WAGENINGEN UNIVERSITY

LABORATORY OF ENTOMOLOGY

# *Chemical espionage on male-specific compounds and anti-aphrodisiacs of Pieris butterflies by the egg parasitoid Trichogramma brassicae*

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Name: J.J.G.P. van Roosmalen

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Supervisor: dr. ir. M.E. Huigens

1e Examinator: dr. ir. J.J.A. van Loon

2e Examinator: prof. dr. M. Dicke

## *ABSTRACT*

Female *Trichogramma brassicae* wasps hitch-hike on mated female cabbage white butterflies to eventually locate their eggs. Indications had been found that male butterflies were also used for hitch-hiking (a.k.a. phoresy). In addition, it was found that the wasps showed no significant preference for mated female over male *Pieris rapae* butterflies during an olfactometer bioassay. In this thesis, research was done to the arrestment behavior of female *T. brassicae* wasps by male-specific *P. rapae* compounds. After performing static dual-choice olfactometer bioassays, it was found that the wasps showed arrestment behavior when detecting wing compounds of male *P. rapae* butterflies on short distance. However, insignificant preferences of the wasps species for paper models of *P. rapae* treated with comparable amounts of this extract were found after performing dual-choice phoretic behavior bioassays, although there seemed to be a tendency towards preference. More work on the arrestment behavior of *T. brassicae* wasps by male-specific compounds of *Pieris* butterflies, a new research line, is needed before an appropriate conclusion can be drawn. Previous studies have demonstrated that *T. brassicae* wasps can exploit the anti-aphrodisiacs of 2 *Pieris* butterfly species. However, it was unknown whether the wasp exploits these pheromones as single compounds or in combination with other female odors. This study shows that *T. brassicae* wasps need to detect other female odors in combination with anti-aphrodisiacs of the same *Pieris* butterfly species to be arrested by mated female *Pieris brassicae* and *P. rapae* butterflies.

Few data are available on the phoresy efficiency of *T. brassicae* wasps under natural conditions. Catching and phoresy efficiency experiments with *T. brassicae* wasps were performed in a gauze tunnel under semi-field conditions. Only several wasps were found back 60 seconds after the release of mated female *P. brassicae* butterflies, with on each of them one wasp. This can indicate that the method of catching butterflies is unsuitable. No egg parasitism by *T. brassicae* wasps after phoretic behavior on mated female *P. brassicae* was found. This can be explained by low oviposition frequencies of and stress among the butterflies, and because of bad weather conditions. No significant attraction was observed when testing the attractiveness of anti-aphrodisiacs of mated female *P. brassicae* butterflies for *T. brassicae* wasps under semi-field conditions. Before an appropriate conclusion can be drawn, more replicates have to be performed and these should only be performed under favorable weather conditions.

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## *INTRODUCTION*

For reducing the detrimental effects of herbivorous insects on crops and for securing our food availability, the release of their natural enemies in the field can be considered as part of an Integrated Pest Management (or IPM) program (Lewis & Nordlund, 1985). Natural enemies occur in many forms, like entomophagous species which derive nutrients to grow and eventually to reproduce. Some entomophagous or parasitoid species parasitize eggs of their host as part of their reproductive cycle. For releasing these natural enemies in the field, one has to keep in mind that beneficial non-target and for the crop species harmless insect species and other arthropods can become the victim of the IPM program (Van Lenteren, 2000). One important and promising insect species which can parasitize the eggs of *Pieris* butterflies, which can form a pest on cabbage crops (Brassicaceae), is the minute wasp *Trichogramma brassicae*: a generalist egg parasitoid on numerous butterfly species (Nordlund, 1994).

Recently, several studies have focused on the behavior of *Trichogramma* wasps towards *Pieris* butterflies (Fatouros *et al*, 2005a & Fatouros *et al*, 2007). The egg stage is the target of the wasps and is an immobile, non-feeding stage that releases much fewer detectable volatile cues than other stages (Hilker & Meiners, 2002 & Fatouros, 2006). This given implies that these wasps face an enormous challenge to find host eggs (Fatouros *et al.*, 2005a). An indirect way to search for potential hosts is to aim at the better detectable source of the target: a mated female butterfly which is ready for oviposition. Butterflies emit many chemical compounds which can be detected and exploited by *Trichogramma* wasps. By means of climbing on and hitch-hiking with these alternative targets, they can arrive at their main target: the eggs. The transport of insect individuals on host bodies for purposes other than direct parasitism is called phoresy (Farish & Axtell, 1971 in Houck & OConner, 1991). Recently, it was found in laboratory bioassays that a female *T. brassicae* can reach eggs of *Pieris brassicae*, the large cabbage white butterfly, after performing phoretic behavior on mated female butterfly and in the best case, the wasp walks off the butterfly when the latter lands at a suitable location for oviposition (Fatouros *et al.*, 2005b). This hitch-hiking behavior seems a logical adaptation given the limited flight ability, the narrow time window for reproduction and their preference for egg patches (Fatouros *et al*., 2005b). Fatouros *et al*. (2005b) have shown that phoretic behavior for a successful egg parasitism can occur under laboratory conditions and reported that 7.1 percent of the hitch-hiking *T. brassicae* wasps successfully parasitized *P. brassicae* eggs during the experiment.

### Host location

The searching behavior of *Trichogramma* wasps consists of several phases: host habitat location, host location and host acceptance (Fatouros *et al.*, 2008a). For host habitat location, *Trichogramma* wasps (and other egg parasitoids) can be arrested by volatiles from plants when entering a specific habitat rather than attracted from a distance (Romeis *et al.*, 1997). For host location, the wasps can locate potential hosts by sensing non-volatile kairomones present on wing scales from adult *Pieris* butterflies or plant synomones induced by the deposition of *Pieris* eggs (Fatouros *et al*., 2005a). Another strategy is to eavesdrop on the intersexual communication between conspecific butterflies (Fatouros *et al*., 2005b). The transfer of anti-aphrodisiacs from male to female butterfly during courtship is one way of intersexual communication and is presumably more reliable for successful host egg localization than the exploitation of sex pheromones during the pre-mating stage (M.E. Huigens, unpublished). Fatouros *et al.* (2005b) have shown that female *T. brassicae* perform phoresy on virgin female *P. brassicae* which was treated with benzyl cyanide, that acts as an anti-aphrodisiac in this butterfly species. The use of mating-related pheromones by parasitoids can form a serious constraint to the evolution of sexual communication in hosts (Fatouros *et al*., 2005b).

There is also some evidence that volatiles emitted by plants after oviposition by potential host species can arrest *Trichogramma* wasps. After the oviposition of *P. brassicae* on plant material, *T. brassicae* is arrested by the changed infochemical production of the plant after 3 days (Fatouros *et al.*, 2005a). Interestingly, the anti-aphrodisiac benzyl cyanide seems to trigger these chemical changes in the plant (Fatouros *et al.*, 2008b). In general, the minute wasps are assumed to be dispersed passively by wind currents (Fatouros *et al.*, 2008a). Therefore, volatile plant chemicals do not attract but arrest wasps and it is assumed that they also respond to general plant volatiles (Fatouros *et al*., 2005a).

**Intersexual behavior of *Pieris* butterflies**

During mating, male *Pieris* butterflies transfer anti-aphrodisiacs to females so that formerly very attractive virgin females are rendered very unattractive after the deed (Andersson *et al.*, 2000 & Andersson *et al*., 2003). In this way, there will be a reduction in the harassment and interruption by males during oviposition. *Pieris brassicae* males transmit a part of their body-specific odor, benzyl cyanide, as anti-aphrodisiac to females. This in contrast to male *Pieris rapae* and *Pieris napi* which transfer respectively methyl salicylate + indole and methyl salicylate as anti-aphrodisiacs to females (figure 1). These compounds are not considered as male-characteristic (Andersson *et al.*, 2003). The amount of anti-aphrodisiacs emitted by mated female *P. napi* will be reduced when they actively adopt the mate-refusal posture when courted by male individuals (Andersson *et al.*, 2004). Mated females become more attractive to males when time after mating increases suggesting the male's capability to sense the differences in the amount of anti-aphrodisiacs released by mated females. For influencing the mating behavior of females, male butterflies release male-specific compounds from their androconia (Bergström & Lundgren, 1973 in Andersson *et al*., 2000), but they seem incapable of forcing females to mate (Svärd & Wiklund, 1989). In general, the female receptivity for mating by butterflies is dependent on visual and olfactory signals of which olfactory stimuli are more important at a short distance and visual stimuli at a relatively long distance (Silberglied, 1984).

**Odor preference and mounting behavior of *Trichogramma* species on *Pieris* butterflies**

In contrast to *T. brassicae*, naïve female *Trichogramma evanescens* wasps are not arrested by mated female over male *P. brassicae* and *P. rapae* (respectively Fotouros *et al.*, 2007 and Van Elven, 2007) and therefore, the wasp must first undergo a rewarding hitch-hiking experience with a mated female butterfly before the wasp is significantly arrested to the odor of mated female butterflies (Pashalidou, 2008). Naïve *T. brassicae* females already show a significant odor preference for mated female over air flow, virgin female and male *P. rapae* (Woelke, 2008). When treating virgin female *P. rapae* with different concentrations of conspecific anti-aphrodisiacs, methyl salicylate and indole, 2 µg of both compounds works best for arresting *T. brassicae*. By using a concentration over 20 µg, the wasps show a preference for untreated control virgin butterflies (Woelke, 2008). A possible explanation for the observation that naïve *T. brassicae* already prefer mated females of *P. brassicae* and *P. rapae* is a longer coevolution with these butterfly species or a more restricted host range than *T. evanescens*.

