Taxi please!

Do *Trichogramma evanescens* wasps specifically hitch-hike with mated female *Pieris rapae* butterflies?

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Do *Trichogramma evanescens* wasps specifically hitch-hike with mated female *Pieris rapae* butterflies?

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Contents

Summary

Egg parasitoids are known to use specific odours of certain hosts or habitats and host plants during the host selection process. It was recently discovered that *Trichogramma brassicae* egg parasitoids are able to spy on the anti-sex pheromone, also called anti-aphrodisiac, of the adult stage of their host, the Large Cabbage White butterfly *Pieris brassicae*, and then specifically hitch-hike (phoresy) with a mated female butterfly to parasitize her freshly laid eggs. This research project is a start to find out whether spying on an anti-aphrodisiac in combination with phoretic transport on a mated female host is a more widespread strategy among phoretic egg parasitoids. In two-choice olfactometer- and phoresy experiments it was tested whether *Trichogramma evanescens* wasps can innately recognize mated *Pieris rapae* females and, if not, if they are able to associatively learn to recognize them after a positive oviposition experience. The wasps did not show an innate preference for mated females, but could learn to respond more strongly towards the odour of mated females than males and tended to mount mated females more often than virgin females and males after an oviposition experience in the presence of mated *P. rapae* females. It seems that *T. evanescens* is able to learn to respond to mated *P. rapae* females. Future experiments should find out if *T. evanescens* indeed spies on the anti-aphrodisiac and if it also has to learn to recognize mated female butterflies in the field.

Samenvatting

Voor de selectie van een gastheer kunnen eiparasieten gebruik maken van specifieke geuren van hun gastheer, diens habitat en gastheerplanten. Recentelijk is ontdekt dat de eiparasiet *Trichogramma brassicae* kan spioneren op het anti-sex feromoon, ook wel anti-aphrodisiac, van het volwassen stadium van haar gastheer, het grote koolwitje *Pieris brassicae*, om vervolgens met een gepaard vlindervrouwtje mee te liften en haar versgelegde eieren te parasiteren. Dit onderzoeksproject is een begin van een groter onderzoek naar of het spioneren op een anti-aphrodisiac in combinatie met het meeliften op gepaarde gastheer vrouwtjes een algemene strategie is onder foretische eiparasieten. In twee-keuze biotoetsen werd getest of de eiparasiet *Trichogramma evanescens* een aangeboren voorkeur heeft voor gepaarde *Pieris rapae* vrouwtjes en, zo niet, of zij in staat zijn dit te leren door een associatieve ervaring met een succesvolle ovipositie in aanwezigheid van gepaarde vlindervrouwtjes. Naïeve wespen toonden geen voorkeur voor gepaarde vlindervrouwtjes, maar bleken wel in staat een voorkeur voor de geur van gepaarde vrouwtjes ten opzichte van mannetjes te kunnen leren na een associatieve leerervaring. Daarnaast neigden de ervaren wespen vaker op gepaarde vlindervrouwtjes te klimmen dan op maagdelijke vrouwtjes of mannetjes. Het lijkt er op dat *T. evanescens* in zekere mate gepaarde *P. rapae* vrouwtjes kan leren herkennen na een associatieve eilegervaring. Vervolgonderzoek moet nu uitwijzen of *T. evanescens* hierbij spioneert op het anti-aphrodisiac en of zij in het veld ook moet leren gepaarde vlindervrouwtjes te herkennen.

Introduction

Chemical communication

Many organisms make use of chemical communication (Wyatt 2003). Chemicals that convey information between two individuals and might evoke a response in the receiver, without being detrimental or beneficial to themselves, are called infochemicals (Dicke and Sabelis 1988; Dicke and Sabelis 1992). There are two different types of infochemicals. The first and most obvious infochemicals are the ones that are used for communication between individuals of the same species. Such chemicals are called pheromones. The first discovery of chemical communication in insects was the discovery of the sex pheromone of the silk moth (Fabré 1911). The second group of infochemicals are the allelochemicals which mediate interactions between individuals that belong to different species. Allelochemicals can be beneficial for the producer (allomones), the receiver (kairomones) or both the producer and receiver (synomones) (for more detail see Dicke and Sabelis 1988). Infochemicals can comprise of different numbers of components. Lepidopteran sex pheromones can, for instance, comprise of single or multiple components that attract a partner (Cardé and Charlton 1984).

Chemical stimuli used in host location by egg parasitoids

Parasitoids often require several stimuli, chemical as well as other stimuli, to find a host or host community (Vinson 1984). Within an ecosystem organisms, plants as well as animals, can interact both inter- and intraspecifically by chemical communication at all trophic levels (Lewis and Martin 1990). In a tritrophic interaction of a plant-host-parasitoid complex, the parasitoid can use several chemicals for host location. It may be attracted by chemicals produced by the host itself or products of the host (for instance faeces) as well as chemicals produced by the plant on which the host is feeding. Egg parasitoids may also be arrested by the odours produced by the plant in response to oviposition on the plant by a herbivorous host (reviewed by Hilker and Meiners 2002).

