.05 as statistically significant.

Result

Olfactometer assays

In all three *Trichogramma* species, naive females spent more time on the control chamber than the chamber containing the infested tomato leaflet (Figs. 2, 3, 4). However, this deterrence from the tomato leaflet was only significant in two trials with female *T. achaeae* (Wilcoxon signed-rank test; $z=2.05$, $p=0.039$, Fig. 2b; $z=2.46$, $p=0.013$, Fig. 3a).

Trichogramma achaeae

EL females spent more time on the control chamber than on the test chamber containing the infested tomato leaflet (Fig. 2a). This difference, however, was not significant. In contrast, ALT females spent significantly more time on the test chamber than on the control chamber ($z=3.12$, $p=0.002$; Fig. 2b). When given a choice between an infested and an uninfested tomato leaflet, ALT females did not show any difference in searching time ($z=0.37$, $p=0.708$; Fig. 2b). When tested 24 h after the learning experience, ALT females spent numerically but not significantly more time on the test chamber than on the control chamber ($z=1.34$, $p=0.178$; Fig. 2c). When tomato leaves were extracted with diethyl ether, ALE females spent significantly more time on the test chamber containing the plant extract than on the control chamber ($z=2.21$, $p=0.002$; Fig. 3a). However, the females did not respond to the test chamber when tomato leaves were extracted with hexane (Fig. 3b).

Trichogramma evanescens and *T. dendrolimi*

ALT female *T. evanescens* spent significantly more time on the chamber containing an infested tomato leaflet than

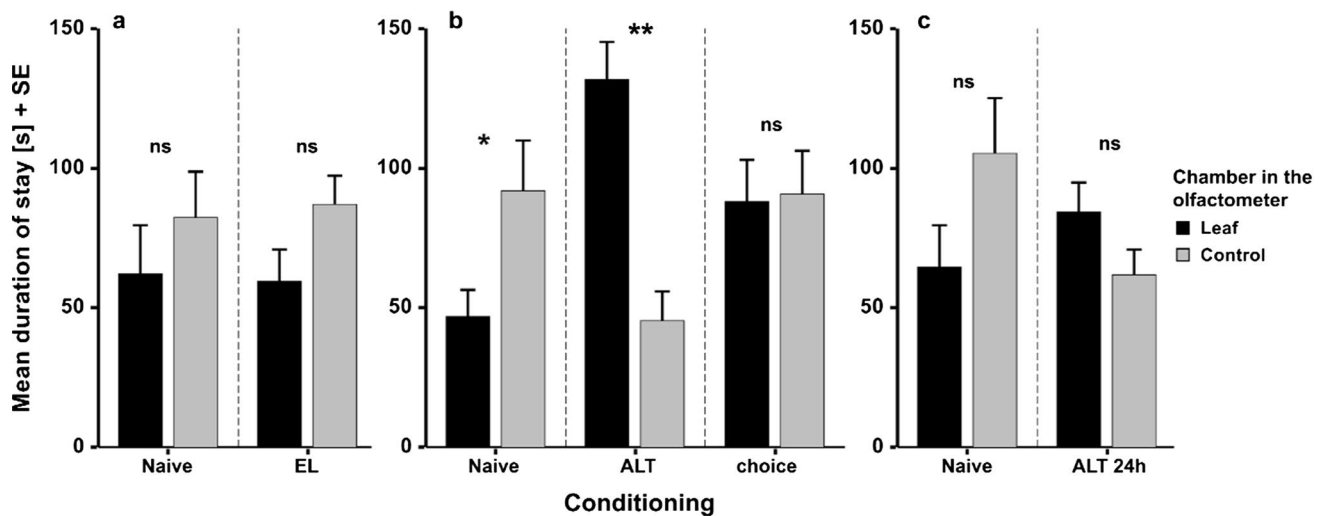


Fig. 2 Walking time (mean+SE) of female *Trichogramma achaeae* in the odor chambers of a four-chamber olfactometer during 5 min. after different conditioning. One chamber contained a tomato leaflet infested with *Tuta absoluta* eggs, and the opposite control chamber a factitious leaflet made from green paper. **a** Naive=control ($n=18$); EL=Emergence learning, ($n=20$). **b** Naive=control ($n=20$); ALT = Associative learning on tomato leaflet ($n=28$); ALT choice

