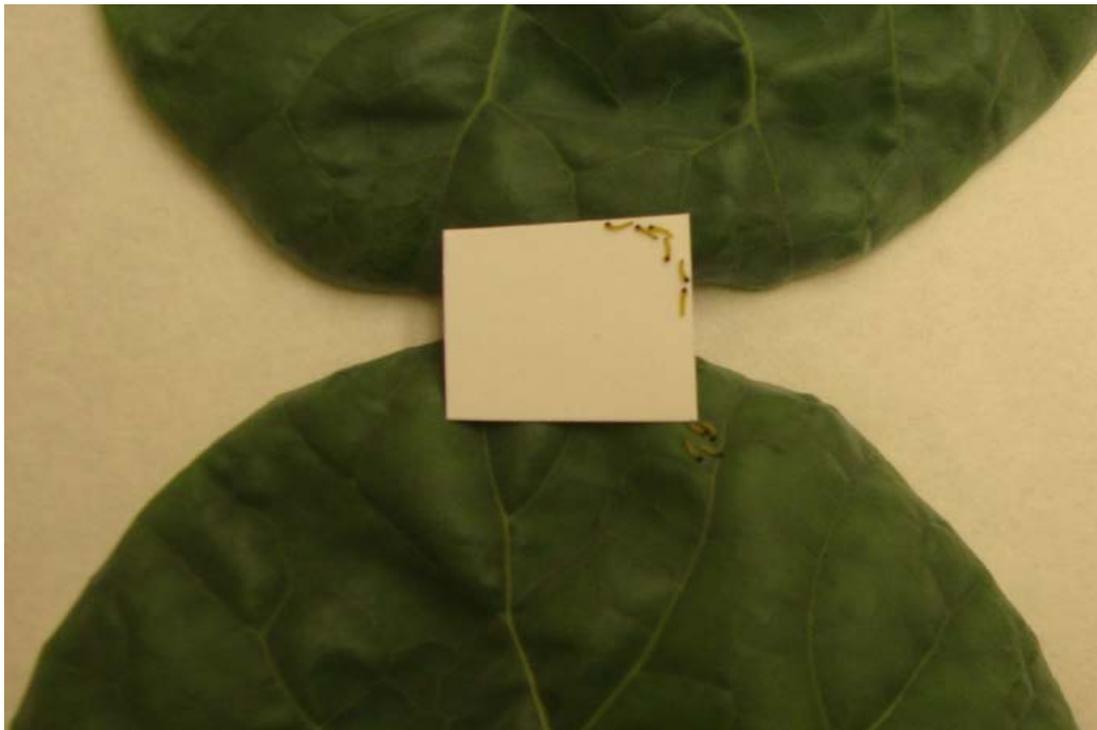


Plant-mediated inter-guild interactions: influence of the phloem feeder *Brevicoryne brassicae* on the host preference of the leaf chewer *Pieris brassicae*, and its parasitoid *Cotesia glomerata*



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Summary

In field conditions, plants are usually attacked by multiple herbivores simultaneously. However, there are limited studies focusing on plant-mediated interactions between two herbivore species co-existing on one host plant. Competition is classically reported to be the most frequent outcome of interactions between two herbivores sharing a common plant, especially when they belong to different feeding guilds. Nevertheless, a recent study on the performance of *Pieris brassicae* larvae on plants previously infested by aphids (*Brevicoryne brassicae*), showed an accelerated development and enhanced body mass compared to larvae reared on undamaged plants. Moreover, the parasitoid *Cotesia glomerata* also gained a bigger size by parasitizing the host feeding on aphid-infested plant rather than the host feeding on undamaged plant. It was concluded that *B. brassicae* facilitated the performance of *P. brassicae* larvae. Molecular studies also suggested facilitation between two inter-guilds herbivores by the antagonism of plant hormones salicylic acid and jasmonic acid signaling pathways involved in plant defenses.

The major research objective of this research was to study if host plant preference of *P. brassicae* females and *P. brassicae* larvae were influenced by an earlier aphid infestation. Based on the improved performance of *P. brassicae* larvae on aphid-infested plants, I expected *P. brassicae* females could choose what were beneficial for their offspring, and larvae could choose the better food sources, which were aphid-infested plants. The effect of aphid infestation on host preference of the parasitoid *Cotesia glomerata* was also investigated. I tested preference of *P. brassicae* gravid females and larvae in dual-choice assays by providing them with aphid-infested plants and undamaged plants. Preference of *C. glomerata* was tested in pair-wise comparisons between plants infested by both caterpillar and aphid and plants solely infested by caterpillar. Overall, I found butterflies did not discriminate between aphid-infested plants and undamaged plants, while larvae could distinguish these two types of plants and strongly preferred aphid-infested plants which were beneficial for their further performance in individual and full clutch, but not in sub-group. Moreover, it took larvae in individual and sub-group less time to choose beneficial plants than larvae in clutch. Additionally, full clutched larvae displayed a diminished split rate when choosing food plants as compared to sub-groups. *Cotesia glomerata* did not show preference between plant infested dually by both aphid and caterpillar and plants infested by caterpillars alone.

In summary, preference of *P. brassicae* female was not always well correlated with performance of *P. brassicae* larvae. I hypothesized that mothers did not necessarily choose better host plants for their offspring at the stage of oviposition, however, larvae were able to choose better food source within limits of mobility. When aphid infestation exceeds the 'breaking point', facilitation between the aphids and the caterpillars was replaced by competition. Facilitation is temporary, competition seems to last forever. As a gregarious species, *Pieris brassicae* larvae were fewer studies on individual behavior. Remarkably, we found larvae in individual and full clutch showed better ability in choosing beneficial food plants compared to larvae in sub-group, as the intra-specific communication was disrupted in sub-groups. Investigations of interactions between two herbivores sharing the same plant should include both bottom-up effect and top-down effect. Since the parasitoid did not show preference to hosts feeding on aphid-infested plants, facilitation between co-existed *P.*

brassicae larvae and aphid *B. brassicae* resulted from less-defended host plant still persisted when pressure from the parasitoid was taken into account. Moreover, Plant volatiles probably played a role in mediating the host selection behavior of adult / larval *P. brassicae* and parasitoid *C.glomerata*.

1 Introduction

Studies on plant-insect interactions were traditionally focused on direct interactions between plants and herbivores or herbivores and carnivores. After the 80's and until now more and more studies tend to research on multi-trophic interactions, considering plants, herbivores and carnivores simultaneously (Price et al., 1980; Dicke & Sabelis 1988; Dicke et al., 2003). It is believed that plants-insects interactions can be better understood when these three trophic levels are included (Turlings et al., 1990; Vet & Dicke, 1992; Denno et al., 1995; Inbar et al., 1999). Herbivory could alter plant physiology and phenology, as primary and secondary metabolites in plants are changed with responses upon herbivory (Dicke et al., 1992; Dicke and Baldwin, 2009; Inbar et al., 1999). Plant direct defenses can directly alter herbivore's biology and result in enhanced mortality, prolonged development period, reduced body mass and/or reduced fecundity predominately by production of secondary compounds. Meanwhile, plant indirect defenses link plants (first trophic level) with carnivores (third trophic level) via herbivores (second trophic level), as plants emit induced volatiles recruiting carnivores as a reaction to herbivory (Vet & Dicke, 1992; Turlings et al., 1995; Geervliet et al., 1994; Dicke 2009). Besides connecting species across trophic levels, plants have been shown to connect species within trophic level. Within the second trophic level, the common outcome of interactions among herbivore species is reported to be competition (Denno et al., 1995; Kaplan & Denno, 2007). Although interference competition and exploitative competition were historically emphasized as mechanisms underlying inter-specific interactions between herbivores, it is notably that indirect interactions mediated by plants and/or natural enemies provide the vast majority of evidences for inter-specific interactions (Denno et al., 1995; Kaplan & Denno, 2007). Herbivore-induced plant defenses not only impact one attacking herbivore but also impact other herbivores sharing the common host plant. Moreover, these long-persisted and systemic plant induced defenses could affect herbivores crossing different growing seasons or herbivores attacking different parts of plants (Dicke 2009).

More recently, the molecular mechanism underlying plant defenses has drawn much attention among ecologists, a lot is known now not only on the ecology of plant-insect interactions but also on the sub cellular mechanisms behind it. There are two major signaling pathways involved in mediating plant defenses, salicylic acid (SA) and jasmonic acid (JA) pathways. Jasmonate and salicylic acid are phytohormones that regulate plant growth, development, reproduction and survival and also serve as primary signals in regulation of plants resistant responses (Pieterse et al., 2009). Evidences about negative crosstalk between JA and SA in plant-insect interactions are accumulating (Zhang et al., 2009; Thaler et al., 2002; Bruessow et al., 2010). Doares et al. (1995) showed SA suppressed the JA-dependent defense gene expression. Similarly, JA pathway induction often inhibits the induction of the SA pathway (Thaler et al., 2002). Plants are proposed to optimize defenses to fight against different types of herbivores through antagonistic interaction of JA, SA pathways, and other

related different pathways (Pieterse et al., 2009). However, JA-SA cross-talk also suggested the possibility of facilitation between insect species from different feeding guilds, compromising plant defenses. Recently, this has been shown in a few model systems. Zhang et al. (2009) indicated infestation of whiteflies interfering indirect defense of Lima bean against spider mites via JA-SA cross-talk, and resulted in reduced attraction to predatory mites. Study from Rodriguez-Saona (2005) showed that aphid feeding has a positive effect on performance of caterpillar *S. exigua*, proved by increased larval survival and food consumption of caterpillar feeding on aphid-infested plants. Soler et al. (2011) demonstrated that *Pieris brassicae* larvae performed significantly better, growing bigger and faster on host plants previously colonized by aphids than on uninfested plants.