Although they parasitize the most inconspicuous host stage, *Trichogramma* egg parasitoids are considered efficient biological control agents and are used worldwide for control of lepidopteran pests in many crops (Smith 1996). Butterfly egg parasitism rates of more than 50% reveal that the parasitic wasps can be successful in finding and parasitizing butterfly eggs in nature (van Heiningen et al 1985). Because eggs are rather inconspicuous hosts, egg

parasitoids have developed several complicated strategies for successful host finding (Vinson 1984).

Although only little is known about host specificity of *Trichogramma* spp. in nature, they are generally assumed to be generalists. *Trichogramma* spp. may have an innate ability to use specific odours of certain hosts or habitats and host plants during the host selection procedure but preferences for certain plants can also be learned (Romeis et al 2005). If the wasps learn chemical stimuli during earlier developmental stages they will express higher specificity with respect to the infochemicals necessary for the location of that certain host than could generally be assumed. Bjorksten and Hoffmann (1998) showed that wasps that emerge from a host on a tomato plant indeed express higher interest in tomato plants and that adult parasitoids can learn from successful oviposition experience (Bjorksten and Hoffmann 1995). Fatouros et al (2005a) found that naïve *Trichogramma brassicae* female wasps were arrested to the changes of *Brassica oleracea* leaves after oviposition by the Large Cabbage White butterfly *Pieris brassicae*. In contact bioassays, the wasps were more arrested to egg-free leaf squares excised from leaves with 72 hours old egg masses than to clean leaves. This effect was not due to odours from scales or other deposits of the adult *Pieris* butterflies. Plant odours alone were not enough to arrest the wasps. Fatouros et al (2005a) also showed that oviposition experienced *T. brassicae* wasps showed more preference to egg deposited leafs than naïve wasps and that butterfly deposits arrest the parasitoids up to 48 hours after oviposition on these leaves.

As mentioned, infochemicals of host insects can also be exploited by parasitoids in order to find them. This is called chemical espionage. However, where parasitoids are willing to detect suitable hosts, the host will try to avoid parasitoids. Therefore parasitoids have evolved many strategies in order to be able to locate potential hosts. There are two main strategies to actually find a host once a host community is located by a parasitoid wasp: the host searching strategy and the ambush strategy (Vinson 1984 and 1985 for more detail). The searching strategy is a strategy in which the parasitoid by itself searches for its host, starting with random search, and should lead to the finding of an appropriate host. The ambush strategy is a strategy in which the parasitoid physically or cryptically hides for the host and is, for instance, used by phoretic egg parasitoids. Phoresy is defined as the transport of certain insects on the bodies of others for purposes other than direct parasitism of the transporting individual(s) (Howard 1927). The female egg parasitoid waits for a transporting host to which they attach to be carried to where the host female lays eggs.

Chemical espionage on anti-aphrodisiacs

To find a host *Trichogramma* wasps can use infochemicals from the environment, the host itself or other odours related to the host. Of the chemicals produced by the adult stage of the host, sex pheromones may be reliable chemicals to spy on since eggs are often deposited soon after mating (Stowe et al 1995). Noldus (1989) demonstrated that *Trichogramma evanescens* females are attracted by volatiles of virgin *P. brassicae* females and calling *Mamestra brassicae* females. Studies on *Trichogramma pretiosum* also demonstrated the use of host pheromones for host detection (Noldus 1988). Eavesdropping on sex pheromone signals from the host, sometimes in combination with phoresy, have been shown for several egg parasitoid species (reviewed by Powell 1999).

Only little research has been done on the attractiveness of host anti-sex pheromones, also called anti-aphrodisiacs, towards egg parasitoids. Anti-aphrodisiacs are pheromones that are transferred from males to females during mating to render the females less attractive to conspecific males (Andersson et al 2003). These chemicals might provide an even more reliable cue for egg parasitoids than sex-pheromones, because mated host females are generally closer to egg deposition than virgin host females.

Fatouros et al (2005b) recently discovered that naïve female *T. brassicae* wasps are attracted to mated females of the Large Cabbage White butterfly *P. brassicae.* The anti-aphrodisiac of *P. brassicae* is benzyl cyanide which is an odour carried by the male. After mating the female carries this male odour with her (Andersson et al 2003). In two-choice bioassays, it was shown that the wasps could smell the anti-aphrodisiac odour of *P. brassicae* and mounted more often on virgin females painted with benzyl cyanide than on virgin controls. Under laboratory conditions 7.1% of the observed transportations on mated *P. brassicae* females led to successful parasitism of *P. brassicae* eggs by *T. brassicae* (Fatouros et al 2005b).

In contrast to *T. brassicae,* naïve *T. evanescens* females did not specifically recognize the odour of mated *P. brassicae* females and also did not specifically mount these mated females (Fatouros et al 2007). It might be possible that *T. evanescens* has another searching strategy than *T. brassicae*, expressing less innate responses to chemical cues than its relative, but in stead has the ability of associative learning. Schöller and Prozell (2002), for example, found that *T. evanescens* has the ability to associatively learn to respond to the sex pheromone of *Ephestia* moths. It might therefore also be able to associatively learn to respond to anti-aphrodisiacs of its hosts.

Like *P. brassicae*, *Pieris rapae* and *Pieris napi* also use anti-aphrodisiacs, respectively a combination of methyl salicylate and indole, and methyl salicylate alone. Other than in *P.*

brassicae, the anti-aphrodisiac odours of *P. rapae* and *P. napi* are not emitted by the male (Andersson et al 2003).