= Associative learning on tomato leaflet ($n=20$). Instead of a factitious paper leaflet, an uninfested tomato leaflet was placed in the control chamber of the olfactometer. **c** Naive=control ($n=20$); ALT 24 h = Associative learning on tomato leaflet happened 24 h before the experiment ($n=20$). ns. not significant; * $P<0.05$; ** $P<0.01$ (Wilcoxon signed-rank test)

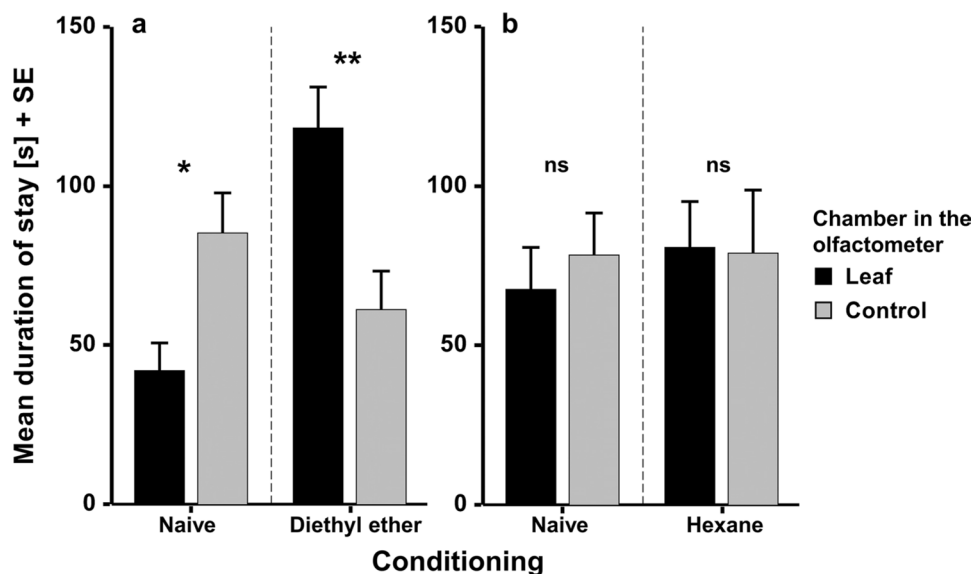


Fig. 3 Walking time (mean+SE) of female *Trichogramma achaeae* in the odor chambers of a four-chamber olfactometer during 5 min. after different conditioning. One chamber contained a tomato leaflet infested with *Tuta absoluta* eggs, and the opposite control chamber a factitious leaflet made from green paper. **a** Naive=control ($n=20$);

Diethyl ether= Associative learning on tomato extract (ALE females) using diethyl ether as solvent ($n=20$). **b** Naive=control ($n=22$); Hexane= Associative learning on tomato extract (ALE females) using hexane as solvent ($n=15$). ns. not significant; * $P<0.05$; ** $P<0.01$ (Wilcoxon signed-rank test)

on the control chamber ($z=2.68$, $p=0.007$; Fig. 4a), while EL female *T. evanescens* spent similar time on the test and control chambers (Fig. 4a). On the opposite,

ALT female *T. dendrolimi* spent similar time on the test and control chambers (Fig. 4b), while EL female