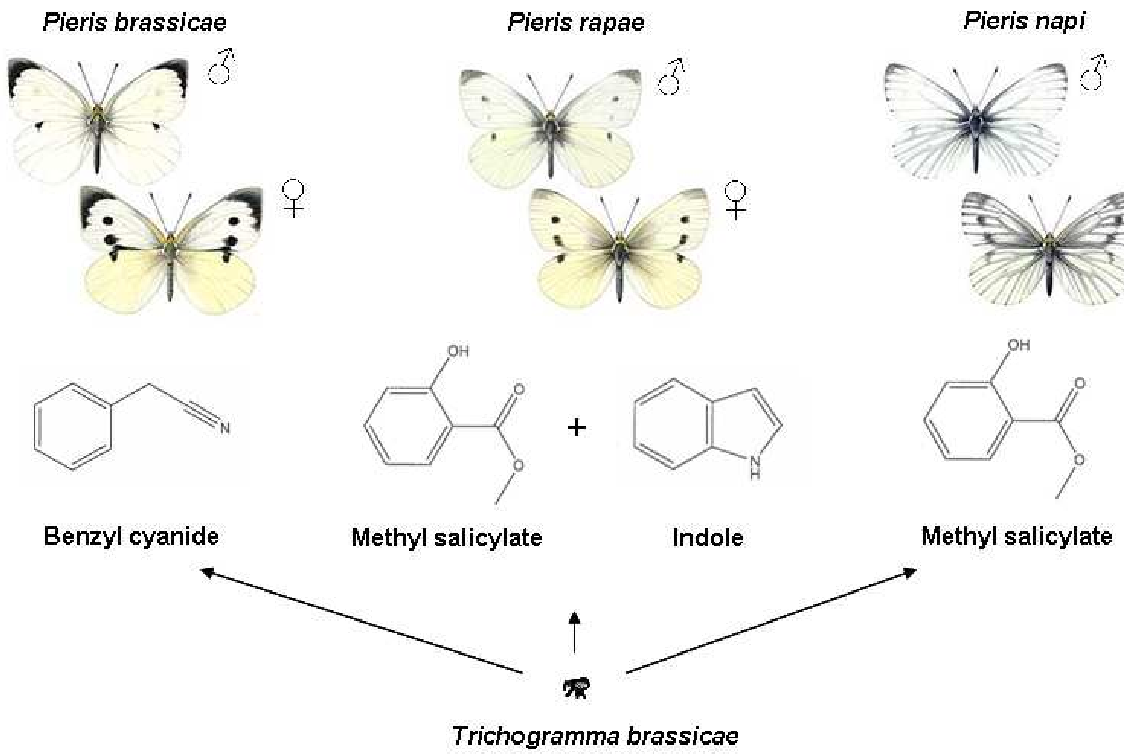



***Figure 1:*** *chemical structure of different anti-aphrodisiacs which are produced by male* Pieris *butterflies and transferred to females during mating to make them less attractive to other conspecific males.*

*Trichogramma brassicae* also shows a significant preference for mated female over virgin female *P. rapae* during mounting behavior bioassays. The wasps also have a preference for virgin females treated with 2 µg methyl salicylate and indole in hexane compared to virgin female *P. rapae* butterflies treated with hexane only. However, naïve *T. brassicae* showed no significant preference for mated female over male *P. rapae* butterflies (Woelke, 2008). The wasps may also hitch-hike on male butterflies to eventually parasitize eggs after the transfer to females during mating. Some egg parasitoid species are known for their phoretic behavior on males as well (Fatouros, 2006). *Trichogramma evanescens* wasps first need a positive hitch-hiking experience before the wasp has a significant mounting preference for mated female over male and virgin female *P. rapae* (Van Elven, 2007).

Pashalidou (2008) also finds that *T. evanescens*, which was given an oviposition experience after a hitch-hiking experience with a mated female butterfly, is not significantly arrested to virgin female *P. rapae* and *P. brassicae* treated with heterospecific anti-aphrodisiacs over untreated virgin females. Only when (a) conspecific anti-aphrodisiac(s) are offered in combination with female butterfly odors with which the wasp had experience, the wasp has a significant preference for the virgin females treated with that anti-aphrodisiac (figure 6). Fatouros *et al.* (2005b) and Woelke (2008) show that *T. brassicae* is significantly arrested by the odor of respectively virgin female *P. brassicae* and virgin female *P. rapae* treated with conspecific anti-aphrodisiacs compared to untreated virgin females.

**Male-specific and sex pheromone-like compounds**

Male butterflies release male-specific compounds to increase their acceptance for mating by females and as a result can act as sex pheromones (Andersson *et al.*, 2000). It is possible that *T. brassicae* wasps might also eaves-drop on these compounds. Andersson *et al*. (2007) were able to derive some male-specific compounds from *P. napi* butterflies. It seems that *P. napi* males release citral when interacting with conspecific males and females. The latter express mating acceptance behavior when they sense male wings with traces of citral and their antennal response is ten times more sensitive to this compound than that of males (Andersson *et al*., 2007). Therefore, some proof has been obtained that *Pieris* butterflies release sex pheromones to attract females. The question now remains if *T. brassicae* can detect and benefit from sex pheromone-like compounds on males of several *Pieris* species.

**Hitch-hiking behavior of *Trichogramma* wasps under natural conditions**

The hitch-hiking behavior of *Trichogramma* wasps on *Pieris* butterflies can be realized in the laboratory. Fatouros *et al*. (2005b) performed a phoresy experiment in a greenhouse in which 7.1% of the *T. brassicae* individuals studied was able to reach the host plant by means of phoresy and to successfully parasitize the freshly laid eggs. However, actual observations of phoretic behavior of the wasps on butterflies are rare in nature. After intensive field work around with butterfly nets in and around Wageningen, just a few female *Trichogramma* wasps were caught hitch-hiking on *Pieris* butterflies (M.E. Huigens, unpublished data) (see appendix I for percentages). It is likely that the method of catching butterflies has to be improved, because hitch-hiking wasps on caught butterflies can easily escape through the gauze of the butterfly net.

**Main topic and research questions**

The aim of this thesis was to further investigate the exploitation of *Pieris* pheromones by the egg parasitoid *T. brassicae*. This wasp species showed no preference for performing first time mounting behavior on mated female over male *P. rapae* butterflies (Woelke, 2008). A reason for this is the possibility that *T. brassicae* is arrested by male-specific compounds. Therefore, olfactometer and phoretic behavior bioassays were done to find out if *T. brassicae* wasps could detect male-specific compounds of *P. rapae* and if they were arrested to them. Pashalidou (2008) showed that *T. evanescens* wasps, after a rewarding hitch-hiking experience with a mated female butterfly, were arrested by virgin *P. brassicae* and *P. rapae* females treated with conspecific anti-aphrodisiacs. To test if this behavior was also performed by *T. brassicae*, the olfactometer was used again to try to answer the question if the wasps need to detect other female compounds in combination with anti-aphrodisiacs to be arrested by mated *P. rapae* and *P. brassicae* females.

The phoresy efficiency of *Trichogramma* wasps in nature and the question if the wasps can detect and are attracted to the anti-aphrodisiac dispersed by mated female and male *P. brassicae* butterflies are unknown. Also, there is some doubt about the butterfly catching method. Therefore, experiments with *P. brassicae* butterflies were done in a gauze tunnel under semi-field conditions to determine the efficiency of the method that was used for trapping hitch-hiking wasps, the phoresy efficiency of *T. brassicae* on *P. brassicae* and if *T. brassicae* was attracted to the anti-aphrodisiac of mated *P. brassicae* females under semi-field conditions.

## *MATERIAL AND METHODS*

**Insects and plants**

For this thesis, *Pieris rapae* L. and *Pieris brassicae* L. also known as small and large cabbage white (Lepidoptera: Pieridae) were used and reared on Brussels sprouts plants at 21±1$^{o}$C, 50-70% relative humidity and L16:D8 light/dark treatment. One day after eclosion, female butterflies were kept separated from the males to maintain their virginity. Mated females and males were taken as mating couples from the breeding cages. The egg parasitoid *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) strain Y175 was reared in glass tubes inside a climate room (23±1$^{o}$C, 50-70% relative humidity, L16:D8 light/dark treatment). New wasp generations were bred by putting *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) egg-cards in the tubes. After 15 days from the moment the egg-cards were put in the tube, the wasps were used and were naïve and ≤ 5 days old when they were used for the experiments. They were fed with a honey solution.

For the performance of experiments under semi-field conditions, *Brassica nigra* (L.) Koch or black mustard (Brassicales: Brassicaceae) was used during their flowering stage. The plants were cultivated at 19.5±0.5$^{o}$C, ±70% relative humidity and L16:D8 light/dark treatment in a greenhouse of Unifarm in Wageningen. Black mustard needed ±28 days to grow from seed to a full-grown flowering plant.

