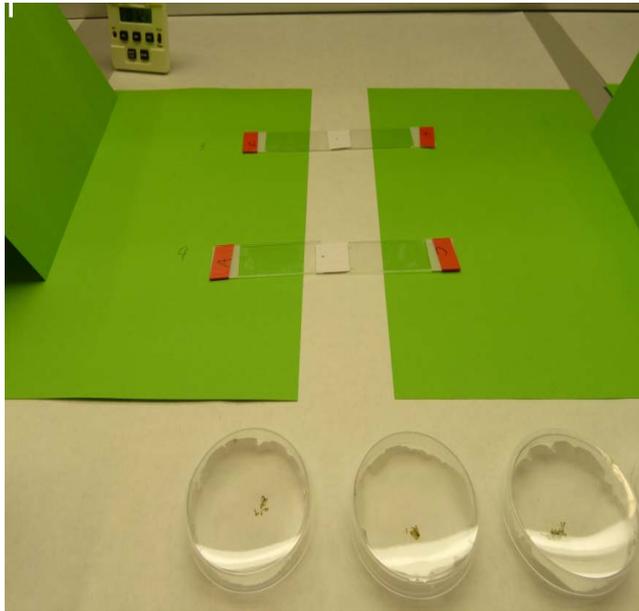# When facilitation shifts to competition: influence of phloem-feeders on the behavior of generalist and specialist leaf chewers

WAGENINGEN UR
For quality of life

Abstract

Herbivorous insects that belong to different feeding guilds may induce different plant defense signaling pathways, for instance, phloem feeders often induce the salicylic acid signaling pathway while leaf chewers predominantly induced the jasmonic acid signaling pathway. The antagonism between the JA and SA signaling pathways, indicated by the recent molecular work, suggested a possibility of facilitation between phloem feeders and leaf chewers. Previous studies revealed an enhanced performance of caterpillars when feeding on aphid-infested plants that were suppressed in defenses. According to the performance-preference hypothesis, phytophagous insects are able to select the host plants which maximize the performance of their offspring. The objectives of this study were firstly to investigate whether adults and larvae of different leaf chewer species are able to discriminate host plants with or without aphid-infestation; secondly to investigate whether the facilitative effect to caterpillar performance provided by aphid feeding extends to more species of leaf chewers across the generalists and the specialists (*Pieris brassicae*, *Pieris rapae*, *Plutella xylostella*, *Mamestra brassicae* and *Spodoptera exigua*).

In this study, I found that generalist and specialist caterpillars exhibited different patterns in preferences between aphid-infested plants and un-infested plants. Under the low density of aphid-infestation, caterpillars of all the five species did not discriminate between aphid-infested plants from un-infested plants. However, when caterpillars were tested with aphid-infested plants at the medium density, *Pieris brassicae* and *Pieris rapae* significantly preferred the aphid-infested plans over un-infested plants, but *Plutella xylostella* and *Mamestra brassicae* reversely preferred un-infested plants, while *Spodoptera exigua* was equally attracted by both types of plants. Under the high density of aphid-infestation, *Pieris brassicae*, *Pieris rapae*, *Plutella xylostella* and *Mamestra brassicae* were repelled by aphid-infested plants, but the trend was not significant for *S. exigua*. The four responded species (PB, PR, PX and MB) all avoided the aphid-infested plants at a high density, and facilitation appeared to shift to competition. I believe that the beneficial less-defended aphid-infested plants turned to nutritionally poorer plants at a high aphid density compared to un-infested plants. In contrast, butterflies and moths showed equal oviposition preferences to both aphid-infested plants in medium density and un-infested plants. In the performance study, larval weights of *P. brassicae*, *P. rapae* and *M. brassicae* on aphid-infested plants in low density did not significantly differ from those on un-infested plants. The performance results co-related to the indiscriminate behavior of leaf chewers between aphid-infested plants in low density and the un-infested plants.

It can be concluded that larvae are better able to discriminate superior host plants than adults. The attraction of aphid-infested plants, which can potentially contribute to the better performance of caterpillars, can be altered with different levels of aphid-infestation. The turning point of the larval preferences suggested that facilitation between phloem feeder and leaf chewers shift to competition in the end. There is a correlation between the larval food range and their choosy behavior of host plants, the broader the host range is, the less choosy caterpillars are.

Table of content

1 Introduction

'Mother knows best' hypothesis predicts that females have evolutionary adapted to oviposite on the host plants which optimize the performance of their offspring (Single et al.,1994; Wennström et al., 2010; Gripenberg et al., 2010). Under this notion, it can be interpreted that mothers are able to select the best food sources, and the ovipostion preference reflects the offspring performance (Shikino et al., 2010). However, the positive correlation between female ovipostion preferences and the larval performance is not always observed. For example, it was evident that the larval survival of *P. machaon* on Scandinavian umbelliferous and rutaceous species was not positively linked with the oviposition preferences of adults (Wiklund, 1974). A study provided by Berdegué et al (1998) showed an absence of correlation between the host plant selection of *Spodoptera exigua* females and the performance of their offspring. It was indicated that *S. exigua* oviposited more frequently on *C. murale* than on *A. graveolens* plants, but the fitness of larvae developed on *A. graveolens* was superior to those reared on *C. murale.* The poor relationships between oviposition preference and performance of offspring is often explained by the different ecological conditions and selecting pressures, including time, patch dynamics hypothesis, parasite versus grazer lifestyle hypothesis, and/or the enemy-free space hypothesis (Thompson 1988).

It is still open to debate that adults, who have limited contact with the plants, could be able to choose the most suitable host plants for the larvae which have to feed and live on the plant throughout the larval period (Wiklund, 1974). There is a variety of studies investigating the preference-performance correlation by comparing the adult oviposition preferences and the larval performance. However, the preference of larvae was not much examined, possibly with regards to the limited mobility of larvae (Wennström et al., 2010). In fact, there are some species of Lepidoptera, larvae are highly mobile and have been suggested to be able to participate in the host plant selection (Berdegué et al., 1998). It was showed that caterpillars of different species exhibited the different movement patterns as adapted to the spatial distribution of food plants (Jones, 1976). As early as in 1940s, experiments already showed that the antennas of Lepidoptera larvae are tactile and olfactory organs (Dethier, 1941). Van Loon (1998) revealed that deterrent chemoreceptors in maxillary styloconic taste sensilla enable *Pieris brasssicae* caterpillars to discriminate between chemically different deterrents when choosing host plants. Therefore, there are plenty of possibilities that larvae engaged in the selection of their own host plants. A Study by Chew (1980) demonstrated plasticity in food plants preferences of *Pieris* spp. In the other words, the broader food range allows caterpillars to resolve the inappropriate choices made by their mothers by accepting alternative food plants that are available at that time. Recently, 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.

Host plant preference of caterpillars has been traditionally studied offering leaf discs of fairly distinct plant species, sometimes even host and non-host plants, comparing the leaf area eaten to rank their preferences (e.g. Portillo 1996). In this scenario, larvae can often

be confronted with choices they would not encounter in nature. Under this framework, mothers often know best. It was well-studied that plant induced defenses upon one attacking herbivore can impact on the performance of other herbivores sharing the common host plant (Dicke 2009). At the molecular level, the antagonism of the two major signaling pathways mediating plant defenses, salicylic acid (predominantly induced by phloem feeders) and jasmonic acid (induced by chewer species) pathways suggested the possibility of facilitation between insect species from different feeding guilds (Pieterse et al., 2009). 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 than on un-infested conspecifics. Soler et al. (2011) showed that *Pieris brassicae* larvae performed significantly better, growing bigger and faster on *B. oleracea* plants previously colonized by the aphid *Brevicoryne brassicae* than on conspecific uninfested plants. As the performance of caterpillars can be enhanced by the presence of a phloem feeder, this might provide an interesting opportunity to study plant selection by caterpillars between choices they might often been confronted to make; conspecific plants that 'just' differ in their defensive state and naturally coexist in space and time, just one next to the other.

In my previous Master thesis, I found that *P. brassicae* larvae preferred *B. oleacea* plants infested by aphids instead of un-infested plants. In this present study, I investigated the preference-performance linkages of the most common Crucifer leaf chewers, between conspecific *B. nigra* under the same nutritional regime that differed in their defensive state caused by the previous infestation of the phloem feeder *B. brassicae*. I expected some differences on the preference pattern of larvae and adults, the specialists and generalists, although they could possibly all benefit from the aphid-infested plants which were inhibited in defenses. Hereby, we asked the following research questions:

1) If the leaf chewers performed better on plants infested by aphids than on undamaged plants, do the adults and larvae also discriminate and prefer aphid-infested plants?
2) If the larvae show the preference to aphid-infested plants, what is the mechanism underlying such preference behavior
3) Is the facilitative effect of aphid infestation on the performance of caterpillars extended to the most of the leaf chewer species, across generalists and specialists?

2 Material and methods

2.1 The model-system

In this study, I explored interactions between phloem feeders and various species of leaf chewers mediated by the common host plant. I investigated whether performance, feeding and oviposition preference of three specialist leaf chewers (*Pieris. rapae*; *Pieris brassicae*; *Plutella xylostella*) compared to two generalist leaf chewers (*Spodoptera exigua*; *Mamestra brassicae*) that facilitated by the specialist phloem feeder (*Brevicoryne brassicae*) on the wild cruciferous plant species *Brassica nigra* (Fig 1).
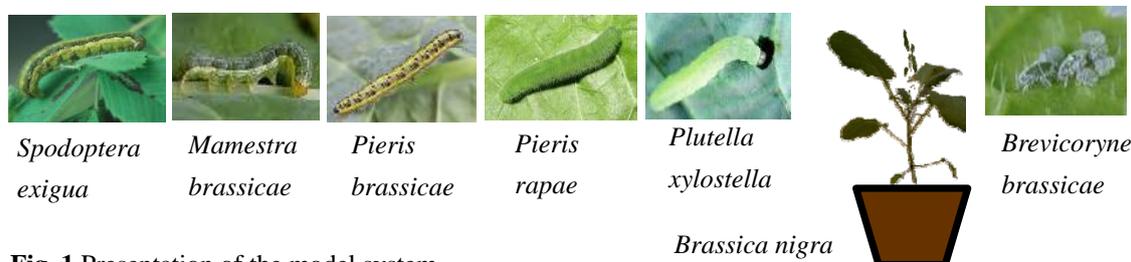
Brassica nigra (Brassicaceae)is a widely distributed annual species in the family

Brassicaceae, commonly known as black mustard. Plants in the Brassicaceae contain diverse phytochemicals to serve defensively. *Brassica nigra* can induce defenses against herbivorous insects providing excellent potential for the study of interactions between herbivore species sharing common host plants.