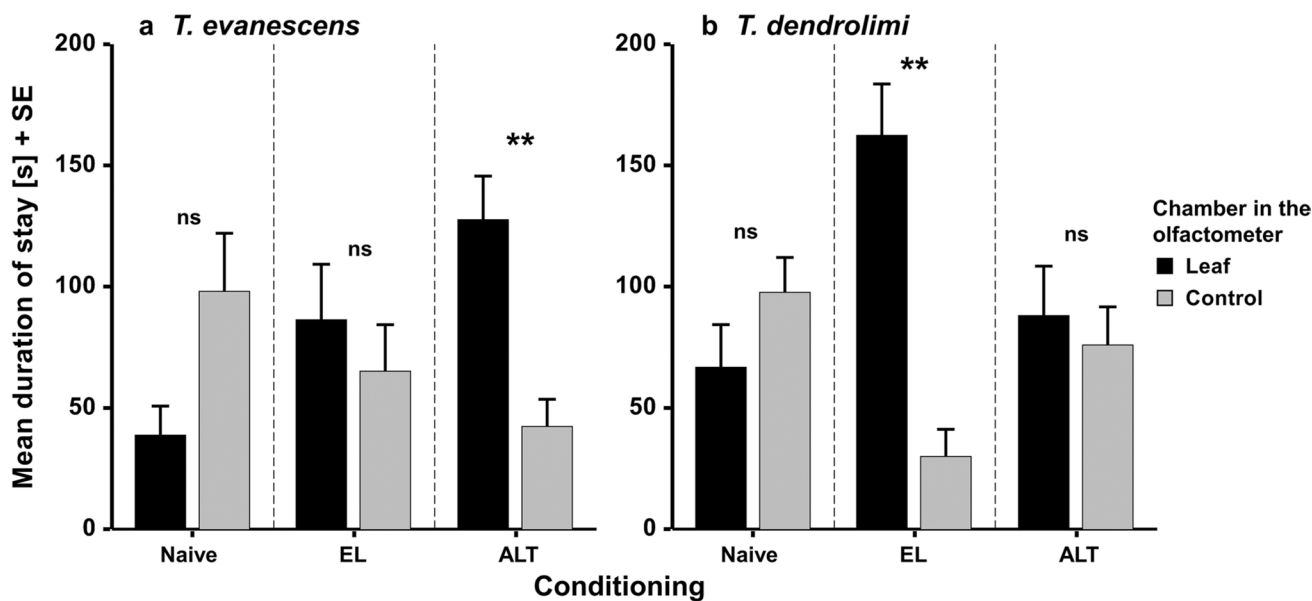


Fig. 4 Walking time (mean+SE) of female *Trichogramma evanescens* and *dendrolimi* in the odor chambers of a four-chamber olfactometer during 5 min. after different conditioning. One chamber contained a tomato leaflet infested with *Tuta absoluta* eggs, and the opposite control chamber a factitious leaflet made from green

paper. **a** Naive=control ($n=20$); EL=Emergence learning ($n=16$); ALT=Associative learning on tomato leaflet ($n=20$). **b** Naive=control ($n=20$); EL=Emergence learning ($n=16$); ALT=Associative learning on tomato leaflet ($n=20$). ns. not significant; $**P<0.01$ (Wilcoxon signed-rank test)

T. dendrolimi spent significantly more time on the test chamber than on the control chamber ($z=3.09$, $p=0.002$; Fig. 4b).

Efficacy test

Tube assays

When *Trichogramma* spp. females were allowed to parasitize *T. absoluta* eggs on a tomato leaflet, ALT female *T. achaeae* were more likely than naive females to parasitize at least one egg ($X^2=9.36$, $df=1$, $N=52$, $p=0.002$); of ALT females, 88% parasitized one or more eggs whereas only 48% of the naive females did. In addition, ALT females parasitized significantly more eggs than the naive ones (unpaired t test of equal variance, $t=2.24$, $N=33$, $p=0.03$; Fig. 5a). In contrast, ALT female *T. evanescens* did not parasitize more eggs than the naive females ($t=-0.19$, $N=34$, $p=0.842$; Fig. 5b); 48% ALT females and 56% naive females parasitized one or more eggs ($X^2=0.138$, $df=1$, $N=61$, $p=0.709$). Also, no difference was observed in the number of eggs parasitized by the naive and EL female *T. dendrolimi* ($t=0.57$, $N=40$, $p=0.567$; Fig. 5c); 66% EL females and 73% naive females parasitized one or more egg ($X^2=0.317$, $df=1$, $N=60$, $p=0.573$).