**<u>I Experiments under laboratory conditions</u>**

The behavioral dual-choice experiments in the laboratory were performed in a room which that had a temperature of 23±2$^{o}$C, apart from that standard room conditions were applied. After testing 3 wasps, the odor source or phoretic object was replaced by another and the setup was turned 180 degrees to compensate for possible directional effects of the surroundings on the experiment. Each replicate with a different wasp was run for 300 seconds and in total 40 replicates were done for each experiment.

**Arrestment by male-specific butterfly compounds**

For performing the bioassay which investigated the arrestment of *T. brassicae* by an odor source, a static dual-choice olfactometer had been used with slight modifications from the one that was described by Fatorous *et al*. (2005a). This setup consisted mainly of a Perspex cylinder which was 6cm high and had a diameter of 12cm, divided by a thin plastic sheet. For preventing the escape of the wasps, a glass plate was put on top of the cylinder. Possible contact of the wasp with an odor source was prevented by a gauze component which was permeable to volatile compounds (figure 2). During the start of each replicate, the wasp was released in the middle of the gauze component.

***Figure 2:*** *on the left, one can see the olfactometer setup in which filter papers on flasks of glass were used for testing odor combinations I to VII. On the right, the arena for testing the phoretic behavior of* T. brassicae *in which* P. rapae *paper models were used for testing phoretic object combinations VIII and IX.*

For testing odor combinations I to VII, filter papers with a diameter of 2.5cm were used of which one was treated with a specific extract in a solvent and the other one, which acted as a control, was treated with the solvent only. These extracts consisted of a mixture of compounds dissolved in hexane or dichloromethane. An extract was derived by dipping wings of 10 male *P. rapae* butterflies 3 times in 5mL solvent. Schulz (unpublished) made *P. rapae* solution A and B by mixing specific wing compounds which he was able to derive from this butterfly species (see appendix II for compounds). An extract of *P. rapae* solution A and B was made by dissolving these mixtures in solvent. The filter papers were put on small flasks made of glass which were 3.5 cm high for decreasing the distance between wasp and odor source. In this way, the wasp was better able to perceive the possible attractive chemical compound combination. For a better response of the wasp, the setup was shielded from the influences of the surroundings by a box in which an artificial light source shone below the odor sources at 6cm distance from the wasp.

The tested compound combinations were:

I. Filter paper treated with 4.0 µg male *P. rapae* wing extract vs. filter paper treated with solvent only (hexane)
II. Filter paper treated with 20 µg male *P. rapae* wing extract vs. filter paper treated with solvent only (hexane)
III. Filter paper treated with 0.20 µg *P. rapae* A solution vs. filter paper treated with solvent only (hexane)
IV. Filter paper treated with 2.0 µg *P. rapae* A solution vs. filter paper treated with solvent only (dichloromethane)

V. Filter paper treated with 20 µg *P. rapae* A solution vs. filter paper treated with solvent only (dichloromethane)

VI. Filter paper treated with 2.0 µg *P. rapae* B solution vs. filter paper treated with 2.0 µg *P. rapae* A + *P. rapae* B solution

VII. Filter paper treated with 2.0 µg *P. rapae* A solution vs. filter paper treated with 2.0 µg *P. rapae* A + *P. rapae* B solution

*Pieris rapae* solution B was tested against a mixture of *P. rapae* solution A and B to test if *T. brassicae* was arrested by body-specific compounds only (combination VI). *Pieris rapae* solution A was tested against a mixture of *P. rapae* solution A and B to test if *T. brassicae* need to detect this combination to be attracted to male-specific compounds (combination VII).

For testing the phoretic behavior of *T. brassicae* wasps on objects treated with male-specific compounds, paper models of *P. rapae* were used and the setup of this kind of experiment was described by Fatouros *et al*. (2007). For this setup, a Perspex arena had been used which was 6 cm high and 12 cm in diameter. The paper models were placed opposite to each other in this arena and each wasp was released about 3 cm from the 2 butterfly models (figure 2). The arena was closed with a glass plate on top of the cylinder to prevent the escape of the wasp during the experiment. For testing phoretic object combinations VIII and IX, one model was treated with a specific extract and the control with the solvent only. These extracts consisted of different concentrations of male *P. rapae* wing extracts dissolved in hexane or dichloromethane. The wasp could choose to climb onto one of the models and when a wasp did not climb at all during the 300 seconds of observation, it was noted as a ‘no response’. Each day, up to 10 responding wasps were tested.

The tested phoretic object combinations were:

VIII. *Pieris rapae* paper model treated with 4 µg male *P. rapae* wing extract vs. paper model treated with solvent only (hexane)

IX. *Pieris rapae* paper model treated with 20 µg male *P. rapae* wing extract vs. paper model treated with solvent only (dichloromethane)

**Arrestment by anti-aphrodisiacs in the odor blend of mated female butterflies**

The static dual-choice olfactometer was used again for testing odor combinations X and XI. For these experiments, 2 living virgin female *P. rapae* or *P. brassicae* were put in each chamber of an olfactometer which was 18cm high (Fatouros *et al*., 2005a) (figure 3). Each species was treated with (a) heterospecific anti-aphrodisiac(s). In this bioassay, the average residence time of the wasp above the olfactometer chambers was calculated. Each day, 10-15 wasps were tested.

The tested butterfly treated with anti-aphrodisiacs combinations were:

X. 2 *P. brassicae* each treated with 2.0 µg methyl salicylate + 2.0 µg indole vs. 2 *P. brassicae* treated with solvent only (dichloromethane)

XI. 2 *P. rapae* each treated with 2.0 µg benzyl cyanide vs. 2 *P. rapae* treated with solvent only (dichloromethane)

***Figure 3:*** *on the left, one can see the olfactometer setup in which 2* P. brassicae *or* P rapae *were put in each chamber for testing combinations X and XI. On the right, the gauze tunnel in which semi-field experiments were done.*

## II Experiments under semi-field conditions

The experiments under semi-field conditions were carried out between April and July 2008 in a gauze tunnel which was 16 meter long and 8 meter wide (figure 3). Because the tunnel mainly consisted of gauze, some biotic and abiotic factors could influence the course of the experiments. *Pieris brassicae* was used during these experiments because it was easy to breed large numbers of this butterfly species, it is known that wasps can detect *P. brassicae*'s anti-aphrodisiac benzyl cyanide in the laboratory and wasps can hitch-hike with mated female *P. brassicae* butterflies in the greenhouse (Fatouros *et al.*, 2005b).

All experiments were only done when the weather was relatively good. Relatively good conditions are no or little wind, it has to be pretty warm and the sun has to shine. Days with bad weather prediction for the following 24 hours were avoided for performing these experiments.

### Catching efficiency

To test the efficiency of the previously used method of catching hitch-hiking *Trichogramma* wasps, a mated female *P. brassicae* with a female *T. brassicae* wasp was released in the middle of the tunnel. In each replicate, the female butterfly was hold by her wings and was released after a wasp was successfully put on her back by using a small brush. Each butterfly was released only once. After 60 seconds, the butterfly was caught with a butterfly net with a small jar inside which was large enough for holding such insect. This jar was kept in the net through an elastic band. After the butterfly was caught in the jar, the screw top was instantly put on. In this way, it was tried to keep the possibility that the wasp would escape during the catch as low as possible. After that, the butterflies were taken back to the laboratory and checked under a microscope for wasps.

### Phoresy efficiency

For testing the phoresy efficiency of *T. brassicae* on mated female *P. brassicae* butterflies, one wasp was put on the back of the butterfly per replicate and was released in the middle of the tunnel. In theory, the butterfly had to land on a leaf of one black mustard plant and had to lay eggs. After the landing of the butterfly on the leaf, the wasp had to descend from the insect on this leaf, had to wait shortly until it was possible to parasitize the freshly laid butterfly eggs (Fatouros *et al.*, 2005b). For each replicate, the time that the butterfly took to land on a plant leaf was written down. Furthermore, it was written down if the butterfly laid eggs, if the wasp successfully reached the habitat of the butterfly by means of phoresy, if the wasp successfully descended from the insect on the plant leaf on which the butterfly had landed, and if the wasp was able to parasitize the freshly laid butterfly eggs. The butterfly was caught 10 minutes after the release using the butterfly net. The jar was checked under a microscope in the laboratory for the presence of the wasp.

### Attractiveness of anti-aphrodisiac of mated female butterflies under semi-field conditions

In the gauze tunnel, 16 flowering black mustard plants were distributed in a 4x4 plot (figure 4 & see appendix III for the scheme) and the flowers acted as a meeting point for butterfly and wasp. For examining the attraction of *T. brassicae* to anti-aphrodisiacs under semi-field conditions, 3 cages were hung on each plant. In each cage either a male, a virgin female, or a mated female of *P. brassicae* was present together with a small patch with >20 *Mamestra brassicae* L. (Lepidoptera: Noctuidae) eggs which were taped on paper. This paper was taped on the bottom of each cage for preventing the escape of the butterfly (figure 4). For each replicate of the experiment, approximately 12,500 wasps (with a sex ratio of approximately 75% females) were released in the middle of the 4x4 plot. After 24 hours the cages were put in bags separately so that possible wasps in cages would not escape. In the vicinity of the gauze tunnel, the weather station of Wageningen University continuously measured the weather conditions. Because of the weather conditions, the experiment was replicated 5 times.