Aphid *Brevicoryne brassicae* (Hemiptera: Aphididae) feed on cruciferous plants and cause damage to plants due to the accelerated reproduction rate. Aphid females give birth to female nymphs in similar appearance to adults. But in autumn, in natural conditions, females respond upon decreases in photoperiod and temperature, males are produced and aphids will then reproduce sexually. Aphids sequestrate glucosinolates from plants to mount defense against their predators.

Larvae of *Pieris rapae* (PR), *Peris brassicae* (PB), *Plutella xylostella* (PX), *Mamestra brassicae* (MB) and *Spodoptera exigua* (SE) are common foliar-feeders of cruciferous plants. *Pieris rapae* (Lepidoptera: Pieridae), commonly known as the small white cabbage butterfly, exploits a variety of wild crucifers. *Pieris rapae* (Lepidoptera: Pieridae) female butterflies lay single egg on their host plants in the field. *Pieris brassicae* is commonly known as the large white cabbage butterfly, and restricts to feeding on plants in the Brassicaceae as well. The female butterflies lay clutches of 40-80 eggs mostly on the backsides of leaves. The eggs take approximately five to seven days to hatch in the rearing room (21-22 ℃). *Pieirs brassicae* larvae are voracious feeders, the first to third instars feed gregariously, and later instars will disperse throughout the host plant. The diomond-back moth *Plutella xylostalla* (Lepidoptera: Plutellidae) is a specialist on Brassicaceae plants, and is one of the most serious pests of cole crops worldwide. They lay eggs singly on the lower leaf surface. The cabbage month *Mamestra Brassica* (Lepdoptera: Noctuidae) is highly polyphagous, and is a main pest of vegetable crops. The eggs of *M. brassica* are laid in batches of up to approximately 200, but the hatched larvae do not feed gregariously, and they pupate in the soil. The beet army worm *Spodotera exigua* (Lepdoptera: Noctuidae) exploit a wide host range, and causes severe damage to cabbage and lettuce by burrowing into the center of the crown. Females often lay batches of 50-150 solitary eggs. The eggs are covered with a layer of whitish scales which give the egg mass a fuzzy or cottony appearance. The pupation also occurs in the soil, and they lay eggs two-three days after mating.

The study system

| *Spodoptera exigua* | *Mamestra brassicae* | *Pieris brassicae* | *Pieris rapae* | *Plutella xylostella* | *Brassica nigra* | *Brevicoryne brassicae* |

Fig. 1 Presentation of the model system.

2.2 The rearing condition of plants and insects

All *B. nigra* plants used in this study were four to five weeks old, at vegetative stage, and were grown in the greenhouse compartment (22±2℃, 50-70% RH, L16:D8). Aphids

Brevicoryne brassicae were reared on *Brassica nigra* plants in a greenhouse compartment in Laboratory of Entomology (Wageningen University, the Netherlands) (22±2℃, 50-70% RH, L16:D8). *Pieris brassicae*, *Pieris rapae*, *Plutella xylostalla, Mamestra brassicae* were reared on *Brassica Oleracea* plants in a climate room at 20-22℃, 50-70% R.H. and a L16:D8 photoregime in Laboratory of Entomology (Wageningen University, the Netherlands). *Spodoptera exigua* were reared in a climate room (22℃, 50-60% R.H. and a L16:D8 photoregime) in Laboratory of Phytoviruology (Wageningen University, the Netherlands). All the newly emerged adults of each species of leaf chewers used in the experiments were reared on artificial diet of honey solution (honey: water = 1: 9).

2.3 General treatment

Plants with the two treatments were provided throughout all the experiments included in this study, the aphid-inoculated plants and the un-infested plants. In order to prepare the aphid-infested plants, the aphids *Brevicoryne brassicae* (either nymphs or adults in different amount, varied with the experiments and is explained in detail below) were placed on the first fully expanded leaf of *B. nigra* plants for 5 days and restricted by clip cages for 24 hours to prevent them from escaping to other leaves (Fig 2). One day after the inoculation, the mortality was checked on all plants, any dead aphids were replaced. In contrary, un-infested plants were kept to be intact without any herbivory nor other damages


Fig 2. The inoculation of aphids (*B. brassicae*) on the first expanded leaves of *B. nigra* plants (left). The necrotic spots found on aphid-infested sites on a few plants.

2.4 Larval preference experiments

In order to investigate the preferences of various leaf chewers, the neonate larvae were tested in a two-choice bioassay consisted of aphid-infested plants and un-infested plants. *Brassica nigra* plants were inoculated with aphids in three different densities: the high, medium and low density were caused by 8 adult aphids (*Brevicoryne brassicae*), foure adults, or 15 nymphs (N1-N2) continuously feeding for identically 5 days respectively. The aphid inoculation in the high density end up with a population of 90-110 aphids on the day of experiments, the medium density with a population of 40-60 aphids, while the low density remained to be 15 adults. Five plants were inoculated in each density, and used for testing feeding preference of caterpillars compared to five un-infested plants for each species of leaf chewers.

In the larval preference experiments, only the neonates of different species of leaf chewers were used, because I tested the innate responses of caterpillars. The neonates were

less than 12 hours old, neither had suffered from starvation nor had contacts with host plants. Therefore, it was ensured that they had no learning experience nor chose in a hurry driving by starvation. In an effort to obtain the newly hatched larvae of each species exactly at the time I carried out the experiments, the eggs synchronization was well-scheduled and performed.
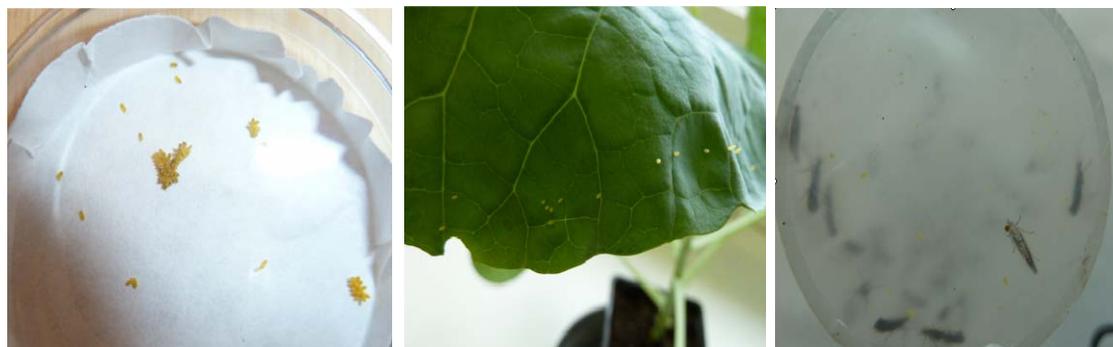
Fig 3. Eggs of *Pieris brassicae*, *Pieris rapae* and *Plutella xylostalla* used in the experiments for manipulating newly hatched larvae without contacting with host plants (from left to right).


Fig 4. Eggs of *Mamestra brassicae* laid on the filter paper (Photo 1); newly hatched larvae of *M. brassicae* (Photo 2); eggs of *Spodoptera exigua* laid on the filter paper (Photo 3); newly hatched larvae of S.exigua (Photo 4) (from left to right).

All the eggs obtained were kept in and kept in a climate cabinet at 23 ℃, L16:D8. Intact egg clutches of *Pieris brassicae* and single eggs of *Pieris rapae* with black head were gently removed from the plants one day before the test and kept in the climate cabinet. *Pieris brassicae* larvae hatched after 16-24 hours, while *Pieris rapae* often hatch after approximately 24-28 hours. Eggs of the *M. brassicae* and *S. exigua* laid on the filter paper hatched 5 days and 4 days respectively after being reared in the climate cabinet. Around 30 *Plutella xylostalla* adults were caught by electrical aspirator 4 days before performing the experiments. They were thus kept in a plastic cylinder with Para film covering the two opening side in a climate cabinet. *Plutella xylostalla* kept mating and laying eggs on the Para film along the winkles and inside the cylinder as well (Picture 3, right).

