# Host species-specific memory duration in the parasitoid wasp *Trichogramma evanescens*

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# **ABSTRACT**

Egg parasitoids are able to find low detectable host eggs by exploiting chemical cues from plants, the adult host stage and/or the eggs themselves. The tiny (0.5 mm long) egg parasitoid wasp *Trichogramma evanescens* can learn to exploit anti-aphrodisiac pheromones emitted by mated females of the gregarious large cabbage white butterfly *Pieris brassicae*, and the solitary small cabbage white butterfly *P. rapae*, by phoretic (hitch-hiking) behavior on a mated female butterfly to reach a host plant and parasitize the butterfly's eggs. This behavior is learned after a single learning "hitch-hiking and oviposition (H+O)" event of hitch-hiking on a mated female butterfly followed by a successful parasitism of one of her eggs. The wasp learns to associate the odor of a mated female butterfly with the reward of parasitizing her eggs. In this study, I investigated mechanisms underlying memory formation in *T. evanescens* after one or more (different) H+O events with the two closely related butterfly species *P. brassicae* and *P. rapae*. Results show that memory duration after one learning event in this wasp species is host species-specific. Memory for the odor of a mated female butterfly lasted at least 24 h after one H+O event with *P. brassicae* whereas memory was lost before 24 h after a single training event with *P. rapae*. Wasp oviposition experiments indicate that the host species-specific memory duration after one H+O event was due to a difference in reward value constituted by the eggs of the two host species. Memory lasted longer after an experience with the high-value host. Memory was present at 24 h after spaced training with *P. rapae*. However, memory for the odor of mated females of both butterfly species was not present anymore at 24 h after two different training events with the two butterfly species spaced by 1 h. Subsequent experiments on the effect of night temperature on memory formation, and on the formation of different memory types, after one learning event with the two butterfly species yielded contradicting results that were difficult to interpret. In conclusion, my results suggest that learning and memory formation in minute egg parasitoids depends on the reward value offered by their hosts.

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# INTRODUCTION

Exploitation of chemical cues to find host eggs with a low detectability is a common strategy in foraging egg parasitoids (for a recent review see Fatouros *et al.* 2008) The infochemicals can come from eggs directly or from several other sources such as plant cues induced by egg deposition, or adult host sex and anti-sex (or anti-aphrodisiac) pheromones.

The cabbage white butterflies *Pieris brassicae* and *Pieris rapae* are known to use anti-aphrodisiac chemicals for sexual communication. The male-produced anti-aphrodisiacs are transferred to females during mating in order to render them less attractive to conspecific males (Andersson *et al.* 2003). Minute (± 0.5 mm long) *Trichogramma* wasps, that parasitize eggs of a range of Lepidoptera, can exploit the anti-aphrodisiac pheromones of *Pieris* butterflies by chemical espionage (Fatouros *et al.* 2005). Female wasps of *T. brassicae* and *T. evanescens* are lured by the anti-aphrodisiac pheromones of two of their hosts, namely benzyl cyanide (BC), emitted by mated females of the large cabbage white *P. brassicae*, and methyl salicylate (MeS) and indole, emitted by mated females of the small cabbage white *P. rapae*. After detecting the anti-aphrodisiacs, the wasps mount mated female butterflies and are transported to wild and cultivated cabbage plants where the butterflies lay their eggs, i.e. a clutch of more than 20 eggs in the case of *P. brassicae* and singly laid eggs in the case of *P. rapae* (Fatouros *et al.* 2005; Huigens *et al.* 2009, 2010). The reward of this phoretic (hitch-hiking) behavior is a successful parasitism of butterfly eggs (Huigens *et al.* 2009, 2010).

Chemical espionage on the anti-aphrodisiac pheromones of the two cabbage white butterflies is an innate behavior in *T. brassicae* (Fatouros *et al.* 2005; Huigens *et al.* 2010) whereas the closely related species *T. evanescens*, probably a more generalist species, is able to "learn" to use the anti-aphrodisiac pheromones as kairomones (Huigens *et al.* 2009, 2010). The latter species can learn to associate BC of *P. brassicae*, as well as MeS and indole of *Pieris rapae*, with the presence of suitable butterfly eggs after a learning event consisting of hitch-hiking on a mated female butterfly and a consecutive oviposition into freshly laid butterfly eggs (Huigens *et al.* 2009, 2010). Such appetitive conditioning in which chemical cues are associated with a reward (oviposition into a host) is known as associative learning (Vet *et al.* 1995). It is expected to be especially adaptive in generalist parasitoid wasps (Vet and Dicke 1992; Geervliet *et al.* 1998). They may gain experience by association of certain stimuli with the presence of suitable hosts, and subsequently use these stimuli in further host-finding activities (Turlings *et al.* 1993; Smid *et al.* 2007).

In the animal kingdom, memory formation after learning is classified into temporally distinct forms based on the time that memory can prevail. Short-term memory (STM, or also called anaesthesia-sensitive memory (ASM)) is generally acquired by parasitoid wasps after just one single training event. It lasts only a few hours, is labile and easily disrupted by other effects such as anaesthesia application shortly after learning (Smid *et al.* 2007). Longer lasting (from several hours to days) forms of consolidated memory that are resistant to anaesthesia are generally formed after several training events separated in time (spaced training); when consolidated memory requires gene expression and/or protein synthesis it is called long-term memory (LTM), if not, it is called anaesthesia-resistant memory (ARM) (Smid *et al.* 2007). As mentioned, LTM is generally formed after several spaced learning events but some parasitoid wasps already form LTM after only one learning event (Collatz *et al.* 2007, Smid *et al.* 2007). The larval parasitoid wasp *Cotesia glomerata* is, for example, able to form LTM after one single parasitization event with its preferred gregarious host *P. brassicae,* whereas the closely related parasitoid *Cotesia rubecula* forms LTM only after several spaced learning events with its solitary host *P. rapae* (Smid *et al.* 2007), showing that the wasps' memory formation is strongly adapted to the ecology (oviposition behavior) of the host.

In the parasitoid wasp *T. evanescens,* despite of its extremely small brain, LTM is present at 24 h after one single learning event with *P. brassicae* consisting of a successful ride on a mated female butterfly leading to an oviposition into a clutch of freshly laid butterfly eggs, which is called a hitch-hiking and oviposition (H+O) experience (Huigens *et al.* 2009, 2010). This type of LTM formation is expected to be adaptive because of the very limited opportunities that a female wasp may get to mount a mated female *P. brassicae* butterfly during its short lifespan in nature (Huigens *et al.* 2009, 2010). A few successful rides on a mated female butterfly should be enough for a female wasp to get rid of all her eggs. Very recently, memory has also been confirmed at 24 h after one training event when *T. evanescens* wasps were offered only a single *P. brassicae* egg as a reward (Pashalidou *et al.* unpublished data). Naïve *T. evanescens* wasps do not respond to the anti-aphrodisiacs of mated *P. brassicae* females (BC) and mated *P. rapae* females (MeS+ indole). However, wasps do respond to these anti-aphrodisiacs at 1 h after an H+O experience with both butterfly species. Memory duration and/or consolidation may, however, not be the same after an H+O experience with both butterfly species because of a difference in reward value. A higher reward value is predicted to improve associative learning (Hutt 1954). Relatively little research has addressed the question how variation in reward value affects associative learning. With respect to learning in parasitoid wasps, Wäckers *et al.* (2006) found the duration of the feeding response of *Microplitis croceipes* to the learned odor to be dependent on the type of sugar that was used as the reward but not on the sugar concentration. Takasu and Lewis (2003), using the same parasitoid species, found that antennation of host frass was a sufficient reward to elicit a response to the learned odor at 30 minutes after the experience, but that 24 h memory was only formed after oviposition in the host.

