WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

Hitch-hiking parasitic wasp can associatively learn to exploit butterfly anti-aphrodisiac

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Abstract

Chemical cues play a significant role in interactions between living organisms. Pheromones are chemical signals that used between conspecific organisms and can be exploited by natural enemies. Males of the cabbage white butterfly species *Pieris brassicae*, *Pieris rapae* and *Pieris napi* transfer antiaphrodisiac pheromones during mating to render female butterflies less attractive to conspecific males. Parasitic *Trichogramma brassicae* wasps spy on the antiaphrodisiac pheromone benzyl cyanide (BC) of the large cabbage white butterfly *P. brassicae* and specifically hitch-hike with mated female butterflies and parasitize their freshly laid eggs. Naive *Trichogramma evanescens* wasps, however, do not distinguish between mated female, male and virgin female *P. brassicae* butterflies. This study investigated whether female *T. evanescens* wasps can learn to associate the odor of a mated female *P. brassicae* butterfly after a rewarding hitch-hiking experience with such a female and then also specifically hitch-hike with her through a combination of laboratory and field studies. In two-choice laboratory bioassays. I showed that female *T. evanescens* are able to learn to detect the butterflies' antiaphrodisiac and then specifically hitch-hike with mated female *P. brassicae* butterflies and not with males or virgins. It is also shown that *T. evanescens* smell the antiaphrodisiac in combination with other compounds in the female butterfly odor blend. *Trichogramma. evanescens* wasps innately recognize adult butterflies because naïve wasps do prefer to hitch-hike with mated females of its hosts *P. brassicae* and *P. rapae* over non-host insects. In the field, 424 *Pieris* butterflies were caught in Wageningen, the Netherlands. In total 4 female *T. evanescens* wasps were found on female and male butterflies of *P. brassicae*, *P. rapae* and *P. napi*. Overall the results indicate that the combination of spying on an antiaphrodisiac and specifically hitch-hiking with mated female butterflies can be learned by the parasitic wasp *T. evanescens*. The reason why this is an innate behavior in some egg parasitoid species (*T. brassicae*) and has to be learned in other species (*T. evanescens*) might be related to their host range in the field.

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1. Introduction

Communication, i.e. the exchange of information, plays an important role in the biology of all organisms (Wyatt, 2003). An organism can alter the behavior of another organism due to chemical signals (chemical communication). These signals are called semiochemicals (Law and Regnier, 1971). Semiochemicals induce behavioral or physiological responses such as searching for food or to locate a mate etc. There are two main categories of semiochemicals, namely pheromones and allelochemicals. In general, the organism that produces chemical signals is called emitter, and the organism that receives the signals, receiver. It is possible that there are more than one receivers and/or more than one emitter of semiochemicals (Wyatt, 2003). A variety of important semiochemicals for species communication have been identified. During the last decades many definitions were given to specify the term pheromones. The word "pheromone" comes from the Greek word "pherein" (φερειν), which means to transfer, and "hormone" (ορμονη), which means to stimulate or to excite. Karlson and Lüscher (1959) proposed to define pheromones as chemicals that are emitted by an organism to the environment and cause a reaction in another organism of the same species (intra-specific interactions). This definition was introduced to separate hormones, which are not secreted to the outside, from pheromones, which are secreted to the outside. Moreover, Law and Regnier (1971) proposed that pheromones are semiochemicals which interfere in chemical communication within a species (intra-specific interactions). They can be a single compound or a mixture of compounds. In 1988, Dicke and Sabelis defined a pheromone as "an infochemical that mediates an interaction between organisms of the same species in which the benefit is to the origin-related organism ([+, -] pheromone), to the receiver ([-, +] pheromone) or to both ([+, +] pheromone)". Pheromones are divided in categories such as: aggregation pheromones, sex pheromones, alarm pheromones, trail pheromones and anti-sex or antiaphrodisiac pheromones (Wyatt, 2003).

Allelochemicals are semiochemicals that interact between different species such as kairomones, allomones and synomones (inter-specific interactions) (Nordlund, 1981). Kairomones are allelochemical signals that benefit the receiver but not the emitter (Dicke and Sabelis, 1988). For example, adult *Drosophila melanogaster* females (emitter) use an aggregation pheromone, which is transferred from male to female during mating to attract conspecific flies (receiver) (Bartelt *et al.*, 1985; Wertheim *et* *al*., 2001). The parasitoid *Leptopilina heterotoma* (receiver), which parasitizes the larvae of *D. melanogaster*, however, eavesdrops on the aggregation pheromone of *D. melanogaster* (Wertheim *et al*., 1994; Delpuech, 2005). Allomones can favor the emitter but will induce a negative behavioral or physiological response in the receiver (Dicke and Sabelis, 1988). For example, plants like *Magnolia grandiflora* defend themselves against enemies by producing a broad range of secondary metabolites with anti-fungal, insect anti-feedant, activities. Synomones are allelochemicals that benefit both the emitter and the receiver (Dicke and Sabelis, 1988). One typical example of a synomone is when a plant emits volatiles that attract predators (Dicke and Sabelis, 1988) or parasitoids (Turlings *et al*., 1990) after a herbivore attack.

When natural enemies exploit pheromones of their prey or host, this is known as "chemical espionage" (Stowe, 1995). Chemical espionage plays an important role in interactions between egg parasitoids and their herbivorous hosts. Cabbage white butterflies of the genus *Pieris* (Lepidoptera; Pieridae) are known to use anti-sex pheromones (antiaphrodisiacs) that are transferred from male to female during mating, in order to enhance female monogamy by rendering females less attractive to other males (Andersson *et al*., 2003). The large cabbage white butterfly *Pieris brassicae* uses benzyl cyanide (BC) as an anti-aphrodisiac whereas the small cabbage white butterfly *Pieris rapae* uses a combination of methyl salicylate and indole (MeS + I). The green-veined white butterfly *Pieris napi* uses methyl salicylate (MeS) (Anderson *et al*., 2003). Egg parasitoids of the genus *Trichogramma* (Hymenoptera; Trichogrammatidae), that parasitize eggs of a wide variety of Lepidoptera and are therefore massively used as biocontrol agents against lepidopteran pests worldwide (Wajnberg and Hassan, 1994; Smith, 1996; van Lenteren, 2000), may, however, exploit these antiaphrodisiacs. In laboratory tests it has been shown that the tiny (± 0.5mm long) egg parasitoid *Trichogramma brassicae* can locate eggs of *P. brassicae* by eavesdropping on the antiaphrodisiac BC of mated female butterflies to hitch-hike with them to their egg-laying site and finally parasitize their freshly laid eggs (Fatouros *et al*., 2005a). This hitch-hiking behavior is called phoresy, i.e. when an organism hitch-hikes with another organism for means of transportation. For *T. brassicae* females this combination of spying on an antiaphrodisiac in combination with phoresy on a mated female butterfly is an innate behavior (Fatouros *et al*., 2005a). This fascinating behavior may be a common strategy in egg parasitoids. Female *Trichogramma evanescens* wasps show a different behavior than *T. brassicae*. Even though naïve *T. evanescens* wasps do climb onto their host *P. brassicae*, they do not discriminate between climbing onto mated female butterflies, virgin females and males. Naive *T. evanescens* also don't discriminate between the odors of a mated female, a male and a virgin female *P. brassicae* butterfly (Fatouros *et al*., 2007). However, *T. evanescens* might be able to specifically learn to recognize the odor of mated female butterflies after a positive experience. Schöller and Prozell (2002) have shown that *T. evanescens* can associatively learn to recognize the female sex pheromone ((Z, E)-9, 12-tetradecadenyl acetate (ZETA)) of pyralid moths after an oviposition experience in the presence of ZETA.