In the bioassay, five aphid-infested plants and five un-infested plants were set up in on a workbench with a daylight TL light source above it in the behavior lab (20-22℃, 50-70% R.H.). Pairs of 1 infested and 1 control plant were placed next to each other as showed in picture 5 (left). From the aphid-infested plants, the first leaves above the aphid-inoculated leaves were used in combination of leaves from undamaged plants in similar age. The neonates of the leaf chewers were placed singly on the 'bridges' made from carton, in between of the two stretched leaves (Fig 5, right). Once placed on the bridge the time which the caterpillars took to make a choice was monitored. A choice was recorded once

caterpillars fully left the bridge to move onto the plant leaves. After that the caterpillar was immediately removed, before feeding on the plant. Bridges were replaces for every caterpillar tested. Larvae that did not respond within 20 minutes were regarded as 'non-response', and were excluded from the statistical analysis. Fifteen to twenty larvae were tested on each pair of plants, using five to six plant pairs. Each species and density (High, Medium and Low) was tested in a separated day.
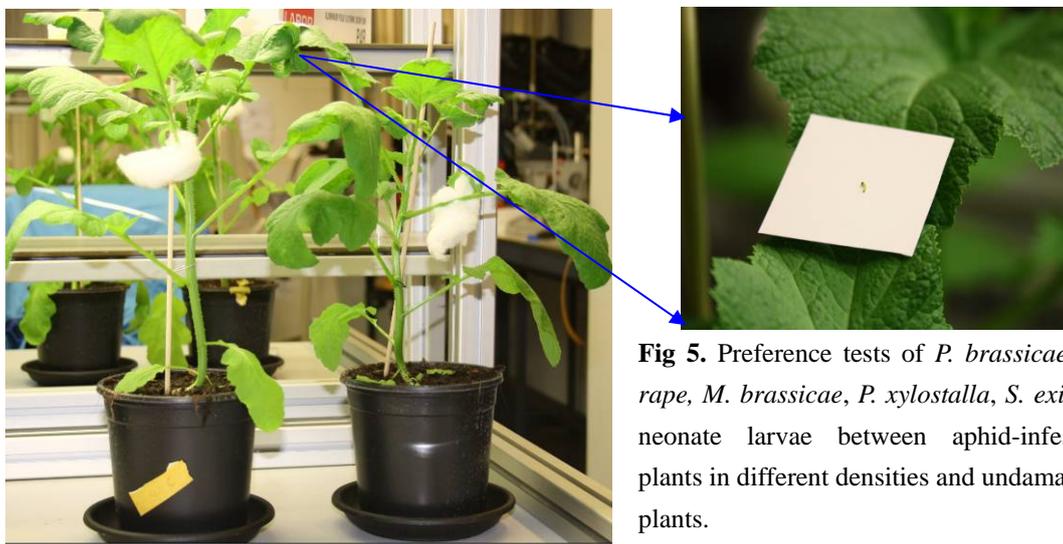
Fig 5. Preference tests of *P. brassicae*, *P. rape, M. brassicae, P. xylostalla, S. exigua* neonate larvae between aphid-infested plants in different densities and undamaged plants.

2.5 Plant cuticular extracts

To explore examine the mechanism underlying the choosing behavior of *P. brassicae* larvae, larvae were tested with the leaf cuticular compounds extracted from both aphid-infested and undamaged *B. nigra* / B. oleracea plants. Aphid-infested plants were inoculated with 4 adult aphids for 5 days in this series of experiments, as this was the density where the caterpillars showed the highest attraction to aphid-infested plants. To test the behavior of larvae in reaction to plant cuticular compounds, the two systemic leaves above the leaf with aphid feeding were cut off by scissors from aphid-infested plants. Two leaves in similar size and order from undamaged plants were used in the experiments. Cuticular extracts were prepared by dipping the leaves vertically into a 200 ml hexane/methanol solution (7:3), for 10 seconds. The chemical compounds were expected to be rinsed off by the solvent and dissolved in it without damaging the plant cell (de Nys et al., 1998). Additionally, the leaves were scanned to measure the leaf area (in the program Image J) before being dipped into the solvent, or alternatively left in the oven at 60 ℃ for 48 h after extracting to measure the dry weight. The solvents were evaporated under nitrogen flow or on the fume-hood, and were then stored in the glass test tubes covered by aluminum paper in the fridge at 4 ℃.

I adjusted the volume and concentration of crude extracts for each time in order to approach the most precise method. In the *first series*, cuticula of 20 leaves from 10 *B. nigra* plants with and without aphids were extracted in one common pool and then evaporated with nitrogen flow till 40 ml (Set A and Set D). In the *second series*, cuticula of six leaves from three *B. nigra* plants were extracted in each sample. Five pairs of the extracts samples (aphid-infested plants VS undamaged plants) were evaporated till 1.5ml, firstly with air flow

on fume-hood and then with gas nitrogen (Set B). However, the other five pairs were completely evaporated, but re-dissolved in 1.5 ml methanol added into each sample afterwards (Set C). In the *third series*, six leaves picked from three *B. oleracea* plants were extracted in each sample as well, but the samples were evaporated to 6 ml (Set D) (Fig 6). I calculated the dose of extracts which equals to cuticula from 5 cm^2 of leaf area based on the equation: Dose = Volume of sample $\times$ 5 (cm^2) / Leaf area of leaved used in the sample. In this way, the crude cutilular extracts of each sample in various does were standardized in cuticula from leaf in 5 cm^2. The cuticular extracts of pair samples coming from plants with and without aphids were transferred with a glass pipette individually on the glass slides, and evaporated before being offered to individual *P. brassicae* neonate larvae.

Fig 6. Method of extracting plant cuticular compounds; *B. oleracea* leaf before (left) and after (right) extracting; *B. nigra* leaf before (left) and after (right) extracting (from left to right).

In the preference test of *P. brassicae* larval choices between leaf cuticular extracts from aphid-infested plants and extracts from undamaged plants, two glass slides applied with extracts from either of the two types of plants were offered in a dual-choice bioassay. Green papers were placed under beneath and beside the glass slides, as offering larvae some visual cues mimicking plants. *Pieris brassicae* neonates were placed singly on the bridge in between of these two glass slides (Fig 7). When the caterpillar body completely reached the glass slides, the final choice was recorded. Both choices of larvae and the times taken by them to make a decision were noted. If a caterpillar does not respond within 20 minutes, 'non-response' was recorded, as then offering entire plants most larvae made a decision within this time frame. In total, sixty to hundred responded larvae were tested, and the 'non-response' proportion was presented in the results section separately.
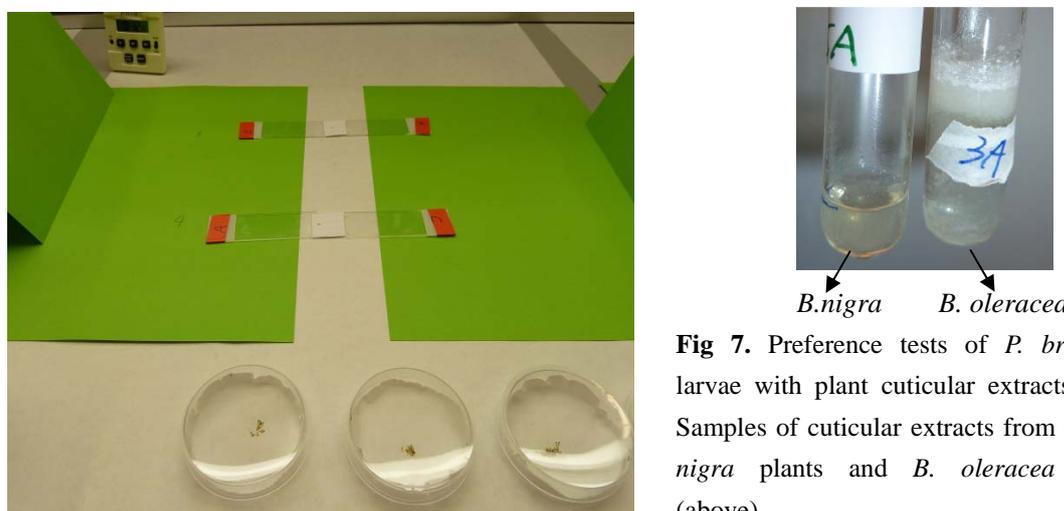
B.nigra   *B. oleracea*

Fig 7. Preference tests of *P. brassicae* larvae with plant cuticular extracts (left). Samples of cuticular extracts from both *B. nigra* plants and *B. oleracea* plants (above).

The samples of leaf cuticular extracts in Set A (a pool from aphid-infested plants VS undamaged plants) were analyzed by GC-MS for identification and quantification of chemical compounds. An Agilent 7890A GC system coupled to a 5975C MSD (Agilent Technologies, Palo Alto, CA) equipped with ZB-MS column (30 ml x 0.250 mm i.d. x 0.25 μm film thickness, Agilent Technologies) was used for the analysis using helium as carrier gas at a flow rate of 1 ml/min in constant pressure mode. The GC oven temperature was initially held at 45 ℃ for 2 min and temperature increased at 10 ℃/min to 280 ℃ (hold 6.5 min). 1 μl sample extract was injected in splitless mode, with a splitless time of 2 min at an injector temperature of 250 ℃. Spectra were recorded in electron impact ionisation mode (EI) at 70 eV in a full scan mode with masses ranged between 45 – 450 amu. Identification of compounds was based on comparison of mass spectra with those of 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.

2.6 Oviposition preference experiments

The adult oviposition preferences of each species were tested between aphid-infested plants and un-imfested plants. Before conducting the experiments, six aphid-infested plants (*B. nigra*) were prepared by inoculating 4 adult *B. brassicae* for 5 days (40-60 aphids of mixed ages at the beginning of the test). I tested the preference of newly emerged butterflies, because egg load can influence oviposition preference of butterflies: butterflies with high egg loads are expected to besignificantly less choosy than young females with relatively low egg loads (Pilson & Rausher, 1988; odendaal & Rausher, 1989). In order to obtain the newly emerged females, forty *S. exigua* pupa were kept in a climate cabinet (22℃, L16:D8) for 5 days till the adults emerged. After emerging, one more day was left for them to mate, and then females were selected separately. I also observed the *M. brassicae* mating couples in the freshest batch, and place every 2 couples in separated boxes one day before doing the experiments. For *P. xylostalla*, the pre-pupa caterpillars were reared in a climate cabinet with leaves of *B. oleracea* (22℃, L12:D12, with light off from 7:00 to 19:00 in the day time and time one from 19:00 to 7:00 in the night) for one week. After they pupated and emerged, the mating couples were observed and selected every one hour. To have the fresh grave females, newly emerged *P. brassicae* and *P. rapae* females and males were kept together in two separated cages for 1-2 days before the experiments.