Mesh cage assay

When *T. achaeae* were allowed to parasitize *T. absoluta* eggs on a compound tomato leaf, ALT females parasitized significantly more eggs than the naive ones ($t=2.9$, $N=31$, $p=0.007$; Fig. 5d). Overall, 80% of females in both treatments (ALT and naive) parasitized at least one egg.

Discussion

Our study provides clear evidence for three species of *Trichogramma* that they can learn odor cues from the host-plant complex of tomato plants infested with *T. absoluta* eggs. While in *T. achaeae* and *T. evanescens*, associative learning resulted in an increased arrestment response in a four-chamber olfactometer, the primary learning mechanism in *T. dendrolimi* was emergence learning. Furthermore, in *T. achaeae*, but not in the other two species, the increase in arrestment response translated into a higher parasitism efficiency of *T. absoluta* eggs on tomato in small-scale laboratory assays.

The odor of tomato leaves infested with *T. absoluta* eggs innately repelled naive females of all three *Trichogramma* species. This is likely one reason explaining the low suitability of the tomato crop for supporting *Trichogramma* spp. parasitoids (Giorgini et al. 2019). That naive

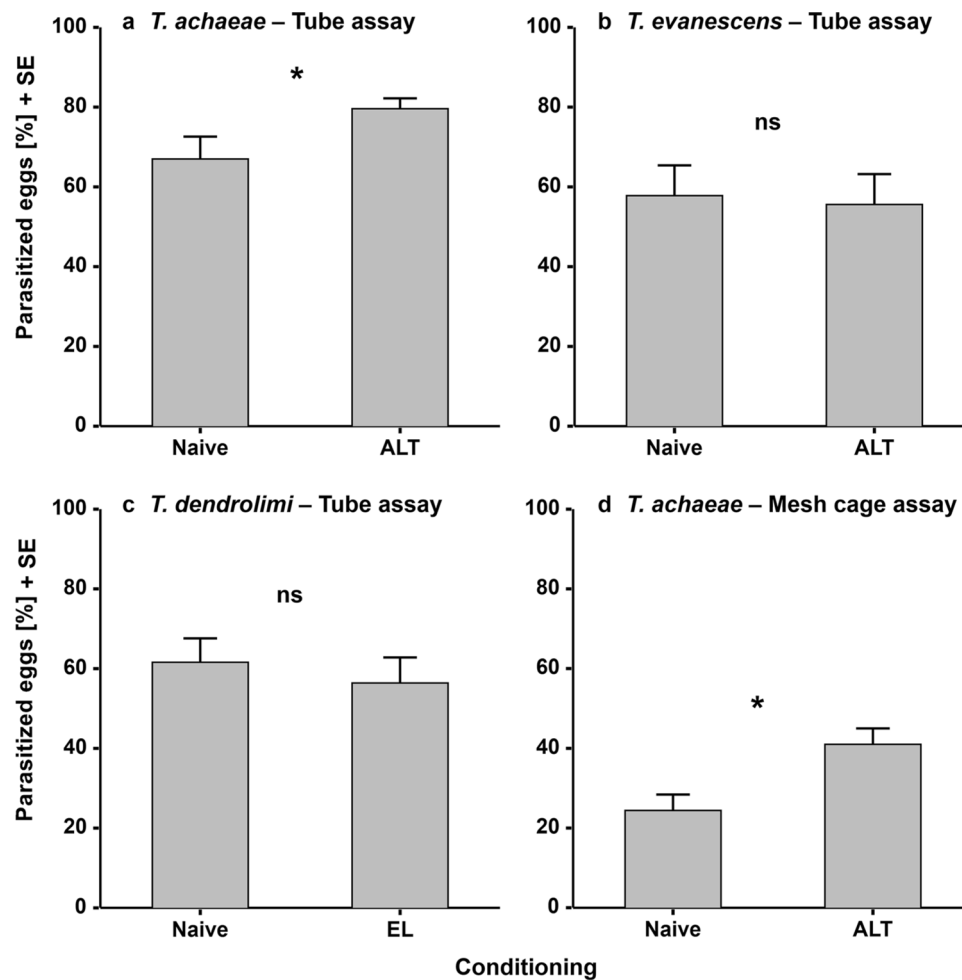


Fig. 5 Parasitism of *Tuta absoluta* eggs under laboratory conditions. Mean percentage (+SE) of parasitized *T. absoluta* eggs per *Trichogramma* species and conditioning. The tube assay consisted of a single tomato leaflet (with 10 eggs) and a single female per replicate; the mesh cage assay of a compound tomato leaf (seven leaflets with 20 eggs) and 10 females per replicate. **a** Number of eggs parasitized after 2 h by *Trichogramma achaeae* in tube assay; Naive=control ($n=27$); ALT=associative learning on tomato leaflet ($n=25$). **b**

Number of eggs parasitized after 6 h by *Trichogramma evanescens* in tube assay; Naive=control ($n=31$); ALT=associative learning on tomato leaflet ($n=30$). **c** Number of eggs parasitized after 6 h by *Trichogramma dendrolimi* in tube assay; Naive=control ($n=30$); EL=Emergence learning ($n=30$). **d** Number of eggs parasitized after 4 days by *T. achaeae* in mesh cage assay; Naive=control ($n=16$); ALT=associative learning on tomato leaflet ($n=16$). ns. not significant; * $P < 0.05$ (two-sample unpaired t test of equal variance)