Research aim

Because anti-aphrodisiacs can provide reliable cues, spying on an anti-aphrodisiac in combination with phoretic transport on a mated female host may be a widespread strategy among phoretic egg parasitoids. This research project now represents a start to find out whether this truly is the case. The work is performed with *Trichogramma evanescens* wasps that emerged from *Pieris rapae* eggs collected in the field. It was tested whether the wasps can innately recognize mated *P. rapae* females, or if not, they are able to associatively learn to recognize them after a positive oviposition experience. The work mainly comprises of two-choice olfactometer and phoresy experiments.

Materials and methods

Insects

Trichogramma wasps were collected in the field from *Pieris rapae* eggs found on *Brussels sprouts* plants near Wageningen. After collection, the wasps were reared on *Ephestia kuehniella* eggs and identified using a molecular method based on the Internal Transcribed Spacer 2 gene (Gonçalves et al (2006), van Rijswijk (2000), Silva et al (1999) and Stouthamer et al (1999) see appendix 1 for the protocols). Subsequently, all experiments were performed with the *Trichogramma evanescens* GD-011 strain. Only mated 2-5 days old female wasps were used in the experiments. I tested four groups of wasps: 1) naïve wasps, 2) wasps that had an oviposition experience in *P. rapae* eggs one night prior to the experiment, 3) wasps that had an oviposition experience in *Mamestra brassicae* eggs in the presence of four mated female butterflies. The experiments were performed between 45 and 65 minutes after the oviposition experience, and 4) wasps that had an oviposition experience in *Mamestra brassicae* eggs in the presence of a synthetic anti-aphrodisiac odour of *P. rapae.* The experiments were performed between 45 and 65 minutes after the oviposition experience.
I used *Mamestra brassicae* eggs because they are laid on paper. In this way, wasps cannot associate an oviposition with plant odours in stead of butterfly odours. *Pieris rapae* adult butterflies were obtained from a laboratory colony maintained on Brussels sprouts plants. Copulating couples were taken from the rearing the afternoon before the experiment. Virgin females were selected from the rearing just after hatching from the pupa.

Olfactometer experiment setup

The experiments were carried out in a cylinder shaped two-chamber-static-air-flow olfactometer of 18 cm in height and 12 cm in diameter (Fatouros et al. 2005b). The cylinder was made of acrylic glass divided into two chambers by a vertical plate and with a removable walking area (2 cm high and 9 cm in diameter) on top of it. The walking area was made of plastic gauze, with mesh size of 0.1 mm, and a plastic rim. During the experiment it was covered with a glass plate.
Per chamber an odour source (0, 2 or 4 butterflies) was offered. For 300 seconds the time spent by the wasps in one of the two offered odour fields was observed. Each day 8-18 wasps were tested for a total of 40 wasps per combination. After each 6^{th} wasp the butterflies were

replaced and halfway each butterfly combination, so after 3 wasps, the olfactometer was rotated 180^{o} to avoid biased results.

Response to butterfly odours

In the olfactometer setup, the response of *T. evanescens* GD-011 to different butterfly odours (virgin female, mated female or mated male) was tested. The response of naïve wasps to the odour of two *P. rapae* butterflies was tested against air or against two other *P. rapae* butterflies. The butterfly combinations that were tested were mated females against virgin females, mated females against males, and mated males against virgin females. The combination of mated females against mated males was also tested with four butterflies. The response towards two mated females against air was also tested with wasps that had an oviposition experience. This experience existed of one *P. rapae* egg on a small (about 2 by 2 mm) cut out piece of Brussels sprouts leave with which the wasp could have free access overnight.

In an additional olfactometer experiment the wasps got an oviposition experience in *Mamestra brassicae* eggs in the presence of four mated *P. rapae* females to find out if the wasps could learn to associate the mated female odour with a successful oviposition experience. The experiment was performed between 45 and 65 minutes after oviposition. The response of oviposition experienced wasps to the odour of four mated females against four male *P. rapae* butterflies and the combination of four mated females against four virgin females of *P. rapae* were tested.

Mounting experiment

The mounting behaviour of the GD-011 *T. evanescens* line was tested in a glass arena made of two large Petri dishes (14,5 cm in diameter and 2 cm in height). Two adult *P. rapae* were cooled down in the refrigerator to decrease their mobility and placed in the arena. A naïve GD-011 *T. evanescens* female was introduced into the arena and continuously observed. When the wasp climbed onto the butterfly, the mounted body part and the time spent on the butterfly were recorded. When the wasp had not made a choice after 300 seconds this was recorded as a "no response". The butterflies were used in a rotating system. After each wasp the butterflies were placed back into the refrigerator. 4 to 6 wasps were tested per butterfly couple. Each time all butterfly couples were tested the setup was rotated 180^{o}. In total, 8 to 16 wasps were tested per day.