The aim of this thesis is to investigate whether female parasitic wasps *T. evanescens* are able to associatively learn to exploit a butterfly anti-aphrodisiac and specifically hitch-hike with mated female butterflies. I will test whether female *T. evanescens* wasps are able to recognize an antiaphrodisiac of *Pieris* butterflies after first hitch-hiking with mated female butterflies and then immediately parasitizing fresh butterfly eggs, and whether female wasps of this species are found on adult butterflies in nature.

2. Material and Methods

A. Laboratory experiments

Insects

Pieris brassicae and *Pieris rapae* (Lepidoptera: Pieridae) butterflies were reared under room conditions (21 ± 1°C, 50-70% r.h., L16:D8). Mated females and males were taken as a mating couple and used the next day after mating. Freshly emerged virgin butterflies were collected from the rearing chamber and kept separated until the day of the experiment.

Trichogramma evanescens (Hymenoptera: Trichogrammatidae) (iso-female strain GD011) was reared on *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs in a climate room. (25 ± 1 °C, 50-70% rh, L16:D8). Two-day old wasps were used for each experiment. The wasps were sexed under a microscope. The wasps that were used for the experiments were either naïve (N) or experienced. The types of experiences are described below:

(O): Oviposition experience: *T. evanescens* wasps had the opportunity to oviposit into freshly laid (<1 day old) eggs of *P. brassicae* or *P. rapae* on Brussels sprouts plants. The wasps were used one hour after the experience.

(H): Hitch-hiking experience: female *T. evanescens* wasps had the opportunity to climb on and then of a mated female *P. brassicae* butterfly. The wasps were used one hour after the experience.

(H+O): Hitch-hiking and oviposition experience: The wasps were given an oviposition experience (O) after a hitch-hiking experience (H) with mated female *P. brassicae* or *P. rapae* butterflies. Again, the wasps were used one hour after the experience.

2.1 Two-choice olfactometer bioassays

Here, the response of female wasps towards different butterfly odors is tested in a static two chamber olfactometer that is carefully described in Fatouros *et al.* (2005a) (Figure 1). Either two *P. brassicae* or four *P. rapae* butterflies were offered as an odor source per chamber. The female wasp was released in the middle of a decision area (Figure 2). The time that the wasps spent in the two odor fields was measured for 300 seconds. A number of 10-15 wasps were tested per day. In total 40 wasps were tested per combination. After 5 wasps the butterflies were replaced by new ones and after every 3rd wasp the olfactometer was rotated 180° in order to avoid biased results.
Similar to this, the response of female *T. evanescens* wasps to virgin female butterflies treated with synthetic antiaphrodisiac was examined. Virgin *P. rapae* females were painted with a 2µl solution of synthetic antiaphrodisiac in hexane or hexane only.
Virgin *P. brassicae* females were painted with a 10 µl solution of synthetic antiaphrodisiac in hexane or hexane only.

The test combinations were:

- <u>(O) *T. evanescens* on *P. brassicae* eggs</u>

Mated females VS males

- <u>(H) *T. evanescens* with *P. brassicae*</u>

Mated females VS males

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Mated females VS males

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Mated females VS virgins

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Virgin females *P. brassicae* painted with 10µl solution of 0.2µg/µl benzyl cyanide (BC) in hexane VS virgin females *P. brassicae* + 10µl hexane

- (N) *T. evanescens*

Mated females *P. brassicae* VS *P. rapae*

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Mated females *P. brassicae* VS mated females *P. rapae*

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Virgin females *P. rapae* painted with 2µl solution of 1µg/µl benzyl cyanide (BC) in hexane VS virgin females *P. rapae* + 2µl hexane

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. rapae*

Virgin females *P. brassicae* painted with 10µl solution of 0.2 µg/µl methyl salicylate (MeS) and indole (I) in hexane VS virgin *P. brassicae* + 10µl hexane

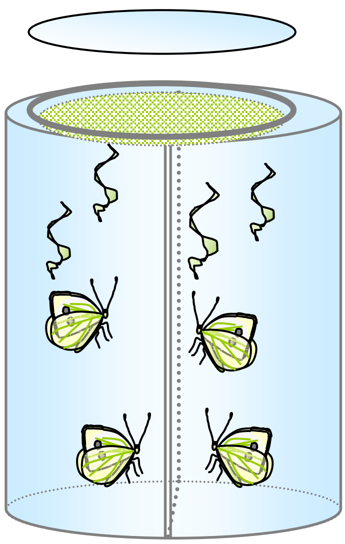

Figure 1: Two-choice olfactometer test. *Pieris brassicae* or *P. rapae* butterflies were placed into the olfactometer chambers as an odor source.

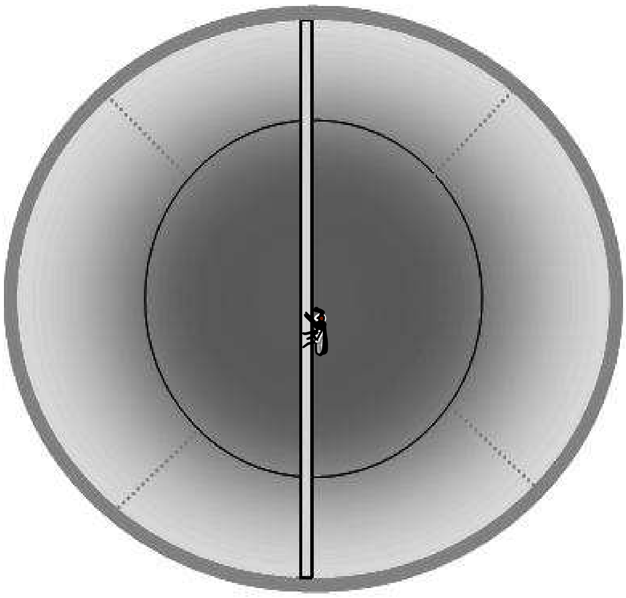

Figure 2: Top view of two-choice olfactometer test. The wasp was released in the middle of the two odor fields (decision area).