In this study, I investigated mechanisms underlying memory formation (duration and/or consolidation) in *T. evanescens* after one or more (different) H+O events, and tested whether it is host species-specific and dependent on environmental conditions such as night temperature. Memory formation is expected to differ after an H+O experience on *P. brassicae* and *P. rapae* because of a difference in egg-laying behavior between the butterfly species. *Pieris brassicae* lays its eggs in clutches whereas *P. rapae* lays single eggs (Feltwell 1982; Tolman 2001). From a wasp's perspective hitch-hiking with a mated *P. brassicae* female is likely to be more rewarding than hitch-hiking with a mated female *P. rapae* butterfly. I therefore expect memory to last longer after a single H+O training with *P. brassicae* than with *P. rapae*. Furthermore, memory may last longer after spaced training with *P. rapae* than after a single training on that host species because spaced training is ecologically relevant and also a much more reliable indicator of the presence of more suitable eggs of this very abundant solitary butterfly than only a single learning event (Tolman 2001). The effect of night temperature was tested because these parasitoids may experience cold nights after a learning event early (spring) and late (summer/autumn) in the season in nature that interfere with memory formation.

### Research questions

1. Is memory duration in *T. evanescens* after learning host species-specific? (R1)
2. If so, can this be explained by a difference in reward value? (R2)
3. Can *T. evanescens* remember two consecutive learning trials with a different host species? (R3)
4. Is there an effect of night temperature on memory duration after a single learning trial in *T. evanescens*? (R4)
5. What type of memory is consolidated in *T. evanescens* between 1 and 24 h after a single learning trial? (R5)

# MATERIALS AND METHODS

## Insects

The two cabbage white butterfly species *Pieris rapae* L. and *Pieris brassicae* L. (Lepidoptera: Pieridae) were reared on Brussels sprouts plants (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) in a climate room (21 ± 1°C, 50-70% rh, L16:D8) of the Laboratory of Entomology, Wageningen University. In the experiment, I used mated females for wasp training and bioassays, and males as control in bioassays. Mated females were obtained by taking mating pairs the day before training or bioassay (Huigens *et al.* 2009, 2010).

The egg parasitoid *Trichograma evanescens* Westwood (Hymenoptera: Trichogrammatidae) (strain GD011) was reared in eggs of the moth *Ephestia kuehniella* under laboratory conditions (23 ± 2°C, 50-70% r.h., L16:D8). The wasps were kept feeding on honey. In the experiments, I used two-days-old, mated female wasps.

## Wasp training and two-chamber olfactometer bioassays

Hitch-hiking and oviposition (H+O) experience: Ten minutes before conditioning, mated female butterflies were put at cold temperature (4°C) to make them lethargic and easy to handle for wasp training.

After cooling, 2 mated female butterflies were put in a hitch-hiking arena, i.e. a plastic cylinder of 10 cm in diameter and 6 cm high (Huigens *et al.* 2009, 2010). Subsequently naïve mated female were introduced into the arena in order to achieve the typical hitch-hiking behavior. Once a butterfly was mounted by some wasps, it was transferred to another "arena" containing pieces of Brussels sprouts leaves with eggs less than 24 hrs-old of the same butterfly species (figure 1a-b).

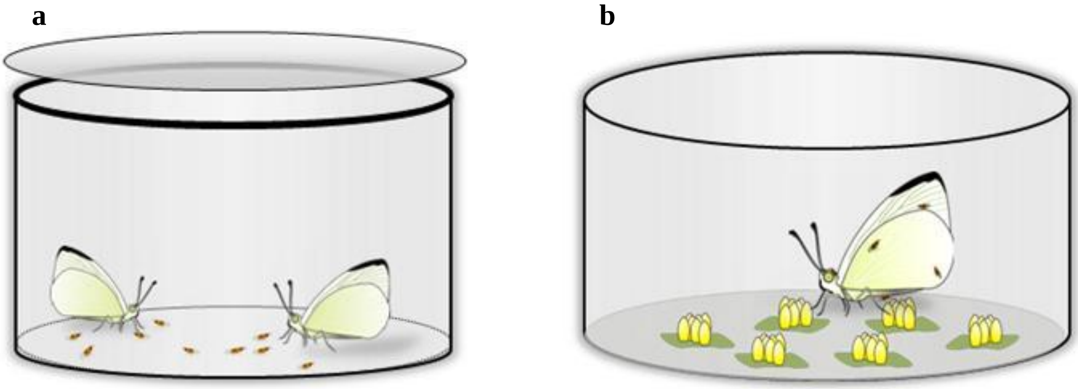

**Figure 1. Schematic drawing of a) hitch-hiking arena with mated female butterflies being mounted by wasps. b) Oviposition arena containing leaf pieces with butterfly eggs to which a 'mounted' butterfly was transferred. Phoretic wasps were able to descend from the butterfly and parasitize the eggs.**

Phoretic wasps were allowed to descend from the host butterfly and move towards the eggs. Oviposition was monitored under a binocular microscope and after contact with, and oviposition into, one egg the wasp was isolated in a tube with a small droplet of honey until use in the olfactometer bioassays. Experienced wasps were kept as such under laboratory conditions (23 ± 2°C, 50-70% r.h., L16:D8).

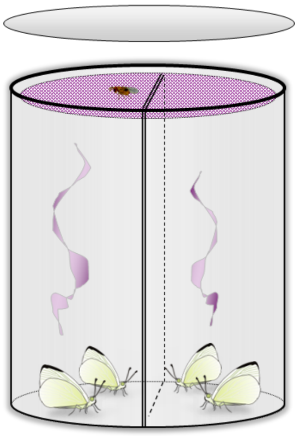

**Figure 2. Schematic drawing of the static 2-chamber olfactometer.**

Two-chamber olfactometer bioassays: Olfactory bioassays were carried out using a two-chamber olfactometer made of one plastic cylinder of 25 cm high divided in two equal spaces with hard plastic sheet. The cylinder is covered with a mesh cap of 100 squares/mm$^2$ (Fatouros *et al.* 2005; Figure 2). Two adult butterflies per chamber were used as odor source. In most of the experiments I introduced 2 mated female butterflies in one chamber (treatment) and 2 males in the other (control). Finally an H+O-experienced wasp was released onto the mesh. The time that a wasp spends above the two odor sources was measured for 300 sec. After every three wasp observations the complete system was rotated 180º and the set of butterflies was exchanged with fresh ones (Huigens *et al.* 2009, 2010). Every day 10 to 15 wasps were tested till achieving 40 wasps tested at the end of a bioassay.

## R1. Host species-specific memory duration

Naïve wasps do not discriminate between the odors of mated female and male butterflies of *P. brassicae* and *P. rapae* (Huigens *et al.* 2009, 2010). Memory (retention) is defined as present when wasps show a significant preference for the odor of mated female butterflies, when compared to the odor of male butterflies, after learning. In order to determine the duration of memory, experienced wasps were kept 4 h, 16 h, 24 h, 72 h and/or 120 h after training until a bioassay.

## R2. Reward value

The reward value for a parasitizing female wasp was determined by the number of wasp eggs that a female wasp allocates to a host egg. The more wasp eggs a female oviposits into a host egg, the higher the reward value. Moreover, I determined the suitability of a host egg in terms of offspring survival and offspring fitness (size and fecundity).

### *Single egg experiment*

Female wasps were able to parasitize single eggs of both butterfly species. I observed the number and sex of the wasp eggs allocated to the butterfly eggs by abdominal movements as described by Suzuki *et al.* (1984) using a video camera setup. Every day 10-15 successful ovipositions were filmed. After parasitism the eggs were isolated in tubes and preserved in a climate chamber until emergence on day 12 after parasitism under the same conditions as described above.

### *Grouped eggs experiment*

Besides the single egg experiment I offered a group of 8 eggs to a single wasp for three days to determine offspring number and offspring fitness, without observing the wasps' oviposition behavior on the 8 eggs. From the emerging offspring, one mated female wasp was transferred in another tube to parasitize a standard over amount (± 150) of *E. kuehniella* eggs on a small sticker (0.5 cm diameter). Three days later, the female was removed and put in the freezer for subsequent measurement of her hind tibia length (HTL). HTL is a widely accepted parameter for the size of *Trichogramma* wasps (Kazmer and Luck 1995). Every frozen wasp was placed on a glass slide, covered with a very thin glass cover and squashed to measure HTL using a special lens with a 1 mm gauge on a monocle microscope. Furthermore, parasitized eggs were counted as the number of black eggs. After parasitism, eggs were placed in a climate chamber until adult emergence (second generation offspring). I recorded both number and sex of the second generation offspring.