In the oviposition preference experiments, six cages were prepared in the greenhouse compartment for each species (22±2℃, 50-70% RH, L16:D8). One aphid-infested plant and one undamaged plant were placed in each cage. To ensure that the females were mated, two females were released in each cage in company of two males. Ovipostion choices of 12 females of each species were tested. Artificial diet was offered in each cage. The experiments terminated 12-24 hours after the first egg, egg clutch or egg batches were detected on the plants. The oviposition choices (the plant with or without aphids) and the number of egg/ clutches were recorded.

Fig 8. Oviposition preference tests set up of PB, MB, PR, PX, SE between aphid-infested plants and un-infested plants.

2.7 Performance experiments

As an important parameter of insect fitness, pupa masses of chewer species should be measured in the performance experiments. In order to figure out the certain date on which the pupa mass is stabilized, a pilot test was performed to measure 15-20 fresh *Pieris rapae, Pieris brassicae, Spodoptera exigua* and *Mamestra brassicae* pupa (pupated within 24 hours) along 4-5 days consecutively before adults emerged.

The performance of various chewer insects *Pieris brassicae* (PB), *Memastra brassicae* (MB), *Pieris rapae* (PR), *Plutella xylostella* (PX) and *Spodoptera exigua* (SE) on aphid-infested plants was compare to performance on un-infested plants (control). Five days in prior to the performance test of leaf chewers, fifteen nymphs were inoculated on 12 individual plants. Cotton balls were placed around the petiole of the aphid-inoculated leaf and two above systemic leaves to prevent the dispersal of aphids to other leaves of the same plants. I also placed cotton balls on the petiole of un-infested plants to avoid the variation (Fig 9). All the plants were placed on a platform filled with water (as deep as 2 cm) to further prevent the crossing of insects from one plant to the other in the greenhouse compartment.

Five days after the aphid inoculation, two neonate larvae of PB, PR and MB, were inoculated on both the first and second systemic leaves after removing all the aphids from the aphid-infested plants. *Mamestra brassicae* were kept in the clip-cages on both systemic leaves, due to the great mobility of this species. *Pieris brasicae* and *P. rapae* larvae are sluggish and do not move from plant to plant as long as leaves are not touching. Thus, larvae were not caged or restricted. Twelve aphid-inoculated plants and twelve un-infested plants were prepared respectively for each species of leaf chewers, thus there were 96 larvae from three species tested in this study. PX and SE were planned to be evaluated after, but due to the high infestation of thrips in the greenhouse compartment, the performance studies were not continued and only the preference tests, which were prepared in a climate cell and performed in the behavioural lab were conducted.

The performance of *Mamestra brassicae* were further evaluated by a separated set of experiments in 20 pairs of plants with or without aphid infestation, in which two neonates were inoculated on the two systemic leaves above the aphid-infested leaves separately, with regards to the fact that this solitary species seldom co-exist on one common host plant in the nature. Apart from the difference in the method of inoculation of larvae, this set of experiment was conducted in the same procedure as described above.
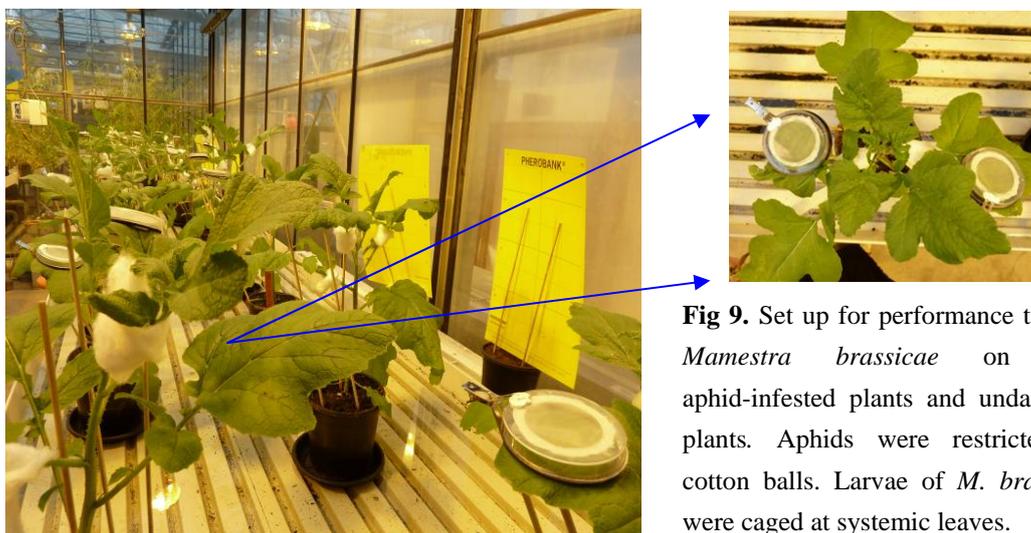
Fig 9. Set up for performance tests of *Mamestra brassicae* on both aphid-infested plants and undamaged plants. Aphids were restricted by cotton balls. Larvae of *M. brassicae* were caged at systemic leaves.

To measure the growth rates of the different species of leaf chewers on aphid-infested plants and un-infested plants, the weight of the larvae along their development was planned to be measured every 48 hours. Larvae feeding on each host plant were gently transferred to separate Petri-dishes. The plants and Petri-dishes were marked. The body mass of larvae in each Petri-dish were weighed individually by a Sartorius balance C2P (maximum of 2g). After being weighed, the larvae were placed back on the plant, on which they were originally feeding. PB, PR and MB larvae were weighed after feeding on either aphid-infested plants or un-infested plants for 4-5 days in the first series of experiments. In the second series of experiments, MB larvae were weighed after having been feeding on both types of plants for 24 hours.

2.8 Statistics

A generalized linear model with binomial distribution and logit link function was fitted to analyze the preference of caterpillars (five species) for plants with and without aphids, at the three densities tests. For the individual preference experiments between cuticular extracts, a binomial test was used to determine whether plant preferences of the insects differed significantly from a non-preference situation (p_ q_ 0.5, two-tailed, a_ 0.05). For the variables number of eggs/ clutches per plant and plants selected by the females a generalized liner model with Poisson distribution was fitted with the logarithm as link function. The variables decision times, weights of larvae were tested using one-way ANOVA. Normality and homogeneity of variance were checked by inspection of the residuals after model fitting.

3 Results

3.1 Feeding preferences

Larval preferences were measured by the proportion of neonates choosing between aphid-infested and un-infested plants. Statistically, there were interactions between the factors of species, density and treatment in the preference tests of larvae (species *density *treatmet, F= 4.62, P< 0.001). Under the low density of aphid inoculation, none of the five species of larvae (PB; PR; PX; MB; SE) discriminate between aphid-infested plants and undamaged plants. However, there was a trend that a higher proportion of *P. brassicae* larvae (61%) were attracted by aphid-infested plants rather than undamaged plants.
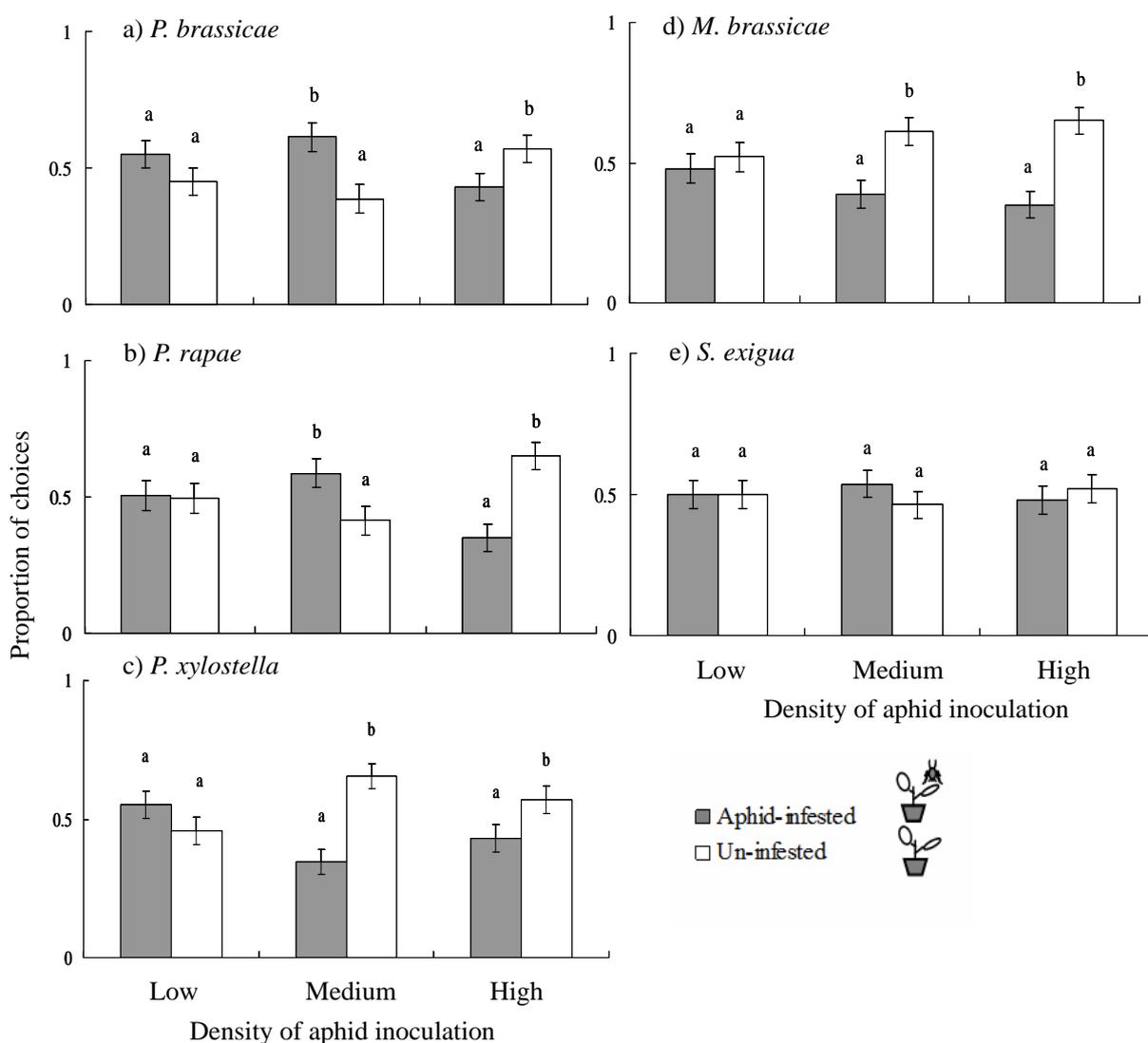

Fig 10. Proportion of choices of *Pieris brassicae* (a); *Pieris rapae* (b); *Plutella xylostella* (c); *Mamestra brassicae* (d) and *Spodoptera exigua* (e) neonate larvae made between aphid-infested plants (in low; medium; high densities) and undamaged plants in dual-choice experiments. Different letters represent the statistically differences within species and densities (P< 0.05) (n= 80-100 larvae per species).