***Figure 4:*** *on the left, one can see the setup of the experiment with* B. nigra *plants in the gauze tunnel. Three cages were hung on each* B. nigra *of a total of 16 plants.* T. brassicae *wasps were released in the middle of the plot. On the right, one of the cages which were hung on each plant of the 4x4 plot. Not shown, a butterfly in this cage.*

In the laboratory, the bottoms of the cages with *M. brassicae* eggs were carefully removed, put in Petri dishes and kept in a climate room (23±1$^{o}$C, 50-70% relative humidity, L16:D8 light/dark treatment). After 6-7 days, these eggs were checked for parasitism by *T. brassicae*. The bags were put in the freezer to kill the butterflies and the possible wasps. After this treatment, the butterfly, the cage and the bag itself were checked for wasps.

**III Statistics**

All dual-choice olfactometer bioassays were analyzed using a Wilcoxon's matched pairs signed rank test in SPSS 15.0. All phoresy bioassays were analyzed using a binomial test in Microsoft Office Excel 2003. The test calculated if the proportion of wasps which firstly mounted the treated model was significantly different from the proportion of wasps firstly mounted the control model. With SPSS 15.0, also a Mann-Whitney U test was performed to test if there was a significant difference in the time the wasps spent on treated and control models.

The results of the experiment with the field cages about the attraction of *T. brassicae* towards the anti-aphrodisiac of mated *P. brassicae* females under semi-field conditions were analyzed using a chi-square test of independence (Thorne, 1989). With contingency tables it was determined if the date had an influence on the distribution of the fraction of parasitized egg patches over the 3 different types of cages with males, virgin and mated females, and if the fraction of parasitized egg patches differed between these 3 types of cages when taking the data of all 5 replicates together.

## *RESULTS*

**I Experiments under laboratory conditions**

**Arrestment by male-specific butterfly compounds**

Figure 5 shows the outcome of the olfactometer bioassay combinations I to VII. Only in one bioassay, female *Trichogramma brassicae* significantly prefer the odor of the treated filter paper above of the control paper. This effective treatment was 20 µg male *Pieris rapae* wing extract on filter paper (P=0.025, mean residence time treatment 177.1s vs. control 122.9s). It seemed that 4.0 µg male *P. rapae* wing extract on filter paper was not enough to arrest the wasp (P=0.521, mean residence time treatment 157.0s vs. control 143.0s). For testing the mixture of compounds derived from male *P. rapae* wings (*P. rapae* solution A), an amount of 2.0 µg on filter paper tended to arrest the wasp mostly compared to the control paper which was treated with solvent only (P=0.190, mean residence time treatment 166.5s vs. control 133.5s). For bioassays VI and VII, also a mixture of wing-derived compounds of male and female *P. rapae (P. rapae* solution B) was used. No significant treatment effects were found. Also insignificant results were obtained for olfactometer bioassay combinations VI and VII (2.0 µg *P. rapae* solution B vs. 2.0 µg solution A+B: P=0.326, mean residence time treatment 159.0s vs. control 141.0s and 2.0 µg *P. rapae* solution A vs. 2.0 µg solution A+B: P=0.850, mean residence time treatment 147.6s vs. control 152.4s).

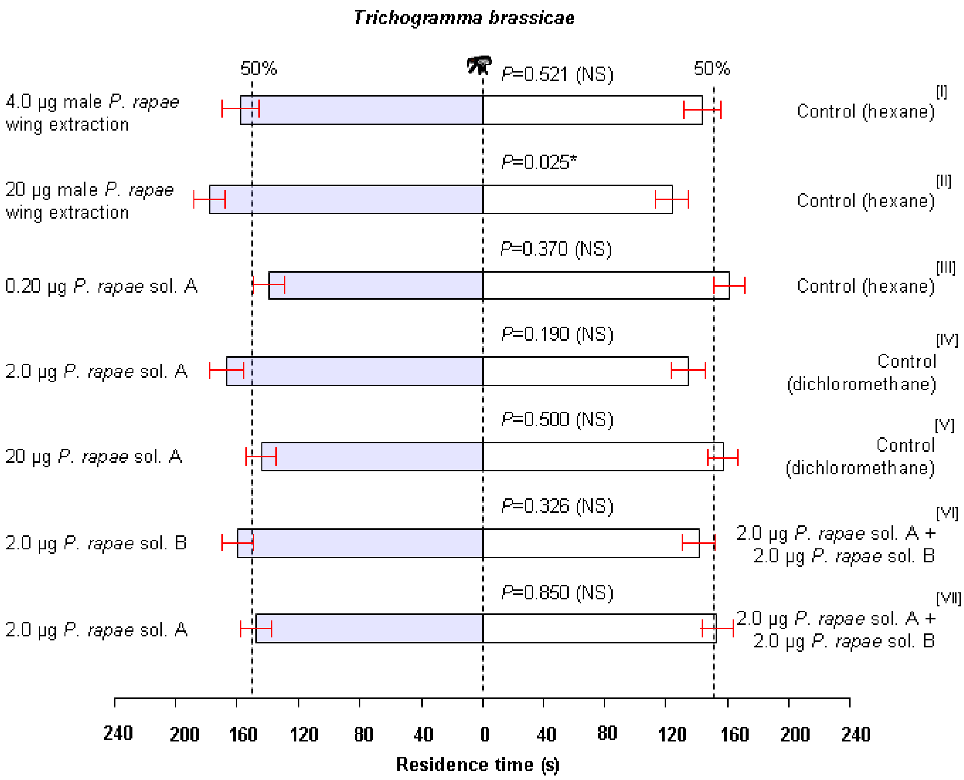



***Figure 5:*** *results of dual-choice olfactometer bioassay combinations I to VII. A blue or white bar represents the mean residence time (± s.e.m.) of* T. brassicae *above the odor source indicated. For each tested combination, 40 wasps were tested and the outcome was analyzed by using a Wilcoxon's matched pairs signed ranks test. Asterisks indicate a significant preference of the wasp for a particular odor source and NS stands for not significant.*

Figure 6 shows the outcome of phoretic behavior bioassays VIII and IX. Paper *P. rapae* models, which were treated with 4.0 µg male *P. rapae* wing extract, were not preferred by *T. brassicae* (P=0.114, first climb treatment 16 wasps vs. control 24 wasps). The wasps also did not significantly prefer to climb onto models treated with 20 µg wing extract compared to the control models (P=0.073, first climb treatment 25 wasps vs. control 15 wasps).

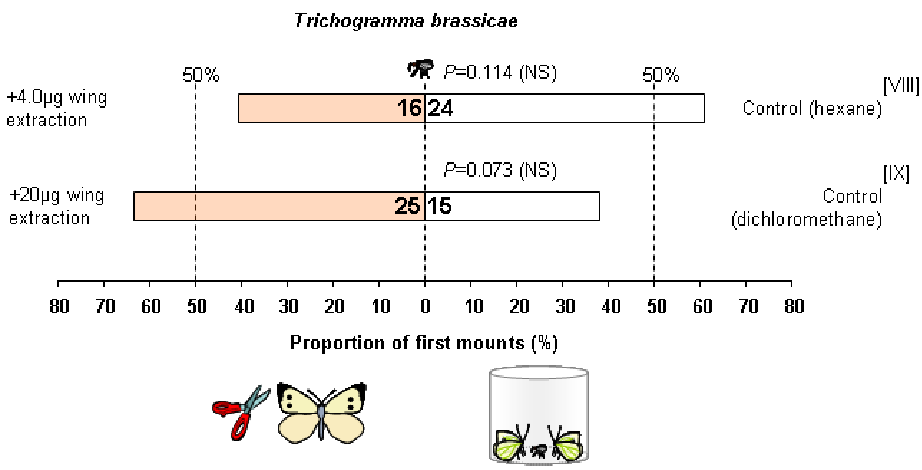



***Figure 6:*** *results of phoretic behavior bioassays which depicts the proportion of first mounts (%) by female* T. brassicae *on one of the paper* P. rapae *models. For each tested combination, 40 wasps were tested and the outcome was analyzed by using a binomial test. NS indicates an insignificant preference of the wasp for a particular phoretic object.*

The mean residence time with standard error mean of female *T. brassicae* on paper *P. rapae* models during bioassay X and XI are depicted in figure 7. Paper models treated with 4.0 µg male *P. rapae* wing extract did not hold wasps significantly longer than untreated models (P=0.135, mean residence time treatment 184.8s vs. control 151.1s). This was also true for *T. brassicae* on paper models with 20 µg wing extract (P=0.459, mean residence time treatment 152.2s vs. control 118.7s).