## R3. Memory after two different learning trials

To test for memory after two consecutive but different H+O experience trials, naïve wasps were first trained on *P. rapae* and then, 1 h later, on *P. brassicae* and vice versa. Memory was assessed at 24 h after the last training in bioassays in which I tested the wasps' response to the following odor combinations: mated *P. brassicae* female against mated *P. rapae* female, mated *P. brassicae* female against *P. brassicae* male, and mated *P. rapae* female against *P. rapae* male.

## R4. Night temperature effect

The effect of night temperature on memory duration was tested by placing experienced wasps in a climate chamber (50-70% r.h.) programmed at a warm day of 22 °C (16 h light) and either a cold night of 10 °C or a warm night of 22 °C (8 h dark). In this experiment, I tested for the presence of memory at 16 h after an H+O experience with *P. brassicae* and *P. rapae*, and at 24 h after an H+O experience with *P. brassicae*.

## R5. Memory types after training

To determine whether memory consolidation is host species-specific, I tested for the presence of ASM, ARM and LTM at 4 h and 16 h after one H+O experience with either *P. brassicae* or *P. rapae*. I tested for the presence of ARM by giving wasps a cold shock 1 h before the bioassay. To do this, tubes with trained wasps were kept for 5 minutes on ice. To test for the presence of protein synthesis-dependent LTM, wasps were first deprived of food and then given the opportunity to feed overnight on 30 μL of a 10 mM solution of the translation-inhibitor Anisomycin (ANI) in 2.5 % sucrose prior to an H+O experience (Huigens *et al.* 2009). In the latter test, control wasps were given almost the same treatment but instead were feeding on a 2.5 % sucrose solution without ANI.

### Statistics

Residence times in the odor field of mated female and male butterflies within the two-chamber olfactometer bioassays were compared using a Wilcoxon matched pairs signed ranks test. A non-parametric test for non-related samples, called the Mann-Whitney U test, was used for comparison of the number of eggs allocated to a host egg, and offspring fecundity and offspring size in the reward value experiments. To compare offspring sex ratio and mortality of wasps developing in eggs of the two different butterfly species in the latter experiments I used a chi-square test with a 2x2 contingency table. To test for the effect of night temperature on memory duration, residence times in the odor field of mated female butterflies between wasps kept at either warm or cold night were compared with the Mann-Whitney U test. To compare the residence times in the odor field of mated female butterflies between ANI-treated-, cold shock-treated and control wasps (i.e. test for memory types present) at 4 and 16 h after an H+O experience with either *P. brassicae* or *P. rapae,* I used the non-parametric Kruskal-Wallis test. In case of significant differences, pair-wise comparisons were done with the Mann-Whitney U test. All tests were carried out at $\alpha = 0.05$ in SPSS version 11.0.1.

## RESULTS

### R1. Host species-specific memory duration

Results show that memory is present at 1 h, 4 h and 16 h after one H+O event with *P. rapae* in the parasitoid *T. evanescens* (P < 0.001 and P = 0.002, resp. - Wilcoxon signed ranks test; Figure 3). However, at 24 h after such a training event this parasitoid does not associate the odor of a mated *P. rapae* female with the reward of parasitizing a butterfly egg, i.e. no memory is present, anymore (P = 0.221, Wilcoxon signed ranks test; Figure 3). Memory was present at 24 h after two consecutive trainings with *P. rapae* with a time interval of 1 h (P = 0.013 - Wilcoxon signed ranks test; Figure 3).

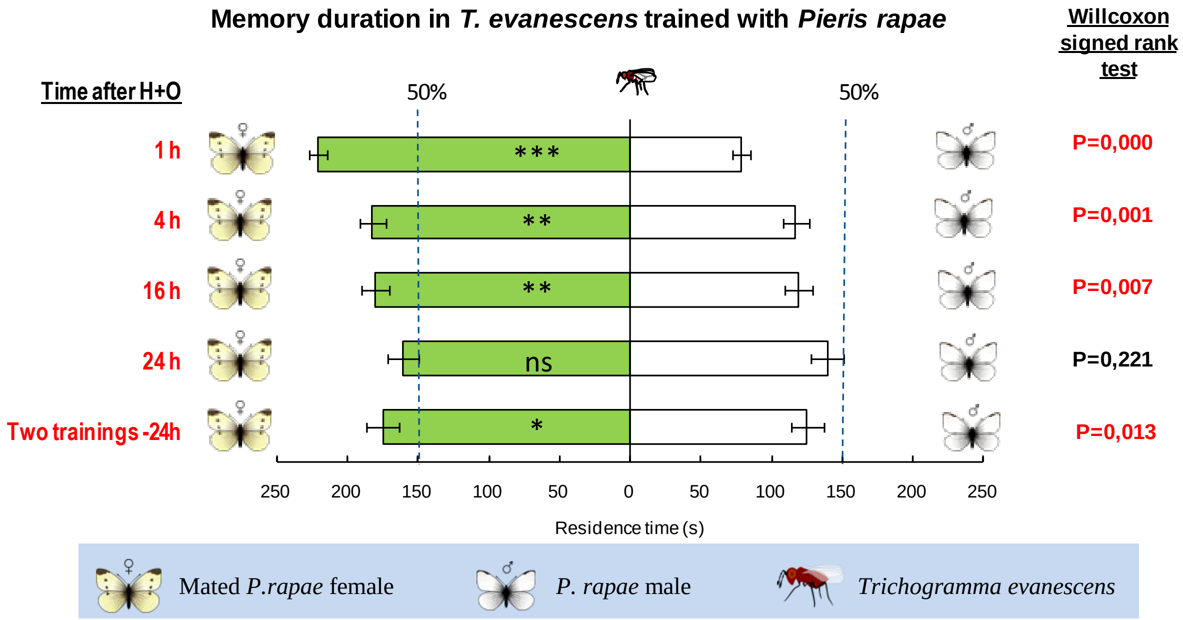

**Figure 3. Response of wasps to odors of mated female and male *P. rapae* butterflies at different time points after one or two consecutive H+O training events with *P. rapae* in a two chamber olfactometer. Each bioassay was done with 40 wasps. Mean residence time (s) ± standard error in the odor fields of mated female and male butterflies are given. H+O = rewarding "hitch-hiking + oviposition" experience consisting of a successful ride on a mated female butterfly leading to an oviposition into a freshly laid butterfly egg. Significant differences *** P<0.001; ** P<0.01; * P<0.05; ns=no significant difference. Red text P-values are given when they show significant differences.**

In the case of wasps trained with *P. brassicae*, memory lasted at least 24 h and was lost somewhere between 24 h and 72 h after one H+O experience (Figure 4).