parasitoids are repelled by particular plant odors has been reported before. For example, Romeis et al. (1997) showed that female *Trichogramma chilonis* Ishii were repelled by volatiles from pigeon pea (*Cajanus cajan*) plants in the reproductive stage. It has been hypothesized that repelling odors will likely be found in systems where the interactants have not coevolved closely (Romeis et al. 1997). In our study, the three strains tested likely have no common origin with *T. absoluta*, which is native to South America (CABI 2021, Invasive Species Compendium). Corroborating this hypothesis, *Trichogramma pretiosum* Riley, from the area of origin of *T. absoluta*, have been reported to innately respond to volatiles from tomato (Nordlund et al.

1985). Therefore, repellent volatiles might be a limiting factor for using new associations in biological control.

Previous studies in y-tube olfactometers with *Trichogramma* spp. and tomato plants infested or not by *T. absoluta* have reported contradictory results. Gontijo et al. (2019) found that naive *T. achaeae* females were attracted to volatiles produced by tomato, regardless of their infestation status. This, however, could not be confirmed by Milonas et al. (2019). Another study has found that *T. evanescens* were innately attracted to cues of infested tomato plants (Alsaedi et al. 2016). Our results cannot directly be compared to those previous studies since entirely different setups were used. A repellency or arrestment response is difficult

to detect in a y-tube olfactometer since non-responsive individuals are excluded. Moreover, since multiple females were tested simultaneously in the studies by Alsaedi et al. (2016) and Gontijo et al. (2019), an aggregation effect cannot be excluded. Another explanation for the observed differences resides in the different strains used. It is acknowledged that biological traits can differ significantly among strains of the same *Trichogramma* species (Hoffmann et al. 2001; El-Wakeil 2007; Chailleux et al. 2012).