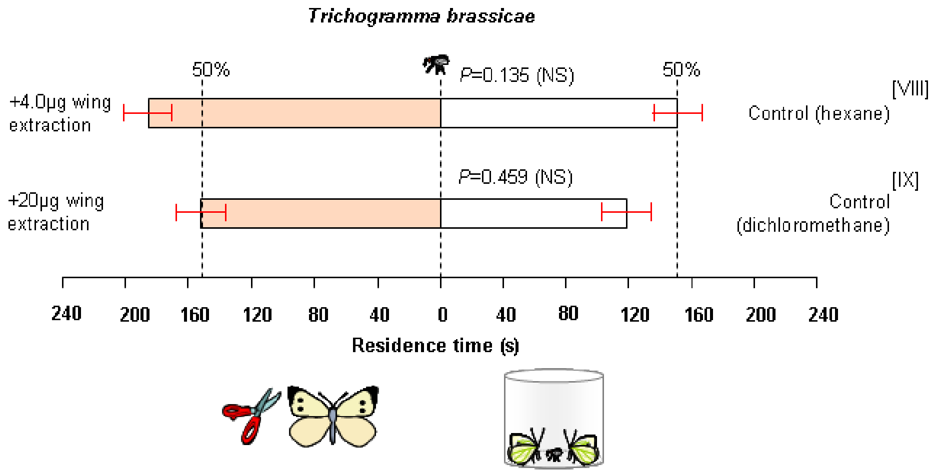



***Figure 7:*** *results of phoretic behavior bioassays which depicts the mean residence time (± s.e.m.) of female* T. brassicae *on the paper* P. rapae *models. Keep in mind that in contrast to olfactometer bioassays, the results of phoretic behavior bioassays were independent so in this way a total mean residence time larger of smaller than 300s could be obtained. For each tested combination, 40 wasps were tested and the outcome was analyzed by using a Mann-Whitney U test. NS indicates an insignificant preference of the wasp for a particular phoretic object.*

**Arrestment by anti-aphrodisiacs in the odor blend of mated female butterflies**

In figure 8, the results of dual-choice olfactometer bioassay combinations VIII and IX are shown for *T. brassicae*. Woelke (2008) found that naïve wasps were significantly arrested by virgin female *P. rapae* treated with anti-aphrodisiacs of conspecific males (P=0.07, mean residence time treatment 175.4s vs. control 124.6s). Fatouros *et al.* (2005b) showed the same significant preference of *T. brassicae* for virgin female *P. brassicae* treated with conspecific anti-aphrodisiacs (P=0.008, mean residence time treatment 174.7s vs. control 125.3s). In accordance with the results of Pashalidou (2008) who worked with *T. evanescens* (figure 9), *T. brassicae* also did not show a significant preference for virgin female *P. brassicae* or virgin female *P. rapae* treated with heterospecific anti-aphrodisiacs during olfactometer bioassays (respectively P=0.686, mean residence time treatment 155.6s vs. control 144.4s and P=0.666, mean residence time treatment 154.6s vs. control 145.4s).

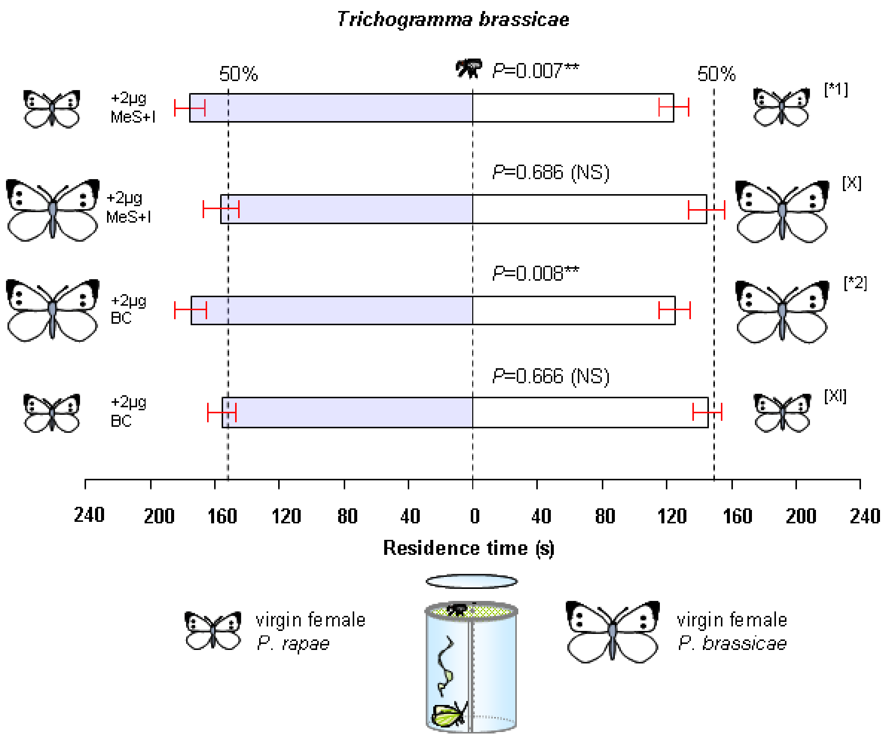



***Figure 8:*** *results of dual-choice olfactometer bioassay combinations VIII and IX. Woelke (2008) [*1] and Fatouros* et al. *(2005b) [*2] performed a bioassay in which respectively virgin female* P. rapae *and virgin female* P. brassicae *were treated with anti-aphrodisiacs from conspecific males. A blue or white bar represents the mean residence time (± s.e.m.) of* T. brassicae *above the odor source indicated. For each tested combination, 40 wasps were tested and the outcome was analyzed by using a Wilcoxon's matched pairs signed ranks test. Asterisks indicate a significant preference of the wasp for a particular odor source and NS stands for not significant.*

Figure 9 shows olfactometer bioassay results of Pashalidou (2008) for *Trichogramma evanescens* was used. These results were shown here in comparison with the results of the experiments which were done during this thesis in which *T. brassicae* wasps were used (figure 8). Virgin female *Pieris* butterflies were treated with 2.0 µg conspecific or heterospecific anti-aphrodisiacs. The figure clearly demonstrates that female *T. evanescens*, which was given an oviposition experience after a hitch-hiking experience with a mated female and a detection of conspecific anti-aphrodisiacs, were significantly arrested by virgin females of the *Pieris* butterfly species. This statement was valid for *P. rapae* (P<0.001, mean residence time treatment 205.5s vs. control 94.5s) as for *P. brassicae* (P=0.002, mean residence time treatment 197.6s vs. control 102.4s) which met both requirements. Bioassays with *T. evanescens*, which was given an experience on mated females and detected heterospecific anti-aphrodisiacs, was not significant arrested by virgin females of this *Pieris* butterfly species. This statement was valid for *P. rapae* (P=0.966, mean residence time treatment 145.6s vs. control 154.6s) as for *P. brassicae* (P=0.955, mean residence time treatment 145.6s vs. control 154.4s).

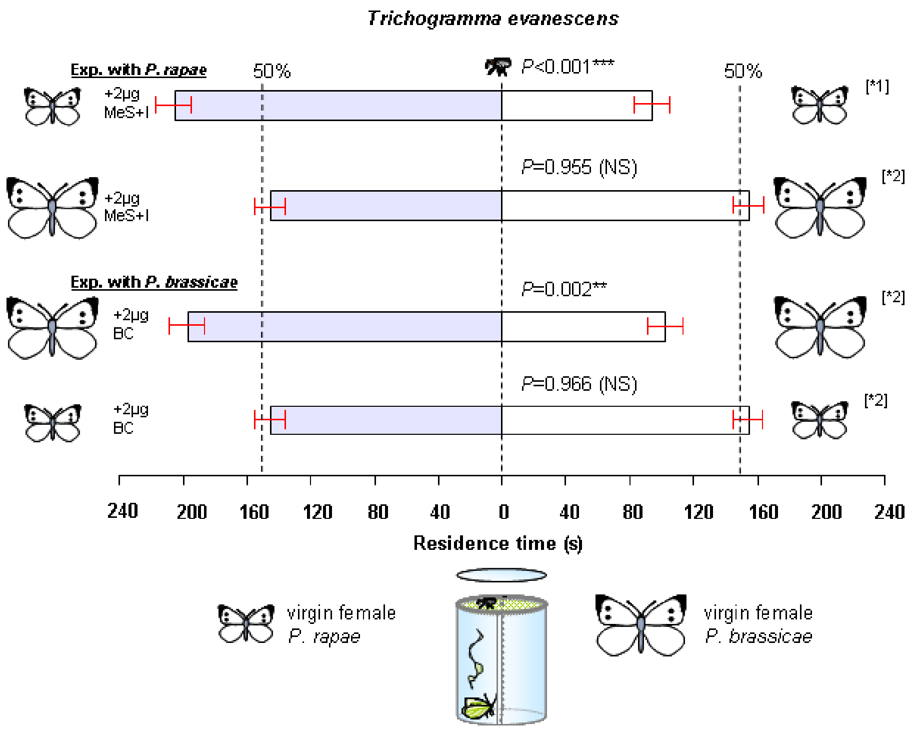



***Figure 9:*** *results of dual-choice olfactometer bioassay combinations performed by Pashalidou (2008) [*2]. The results of one bioassay was never published [*1].* Trichogramma evanescens *was used during these bioassays and the results are shown here for comparison with figure 8. Pashalidou gave all wasps an oviposition experience after a hitch-hiking experience with mated female* P. rapae *and mated female* P. brassicae*. A blue or white bar represents the mean residence time (± s.e.m.) of* T. evanescens *above the odor source indicated. For each tested combination, 40 wasps were tested and the outcome was analyzed by using a Wilcoxon's matched pairs signed ranks test. Asterisks indicate a significant preference of the wasp for a particular odor source and NS stands for not significant.*

## II Experiments under semi-field conditions

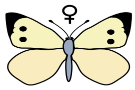
### Catching efficiency

During each repeat of this experiment, one mated *P. brassicae* female with a *T. brassicae* wasp on the thorax was released. After 60 seconds, the female butterfly was caught when she was still in the air. After performing 42 catches, on 7.1% of the released butterflies a wasp was found in the jar when checked under a microscope.