Response to butterflies

In the mounting setup, the mounting choice preference of *T. evanescens* GD-011 to the different butterflies (virgin female, mated female, mated male or female dummy butterfly, constructed of paper and iron wire) was tested. First the innate mounting preference of naïve *T. evanescens* GD-011 virgins was tested in the combinations mated female against mated male, mated female against virgin female, mated male against virgin female and mated female against virgin dummy. After that the butterfly combinations of mated female against mated male, mated female against virgin female and mated female against female dummy butterfly were tested with wasps that had an oviposition experience in *Mamestra brassicae* eggs in the presence of four mated *P. rapae* females.

The response to virgin and mated females was also tested with wasps that had the same oviposition experience, but in stead of four *P. rapae* butterflies, four treated female *P. rapae* dummies were offered. The dummies were treated with a synthetic anti-aphrodisiac odour blend, 2 µl (1 mg methyl salicylate (Sigma 99%) and 1 mg indol (Aldrich 99+ %) per ml hexane (Merck)) solution (Andersson et al. 2003) were used per dummy.

Results

Trichogramma identification

From the 154 collected *Pieris rapae* eggs, 37 were parasitized by *Trichogramma* spp. A total of 107 wasps emerged, of which 34 were males and 73 were females (68.2% females). An amount of 84 wasps were identified, of which 96.4% were identified as *Trichogramma evanescens* and 3,6% were identified as *Trichogramma brassicae.* The *T. brassicae* wasps all emerged from a *P. rapae* egg together with one or more *T. evanescens* wasps.

Olfactometer experiments

In the two-choice olfactometer bioassay the naïve wasps of the GD-011 *T. evanescens* line showed an innate preference for the odour of mated *P. rapae* females in comparison with clean air (figure A). After an overnight oviposition experience in a *P. rapae* egg, this preference disappeared. The wasps did not show an innate response towards males neither to virgin females of *P. rapae* compared to air. Also when males were offered against virgin females, the wasps did not have a preference for one of the two odour fields, although there was a tendency towards the virgin females. Naïve wasps did not distinguish between the odour field of mated and virgin *P. rapae* females. However, after an oviposition experience in the presence of mated *P. rapae* females they tended to prefer the odour of four mated females above four virgin females (Pashalidou and Michelaiki, unpublished data). These wasps even significantly preferred the odour of four mated *P. rapae* females above the odour of four males, whereas naïve wasps did not distinguish between these two odour fields.

