Attraction of the larval parasitoid *Cotesia glomerata* to *Pieris brassicae* egg-infested Black mustard plants

Abstract

Plants are threatened by various enemies. They can defend themselves against herbivore attack by direct defense mechanisms that negatively affect herbivores or by indirect defenses that attract natural enemies. A characteristic example of a direct defense response induced by eggs is shown in the black mustard *Brassica nigra* that develops a necrotic zone around the eggs of the Large cabbage White butterfly *Pieris brassicae*, i.e. the so called hypersensitive response (HR), which inhibits further egg development. *B. nigra* plants can also indirectly defend themselves against herbivorous attacks through natural enemies' attraction (i.e. by attracting the larval parasitoid wasp *Cotesia glomerata* to *P. brassicae* feeding caterpillars). The objective of this research was to investigate whether *B. nigra* volatiles induced by eggs of the Large cabbage White butterfly *Pieris brassicae* are exploited by *C. glomerata* for host location in order to be able to parasitize young caterpillars after hatching and whether those egg-induced volatiles are host specific. Interestingly, I discovered that *C. glomerata* wasps were significantly attracted to egg-induced plant volatiles 24 h after *P. brassicae* oviposition regardless of HR developemnt around the eggs, when tested against clean plants in two-choice tests conducted in the windtunnel. On the contrary, plants induced by 96 h-old *P. brassicae* eggs were attracted to the *C. glomerata* wasps only when HR was developed around the eggs. I also found out that the egg-induced volatiles can be host specific, since there was no attraction to plants infested by eggs of the non-host moth species *Mamestra brassicae* when tested against clean *B. nigra* plants. Moreover, I tested the parasitoid performance (parasitization) in caterpillars just before hatching. Although the wasps showed a strong interest in the eggs by probing and trying to sting them, this did not result in successful parasitism of caterpillars inside the eggs. The outcomes demonstrate that *C. glomerata* wasps can make use of plant volatiles induced by *P. brassicae* eggs to arrive on time before the host caterpillars hatch. Hereby, it seems that the direct (hypersensitive response) and indirect defensive mechanisms (*C. glomerata* recruitment) 'act in concert' in the studied population of *B. nigra* plants.

Keywords: Parasitoids, *Cotesia glomerata*, *Brassica nigra*, *Pieris brassicae*, hypersensitivity response (HR), direct/indirect defence, herbivore-induced plant volatiles (HIPVs).

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Introduction

Plants have the ability to defend themselves directly and indirectly against herbivorous insects. Direct defensive responses negatively affect the herbivores. Some plants were shown to react in the presence of the eggs by a) the formation of neoplasms, b) a hypersensitive response (HR), and c) the production of oviposition deterrents. The latter can be a local or systemic response (Hilker and Meiners 2002). For example, the black mustard, *Brassica nigra,* shows HR against *P. brassicae* eggs: a necrotic zone is being developed around the eggs 24 hours after oviposition and after 72 h some of the eggs dry out and often fall on the ground (Shapiro and De Vay 1987). By indirect defense mechanisms, plants can enhance the performance of parasitoids that are the herbivores' natural enemies. In order to defend themselves against herbivorous insects and their activities (oviposition and larval feeding), plants emit volatile organic compounds (VOCs) induced by herbivore egg deposition or feeding that guide the natural enemy to the infested host plant (Dicke 2009). Studies on tritrophic systems shown that egg-deposition leads to plant-volatile emission that attracts egg-parasitoids (Hilker and Meiners 2006). For example, contact *Brassicae* plant-cues induced by eggs of the Large cabbage White butterfly *Pieris brassicae* arrests *Trichogramma* parasitoids (Fatouros et al. 2005).