2.2 Two-choice mounting bioassays

The phoretic behavior of female wasps was tested in a mounting arena. Two butterflies were placed in the arena (9 cm high, 13.5 cm diameter). Afterwards, a wasp was released between the two butterflies (Figure 3) and was observed for 300 seconds. The time that the wasp climbed onto one of the two butterflies was measured (duration of phoresy). When the wasp did not climb on any butterfly within the 300 seconds it was reported as a “no response” observation. The body part of the butterfly where the wasp climbed onto was also reported. Before every experiment the butterflies were cooled down in the refrigerator in order to have inactive butterflies during the experiment. Forty climbing wasps were tested per combination. After 5 wasps the butterflies were replaced by new ones and after 5 wasps the position of the butterflies was changed, in order to avoid biased results. Moreover, mated female *P. brassicae* and *P. rapae,* were offered against young desert locusts of the species *Schistocerca gregaria* (Orthoptera: Acrididae). The locusts were chosen to be of similar size as the butterflies. They were frozen before the experiments.

The tests combinations were:

- (H +O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Mated females VS males

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Mated females VS virgins

- (N) *T. evanescens*

Mated females *P. brassicae* VS mated females *P. rapae*

- (H+O) *T. evanescens* with the odor of mated female butterfly *P. brassicae*

Mated females *P. brassicae* VS mated females *P. rapae*

- (N) *T. evanescens*

Mated females *P. brassicae* VS *S. gregaria*

- (N) *T. evanescens*

Mated females *P. rapae* VS *S. gregaria*

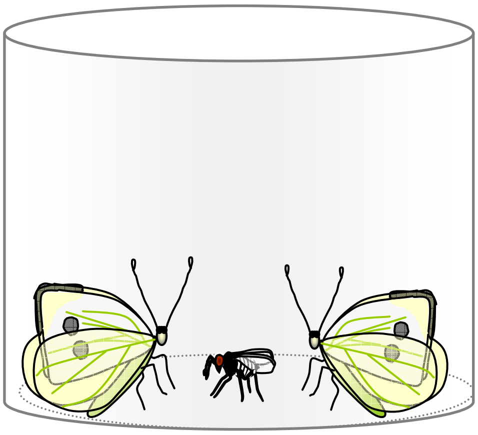

Figure 3: Two choice mounting bioassay where a wasp gets the opportunity to climb onto two butterflies.

2.3 One-choice mounting bioassays

I also investigated if hitch hiking is a female-specific strategy or also a male wasp strategy. Naive female and naive male wasps were given the opportunity to climb onto a mated *P. brassicae* female in an arena (9 cm high, 13.5 cm diameter). The behavior of the wasp was observed for 300 seconds. Whenever it climbed onto the butterfly the duration of phoresy was reported. In case the wasp didn't climb within the period of 300 seconds, the observation reported as a "no response". The body part of the butterfly where the wasp climbed onto was also reported. The butterfly was replaced by another one after every 5 wasps or when she started flying. Ten female and ten male *T. evanescens* wasps were tested per day until a total of 40 wasps of each sex. The butterflies were cooled down in the refrigerator (4˚C) in order to reduce their activity.

B. Field work - Butterfly catches

In the period from May to September 2007 cabbage white butterflies of *P.brassice*, *P. rapae* and *Pieris napi* were collected around Wageningen.

1 = Entomology garden, Wageningen

2 = Marijkeweg, Wageningen

3 = Pabstsendam, Wageningen

4 = Klein arboretum, Wageningen

5 = Groot arboretum, Wageningen

Figure 4: Wageningen map. Locations where butterflies were trapped

The butterflies were collected with a net containing a plastic vial in the bottom in order to avoid loosing a wasp while catching a butterfly (Figure 5). After each butterfly catch the plastic vial was replaced by another one.

Figure 5: Butterfly net with a plastic vial in the bottom in order to capture butterflies with hitch-hiking *Trichogramma* wasps.

The vials with the butterflies were placed in the refrigerator for an hour, in order to reduce butterfly activity. Afterwards, the butterflies species and sex were determined. The butterflies and the vials were then examined under the microscope for the presence of *Trichogramma* wasps. The wasps were put in small vials with 95% alcohol for molecular species identification based on the sequence of the internal transcribed spacer 2 (ITS-2) region of the rDNA (Stouthamer *et al.*, 1999). All butterflies were released back into the field again.

2.4 *Trichogramma* species identification

2.4.1 DNA extraction

The wasps that were stored in alcohol were dried on a filter paper. Afterwards, they were crushed in a 0.5 ml Eppendorf tube with a closed pasteur pipet. Then, 50 μl of Chelex solution (5%) and 4 μl of proteinase K (20mg/ml) were added. The samples were incubated overnight at 56 ºC, followed by 10 min at 95 ºC.

2.4.2 PCR amplification

For every sample a PCR reaction was performed. PCR reactions were performed in a 25 μl volume consisting of 18.43 μl of distilled water, 2.5 μl 10xPCR reaction buffer (HT Biotechnologies Ltd., Cambridge, UK), 2.5 μl DNA template, 0.5 μl dNTP (10mM), 0.5 μl forward primer, 0.5 μl reverse primer and 0.07 μl Taq polymerase (5 units/µl) (HT Biotechnologies Ltd., Cambridge, UK) within a 0.2 ml Eppendorf tube. The primers used to amplify the ITS-2 region were:

5’-TGTGAACTGCAGGACACATG-3’ (forward) and

5’ -GTCTTGCCTGCTCTGAG-3’ (reverse).

The PCR cycling program was 3 min at 94 ºC, 33 cycles of 40 sec at 94 ºC, 45 sec at 53 ºC and 45 sec at 72 ºC, followed by 10 min at 72 ºC after the last cycle. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide.

2.4.3 Cloning and sequencing

After conducting a PCR reaction and running the samples on an electrophoresis gel, the ITS-2 products were excised from the gel using the MinElute Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) for DNA fragment purification. The PCR fragment was ligated to a pGEM-T vector (Promega, Madison, WI, USA) and transformed into Escherichia coli xl2 cells (Stratagene, La Jolla, CA, USA). Correct insertion of the ITS-2 fragments was confirmed by PCR. To purify the plasmid, a GeneElute Plasmid Miniprep Kit (Sigma-Aldrich Chemie, Steinheim, Germany) was used. ITS-2 fragments were sequenced using an Applied Biosystems automatic sequencer. Finally, the ITS-2 sequences were aligned and matched against sequences present in GenBank.

C. Statistical analysis

Residence times of wasps in the two odor fields in the olfactory bioassays were compared with a Wilcoxon’s matched pairs signed rank test ($\alpha < 0.05$). A binomial test ($\alpha < 0.05$) was performed to compare the proportions of first mounts on the butterflies by wasps in the two-choice mounting bioassays. Comparisons between proportions of climbing male and female wasps were done with a Chi-square test with a 2x2 contingency table ($\alpha < 0.05$).

3 Results

A. Laboratory experiments

3.1 Two-choice olfactometer bioassays

It has been shown that naïve female *T. evanescens* wasps cannot discriminate among the odor of a mated female, a virgin female and a male *P. brassicae* butterfly (Fatouros *et al*., 2007).