The consequences of cross talk, occurring when more than one herbivore from contrasting feeding guilds attacked the plants, just started to be explored (Rodriguez-Saona et al. 2005, Zhang et al. 2009; Soler et al. 2011). So far it has been shown that there can be facilitation between insect species from contrasting feeding guilds, but if this brings consequences on insect behavior remain unexplored. This study is based on the previous study by Soler et al. (2011) that showed that *Pieris brassicae* grow bigger and develop faster on plants previously colonized by aphids than on uninfested plants, at remarkably low level of attack. Moreover, its parasitoid *Cotesia glomerata* also benefit attaining a bigger size when it parasitized hosts feeding on aphid-infested plants as compared to those hosts feeding on uninfested plants. This is believed to be partly explained by interference in signal transduction, as JA level in plants exposed to aphids was 10 times lower than in undamaged plants or plants attacked by caterpillars. The main aim of this study is to investigate whether *P. brassicae* prefer to feed on plants previously colonized by aphids (*B. brassicae*) in order to maximize their fitness, and whether its parasitoid *C. glomerata* might compromise the facilitation that the aphid provided to the caterpillar by preferring to parasitize hosts feeding on aphid-infested plants, which also facilitates their performance. I hypothesize firstly that *P. brassicae* adults are capable to choose plants which benefit them, in this case, plants infested by *B. brassicae* via different volatiles emitted by undamaged plants and aphid-infested plants, while larvae might hardly choose the beneficial plants as limited by their immobility. Secondly, I hypothesize that *C. glomerata* will prefer plants infested by both aphid and host over plants infested by host solely, through different volatiles produced by these two types of infested plants, since this facilitates its performance as well. To test our hypothesis, we asked the following specific questions:

- (1) Do *P. brassicae* butterflies prefer to oviposit on aphid-infested plants over undamaged plants?
- (2) Do *P. brassicae* larvae prefer aphid-infested plants over undamaged plants as food source?
- (3) Does the behavior of the individual larvae differ from the behavior of the larval groups?
- (4) Does exogenous salicylic acid (SA) application have the similar effect on preference of *P. brassicae* larvae as aphid infestation?
- (5) Do *C. glomerata* females discriminate between plants infested by hosts and plants infested by both hosts and aphids?
- (6) Could herbivore-induced plant volatiles play a role in the interactions?

2 Material and methods

2.1 Plants and insects

Brassica oleracea is a species from the family *Brassicaceae*, distributed natively in coastal southern and western Europe. *Brassica oleracea* has been widely used as material in studies of tritrophic interactions particularly in our lab, so it is an excellent model system to further study plant-insect interactions. *Brassica oleracea* plants were grown in a greenhouse compartment for 4 weeks after transplanting ($22\pm 2^{\circ}\text{C}$, 50-70% RH, L16:D8). All the plants used for the experiment were 5-6 weeks old, at the vegetative stage.

Several species of herbivores coexist on this plant species with different feeding behavior. *Pieris brassicae* (Lepidoptera: Pieridae), a specialist leaf chewer, and *Brevicoryne brassicae* (Hemiptera: Aphididae), a specialist phloem feeder, are common herbivores can be found in nature feeding on *Brassica oleracea*. *P. brassicae* feed on several species of wild and cultivated plants in the Capparales. Female butterflies lay clutches of 7-100 eggs on the backside of leaves. Eggs attached on the leaf surfaces need 5-7 days to hatch, after that larvae feed on plant foliar aggregately until the third instar, and then they disperse on the host plant. Aphid *B. brassicae* feed on cruciferous plants and cause predominant damage to plants due to the accelerated reproduction rate. Aphids have the special way of reproduction named parthenogenetic reproduction, females give birth to female nymphs in similar appearance as adults without mating. But in autumn, females respond upon decreases in photoperiod and temperature, males are reproduced and mating occurs. Aphids accumulate glucosinolates from plants as defenses against their predators. In this study, both *P. brassicae* and *B. brassicae* were involved in the experiments. Larvae of *Pieris brassicae* and aphids *Brevicoryne brassicae* were reared on *Brassica oleracea* plants in a climate room at $20-22^{\circ}\text{C}$, 50-70% R.H. and a L16:D8 photoregime in Laboratory of Entomology (Wageningen University, the Netherlands).

The genus *Cotesia* (Hymenoptera: Braconidae) is one of the largest genera of parasitoid wasps, and has been used in biological control to fight against phytophagous insects, as well as model insects in entomological studies. *Cotesia glomerata* is a gregarious endoparasitoids that commonly parasitize larvae of a few genera of the Pieridae in first to third instar, and prefers *P. brassicae* as host plants in Europe. Larvae of *Cotesia glomerata* feed on larval host inside, but allow them to grow and feed until their final instar (L5). Pupae of *Cotesia glomerata* develop outside the body of larval hosts under the protection of larval hosts. Adults can be mated as soon as emerged, and fertilized females start search for hosts. Females lay approximately 20-30 eggs per larval host and can reproduce 1000 to 2000 eggs in total.

Larvae of *Pieris brassicae* and aphids *Brevicoryne brassicae* were reared on *Brassica oleracea* plants in a climate room at $20-22^{\circ}\text{C}$, 50-70% R.H. and a L16:D8 photoregime in Laboratory of Entomology (Wageningen University, the Netherlands). *Cotesia glomerata* was reared in *Pieris brassicae* feeding on *Brassica oleracea* plants. Cocoon of *Cotesia* were obtained from the insects culture maintained at Laboratory of Entomology (Wageningen University, the Netherlands), and then kept in the cages (40cm×30cm×30cm) placed in climate cabinet at 22°C . Adults of parasitoids *Cotesia glomerata* emerged from cocoons 3-4 days later, and they were maintained in the same cage by supplying them with honey drops on the cage ceiling and water.

2.2 Treatments and bioassays

2.2.1 Pre treatments

In the two-choice experiments designed in this study, I provided the aphid-infested plants *B. oleracea* (A) in pair-wise comparison with control plants (C) and observed preference behavior of targeted insects. For tests of behavior of the *P. brassicae*, 4-6 adult aphids were allowed to colonize or reproduce on the plants for 5-6 days, and then there was a population colony of 60-80 aphids on each aphid-inoculated plant afterwards. For tests of behavior of the parasitoid *C. glomerata*, plants were inoculated with aphid *B. brassicae* nymphs N1 or adults in different densities for 5 days (described below). The aphid density and pre-infestation period selected was based on the previous study that framed my thesis project (Soler et al. 2011). In order to re-evaluate this conclusion, we tested the oviposition preference of *P. brassicae* butterflies with aphid-infested plants in increased density of aphid inoculation. Aphids were only placed on the first fully expanded leaf of plant in treatment of aphid-inoculation. The leaf given for the choice of *P. brassicae* larva was the one above the aphid-inoculated leaf, as the effect of aphid infestation is systemic (A leaf). Leaf of the same order as A leaf in undamaged plant was used in pair-wise comparison for larva (C leaf). The undamaged plants without any herbivory were explored as control plants in the experiments.