Infochemicals play an important role during the host selection of parasitoid wasps (Vet et al. 1992). The parasitoids' foraging behavior has been evolved according to the chemical interaction between their herbivore victim and its food plant. Infochemicals that are emitted by one species and received by another species in favor of the receiver but not at the cost of the emitter are called kairomores, whereas those that benefit both emitter and receiver are called synomones (Dicke and Sabelis 2008). Within a tritrophic context, parasitoids (3rd trophic level) can use infochemicals emitted by the herbivore victim (2nd trophic level, i.e. kairomones) and/or the host plant (1st trophic level, i.e. synomones) (Vet and Dicke 1992). For parasitoids plant-derived cues are easily detectable from a distance but not so reliable like host-derived cues which are more reliable indicators for their presence but not easily detectable in long distances. The reliability and detectability of the information from the food of the host, is influenced by the degree of the infestation of the host plant (Vet et al. 1991). To overcome this problem of low detectability-reliability, parasitoids can make use of the highly detectable herbivore-induced plant volatiles (HIPVs) that reliably indicate the presence of the herbivore due to their specificity. Certain herbivores induce certain chemical release from their food plant, which can be a specific signal for the parasitoid (Vet et al. 1991).

A number of investigations have been conducted to explore the *Brassica-Pieris-Cotesia* interaction. As already mentioned, *B. nigra* directly defends itself against *Pieris* eggs showing HR (Shapiro and De Vay 1987). As an indirect response *Brassica* plants were shown to release HIPVs in the presence of *Pieris* eggs or caterpillars that attract parasitoid wasps (Dicke 2009; Fatouros et al. 2008; 2009; Lucas Barbosa 2009). Volatiles of Brussels sprouts plants induced by 1st and 5th instar *P. brassicae* caterpillars are equally attractive to their larval parasitoid *Cotesia glomerata*. There is no indication so far that *C. glomerata* can discriminate the herbivore induced synomones related to different host instars (Mattiacci and Dicke 1995). However, the duration of searching by the parasitoids after reaching the leaf is affected by several close-range cues (Mattiacci and Dicke 1994). The time the parasitoid spent searching

was longer on Brussels sprouts leaves infested by 1st instar larvae than on those infested by 5th instar larvae (Mattiacci and Dicke 1995). Neonates cannot defend themselves against parasitization by *C. glomerata* wasps as much as older instars which have an aggressive attitude towards the attacking wasps, by hitting, biting or regurgitating (see figure 1) often leading to the death of the wasps. Therefore, parasitization of neonate caterpillars by *C. glomerata* would be advantageous from the parasitoids' perspective (Mattiacci and Dicke 1994).

Figure 1 L2 *P. brassicae* caterpillar attacking *C. glomerata*; Photo: Hans Smid

In this research I studied the tritrophic system consisting of the black mustard (*Brassica nigra*), the Large cabbage White butterfly *Pieris brassicae* (Lepidopter: Pieridae) and its larval parasitoid *Cotesia glomerata* (Hymenoptera: Braconidae). I investigated whether plant volatiles induced by *P. brassicae* eggs are exploited by *C. glomerata* for host location, in order to parasitize neonate caterpillars at the moment of hatching, and if those egg-induced plant volatiles are host specific. Furthermore, I observed the possible parasitization of *Pieris* eggs just before hatching by *C. glomerata* and examined whether this resulted in successful parasitism of the caterpillars.

Research aim and questions

The objective of this research was to investigate whether *C. glomerata* wasps are able to use volatiles of *B. nigra* plants induced by *P. brassicae* eggs and whether the wasps can discriminate induced volatiles at different time points after butterfly oviposition.

Under the following hypothesis, three research questions were derived:

H: The larval parasitoid *C. glomerata* is recruited by *P. brassicae* egg-induced volatiles before caterpillars hatch.

Q1: Is *C. glomerata* attracted to *P. brassicae* egg-induced volatiles of *B. nigra* plants?

Q2: Does *C. glomerata* prefer volatiles of plants infested with old eggs (96 h) close to hatching?

Further questions as a responce to questions 1 and 2.

Q3: Are the egg-induced volatiles host specific?

Q4: Is *C. glomerata* able to parasitize *P. brassicae* eggs just before hatching?