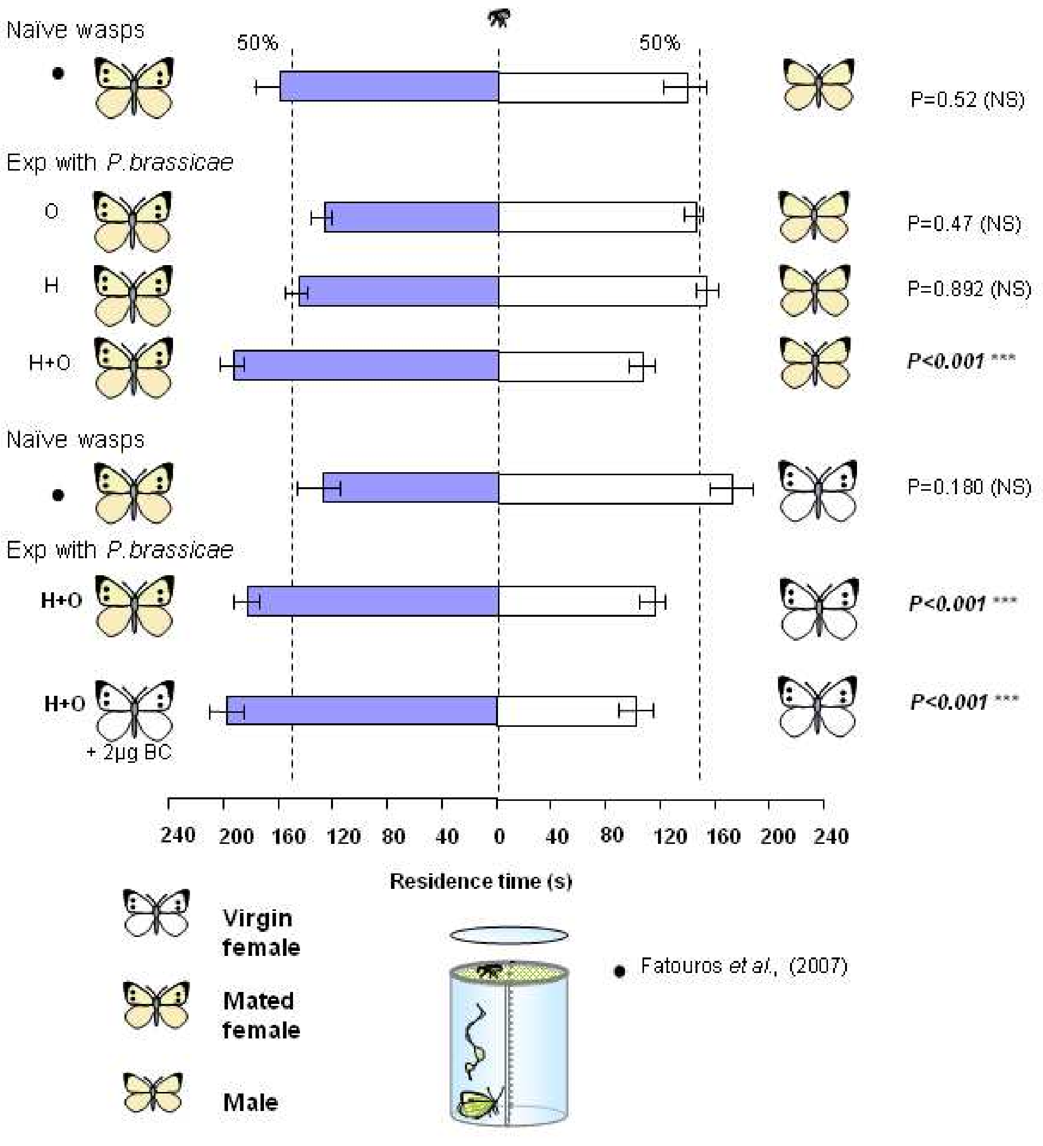

Figure 6: Olfactometer bioassay results. Mean residence time (± s.e.m.) that female wasps spent in two odour fields in a static olfactometer; *n=40* wasps tested per combination (Wilcoxon's matched pairs signed ranks test). O: Experienced female *T. evanescens* wasps, only with oviposition. H: Experienced female *T. evanescens* wasps, only with hitch-hiking with a mated female butterfly. H+O: The wasps were given an oviposition experience (O) after a hitch-hiking experience (H) with mated female butterflies. Asterisks indicate significant differences within the choice test; NS, not significant.

Female *T. evanescens* wasps do not respond to the odor of mated *P. brassicae* females after an oviposition experience (O) in freshly laid eggs of *P. brassicae,* when tested against the odor of male butterflies (P= 0.470, AVG: 145.2sec VS 154.8sec). In addition, the wasps cannot discriminate between the odor of male and mated female *P. brassicae* butterflies after a hitch-hiking experience (H) with a mated female butterfly (P= 0.892, AVG: 153.5sec VS 146.5sec). However, the wasps do show a significant preference for the odor of mated *P. brassicae* females after they had hitch-hiked with a mated *P. brassicae* female and oviposited into freshly laid eggs (H + O) when tested against male odor (P< 0.001, AVG: 194.0sec VS 106.0sec). The same experienced wasps (H + O) do also prefer the odor of the mated female *P. brassicae* compared to the odor of virgin female butterflies (P< 0.001, AVG: 184.5sec VS 115.5sec). Furthermore, *T. evanescens* female wasps, after an H + O experience, make a significant distinction between virgin *P. brassicae* butterflies treated with 2µg BC (synthetic antiaphrodisiac) and solvent-treated virgins (P< 0.001, AVG: 197.6sec VS 102.4sec) (Figure 6).

I also performed tests with 2 butterfly species (*P. brassicae* and *P. rapae*). Naïve female *T. evanescens* wasps cannot discriminate between the odors of mated females of *P. brassicae* and *P. rapae* (P= 0.750AVG: 143.5sec VS 156.6sec). However, the wasps do show a significant preference for the odor of mated *P. brassicae* females compared to the odor of mated *P. rapae* females after a rewarding hitch-hiking experience (H+O) with mated *P. brassicae* females (P= 0.001, AVG: 186.0sec VS 114.0sec). The same experienced wasps did not show a preference for the odor of virgin *P. rapae* females painted with the synthetic antiaphrodisiac of *P. brassicae* (2µl solution of 1µg/µl BC) over solvent treated virgin *P. rapae* females (P= 0.480, AVG: 145.6sec VS 154.6sec). Similarly, wasps that had an H+O experience with mated *P. rapae* females did not show preference for the odor of virgin *P. brassicae* females painted with the synthetic antiaphrodisiac of *P. rapae* (10µl solution of 0.2 µg/µl methyl salicylate (MeS) and indole (I)) over solvent-treated virgin *P. brassicae* females (P= 0.470, AVG: 154.6sec VS 145.6sec) (Figure 7).