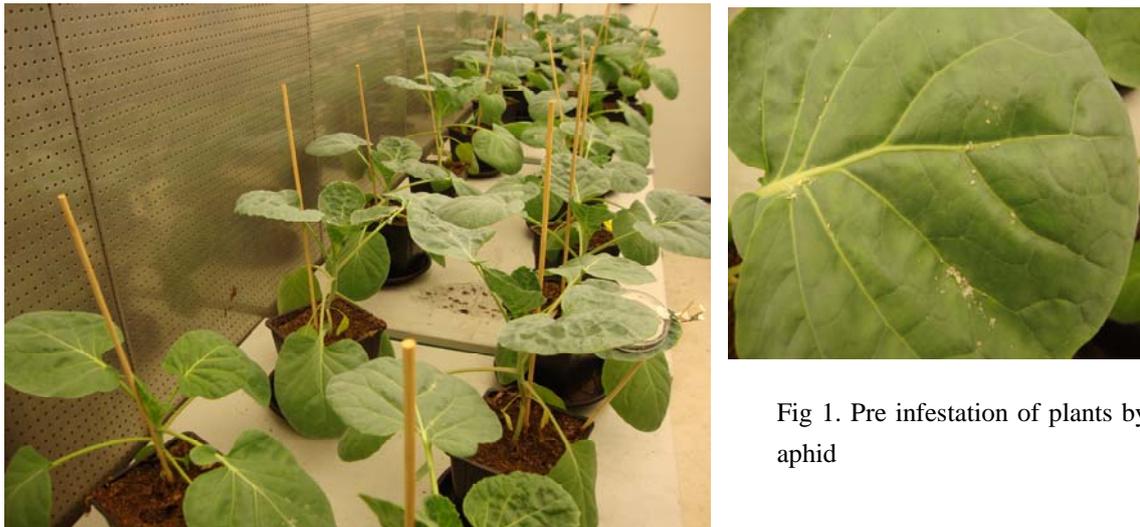


Fig 1. Pre infestation of plants by aphid

2.2.2 Oviposition preference of *P. brassicae* females

To study whether aphid colonization influences the oviposition preference of *P. brassicae* butterflies, two-choice experiments were performed. Experiments were conducted in a large netted and transparent tent (3m×2m×3m) containing aphid-infested plants and undamaged plants. Seven aphid-infested plants and seven undamaged plants were placed alternated in the tents, and plants were approximately 50 cm apart with each other. One or two days old virgin butterflies were used in the experiments, as older gravid butterflies with large egg load were likely to be less picky about the host plants they choose. Five *P. brassicae* females and 7-10 males (2 days old) were then released in each test in the tent, and allowed to forage freely over the 14 plants for 3-4 days, until half of the plants were selected for oviposition. These

males were added to insure mating in the tent during the experiments. Butterflies in the cage were supplied with cotton balls filled with 20% honey solution and water continuously during the experiments. The experimental plants were checked for eggs of *P. brassicae* every 2 hours in the day time. Host preference of butterflies was measured by noting the plant chosen for the oviposition. Additional indicators that were measured were the total number of clutches per plant, the number of eggs per clutch as well as the distribution of the clutches on the control and treated plants over the duration of the experiment. Experiments were terminated when 8 plants were observed having egg clutches. The experiments were repeated five times, thus responses of 25 *P. brassicae* females were observed.

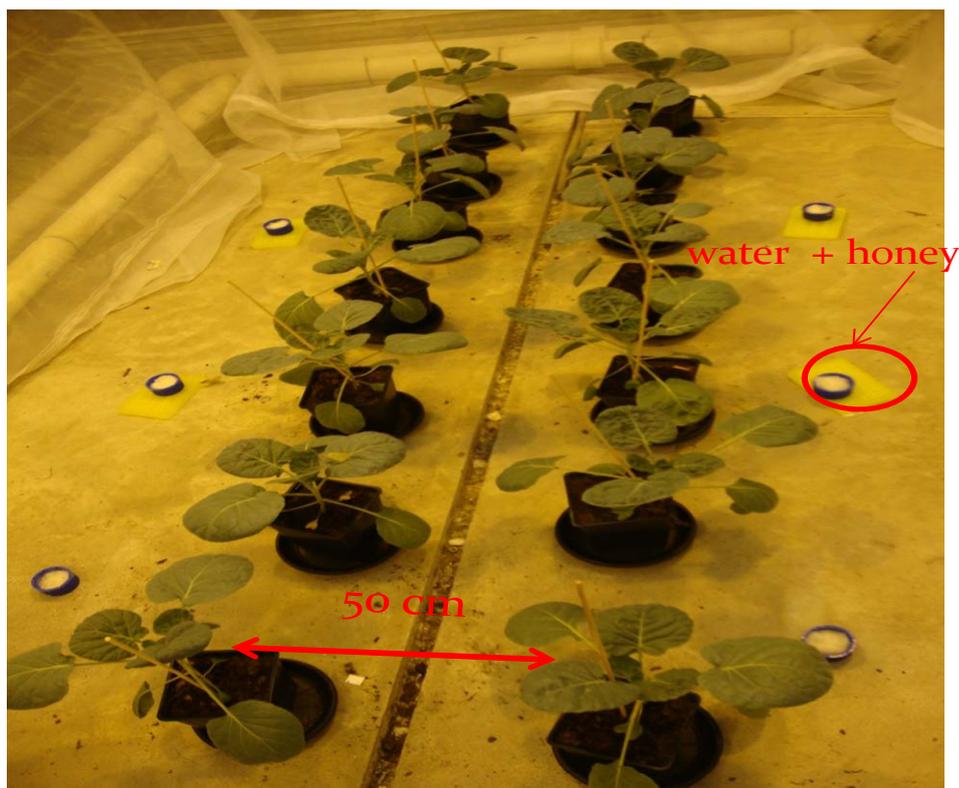


Fig 2. Oviposition preference tests of *P. brassicae* females between aphid-infested plants and undamaged plants.

2.2.3 Feeding preference of *P. brassicae* larvae

To evaluate preference of *P. brassicae* larvae between aphid-infested plants and undamaged plants, we examined the choice of the caterpillars individually, in sub-groups (10 individuals taken from one clutch) and in entire (complete) clutches. Noteworthy, behavior tests of clutched larvae were divided into two parts, larval clutches tested with 6 days inoculated plants and 7 days inoculated plants. To examine if salicylic acid mimics has the similar effect on preference behavior of individual larvae as aphid-inoculation, we exogenously applied SA (1 Mm + 0.1 % Tween 20) solution fully on the first expanded leaves until run-off. In order to do this, plants were covered by plastic paper, only allowing first expanded leaves exposed. Separated experiments were proceeded with SA-treated plants in the same way as using aphid-inoculated plants.

Entire plants were provided for behavior tests of individual larvae, whereas leaves were

excised for behavior test of grouped larvae. Leaves were cut at the base of stem just prior to experiments. In order to keep leaves further, leaves were placed in glass tubes with water, and concealed with cotton. ‘Bridges’ were built up in between A leaf and C leaf. These ‘bridges’ were made from hard paper cut in to squares (1.5 cm×1.5 cm), and they made both A leaves and C leaves equally accessible for larvae. Egg clutches of *P. brassicae* were removed from rearing plants and kept in separated petri dishes in room temperature before the experiments. These larvae were hatched in the petri dishes without any contact with plants in order to avoid previous learning, as we tested the innate response of caterpillars. Newly hatched larva of *P. brassicae* from each clutch, either in terms of individual, sub-group, or full clutch were placed on the “bridge” in between of two leaves (A leaf VS C leaf). Afterwards, we observe the behavior of neonate caterpillars and their choices between these two leaves, and recorded the time they needed to reach either one of the leaves as well.



Fig 3. Preference tests of individual *P. brassicae* larvae (L1) between aphid-infested plants and undamaged plants (Left). Preference tests of grouped *P. brassicae* larvae (L1) (sub-groups or full clutches) between leaves excised from aphid-infested plants and undamaged plants (Right).

2.2.4 Host preference of the parasitoids *C. glomerata*

To examine preference of *C. glomerata* between plants infested by both aphid and hosts and plants singly infested by hosts, two-choice tests were conducted in 4 sets of comparisons with various densities of aphid inoculations:

- 1) Plants infested by 10 caterpillars (L1) and 15 aphid nymphs (N1) VS Plants infested by 10 caterpillars (L1)
- 2) Plants infested by 10 caterpillars (L1) and 30 aphid nymphs (N1) VS Plants infested by 10 caterpillars (L1)
- 3) Plants infested by 5 caterpillars (L1) and 30 aphid nymphs (N1) VS Plants infested by 5

caterpillars (L1)

- 4) Plants infested by 10 caterpillars (L1) and 6 adult aphids VS Plants infested by 10 caterpillars (L1)

After aphids in various densities were inoculated on plants, *P. brassicae* hosts (5 or 10 larvae) were placed on the first leaf above the aphid-inoculated leave 24 h prior to test, and the same caterpillar inoculation was applied to undamaged plants. Therefore, we manipulated pair-wise plants for preference tests of the parasitoids *C. glomerata*, plant with both aphids infestation and hosts feeding (A plants) VS plants with only hosts feeding (C plants). Experiments were conducted with a pair of plants daily, and repeated over 5 days. We carried out the tests by using a flight cage in behavior room (with temperature of 20-21°C). Flight cage was in size of 0.95×0.9×0.7 m, covered with nylon net. Two ventilators detached from computer with wind speed of 0.2 m/s were places at the backside of each plant to help parasitoids tracking plant volatiles. Some replicates of experiments were carried out in Wind Tunnel with similar results.

Cotesia glomerata females (4 to 7 days old) were separated and released in group of 3 individuals from a centre point in the flight cage with a distance of about 60 cm from the plants. Complete oriented flights that ended in landings on one of the two treated plants were recorded as 'choice'. Landing somewhere else in the set up, did not approach plants or wasps that did not take off within 40 minutes were recorded under 'no response', and excluded from statistics. Plants in the flight cage were 60 cm apart from each other, and plant positions were switched after every 3 groups of females have been tested to minimize biases. The test for each pair of plants was ended up with at least 20 parasitoids made their choice and showed first landings on the plants. For the first three sets of treatments, around 100 responded wasps were recorded on testing five pairs of plants in total. However, due to the shortage of wasps when doing the last set of experiments, we terminated each single test on one pair of plants with 10-15 responded wasps.