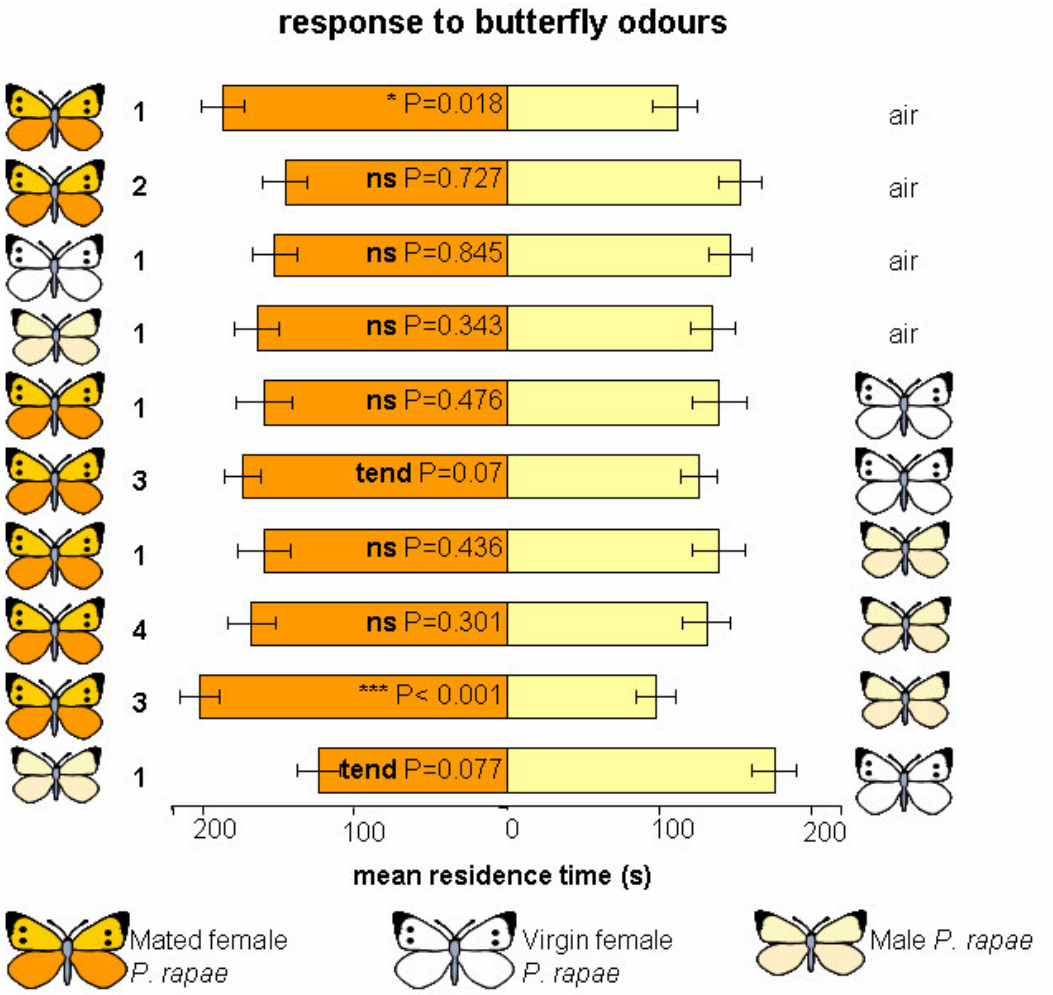

Figure A: The response of *T. evanescens* wasps to the offered *P. rapae* odour fields in a two-chamber olfactometer. The numbers on the left side represent the experimental conditions 1) naïve wasps, tested with two butterflies per treatment, 2) wasps that had an oviposition experience in one *P. rapae* egg offered overnight, tested with two butterflies per treatment, 3) wasps that had an oviposition experience in *M. brassicae* eggs in the presence of four mated *P. rapae* females, tested with four butterflies per treatment, 4) naïve wasps, tested with four butterflies per treatment. The mean residence times (± s.e.) are shown, N=40 tested wasps per experiment. Significances are calculated with a Wilcoxon's matched pairs signed rank test.

Mounting experiments

The naïve GD-011 *T. evanescens* wasps did not discriminate between mated and virgin female *P. rapae* butterflies, nor between mated females and males, males and virgin females or even mated females and female dummies (figure B). Wasps that had an oviposition experience in the presence of four mated *P. rapae* females tended to mount mated females more often than virgin females (Tribuhl & Munneke, unpublished data) or males (Pashalidou

& Michelaiki, unpublished data) and had a clear preference for mated females compared to *P. rapae* dummies (Pashalidou & Michelaiki, unpublished data). Wasps that had an oviposition experience in the presence of dummy butterflies treated with the *P. rapae* anti-aphrodisiac blend of methyl salicylate and indol dissolved in hexane did not express any preference for mated females when tested against virgin females.

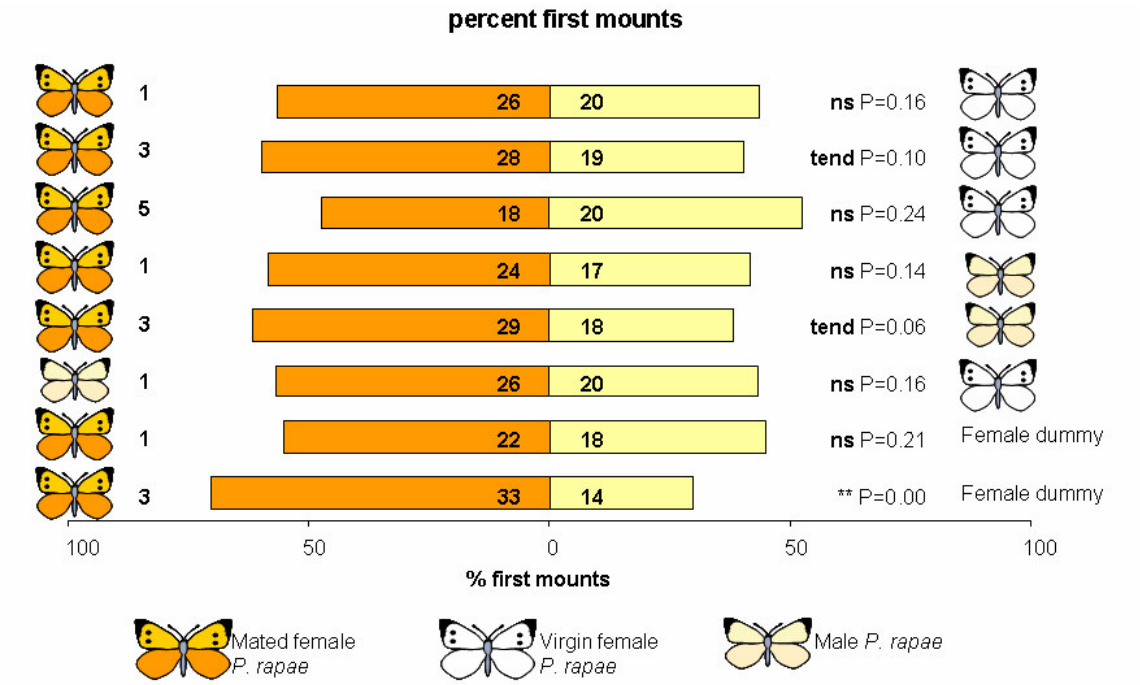

Figure B: Percentage of first mounts by *T. evanescens* on *P. rapae* butterflies. Number of responding *T. evanescens* is written inside the bars. The numbers on the left side represent the experience of the tested wasps 1) naïve wasps, 3) wasps that had an oviposition experience in *M. brassicae* eggs in the presence of four mated *P. rapae* females, 5) wasps that had an oviposition experience in *M. brassicae* eggs in the presence of four dummies treated with methyl salicylate and indol dissolved in hexane. Significances are calculated with a binominal test.