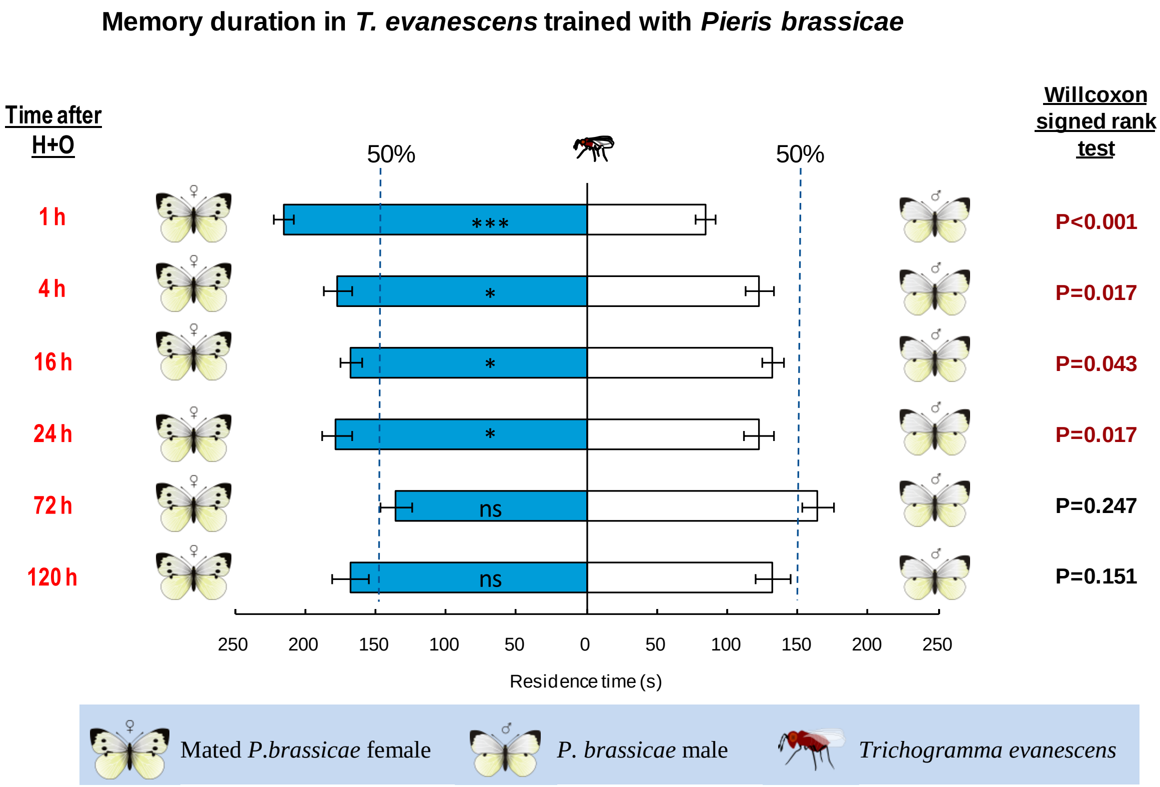

**Figure 4. Response of wasps to odors of mated female and male *Pieris brassicae* butterflies at different time points after one H+O training event with *P. brassicae* in a two chamber olfactometer. Each bioassay was done with 40 wasps. Mean residence time (s) ± standard error in the odor fields of mated female and male butterflies are given. H+O = rewarding "hitch-hiking + oviposition" experience consisting of a successful ride on a mated female butterfly leading to an oviposition into a freshly laid butterfly egg. Significant differences *** P<0.001; * P<0.05; ns=no significant difference.**

## R2. Reward value

***Single egg experiment***: The results show that significantly more wasp eggs were laid in single *P. brassicae* eggs than in single *P. rapae* eggs (P < 0.001 – Mann Whitney-U test; Figure 5). Each wasp egg clutch always contained at least one male according to Local Mate Competition theory (Hamilton 1967). On the other hand, immature mortality is significantly higher in *P. brassicae* eggs than in *P. rapae* eggs ($\chi^2 = 16.22$, P < 0.001; Figure 6).

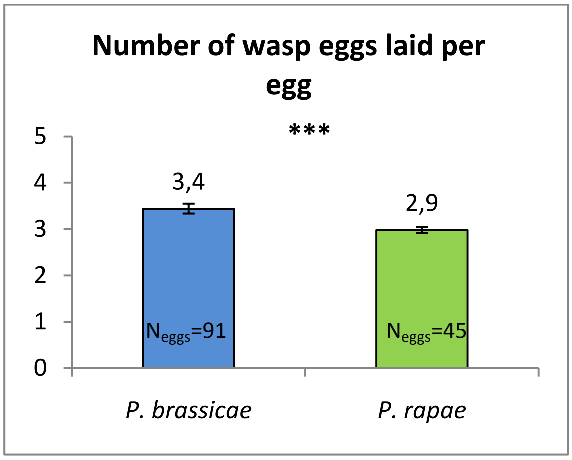

**Figure 5. Mean number of wasp eggs laid per egg of *P. brassicae* and *P. rapae* ± standard error. N = number of butterfly eggs recorded. *** P<0.001.**

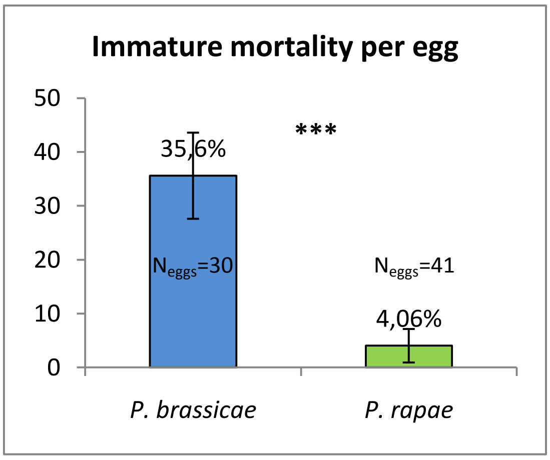

**Figure 6. Mean mortality of immatures in single *P. brassicae* and *P. rapae* eggs ± standard error. N = number of butterfly eggs that resulted in wasp emergence. *** P<0.001.**

***Grouped eggs experiment*** : The results on the number of offspring emerging per egg confirmed the results in the single egg experiment, with significantly more wasps emerge from a single *P. rapae* egg than from a single *P. brassicae* egg ($P < 0.001$ – Mann-Whitney U test; Figures 7 & 8).

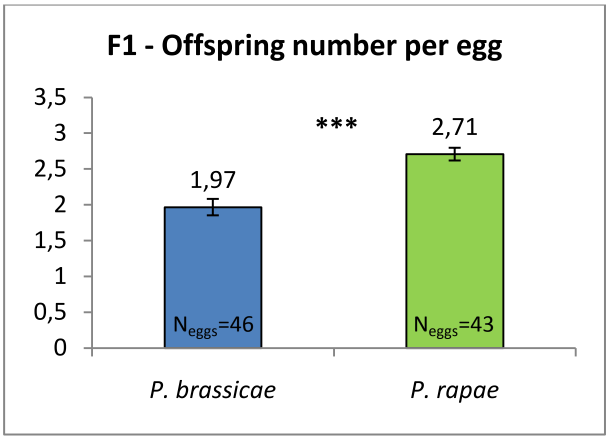

**Figure 7. Mean number of wasps emerging per egg of *P. brassicae* and *P. rapae* in a group of 8 eggs ± standard error. N = number of parasitized egg groups. *** P<0.001.**

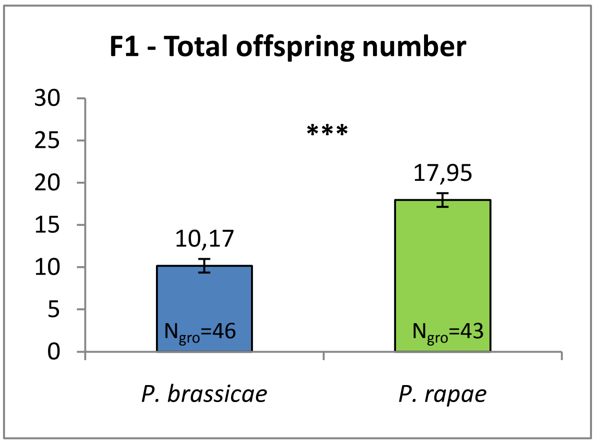

**Figure 8. Mean total number of wasps emerging per group of 8 eggs of *P. brassicae* and *P. rapae* ± standard error. N = number of butterfly egg groups resulting in wasp emergence. *** P<0.001.**

The sex ratio (fraction of females) of wasps emerging from *P. brassicae* eggs was significantly lower than of those emerging from *P. rapae* eggs ($\chi^2 = 10.22$ $P = 0.001$; Figure 9). Furthermore, female wasps emerging from *P. brassica* eggs were significantly larger than those emerging from *P. rapae* eggs ($P < 0.001$ – Mann Whitney-U test; Figure 10).