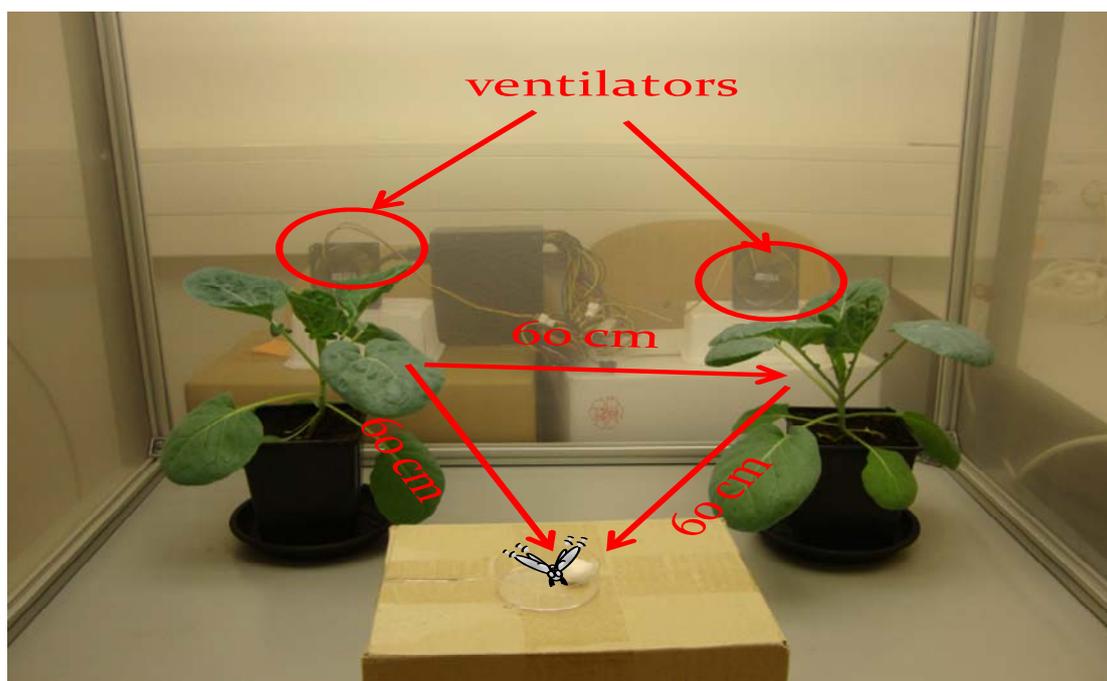


Fig 4. Preference tests of parasitoids *Cotesia glomerata* between plants infested by both aphids *B. brassicae* and hosts *P. brassicae* and plants infested by hosts alone.

2.2.5 Plant volatiles

To assess whether plants infested by both aphid and hosts emit distinct volatile blends from plants infested singly by either aphids or caterpillars, we collect plant volatiles simultaneously to preference tests of parasitoids. Volatiles were collected from plants exposed to 4 aphid herbivory (2AA), 6 aphid herbivory (3 AA), 10 larval herbivory (CCP), a combination of 6 aphid and 10 larval herbivory (AC) or no herbivory (CP). Aphid-infested plants were inoculated 5 days prior to the collection of volatiles. Plants with caterpillar herbivory were inoculated 24 h prior to volatile collection. There were 5 replicates of plants for each experimental treatment, as well as 10 replicates of undamaged plants (no herbivory).

Dynamic headspace sampling was carried out in a climate room (20-22°C, 50-70% r.h.). Prior to sampling, the pots were removed, roots and soil were carefully wrapped with aluminum foil and the plant was placed into a 25 liter glass jar. The jar was sealed with a viton-lined glass lid with an inlet and outlet. Compressed air was filtered by passing through charcoal and Tenax TA, respectively, before reaching the glass jar with plant. Volatiles were trapped by sucking air out of the glass jar at a rate of 200 ml min⁻¹ through a stainless steel tube filled with 200 mg Tenax TA (Markes, Llantrisant, UK) for 4 hours.

Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, USA) coupled with Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, USA) quadrupole mass spectrometer (MS) were used for separation and detection of plant volatiles. Identification of compounds was based on comparison of mass spectra with those in the NIST 05, Wiley and Wageningen Mass Spectral Database of Natural Products MS libraries. Experimentally calculated linear retention indices (LRI) were also used as additional criterion for confirming the identity of compounds. Relative quantitation (peak areas of individual compounds) was performed using a single (target) ion, in selected ion monitoring (SIM) mode. These individual peak areas of each compound were further used for characterization of the different plant using statistical approach. Differences and similarities in the volatile blends between treatments were analyzed by principle component analysis (PCA).

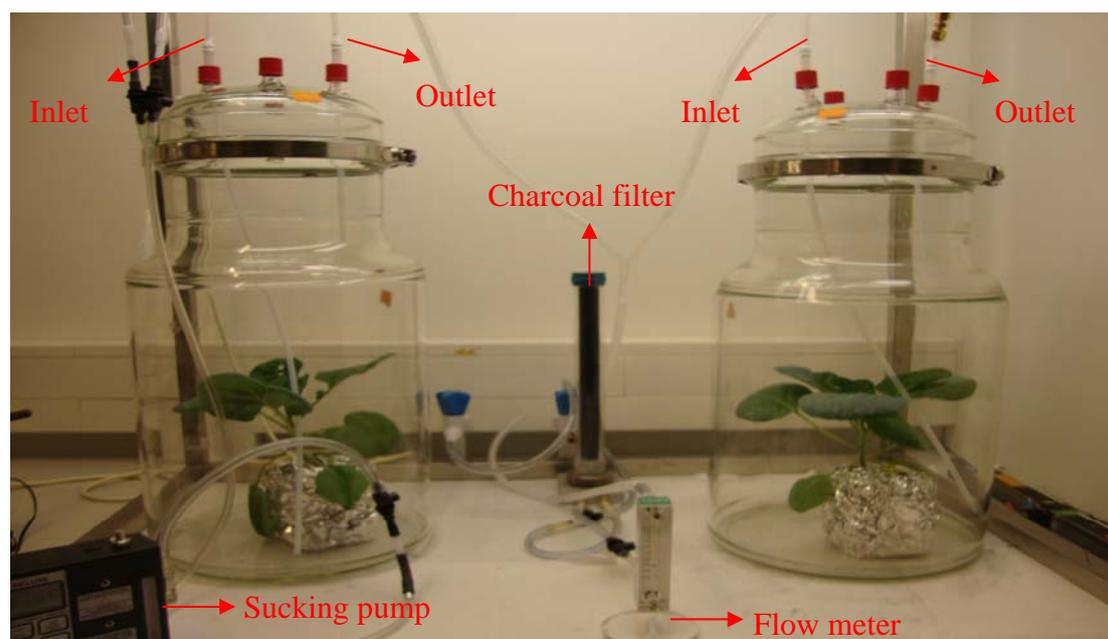


Fig 5. Set up of plant volatiles collection.

2.3 Statistical analysis

For all the preference experiments, a binomial test was used to determine whether plant preferences of the insects differed significantly from a non-preference situation ($p < 0.5$, two-tailed, $\alpha = 0.05$). For the variables number of egg clutches per plant and plants selected by the females a Generalized linear model with Poisson distribution was fitted with the logarithm as link function. For the variable proportion of plants selected for oviposition by the females a Generalized linear model with binomial distribution with the logit link function was used. The variables decision times and number of eggs per clutch was tested using one-way ANOVA. Normality and homogeneity of variance were checked by inspection of the residuals after model fitting.