Our olfactometer assays showed that the learning behavior is species-specific. Female *T. dendrolimi* learned the cues of the host-plant complex at emergence; female *T. evanescens* likewise, but to a lesser extent. However, female *T. achaeae* did not learn the cues from the host-plant complex during development or emergence. This latter result contrasts with a study by Cascone et al. (2015), who reported that rearing *T. achaeae* on *T. absoluta* eggs laid on tomato plants for one generation significantly improved the attack rate against *T. absoluta*. However, in this study, the attack rate was tested in small-scale assays, and the eggs were not provided on tomato leaves.

It is generally accepted that egg parasitoids can learn odors from the host-plant complex at the time of emergence (Dukas and Duan 2000; Meiners et al. 2005; Pashalidou et al. 2010). However, few studies have compared this learning process between species. Like our result, Bjorksten and Hoffmann (1995) found that the rearing host's effects were weak or absent in the Australian egg parasitoid, *Trichogramma* nr. *ivelae* Pang & Chen. In contrast, the host chosen initially for oviposition (associative learning) substantially impacted the later host preference. However, another study found that when reared on a tomato plant, *Trichogramma brassicae* Bezdenko modified their behavior, prolonging searching time compared to individuals that emerged from the same host eggs placed directly in glass vials (Bjorksten and Hoffmann 1998) demonstrating that even in the same genus, species may show very different learning mechanisms. In our study, it is possible that another rearing host than *E. kuehniella* may have influenced the response of *T. dendrolimi* since this species was attracted by the tomato plants after having been reared for just one generation on *T. absoluta* eggs. We think that this is less likely for the two other species since they did not respond positively to a change of hosts.

Associative learning increased the arrestment response of female *T. achaeae* and *T. evanescens* to *T. absoluta*-infested tomato leaves. Since naive females showed no arrestment response and did not learn the host plant's odor at emergence, associative learning seems to be the key to improving their arrestment response to the tomato plant's odor. This finding is consistent with the results from previous studies. *Trichogramma* species have been reported to learn different components of host plant volatiles by associative learning

and to adapt their behavior accordingly (Pashalidou et al. 2010; Wilson and Woods 2016). *Trichogramma* spp. have many different hosts. Therefore, their learning ability corresponds to the generalist theory, which declares that generalist parasitoids, compared to specialists coevolved with a specific host, rely more strongly on learning plant cues to adjust their foraging behavior (Vet and Dicke 1992; Steidle and Van Loon 2003). Overall, the low innate response to tomato plants and the learning ability match the environmental unpredictability hypothesis, suggesting that it is best to learn odor cues unique to a specific time and place (Dukas 2008) rather than evolve innate responses (Papaj and Prokopy 1989). In contrast to *T. achaeae* and *T. evanescens*, the behavior of *T. dendrolimi* was not affected by associative learning. For associative learning, the small *E. kuehniella* eggs might have been considered a low-quality host for the relatively large *T. dendrolimi*. Indeed, female *Trichogramma* spp. discriminate between high- and low-quality hosts. They often visit and oviposit in fresh eggs of high-quality and use the old ones of low-quality for feeding (Kishani Farahani et al. 2015). We assume all eggs used in our experiments were adequately fresh. In our study, the associative learning experience in *T. achaeae* females was weaker after 24 h than after 6–12 h. Memory is sensitive to differences in conditioning, such as the number and type of conditioning trials, and more subtle differences, such as reward value (Kruidhof et al. 2012). Thus, only one conditioning trial is likely not enough to produce a long-lasting memory. Numerous and spaced training often leads to longer forms of memory in parasitic wasps, but there are also exceptions where a single training resulted in memory lasting for five and more days (Collatz et al. 2006).