At the medium density, there was a discrepancy in choices of leaf chewers

evaluated. *Pieris brassicae* and *P. rapae* preferred aphid-infested plants inoculated in medium density over un-infested plants (P=0.001, mean LSD= 0.14 (a); P< 0.001, men LSD= 0.145 (b)). However, both *P. xylostella* and *M. brassicae* neonates were significantly attracted by un-infested plants rather than aphid-infested plants in medium density (P< 0.001, mean LSD= 0.138 (c); P= 0.026, mean LSD= 0.133(d)). *Spodoptera exigua* did not show any preferences between aphid-infested plants and undamaged plants, irrespectively to densities of aphid-inoculation (P= 0.471(e)). Interestingly, at the high density of aphid-infestation, *Pieris brassicae*, *Pieris rapae* , *Plutella xylostella* and *Mamestra brassicae* larvae avoided the aphid-infested plants in high density and switched their preferences to un-infested plants, but for *Spodoptera exigua*, it was not significant (Fig 10).

The mean times taken by larvae to choose between aphid-infested plants and un-infested plants varied among species. *Pieris brassicae* always slower, while *Mamestra brassicae* always the faster than all the other species when tested in different series of experiments. On average, *Pieris brasicae* made a choice approximately on 8.6 minutes, but *M. brassicae* made a decision on 1.1 minutes to between host plants. Nevertheless, *Spodoptera exigua* and *Plutella xylostella* took moderate time to respond in the experiments. It is remarkable that *P. brassicae*, *M. brassicae*, *S. exigua* and *P. xylostella* used similar amount of time to make a choice between aphid-infested plants and undamaged plants, regardless the different densities in aphid-infestation, however, *Pieris rapae* was an exception. *Pieris rapae* was always the second slower species after *P. brassicae*, though, it took significant less time, 2.9 minutes on average to choose between aphid-infested plants in low density and undamaged plants, compared to averaged 4.7 minutes when being tested with aphid-infested plants in medium and high density.
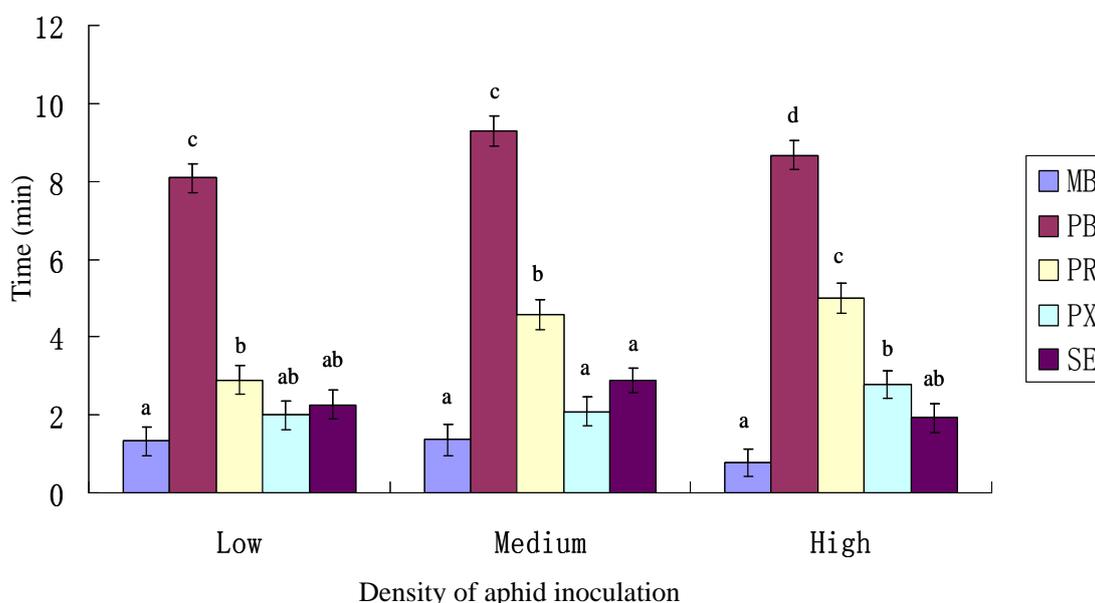
Fig 11. Mean (±SE) time taken by larvae of *Mamestra brassicae* (MB), *Pieris brassicae* (PB), *Pieris rapae* (PR), *Plutella xylostella* (PX) and *spodoptera exigua* (SE) responded to aphid-infested plants (in low; medium; high densities) or undamaged plants. Significant differences within densities were indicated by different letters (P< 0.05, P= 0.007).
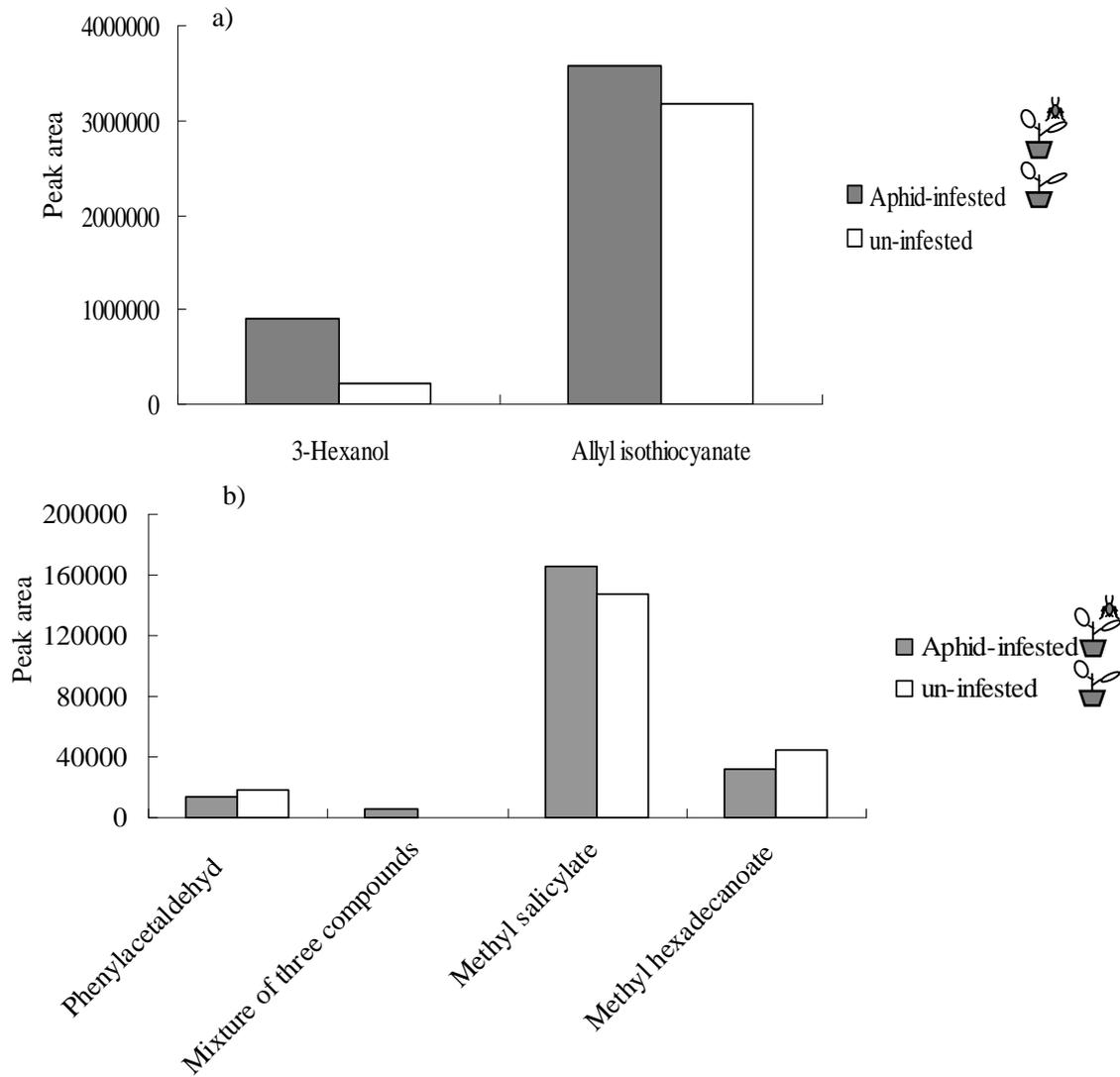
3.2 Cuticular extracts