Materials and methods

Plants and insects

Plant seeds of *Brassica nigra* were used from the Center for Genetic Resources (CGR, Wageningen, The Netherlands), from an earlier flowering accession CGN06619 (feral population collected in 1975 from Peloponnesos, Greece). A seed mixture of 25 individuals has represented this plant population. *B. nigra* were reared in a greenhouse compartment (22 ± 2°C, 70% r.h., L16:D8). Four and five week old plants have been used for the experiments. Entomopathogenic nematodes were applied in the soil to avoid sciarid fly larvae infestation.

Pieris brassicae caterpillars were reared on Brussels sprout plants in a large cage in a climate room (22 ± 1°C, 50-70% r.h., L16:D8). The adults were feeding on saturated sugar solution. Each of the *B. nigra* plants that were used in the experiment were placed in the cage allowing the adult *P. brassicae* to oviposit. Later, the plants were transferred in the greenhouse compartment (22 ± 2°C, 50-70% r.h., L16:D8) and kept separately from the clean plants that were used as control, for 24 to 96 hours, depending on the experiment.

Cotesia glomerata were reared on *P. brassicae* larvae, feeding on Brussels sprouts plants under greenhouse conditions (22 ± 1°C, 50-70% r.h., L16:D8). *C. glomerata* pupae were collected and reared in gauze cages in a climate chamber (23± 1°C, 50-70% r.h., L16:D8). After emergence wasps were provided with water and honey. There was no contact between them and the plant material or caterpillars before the experiments. They were referred to as naive wasps.

Mamestra brassicae caterpillars were reared on Brussels sprouts plants in large cages in a climate room (22 ± 1°C, 50-70% r.h., L16:D8). The adult moths were feeding on saturated sugar solution. Adult mated females were collected for the experiments in order to lay eggs on the *B. nigra* plants in a separate cage. Later, the plants were placed in a cage separately from the clean plants that were going to be used as control, for 24 hours.

Plant treatments

The tested plant treatments were *P. brassicae* egg-infested plants 24 h and 96 h after oviposition and *M. brassicae* egg-infested plants 24 h after oviposition. The plants for *Pieris* infestation were placed in the butterfly cage for about 10 min allowing *P. brassicae* for oviposition. The butterflies were allowed to lay 3-5 egg-clutches on each plant. The plants for *Mamestra* infestation were placed in the *M. brassicae* cage early in the morning for about 1 h, allowing the moths to oviposite 3-5 egg clutches on each plant. The control plants in each treatment were always clean *B. nigra* plants.

Hypersensitive response

The HR phenomenon is not universal for all *B. nigra* plants; therefore I tested the HR+ *B. nigra* plants separately from the HR- plants. Both *P. brassicae* and *M. brassicae* egg-induced *B. nigra* plants were checked for hypersensitive response 24 h and 96 h after oviposition. The tested plants with 24 h-old and 96 h-old *P. brassicae* eggs with HR (HR+) and without HR (HR-) were analyzed separately.

Preference test/ host location test in the windtunnel (questions 1, 2 and 3)

To test whether *C. glomerata* wasps are attracted to *P. brassicae* egg-induced volatiles of *B. nigra* (q. 1-2) a two-choice test was conducted in a windtunnel measured 200 x 60 x 60cm, (26% ± 1°C, 60% r.h., velocity 10). Similarly, the windtunnel was also used for research question 3, testing the preference of *C. glomerata* towards *M. brassicae* egg-induced volatiles of *B. nigra*. The wind blows from behind the plants and the distance between the wasp release point and the plants was about 70 cm. Fifteen adult female wasps were released one by one per day for 10 min to let them choose between the volatile blends of the two plants offered in the windtunnel. Each treatment was carried out in different experimental days. The number of wasps that had chosen any plant has been reported. The non-responding wasps were counted but excluded from the statistical analysis. The non-responding wasps were those who did not make a choice in the given time of 10 min. Only the first landing of the wasps was counted, also the time they spent until they make a decision was recorded. Each wasp and plant was used only once and then discarded.

Table 1 Overview of the two-choice preference tests in the windtunnel.