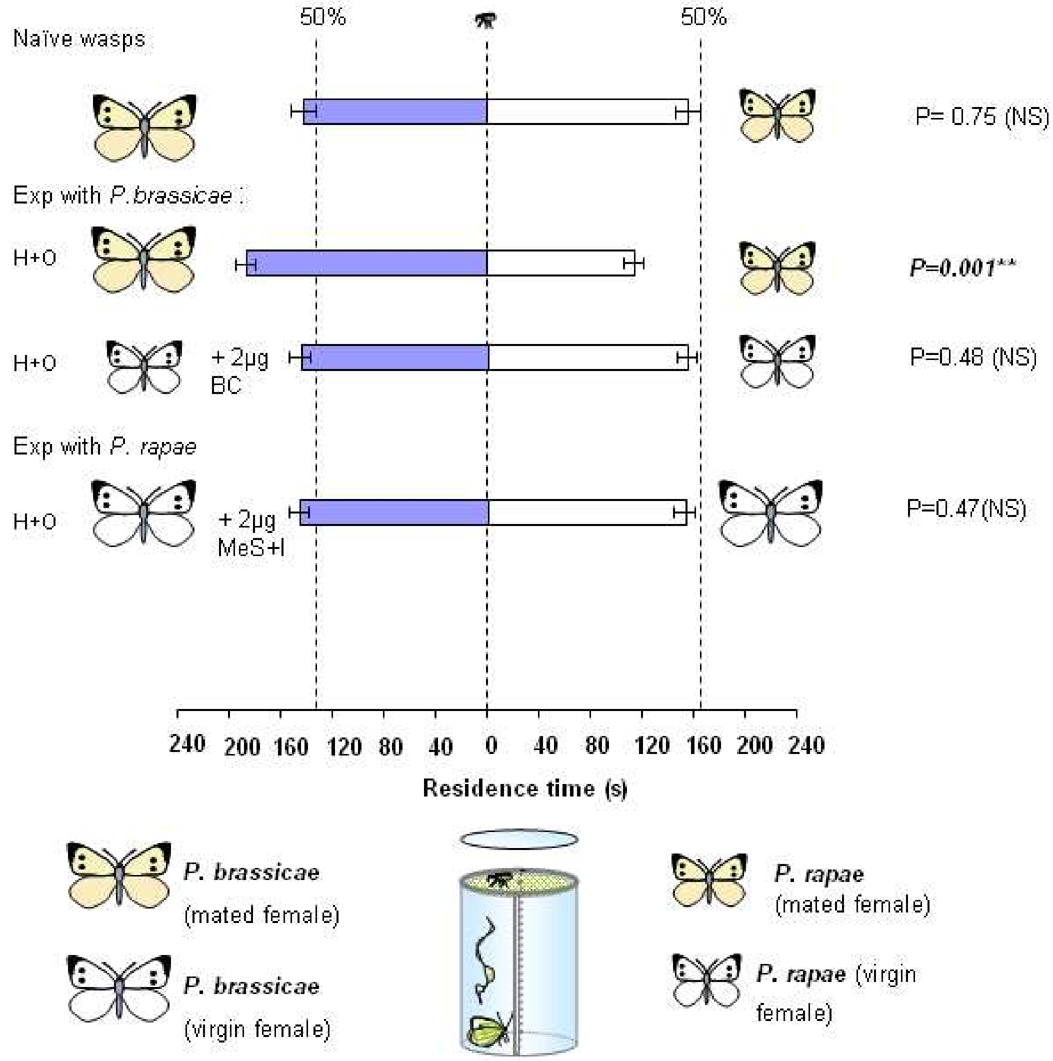

Figure 7: Two-choice olfactometer results when using two butterfly species *P. rapae* and *P. brassicae*. Mean residence time (± s.e.m.) that female wasps spent in two odor fields in a static olfactometer; *n*=40 wasps tested per combination (Wilcoxon's matched pairs signed ranks test) H+O: The wasps were given an oviposition experience (O) after a hitch-hiking experience (H) with mated female butterflies. Asterisks indicate significant differences within the choice test; NS, not significant.

3.2 Two-choice mounting bioassays

T. evanescens female wasps do not distinguish between climbing onto mated females males or virgin female *P. brassicae* butterflies (Fatouros *et al.*, 2007). However, they do have a preference to climb onto mated female *P. brassicae* butterflies over males (P= 0.004) and virgin females (P= 0.010) after an H + O experience (Figure 8).

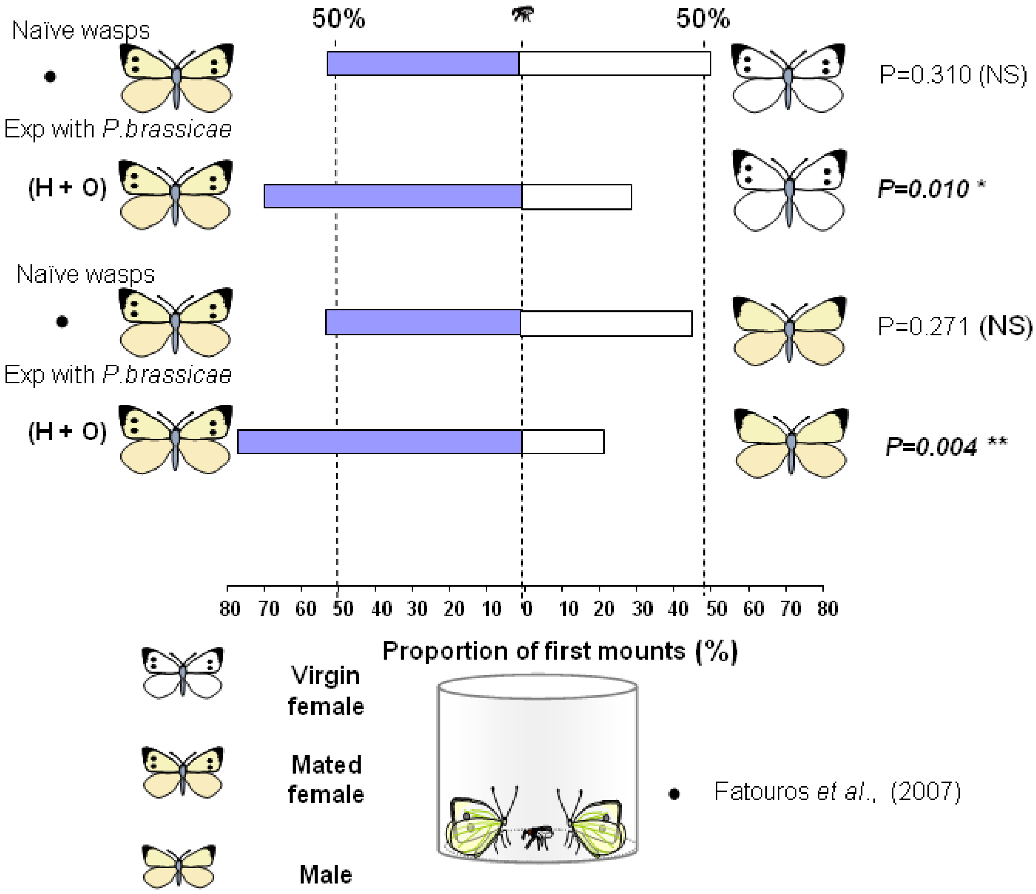

Figure 8: Two-choice mounting results. Proportion of first mounts (%) by female *T. evanescens* wasps. *n*=40 wasps tested per combination (χ^2 test). H+O: The wasps were given an oviposition experience (O) after a hitch-hiking experience (H) with mated female butterflies Asterisks indicate significant differences within the choice test; NS, not significant.