3 Results

3.1 Oviposition preference of *P. brassicae* females

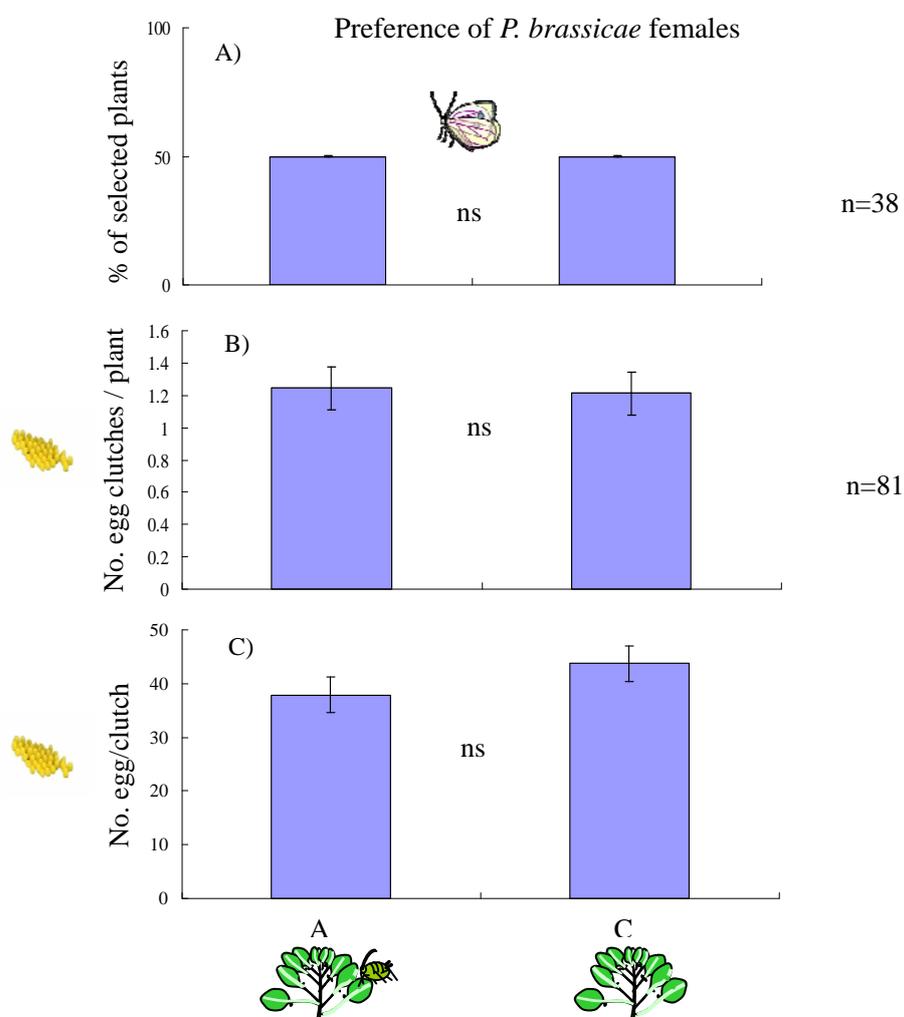


Figure 6. Back transformed means (\pm SE) of (A) the proportion of plants selected for oviposition by *Pieris brassicae* females (total $n=25$ females), (B) number of egg clutches, and (C) number of eggs per clutch, on aphid-infested plants (A plants) and on plants without aphids (C plants). of egg clutches, nor sizes of egg clutches (ns).

Pieris brassicae selected a similar proportion of plants with and without aphidson which to oviposit (Fig 6 A). Moreover, there was no significant difference between numbers of eggs clutches laid by *P. brassicae* on aphid-infested plants and undamaged plants. As indicated in Fig 6 B, we found 1.24 clutches on aphid-infested plants and 1.21 clutches on undamaged plants ($P = 0.873$). *Pieris brassicae* oviposited slightly smaller egg clutches on aphid-infested plants compared to undamaged plants, but this was not significant ($P = 0.212$, Fig 6 C).

3.2 Feeding preference of *P. brassicae* larvae

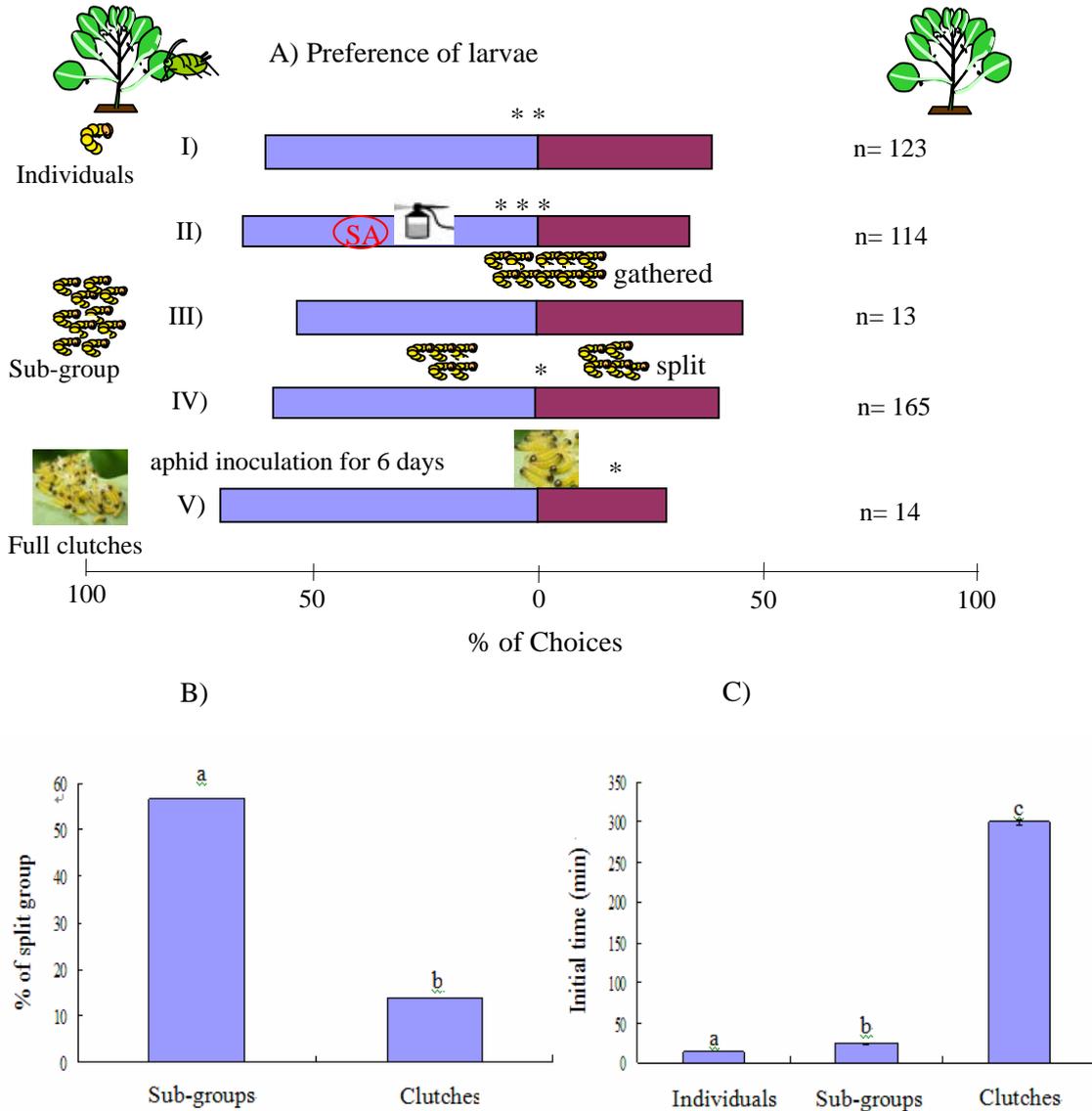


Figure 7. (A) Responses of *P. brassicae* larvae in individual (I, II); in sub-group(III, IV); in clutch(V) to aphid-infested plants tested against undamaged plants (control). Asterisks indicate a significant difference within a choice test: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. (B) Mean (\pm SE) percentage of larvae groups (*P. brassicae*) either in sub-groups or in full clutches that split when tested. Different letters indicate significant differences ($P < 0.05$). (C) Mean (\pm SE) initial time taken by larvae in individuals (*P. brassicae*) and first larva in group responded to aphid-infested plants or undamaged plants in preferences tests. Significant differences ($P < 0.05$) between individuals, sub-groups and clutches are indicated with different letters.

Overall, *Pieris brassicae* larvae showed a preference to *B. oleracea* plants attacked by aphids in terms of individuals behavior, sub-groups behavior and clutches behavior (Fig 7 A I; II; III; IV; V). Of the *P. brassicae* individuals larvae tested, 61 % individuals chose aphid-infested plants ($P = 0.01$), and similarly 66% individuals chose SA applied plants over uninfested plants (Fig 7 A II) ($P < 0.001$). In tests of larvae in sub-group, it showed evenly distributed choices of gathered larvae between aphid-infested plants and undamaged plants (Fig 7 A III) ($P = 1.0$), however, when they split, most of larvae (59%) selected aphid-infested plants as well (Fig 7 A IV) ($P = 0.029$). When entire clutches were confronted to choose, they significantly preferring aphid infested plants (Fig 7 A V), and they mostly did not split but behaved as a unit.

Fig 7 B showed a clear difference in percentage of split groups between sub-groups and clutches, with 57 % of sub-groups ($P = 0.585$) and only 14 % of clutches ($P \leq 0.001$) split respectively when they responded to two types of plants in the dual-choice experiments. On average, individual larvae took 13.6 minutes to respond upon food plants, first larvae in sub-groups took 24.1 minutes, and it cost initial larvae in clutches 300 minutes to respond. These time differences among individuals, sub-groups, clutches were statistically significant ($P \leq 0.001$) (Fig 7 B).

In contrast to tests with 6 days inoculated plants, 7 days inoculated plants evoke a reversed preference of larvae clutches. As 88 % gathered clutches responded to undamaged plants when tested with 7 days inoculated plants, it is conversed to results from tests with 6 days inoculated plants ($P = 0.002$) (Fig 8 A).