No	Treatment	Induction time (h)
1.	*Pieris* egg-infested HR+ *B. nigra* vs intact plant	24
2.	*Pieris* egg-infested HR- *B. nigra* vs intact plant	24
3.	*Pieris* egg-infested HR+ *B. nigra* vs intact plant	96
4.	*Pieris* egg-infested HR- *B. nigra* vs intact plant	96
5.	*Mamestra* egg-infested *B. nigra* vs intact plant	24

Performance test (question 4)

To observe the parasitization behavior of *C. glomerata* and measure the parasitism rate, leaves of *B. nigra* and /or Brussels sprouts with 96 h and 120 h old eggs close to hatching were collected. I offered to 10 female *C. glomerata* wasps 10 *Pieris* eggs of the same leaf and age and allowed them to parasitize for 1 hour. Two days later, I measured the parasitism rate through dissection of L2 instars and checked for *Cotesia* larvae under the microscope.

Statistics

The choice of *C. glomerata* wasps between the two odor sources in a windtunnel bioassay was analyzed by a two-sided binomial test (a=0.05; *,P <0.05; **,P<0.01; ***,P<0.001; ns, not significant).
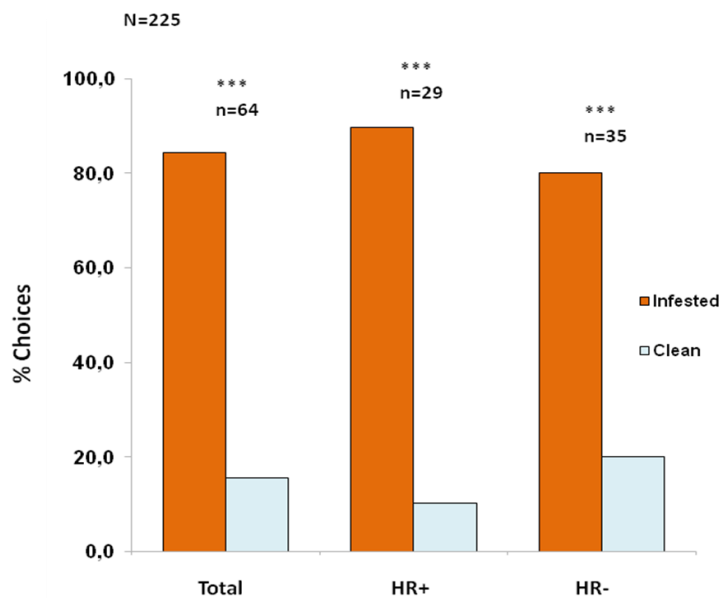
Results

N=225

Figure 2. The percentage of choices of *C. glomerata* between *B. nigra* plants induced by 24 h-old *Pieris* eggs (HR+/-) and clean plants. The 'total' includes the percentage of the choices of all treatments both with HR+ and HR-. Binomial test; ***, P<0,001. N: number of wasps tested; n: number of wasps responded.
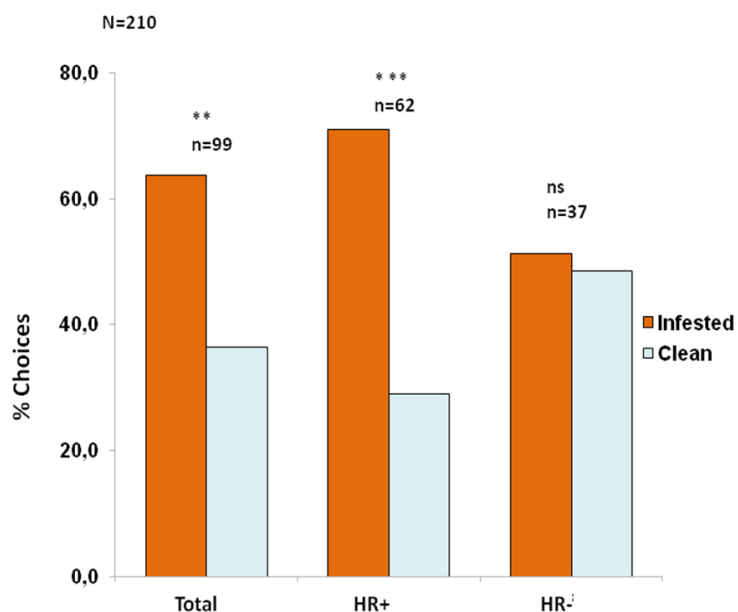
Host location behavior 24 h after *Pieris* oviposition