Conclusion and Discussion

This research gives a good view on how *Trichogramma evanescens* GD-011 females respond to *Pieris rapae* butterfly odours. Although the tested *T. evanescens* females do show an innate response towards the odour field of mated *P. rapae* females compared to air, it does not show an innate response to mated female odours when compared with other butterfly odours. Only after an oviposition experience in the presence of mated females the wasps respond to the odour of mated females more strongly than to male butterfly odours, tend to prefer the odour of mated females above virgin females, tend to mount mated females more often than virgin females or male butterflies and significantly prefer to mount mated females more often than female dummies. An oviposition experience alone, or an oviposition experience in the presence of dummies treated with a synthetic anti-aphrodisiac blend dissolved in hexane, did not influence the wasps' preference. When naïve wasps were offered the opportunity to climb onto female butterflies and a 3rd stage instar female desert locusts, they even showed no preference (van Elven, unpublished data). Because we used an isofemale *T. evanescens* line the genetic variation within the colony is minimized. This makes the colony poorly representative for the natural population of *T. evanescens*, but it also minimizes the variation in response to butterflies within the colony (Thomson and Stinner 1990). It would now be interesting to do the same experiment with different *T. evanescens* lines.
Obviously, this *T. evanescens* line does not innately respond specifically to the odours or appearance of mated *P. rapae* females. This is consistent with the results of Fatouros et al (2007) who found no specific innate response of *T. evanescens* towards mated *Pieris brassicae* females. In contrast to *Trichogramma brassicae, T. evanescens* is not able to innately recognize butterfly anti-aphrodisiac odours. They, however, do seem to be able to learn and associate mated female butterflies with the positive experience of an oviposition in a host egg. Schöller and Prozell (2002) found that *T. evanescens* has the ability to associatively learn to respond to an artificial sex pheromone of *Ephestia* spp. and *Plodia interpunctella.* This was, however, only tested in a combination of the synthetic pheromone against clean air. It could be possible that *T. brassicae* has another searching strategy because, in contrast to *T. evanescens*, it does express an innate response towards butterfly odours. This innate response might be because of a more narrow host range, and thus specificity. The absence of an innate specificity in *T. evanescens* suggests a wider host range which makes it important to have learning abilities. It would be interesting to find out if the innate response or learning

possibilities are indications of a small or wide host range. If they are so and *T. brassicae* is more specified than *T. evanescens* this would mean that *T. brassicae* can poorly learn to associatively learn to respond to odours.

Host specificity might contribute to the distribution of *Trichogramma* spp. among potential hosts within an area. Many *Trichogramma* wasps respond to the wing scales of Lepidopteran hosts. Lewis et al (1971, 1972) showed that *T. evanescens* was more likely to parasitize host eggs in the presence of hosts as a consequence of the presence of host scales. Thomson and Stinner (1990) found that contact with host scales leads to increased searching behaviour in four *Trichogramma* species, which is likely to increase the likeliness of finding a host in the searched area. They found that a positive oviposition experience in the presence of scales of a certain Lepidopteran species enhanced the responsiveness to scales in general. Although the experienced wasps tended to mount mated females more often than virgin females and males, they might have to experience the mated female odour plus the mounting experience to specifically hitch-hike with mated females. Preliminary experiments with wasps that had an oviposition experience after climbing onto mated female butterflies support this hypothesis (Tribuhl and Munneke, unpublished data). It would be interesting to test whether *T. evanescens* in the field really use their learning ability in order to find mated female butterflies. This could for instance be examined by measuring the efficiency with which experienced and inexperienced *T. evanescens* wasps can find mated female butterflies and parasitize butterfly eggs in a cage with *P. rapae* butterflies and Brussels sprouts plants on which the butterflies can lay their eggs.

Fatouros and Huigens (unpublished data) have found several *T. evanescens* wasps on mated females of the Large Cabbage White *P. brassicae* in the field. Considered that these wild *T. evanescens* use the strategy of phoresy on mated females to find their host, the butterfly egg, would they, like the laboratory colony, have to learn to respond to the odours of mated females of *P. brassicae*? And if so, do they recognize them by the smell of the anti-phrodisiac? Andersson et al. (2000, 2003) demonstrated the existence of the male-contributed anti-aphrodisiac system in *Pieris napi*, *P. brassicae* and *P. rapae*., and demonstrated that the anti-aphrodisiac titre of *P. napi* (MeS) does not decrease for at least six days after mating when the female is not courted (Andersson et al 2004). These data are not present yet for *P. rapae* and *P. brassicae* but they suggest that, in the field, wasps would have sufficient time to spy on the anti-aphrodisiac of *P. napi*, and that the anti-aphrodisiac of our tested butterflies was certainly still present during the experiment. In the future, one should now try to determine whether *T. evanescens* associatively learns to respond to the anti-aphrodisiac odour

blend of *P. rapae*. Experiments should use the synthetic anti-aphrodisiac blend of butterflies dissolved in hexane and test virgin female butterflies treated with this blend against virgin female butterflies treated with the solvent hexane only. In my experiments, the wasps may have associated the oviposition experience with the solvent hexane in stead of the anti-aphrodisiac blend.

The use of adult host stage stimuli for host finding is widely spread among egg parasitoids (Vinson 1991). Because of their size and limited flight ability the use of anti-aphrodisiacs in combination with phoresy could be an adaptive strategy among *Trichogramma* species. Preliminary data now suggest that this behaviour is certainly not just restricted to one *Trichogramma* species.