Many parasitoids of herbivorous insects rely on odors emitted by plants (volatile organic compounds—VOCs) to find hosts (McCormick et al. 2014). Firstly, plants are in general large and produce more volatiles compared with host insects. Secondly, plants may produce volatiles in response to herbivory and egg deposition to alert natural enemies (Heil 2008). Interestingly in our study, female *T. achaeae* showed no difference in arrestment response between *T. absoluta* egg-infested and uninfested tomato leaves. This finding corresponds to a recent study by Gontijo et al. (2019). The same result was also found for other natural enemies of *T. absoluta*, such as the mirid predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) (Naselli et al. 2017). However, there is evidence that egg deposition from *T. absoluta* on tomato induces volatiles (Faria et al. 2008; Gontijo et al. 2019). Likely, the low amount of volatiles produced by a single *T. absoluta* egg-infested leaflet or the fact that the leaflet used in our experiment had been cut off from the plant may have impaired the ability of *T. achaeae* females to distinguish between infested and uninfested leaves. Laboratory studies have shown that

a higher density of *T. absoluta* infestation results in releasing larger amounts of VOCs (Silva et al. 2017), which arrested more *Trichogramma* spp. parasitoids (Alsaedi et al. 2016).

To disentangle the different stimuli learned within the host-plant complex was not the particular aim of our study. During emergence learning, females may have learned the tomato plant's cues from remainders of the host, induced volatiles from egg deposition, or everything simultaneously. Also, to conduct associative learning, we used factitious eggs and not eggs from *T. absoluta*. Therefore, we might have obtained a better result in our efficacy test if eggs of *T. absoluta* were used for the conditioning. However, we think this is unlikely and that, in general, *Trichogramma* spp. are more prone to learn stimuli of the plant than the host egg. For instance, Kaiser et al. (1989) showed that *T. brassicae* (as *Trichogramma maidis* Pintureau and Voegelé) can learn the odor of maize extract through associative learning but cannot learn the odor of host eggs or the sex pheromone of the maize pest, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae). Since eggs produce no long-range cues and parasitoids rely primarily on the host plant cues for long-range detectability (reviewed by Colazza et al. 2009), we speculate that the tomato plant's odor is the strongest or only stimuli in this learning process.

While primarily laboratory-based evidence shows that parasitoids can learn cues of the plant-host complex, few attempts have been made to link it to efficacy (Kruidhof et al. 2019). Our result demonstrates that for *T. achaeae*, associative learning increases efficacy at the leaf and plant level under small-scale, confined conditions. Conditioning the parasitoids before their release in a biological control program might thus improve their efficiency against *T. absoluta* in the field. However, even under the confined conditions, *T. achaeae* did not achieve more than 50% parasitism of host eggs within a four days period. Whether this results from a lack of short range host detection cues as discussed above or points to a limited efficacy of *T. achaeae* in the tomato system in general remains to be determined. A comparable link between conditioning and efficacy was reported by Papaj and Vet (1990) where *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) experiencing the host habitat odor in the presence of *Drosophila* spp. host larvae had a 3–4 times higher chance of finding a host habitat than inexperienced females. Furthermore, *T. achaeae* females responded to diethyl ether extracts of tomato leaves, but not to hexane extracts, indicating that the plant odor cues involved have a low molecular weight and are water-soluble compounds (Schultz et al. 1977). Therefore, future studies should prefer diethyl ether as solvent to extract the tomato plant odor cues. The plant extracts might ultimately be used to prime parasitoids before their release in a biological control program to increase their efficacy.

In contrast to *T. achaeae*, associative learning did not increase the parasitism rate of *T. evanescens* in our tube experiment, nor did emergence learning for *T. dendrolimi*. Possibly, these two species have a lower capacity to cope with the tomato trichomes than *T. achaeae* or are more prone to reject *T. absoluta* eggs as host. *Trichogramma* spp. are known to be less effective on plants bearing trichomes (Romeis et al. 2005); therefore, females may have been arrested by the tomato leaf in the olfactometer but may have avoided foraging on tomato leaves or being hampered in their movement when released into tubes. Furthermore, Roriz et al. (2006) showed that *Trichogramma* spp. prefer larger eggs as hosts. Eggs of *T. absoluta* are much smaller than the *E. kuehniella* eggs used in the rearing. Thus, *T. evanescens* and *T. dendrolimi* may have avoided *T. absoluta* eggs for the simple reason that they were too small.