The results show that *C. glomerata* wasps were significantly attracted to egg-induced volatiles 24 h after *P. brassicae* oviposition when tested against clean *B. nigra* plants (total: ***, P<0,001; binomial test, Figure 2), regardless of HR around the eggs (HR+: ***, P<0,001; HR-: ***, P<0,001). From the 225 wasps tested in the experiment, only the 30% responded.

N=210

Figure 3. The percentage of choices of *C. glomerata* between *B. nigra* plants induced by 96 h-old *Pieris* eggs (HR+/-) and clean plants. The 'total' includes the percentage of choices of all treatments both with HR+ and HR-. Binomial test; ***, P<0,001; **, P<0,01; ns, P>0,05 N: number of wasps tested; n: number of wasps responded.

Host location behavior 96 h after *Pieris* oviposition

The results show that *C. glomerata* wasps were significantly attracted to egg-induced volatiles 96 h after *P. brassicae* oviposition over the intact *B. nigra* plants, only when showed HR+. (Total: **, P=0.004; HR+: ***, P<0.001, binomial test Figure 3). When *B. nigra* plants showed no HR the wasps could not discriminate between the two offered plants (ns, P=0.257; binomial test, Figure 3). From the 210 wasps tested in the experiment only the 47% responded.
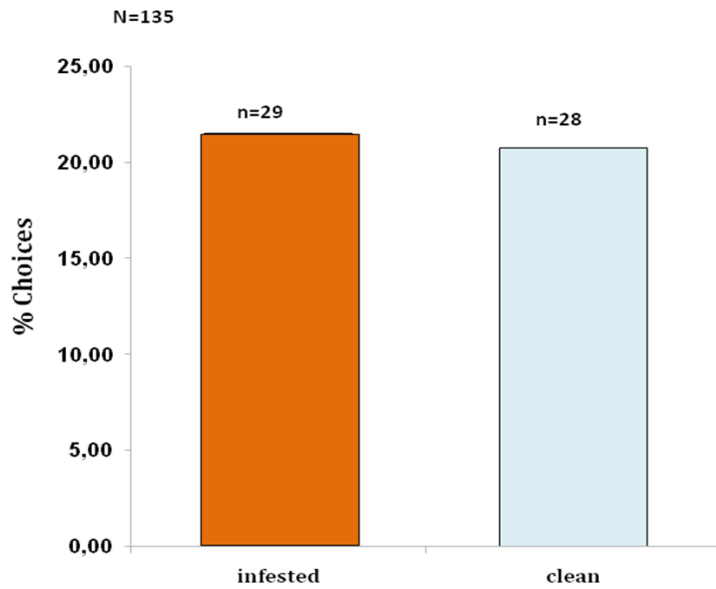
N=135

Figure 4. The percentage of choices of *C. glomerata* between *B. nigra* plants induced by 24 h-old *Mamestra* eggs tested against clean plants. Binomial test: ns, P>0,05. N: number of wasps tested; n: number of wasps responded.

Host location behavior 24 h after *Mamestra* oviposition

The results show that *C. glomerata* wasps could not discriminate *M. brassicae* egg-infested plants when tested against clean *B. nigra* plants (ns, P=0.208; binomial test, Figure 4).

After testing *M. brassicae egg*-infested plants were checked for HR development. The *B. nigra* plants did not show a hypersensitive response either 24 h or 120 h after *M. brassicae* oviposition. From the 135 wasps tested in the experiment, only the 42% responded.