The phoretic behavior of naïve female wasps, on different butterfly species and non-hosts (desert locusts) was also tested. Naive *T .evanescens* female wasps significantly prefer to climb onto mated female *P. brassicae* (P= 0.042, α <0.05) and *P. rapae* (P= 0.010, α <0.05) butterflies when tested against *S. gregaria* locusts. Naïve female *T. evanescens* wasps do not distinguish between climbing onto mated female *P. brassicae* or mated female *P. rapae* butterflies (P= 0.206, α < 0.05). However, after a H + O experience with mated female *P. brassicae* butterflies, the wasps do show significant preference climbing onto mated *P. brassicae* females compared to mated *P. rapae* females (P= 0.010, α < 0.05). (Figure 9)

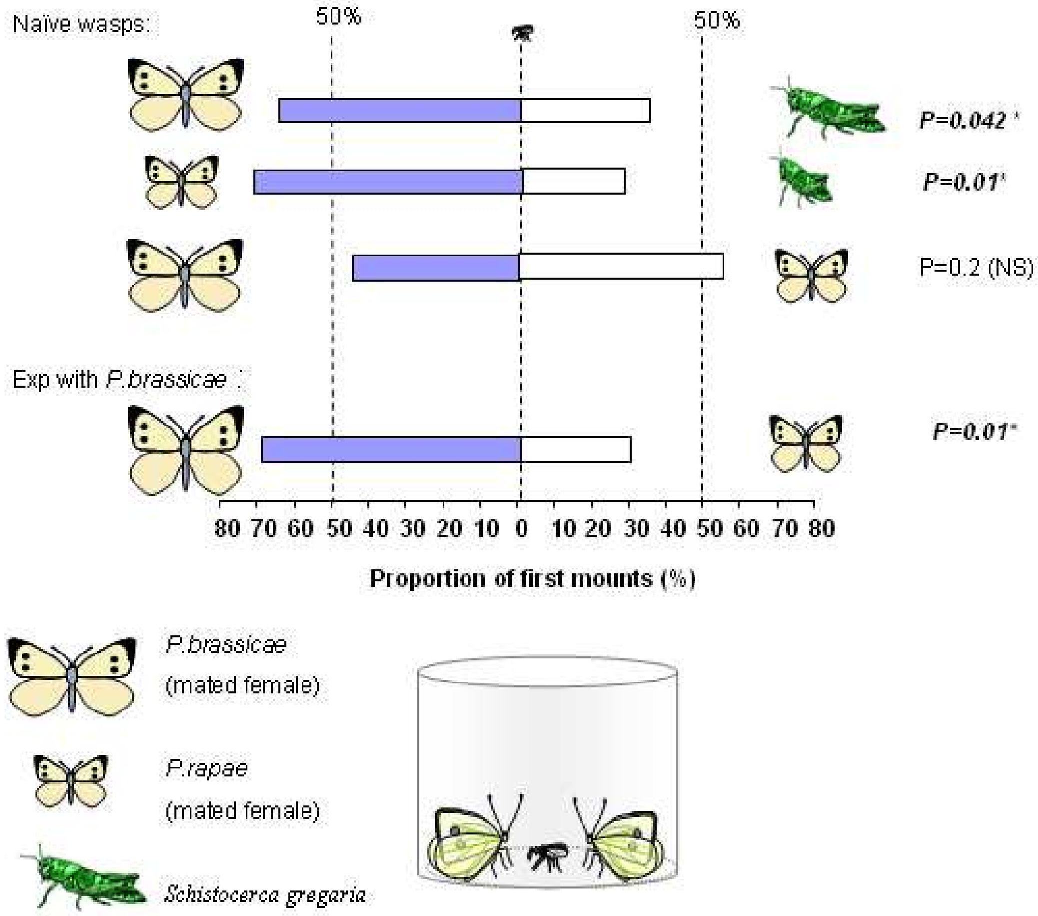

Figure 9: Two-choice mounting results. Proportion of first mount (%) by female *T. evanescens* wasps. *n*=40 wasps tested per combination (χ^2 test). H+O: The wasps were given an oviposition experience (O) after a hitch-hiking experience (H) with mated female butterflies Asterisks indicate significant differences within the choice test; NS, not significant.

3.3 One-choice mounting bioassay

In a one-choice mounting bioassay, 18 of 40 (45%) female wasps climbed and only 6 of 40 (15%) male wasps climbed onto a mated female *P. brassicae* butterfly. Female wasps climbed significantly more often onto mated female *P. brassicae* butterflies than male wasps (P=0.003) (Figure 10).

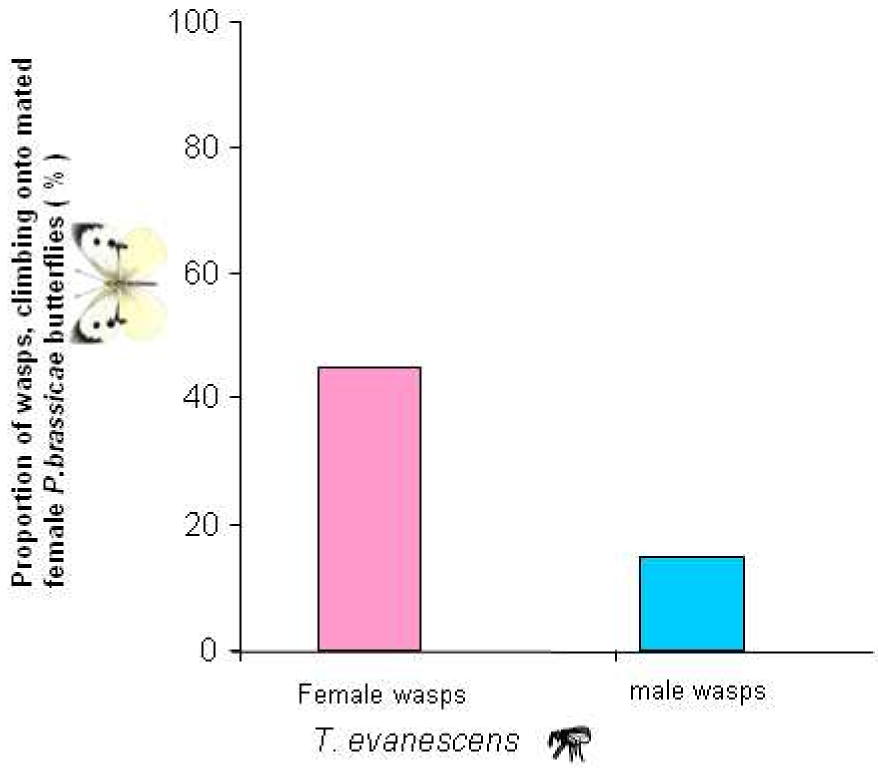

Figure 10: One-choice mounting test results. Pink bar represents the proportion of female wasps, and the blue bar the proportion of the male wasps that climbed onto mated female *P. brassicae* butterflies. In total 40 wasps of each sex were tested.