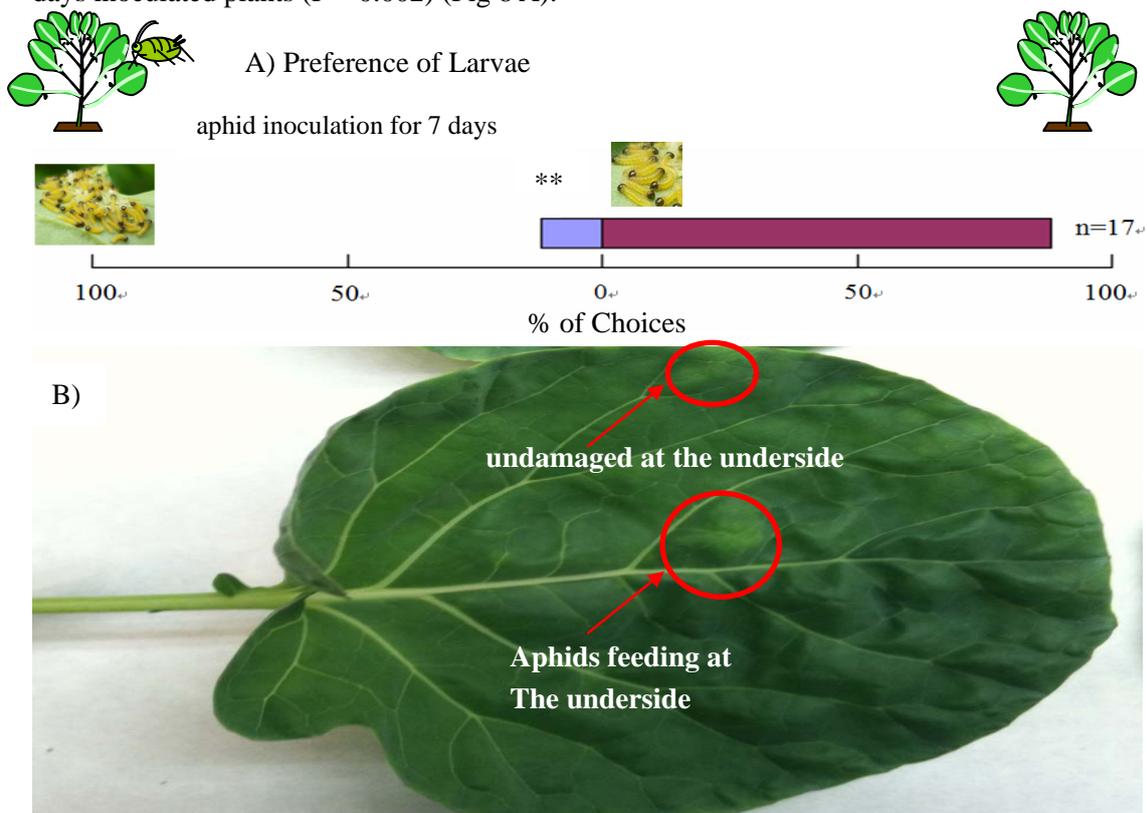


Figure 8. (A) Feeding preference of larval clutches (*P. brassicae*) between aphid-infested plants and undamaged plants (control). Aphid-infested plants were inoculated by 4-6 adult aphids for 7 days. Asterisks indicate a significant difference within a choice test: ** $P < 0.01$ (B) Plants infested by 4-6 adult aphids for 7 days showed over defense or nutritional decline (yellow spots).

3.3 Preference of the parasitoids *C. glomerata*

Cotesia glomerata did not distinguish hosts *P.brassicae* feeding on aphid-infested plants from hosts feeding on undamaged plants without herbivory. There was no apparent observation that aphid-inoculation regardless densities affect preference of parasitoids (Fig 9 a, $P = 0.137$; Fig 9 b, $P = 0.368$; Fig 9 c, $P = 0.488$; Fig 9 d, $P = 0.104$). Parasitoids showed evenly choices frequency to plants with solely *P. brassicae* larval herbivory and plants with both larval and aphid herbivory, with approximately fifty percent of females alight on each plant choice. Overall in the first three series of experiments with aphid nymphs induction, 82% of the released parasitoids alighted on plants, independent of choices, which confirming the flight cage set up was appropriate to examine preference of parasitoids. Probably as a consequence of problems in the rearing, in the last series of tests with adult aphid inoculation (6 adults + 10 hosts), a smaller proportion of parasitoids responded to plants (44%), whereas sample size of this series of tests was diminished compared to first three series ($n = 46$).

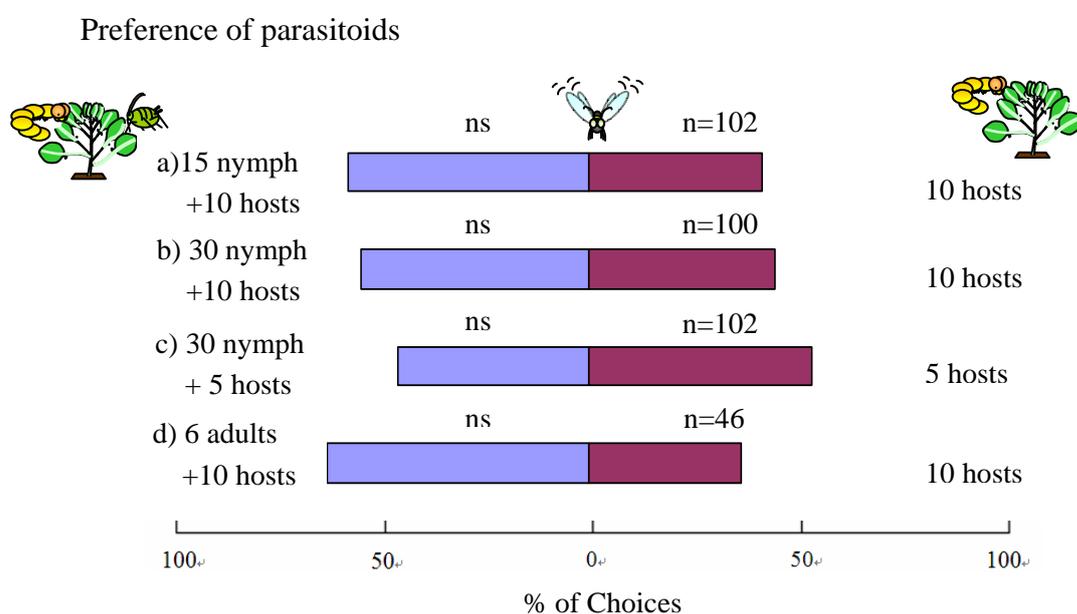


Figure 9. Percentage of choices of *C. glomerata* females, in the two-choice experiment, between plants *Brassica oleracea* attacked by both hosts *P. brassicae* [(10 L1); (5 L1)] and aphids *B. brassicae* [(by 15 N1); (by 30N1); (by 30N1); (by 6 adults)], and plants attacked by hosts alone. Aphid-infested plants were created by inoculation of *B. brassicae* N1 nymphs or adults.

3.4 Plant volatiles

We examined volatile blends of plants exposed to aphid herbivory with density of 4 adults or 6 adults *B. brassicae* (2AA, 3AA), or caterpillar herbivory with density of 10 caterpillars *P. brassicae* (CCP), both types of herbivory (AC), or no herbivory (control). The results of principle components analysis showed a separation of treatments between aphid-infested plants (AA), caterpillar-infested plants (CCP) and plants infested by both types of herbivory (AC) (Fig 10). However, aphid-infested plants (2AA; 3AA) overlapped with undamaged plants (CP) without herbivory, and the similarity in volatile blends between these two groups were convinced by the minuteness distance between them, compared to larger distance recorded between caterpillar-infested plants and undamaged plants (Fig 10).