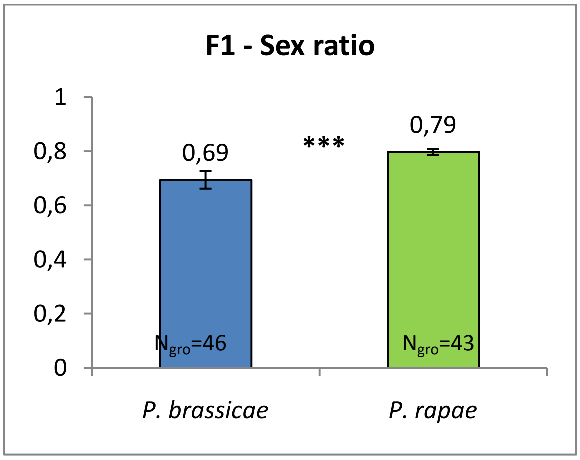

**Figure 9. Mean sex ratio (fraction of females) of offspring emerging per group of 8 parasitized egg of *P. brassicae* and *P. rapae* ± standard error. N = number of butterfly eggs resulting in wasp emergence. *** P<0.001.**

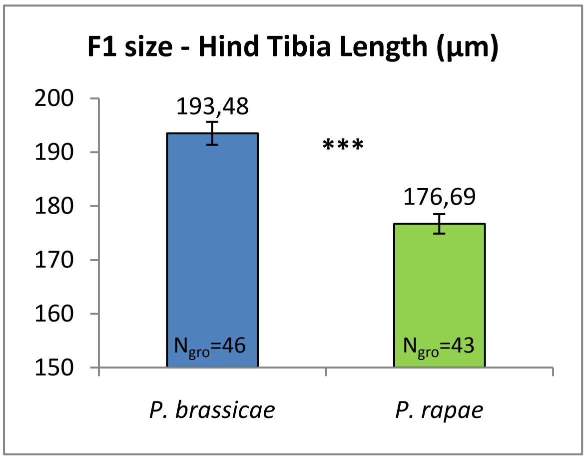

**Figure 10. Mean size (Hind Tibia Length) of female wasps per group of 8 parasitized egg of *P. brassicae* and *P. rapae* ± standard error. N = number of female wasps. *** P<0.001.**

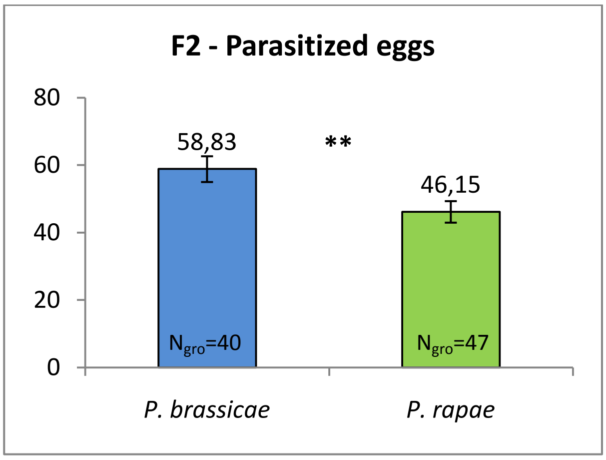

**Figure 11. Mean number of parasitized *E. kuehniella* eggs by female wasps emerging from *P. brassicae* and *P. rapae* eggs ± standard error. N = number of female wasps. ** P<0.01.**

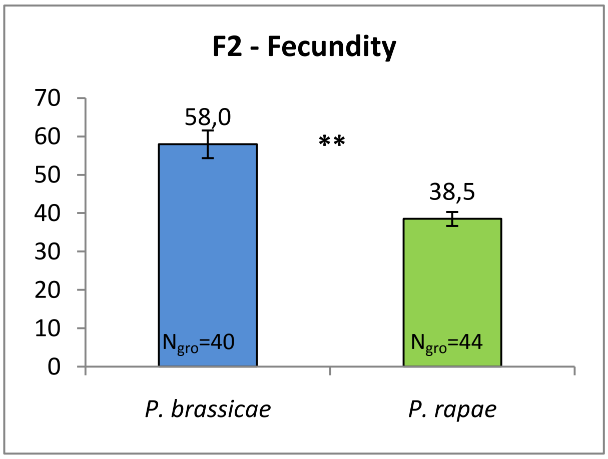

**Figure 12. Fecundity of female wasps emerging from eggs of *P. brassicae* and *P. rapae* ± standard error. N = number of female wasps. ** P<0.01.**

The larger female wasps emerging from *P. rapae* eggs also significantly parasitized more *E. kuehniella* eggs (P = 0.002 – Mann-Whitney U test; Figure 11) and had a higher fecundity (P = 0.006 – Mann-Whitney U test; Figure 12) than the smaller wasps emerging from *P. rapae* eggs. Clearly the fecundity of female wasps increases with size (Figure 13).

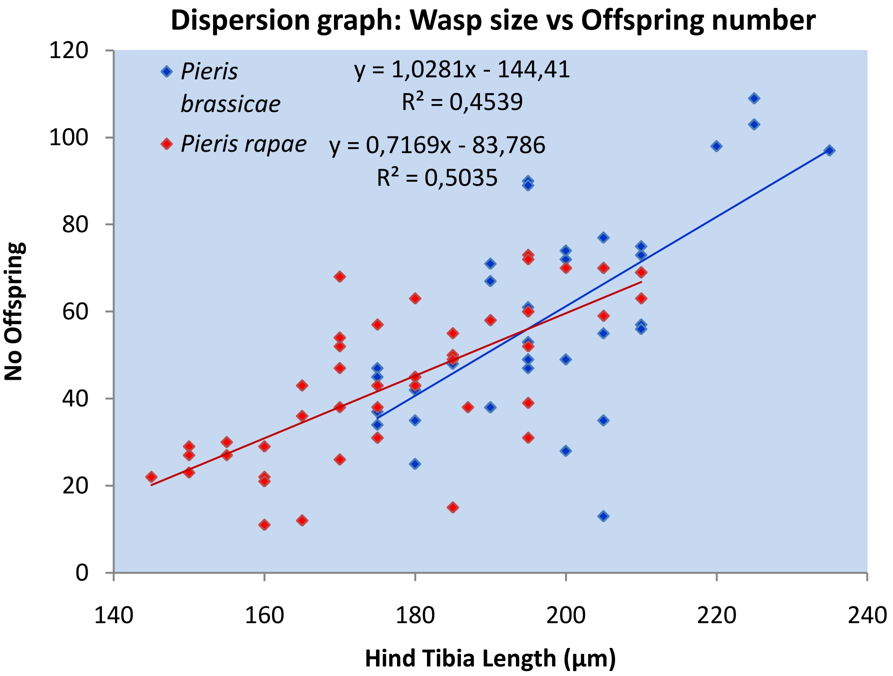

**Figure 13. Relationship between female wasp size (hind tibia length) and fecundity (offspring number) for wasps emerging from *P. brassicae* and *P. rapae* eggs in a dispersion graph. Blue dots represent wasps emerging from *P. brassicae* eggs and red dots are those emerging from *P. rapae* eggs. $R^2$ and trend line equation are shown.**

## R3. Memory after two different learning trials

Results of experiments in which female wasps received two different trainings spaced by one hour, show that memory for both the first and second training is completely absent at 24 h after the second training independent on which butterfly species the wasps were trained first or second (Figure 14).