Preferences tests of *P. brassicae* between cuticular extracts from aphid-infested plants and undamaged plants indicated a significant attraction for the crude extracts from aphid-infested plants (set A) (Fig 12 *i*, P= 0.008). However, in the B, C, D sets of experiments with cuticular extracts from *B. nigra* plants and in particular, the last set of tests with extracts from *B. oleracea* leaves, *Pieris brassicae* larvae did not exhibit any significant preferences to aphid-infested plants or un-infested plants (P= 0.841; 1; 0.152; 0.576). Interestingly, when a different plant species, *B. oleracea* was evaluated, *Pieris brassicae* did not show preferences neither, but there was a much higher non-response rate of 66.2%, as compared to averaged 9.4% of the non-response rate on *B. nigra* extracts.
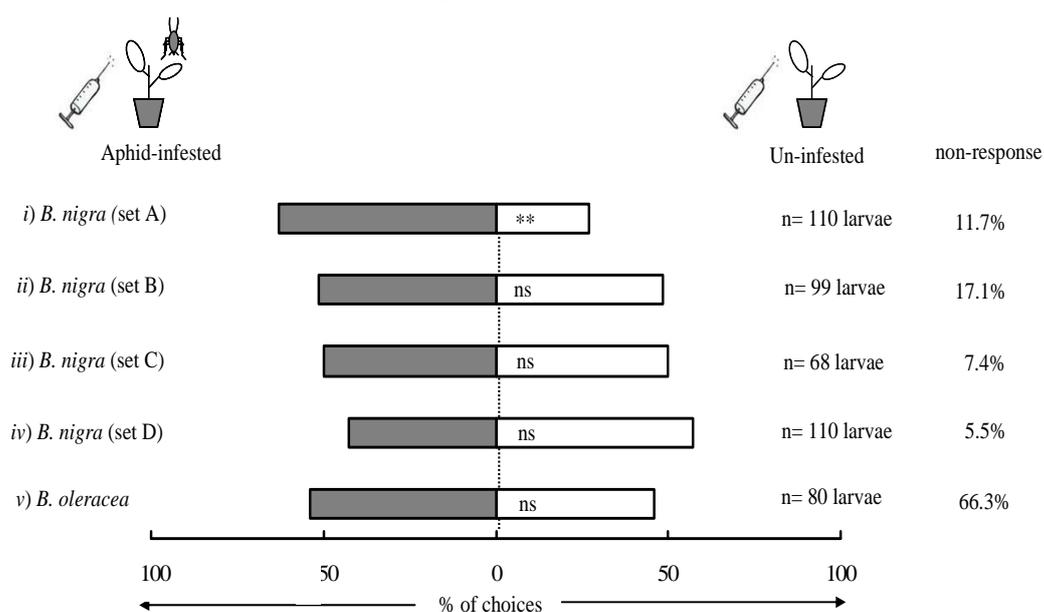
Fig 12. Feeding preferences of *Pieris brassicae* (PB) neonate larvae, in dual-choice experiments, between leaf cuticular extracts from *B. nigra* plants exposed to aphid herbivory and crude cuticula extracted from un-infested *B. nigra* plants (*i*, *ii*, *iii*, *iv*); or between cuticular extracts from aphid-infested *B. oleracea* plants and un-infested ones (*v*). Asterisks represent a significant difference within a choice test: ** P < 0.01.

Chemical compounds contained in the leaf cuticular extracts from *B. nigra* plants in samples of set A, were identified and quantified by GC-MS. Both cuticular extracts from aphid-infested and un-infested plants contained Allyl isothiocyanate and 3-Hexanol (Fig13 a), and in a highest amount as compared to the other chemical components. However, extracts from aphid-infested plants contained more 3-Hexanol, Allyl isothiocyanate and Methyl salicylate than extracts of undamaged plants, but less Phenylacetaldehyde and Methyl salicylate (Fig 13 b). It was notably that the 'mixture of three compounds', which was a mixture of an unknown compound, 4-Methylguaiacol and 6.2,5-Dimethyl-3-acetylfuran was completely absent in un-infested plants but only present in aphid-infested plants. Since only one sample pool of cuticular extracts from aphid-infested plants and undamaged plants (Set A) was roughly analyzed, there was no variation of the quantification of the chemical compounds, thus the statistical analysis was not performed.

Fig 13. Quantification of major chemical compounds of leaf cuticular extracts pool from aphid-infested plants and undamaged plants (sample of set A). 'Mixture of three compounds' was the mixture of an unknown compound, 4-Methylguaiacol and 6.2,5-Dimethyl-3-acetylfuran.

3.3 Oviposition preferences

In the oviposition test, approximately 60% of the eggs laid by *M. brassicae* were found on cages or pots, instead of plants. Moreover, *Spodoptera exigua* almost laid all the eggs on the cages (98%) (Fig 14; A). Based on that, it is not functional to analyze the oviposition preferences of these two species between aphid-infested plants and un-infested plants. *Pieris brassicae*, *Pieris rapae* and *Plutella xylostella* females did not show significant oviposition preferences on either aphid-infested plants or un-infested plants, since they laid equal amount of eggs/ clutches on both types of plants (P= 0.574) (Fig 14; B).
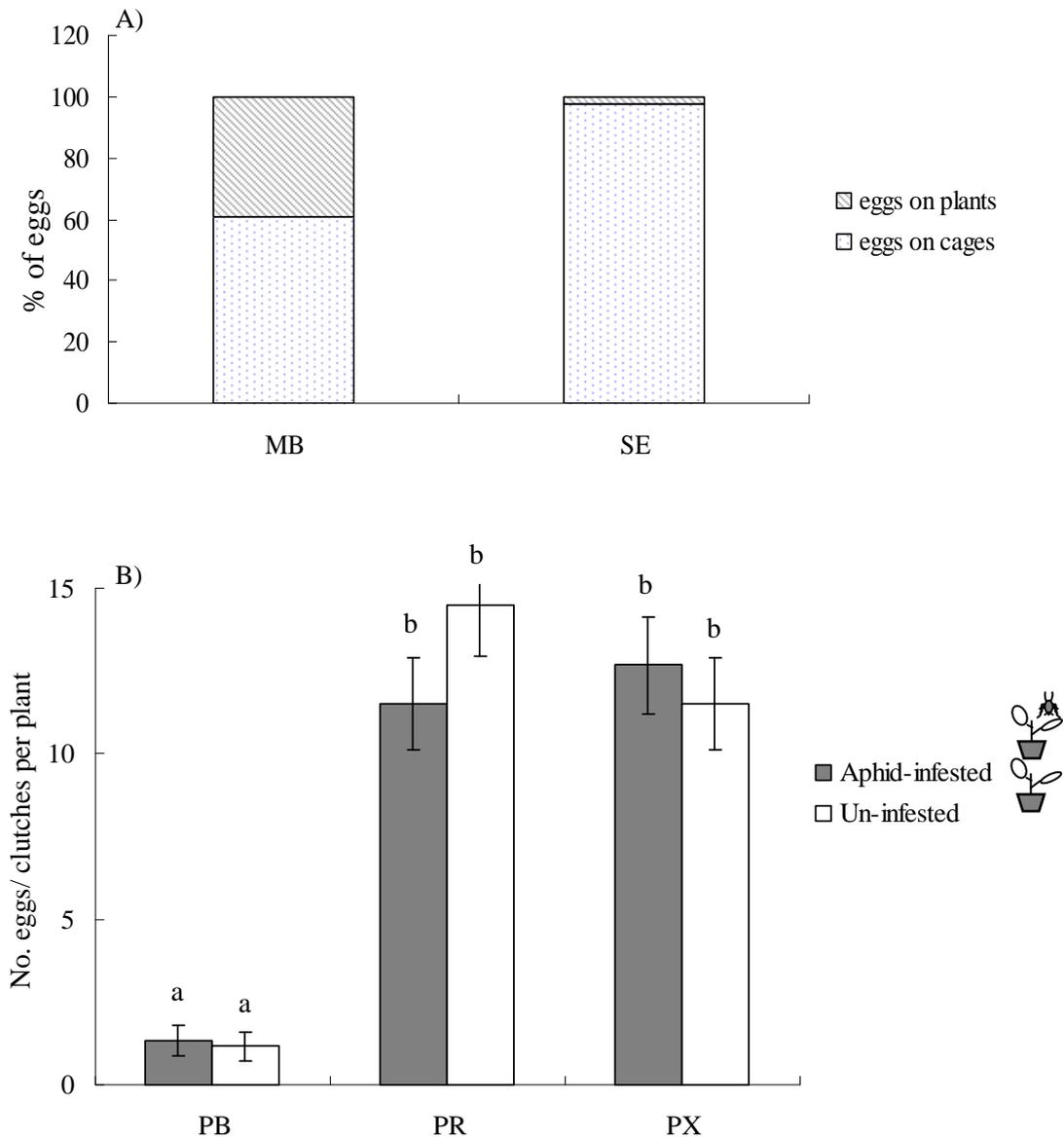
Fig 14. Percentage of eggs laid on plants or cages by *Mamestra brassicae* (MB) and *Spodoptera exigua* (SE) (A). Amount of eggs/ clutches of *Pieris brassicae* (PB), *Pieris rapae* (PR) and *Plutella xylostella* (PX) laid on aphid-infested plants and undamaged plants (P< 0.05, P= 0.574, F= 0.32) (B) (n= 12 females per species).