Parasitoid performance

In this treatment I have checked the parasitization performance of 10 naive female *C. glomerata* into 10 *P. brassicae* eggs either 96 or 120 h-old. The results show that *C. glomerata* were not able to parasitize *P. brassicae* caterpillars inside eggs close to hatching. No *C. glomerata* eggs or larvae were found in L2 caterpillars. The observation of the parasitization behavior revealed that *C. glomerata* were strongly attracted to *P. brassicae* eggs and that they spend long time (more than 40 min) searching around them and trying to probe and oviposit (see figure 5a). However, it was different from the parasitization behavior when caterpillars were offered (see figure 5b). The wasps' legs and wings were not standing upwards and the abdomen was less bended.

Figure 5a *C. glomerata* trying to probe and parasitize *Pieris eggs*. Photo: Nina Fatouros

Figure 5b *C. glomerata* parasitizing L2 instar *Pieris* caterpillars. Photo: Hans Smid

Discussion

Cotesia glomerata wasps were clearly attracted to volatiles of *Pieris brassicae* egg-infested *Brassicae nigra* plants. According to Mattiacci and Dicke (1994), *C. glomerata* were not able to discriminate between volatiles of *B. oleracea* induced by 1st instar caterpillars or 5th instars although young host larvae are more suitable for parasitization and less aggressive than older caterpillars (Mattiacci and Dicke 1995). The clear attraction of *C. glomerata* to volatiles of *P. brassicae* egg-infested plants is in agreement with previous research shown that *B. nigra* emit oviposition-induced volatiles in response to *P. brassicae* eggs that attract the egg parasitoid *Trichogramma brassicae* (Lucas Barbosa 2009). Interestingly, the hypersensitive response was not a restricting factor for *C. glomerata*'s foraging decision, since both HR+ and HR- plants were equally attractive 24 h after oviposition. *B. nigra* emit egg-induced volatiles even 96 h after oviposition. However, in this case unlike the 24 h treatment, the HR is a limiting factor for the wasps' foraging behavior. *C. glomerata* showed a clear preference for the 96 h-old eggs on plants with HR+, while they could not discriminate the HR- egg-infested plant from the clean plant. Lucas Barbosa (2009) has shown in her research that volatiles induced by 72 h-old *P. brassicae* eggs were attractive to *Trichogramma brassicae* only when there was HR+. HR indeed plays a significant role in the *C. glomerata*'s decision when eggs are 96 h-old. The differences in the volatile blend between HR+ and HR- plants and the exact time that the *B. nigra* plants stop emitting cues when no HR (HR-) is observed, is not yet known. Analysis of volatile blends of HR+ and HR- plants are clearly needed. Moreover, it would also be interesting to discover the exact time at which *B. nigra* plants stop emitting those volatiles.

Direct defense seems to "help" indirect defense. Kahl et al. (2000) state that in some cases the production of herbivore-induced plant volatiles (HIPVs) happens due to the inability of direct defenses to act alone. Usually those HIPVs are responsible for the recruitment of beneficial parasitoids. For example, some plants cause reduction of growth rate of feeding herbivores as direct defense. However, this direct defense has no influence on fitness of neither the plant nor the herbivore without the involvement of the 3rd trophic level because the herbivore is still feeding on the plant until the end of its development (Baldwin and Preston 1999). Another example of cooperation between direct and indirect defense mechanisms is the production of VOCs induced by spider mites on tomato plants as an indirect defense that enhances the direct defenses of tomato. In this case, a significant increase in signaling compounds results in local and systemic changes such as the increase of wound-inducible proteinase inhibitors. Due to these changes the production of herbivore-induced volatiles is increased over time, which results in the attraction of predatory mites to the infested plants (Kant et al. 2004). In my research the development of HR (direct defense) seems responsible for the systemic volatile change of the plant that attracts *C. glomerata* wasps, (indirect defense). In the case of the used population, the hypersensitive response is neither killing all the eggs nor restricting herbivore feeding; a percentage of 80-90% of the eggs survive (Lucas Barbosa 2009). Therefore, it seems that in the tested *B. nigra* population there is no conflict between direct and indirect defense but they rather work in concert.