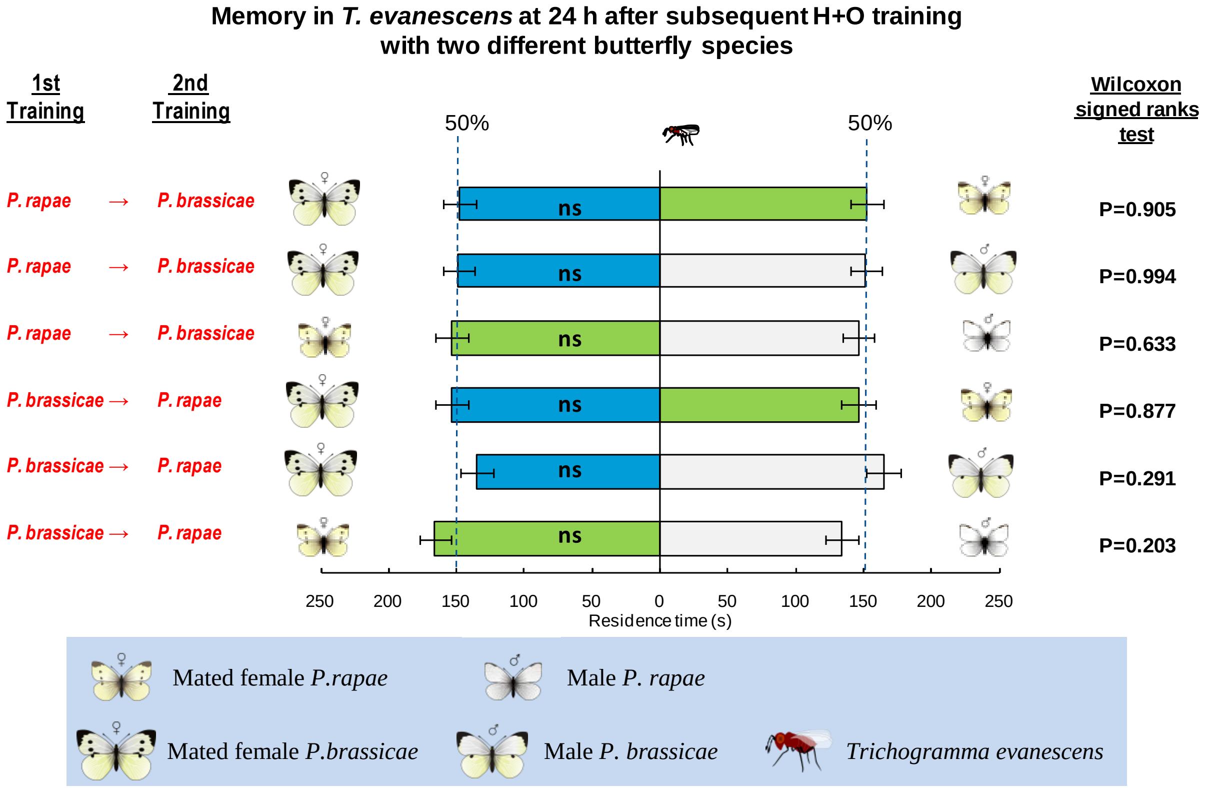

**Figure 14. Response of wasps to mated female and male butterfly odors at 24 h after H+O training with the two butterfly species *P. rapae* and *P. brassicae* with one hour time interval in a two chamber olfactometer. Each bioassay was done with 40 wasps (replicates). Mean residence time (s) ± standard error in the odor fields of mated female and male butterflies are given. H+O = rewarding hitch-hiking experience consisting of a successful ride on a mated female butterfly leading to an oviposition into a freshly laid butterfly egg. ns=no significant difference.**

## R4. Night temperature effect

Keeping the wasps at 10 $^{o}$C overnight did not affect memory at 16 h after an H+O experience with *P. rapae* when compared to wasps that spent the night at 23$^{o}$C (P = 0.617 – Kruskal-Wallis test; Figure 15). However, memory was not present at 16 h (P = 0.497 – Wilcoxon signed ranks test; Figure 15) and 24 h (P = 0.488 – Wilcoxon signed ranks test; Figure 15) after an H+O experience with *P. brassicae* when the wasps were kept at 10 $^{o}$C overnight, whereas it was present after a night of 23 $^{o}$C (P = 0.043 and P = 0.018, resp., Wilcoxon signed ranks test; Figure 15). There were no significant differences in the residence times in the odor field of mated female butterflies between wasps kept at a cold and warm night at 16 h and 24 h after an H+O experience with *P. brassicae* (P = 0.547 and P = 0.238, resp. – Kruskal-Wallis test; Figure 15).

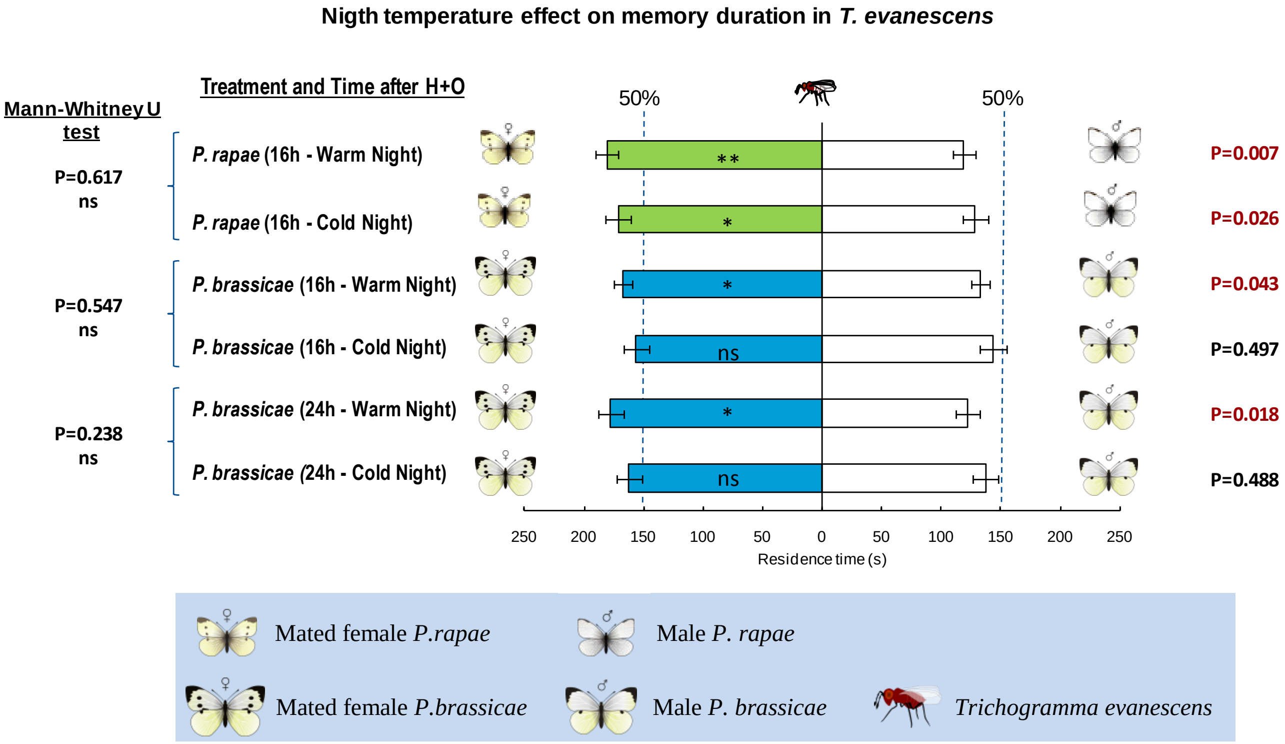

**Figure 15. Effect of cold night temperature on the response of wasps to mated female and male butterfly odors after an H+O experience with *P. brassicae* and *P. rapae* in a two chamber olfactometer. Cold night treatment was done for 8 h at 10 °C, warm night for 8 h at 23°C. Each bioassay was done with 40 wasps (replicates). Mean residence time (s) ± standard error in the odor fields of mated female and male butterflies are given. H+O = rewarding hitch-hiking experience consisting of a successful ride on a mated female butterfly leading to an oviposition into a freshly laid butterfly egg. Significant differences ** P<0.01; * P<0.05; ns=no significant difference.**

## R5. Memory types after training

Memory was only present at 4 h after an H+O experience with *P. rapae* in control wasps, and not in ANI- and cold shock-treated wasps (Paired comparisons with Wilcoxon signed ranks test; Figure 16). However, there was no significant difference in the time spent in the odor field of mated female butterflies between differently treated wasps (control, cold-shock and ANI treatment) (P = 0.177 – Kruskal-Wallis test; Figure 16).

At 16 h after such a training event, memory was also only present in control wasps, and not in ANI- and cold shock-treated wasps (Paired comparisons with Wilcoxon signed ranks test; Figure 16). But also there was no significant difference in residence times in the odor field of mated female butterflies between differently treated wasps (control, cold-shock and ANI treatment) (P = 0.163 – Kruskal-Wallis test; Figure 16).