B. Field work

From the 424 *Pieris* butterflies that were collected, 37 were *P. brassicae*, 277 were *P. rapae* and 110 were *P. napi* (Figure 11). In total 4 female *T. evanescens* wasps were found. One *T. evanescens* wasp was found on a *P. brassicae* female (6.3%), one on a *P. rapae* female (1.1%) and one on a *P. rapae* male (0.5%), and one a *P. napi* male (1.1%)

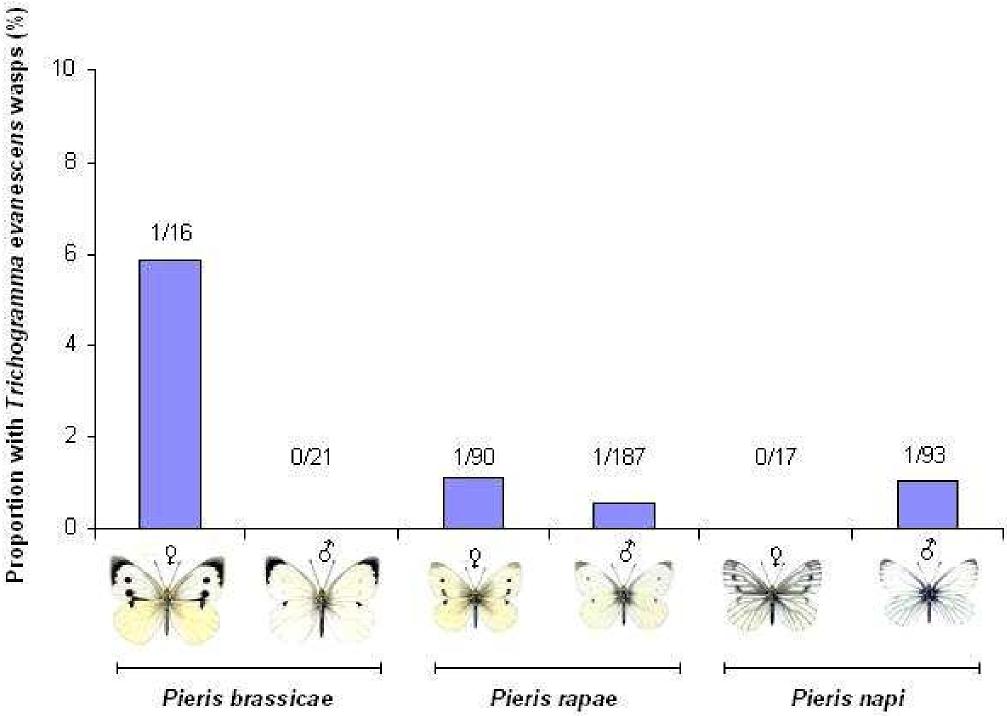

Figure 11: Field results from butterfly catches.

4. Discussion

The findings presented in this research show that female *T. evanescens* wasps can associatively learn to exploit a butterfly anti-aphrodisiac of mated female butterflies and specifically hitch-hike with them, after a rewarding hitch-hiking experience with such females. Naïve wasps do not respond to the odor of mated female *P. brassicae* butterflies, neither do wasps after only a hitch-hiking or an oviposition experience. They do, however, respond to mated female butterflies and virgin female butterflies painted with the antiaphrodisiac BC when they get an oviposition reward after the hitch-hiking experience. These results are in accordance with a previous study on the response of *T. evanescens* to a moth sex pheromone. Inexperienced, naïve *T. evanescens* females do not recognize and respond to the synthetic female sex pheromone ZETA of pyralid moths, whereas females that previously had a rewarding learning experience by parasitizing female eggs in the presence of the female sex pheromone do respond to this pheromone (Schöller and Prozell, 2002).

This type of associative learning requires the capacity to form memory. Memory is divided in several forms according to its temporal duration. Short term memory (STM) is an early stage of memory that is unstable and can be disturbed. Hours after learning, memory becomes more stable. Stable memory can be divided in long term memory (LTM), that requires the expression of genes and/or protein synthesis, and anaesthesia-resistant memory (ARM) (Margulies, 2005; Smid *et al.,* 2007). Here, it is shown that female *T. evanescens* wasps have short term memory because we only tested the wasps 1 h after the experience. Because of their expected short life-span and their restricted opportunities of finding mated female butterflies it, however, seems very useful for the wasps to adapt any behavior that assures quick host location, or quick elimination of non-hosts or host like objects (Lobdell *et al.*, 2005). Long term memory would be advantageous, because it can provide the wasps with the right information considered host location. Life-span not only depends on temperature and humidity but also on the type of selected host. In the laboratory at 4° C and 95 + 2% R.H., the life-span of *T. evanescens* wasps emerging from eggs of *L. botrana* varied from 10 ± 0.18 days for males and 11.93 ± 0.28 days for females whereas the life span varied from 8.86 ± 0.30 days for males and 9.67 ± 0.33 days for females when the wasps emerged from *S. cerealella* (Nasr *et al.,* 1995). In case of a short life-span in the field, the wasps shouldn't make many 'mistakes' in their short life by climbing on males or virgin females. Future research should test the wasps >24 hrs after a rewarding hitch-hiking experience with mated female butterflies. If the wasps then still discriminate mated female butterflies from males and virgin females, they may have formed long term memory. To confirm the presence of long term memory in *T. evanescens,* one should test whether wasps that are fed with translation and transcription inhibitors prior to the rewarding hitch-hiking experience are not able to do not discriminate mated female butterflies from males and virgin females (Smid *et al.,* 2007). The fruitfly *Droshophila melanogaster* is an important example which relates memory and learning in insects. Their brain plasticity makes them capable of learning to recognize specific plant odors. *Droshophila* are acting like Pavlovian dogs. When they are repeatedly offered a conditioned stimulus (CS, specific plant odors) and an unconditioned stimulus (US, food) simultaneously, they eventually associate the two stimuli and a behavioral response (CR) is triggered by only offering a CS (Margulies, 2005).