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Appendix 1

Protocols after Gonçalves et al (2006), van Rijswijk (2000), Silva et al (1999) and Stouthamer et al (1999)

DNA-extraction using the CHELEX method.

Materials

- DNA material (wasp)
- autoclaved pasteurpipet with a closed tip which is melted into a round bulb.
- 5% CHELEX solution
- proteinase K (20mg/ml) stored at -20°C and kept on ice while using.

Methods

All samples should be kept on ice

1. Place the wasp in an eppendorf tube and ground it with the closed Pasteur pipette.
2. Add 50 µl of the 5% CHELEX solution. In order to be able to take up the glass balls the tips of the pipette should be cut off.
3. Add 4 µl proteinase K
4. Samples are incubated at least overnight at 56°C
5. Samples are incubated at 95°C for 10 minutes
6. Samples are centrifuged for a short time in order to collect the glass balls at the bottom of the eppendorf tubes
7. The samples, containing the template DNA, are stored at -20°C. Thaw and centrifuge before use.

Polymerase Chain Reaction

Materials

- Taq polymerase, 5 units μl^{-1} which should be kept as cold as possible
- 10 x PCR reaction buffer, which should be vortexed before use
- Forward primer
- Reverse primer
- 10 mM dNTP set
- Template DNA

Methods

1. Make the PCR cocktail mix. It should be enough to carry out the number of reactions needed (number of samples plus a positive and negative control and two extra) which should consist the following ingredients per sample;

	Super-taq cocktail	Go-taq cocktail
Water	17.43 µl	33.75 µl
PCR buffer	2.5 µl	10 µl
ITS2-primer forward	0.5 µl	1 µl
ITS2-primer reverse	0.5 µl	1 µl
10 mM dNTP	0.5 µl	1 µl
Taq polymerase	0.07 µl	0.25 µl
MgCl	-	3 µl
Template DNA	2.5 µl	5 µl

Go-taq contains loading buffer, which should, thus, not be added during the preparation of the gel electrophorese

2. A 0.2 ml thin-wall eppendorf tube is marked for each sample
3. 22.5 µl of the PCR-cocktail is added to each eppendorf tube
4. template DNA is added to each eppendorf tube
5. The eppendorf tubes are placed in the PCR machine and have to follow the following program (Stouthamer et al 1999)
    - First the samples are placed at 94^{o}C for 5 minutes to make all DNA single stranded
6. Than a cycle (after Gonçalves et al 2006) is carried out 35 times, consisting of;
    - A high temperature (94^{o}C) for 40 seconds to make all template DNA single stranded
    - 45 seconds at the annealing temperature of 53 which is specific for the ITS2 primer
    - 45 seconds at 72^{o}C during which the polymerase complements the single stranded DNA piece after the primer
7. Finally the sample is placed at 72^{o}C for 10 minutes
8. The samples are stored at -20^{o}C

Agarose Gel Electrophoresis

Materials

- 1 x TAE, 0.04 tris-acetate 0.001 M EDTA electrophoresis buffer
- Agarose
- 10 mg/ml Ethidium-Bromide (carcinogenic, use gloves and be carefull, only handle in zuurkast, throw away gloves after handling Ethidium-Bromide)
- 6 x loading buffer containing 30% glycerol, 0.25% orange G. (+4^{o}C), or 30% glycerol, 0.25% broomphenolblue
- DNA-ladder

Methods

1. For a 15 x 10 cm gel tray use 100 ml 1-3% agarose / 1 x TAE buffer. Weigh it out in an erlenmeyer and heat it in the microwave until you get a clean solution. Let it cool till about 60^{o}C (just touchable with your hands) and add 3.5 µl Ethidium-Bromide solution.
2. While the agarose solution is cooling down, the geltray is prepared by closing the open ends with tape. A comp is placed to make slots.
3. Poor the agarose solution in the geltray and let it harden for 20 minutes.
4. Put the hardened gel in the running box and submerge it under 1 x TAE
5. Prepare samples by applying 4 µl loading buffer on parafilm and add 10 µl PCR-sample, mix and apply it in the gel slots. Apply DNA-ladder at both sides
6. Run the gel at about 70 V until the dye has moved ¾ in the gel.
7. Look at the results under UV-light and take a picture.

Mse1 restriction

Materials

- Distilled water
- Reaction buffer
- BSA
- Mse1
- PCR product

1. Make the Mse1 restriction mix. It should be enough to carry out the number of reactions needed (number of samples and two extra) which should consist the following ingredients per sample;
   - 5µl distilled water
   - 2µl restriction buffer
   - 2µl BSA
   - 1µl Mse1
2. A 0.2 ml thin-wall eppendorf tube is marked for each sample
3. 10µl restriction mix is added to each eppendorf tube
4. 5-10µl PCR product is added to each eppendorf tube
5. The eppendorf tubes are incubated in the PCR machine for two hours at 37^{o}C (after Silva et al 1999).
6. The digestions are checked by running them on agarose gel