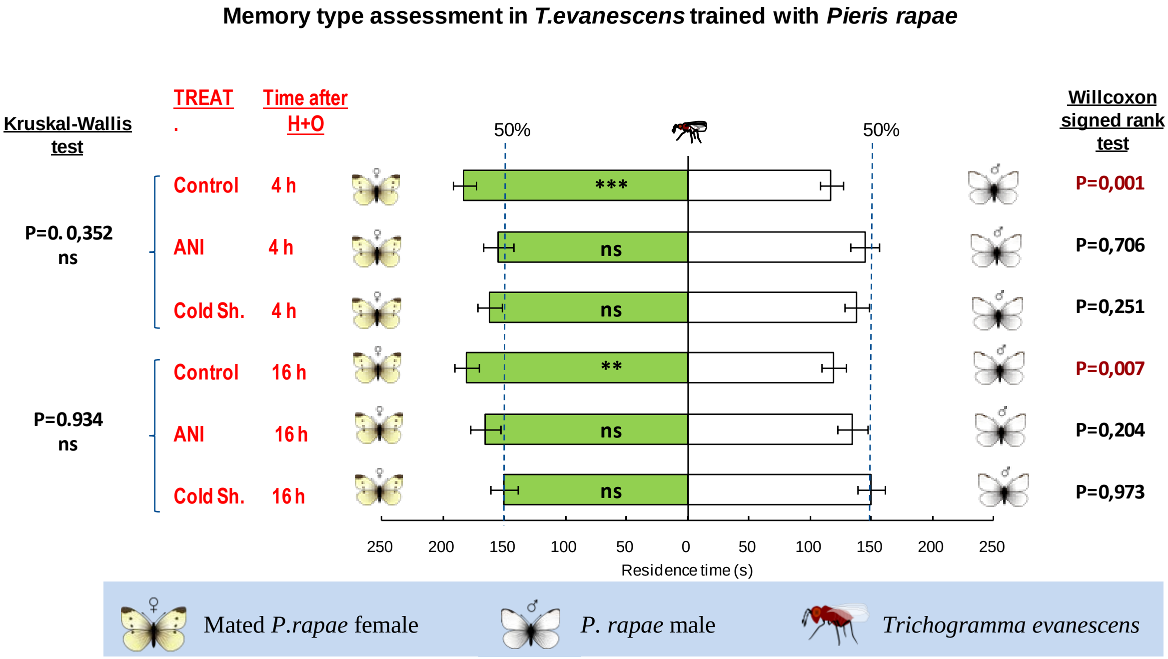

**Figure 16. Assessment of the presence/absence of Anaesthesia Sensitive Memory (ASM), Anaesthesia Resistant Memory (ARM) and/or protein synthesis-dependent Long Term Memory (LTM) at 4 h and 16 h after an H+O experience with *P. rapae* by testing the response of wasps to mated female and male butterfly odors in a two chamber olfactometer. Anisomycin (ANI) treatment was done at 24 h before training. Cold shock treatment was done one hour before a bioassay. Mean residence time (s) ± standard error in the odor fields of mated female and male butterflies are given for every bioassay. H+O = rewarding hitch-hiking experience consisting of a successful ride on a mated female butterfly leading to an oviposition into a freshly laid butterfly egg. P-values for pair wise comparison (Wilcnxon signed ranks test) and mean comparison between treatments (Kruskal-Wallis test) are given. Significant differences *** P<0.001; ** P<0.01; ns=no significant difference.**

In the case of wasps trained with *P. brassicae*, the same pattern was observed. Memory was only present at 4 and 16 h after an H+O experience in control wasps, and not in ANI- and cold shock-treated wasps (Paired comparisons with Wilcoxon signed ranks test; Figure 17), but there was no significant difference in the time spent in the odor field of mated female butterflies between differently treated wasps (control, cold-shock and ANI treatment) (P = 0.352 and P = 0.934, resp. – Kruskal-Wallis test; Figure 17).

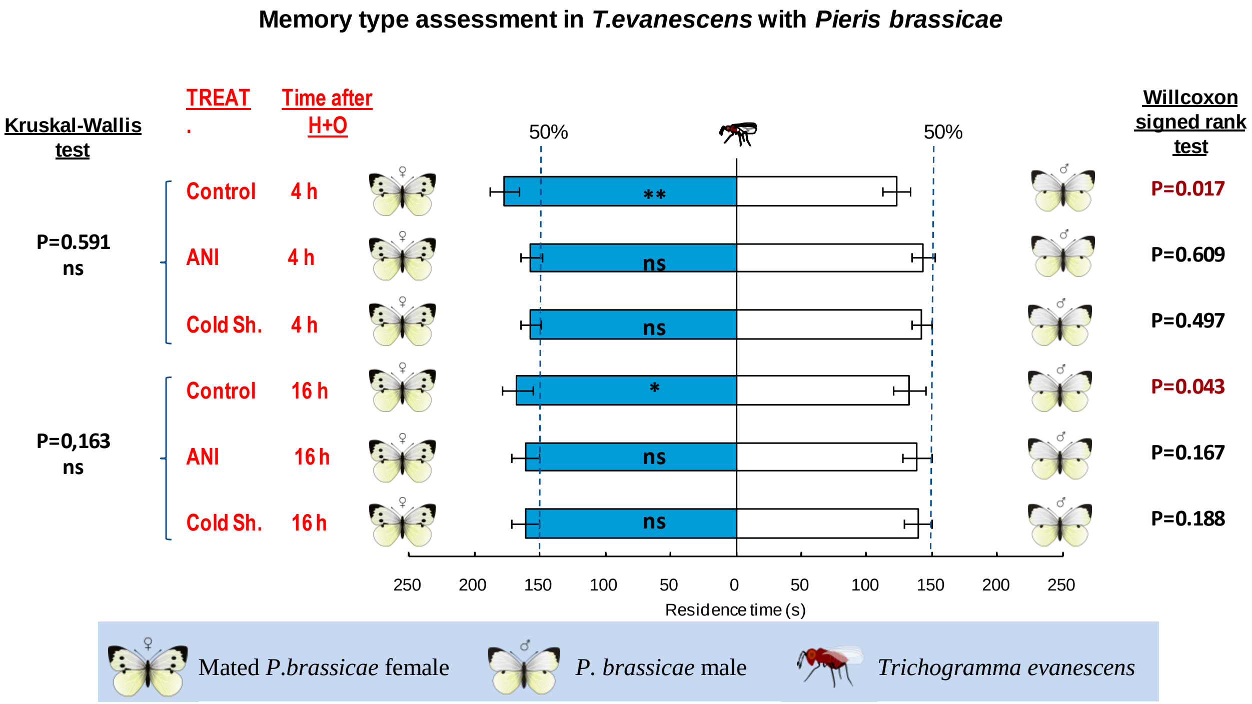

**Figure 17. Assessment of the presence/absence of Anaesthesia Sensitive Memory (ASM), Anaesthesia Resistant Memory (ARM) and/or protein synthesis-dependent Long Term Memory (LTM) at 4 h and 16 h after an H+O experience with *P. brassicae* by testing the response of wasps to mated female and male butterfly odors in a two chamber olfactometer. Anisomycin (ANI) treatment was done at 24 h before training. Cold shock treatment was done one hour before a bioassay. Mean residence time (s) ± standard error in the odor fields of mated female and male butterflies are given for every bioassay. H+O = rewarding hitch-hiking experience consisting of a successful ride on a mated female butterfly leading to an oviposition into a freshly laid butterfly egg. P-values for pair wise comparison (Wilcoxon signed ranks test) and mean comparison between treatments (Kruskal-Wallis test) are given. Significant differences ** P<0.01; * P<0.05; ns=no significant difference.**

## DISCUSSION AND CONCLUSIONS

### R1 & R2. Host species-specific memory duration and reward value

Memory duration in the generalist egg parasitoid wasp *T. evanescens* after a single rewarding hitch-hiking (H+O) experience was shown to be host species-specific. At 24 h after a single conditioning trial, memory is still present in wasps that were trained with *P. brassicae* but not in those that were trained with *P. rapae*. My results indicate that memory waned somewhere between 16 h and 24 h after a single training with *P. rapae*, whereas memory after a single training with *P. brassicae* is only lost somewhere between 24 h and 72 h. Interestingly, memory duration increased to at least 24 h after training with *P. rapae* when wasps received two learning trials on that butterfly species spaced in time (Figure 3). This is similar to learning and memory formation in the specialist larval parasitoid *Cotesia rubecula* after training with its host *P. rapae* (Smid *et al.* 2007). Such spaced training on *P. rapae* is ecologically relevant as this abundant butterfly lays only a single egg on a plant at a time. Only after hitch-hiking with a few mated female *P. rapae* butterflies, and parasitizing a single butterfly egg (i.e. allocating 3 wasp eggs to a *P. rapae* egg; Figure 5) after each hitch-hiking event, approaching more mated female butterflies would reliably indicate the presence of more suitable host eggs for a female *T. evanescens* wasp to eventually be able lay a large proportion of her eggs (Doyon and Boivin 2005; this study).