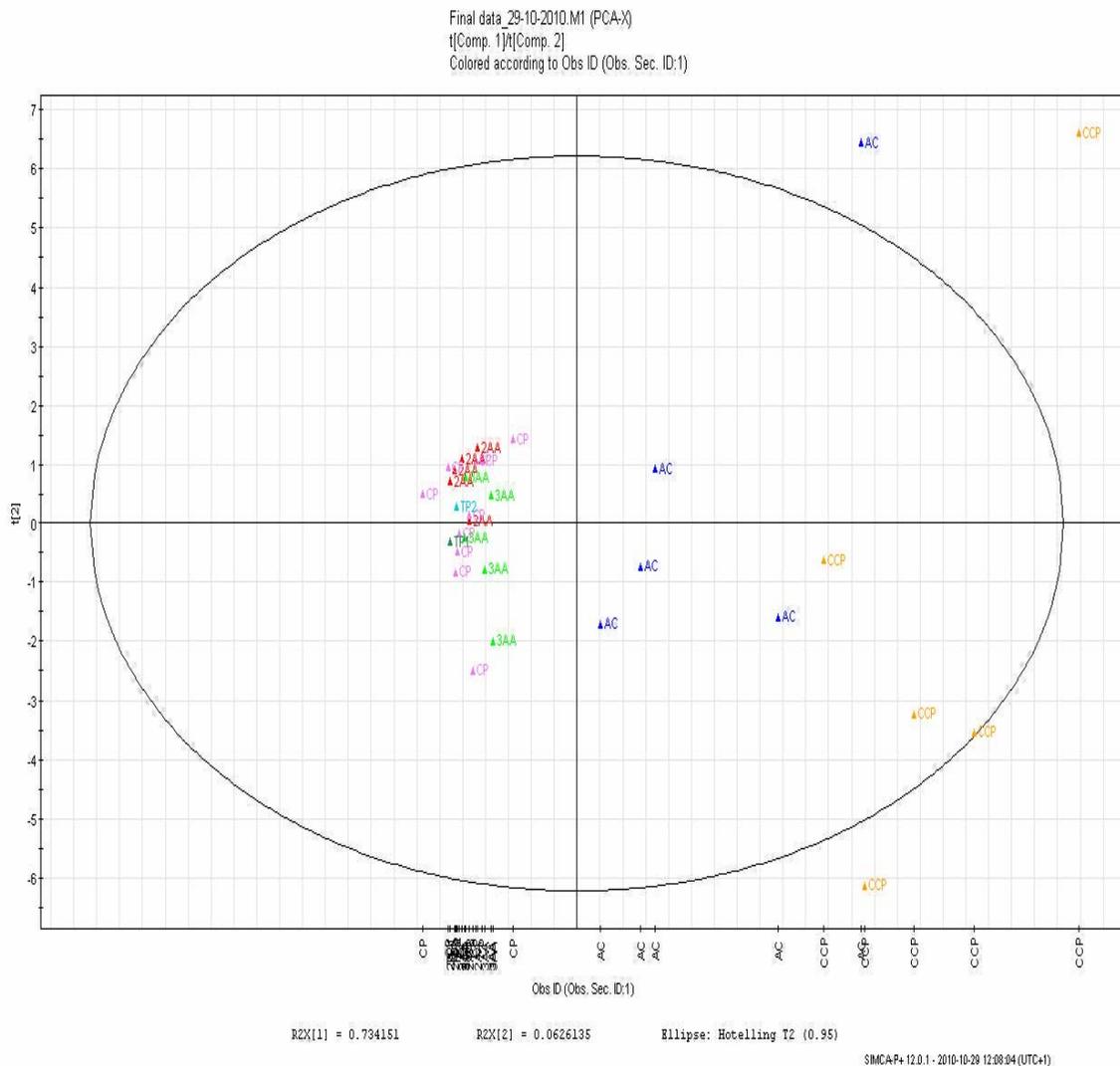


Figure 9. Principle components analysis (PCA) plots of volatile blends of plants exposed to aphid herbivory of 4 adults *B. brassicae* (red letters, 2AA) or 6 adult aphids (green letters, 3AA), 10 *P. brassicae* larval herbivory (yellow letters, CCP), both types of aphid herbivory of 6 adult and 10 *P. brassicae* larval herbivory (blue letters, AC) or no herbivory (control, purple letters, CP).

4 Discussion

In this study, I found that the presence of aphids influenced, in different ways, the feeding and oviposition preferences of *P. brassicae* butterflies, larvae and their parasitoid *C. glomerata*. Overall, *Pieris brassicae* butterflies did not discriminate between aphid-infested plants and undamaged plants, as we observed the evenly distributed egg clutches on both aphid-inoculated plants and undamaged plants, in similar amount of egg clutches and clutch size. *Pieris brassicae* larvae showed a preference to aphid-infested plants over undamaged plants overall, either in terms of individuals, sub-groups, or clutches. Results from this study revealed parasitoids *C. glomerata* did not discriminate between aphid-infested plants or

aphid-free plants, in presence of hosts *P. brassicae* on both types. This indicated that the benefits for the caterpillars provided by the aphids persisted even considered the influence of the parasitoids, since they did not suffer from greater pressure from the parasitoids.

Soler et al. (2011) showed that *P. brassicae* larvae developed better on aphid-infested plants. However, results in this study did not support the classical theory that ‘mother knows the best’, in the context of mothers are more talented in selecting suitable host plants which positively affect survival of their offspring, as they have better mobility and advanced chemosensory systems (Wennstrom et al., 2010). Nevertheless, oviposition preference and performance of offspring are not always well correlated. Observations of poor relationships between oviposition preference and performance of offspring could be explained by four hypotheses: time, patch dynamics, parasite versus grazer lifestyle, and the enemy-free space (Thompson 1988). Thompson (1988) revealed that when novel plant species were added to a community, insects lack the time to modify preference to performance, besides they may follow the geographic variation in relative abundance of host plants. Furthermore, the selection of host plants also depends on different life style (parasite or grazer) and the influence of natural enemies. I assumed that conditions in the microhabitat which mothers oviposited altered after larvae hatching. Therefore, it is overcritical for mothers to estimate host plants with high quality and low risks of recruiting parasitoids in the long term rather than the moment they oviposited. There are possibilities that mothers select sub-optimal host plants for their offspring. Rodriguez-Saona et al. (2005) found that adults *S. exigua* did not reject to oviposit on plants with dual herbivory when they were given the chance to choose, despite these plants were poor sources for their offspring compared to undamaged plants.

Pieris brassicae females were showed to be very selective on host plants for oviposition. *Pieris* prefer plants with no eggs or conspecifics, as avoiding inter-specific competition and attraction of natural enemies (Rothschild & Schoonhoven, 1977). *Pieris brassicae* avoids plants infested by root herbivores, which negatively impact larval performance (Soler et al., 2010). Therefore, I expected that *P. brassicae* would prefer aphid-infested plants which are beneficial for the performance of their off-springs. As discussed above, that was not the case and young butterflies equally accepted plant with and without aphids for oviposition. I hypothesized the great similarity between plant volatile blends emitted by aphid-infested plants and undamaged plants lead to the unselective oviposition of *P. brassicae* on both types of plants in this study. Although butterflies may use a combination of visual cues and olfactory cues to locate host plants, plant volatiles were showed to mediate host plants orientations of gravid females in Lepidoptera and their avoidance of unsuitable plants (Hern et al., 1996). Hern et al. (1996) reviewed that *P. rapae* was able to perceive general plant odours and isothiocyanates, indicated by electrophysiological studies. Since there was nearly no difference between volatile blends emitted by aphid-infested plants and undamaged plants, *Pieris brassicae* were unable to distinguish these two types of plants through olfactory cues.

Study provided by Bruessow et al. (2010) demonstrated that *P. brassicae* oviposition on *A. thaliana* triggered SA accumulation locally at the site of oviposition and the application of egg extracts also showed the suppression of plant defense against herbivores. Therefore, they

concluded that insects have evolved a way to suppress the induction of defensive genes by elicitors from eggs through SA accumulation which negatively interfere JA pathway. This might be an alternative logical explanation to our observations that *P. brassicae* butterflies did not choose aphid-infested plants. As eggs are the very first stage of offspring and are relatively susceptible, butterflies may adapt to protect their eggs by deploying eggs elicitors. Since then, helps from aphids seem to be redundant to butterflies, as they manage SA accumulation by the eggs elicit as well. However, Bruessow et al. (2010) also demonstrated that plants treated with eggs extract enhanced performance of generalist *S. littoralis* larvae but not larvae of specialist *P. brassicae*.

A Study by Chew (1980) demonstrated plasticity in food plants preferences of *Pieris* caterpillars permit them to resolve the inappropriate choices made by their mothers by accepting alternative food plants that are available at that time. Shikano et al. (2010) confirmed that neonate larvae were better able than adults to rank plants according to larval performance, and that larval diet breadth was wider than the range of plants accepted by adults. So far it was showed that caterpillars discriminate between plant species. However, it has not been pointed out that caterpillars are able to discriminate between differences within plant species under the same nutritional regime, but printed by the infestation of a second herbivore species, even at densities exerting negligible physical damage. In this study I found that *P. brassicae* larvae did distinguish *B. oleracea* plants infested by aphid and undamaged *B. oleracea* plants, and preferred aphid-infested plants. Conversely, *Pieris brassicae* butterflies did not show a preference for aphid-infested plants where their off-spring perform better. According to these observations, we hypothesized that butterflies limited themselves in selecting proper host habitat, but they were not necessarily go further on selecting better host plants, because larvae were capable to relocate to more beneficial plants in the micro habitat with attached neighboring plants, by their locomotory prowess within a certain limit. The original choices made by their mothers were not optimal any longer since habitat patches changed from oviposition to larvae hatched, as well as the coming pressure from natural enemies. Therefore, it makes sense from the ecological meaning that larvae have to make their own decisions instead of relying on what their mothers chose for them, which is determined by dynamic micro habitats.