### Phoresy efficiency

Table 1 shows the results of an experiment under semi-natural conditions of which the phoresy efficiency of *T. brassicae* was investigated. In total, 48 mated female *P. brassicae* with a wasp on her back were released and landed eventually on *Brassica nigra* within 10 minutes. Only 8 butterflies laid eggs after the landing on the plant. 12.5% of the wasps on the butterflies that did not lay any eggs reached the host habitat and 5% of the wasps was able to descend from the butterfly and walked onto a plant leaf after performing phoretic behavior. Of the butterflies that laid eggs after landing on the plant, 12.5% of the wasps descended from the butterfly and walked on a leaf. During this experiment, no parasitism behavior of *T. brassicae* on the freshly laid eggs of *P. brassicae* was observed.

***Table 1:*** *results of a semi-environment experiment in which the phoresy efficiency of* T. brassicae *was tested. In total, 48 mated female* P. brassicae *landed on the plant of which 8 butterflies also laid eggs.*

|  | Total (N) |  | | | |
|---|---|---|---|---|---|
| | | **Reaches host habitat (N)** | **Reaches host plant (N)** | **Observed parasitism (N)** | **Lost (N)** |
| **Landing on plant** | **40** | 5 | 2 | 0 | 33 |
| **%** | | *12.5* | *5.0* | *0* | *82.5* |
| **Landing on plant + oviposition** | **8** | 0 | 1 | 0 | 7 |
| **%** | | *0* | *12.5* | *0* | *87.5* |

**Attractiveness of anti-aphrodisiac of mated female butterflies under semi-field conditions**

In total, 5 replicates of the experiment were carried out. Table 2 shows the results of the number of parasitized egg patches by *T. brassicae* in the cages of each *P. brassicae* group during each replicate. The date of the replicate did not influence the distribution of the parasitized egg patches over the three types of *P. brassicae* groups (males, mated female and virgin female) ($\chi^2(8) = 11.9$, $p > 0.05$).

***Table 2:*** *results of the experiment which investigated the attraction of* T. brassicae *to anti-aphrodisiacs of mated female butterflies under semi-field conditions. Each number represents the number of cages of the above mentioned* P. brassicae *group in which a parasitized egg patch was found on that day. For each experiment replicate, 16 cages with inside one butterfly in combination with an egg patch were present per butterfly group. During this thesis, 5 replicates were done.*

| **Date** | **# parasitized egg patches** | | | |
|---|---|---|---|---|
| | Male | Mated female | Virgin female | Total |
| 22 May | 6 | 14 | 7 | 27 |
| 28 May | 10 | 14 | 10 | 34 |
| 9 June | 1 | 0 | 4 | 5 |
| 13 June | 6 | 12 | 7 | 25 |
| 2 July | 3 | 0 | 2 | 5 |
| Total | 26 | 40 | 30 | 96 |

When taking all data from all 5 different experimental dates together, there was no significant difference between the fraction of parasitized egg patches in the three *P. brassicae* groups ($\chi^2(2) = 5.4$, $p > 0.05$) (table 3).

***Table 3:*** *results of the experiment which investigated the attraction of* T. brassicae *to anti-aphrodisiacs under semi-field conditions in another perspective. This time, each number represents the number of parasitized or unparasitized egg patches in the cages of each* P. brassicae *group after 5 replicates of this experiment.*

| **Butterfly group** | **Parasitized egg patches** | **Unparasitized egg patches** | **Total** |
|---|---|---|---|
| Male | 26 | 54 | 80 |
| Mated female | 40 | 40 | 80 |
| Virgin female | 30 | 50 | 80 |
| Total | 96 | 144 | 240 |

After each replicate, the cages were put in bags and were brought to the laboratory to check them for wasps. The numbers of female wasps found for each *P. brassicae* group after each replicate are shown in table 4.

***Table 4:*** *number of wasps found in the bags of a specific P. brassicae group during each experimental date.*

| **Date** | **# wasps found in bags** | | | |
|---|---|---|---|---|
| | Male | Mated female | Virgin female | Total |
| 22 May | 3 | 3 | 2 | 8 |
| 28 May | 3 | 3 | 1 | 7 |
| 9 June | 0 | 1 | 1 | 2 |
| 13 June | 0 | 0 | 0 | 0 |
| 2 July | 1 | 0 | 1 | 2 |
| Total | 7 | 7 | 5 | 19 |

## *DISCUSSION*

### Exploitation of male-specific butterfly compounds

Of all static dual-choice olfactometer bioassays, only combination II, one filter paper treated with 5x 4.0 µg wing extract of male *Pieris rapae* butterflies against filter paper treated with solvent only, resulted in a significant arrestment of *Trichogramma brassicae* on short distance (figure 5). This can indicate that this wasp species can be attracted to and can perform phoresy on male butterflies in nature. Also Fatouros (2006) suggested that some egg parasitoid species were known for their phoretic behavior on males. The outcome of this part of the thesis is in favor of this statement. An amount of 20 µg body-specific compounds is found on the wings of a single male *P. rapae* butterfly (Schulz, unpublished) and this indication seems plausible when taking this outcome into account.

When testing olfactometer bioassay combinations in which only *P. rapae* solution A was used, no single significant preference of *T. brassicae* wasps for an odor source was encountered. A possible explanation is that the amount of solution on the treated filter paper was not the correct amount to arrest wasps. However, when taking the approximations of Schulz (unpublished) into account, 0.20 µg of *P. rapae* solution A represents the amount present on a male individual. Therefore, a possible better explanation assuming that *T. brassicae* wasps are attracted to some male-specific compounds, is that they need to encounter a different combination of male-specific compounds of this butterfly species to be significantly arrested by a treated filter paper. Notice that Schulz made *P. rapae* solution A by mixing male-specific compounds which he thought were most important and most abundant on male *P. rapae* butterflies (see appendix II for compounds). However, this selection of compounds was just the tip of the iceberg because many more were found during the extraction process. A third explanation is that *T. brassicae* wasps also need to encounter adult *Pieris*-specific compounds which can be found on both male and female butterflies of the same species in combination with male-specific compounds in *P. rapae* solution A to be arrested by treated filter papers.

For testing olfactometer bioassays in which was questioned if *T. brassicae* also need to detect body-specific compounds (*P. rapae* solution B) which can also be found on conspecific females, in combination with male-specific compounds in *P. rapae* solution A, no significant arrestment behavior of the wasps by treated filter papers was found. A possible reason for the insignificance of this outcome is that also *P. rapae* solution B lacks some important compounds by which *T. brassicae* wasps are arrested. *Pieris rapae* solution B was a mixture of the most abundant body-specific compounds which were found on both male and female butterflies (see appendix II for compounds).

Also there is this possibility that both *P. rapae* solution A and B do not contain those components which are necessary for arrestment by male-specific compounds in combination with body-specific compounds.

A recommendation for further research on this topic is to test a combination of male and body-specific compounds in those amounts which are approximated by Schulz. If one wants to perform this dual-choice olfactometer bioassay, one filter paper has to be treated with 0.20 µg of *P. rapae* solution A and 20 µg of *P. rapae* solution B against another filter paper treated with *P. rapae* solution A or B only. If an insignificant result is obtained, one can choose to make new mixtures to investigate whether other male and/or body-specific compound combinations derived from *P. rapae* butterflies trigger arrestment. The possible attraction of *T. brassicae* wasps to these kind of odors other than anti-aphrodisiacs is a new line in the research about the exploitation of *Pieris* butterflies compounds by these egg parasitoids.

When testing dual-choice phoretic behavior bioassays, insignificant results for both combinations in which the proportion of first mounts of *T. brassicae* on one of the *P. rapae* paper models, were obtained. However, *T. brassicae* wasps tended to mount the paper butterfly model which was treated with 4x 5 µg male *P. rapae* wing extract (figure 6). Woelke (2008) found that *T. brassicae* wasps showed no first time mounting preference on mated female over male *P. rapae*. This can indicate that males have some compounds which are also attractive to this wasp species. Therefore, if one chooses to continue with these experiments, different concentrations than the few tested thus far can be investigated, possibly revealing a significant preference.

The results of the phoretic behavior duration of the wasps on *P. rapae* paper models were also insignificant (figure 7) and therefore it is not proven that *T. brassicae* wasps stayed longer on phoretic objects treated with male *P. rapae* wing compounds compared to models treated with solvent only. Again, a possible explanation is that the amounts of wing compounds on treated models are not optimal for a longer arrestment of the wasps. Therefore, a recommendation for future research is to use other concentrations.