The longer lasting memory in *T. evanescens* after a single learning trial with *P. brassicae* is in accordance with very recent work on a larval parasitoid of the two cabbage white butterflies, *Cotesia glomerata.* This parasitoid also exhibits memory at 24 h after a single conditioning with a caterpillar of its preferred host *P. brassicae*, whereas it does not after a similar learning trial with its less preferred host *P. rapae* (Kruidhof *et al.* unpublished data). The host-species specific memory duration in both parasitoid species can be explained by a different reward value offered by the two hosts. For *C. glomerata,* that has closely coevolved with the two *Pieris* species; the reward value of parasitizing just one *P. brassicae* caterpillar on a plant is higher than that of parasitizing a *P. rapae* caterpillar on a plant in two ways. First of all, one *P. rapae* caterpillar is a more suitable host because they have larger clutch size, developmental rate and adult size when developing in *P. brassicae* (Harvey 2000). Second, parasitizing a *P. brassicae* caterpillar on a certain plant species is more reliable predictor of the presence of more *P. brassicae* caterpillars on that plant species because *P. brassicae* butterflies tend to lay their clustered eggs in dense stands of the same plant species (Lemasurier 1994), whereas *P. rapae* travels long distances to distribute its singly laid eggs over different plant species (Root and Kareiva 1984). This is different for *T. evanescens*. This generalist wasp parasitize a wide range of hosts (Hase 1925) and the duration of memory after a rewarding hitch-hiking experience thus most likely only depends on the value of the host egg(s) perceived while parasitizing. A clutch of *P. brassicae* eggs should represent a larger reward than a single *P. rapae* egg. Eventhough a female *T. evanescens* wasp only had the opportunity to parasitize just one *P. brassicae* egg in a whole clutch during a conditioning trial in my study, she did first walk over more eggs in the clutch and thus ‘sensed’ the presence of other eggs. Recent work has, however, shown that even when *T. evanescens* wasps are offered only a single *P. brassicae* egg as a reward after hitch-hiking with a mated *P. brassicae* female, memory lasts longer than after a similar training with a single *P. rapae* egg: memory is still present at 24 h after training in the first case but not in the latter (Pashalidou *et al.* unpublished data). I therefore hypothesized that even a single *P. brassicae* egg has a higher reward value (higher fitness gain) for *T. evanescens* than a single *P. rapae* egg. The results clearly show that female wasps lay more eggs into a *P. brassicae* egg than into one of *P. rapae* meaning that they perceive a *P. brassicae* egg as a larger reward. This difference in reward value can thus explain the host species-specific memory duration after a single H+O event in which the wasps received only a single egg of both wasp species as a reward. When following immature development inside the eggs of both butterfly species, it became evident that immature wasps have a higher mortality in *P. brassicae* eggs than in those of *P. rapae*, and consequently fewer adult wasps emerge from *P. brassicae* eggs. The higher mortality within *P. brassicae* egg may be due to an immunity response similar to the immunity response to *T. evanescens* observed in eggs of the tobacco hornworm *Manduca sexta* (Abdel-Latief *et al.* 2008). Although fewer wasps emerged from *P. brassicae* eggs, the emerging wasps were larger, and consequently also had a higher fecundity during three days of parasitizing an overamount of host eggs. *Trichogramma* wasps are known to lay by far most eggs during the first three days under such conditions (Doyon and Boivin 2005).

In this study, I have compared the difference in reward value of single eggs of both species. However in nature, a clutch of *P. brassicae* eggs should even represent a much higher reward value than a single *P. rapae* egg. In future experiments, it would be interesting to test whether parasitizing multiple *P. brassicae* eggs in the clutch even increases the difference in host species-specific memory duration.

### R3. Memory after two different learning trials

Two H+O training events with a different host species spaced by 1 h resulted in a complete absence of memory for both training events in *T. evanescens* at 24 h. Memory is known to be present at 24 h after one training event with *P. brassicae* (Huigens *et al*. 2009; this study). However, when such a learning trial is followed, or preceded, by a single training event with *P. rapae*, this memory trace is not present anymore at 24 h. A second training with *P. rapae* either results in the extinction of memory for the first training with *P. brassicae* or in a memory trace for that first training that lasts shorter than 24 h. Surprisingly, a second training with *P. brassicae* is also not remembered anymore at 24 h when it was preceded by a single training event with *P. rapae*. Female *T. evanescens* wasps seem to be ‘confused’ by two different trainings. In nature a female egg parasitoid that has had one H+O experience with a mated female of one butterfly species is, however, searching for other mated females ofthat particular butterfly species and is thus not likely to mount a mated female butterfly of another species within 1 h.

### R4 & R5. Night temperature effect and memory types after training

Memory formation in *Trichogramma* wasps is only recently reported (Huigens *et al.* 2009, 2010). My study represents the first step to unravel mechanisms underlying memory formation. The results indicate that a cold night temperature ($10^0$C for 8 hours) has a negative effect on memory duration in *T. evanescens* after one single training event with *P. brassicae*. Memory was present at 16 h and 24 h after a warm night but not after a cold night. A low night temperature thus seems to act as an abiotic memory suppressing factor. A cold night is common in nature and may disturb the capability of *T. evanescens* to remember a prior learning experience. However, there were no significant differences in residence times in the odor field of mated female butterflies between trained wasps kept at either a warm or a cold night (Figure 15). Interestingly, a cold night did not affect 16 h memory after an H+O event with *P. rapae,* thereby suggesting that this 16 h memory differs at least in one way from 16 h memory after training with *P. brassicae*. However, I cannot draw a firm conclusion about the effect of night temperature after training with *P. brassicae*.

Unfortunately, my study did not elucidate which memory types are present in *T. evanescens* at 4 and 16 h after training with *P. rapae*, as well as with *P. brassicae.* Irrespective of the butterfly species used for conditioning, memory is only present at both timepoints after training in control wasps, and not in ANI- and cold-shock-treated wasps, thereby suggesting both LTM and ASM to be present. This would be in contrast with other studies on memory formation in other (much larger) animals that have shown ASM to last only for a few hours (Smid *et al*. 2007). Only a recent study indicate an ASM trace to last for more than 24 h in the fruit fly *Drosohila melanogaster* (Shuai *et al*. 2010). However, there was no significant difference in the time spent in the odor field of mated female butterflies by control, ANI- and cold-shock-treated *T. evanescens* wasps which suggests only ARM to be present at 4 and 16 h after training with *P. brassicae* and *P. rapae*. A recent study by Kruidhof *et al*. (unpublished data) showed that the larval parasitoid *C. glomerata* consolidated LTM after single-trial conditioning with *P. brassicae* caterpillars (high-value host), but consolidated only shorter-lasting ARM with *P. rapae* caterpillars (low-value host). This suggests that memory consolidation is a plastic trait that is adjustable to the reward value. Energetically costly LTM (Mery and Kawecki 2005) is formed after obtaining a bigger reward whereas cheaper ARM is formed after receving a smaller reward. It will be interesting to find out whether the same applies to memory consolidation in *T. evanescens* after single-trial learning with both butterfly species. At least I also found a shorter lasting memory type in this wasp species after one training event with *P. rapae* than with *P. brassicae*.

Obviously, it is difficult to draw any firm conclusions about the effect of night temperature, ANI and/or cold-shock-treatment on memory formation in *T. evanescens*. I believe that this is mainly due to the fact that the two-chamber olfactometer used in my experiments does not have a strong discriminative power between a wasp’s response to treatment and control odors. Future studies on the effect of different treatments on memory formation in *Trichogramma* wasps should therefore be carried out using setups that have a larger discriminative power. Y-tube olfactory bioassays in which groups of *Trichogramma* wasps can be released at a time might provide a solution to this problem (Lucas-Barbosa 2010). In that case it would be essential to improve the efficiency of the “associative learning experience” step, because the current training methodology does not allow getting more than 30 experienced wasps per day.

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Finally I want to dedicate this work to my coming treasure…

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