Individual larvae presented a similar preference to plants treated with salicylic acids over undamaged plants. It is clearly demonstrated that exogenous application of SA can inhibit plant resistances induced by JA (Thaler et al., 2002). This suggested that mechanism underlying larval preference is antagonism of SA-JA pathways. In a number of model systems, aphid infestation has been shown to suppress JA, which predominantly activated as resistance against leaf chewers (Thaler et al., 2002; Soler et al., 2011). It is well studied that there is a negative cross talk between SA signaling pathway induced by phloem feeders and JA signaling pathway induced by leaf chewers (Maleck & Dietrich, 1999; Thaler et al., 2002; Koornneef et al., 2008). However, plants are living in hostile circumstances and often invaded by more than one organism, if there is only antagonism between signaling pathways, it is difficult for them to cope with the various species of herbivores. In fact, signal pathways also worked synergistically or compensatory with each other, and may minimize expression of

ineffective and costly defenses. Therefore, plants integrated the suite of signals and deployed interacted signal transduction pathways to provide the best defense against intruders (Walling 2008). Recent molecular analysis by Rodriguez-Saona (2010) indicated that no gene was inducible by a single transcription factors or signaling pathway. Furthermore, it is revealed there are more other signal-transduction pathways involved in plant volatiles emission besides JA (Van Poecke et al. 2003).

In preference tests of clutched larvae with plants infested by aphid for 7 days, 88.2 % of the larvae chose undamaged plants, reversed to the preferences of clutched larvae tested with plants inoculated for 6 days. The preferences toward undamaged plants could result from reduction of nutritional quality of plants colonized by aphids. This argument is supported by the facts that chlorosis was found in most of the leaves from plants colonized by aphids for 7 days. Denno et al. (1995) suggested positive interactions between herbivores are transient, and at certain thresholds give way to competitive relationships. In this study, the over inoculated plants with longer period resulted in the ‘breaking point’ of relationship between aphids and caterpillars. We expected the breaking point turned facilitation between aphids and caterpillars into competition as plants nutrition declined dramatically and defenses augmented. Nutritional content should be included in explanation of caterpillar behavior with balances of plant defense.

It is still not clear which cues *P. brassicae* larvae track in selecting host plants. Although volatiles are major and reliable indicators of host plant quality (Dicke 1999; Herrmann 2010), it is likely that larvae explore other cues, besides using olfactory cues exclusively. As in this study, we did not find significant differences between volatile blend emitted by aphid-infested plants and undamaged plants. Insects are capable to perceive various integrated cues such as visual cues, mechano-sensory cues, and gustatory cues in different phases of host selection behavior (Schoonhoven et al., 2005). In the contact evaluation phase, insects do the test bites, antennation, tarsal drumming, oviposition probing *etc*, in order to assess plants texture and quality before accepting it as a host plant (Schoonhoven et al., 2005). Since larvae were not observed to bite the plant before making decision, I assume they used mechano-sensory, olfactory cues or in combination of these two in evaluating aphid-infested plants with comparison of undamaged plants, although it is not investigated specifically yet. Moreover, it is demonstrated that specialists rely more on a few components in volatile blends, whereas generalists are attracted by basic volatile signaling or general compounds in volatile bouquet (Herrmann 2010). Since only general volatile blends were analyzed in this study, I can not exclude the possibility that specialists *P. brassicae* tracked the differences between particular compounds in volatiles emitted by aphid-infested plants and undamaged plants.

During the experiments carrying out, it was often observed one ‘leader’ moved towards either one of the leaves, subsequently the rest of group members follow the movement of the ‘leader’. Studies from Roessingh (1989; 1990) about the trail following behavior of caterpillars *Yponomeuta cagnagellus* suggested that caterpillars responded to tactile cues provided by persisted chemical makers in silk secretion from con-species. Intra-specific

communications were concluded to be either co-operative or competitive (West-Eberhard 1984). *Pieris brassicae* are gregarious larvae and they explore food plants aggregately as a group, so they are expected to follow the trails made by con-specific larvae. However, *P. brassicae* are only gregarious until 4th instar, they might not keep staying aggregately or exhibiting the trail following. Intra-specific communications among *P. brassicae* possibly play a less important role in coordinating the activities in the performance of a task. As indicated in the results, individual larvae and full clutches (6A 6 days) significantly preferred aphid-infested plants or SA-induced plants over undamaged plants. However, sub-groups with 10 individuals extracted from one clutch, did not show the preferences, although we found a larger proportion of larvae in split groups chose aphid-infested plants. More interesting, larvae in clutch spend much more time in selecting host plants than individual larvae, whereas larvae in individual and sub-group took less time. Grouped larvae were less efficient in selecting host plants might be attributed to two stimuli guiding the behavior of caterpillars, to remain gregarious or to move as a pioneer towards to the plants, which made them confusing. Furthermore, larvae in sub-groups presented higher rate of split than larvae in clutches. Thus, I hypothesized that intra-specific communications were partly lost in sub-groups, might due to the exclusion of inborn 'leader'.

Females of *C. glomerata* showed approximately equal preferences to hosts feeding on aphids previously attacked plants and hosts feeding on undamaged plants, which in contrast of my hypothesis. I hypothesized that *C. glomerata* would prefer hosts co-existed with aphids, since they attained a bigger size when parasitized caterpillars feeding on plants previously infested by aphids (Soler et al., 2011). Furthermore, body size is a key parameter of selection in parasitoids, as it is correlated with fitness, for instance, it affects the searching and mating efficiency, longevity, and fecundity (Harvey 2005). Although I tested lower and higher infestation densities, but none of them influenced the preference of the parasitoid. There are various theories to explain observations in our study that parasitoids did not discriminate between plants infested by hosts alone and plants exposed to double herbivory from both aphid and host. Forage efficiency of parasitoids are effected by the interactions of genetic variation, phenotypic plasticity, physiological state, previous learning experience, as well as external factors, e.g. climate conditions, habitat types or hosts density (De Moraes et al., 2000; Geervliet et al. 1996; Geervliet et al. 1998; Girling et al. 2006; Gols et al., 2008). The climate condition when we carried out the experiments was not ideal and wasps we used were under sub-optimal fitness. Thus, we can hardly estimate the influence from these aspects.

Turlings et al. (1995) concluded that specificity is limited when different herbivores feed on the same plant species. A study provided by Rodriguez-Saona et al. (2010) indicated there was greater similarity in gene expressions between dual damaged plants and undamaged plants than to those only damaged by aphids or caterpillars. As there was more varied volatiles production between CCP (caterpillar-attacked plants) and CP (undamaged plants) compared to differences between AC (plants damaged by both caterpillars and aphids) and CCP, I assume that it is easier for *C. glomerata* to distinguish between CCP and CP rather than AC and CCP. It is possible that difference between AC and CCP is too slight to be detected by parasitoid. Moreover, Turlings et al. (1990) showed the quantities of volatiles

emitted in response of phloem feeders are lower than responding upon leaf chewers, due to the minimal damage inflicted or introduction of saliva (Walling 2008; McCloud & Baldwin, 1997). Wasps are not attracted to plants infested with herbivore that hardly damages leaf tissue such as aphids (van Poecke et al. 2003). In general, relying on general volatile blends may benefit generalists like *C. glomerata* to find multiple preys/ hosts species on a variety of plant species. However, it also leads to the cost of being attracted to unsuitable or less suitable hosts (Herrmann 2010).

In this study, I hypothesized that the little difference in size between hosts feeding on aphid-infested plants and hosts feeding on undamaged plants was not evaluated by parasitoid *C. glomerata* in the phase of long-range foraging. The little difference in host size probably does not translate into the better fitness for the parasitoid, hence it does not evoke the preference behavior of the parasitoid. As well studied, size is correlated with host fitness, but it is not the crucial fitness indicator. Fitness is reflected most directly by the number of granddaughters rather than other related indicators (Harvey 2005). Possibly parasitoids may not care the differential sizes of hosts, because it has limited influence on their fitness. Parasitoid might discriminate between hosts and non-hosts in the pair-wise comparison, but they do not distinguish larger hosts from smaller hosts, since the choices between hosts and non hosts are determinate to their survival whereas choices between larger hosts and smaller hosts only impact slightly on their performances.

In conclusion, it appeared *Pieris brassicae* butterflies were unable to distinguish aphid-infested plants from undamaged plants, whereas larvae showed better ability to choose aphid-infested plants which are beneficial for their growth. This observation suggested addressing with care the classical conclusion that mothers select optimal host plants for their offspring. Individual larvae and clutched larvae could choose better for themselves than sub-grouped larvae, as I hypothesized that intra-specific communication was interrupted by dividing inborn entire clutched into sub-groups. *Cotesia glomerata* did not discriminate plant infested by both aphid and caterpillar and plant solely infested by caterpillar. It indicates that facilitation between aphids and caterpillars initiated by the bottom-up effects (host plants) is not compromised by the top-down effects (pressure from natural enemies). However, when the aphid infestation lasted in longer period, facilitation was taken place by competition for food source. It is likely that in insect community, facilitation is temporary, but competition is forever. Volatiles emitted by aphid-infested plants showed a great similarity with undamaged plants, plants infested by both herbivores (the aphids and the caterpillars) produced varied volatile blends, whereas plant infested by caterpillars alone emitted even more varied volatiles. Since only general volatile blends were analyzed in this study, further study should investigate differences in particular compounds in volatile blends when it is associated with behavior of specialists. Yet the induction process, transcriptional patterns and interactions between different signaling pathways in plants attacked by multiple herbivores are still far been investigated in future molecular study.

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