**Exploitation of anti-aphrodisiacs in the odor blend of mated female butterflies**

The results of the static dual-choice olfactometer bioassays which tested if *T. brassicae* wasps were arrested by virgin female *Pieris brassicae* and *P. rapae* butterflies treated with heterospecific anti-aphrodisiacs, were insignificant (figure 8). Fatouros *et al.* (2005b) and Woelke (2008) showed that *T. brassicae* wasps were arrested by virgin female *P. brassicae* and *P. rapae* butterflies respectively treated with conspecific anti-aphrodisiacs. Therefore, when taking the results of the above mentioned authors in account, the conclusion is that *T. brassicae* wasps are only arrested by the odor of virgin female *P. brassicae* and *P. rapae* when these are treated with conspecific anti-aphrodisiacs. In other words, the detection of anti-aphrodisiacs alone on these butterfly species is not enough to arrest these wasps, because they need to encounter these compounds emitted by mated females in combination with other female compounds. Pashalidou (2008), working with experienced *Trichogramma evanescens*, performed comparable olfactory bioassays and showed also comparable results (figure

9). The reason for this outcome seems logical, because in nature, egg parasitoids as *T. brassicae* wasps perceive a rich combination of plant and host volatiles. An anti-aphrodisiac like benzyl cyanide is also produced by other organisms, like plants (Piñero & Dorn, 2007) and grasshoppers (Seidelmann *et al.*, 2000), and therefore, only a preference for benzyl cyanide in combination with other female odors will lead them to this mated female *P. brassicae* butterfly. In the future, it is interesting to investigate which female compounds are exploited in combination with the anti-aphrodisiacs belonging to the same butterfly species by female *T. brassicae* wasps. An important question to resolve is if the detection of a complete odor blend is necessary for a significant preference of the wasps for mated females of a given butterfly species or is just a couple of the compounds responsible for this behavior.

**Efficiency of the catching method and phoresy under semi-field conditions**

*Trichogramma brassicae* wasps were found on 7.1% of the released mated female *P. brassicae* after 60 seconds and this outcome seems not very high regarding the butterflies did not land on a plant leaf yet. Fatouros *et al.* (2005b) stated that phoretic behavior by *Trichogramma* wasps in nature is rarely observed and M.E. Huigens (unpublished) found low frequencies of caught wasps on wild butterflies (see appendix I for percentages). Therefore, one possible explanation is that this strategy performed by *T. brassicae* is not very common in nature. Results of this thesis showed that phoretic behavior tests in the laboratory took approximately 150 seconds (figure 7) which should be enough to witness such events in nature. A more likely alternative explanation is that the method of catching butterflies involves the escape of many hitch-hiking wasps, because they can fly through the gauze of the butterfly net. It was tried to overcome this problem by placing a jar in the net in which the butterfly will end up during the catch. To prevent the escape of the wasp, the screw top was instantly put on the jar. However, this method is not perfect, because the caught butterfly will not always end up in the jar and there is some time between the actual catch and putting the screw top on the jar allowing the wasp to escape. For catching butterflies, one can choose for a kind of device which can instantly suck up and put away the butterfly in a compartment to overcome these problems. Such devices have yet to be developed. A recommendation is to further test if the method of catching butterflies is good enough for obtaining reliable hitch-hiking frequencies. This can be done by performing an experiment in the gauze tunnel in which one is absolutely sure that the wasp is still on the female butterfly. Firstly, the mated female butterfly has to be cooled and then she has to be instantly caught after putting a wasp on her thorax (M.E. Huigens, personal communication). When the outcome of this experiment proves that this method is not reliable and has a major influence on the catching efficiency, higher hitch-hiking frequencies in nature are expected when using better equipment.

After 48 replicates, no wasps were found which successfully parasitized freshly laid eggs of *P. brassicae* butterflies after their arrival on host habitats (table 1). This in contrast to the phoresy results of Fatouros *et al.* (2005b) which showed that in 7.1% of the cases, *T. brassicae* wasps were able to successfully parasitize eggs after performing phoresy behavior. Notice that this percentage was based on the situation that each released butterfly laid eggs and that this experiment was performed in a greenhouse in which possible biotic and abiotic influences during semi-field conditions prevailing on the behavior of both insect species were negligible. However, no conclusion can be drawn that *T.*

*brassicae* wasps cannot successfully hitch-hike on *Pieris* butterflies under semi-field conditions. A possible explanation for this outcome is that the butterflies endure stress when transporting them in jars to the tunnel and this can have an postponing effect on laying eggs. Furthermore, escape behavior of the butterflies was observed, because some them were flying directly away from the setup, landed on the net of the tunnel and stayed there for a while. Other specimens flew directly to the flowers of the plant, which indicated that they were not fed properly. In other words, the well-being of the *P. brassicae* butterflies is important and can have a major impact on the oviposition behavior and on the phoresy efficiency of the wasps on this butterfly species during the experiment. Only few mated butterflies laid eggs on the *Brassica nigra* plants in these experiments. To enhance oviposition on the plants, one should use only those female butterflies which were laying eggs on other plants in the butterfly cultures just before the experiment (N.E. Fatouros, personal communication). Also the weather can have an influence on the oviposition behavior of *P. brassicae* and therefore, a recommendation is to perform only replicates in the late morning or early afternoon in combination with moderate sunny and warm (18-24$^{o}$C) weather conditions (Root & Kareiva, 1984).

**Attractiveness of anti-aphrodisiac of mated female butterflies under semi-field conditions**

Results of the experiment which investigated the attraction of *T. brassicae* wasps to anti-aphrodisiacs of mated female *P. brassicae* under semi-field conditions showed that the wasps parasitized *Mamestra brassicae* eggs in the cages (table 2 & 3). Even some wasps were found in the bags with the cages when checking them under a microscope back in the laboratory after 24 hours (table 4). This indicates that the released wasps can reach hosts in these cages under semi-field conditions. However, the number of found wasps is low compared to the number of released wasps, approximately 12,500 individuals of which approximately 75% was female, during each replicate. When taking all cages of one replicate in account, only few wasps were found in contrast to many parasitized egg patches. Therefore, one possible explanation is that wasps do not stay long in the vicinity of their hosts. Notice that putting cages in the bags after 24 hours is just a moment in time and that more wasps could have been inside the cages within this period. Therefore, a recommendation is to put the cages in bags after 6, 12, 18 and 24 hours.

No significant differences in the parasitism fractions of egg patches between the cages with male, mated female, and virgin female *P. brassicae* butterflies were found. It was expected to find differences between virgin female and mated female, and virgin female and male *P. brassicae* in accordance to the laboratory results of Fatouros *et al.* (2005b). Notice that only 5 replicates were done during this project and for that reason, no conclusions can be drawn yet. More replicates are recommended for finding possible differences between these butterfly groups.

Decent numbers of parasitized egg patches were found on those days with no rain and with temperatures between 19 $^{o}$C and 22 $^{o}$C during daylight. Results showed that sun minutes are not even required for obtaining decent results. During daylight, temperature has the strongest influence on the distribution behavior of *Trichogramma* wasps (Suverkropp, 1997), minimum flight temperatures are 18 $^{o}$C for *T. brassicae* and the wasps cannot fly against wind currents stronger than 2 m $s^{-1}$ (authors in Suverkropp, 1997). Normally, *Trichogramma* wasps disperse on short-distance by walking and short jumps (authors in Romeis *et al.*, 2005). Wind currents stronger than 2.0 m $s^{-1}$ were measured on some successful days which can indicate that the dispersal of *T. brassicae* especially occurs by walking and/or short jumps. The influence of the rain on those minute insects is not well-known, but when keeping the size of the wasps in mind, one can realize that rain is able to wash them away. Therefore, it can have a major impact on the dispersal of *T. brassicae* during the experiment. On 9 June and 2 July, low numbers of parasitized egg patches were found. When taking data of the weather conditions during those dates in account, high temperatures (25-28$^{o}$C) in combination with many sun minutes were measured. Minutes after the preparation of the setup, dead butterflies were already seen in the cages because of the intense heat. On 2 July, also rain was measured after 2 hours of the preparation. For further research on the attractiveness of the anti-aphrodisiac of mated female butterflies under semi-field conditions, one has to keep the weather conditions in mind. To obtain decent results, rain has to be avoided just as very high temperatures (>24$^{o}$C).

It is questioned if *B. nigra* plants which are introduced in the tunnel, can really form meeting points between butterfly and wasp. In nature, this plant species can be a part of the habitat in which *Trichogramma* wasps will meet *Pieris* butterflies for performing phoresy. The flowers of these plants can provide nectar for *Trichogramma* wasps so that these insects are able to do physiological processes during their adult stage (Wäckers, 2005). In addition, *B. nigra* is a suitable plant for *Pieris* butterflies to lay eggs on, the plant species flowers for a couple of weeks and can be easily cultivated (M.E. Huigens, personal communication). Therefore, it seems logical to hang butterfly cages on these plants for attracting the released wasps, but it is not proven that they can really form this meeting point during this project because only several wasps were found in the bags. For testing that *B. nigra* plants has really an influence on the attraction of *T. brassicae* wasps, one can choose to perform experiments by hanging butterfly cages on sticks instead on these plants.