QIAEX II Agarose Gel Extraction

Material

- QX1 buffer
- QIAEX II
- Buffer PE
- Tris-Cl

Methods

1. carry out PCR with the to-be used DNA sample and carry out a gel electrophoresis but do not place the gel under UV light yet
2. Label 1.5 ml eppendorf tubes with the different DNA-samples to be used
3. Place the gel under 70% UV light and slice out the DNA-bands and put them in the eppendorf tubes. The UV-light should be on for a period as short as possible because UV light breaks down DNA.
4. Weigh the gel slices, select the heaviest gel slice and add to each gel slice 300 µl of buffer QX1 to each 100 mg of the heavies gel slice.
5. Vortex the mixture of gel and buffer for 30 seconds
6. Add 20 µl of QIAEX II and vortex for 30 seconds
7. Incubate the solution at 50^{o}C for 10 minutes and vortex every 2 minutes
8. Centrifuge for 30 seconds and remove the supernatant with a pipette
9. Wash the pellet with 500 µl of QX1 – vortex – centrifuge and remove the supernatant with a pipette
10. Wash the pellet with 500 µl of Buffer PE – vortex – centrifuge and remove the supernatant with a pipette
11. repeat set 10
12. After removing the supernatant for a second time air dry the pellet until it becomes white
13. Add 20 µl of 10 mM Tris-Cl – vortex and incubate at room temperature for 30-60 minutes
14. Centrifuge for 30 seconds and pipette the supernatant, which now contains the PCR-product, into a clean tube

Ligation in PGem-T vector

1. Mark tubes with the sample name
2. add to each of the tubes
   - 5 µl 2x ligase buffer (vortex before use)
   - 1 µl pGem-T vector
   - 1 µl T4 DNA ligase
   - 3 µl own PCR product
3. Incubate overnight in the fridge at 4^{o}C
4. Now ready to be heat shocked (transformation)

Transformation using the pGem-T vector ligation reactions

Materials

- XL2 blue competent cells
- SOC solution at room temperature
- AXI/LB plates (see below)
- LB medium containing 1% ampicillin

Methods

1. Centrifuge the ligation product to collect the contents at the bottom of the eppendorf.
2. Remove the frozen competent cells from the -70^{o}C and place them on ice to thaw for 5 minutes.
3. Mark the tubes with the sample name/number
4. Add 20 µl of the competent cells to each of the tubes
5. Add 2 µl of the ligation mixture and gently mix the contents of the tube, leave on ice for half an hour, after which the cells will be heat shocked
6. Get water bath ready, it should be 42^{o}C exactly
7. The heat shock has to be carried out precisely. Place the tube in the 42^{o}C bath for exactly 30 seconds and place it back on ice for 2 minutes.
8. Add 970 µl SOC to each of the tubes and incubate for an hour at 37^{o}C shaking 225 rpm.
9. Plate 100 µl of each transformation on an AXI/LB plate under sterile conditions
10. Centrifuge the solution to collect contents at the bottom, remove 700 µl of the supernatant and dissolve the pellet in the remaining supernatant
11. Plate 100 µl on an AXI/LB plate
12. Incubate the plates at 37^{o}C overnight (not longer)
13. Put 3.5 ml 1% ampicillin LB medium in an autoclaved tube
14. Pick a white colony from the plate with a sterile toothpick and place it in the tube, do not take any agar with it.
15. shake the tubes at 250 rpm overnight at 37^{o}C in the incubator.

For 30 1.5% agar AXI/LB plates

- 5 g Trypton
- 2.5 g yeast
- 2.5 g NaCl
- 7.5 g agar
- 0.5 L water

1. All ingredients should be placed in a bottle and autoclaved
2. Let the bottle cool down to 60^{o}C and add the following ingredients per 100 ml medium
   - 100 µl ampicilline (100 mg/ml)
   - 200 µl X-gal (20 mg/ml)
   - 100 µl IPTG (100mM)
3. Pour the mixture into the Petri dishes (2 plates per ligation reaction) and dry the plates at 37^{o}C for 30-60 minutes, leave the tops off at an angle. The plates can be stored upside down in the fridge at 4^{o}C.

MiniPrep

Materials

- P1 buffer (in 4°C fridge)
- Buffer2
- Buffer N3
- PE buffer
- EB buffer

Methods

1. Centrifuge the tubes with the bacteria colonies for 10 minutes at 2000g
2. pour out the liquid and turn the tubes up side down on filter paper to remove as much supernatant as possible
3. Add 250 µl buffer P1 mix, carefully dissolve the bacteria and transfer the mixture to a 1.5 ml eppendorf tube
4. Add 250 µl buffer2 and gently invert the tubes 4-6 times
5. Add 350 µl buffer N3 and immediately but gently invert the tubes 4-6 times
6. centrifuge for 10 minutes at 14.000 rpm
7. pour the supernatant into a special blue-white spin column eppendorf, centrifuge for 30 seconds and throw away the flow through
8. Add 750 µl PE buffer, centrifuge for 30 seconds and throw away the flow through
9. Centrifuge for 1 minute, remove the blue part of the eppendorf tube and place it in a 1.5 ml microcentrifuge tube
10. Add 30 µl EB buffer and let it stand for 20 minutes at room temperature
11. Centrifuge for 1 minute on soft
12. Remove the blue part, which can be thrown away, but keep the eppendorf. The content of the eppendorf can now be used for different processes;
    - A small portion can be used for PCR
    - A portion can be used to measure the concentration and cleanliness of the DNA
    - If the samples contain enough DNA and they are clean enough they can be sent of for sequencing