Currently, *T. achaeae* is mass-reared and commercially available for use against *T. absoluta* in Europe. However, the low suitability of the tomato - *T. absoluta* system for supporting *Trichogramma* spp. parasitoids results in high costs limiting their use (Cabello et al. 2009; Chailleux et al. 2012, 2013). Furthermore, the effectiveness of parasitoids to locate their host might be constrained by an initially weak response to foraging cues emanating from the crop environment and an innate tendency for dispersal upon release (Kruidhof et al. 2019). Our findings confirm the tomato plant system's low suitability for commercial *Trichogramma* spp. releases in its current form. Since our efficacy tests with associative learning show promising results, we can conclude that learning is a possible means of improving this suitability and will likely help retain the parasitoid *T. achaeae* in the field after release. However, we showed that learning is species-dependent and must therefore be adapted for each parasitoid species. Moreover, we showed that *T. achaeae* and *T. evanescens* do not learn the host-plant complex's odor at emergence, which is considered an attractive characteristic for mass rearing since it does not condition them on factitious hosts (Bjorksten and Hoffmann 1995).

Trichogramma spp. have been used effectively in various parts of the world through inundative releases. China has achieved successful wide-scale use thanks to its *Trichogramma* spp. manufacturing. The country has industrialized mass-rearing systems for cost-effective *Trichogramma* spp. production, which depends on mechanized processes, complex expertise to manipulate diapause, optimal use of factitious hosts, genetic methods, and novel application approaches. In the country's biggest maize production region, 35% of the area relies on *Trichogramma* spp. releases to control corn borers (reviewed by Zang et al. 2021). So far, up to 24 species have been identified as potential biological control agents and can target over 120 different pests in China (Wang et al. 1998). Meanwhile, at least ten species have been further developed for field applications, such as

T. dendrolimi (maize, rice, cotton), *Trichogramma japonicum* Ashmead (rice), *T. chilonis* (sugarcane, rice, cotton), *Trichogramma ostriniae* Pang & Chen (maize), and *Trichogramma leucaniae* Pang & Chen (soybean) (Lin 1994; Wang et al. 1998). China is the largest tomato producer worldwide, and *T. absoluta* has made its apparition (Zhang et al. 2020a). The country will likely develop a *Trichogramma*-based control strategy against this devastating pest (Zang et al. 2021). Since the three species tested in the present study are commercially reared in China (Li 2007), our findings can be implemented to develop this strategy.

We believe that besides assessing associative learning on a larger scale, future research should look at how this associative process can be implemented for mass production and release. Since *Trichogramma* spp. parasitoids are shipped as pupae in parasitized eggs, conditioning of adults can only occur at the release site providing access to factitious hosts, i.e., sterile eggs or alternative hosts that are not harmful to the crop. Plant extracts can be a successful way of mimicking the crop odor if this is missing or not strong enough to elicit an associative response. For example, the producer could provide the farmers with the parasitoid pupae and a conditioning package that contains an alternative reward and plant extract (reviewed by Kruidhof et al. 2019).

In conclusion, our study provides strong support for the learning ability of *Trichogramma* spp., thereby contributing to our understanding of learning processes for the improvement of biological control programs. A substantial increase in the arrestment response to the odor of tomato plants can be achieved through conditioning; however, the learning process differs between *Trichogramma* species. Overall, associative learning is very likely to increase the efficiency of *T. achaeae* against *T. absoluta*. Our observations nevertheless create demand for field studies on the efficiency of such conditioning at a larger scale.

Authors' contributions

JG, JR and JC designed the research. JG led the trials. YZ and GZ provided insight information regarding the pest and the parasitoids in China. JG, JR and JC analyzed the data. JG, JR and JC wrote the manuscript. All authors read, edited and approved the manuscript.

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Data availability The complete raw data can be found at the Dryad repository. See (<https://doi.org/10.6084/m9.figshare.14747949>).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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