3.4 Performance experiments

3.4.1 Pilot test: variation of pupa mass with time

The results displayed that the pupa mass of all the four species hardly change with 4-5 days, and the weights remained similar afterwards. Therefore, any dates between the first days after pupating and the fifth days before emerging are assumed to be suitable for weighing the pupa mass.
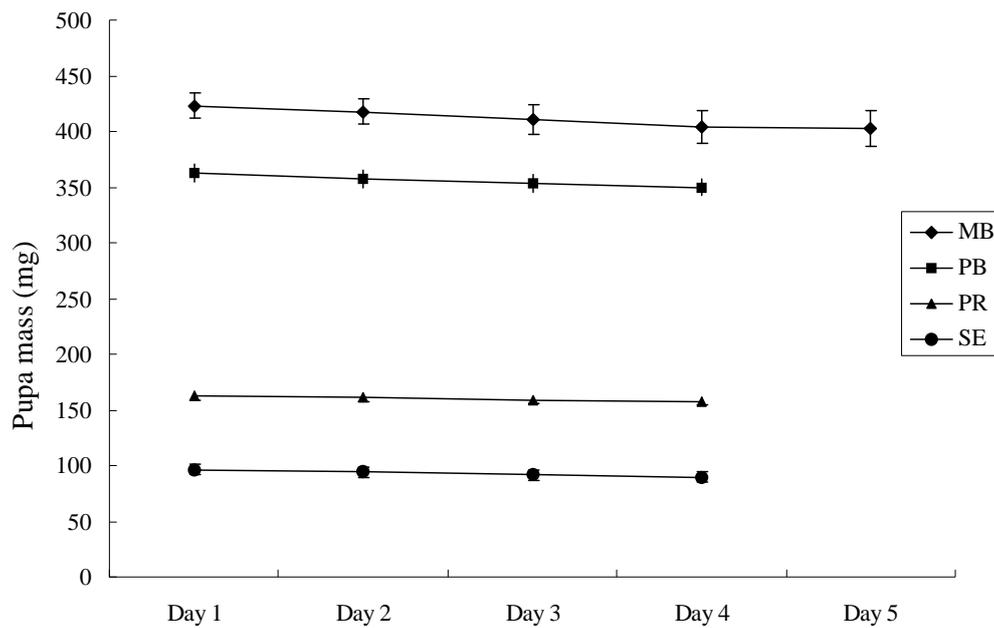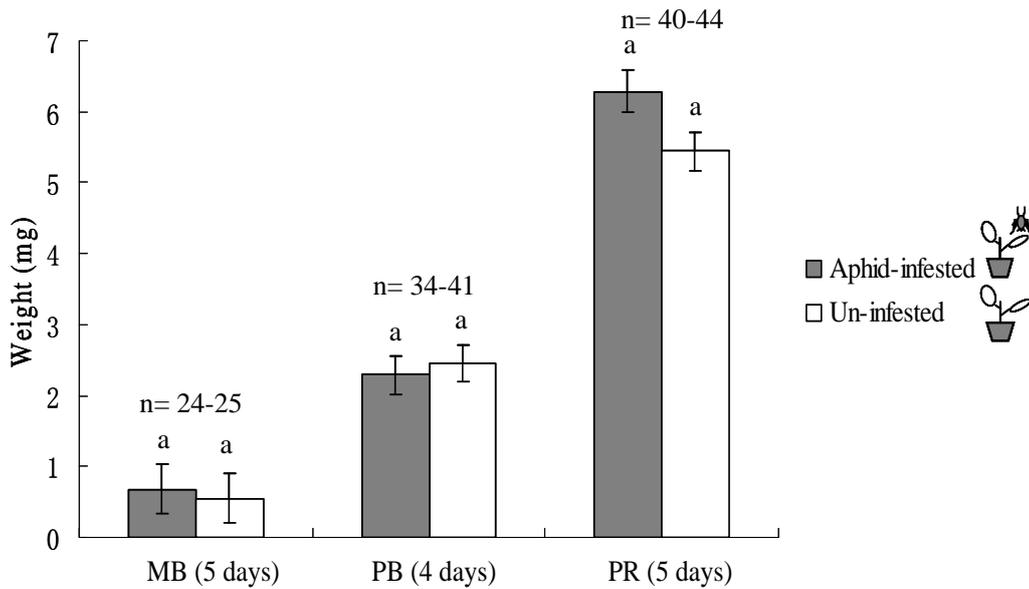
Fig 15. Mean (±SE) pupa mass of *Mamestra brassicae* (MB), *Pieris brassicae* (PB), *Pieris rapae* (PR) and *Spodoptera exigua* (SE) in 4-5 days before emerging (n=15-20 pupa per species).
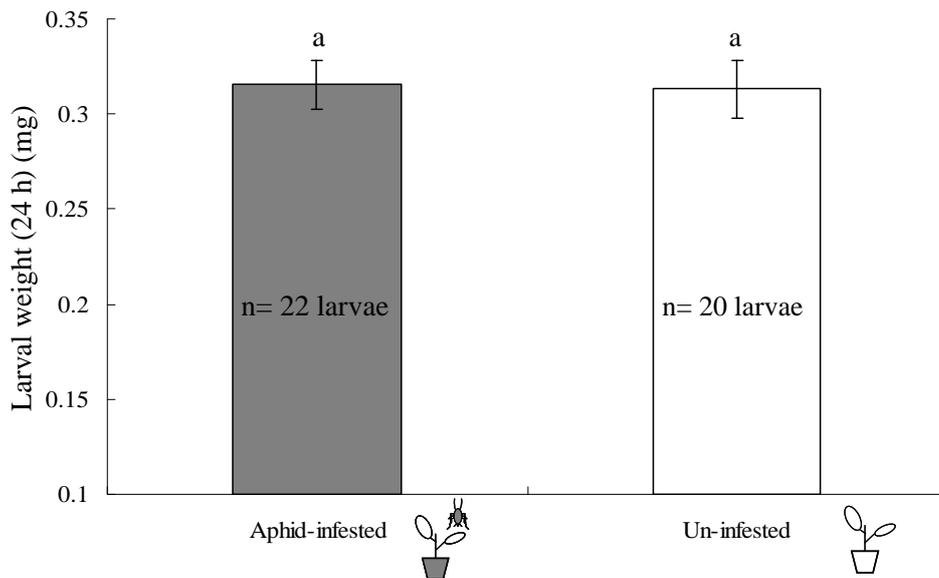
3.4.2 Larval performance

Due to the contamination of greenhouse compartment where the plants used to be kept, the performance tests studying the larval weight of *Pieris rapae* (PR)*, Pieris brassicae* (PB)*, Plutella xylostella* (PX)*, Spodoptera exigua* (SE) and *Mamestra brassicae* (MB) had to be stopped 4-5 days after the beginning of experiment. Therefore, only the few initial measurements of larvae weight performed on PR, PB and MB are reported here, that can actually came from plants with thrips infestation.

The performance of the *Mamestra brassicae* (MB), *Pieris brassicae* (PB), and *Pieris rapae* (PR) was not affected by the presence of the phloem feeder *Brevicoryne brassicae* during the short period that the experiment lasted. After 4-5 days of feeding on either plants previously infested by 15 aphid nymphs or un-infested plants, there were no significant differences among the larval weight of the three species (Fig 16, P= 0.180). Among these three species of leaf chewers, MB obtained the biggest benefit by feeding on aphid-infested plants for 5 days, as the averaged weight of MB larvae on aphid-infested plants (0.679 mg) was 23.5% higher than the weight of MB on un-infested plants (0.550 mg). PR harvested a 15.5% higher weight on aphid-infested plants, with a mean of 6.283 mg (Fig 16).

Based on the trend that *M.brassicae* larvae attained higher weight by feeding on plants infested by 15 aphid nymphs as compared to feeding on un-infested plants, a separated experiment was repeated to further investigate the performance of this species on these two types of plants. The experiments terminated after larvae feeding. However, my results of 24h did not show the differed body mass of larvae feeding on aphid-infested plants from the larvae feeding on un-infested plants (Fig 17, P= 0.906).

16

Fig 16. Mean (±SE) larval weights of *Mamestra brassicae* (MB); *Pieris brassicae* (PB); *Pieris rapae* (PR) after 4 or 5 days feeding on *Brassicae nigra* plants with or without previous aphid infestation. The same letters represent the insignificant differences (P= 0.180).

Fig 17. Means (±SE) of larval weight of *Mamestra brassicae* after 24 h feeding on aphid-infested plants and on undamaged plants. The same letters represent the insignificant differences (P= 0.906).

4 Discussion

The performance-preference hypothesis outlines that phytophagous insects are able to select the host plants that better fulfill the performance requirements of their offspring (Wiklund, 1975; Chew, 1977; Thompson, 1988; Wennström et al., 2010; Shikano et al., 2010; Gripenberg et al., 2010). Most studies have investigated the preference of lepidopterous insects between different host plant species. Nevertheless, in nature many wild crucifers are

patchily distributed (Chew, 1977; Berdegué et al., 1998), and an individual plant is surrounded by conspecific plants (Chew, 1977). Therefore, it is less costly for female butterflies to choose conspecific plants for consecutive oviposition. Additionally, some highly mobile lepidopterous larvae which need more than one individual plant to complete their larval stages, are likely to move and search around within a patch of conspecific host plants, and encounter choices between plants infested by other insect species and uninfested conspecific plants (Chew, 1977; Jones, 1976). In this context of choices between conspecific plants, I have investigated the larval and adult preferences between plants (*Brassica nigra*) previously infested by phloem feeders *Brevicoryne brassicae* or un-infested plants.

It was found out in this study that at low density of aphid-infestation, none of the five leaf chewer species (PB, PR, PX, MB, SE) discriminate aphid-infested and un-infested plants of the wild species *B. nigra*. It is possible that the feeding damage caused by aphid-infested plants at low density is too negligible for caterpillars to detect. Another possibility is the benefits of aphid-infested plants at low density for caterpillars were not pronounced enough to evoke the discriminative behavior of caterpillars. Notably, it was previously found in *B. oleraceae,* that *P. brassicae* preferred the leaf discs from aphid-infested plants at this density over the leaf discs from un-infested plants (Soler et al., unpublished). This shows that the same aphid-infestation density might result in different extents of plant defenses in different plants species.