Obviously, female *T. evanescens* wasps smell an anti-aphrodisiac in combination with other butterfly odors: When I tested a synthetic anti-aphrodisiac in combination with female odors of a butterfly species other than the one with which they received the experience, the wasps did not respond. Female wasps that had a rewarding hitch-hiking experience with a mated female *P. brassicae* butterfly have a preference for virgin *P. brassicae* females treated with the synthetic antiaphrodisiac pheromone BC in hexane over the odor of virgin *P. brassicae* females treated with hexane only. However, the same wasps do not discriminate between the odor of virgin *P. rapae* females painted with hexane and virgin *P. rapae* females treated with the BC in hexane. Similarly, wasps that received a rewarding hitch-hiking experience with a mated female *P. rapae* butterfly do not discriminate between the odor of virgin *P. brassicae* females treated with hexane only and virgin *P. brassicae* females treated with the synthetic antiaphrodisiac pheromone MeS + I of *P. rapae* in hexane. Parasitoids in general smell a complete odor blend and not only specific compounds. The reaction towards a certain compound in a blend depends on the presence/absence of the other compounds in that blend. In a recent study by Mumm *et al.,* (2005), they show that the egg parasitoid *Chrysonotomyia ruforum* has different behavioral responses against a phytohormone applied together with different background plant odors.

In order to obtain a rewarding hitch-hiking experience, naïve female wasps first have to climb onto butterflies. Obviously, they do climb on butterflies but they do not discriminate between butterfly sexes and different butterfly species. However, they do prefer to climb onto mated females of their hosts *P. brassicae* and *P. rapae* over non-host insects suggesting that they are able to recognize adult butterflies but not species, sex or mating status. Butterfly recognition may be based on visual cues (Keasar *et al.,* 2000; Lobdell *et al.,* 2005). Future studies should test whether wasps also prefer to climb onto male and virgin female butterflies over non-hosts. From an applied perspective, it is important to understand the hitch-hiking behavior of *Trichogramma* species to improve the efficiency of mass releases of *Trichogramma* wasps in biological control. Timing of release is crucial when a wasp species would rely on hitch-hiking to find host eggs.

Hitch-hiking with adult butterflies is expected to be a female wasp strategy because only female *Trichogramma* wasps can parasitize butterfly eggs (Herz *et al.,* 2007; Monje, 1999). In the one-choice bioassays, I show that naïve female *T. evanescens* wasps climb significantly more often on mated *P. brassicae* females than male wasps. Similar one-choice tests with *T. brassicae* on *P. brassicae* and *P. rapae* also showed that females climb significantly more often on butterflies than males (Woelke, 2008).

The fact that some males also climb in the bioassays is likely to be a chance effect due to the small arena. Male wasps may, however, also climb on butterflies to locate female wasps.

In the field, only female wasps were found on adult cabbage white butterflies. All wasps were females and can be found on both butterfly sexes. Female wasps on male butterflies are likely to be naïve wasps. The low number of hitch-hiking wasps probably is a strong underestimation of the actual frequency of hitch-hiking wasps on adult butterflies. Wasps may stay on butterflies only for a short time, which decreases the chance of trapping them. Also, wasps may have fallen off the butterflies when they were caught with the net. The field work needs to be extended in future years in order to get a good estimate of the actual frequency of hitch-hiking wasps.

In general, behavioral responses of parasitoids to infochemicals are species-specific (Geervliet *et al*., 1998; Smid *et al*., 2007). It has now been shown that both *T. brassicae* and *T. evanescens* can exploit the antiaphrodisiac pheromones of *P. brassicae* and *P. rapae* (Fatouros *et al*., 2005a; Woelke, 2008; my thesis). Responding to an antiaphrodisiac is an innate behavior in *T. brassicae*, whereas the exploitation of the antiaphrodisiac and phoretic behavior requires learning in *T. evanescens*. It might be that *T. evanescens* is more of a generalist parasitoid than *T. brassicae*. *Cotesia glomerata* and *Cotesia rubecula* are closed related parasitoids that differ in their degree of specialization and show significant differences in their host-finding behavior. *C. glomerata* is considered a generalist whereas *C. rubecola* is considered more of a specialist. Experiments by Geervliet *et al*., (1998) showed that learning plays a much more important role in the generalist *C. glomerata* than in the specialist *C. rubecula*.

Host location behavior may not only differ between species. There might also be natural variation in this behavior within a species. Host selection and acceptance can, for example, differ between *Trichogramma* strains and species (Pak *et al*., 1986; Pak and De Jong, 1987; Pak *et al*., 1990). In my study only one strain of *T. evanescens* was tested. Future experiments should be conducted with multiple strains in order to assure that the exploitation of antiaphrodisiacs is a species specific strategy. As mentioned, Fatouros *et al*., (2006) proved that *T. evanescens* cannot innately discriminate between the odor of mated female, virgin female and male *P. brassicae* butterflies. However, there are also descriptions by Noldus and van Lenteren (1985) that female *T. evanescens* prefer the odor of virgin female *P. brassicae* butterflies above the odor of mated females and males. Noldus and van Lenteren (1985) at least tested another *T. evanescens* strain than Fatouros *et al*., (2007) but maybe even another wasp species because *Trichogramma* identification was really unreliable in the 1980's (Stouthamer *et* al., 1999).

Minute egg parasitoids like *Trichogramma* spp. face a tremendous challenge to find host eggs (immobile source). Because of their small size and their reduced ability to fly, and the fact that they do prefer to parasitize freshly laid eggs (Wang *et al*., 1997; Olson, 1998; Hou, 2006), phoresy seems an adaptive strategy for *Trichogramma* wasps. In order to increase their chances of finding eggs, they may also rely on oviposition-induced plant cues or the detection of butterfly or moth wing scales (Fatouros *et al*., 2005b; Hou, 2006). There are *Trichogramma* spp. like *Trichogramma japonicum* that use only short-range cues like cuticular extracts (mostly wing scales) of adult moths to detect its host. There are also other *Trichogramma* spp. that shown phoretic behavior (Fatouros, 2006). Till now, the combination of chemical espionage on an antiaphrodisiac with phoresy is only shown in *T. evanescens* and *T. brassicae*. Yet, this strategy may have evolved frequently in tiny egg parasitoids in general. Another combination of chemical espionage and phoresy is also known from other egg parasitoids like *Telenomus* spp. which can detect a sex pheromone of a female calling moth to locate it, and hitch-hike with it to the site where it will lay its eggs (Arakaki *et al*., 1995; 1996).

The exploitation of *Pieris* antiaphrodisiac pheromones by *Trichogramma* wasps puts the use of these pheromones by the butterflies under pressure. The potential advantages of using an antiaphrodisiac for male butterflies are obvious. The advantage for female butterflies may be a reduced harassment by other males. Andersson *et al*., (2003) describe that some females can control their attractiveness by controlling the amount of the antiaphrodisiac that they release. In that way they have more time to oviposite. To understand whether *Trichogramma* wasps actively select against the use of an antiaphrodisiac by *Pieris* butterflies, future studies should try to determine whether natural variation in the production, emission or use of an antiaphrodisiac is correlated with the presence of parasitoid wasps at certain locations.

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