## *CONCLUSION*

There are indications that *Trichogramma brassicae* wasps can detect and hitch-hike with male *Pieris* butterflies. After the performance of static dual-choice olfactometer bioassays, a significant preference of *T. brassicae* wasps was found for filter papers treated with 5x 4.0 µg extract which consisted of wing compounds of male *Pieris rapae* butterflies from a short distance. Therefore, one can conclude that female *T. brassicae* wasps are arrested by male-specific compounds of *P. rapae* butterflies. After the performance of dual-choice phoretic behavior bioassays, the wasps had a tendency to prefer to mount paper models of *P. rapae* butterflies treated with 5x 4.0 µg extract which consisted of wing compounds of male *P. rapae* butterflies. *Trichogramma brassicae* wasps are also known to exploit anti-aphrodisiacs emitted by mated female butterflies. In this study it was shown that these wasps need to encounter other female compounds in combination with conspecific anti-aphrodisiacs to be arrested by mated *P. rapae* and *Pieris brassicae* females during static dual-choice olfactometer bioassays. Furthermore, it was found in a gauze tunnel that after 60 seconds of the release of mated female *P. brassicae* butterflies of which each was carrying a wasp, 7.1% of the butterfly re-catches also resulted in finding this wasp. For that reason the method of catching *Pieris* butterflies with possible hitch-hiking *Trichogramma* wasps on their bodies seemed not reliable for acquiring a good indication of the frequency of phoretic behavior by these wasps in nature. No parasitism of freshly laid *P. brassicae* butterfly eggs by *T. brassicae* wasps was found after the performance of phoresy under semi-field conditions. No significant attraction of *T. brassicae* wasps towards mated female over virgin female and male *P. brassicae* butterflies was found under semi-field conditions.

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## *REFERENCES*

Andersson J, Borg-Karlson A-K & Wiklund C. (2000) Sexual cooperation and conflict in butterflies: a male-transferred anti-aphrodisiac reduces harassment of recently mated females. *Proceedings of the Royal Society B* **267**: 1271-1275.

Andersson J, Borg-Karlson A-K & Wiklund C (2003) Antiaphrodisiacs in Pierid butterflies: a theme with variation! *Journal of Chemical Ecology* **29 (6)**: 1489-1499.

Andersson J, Borg-Karlson A-K & Wiklund C (2004) Sexual conflict and anti-aphrodisiac titre in a polyandrous butterfly: male ejaculate tailoring and absence of female control. *Proceedings of the Royal Society B* **271**: 1765-1770.

Andersson J, Borg-Karlson A-K, Vongvanich N & Wiklund C (2007) Male sex pheromone release and female mate choice in a butterfly. *The Journal of Experimental Biology* **210**: 964-970.

Elven JE van (2007) Taxi please! Do *Trichogramma evanescens* wasps specifically hitch-hike with mated female *Pieris rapae* butterflies? *MSc thesis.* Department of Entomology, Wageningen University.

Fatouros NE, Bukovinszkine'Kiss G, Kalkers LA, Gamborena RS, Dicke M & Hilker M (2005a) Oviposition-induced plant cues: do they arrest *Trichogramma* wasps during host location? *Entomologia Experimentalis et Applicata* **115**: 207-215.

Fatouros NE, Huigens ME, Loon JJA van, Dicke M & Hilker M (2005b) Chemical communication: butterfly anti-aphrodisiac lures parasitic wasps. *Nature*: **433**: 704.

Fatouros NE (2006) Parasitic wasps on butterfly expedition. foraging strategies of egg and larval parasitoids exploiting infochemicals of Brussels sprouts and their *Pieris* hosts. *PhD thesis:* 181p. Freie Universität Berlin, Berlin.

Fatouros NE, Bukovinszkine'Kiss G, Dicke M & Hilker M (2007) The response specificity *Trichogramma* egg parasitoids towards infochemicals during host location. *Journal of Insect Behavior* **20**: 53-65.

Fatouros NE, Dicke M, Mumm R, Meiners T & Hilker M (2008a) Foraging behavior of egg parasitoids exploiting chemical information. *Behavioral Ecology* **19 (3)**: 677-689.

Fatouros NE, Broekgaarden C, Bukovinszkine'Kiss G, Loon JJA van, Mumm R, Huigens ME, Dicke M & Hilker M (2008b) Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. *Proceedings of the National Academy of Sciences* **105**: 10033-10038.

Hilker M & Meiners T (2002) Chemoecology of insect eggs and egg deposition. Blackwell, Berlin.

Houck MA & OConnor BM (1991) Ecological and evolutionary significance of phoresy in the Astigmata. *Annual Review of Entomology* **36**: 611-636.

Lenteren JC van (2000) Success in biological control of Arthropods by augmentation of natural enemies. In: *Biological Control: Measures of Success* (eds. Gurr G & Wratten S): 77-103. Kluwer Academic Publishers, Dordrecht.

Lewis WJ & Nordlund DA (1985) Behavior-modifying chemicals to enhance natural enemy effectiveness. In: *Biological Control in Agricultural IPM Systems* (eds. Hoy MA & Herzog DC): 89-101. Academic Press, Orlando.

Nordlund DA (1994) Habitat location by *Trichogramma*. In: *Biological Control with Egg Parasitoids* (eds. Wajnberg E & Hassan SA): 155-163. CAB International, Wallingford.

Pashalidou F (2008) Hitch-hiking parasitic wasp can associatively learn to exploit butterfly anti-aphrodisiac. *MSc thesis.* Department of Entomology, Wageningen University.

Piñero JC & Dorn S (2007) Synergism between aromatic compounds and green leaf volatiles derived from the host plant underlies female attraction in the oriental fruit moth. *Entomologia Experimentalis et Applicata* **125(2)**: 185-194.

Romeis J, Shanower TC & Zebitz CPW (1997) Volatile plant infochemicals mediate plant preference of *Trichogramma chilonis*. *Journal of Chemical Ecology* **23 (11)**: 2455-2465.

Romeis J, Babendreier D, Wäckers FL & Shanower TG (2005) Habitat and plant specificity of *Trichogramma* egg parasitoids-underlying mechanisms and implications. *Basic and Applied Ecology* **6**: 215-236.

Root RB & Kareiva PM (1984) The search for resources by cabbage butterflies (*Pieris rapae*): ecological consequences and adaptive significance of Markovian movements in a patchy environment. *Ecology* **65(1)**: 147-165.

Seidelmann K, Luber K & Ferenz H-J (2000) Analysis of release and role of benzyl cyanide in male desert locusts, *Schistocerca gregaria*. *Journal of Chemical Ecology* **26(8)**: 1897-1910.

Silberglied RE (1984) Visual communication and sexual selection among butterflies. In: *The Biology of Butterflies* (eds. Vane-Wright RI & Ackery PR): 207-223. Academic Press, London.

Suverkropp BP (1997) Host finding behaviour of Trichogramma brassicae in maize. *PhD thesis:* 249p. Wageningen Agricultural University, Wageningen.

Svärd & Wiklund (1989) Mass and production rate of ejaculates in relation to monandry/polyandry in butterflies. *Behavioral Ecology and Sociobiology* **24**: 395-402.

Thorne BM (1989) Statistics for the behavioral sciences. 363p. Mayfield Publishing Company, Mountain View.

Wäckers FL (2005) Suitability of (extra-)floral nectar, pollen and honeydew as insect food sources. In: *Plant-provided food for carnivorous insects: a protective mutualism and its applications* (eds. Wäckers FL, Rijn PCJ van & Bruin J): 17-74. Cambridge University Press, Cambridge.

Woelke JB (2008) Hitch-hiking behavior of *Trichogramma* wasps on cabbage white butterflies and moths. *MSc thesis.* Department of Entomology, Wageningen University.

## *APPENDIX I*

The hitch-hiking percentages of *Trichogramma* wasps on 3 *Pieris* butterfly species and *Maniola jurtina* L., a.k.a. Meadow Brown (Lepidoptera: Nymphalidae) (M.E. Huigens, unpublished data).

| Butterfly species | Sex | Total caught butterflies | Hitch-hiking wasps (%) |
|---|---|---|---|
| *Pieris rapae* | female | 90 | 1.1 |
| *Pieris rapae* | male | 187 | 0.53 |
| *Pieris brassicae* | female | 16 | 6.3 |
| *Pieris brassicae* | male | 21 | 0 |
| *Pieris napi* | female | 17 | 0 |
| *Pieris napi* | male | 93 | 1.08 |
| *Maniola jurtina* | female | 116 | 0.86 |
| *Maniola jurtina* | male | 48 | 0 |

## *APPENDIX II*

***Pieris* cuticular compounds from Prof. Dr. Stefan Schulz**

(Technical University Braunschweig)

Schulz (unpublished) derived many chemical compounds from the wings of *Pieris rapae*. A mixture was made of those compounds which were found at large quantities on the wings of males only (*P. rapae* solution A) or on wings of males and females (*P. rapae* solution B). As solvent, Schulz used dichloromethane.

*P. rapae* mixture A:

| Derived chemical compound | Weight units in extract |
|---|---|
| Hexahydrofarnesylacetone | 12 |
| Ferrulactone | 21 |
| Phytol | 1 |

*P. rapae* mixture B:

| Derived chemical compound | Weight units in extract |
|---|---|
| 1-Tetracosanol, 1-Hexacosanol, 1-Heneicosene (1:1:1) | 0.5 |
| 4,8,12,16-Tetramethyl-4-heptadecanolide | 0.5 |
| Tricosane | 0.9 |
| Pentacosane | 3.4 |
| Heptacosane | 36.4 |
| Heptacosan-7-ol | 3.4 |

*APPENDIX III*

**Setup semi-field experiment in which the attractiveness of the anti-aphrodisiac of mated female *Pieris brassicae* butterflies was tested** *(see also figure 3 and 4 for pictures of the setup)*