Under medium aphid density, *Pieris brassicae* and *P. rapae* preferred the aphid-infested plants over un-infested plants, but *P. xylostella* and *M. brassicae* was repelled by aphid-infested plants, while *S. exigua* did not discriminate these two plant types. Bernays (1998) showed that neural constraints limited the amount of information insects could process in the environment, thus there was a trade-off between diet breadth and the ability to distinguish among hosts. Renwick & Radke (1990) compared the host range on which *P. xylostella* and *P. rapae* oviposited, and they found that *P. xylostella* adults had broader host range for oviposition than *Pieris* species. Generalist *M. brassicae* has a narrower host range than the 'very generalist' *S. exigua*, and actually frequently prefers cabbage plants, especially the lab population I tested were reared on cabbage for generations (Rojas et al., 1999). This could possibly explain the distinct choosing pattern of *P. xylostella* and *M. brassicae* compared to *P. brassicae* and *P. rapae*. A study provided by Van Loon & Schoonhoven (1998) demonstrated the specialist deterrent chemoreceptors enable the *Pieris* caterpillars to discriminate between chemically different deterrents. Therefore, it was supposed that *P. brassicae* and *P. rapae* which are very specialized on cruciferous plants could more accurately than other species identify the differences between aphid-infested plants (medium density) and un-infested plants.

At the high density of aphid-infestation, four responded leaf chewer species (PB, PR, PX, MB) avoided the aphid-infested plants, but *S. exigua* did not discriminate these two types of plants. Denno et al (1995) revealed that facilitation between herbivore species mediated by host plants was transient, and would be taken over by competition in the end. The study provided by Rodriguez-Saona et al (2005) indicated that the transient benefits to a

caterpillar feeding on aphid-infested plants would be compromised by the dense conspecific competition and enhanced risks from natural enemies. It was possible that moderated aphid-infestation plants which were less defensive would turn out to be nutritionally poor plants with the increase of aphid density. Zehnder and Hunter (2007) showed severe aphid infestation led to a decrease in foliar nitrogen concentration. In this study, *B. brassicae* population on aphid-infested plants in high density increased to 90-110 aphids on the day of experiments, and some observed necrotic spots at the aphid-feeding sites on aphid-infested plants at high density also indicated their poor nutritional value. The repellent effect of the aphid-infested plants in high density on *P. brassicae*, *P. rapae*, *P. xylostella* and *M. brassicae* were assumed to be resulted from the declined nutritional quality caused by the severe aphid damage.

Chew (1977; 1980) pointed out that failure of oviposition preferences on unsuitable plants both in quality and quantity may lead to powerful selection pressure in respect to food plant seeking ability of the larvae. Shikano et al (2010) concluded that larvae, which have a broader diet range compare to adults with limited oviposition, the neonates were better able to rank plants rather than adults, according to the larval performance. In this study, larvae did exhibit a choosing ability, even between conspecific host plants only differed in defense or nutrition state. There are three patterns that can be concluded in larval preference behavior with correlation of host range of different species. The very specialized *P. brassicae* and *P. rapae* did not discriminate the aphid-infested plants in low extent of aphid damage from un-infested plants, but preferred plants aphid-infested plants in medium density, and avoided the aphid-infested in high density. Broadly specialized *P. xylostella* and the 'specialized generalist' *M. brassicae* did not discriminate aphid-infested plants in low density and un-infested plants, but significantly repelled by moderated and high aphid-infested plants. The strong generalist *S. exigua* did not discriminate aphid-infested plants and un-infested plants, irrespectively to the densities of aphid-infestation. Additionally, it was remarkably that the choosing behavior of each species tested was well-correlated with the time used by them. *Pieris brassicae* and *P. rapae* were the two slowest species and most choosy species, whereas the generalists *M. brassicae* and *S. exigua* were the fastest species and less choosy.

Espelie et al (1991) proposed that the behavior of herbivorous insects were affected by the diverse plant cuticular lipids and some specific cuticular components attracted or deterred herbivory. Moreover, there are examples where co-evolution has favored a similarity of cuticular lipids between a caterpillar and its host plant, and provided the caterpillar double advantages, protection against predation and reduced cost of sequestering toxic compounds from host plants (Portugal and Trigo, 2005). Therefore, plant cuticular compounds might play a role in driving the choosing behavior of caterpillars. Leaf cuticular extracts from both aphid-infested plants and un-infested plants were offered to *P. brassicae* caterpillars. In one set of experiments, *Pieris brassicae* was significantly attracted by the cuticular extracts from aphid-infested plants, suggesting an important role of those compounds on the larval preference behavior. However, in other sets of experiments, *Pieris brassicae* did not show preference to either of the plant types. I assume that the dosage and the concentration of cuticular extracts partly determined the attraction effects on caterpillars,

and it is required to adjust and find out the appropriate volume. Notably, the high non-response rate of caterpillars upon *B. oleracrea* cuticular extracts were possibly related to the much thicker wax layer that negatively influenced the release of the volatile compounds.

The chemical analysis suggested some quantitative differences on the compounds. There was more 3-Hexanol (Green leaf volatile compound), and Allyl isothiocyanate (breakdown products of glucosinolates) in the aphid-infested plants. It was notably that Methyl salicylate was slightly higher on aphid-infested plants and 4-Methylguaiacol and 6.2,5-Dimethyl-3-acetylfuran was completely absent on the un-infested plants. Since the chemical analysis was only done with one sample, more replicates are needed to further analyze the composition of cuticular compounds on both aphid-infested plants and un-infested plants.

Previous studies reported that oviposition behavior of phytophagous insects was influenced by host plant attacking of other herbivore species. *Spodoptera exigua* females laid 2.3 times more egg mass and 1.6 times more eggs on aphid-infested plants compared to control plants (Rodriguez-Saona et al., 2005); oviposition of leafminer *L. trifolii* was reduced 30% on whitefly-damaged plants compared to control plants (Inbar et al., 1999). However, in this present study, female butterflies and moths of all the five species (PB, PR, PX, SE, MB) appeared to be unselective between aphid-infested plants and un-infested plants in oviposition preferences. It should be noticed that these insect populations have been reared in the laboratory for several generations, and *M. brassicae* and *S. exigua* in particular, were not even allowed to oviposite on plants but alternatively on paper. Thus, they might have partly lost the ability to associate the oviposition with plants, as observed in the experiments that *M. brassicae* and *S. exigua* laid most of their eggs on cages. The observation of indiscriminate behavior of females in this study might also be related to the insufficient density of aphid-infestation, as Rodriguez-Saona et al (2005) found the positive effect of aphid-infested plants inoculated by 40 adult aphids (*M. euphorbiae*) for 5 days, on oviposition behavior of *S. exigua*, and in this study plants were inoculated with 4 adult aphids for 5 days.

The performance of multiple species of leaf chewers (*Pieris rapae, Pieris brassicae,* and *Mamestra brassicae*) feeding on aphid-infested plants or un-infested plants was investigated in this study as well. There was no significant differences on larval weight of MB, PB, or PR when feeding on aphid-infested plants or un-infested plants. This supported the indiscriminate behavior of different leaf chewer species between aphid-infested plants in low density and un-infested plants. Caterpillars were possibly not motivated to select between these two types of plants, because there was no performance benefits for them. However, the plants were accidentally infested by thrips, and this many have overrided the effect of aphid infestation.

Several studies have displayed the influence of previous herbivory on the performance of other subsequent herbivore species mediated by plant induced defenses, even crossing the seasons and covering the neighboring plants (Dicke 2009; Inbar et al., 1999). Zhang et al (2009) showed that infestation of whiteflies promoted the fecundity of spider

mites by interfering the plant defense. Rodriguez-Saona et al (2010) showed that *S. exigua* caterpillars were 46% heavier and had 5% lower mortality on aphid-infested plants compared to control plants. The previous work of Soler et al (2010) found that *P. brassicae* larvae developed faster and achieved bigger size on aphid-infested *B. oleracea* plants rather than un-infested ones. The enhanced performance of the subsequent herbivore was most likely resulted from JA- SA crosstalk in plant immune system (Thaler et al., 2002 ; Pieterse et al., 2009). It was found that aphid feeding attenuated many jasmonate pathway-regulated gene induction (Rodriguez-Saona et al., 2010), and the JA production was inhibited by aphid infestation (Soler et al., 2010). Therefore, leaf chewers attacking the plants subsequently could benefit from the less-defended plants created by previous infestation of phloem feeders. Hereby, the insignificant benefit of aphid-infested plants for caterpillars found in this study probably due to the insufficient density of aphid-infestation.

In contrary to the classical theory that 'mother knows best', this study showed that larvae are better able to choose the beneficial plants than adults, and the choosing ability was positively correlated with their host range. Wiklund (1974) indicated that adult and larval host plant preferences were determined by separated gene complex, so that the larval choosing pattern could differ from the mothers'. The very specialized *P. brassicae* and *P. rapae* are able to recognize the short-living benefits of aphid-infested plants in medium density, but also avoided the nutritionally poor plants infested by aphid in high density. The broadly specialized *P. xylostella* and 'specialized generalist' *M. brassicae* were less precise than *Pieris* species in preferences. They were repelled by the potential disadvantages of aphid-infested plants in medium to high density. The generalist *S. exigua* did not appear to discriminate aphid-infested plants and un-infested plants, no matter the aphid-infested plants in different densities were tested with. It was illustrated by the larval preference behavior that the facilitative effect of aphid-feeding on caterpillar performance was transient and can only be utilized by highly specialists. Facilitation was trigger by the 'right' extent of aphid-infestation only, and taken over by competition in the end.

The performance study of the five leaf chewer species on aphid-infested plants (in different densities) and un-infested plants should be conducted in the future and linked with the preference data in this study. The female oviposition preference should be repeated with more replications and performed with aphid-infested plants in various densities. Thus, the framework of performance-preference study can be completed with performance of larvae correlated with both female oviposition and larval feeding preferences. With regard of the mechanism underlying the larval preferences, plant volatiles, other physical characteristics, such as protein content, leaf toughness can be investigated (Berdegue & Trumble, 1996).

5 Acknowledgment

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