C. glomerata is not able to develop in *M. brassicae* caterpillars and thus was chosen to test the specificity of the oviposition-induced synomones for the wasps. In the two-choice tests in the

windtunnel I observed that the wasps showed no interest in volatiles of *B. nigra* plants infested with 24 h-old *M. brassicae* eggs when tested against uninfested plants. Therefore, it seems that *M. brassicae* eggs either do not induce any chemical changes in the volatile blend of *B. nigra* or the induced blend is different from the blend induced by *P. brassicae* eggs, and thereby not attractive to *C. glomerata* wasps. Under this observation I assume that the egg-induced volatiles can be host specific. Moreover, there was no HR development on *B. nigra* plants around *M. brassicae* eggs even after 120 h. In a reverse situation, if *B. nigra* reacted with HR against any herbivore this would probably mean an enormous fitness cost. However, in this research I have only observed *C. glomerata*'s behavior on 24 h-old *M. brassicae* eggs. Further research on *B. nigr*a plants induced by older *Mamestra* eggs may reveal a different behavior of *C. glomerata*.

The egg-induced volatiles are reliable enough for the parasitoid recruitment. Due to the fact that *C. glomerata* is a larval parasitoid, I would expect that they would show no interest in the eggs of their host. Interestingly, *C. glomerata* was attracted to *B. nigra* volatiles induced by 24 h and 96 h-old *P. brassicae* eggs, a result that gave rise to the question if *C. glomerata* was able to parasitize caterpillars inside the eggs. No offspring was found in the hatching caterpillars, which could be because the wasps' ovipositor was too short to reach the caterpillar in the eggs. However, the strong interest of the female wasps in the eggs is another proof for the reliability of egg-induced volatiles for host location. These observations were done under laboratory conditions and the tested materials were enclosed in small petri dishes. It would not be surprising if we could see *C. glomerata* waiting for its host to emerge from the egg, taking as an example the case of some perilampid wasps, which are larval parasitoid of the lacewing *Chrysopa*. These wasps were observed to stay next to the lacewing eggs waiting for the caterpillars to emerge (Godfray 1994). However, such behavior has not been yet observed in *C. glomerata* in nature.

Conclusions

Cotesia glomerata were attracted to volatiles of *B. nigra* plants induced by the deposition of *P. Btassicae* eggs 24 h and 96 h after oviposition. However, the wasps were not attracted to volatiles induced by eggs 96 h after ovipostition when no HR was developed on *B. nigra* plants. When the eggs are young, apart from the HR development, which is a strong chemical change attractive to wasps, I assume that *B. nigra* emit also other chemical cues responsible for *C. glomerata*'s recruitment. However, those chemical cues that gradually fade away with time, have not been analysed yet.

C. glomerata wasps can thus make use of the oviposition induced plant volatiles to arrive on time before the host caterpillars hatch. Despite the attractiveness of *P. Brassiace* eggs, *C. glomerata* cannot successfully parasitize caterpillars inside the eggs even when they are close to hatching.

The hypersensitive response is a direct defensive mechanism of *B. nigra* against *P. brassicae* oviposition. The parasitic wasps were even attracted to plants with a hypersensitive response. Therefore, the direct and indirect mechanisms seem to work in concert.

In my research I have found out that the egg-induced volatiles can be host specific. The plants' direct and indirect response was specific for the Large cabbage White butterfly that is host of *C. glomerata*. Neither the non-host *M. brassicae* induced any chemical changes in the plant that attracts *C. glomerata* nor did the plant directly defend its eggs 24 h after oviposition.

Future perspectives

Further research is needed in order to identify the chemical cues that take part in parasitoid recruitment, as well as the chemical changes that occur in the plant shown to develop HR. Moreover, it would also be interesting to test egg-infested HR+ *B. nigra* plants against HR- plants 24 h after *P. brassicae* oviposition, to see which of the two blends of chemicals is more attractive to *C. glomerata*. It would also be interesting to examine the attractiveness of plants with *P. brassicae* eggs to *C. glomerata* in the field in a more complex odorous environment. Also, another possible experiment would be to examine the choice of *C. glomerata* between plants with old *P. brassicae* eggs close to hatching against those with old caterpillars.

Acknowledgments

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